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TLR2 and TLR9 Synergistically Control Herpes Simplex Virus Infection in the Brain[1]

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Viruses are recognized by the innate immune system through pattern recognition receptors (PRRs). For instance, HSV virions and genomic DNA are recognized by TLR2 and TLR9, respectively. Although several viruses and viral components have been shown to stimulate cells through TLRs, only very few studies have defined essential roles for single TLRs in innate immune defense in vivo. This could suggest that PRRs act in concert to mount the first line of defense against virus infections. To test this hypothesis we have examined the host response of C57BL/6, TLR2$^{-/-}$, TLR9$^{-/-}$, and TLR2/9$^{-/-}$ mice toward HSV-2 infection. After a systemic infection, the cytokine serum response was markedly reduced in the double knockout mice, but only partly affected in either strain of the single knockout mice. This was supported by in vitro data showing that HSV-induced cytokine expression relayed on TLR2 and TLR9 in a cytokine- and cell type-dependent manner. With respect to the cellular response to infection, we found that recruitment but not activation of NK cells was impaired in TLR2/9$^{-/-}$ mice. Importantly, the viral load in the brain, but not liver, was significantly higher in the brain of TLR2/9$^{-/-}$ mice whereas the viral loads in organs of single knockout mice were statistically indistinguishable from C57BL/6 mice. In the brain we found that TNF-α and the IFN-stimulated gene CXCL9 were expressed during infection and were dependent on either TLR2 or TLR9. Thus, TLR2 and TLR9 synergistically stimulate innate antiviral activities, thereby protecting against HSV infection in the brain. ***The Journal of Immunology,*** **2008, 181: 8604–8612.**

The early innate host response to viral infections is characterized by production of type I IFNs, proinflammatory chemokines, and cytokines (1) as well as direct antiviral cellular activities. This first line of host defense, which has been ascribed important roles in defense against virus infections (2–4), is mediated both through direct inhibition of viral replication by IFNs (1) and, among others, by cytotoxic activity of NK cells (5). Additionally, to the direct virucidal functions of the innate antiviral response, IFN-α/β together with, for example, IL-12 stimulate development of the subsequent adaptive immune response, which is important for viral elimination and development of protective immunity (6). Therefore, activation of an innate antiviral response raises both an early barrier against the infection and shapes the subsequent specific immune response.

The antiviral response is initiated by cellular germline-encoded pattern recognition receptors (PRRs)[3] that recognize viral pathogen-associated molecular patterns (PAMPs) and activate intracellular signal transduction, leading to stimulation of antiviral functions (7, 8). TLRs represent one class of membrane-bound PRRs, which recognize PAMPs in the extracellular or endosomal environment, whereas other PRR systems seem to be responsible for detection of pathogens in the cytoplasm (8–12). Given the existence of many PRRs it is of no surprise that pathogens are often recognized by multiple receptors (13–16), for which reason it is important to understand how PRRs work together in coordinating the host response to a given pathogen.

HSV, which is an enveloped DNA virus belonging to the class of alphaherpesviruses, is detected by TLR2 and TLR9 through recognition of a still unidentified viral surface component and viral genomic DNA, respectively (17–20). Mice lacking TLR2 are resistant to lethal HSV-1 encephalitis despite similar viral load in brains compared with wild-type (WT) mice (17, 21), and they evoke a weaker inflammatory cytokine response after infection with HSV-1. In vitro, most studies show that HSV-induced expression of proinflammatory cytokines is diminished when TLR2 is not functional (17, 18, 21). Plasmacytoid dendritic cells (pDC)s recognize HSV through TLR9 (19, 20), and mice lacking this receptor produce little type I IFN or IL-12p40 at early time points after HSV infection (15, 19). However, for both single knockout (KO) mouse strains, most studies published until now show no strong phenotype with respect to innate defense against HSV infection (17, 20–22). It has been shown previously that MyD88$^{-/-}$ mice are highly susceptible to lethal HSV encephalitis (21, 23), and it was recently shown that TLR2 and TLR9 act in a cooperative manner to activate conventional DCs (cDC)s during infection with HSV (13). Therefore, there are data to suggest that these

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[3] Abbreviations used in this paper: PRR, pattern recognition receptor; BM-DC, bone marrow-derived dendritic cell; cDC, conventional dendritic cell; DC, dendritic cell; DKO, double knockout; ISG, IFN-stimulated gene; KO, knockout; pDC, plasmacytoid dendritic cell; p.i., postinfection; SL, spleen leukocyte; WT, wild type.

TLRs work together to orchestrate the host response to HSV infection. Interestingly, TLR2 and TLR9 have specifically been reported to synergize in activation of the host response bacteria and parasites (14, 24, 25).

Here we have used TLR2 and TLR9 single KO and double knockout (DKO) mice together with MyD88$^{-/-}$ mice to show that these two PRRs are responsible for the MyD88-dependent portion of the innate antiviral response against HSV. TLR2 and TLR9 act in synergy to induce an early cytokine and cellular response, thereby restricting viral load in the brain.

Materials and Methods

Reagents

Growth media used were MEM, DMEM, IMDM, and RPMI 1640 (all from BioWhittaker), which were supplemented with antibiotics (penicillin 200 IU/ml, streptomycin 200 μg/ml) and LPS-free FCS (BioWhittaker). BSA was obtained from Sigma-Aldrich, Depo-Provera from Pfizer, and GM-CSF from R&D Systems. Heparin was obtained from Leo Pharma, isoflurane from Baxter, and Lympholyte from Cedarlane Laboratories. CFSE was from Molecular Probes, and NK1.1-FITC, CD3-PE, CD69-PerCyP5.5, and 7-aminoactinomycin D were from BD Biosciences. TLR ligands used were Pam_3CSK_4 (TLR2) and ODN1826 (TLR9) were from InvivoGen.

Mice

Inbred, specific pathogen-free mice used in this study were 8–12-wk-old, age-matched, female C57BL/6J, TLR2$^{-/-}$, TLR9$^{-/-}$, TLR2/9$^{-/-}$, and MyD88$^{-/-}$. All knockout mice strains were on a C57BL/6J background. C57BL/6J, TLR2$^{-/-}$, TLR9$^{-/-}$, and TLR2/9$^{-/-}$ mice were bred at Taconic M&B, and MyD88 mice were bred at the animal facility at the University of Copenhagen. TLR2$^{-/-}$ and TLR9$^{-/-}$ mice were obtained from Oriental Yeast. All animal experiments described have been reviewed and approved by Danish Government authorities, and hence comply with Danish law.

