The Innate Immune Adaptor Molecule MyD88 Restricts West Nile Virus Replication and Spread in Neurons of the Central Nervous System[▽]

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Type I interferons (IFN- α/β) control viral infection by triggering the expression of genes that restrict transcription, translation, replication, and assembly. Many viruses induce IFN responses after recognition by cytoplasmic or endosomal RNA sensors (RIG-I-like RNA helicases [RLR] and Toll-like receptors [TLR]), which signal through the cognate adaptor signaling molecules IPS-1, TRIF, and MyD88. Recent studies have demonstrated that IPS-1-dependent induction of IFN- α/β downstream of RLR recognition restricts West Nile virus (WNV) infection in many cell types, whereas TRIF-dependent TLR3 signaling limits WNV replication in neurons. Here, we examined the contribution of MyD88 signaling to the control of WNV by evaluating IFN induction and virus replication in genetically deficient cells and mice. *MyD88*^{-/-} mice showed increased lethality after WNV infection and elevated viral burden primarily in the brain, even though little effect on the systemic type I IFN response was observed. Intracranial inoculation studies corroborated these findings, as WNV spread more rapidly in the central nervous system of *MyD88*^{-/-} mice, and this phenotype preceded the recruitment of inflammatory leukocytes. *In vitro*, increased WNV replication was observed in *MyD88*^{-/-} macrophages and subsets of neurons but not in myeloid dendritic cells. MyD88 had an independent effect on recruitment of monocyte-derived macrophages and T cells into the brain that was associated with blunted induction of the chemokines that attract leukocytes. Our experiments suggest that MyD88 restricts WNV by inhibiting replication in subsets of cells and modulating expression of chemokines that regulate immune cell migration into the central nervous system.

West Nile virus (WNV) is a neurotropic enveloped, positive polarity RNA virus of the *Flaviviridae* family and is related to other globally important pathogens, including Dengue, yellow fever, Japanese encephalitis, and tick-borne encephalitis viruses (40). WNV is maintained in an enzootic cycle between mosquitoes and birds and has emerged as an important cause of epidemic encephalitis in humans. Since its entry into the United States in 1999, approximately 29,000 cases of symptomatic WNV infection have been confirmed, and seroprevalence studies suggest that several million people have been infected (9).

Innate immune responses are required for the control of WNV infection (reviewed in reference 47). Alpha and beta interferon (IFN- α and - β) gene induction and signaling are essential components of innate immune programs that control virus infection and are required for the host control of WNV infection. Indeed, IFN- α/β receptor-deficient (*IFN- α BR*^{-/-}) mice rapidly succumb to WNV infection, with expanded tissue tropism, rapid dissemination to the central nervous system (CNS), and uniform death (48). Studies in genetically deficient

cells or mice suggest that IFN- α/β production after WNV infection is triggered after recognition of viral pathogen-associated molecular patterns (PAMP) by the cytoplasmic helicases RIG-I and MDA5 (14, 20). An absence of these molecules, their downstream signaling molecules (e.g., IPS-1), or transcriptional activators (e.g., IRF-3 and IRF-7) results in enhanced WNV infection in many cell and tissue types (7, 11, 13, 14, 20, 21). Recent *in vivo* studies have established that IPS-1 is required for effective development of innate and adaptive immunity against WNV (14, 58). Infection of *IPS-1*^{-/-} dendritic cells, macrophages, and neurons in culture resulted in marked defects in type I IFN responses. However, *IPS-1*^{-/-} mice infected with WNV developed uncontrolled inflammation with elevated levels of proinflammatory cytokines, enhanced humoral responses marked by loss of virus neutralization activity, increased numbers of virus-specific CD8⁺ T cells, and nonspecific immune cell proliferation in the periphery and the CNS. This uncontrolled inflammatory response was associated with a lack of the regulatory T-cell expansion (58) that normally occurs during acute WNV infection (34). Thus, IPS-1 signaling downstream of RIG-I-like RNA helicases (RLRs) regulates the quantity, quality, and balance of the immune response to WNV infection.

The role of Toll-like receptors (TLR) and their downstream signaling molecules TRIF and MyD88 in restricting WNV infection and modulating immune responses remains less well understood. TLR3 and TLR7/8 are endosomal sensors that

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recognize viral PAMPs, such as double-stranded RNA (dsRNA) and single-stranded viral RNA containing uridine-rich motifs, respectively (reviewed in reference 65). TLR3 recruits the adaptor molecule TRIF to induce type I IFN genes via interactions with TRAF3, TRAF6, TBK1, and IKK ϵ , whereas TLR7/8 associate with the adaptor protein MyD88, which forms a complex with TRAF3, TRAF6, IRAK1, and IRAK4 to activate kinases that regulate IRF-3, IRF-5, and/or IRF-7. Despite studies showing that ligation of TLR3 by dsRNA *in vitro* regulates IFN responses (63), its role *in vivo* in inducing IFN and protecting against WNV infection remains less clear. Two studies using the same *TLR3*^{-/-} mice described varying phenotypes: one reported a detrimental role of TLR3, as deficient mice had improved survival rates after WNV infection, likely due to a decreased pathological inflammatory cytokine response (tumor necrosis factor alpha [TNF- α] and/or interleukin-6 [IL-6]) that diminished blood-brain barrier permeability and viral entry into the brain (64). A second study showed a protective role with decreased survival of *TLR3*^{-/-} mice after WNV infection, mildly elevated viral titers in peripheral tissues, and early viral entry in the CNS (12). *Ex vivo* studies showed a dispensable role of TLR3 in regulating the IFN response and controlling WNV replication in fibroblasts, dendritic cells, and macrophages. Instead, TLR3 appeared to have a more significant function in restricting WNV replication in neurons (12). The exact contribution of TLR3 for WNV protection remains controversial but likely involves both cell-intrinsic and -extrinsic effects.

TLR7 and TLR8 were initially identified as triggers of the IFN- α response after exposure to single-stranded viral RNA (16, 28, 62). TLR7 has also been defined as the primary sensor responsible for IFN production by plasmacytoid dendritic cells (pDC) through a MyD88-dependent IRF-7 pathway (4). A protective role of TLR7 and MyD88 in WNV infection *in vivo* was suggested, as deficient mice were more vulnerable to infection (61). These mice showed a defect of leukocyte migration to WNV-infected tissues that correlated with decreased levels of IL-23. Interestingly, systemic levels of proinflammatory cytokines (IL-6, TNF- α , and IL-12) and type I IFN were paradoxically higher in *TLR7*^{-/-} mice than wild-type animals. This result suggested that abrogation of the TLR7 pathway, which should eliminate IFN- α production in pDC, had little systemic impact on IFN production after WNV infection. In contrast, studies with *IRF-7*^{-/-} mice with WNV infection showed blunted levels of systemic IFN, enhanced viral burden and dissemination, and markedly diminished IFN- α responses in several cell types (13).

Because of this disparity, we evaluated the pathogenesis and host immune response against virulent WNV infection in *MyD88*^{-/-} mice and compared this response to our prior studies with *TLR3*^{-/-} and *IPS-1*^{-/-} mice to gain a comprehensive portrait of how different pathogen recognition receptors and their signaling pathways modulate WNV infectivity. *MyD88*^{-/-} mice were more susceptible to lethal infection and demonstrated modestly enhanced viral replication in peripheral tissues, even though the absence of *MyD88*^{-/-} did not alter substantially the induction of a systemic IFN- α / β response. The increased mortality in *MyD88*^{-/-} mice after WNV infection was associated with elevated viral infection in the brain and diminished trafficking of monocyte-derived macrophages

and T cells, likely because of regional defects in chemokine production. Combined with our *in vitro* infection studies, these results suggest that MyD88 has both cell-intrinsic and cell-extrinsic protective roles against WNV in the CNS.