Cells

Bone marrow-derived dendritic cells (BM-DCs) were obtained as follows: femurs and tibia were surgically removed, freed of muscles and tendons, and briefly suspended in 70% ethanol. Ends were cut, the marrow was flushed with 10% RPMI 1640, and cell suspension was filtrated over a 70-μm cell strainer (BD Falcon) and centrifuged for 5 min at 1330 rpm. After 2 washes, cells were resuspended at 2×10^5/ml in RPMI 1640 with 10% FCS and GM-CSF (40 ng/ml) and seeded in bacteriological petri dishes and incubated at 37°C with 5% CO_2. At day 3 the same fresh media were added, and at days 5 and 7 media were replaced with fresh media. At day 10 of culture nonadherent cells were harvested, centrifuged, and vial cells were counted in trypan blue and resuspended in RPMI 1640–10% FCS, GM-CSF (20 ng/ml) and seeded at 1×10^6/ml in cell culture dishes. At day 11 nonadherent cells were harvested and used in assays. Cells were examined by flow cytometry, showing a myeloid dendritic cell type (>95% were CD11c$^+$CD11b$^+$) (data not shown). For isolation of primary cells from mouse spleens, organs were surgically removed and transferred to RPMI 1640 with 5% FCS. The spleens were then transferred to a 1 mg/ml suspension of collagenase D (Roche). The enzyme was injected into the organ, which was subsequently cut into small pieces, followed by incubation in the collagenase D suspension for 30 min at 37°C. The suspension was filtrated over a 70-μm pore size cell strainer, spun down, and suspended in RPMI 1640–5% FCS, and the cells were counted. After centrifugation, the cells were resuspended in PBS with 2 mM EDTA-0.5% BSA (MACS running buffer) in concentrations according to the manufacturer's instruction (Miltenyi Biotec). Anti-mPDCA-1 microbeads were added, and after incubation for 15 min at 4°C the suspension was spun down and suspended in running buffer. pDCs were then isolated in an autoMACS separator by positive selection. The negative selected cell fraction was incubated with anti-CD11c, and cells were isolated by both positive and negative selection. Isolated cell fractions were spun down, suspended in RPMI 1640–5% FCS, and counted. For determination of ex vivo cytokine production, the cells were cultured for 24 h at a concentration of 3.0×10^6 cells in 100 μl of RPMI 1640 with 5% FCS, and supernatants were eventually harvested for measurement cytokines. Spleen leukocytes (SLs) for flow cytometry and NK assay were obtained as follows. Spleens were harvested as described above, homogenized in 5% RPMI 1640 and filtrated over a 70-μm cell strainer. Leukocytes were isolated in Lympholyte according to the manufacturer's recommendation. Peritoneal cells were harvested with PBS containing 5% FCS and 20 IU/ml heparin. Cells were washed, counted, and resuspended in RPMI 1640-5% FCS.

Viruses

The viruses used were HSV-2 (strains MS and 333). Viruses were grown on a Vero cells monolayer to complete cytopathic effect, prepared by one cycle of freezing and thawing, followed by centrifugation for 30 min at $5000 \times g$ for removal of cellular debris.

Treatment of mice in vivo

For vaginal HSV-2 infection, 8- to 9-wk-old mice were pretreated by s.c. injection of 2 mg Depo-Provera. Five days later, mice were anesthetized with isoflurane and inoculated intravaginally with 6.7×10^4 PFU of HSV-2 strain 333 suspended in 20 μl IMDM. The mice were placed on their backs and maintained under anesthetics for at least 10 min. Genitally infected mice were examined daily and scored for genital inflammation, neurological illness, and death. The severity of disease was graded using the following scores: 0, healthy; 1, genital erythema; 2, moderate genital inflammation; 3, purulent genital lesion and/or generally bad condition; and 4, hind limb paralysis (mice were sacrificed). For systemic infections, 8- to 12-wk-old female mice were infected i.p. with 1×10^6 and 5×10^6 PFU of HSV-2 (strain MS). The animals were sacrificed on the indicated time points postinfection (p.i.), and viral load was determined in infected organs.

NK assay

NK cell cytotoxity was assayed in a flow-based killing assay (FloKA) developed from a method described by Lecoeur and colleagues (26). Briefly, mice were infected i.p. with HSV-2 24 h before the assay. Peritoneal cells (10^6 cells/ml) were labeled with 500 nM CFSE in PBS-0.1% BSA for 10 min at 37°C. Cells were then washed twice and used immediately in the assay. CFSE-labeled cells were incubated with YAC-1 target cells (50,000 cells/well) at various ratios (E:T ratios of 15:1, 5:1, and 2:1) and target cells were also incubated alone to measure spontaneous apoptosis in 96-well microplates in 150 μl RPMI 1640-10% FCS for 4 h in 5% CO_2 and 37°C. Cell suspensions were then washed in PBS-0.1% BSA and incubated with 7-aminoactinomycin D at 20 μg/ml for 20 min at 4°C in the dark. Cell samples were washed in PBS, resuspended in PBS-1% paraformaldehyde, and analyzed immediately on a Beckman Coulter FC500 (20,000 cells/sample). For analysis of NK1.1 or CD69 expression, cells were incubated with the following Abs: NK1.1-FITC, CD3-PE, and CD69-PerCyP5.5 for 20 min at 4°C in the dark. Cell samples were washed in PBS, resuspended in PBS-1% paraformaldehyde, and analyzed on a Beckman Coulter FC500 (20,000 cells/sample).

Viral plaque assay

Samples of liver and brain were weighed, thawed, and homogenized for 3×5 s in MEM media supplemented with 5% FCS just before use in the plaque assay. After homogenization the organ suspensions were pelleted by centrifugation at $1620 \times g$ for 30 min, and the supernatants were used for plaque assay, which were done on Vero cells seeded in MEM supplemented with 5% FCS at a density of 1.2×10^6 in 5-cm-diameter plates and left overnight to settle. The cells were infected by incubation for 1 h at 37°C with 100 μl of serial dilutions of the organ suspension and 400 μl of medium, during which the tissue culture plates were rocked every 15 min to ensure even distribution of the virus. Subsequently, the organ suspensions were removed and 8 ml of MEM was added to the plates. The medium was supplemented with 2.5–5% FCS, depending on how confluent the cells were at the time of incubation, and with 0.2% human Ig. The cells were incubated at 37°C for 2 days, stained with 0.03% methylene blue, and finally plaques were counted.

Measurement of cytokine

Cytokine levels in serum and supernatants were measured by Luminex using kits from Bio-Rad. Briefly, the filter plate was washed with assay buffer and 50 μl of freshly vortexed Ab-conjugated beads was added to each well. The plate was washed with assay buffer and samples and standards were added. After a brief shake (30 s at 1100 rpm), the plate was incubated at room temperature in the dark for 2 h with light shaking (300 rpm). After one wash step, 25 μl of the detection Ab was added to each well, and the plate was shaken and incubated as above. Subsequently, the plate was washed and incubated for 30 min with 50 μl of a streptavidin-PE solution with shaking (30 s at 1100 rpm, 10 min 300 rpm). Finally, the plate was washed and 125 μl of assay buffer was added to

each well and the plate was shaken for 10 s at 1100 rpm and read immediately on the Bio-Plex machine.

IFN-α/β bioassay

IFN-α/β bioactivity was measured by a L929 cell-based bioassay. L929 cells (2×10^4 cells/well in 100 μl) in MEM with 5% FCS were incubated overnight at 37°C in successive 2-fold dilutions of samples or murine IFN-α/β as standard. Subsequently, vesicular stomatitis virus (VSV/V10) was added to the wells and the cells were incubated for 2–3 days. The dilution mediating 50% protection was defined as 1 U/ml of IFN-α/β.

Quantitative RT-PCR

Total RNA was extracted with TRIzol (Invitrogen) according to the recommendations of the manufacturer. Briefly, organs were homogenized in TRIzol and chloroform was added, followed by phase separation by centrifugation. RNA was precipitated with isopropanol and pelleted by centrifugation. Pellets were washed with 80% ethanol and redissolved in RNase-free water. For cDNA generation, RNA was subjected to reverse transcription with oligo(dT) as primer and Expand reverse transcriptase (both from Roche). Before quantitative RT-PCR, RNA was treated with DNase I (Ambion) to remove any contaminating DNA, the absence of which was confirmed in control experiments in which the reverse transcriptase enzyme was omitted (data not shown). The cDNA was amplified by PCR using the following primers: β-actin, forward: 5′-TAGCACCAT GAAGATCAAGAT-3′, reverse: 5′-CCGATCCACACAGAGTACTT-3′; CXCL9, forward: 5′-GAA CGG AGA TCA AAC CTG CCT-3′; reverse 5′-TGT AGT CTT CCT TGA ACG ACGA-3′; TNF-α, forward: 5′-ATC GGC TGG CAC CAC TAG TT-3′, reverse: 5′-GTA GCC CAC GTC GTA GCA AAC-3′. Products were measured using SYBR Green I (Qiagen).

Statistics

The data are presented as means ± SD. The statistical significance was estimated with the Wilcoxon rank sum test (p-values of <0.05 were considered to be statistically significant).

Reproducibility of data

The results shown in this work are derived from data that are representative for the results obtained. For each series of experiments, two to five independent repetitions were performed.

Results

The dependency on TLR9 alone distinguishes pDCs from other APCs

It has previously been shown that pDCs are the main bone marrow-derived cell type producing type I IFNs after HSV-2 infection and that this induction is almost totally dependent on TLR9 (19). Lund and colleagues also showed that depleting mice of pDCs rendered them susceptible to a vaginal HSV-2 infection (27). To further examine the role of different TLRs and DC subset in the host response to HSV-2 infection, we examined the induction of proinflammatory cytokines in BM-DCs, which display a myeloid DC-like phenotype, as well as in splenic pDCs. Initially, we examined IL-6 induction by TLR2 agonist Pam_3Csk_4 and TLR9 agonist ODN1826 in BM-DCs to confirm the genotyping of our TLR-deficient mice strains and, as seen in Fig. 1*A*, IL-6 production was totally abrogated in the corresponding TLR-deficient cells.

In BM-DCs IL-6, IFN-α/β, and IL-12p40 were induced by HSV-2 strain 333 (Fig. 1, *B–E*). As the dependency on TLRs varies with different HSV strains (13), we also examined HSV-2 strain MS, which induced all cytokines measured (Fig. 1, *F–I*). We also noted that the MS strain induced cytokines only if the virus was UV-inactivated before addition to cell culture (data not shown), suggesting that a mechanism of recognition, independent of virus replication, is operative in this cell type, and that the virus inhibits this cellular response during replication. HSV-induced expression of IL-6, RANTES, and IL-12p40 was independent of both TLR2 and TLR9, and the cytokine response in TLR2/TLR9$^{-/-}$ BM-DCs was comparable to the response in MyD88$^{-/-}$ cells. Common for both virus strains, the induction of IFN-α/β was significantly reduced in TLR9$^{-/-}$ and TLR2/9$^{-/-}$ BM-DCs. Similar observations were also done when the infectious dose was increased 5-fold (data not shown).

It has previously been reported that different DC populations display different TLR dependency with respect to HSV-induced cytokine expression (15, 19, 28). Therefore, we wanted to compare the in vitro cytokine response of the BM-DCs with that of cDCs harvested from mouse spleens. Like the BM-DCs, splenic cDCs responded to HSV-2 infection with elevated expression of IL-6, RANTES, and type I IFN (Fig. 1, *J–L*), but did not stimulate expression of IL-12 p40 (Fig. 1*M*). None of the cytokines was induced in a manner dependent on TLR2 or TLR9 in cDCs. Thus, both BM-DCs and splenic cDCs induce expression of IL-6 and RANTES in response to HSV-2 infection independent of TLR2 and TLR9, whereas expression of type I IFN is partly dependent on TLR9 in BM-DCs and is independent of this receptor in splenic cDCs.

In pDCs, IL-6, RANTES, IFN-α/β, and IL-12p40 were produced in response to live MS strain, and for all four cytokines this production was strongly diminished in TLR9$^{-/-}$ but not TLR2$^{-/-}$ pDCs (Fig. 1, *N–Q*). TLR2/TLR9 double-deficient pDCs showed no further reduction in cytokine levels compared with TLR9 single KO pDCs. Thus, production of antiviral and inflammatory cytokines and chemokines in response to HSV-2 is induced in both BM-DCs and pDCs. In BM-DCs only IFN-α/β was dependent on TLR9, whereas in pDCs we observed TLR9 dependency for all cytokines tested.

During an acute HSV infection, macrophages play important roles in both the innate response as well as in activating and shaping the adaptive immune response, at least partly through the production of chemokines, IFNs, and proinflammatory mediators such as RANTES, IL-6, and IL-12 (29–32). We therefore also examined the production of cytokines by peritoneal macrophages during infection with HSV-2 strains MS and 333. As seen in Fig. 2, *A–C*, infection with HSV-2 strain 333 induced production of IL-6, RANTES, and IFN-α/β in macrophages. Regarding the dependency on TLRs or MyD88, levels of IL-6 and RANTES were significantly decreased in all TLR- as well as MyD88-deficient macrophages. HSV-2 strain MS also induced IL-6, RANTES, and IFN-α/β, but here the dependency on TLRs varied: IL-6 induction was partly dependent on TLR2, IFN-α/β on TLR9, and full induction of RANTES seemed to rely on both TLR2 and TLR9. Hence, for peritoneal macrophages, both TLR2 and TLR9 are necessary for a complete cytokine response toward HSV-2, but the role of the specific TLRs is cytokine and virus strain specific.

Conclusively, we observed that only pDCs depend entirely on TLR9 in HSV-induced cytokine production, whereas other APCs use both TLR2, TLR9, and other recognition systems to induce this response.