MATERIALS AND METHODS

Viruses. The WNV strain (3000.0259) was isolated in New York in 2000 (17) and passaged once in C6/36 *Aedes albopictus* cells to generate an insect cell-derived stock. The stock titer was determined by viral plaque on BHK21 cells as previously described (15).

Mouse experiments and quantitation of viral burden. C57BL/6 wild-type inbred mice were commercially obtained (Jackson Laboratories, Bar Harbor, ME). The congenic, backcrossed, and rederived *MyD88*^{-/-} mice (1) were obtained from R. Schreiber (Washington University School of Medicine, St. Louis, MO). All mice were bred in the animal facilities of the Washington University School of Medicine, and experiments were performed in accordance with and approval of the Washington University Animal Studies guidelines. Matched 8- to 10-week-old mice were used for all *in vivo* studies unless otherwise indicated. WNV (10² PFU) was diluted in Hanks balanced salt solution (HBSS) supplemented with 1% heat-inactivated fetal bovine serum (FBS) and inoculated by footpad injection in a volume of 50 μ l, respectively. Intracranial (i.c.) inoculation was performed by injecting 10¹ PFU of WNV diluted in 10 μ l of HBSS with 1% heat-inactivated FBS.

Quantification of tissue viral burden and viremia. To monitor viral spread *in vivo*, mice were infected with WNV by footpad or intracranial inoculation and sacrificed at specific time points. After extensive cardiac perfusion with phosphate-buffered saline (PBS), organs were harvested, weighed, and homogenized, and virus was titrated by standard plaque assay as described previously (15). Levels of WNV infection in serum and lymph nodes were measured by analyzing levels of positive-strand viral RNA levels using fluorogenic quantitative reverse transcription-PCR (qRT-PCR) as described previously (49) using the following primers and probe specific for the E gene of WNV: forward primer, 5'-TCAGCGATCTCTCCACCAAG-3'; reverse primer 5'-GGGTCAGCAGCTTTGTCATTG-3'; probe, 5'-6-carboxyfluorescein-TGCCCGACCATGGGAGAAGCTC-6-carboxytetramethylrhodamine-3' (33).

Quantification of IFN activity. IFN activity was quantified via two methods: (i) L929 bioassay and (ii) detection of IFN- α and IFN- β mRNA by qRT-PCR. For the L929 bioassay, relative levels of biologically active type I IFN in serum were determined using an encephalomyocarditis virus (EMCV) L929 cytopathic effect bioassay (11). The concentrations of type I IFN were expressed as international units of IFN per ml (IU/ml) and calculated by using a standard curve of recombinant IFN- α (PBL Biomedical Laboratories, NJ) run in parallel in the bioassay. For the IFN- α and - β mRNA determination by qRT-PCR, RNA was isolated from primary cells using the RNeasy kit (Qiagen). IFN- α and - β mRNA were measured by qRT-PCR using previously published primer sets (14). To analyze the relative level of induction of IFN- α and - β mRNA, 18S rRNA expression levels were also determined for normalization by using the $\Delta\Delta CT$ method (38).

WNV-specific antibody and CD8⁺ T-cell responses. The levels of WNV-specific IgM and IgG were determined using an enzyme-linked immunosorbent assay (ELISA) against purified WNV E protein (41). Intracellular IFN- γ and TNF- α staining was performed on splenocytes from day 8 postinfection animals in a *D^b*-restricted NS4B peptide restimulation assay as previously described (45). Samples were processed by multicolor flow cytometry on a FACSCalibur flow cytometer (Becton Dickinson) using FlowJo (Tree Star) software.

Leukocyte isolation from the CNS. Quantification of infiltrating CNS lymphocytes was performed as previously described (59). Briefly, brains from wild-type or *MyD88*^{-/-} mice were harvested on day 9 after infection, dispersed into a single-cell suspension with a cell strainer, and digested with 0.05% collagenase D, 0.1 μ g/ml trypsin inhibitor *N* α -p-tosyl-L-lysine chloromethyl ketone, 10 μ g/ml DNase I, and 10 mM HEPES (Life Technologies) in HBSS for 1 h. Cells were separated by using discontinuous Percoll gradient (70/37/30%) centrifugation for 30 min (850 $\times$ g at 4°C). Cells were then counted and stained for CD3, CD4, CD8, CD45, B220, CD19, NK1.1, and CD11b with directly conjugated antibodies (BD Pharmingen) for 30 min at 4°C and then fixed with 1% paraformaldehyde. In some experiments, WNV-specific CD8⁺ T cells were identified using a *D^b*-restricted NS4B peptide restimulation assay and intracellular IFN- γ and TNF- α staining. Data collection and analysis were performed with a Becton Dickinson LSR flow cytometer using FlowJo software.

Real-time quantitative RT-PCR for chemokines and IFN- γ . Total RNA was prepared from the brains of WNV-infected wild-type and *MyD88*^{-/-} mice using the RNeasy kit (Qiagen) according to the manufacturer's instructions. Following

DNase I treatment (Invitrogen), total RNA was quantitated by using Ribogreen (Molecular Probes). cDNA (50 μ l) was synthesized using oligo(dT)₁₅, random hexamers, and Multiscribe reverse transcriptase (Applied Biosystems). All samples were reverse transcribed from a single master mix to minimize differences in reverse transcription efficiency. Reverse transcription was carried out under the following conditions: 25°C for 10 min, 48°C for 30 min, and 95°C for 5 min. All oligonucleotide primers used for quantitative PCR were designed with Primer Express v2.0 (Applied Biosystems) and have been previously reported (31, 66).

Each 25- μ l PCR mixture contained 2 μ l cDNA, 12.5 μ l of 2 $\times$ SYBR green PCR master mix (Applied Biosystems), and 12.5 pmol of each primer. Quantitative PCR was performed in 96-well optical reaction plates (Applied Biosystems) on the ABI 7500 real-time PCR system under the following conditions: 50°C for 2 min, 95°C for 10 min, and then 40 cycles of 95°C for 15 s and 60°C for 1 min. Emitted fluorescence for each reaction was measured at the annealing/extension phase. Calculated copies were normalized against copies of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase.

Primary cell culture and infection. For primary cell infection experiments with (i) macrophages and dendritic cells, (ii) cortical neurons, and (iii) cerebellar granular neurons, a low multiplicity of infection (MOI) was used so that multistep growth kinetics could be evaluated. This was by design, as MyD88 could have an antiviral effect through its ability to induce IFN- α/β , which acts most efficiently in a cell-extrinsic manner. (i) Bone marrow-derived macrophages (M ϕ) and myeloid dendritic cells (mDC) were generated as described previously (11). Briefly, cells were isolated from the marrow of the femur of wild-type and *MyD88*^{-/-} mice and cultured for 7 days either in the presence of 40 ng/ml macrophage colony-stimulating factor (M-CSF; PeproTech) to generate M ϕ or 20 ng/ml granulocyte-macrophage colony-stimulating factor (GM-CSF) and 20 ng/ml IL-4 (PeproTech) to generate mDC. Multistep virus growth curves were performed after infection at an MOI of 0.01. Supernatants were titrated by plaque assay on BHK21 cells. (ii) Primary cortical neurons were prepared from wild-type and *MyD88*^{-/-} embryos (15 days old) as described previously (49). Cortical neurons were seeded in 24-well poly-D-lysine-laminin-coated plates in Dulbecco's modified Eagle's medium containing 5% heat-inactivated FBS and 5% horse serum for 24 h and then cultured for 4 days with Neurobasal medium containing B27 and L-glutamine (Invitrogen). Multistep virus growth curves were determined after infection at an MOI of 0.001. (iii) Primary cultures of purified granule cell neurons from neonatal wild-type and *MyD88*^{-/-} mice were prepared as described previously (32). Purification of neurons by Percoll step gradient centrifugation yields cultures containing 97% granule cells and 3% Purkinje neurons. Multistep virus growth curves were performed after infection at an MOI of 0.001.