TLR2 and TLR9 cooperatively induce cytokine production during HSV-2 infection in vivo

A HSV-2 infection in vivo normally leads to a robust cytokine response with induction of type I IFNs, IL-6, IL-12, RANTES, and TNF-α among others (17, 33–35). The data presented in Figs. 1 and 2 demonstrate that different cell types exhibit different requirement for TLR2 and TLR9 in induction of cytokines during HSV-2 infection in vitro. During an in vivo infection, many cell types could contribute to the cytokine response (15), and to address this further, we isolated splenic pDCs, cDCs, and non-DCs from WT mice infected for 8 h with HSV-2 for measurement of ex vivo cytokine production. The pDCs produced large amounts of type I IFN and RANTES after in vivo HSV-2 infection, and they were the only cell type displaying elevated expression of these cytokines

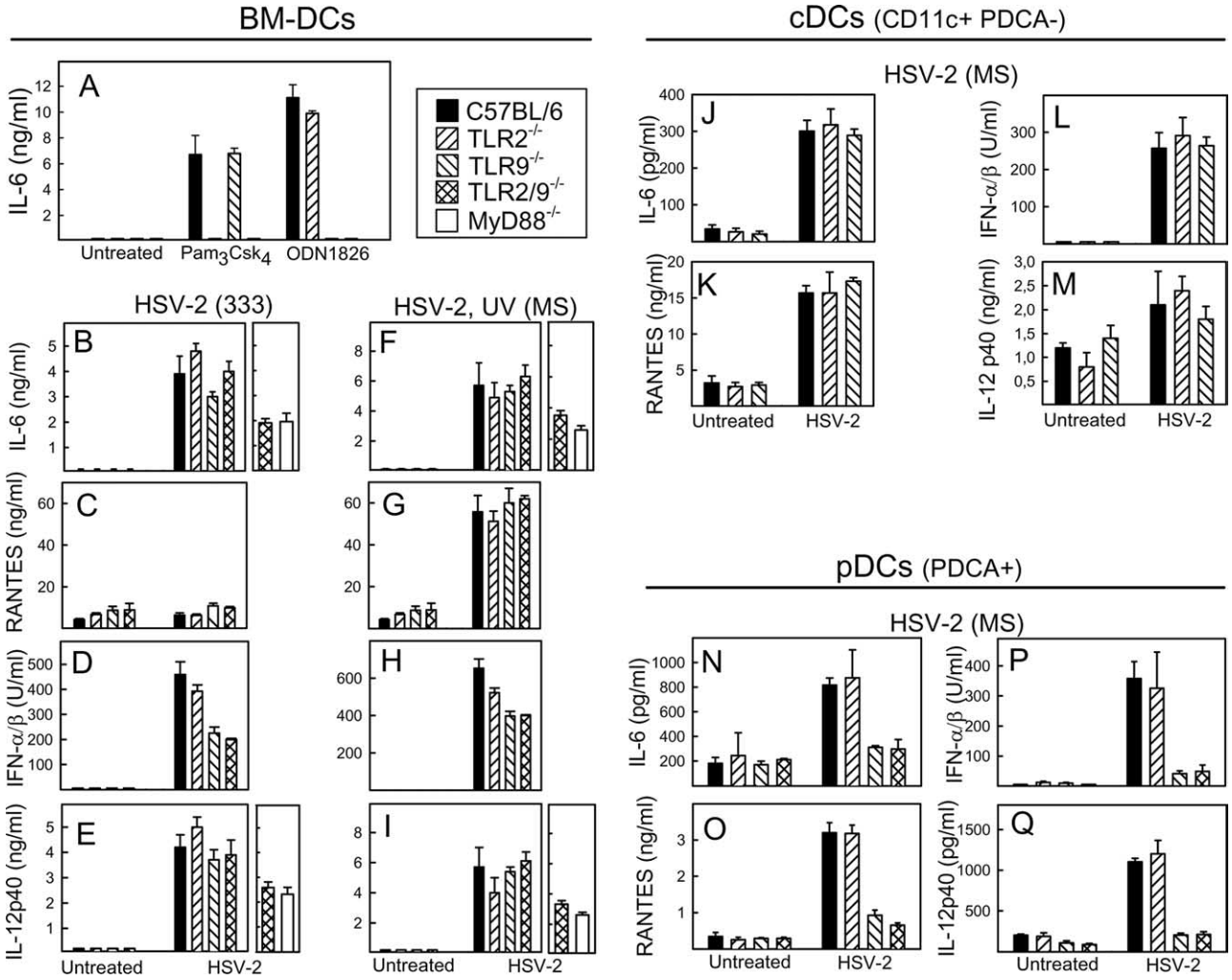

FIGURE 1. Induction of cytokines and chemokines in DC subtypes after stimulation with TLR ligands or HSV-2 infection. *A–I*, BM-DCs were generated from C57BL/6, TLR2$^{-/-}$, TLR9$^{-/-}$, TLR2/9$^{-/-}$, and MyD88$^{-/-}$ mice as described and treated with Pam_3Csk_4 (200 ng/ml), ODN1826 (1 μM), and infected with HSV-2 strain 333 (3×10^6 PFU/ml) or UV-inactivated strain MS (3×10^6 PFU/ml). Conventional DCs (*J–M*) and pDCs (*N–Q*) were isolated from spleen cells as described, cultured, and infected with HSV-2 strain MS (3×10^6 PFU/ml). Supernatants were harvested 24 h posttreatment, IFN-α/β was measured by bioassay, and IL-6, IL-12p40, and RANTES were measured by Luminex assay. Data are shown as means of measurements from triplicate cultures $\pm$ SEM.

after infection (Fig. 3, *A–I*). IL-6 production was induced in all cell types after HSV-2 infection in vivo, with the strongest induction observed in cDCs (Fig. 3, *C*, *F*, and *I*). To examine the role of TLR2 and TLR9 in induction of the cytokine response in vivo, we therefore measured levels of cytokines and chemokines in serum of WT and KO mice infected with HSV-2. As shown in Fig. 3, *J–U*, the levels for IFN-α/β, IL-6, RANTES, and KC were all significantly decreased in TLR2/9 DKO mice. For IFN-α/β, this was likely due to the lack of TLR9, as a similar reduction was seen in TLR9 single KO mice. RANTES induction seemed to be dependent on both TLR2 and TLR9, as the levels were significantly diminished in both single KO strains as well. For induction of IL-6 and KC, TLR2 and TLR9 act in a redundant manner, since reduced cytokine production was observed only in DKO mice Collectively, these data show that both TLR2 and TLR9 are necessary for a full cytokine response to a HSV-2 infection in vivo, and that lack of these two PRRs leads to a substantial reduction in the systemic cytokine levels. However, all of the measured chemokines and cytokines were still induced in serum in TLR2/9 DKO mice, indicating that PRRs other than TLR2 and TLR9 are important for this early response toward HSV-2 infection.