Immunohistochemistry and confocal microscopy. Mice were infected with 10² PFU of WNV and sacrificed at day 9 postinfection. Following perfusion with 20 ml PBS and 20 ml 4% paraformaldehyde (PFA), brains were harvested and fixed in 4% PFA overnight at 4°C. Tissues were cryoprotected in 30% sucrose, and frozen sections were cut. Tissue staining was performed as previously described (31, 59). Briefly, frozen brain sections were hydrated in PBS containing 10% normal goat serum and permeabilized with 0.1% Triton X-100. Staining was performed by incubating sections overnight at 4°C with the following primary antibodies: CD11b, CD3, and CD31 (BD Pharmingen); CXCL10 (PeproTech); NeuN (Abcam); MAP2 (Chemicon); anti-WNV antibody (from hyperimmune rat sera). Primary antibodies were detected with secondary Alexa 488- or Alexa 555-conjugated goat anti-mouse, -rat or -rabbit IgG (Molecular Probes). Nuclei were counterstained with To-Pro3 (Molecular Probes). Fluorescence staining was visualized with a Zeiss 510 Meta LSM confocal microscope.

Statistical analysis. For *in vitro* experiments, an unpaired two-tailed *t* test was used to determine statistically significant differences. For viral burden and immune cell analysis, differences were analyzed by the Mann-Whitney test. Kaplan-Meier survival curves were analyzed by the log rank test. All data were analyzed using Prism 4 software (GraphPad).

RESULTS

MyD88 is required for the control of lethal WNV infection. Mice lacking IRF-3 and/or IRF-7 were vulnerable to WNV infection due to enhanced viral replication, altered tissue tropism, and early CNS dissemination (13). Whereas a similar phenotype of enhanced susceptibility to WNV infection was apparent in *IPS-1*^{-/-} mice, the lack of RLR signaling induced immune system dysregulation with qualitative and quantitative defects in adaptive immune responses (58). Because of these

findings, we evaluated the role of MyD88 signaling in the regulation of WNV infection and immunity. MyD88 is the common adaptor molecule that links TLR7/8 signaling with IRF signaling but operates independently of IPS-1 and the RLR pathways. We challenged *MyD88*^{-/-} mice with 10² PFU of a highly pathogenic North American strain of WNV (strain 3000.0259; New York, 2000). After footpad inoculation, *MyD88*^{-/-} mice showed an increased rate and severity of clinical signs and symptoms of illness. Whereas 65% of wild-type mice survive infection with WNV, *MyD88*^{-/-} mice were more vulnerable, with only a 29% survival rate (*P* < 0.05) (Fig. 1A). This susceptibility pattern was similar to a 15% survival rate observed by another group after infection with 2 $\times$ 10³ PFU of WNV by the intraperitoneal route (61). In comparative historical studies, congenic *IRF-7*^{-/-} mice had a 100% mortality rate after subcutaneous infection with 10² PFU of WNV (13). Thus, although WNV infection causes a more severe phenotype in *MyD88*^{-/-} mice, it does not recapitulate that observed with *IRF-7*^{-/-} mice, suggesting that at least some of the pathogen recognition signal through IRF-7 occurs via a MyD88-independent pathway.

***MyD88*^{-/-} mice show enhanced WNV replication.** As MyD88 transmits an activating signal from endosomal TLR7/8 to IRF-7, we hypothesized that the increased lethality in *MyD88*^{-/-} mice should correlate with higher viral infectivity in tissues. To evaluate this, mice were infected with WNV and viral burden was examined at different days after infection in (i) serum and peripheral organs (draining lymph nodes, spleen, and kidney) and (ii) the brain.