Reduced NK cell recruitment in TLR2 and TLR9 double-deficient mice

NK cells are important for the first line of defense against many viruses including HSV (5, 36, 37). NK cells are activated by cytokines including IFN-α/β and IL-12 (38), and studies have shown that lack of either TLR2 or TLR9 decrease the NK cell response during viral infection (39, 40). We therefore examined the early NK cell response in spleens of WT and TLR2/9$^{-/-}$ mice during systemic HSV-2 infection. We first examined how TLR2 and TLR9 double deficiency affected the size of the spleen and found that organs from C57BL/6 and TLR2/9$^{-/-}$ mice were indistinguishable in terms of weights (at days 0 and 1 p.i.) and total number of cells recovered (data not shown). Interestingly, spleens from TLR2/9 DKO mice infected with HSV-2 contained significantly less NK cells than did their WT counterparts (Fig. 4*A*). However, when examining the activation state of the NK cells, we observed that expression of the early activation marker CD69 was comparable between splenic NK1.1$^+$ cells from WT and TLR2/9 DKO (Fig. 4*B*). Similar results were found when examining CD69 expression in peritoneal cells harvested from infected mice (data not

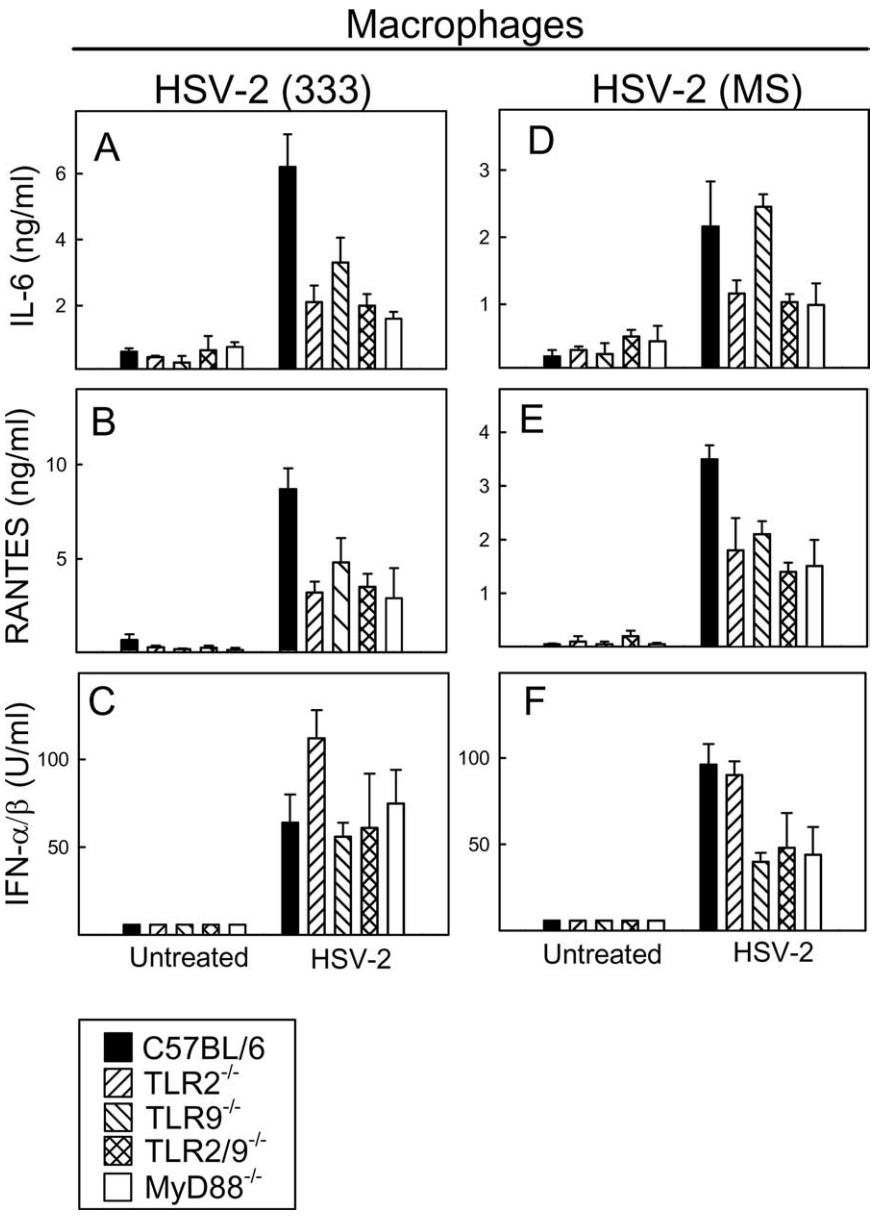

FIGURE 2. Induction of cytokines and chemokines in peritoneal macrophages after HSV-2 infection. Peritoneal cells were harvested from C57BL/6, TLR2$^{-/-}$, TLR9$^{-/-}$, and TLR2/9$^{-/-}$ mice as described, and treated with HSV-2 strain 333 or strain MS (3×10^6 PFU/ml). Supernatants were harvested 24 h posttreatment, IFN-α/β was measured by bioassay, and IL-6 and RANTES were measured by Luminex. Data are shown as means of measurements from triplicate cultures ± SEM.

shown). NK cell cytotoxic activity was measured in a flow-based killing assay, and again we found no significant differences in the activity of the NK cells between WT and TLR2/9 double-deficient mice (data not shown). Thus, although the NK cells present at the site of infection are activated to the same extent in C57BL/6J and TLR2/9$^{-/-}$ mice, the TLR deficiency leads to reduced NK cell recruitment to the spleen and thus potentially an overall decreased NK cell response in the foci of infection.

TLR2 and TLR9 synergistically mediate resistance against HSV-2 in the brain

The results shown above indicate that TLR2 and TLR9 cooperate in shaping the very early immune response toward HSV-2 both in vitro and in vivo. This led us to ask whether the combined function of TLR2 and TLR9 controls HSV-2 replication in the organism. We compared the ability of WT and TLR2-, TLR9-, and TLR2/TLR9-deficient mice to resist a systemic HSV-2 infection induced by i.p. infection. As seen in Fig. 5, *A*, *B*, *D*, and *E*, both TLR2 and TLR9 single KO strains displayed levels of viral load in livers and brains similar to WT mice. By contrast, TLR2/TLR9 double-deficient mice contained significantly higher levels of virus in brain compared with WT mice but showed no difference from WT mice with respect to liver infection (Fig. 5, *C* and *F*).

Work by others has shown that lack of MyD88 is associated with high susceptibility to many pathogens, including HSV, and that a HSV infection leads to severe encephalitis in MyD88$^{-/-}$ mice (21, 41, 42). We therefore examined the resistance of MyD88-deficient mice toward the systemic HSV-2 infection and found a phenotype comparable to the TLR2/TLR9 double-deficient strain (Fig. 5*G*). This suggests that TLR2 and TLR9 together mediate the MyD88-dependent part of the defense against HSV-2 infection in the brain.

To examine the role of TLR2 and TLR9 in a different model of HSV-2 infection, we turned to a model for herpes genitalis, where the mice are infected in the vagina leading to a local infection, with subsequent spread to the CNS. Also in this model the TLR2/TLR9$^{-/-}$ mice displayed higher susceptible to encephalitis than did their WT counterparts both in terms of pathological signs and survival (Fig. 5, *H* and *I*). With respect to viral load, viral titers in vaginal washes and the spinal cord were comparable in WT and

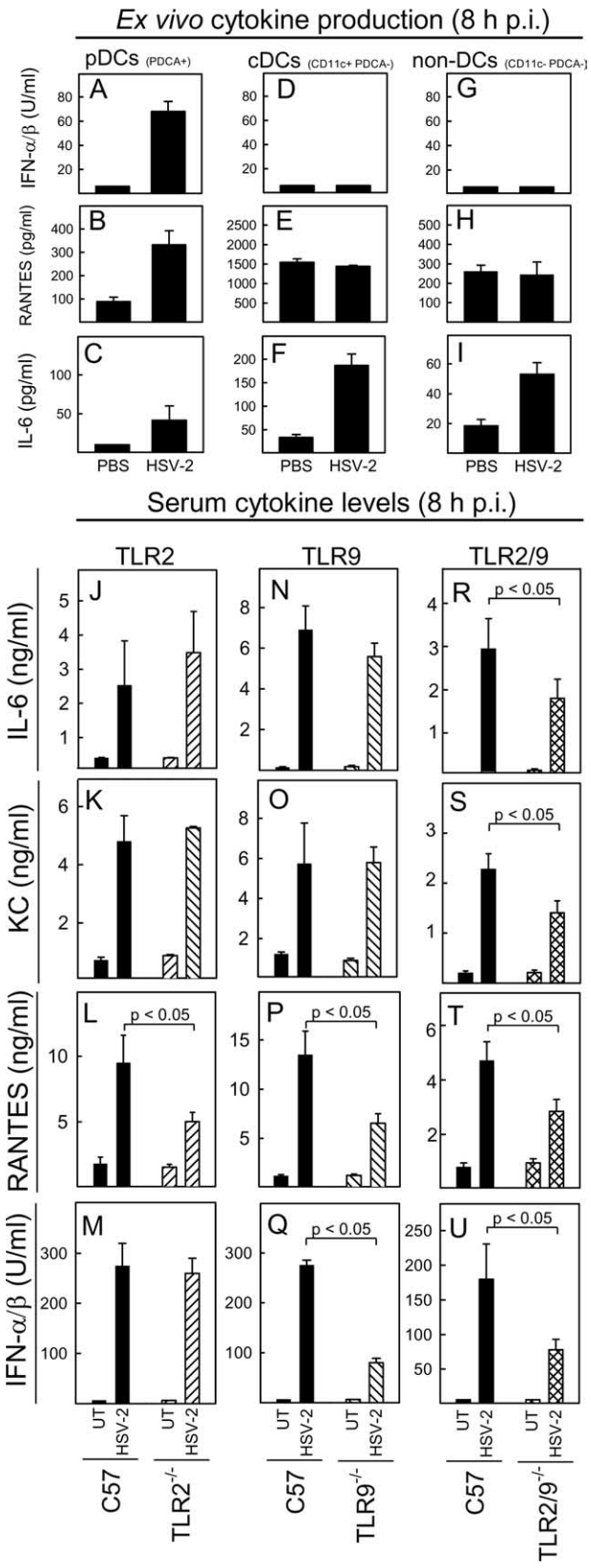

FIGURE 3. Production of cytokines in vivo after systemic HSV-2 infection. *A–I*, Ex vivo cytokine production. Mice were injected i.p. with PBS or 1×10^7 PFU of HSV-2 (strain MS). Eight hours later, splenic pDCs ($PDCA^+$), cDCs ($CD11c^+PDCA^-$), and non-DCs ($CD11c^-PDCA^-$) were isolated and sat in culture for 24 h. Supernatants were harvested for measurement of cytokines. *J–U*, Levels of cytokines in serum during a systemic HSV-2 infection. C57BL/6, $TLR2^{-/-}$, $TLR9^{-/-}$, and $TLR2/9^{-/-}$ mice ($n = 6–8$) were infected i.p. with HSV-2 strain MS ($1–2 \times 10^7$ PFU/mouse). Eight hours after infection, blood samples were collected, serum was fractionated, and samples were analyzed by Luminex (IL-6, KC, and RANTES) or bioassay (IFN-α/β). Data are shown as means ± SEM. *p*-values <0.05 were considered significant and are shown in the figure.

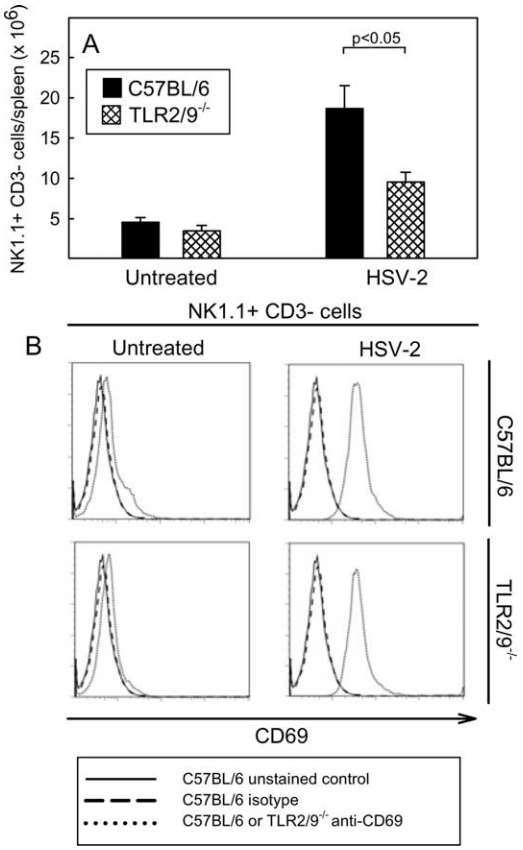

FIGURE 4. Accumulation and activation of NK cells in the spleen after systemic HSV-2 infection. SCs were harvested from naive mice and mice infected with HSV-2 (5×10^6 PFU i.p.) for 24 h. The cells were incubated with labeled Abs as described. Cells were gated as $CD3^-$ and $NK1.1^+$ for estimation of NK cell percentage. *A*, Absolute numbers of NK cells per spleen were calculated and are presented as means ± SEM. $n = 4–6$. *B*, CD69 expression was analyzed on $CD3^-NK1.1^+$ cells. The data are shown as fluorescence intensity histograms for one representative sample from each group.

$TLR2/TLR9^{-/-}$ mice (Fig. 5, *J* and *K*). In contrast, we found significantly higher levels of virus in the brainstem of $TLR2/TLR9^{-/-}$ mice as compared with WT mice on day 7 after infection (Fig. 5*L*). Hence, the combined lack of both TLR2 and TLR9 reduces the in vivo resistance toward HSV-2 infection in the brain.