(i) Blood, lymph node, spleen, kidney, liver, and lung. In contrast to that observed in *IPS-1*^{-/-} or *IRF-7*^{-/-} mice (13, 58), differences in viremia were not observed between wild-type and *MyD88*^{-/-} mice at days 1, 2, 4, and 6 after WNV infection (Fig. 1B) (*P* > 0.2). In comparison, a difference in viral replication was observed in the draining lymph node at day 3 (*P* < 0.02) with a trend toward significance at day 6 (*P* = 0.07) (Fig. 1C). In the spleen, a difference in WNV infection in *MyD88*^{-/-} mice also was seen. Infectious WNV was not detected in the spleen at day 2 after infection in either wild-type or *MyD88*^{-/-} mice. By day 4, 67% (6 of 9) of *MyD88*^{-/-} or wild-type mice had measurable infection levels that were not statistically different from one another (mean titer, 10⁴ PFU/g versus 10^{3.5} PFU/g; *P* > 0.4) (Fig. 1C). By day 6, however, we observed higher (25-fold) WNV titers in *MyD88*^{-/-} mice (mean titer, 10^{3.5} PFU/g versus 10^{2.1} PFU/g; *P* < 0.002). Infectious WNV was recovered from subsets of *MyD88*^{-/-} mice at days 8 and 10 after inoculation, whereas virus was not detected in the spleens of wild-type animals after day 6. In comparison, a previous study observed a small, 2-fold increase in WNV RNA at day 3 in the spleen in *MyD88*^{-/-} mice (61); no other time points were assessed in that study. Thus, in the spleen, a deficiency of MyD88 was associated with greater replication at the peak of infection and a delay in WNV clearance in a subset of animals.

The kidney, lung, and liver in wild-type C57BL/6 mice are resistant to WNV infection, as infectious virus is not usually detected in these organs. However, infection with WNV is observed in many of these tissues in *IFN- α/β R*^{-/-} (48), *IRF-3*^{-/-} (11), *IRF-7*^{-/-} (13), and *IPS-1*^{-/-} (58) mice as well as in hamsters (60) and monkeys (44). In *MyD88*^{-/-} mice, infectious

FIG. 1. Survival and viral burden analysis for wild-type and *MyD88*^{-/-} C57BL/6 mice. (A) Eight- to 12-week-old mice were inoculated with 10^2 PFU of WNV by footpad injection and followed for mortality over 30 days. Survival differences were statistically significant ($P < 0.05$). (B to F) Viral burden in peripheral and brain tissues after subcutaneous WNV infection. WNV RNA in serum (B) or lymph nodes (C) and infectious virus in the spleen (D), kidney (E), and brain (F) were determined from samples harvested on days 1, 2, 3, 4, 6, 8, and 10 using qRT-PCR (B and C) or viral plaque assay (D to F). Data are shown as viral RNA equivalents or PFU per gram of tissue for 8 to 10 mice per time point. For all viral data, the dotted line represents the limit of sensitivity of the assay. Asterisks indicate values that are statistically significant (*, $P < 0.05$; **, $P < 0.005$) compared to wild-type mice as judged by the Mann-Whitney test.

virus was recovered from the kidneys at several different days, although it failed to attain statistical significance compared to wild-type mice, due to the incomplete penetrance of the phenotype (Fig. 1D). In comparison, virus was not recovered from liver or lung at any of the time points in wild-type or *MyD88*^{-/-} mice (data not shown). Overall, our experiments showed that MyD88-dependent recognition pathways contribute to restricting WNV infection only in selective visceral and lymphoid tissues.