Reduced local cytokine and chemokine response in the brain of HSV-infected $TLR2/9^{-/-}$ mice

As TLR2/TLR9 deficiency affected only the viral load in the brain and not the liver or vagina, we examined the local cytokine and chemokine response in brains of mice after vaginal infection. Although we did not detect significant induction of IFN-α or IFN-β in the brains of infected mice (data not shown), mRNA levels of the IFN-stimulated gene (ISG) CXCL9 was up-regulated on day 6 in brains of infected WT mice (Fig. 6*A*). Importantly, no induction of CXCL9 was observed on day 6 in the brains of $TLR2^{-/-}$, $TLR9^{-/-}$, or $TLR2/9^{-/-}$ mice. On day 9, CXCL9 was also induced in the brains of $TLR2^{-/-}$ mice, although not to the same extent as in WT mice, and no induction was found in mice lacking

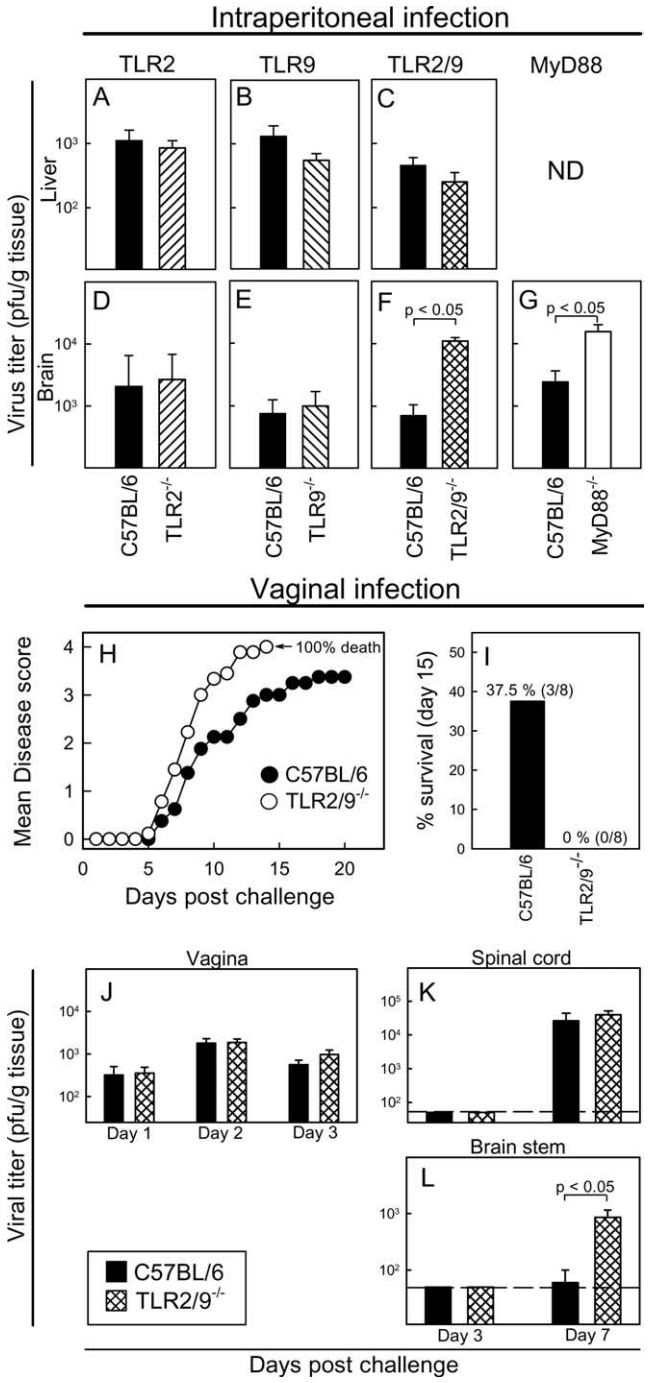

FIGURE 5. Disease development and viral load in organs after systemic HSV-2 infection. Mice were infected i.p. with 5×10^6 PFU (*A*, *B*, *D*, and *E*) or 1×10^6 PFU (*C*, *F*, and *G*) of HSV-2 strain MS or intravaginally with 6.67×10^4 PFU of HSV-2 strain 333 (*H–L*) (for *A–G*, $n = 5–6$; for *H* and *I*, $n = 10$, for *J–L*, $n = 5$). After i.p. infection, organs were harvested for quantification of viral load in the liver on day 4 (*A–C*) or in the brain on day 6 (*D–G*). After genital infection, vaginal washes, spinal cords, and brain stems were harvested at the indicated days postchallenge. Data are shown as means ± SEM. *p*-values <0.05 were considered significant and are shown in the figure. *H* and *I*, Vaginally infected animals were observed for 20 days for disease score and survival. *K* and *L*, Dashed line represents the limit of detection in the virus plaque assay.

TLR9. TNF-α mRNA was induced in infected WT mice at day 9 p.i. and to a lesser extent in TLR2$^{-/-}$ mice (Fig. 6*B*), whereas this induction was totally abrogated in brains of TLR9$^{-/-}$ and TLR2/9$^{-/-}$ mice. Thus, expression of inflammatory cytokine and

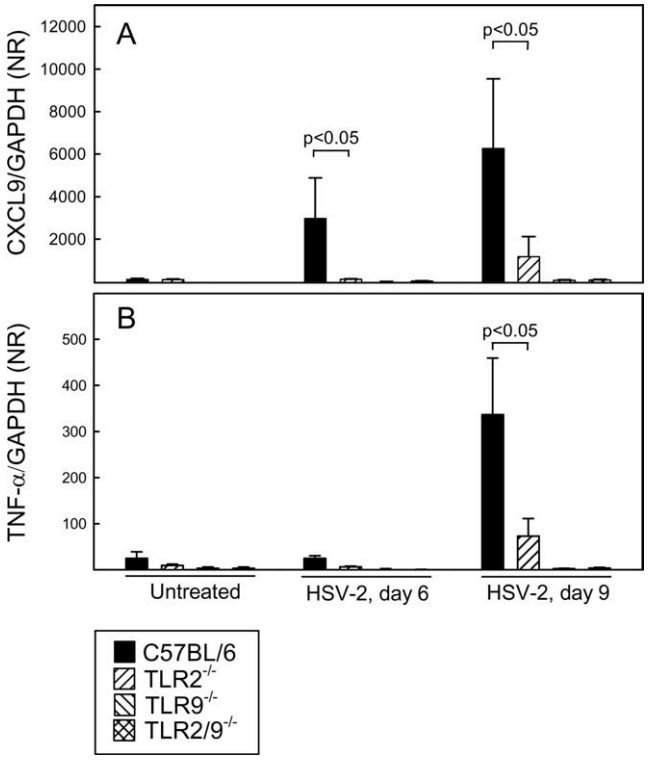

FIGURE 6. Cytokine and chemokine mRNA expression in brains during HSV-2 encephalitis. Mice were infected intravaginally with 6.67×10^4 PFU of HSV-2 strain 333 and brains were harvested at the indicated days p.i. Brains were homogenized and total RNA was extracted as described. Expression of CXCL9 and TNF-α mRNA were normalized to GAPDH mRNA. *p*-values <0.05 were considered significant and are shown in the figure.

chemokines in the brain during HSV-2 infection is dependent on both TLR2 and TLR9.

Discussion

Several studies have shown that TLR2 and TLR9 are activated by HSV components: TLR2 by an unidentified virion molecule, and TLR9 by unmethylated CpG dinucleotides (17–19, 40). The role of pDCs in innate recognition of HSV has been particularly well studied, showing that this cell type is responsible for HSV-induced IFN-α production in bone marrow-derived cells and that pDCs are dependent entirely on TLR9 for this activity (19, 20). We herein show that cell types other than pDCs produce type I IFN after an HSV-2 infection, and that this occurs primarily through TLR9-independent mechanisms. Furthermore, expression of proinflammatory cytokines and chemokines was strongly induced in all cell types tested, with a clear cell type-dependent requirement for TLR2 and TLR9 as well as other recognition systems (15). However, we did not observe a pronounced synergy between TLR2 and TLR9 in induction of specific cytokines. For our in vitro work with cDCs we used BM-DCs differentiated with GM-CSF as well as primary splenic CD11$^+$PDCA$^-$ cells. Note that previous work by others has shown that different primary cDC populations display differential requirement for TLR9 during HSV infection (13, 19, 28), and we can therefore not conclude that our findings with cDCs are general phenomena for all cDC populations. Additionally, it is interesting that the previously reported role for TLR2 and TLR9 in recognition of HSV-1 by BM-DCs was not observed in our system with HSV-2 (13, 28). Our data, together with other studies, thus demonstrate that the PRR requirement to evoke a cytokine response after HSV infection is highly dependent on the cell type,

HSV type and strain, mouse strain, and the organ from which the cells are derived (13, 19, 20, 28). Finally, the presented data on cytokine expression in vitro confirm that only pDCs rely entirely on TLR9 to trigger production of type IFN and inflammatory cytokines.

Studies on the role of TLR2 and TLR9 in HSV infection in vivo have so far focused on either TLR2 or TLR9 and not addressed the potential cooperative action of these two PRRs. Studies on TLR2 KO mice have shown unaltered or even improved resistance to HSV replication and survival comparable to WT mice. Additionally, the TLR2$^{-/-}$ mice responded to the infection with an impaired inflammatory response (17, 21, 23). TLR9 has been examined in the vaginal infection model, with several studies showing a protective effect of locally administered TLR9 ligand CpG before HSV infection (33, 43–47). For TLR9$^{-/-}$ mice one study found these mice to be more susceptible to HSV-2 (27), yet another found no differences (22). Again, these diverse observations could be due to different HSV and mouse strains, as this has been reported (48). However, for both single KO mice strains, most studies published thus far show no strong phenotype with respect to innate defense against HSV infection, and so far there have been no studies on the combined role of TLR2 and TLR9 in HSV infections in vivo (17, 20–22). We found higher viral load in the brains of TLR2/TLR9 DKO mice, coinciding with exacerbated symptoms of encephalitis and decreased survival. Additonally, we examined the NK cell response and found decreased accumulation in the spleen after infection. Finally, we measured cytokine and chemokine levels in serum to evaluate the response of the whole organism and found lowered levels in DKO serum for all cytokines measured. Some cytokines are dependent on only one TLR (e.g., IFN-α/β on TLR9), but for a complete response in vivo, both TLR2 and TLR9 are necessary. However, cytokine induction could still be observed in DKO mice, indicating that yet other PRRs than TLR2 and TLR9 are important factors in a systemic HSV-2 infection. TLR3 represents an alternative candidate, since it has recently been suggested that TLR3 is important for resistance against HSV encephalitis in human (49). Such a multiple TLR dependency has also been shown for other pathogens (50).

As the increased viral load was detected only in brains, we examined the local cytokine mRNA expression and found induction of TNF-α and CXCL9 in brains from WT mice. Many cell types in the brain express TLR2 and TLR9 in constitutive and inducible manners, including microglia cells (51). Lokensgard and colleagues have demonstrated that microglia cells produce a range of cytokines after HSV infection, and that TLR2 plays an important role in this response (18). In the present study, we observed reduced induction of TNF-α and CXCL9 in the brains of TLR2$^{-/-}$ mice and no detectable induction in TLR9$^{-/-}$ and TLR2/9$^{-/-}$ mice. Thus, HSV-induced expression of inflammatory cytokine and chemokines in the brain is totally dependent on TLR9, which in the case of the ISG CXCL9 could be due to decreased IFN-α/β, as HSV-induced type I IFNs production is dependent on TLR9 in many cell types (52). The reduced levels of ISGs such as CXCL9 and inflammatory cytokines such as TNF-α are likely to contribute to the observed phenotype in the TLR2/TLR9$^{-/-}$ mice. Mice lacking the type I IFN receptor display higher viral load in the brain (data not shown) and develop CNS symptoms much more rapidly than do WT mice after genital HSV-2 infection (44). Additionally, it has recently been reported that TNF-$\alpha^{-/-}$ and also IL-1$\beta^{-/-}$ mice exhibit impaired protection against HSV encephalitis after intranasal infection (53). The KO mice had elevated viral load in the brain with dissimulated replication in specific regions of the brain stem, pons, and medulla. Thus, it seems likely that the reduced expression of ISGs and inflammatory genes facilitates viral replication and spread within the brain, thus causing damage in the CNS.

MyD88 is a common adaptor molecule for most TLRs, except TLR3 (54). A model for intranasal HSV-1 infection showed that MyD88$^{-/-}$ mice develop lethal encephalitis, whereas WT and TLR2$^{-/-}$ mice are resistant (21). In the vaginal infection model MyD88$^{-/-}$ mice also showed less resistance than did WT mice (55). Our data strongly suggest that TLR2 and TLR9 are responsible for activation of the MyD88-dependent anti-HSV activity during an in vivo HSV-2 infection. This requirement for both TLR2 and TLR9 could be due to a need for sequential recognition of HSV by DCs as suggested by Sato and colleagues, since an important role for DCs in vivo has been demonstrated (13, 27, 56). Alternatively, and more in line with our data, TLR2 and TLR9 act through a more broad spectrum of cell types to affect multiple innate defense mechanisms that together mount an early host defense against HSV-2.

Our results also indicate that the role of individual TLRs varies in different organs, as viral load was only elevated in brain and not liver or vagina of TLR2/TLR9$^{-/-}$ mice. A parallel observation was done in a study of murine cytomegalovirus, where the early immune response was dependent on TLR9 only in spleen and not in liver (48). The reason for this could be that TLRs are not evenly distributed between or within different organs (57–59) and, furthermore, that the TLR dependency for a certain cell type can differ between organs (28). In this study the increased viral load in the brain of TLR2/TLR9$^{-/-}$ mice could be due to an insufficient systemic response before virus enters the CNS or an impaired control of viral replication in the brain. Our findings that WT and TLR2/TLR9$^{-/-}$ mice display comparable viral load in the vagina and spinal cord but not in the brainstem after vaginal infection strongly support the conclusion that TLR2 and TLR9 control HSV replication within the CNS. In agreement with this conclusion, Sarangi and colleagues have shown that virus is found in the brain of WT, TLR2$^{-/-}$, TLR9$^{-/-}$, and MyD88$^{-/-}$ mice on day 4 p.i. after a corneal HSV-1 infection, but only in MyD88$^{-/-}$ mice on day 7 (23). This indicates that although the amount of virus that reaches the CNS is comparable with WT mice, the combined lack of TLR2 and TLR9 leads to an uncontrolled local replication and subsequent lethal encephalitis.

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Disclosures

The authors have no financial conflicts of interest.

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