(ii) Brain. Consistent with a lack of effect on viremia, we observed no difference in the time of onset of WNV replication in the brain in *MyD88*^{-/-} mice. Infectious virus was first detected in the brain of wild-type and deficient mice at day 6 (Fig. 1E). However, once WNV entered the brain, it replicated more efficiently in *MyD88*^{-/-} mice. For example, at day 6, no difference in viral titers in the brain was observed ($10^{3.9}$ PFU/g for *MyD88*^{-/-} versus $10^{4.2}$ PFU/g for wild type; $P > 0.5$). However, by days 8 and 10, we observed 40 to 55-fold higher levels of WNV in the brains of all *MyD88*^{-/-} mice (day 8, $10^{5.5}$ PFU/g for *MyD88*^{-/-} versus $10^{3.9}$ PFU/g for wild type [$P = 0.009$]; day 10, $10^{6.1}$ PFU/g versus $10^{4.3}$ PFU/g [$P = 0.02$]). Thus, a deficiency of MyD88 did not affect WNV entry into the brain but rather controlled its spread once infection was established.

A deficiency of MyD88 does not blunt the IFN- α/β levels in circulation and cell culture after WNV infection. We hypothesized that the enhanced replication and spread of WNV in peripheral tissues in *MyD88*^{-/-} mice was due to blunted pro-

duction of type I IFN. We reasoned this because pDC in circulation produce IFN- α after signal transduction through a TLR7-MyD88-IRF-7 axis (4) and because systemic levels of type I IFN were decreased in *IRF-7*^{-/-} mice after WNV infection (13). To evaluate this, *MyD88*^{-/-} and wild-type mice were infected with WNV, and the levels of biologically active type I IFN in serum were monitored using a sensitive EMCV-L929 cell protection bioassay (5). Type I IFN activity in the serum of infected wild-type mice peaked at 72 h and then slightly decreased at 96 h. The specificity of the assay for measuring type I IFN activity was confirmed with a neutralizing monoclonal antibody (MAb) against the IFN- α/β receptor (data not shown). Notably, we observed no decrease in IFN- α/β levels in serum of *MyD88*^{-/-} mice relative to the wild-type mice; instead, as was observed previously in *IPS-1*^{-/-} mice after WNV infection (58), statistically significant higher levels of IFN were observed at several time points, possibly due to enhanced viral replication in the immunodeficient mice (Fig. 2A) ($P < 0.007$).

To begin to understand why decreases in IFN levels were not observed after WNV infection of *MyD88*^{-/-} mice, we assayed IFN- α/β production in primary M ϕ and mDC. Previous studies with *IRF-7*^{-/-} cells had established significant phenotypes with loss of induction of IFN- α (13). Notably, IFN- α and - β induction in mDC was virtually identical between wild-type and *MyD88*^{-/-} cells (Fig. 2B and C), consistent with no difference in viral replication (Fig. 2D) ($P > 0.1$). In contrast, IFN- α and - β levels were paradoxically higher in *MyD88*^{-/-} M ϕ at 48 h

FIG. 2. Relative IFN- α/β levels in serum and cells of wild-type and *MyD88*^{-/-} mice and cells after infection with WNV. (A) Mice were inoculated with 10² PFU of WNV by footpad injection and sacrificed at the indicated times. Type I IFN activity was determined from serum collected on days 1 to 4 after WNV infection in an EMCV bioassay in L929 cells. Data reflect the averages of serum samples harvested from eight mice per time point. (B to D) mDC generated from wild-type or *MyD88*^{-/-} mice were infected at an MOI of 0.01, and IFN- α (B), IFN- β (C), and virus production (D) were evaluated at the indicated times by quantitative RT-PCR and plaque assay. Values are averages of quadruplicate samples generated from at least three independent experiments. The dotted line represents the limit of sensitivity of the virologic assay. (E to G) Mφ generated from wild-type or *MyD88*^{-/-} mice were infected at an MOI of 0.01, and IFN- α (E), IFN- β (F), and virus production (G) were evaluated at the indicated times by quantitative RT-PCR and plaque assay. Values are averages of quadruplicate samples generated from at least three independent experiments. The dotted line represents the limit of sensitivity of the assay. For IFN RNA measurements, results were normalized to 18S rRNA and are expressed as the relative fold increase over RNA from uninfected controls. Asterisks (*, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$) indicate values that are statistically different.

after WNV infection (Fig. 2E and F) ($P < 0.03$); this was likely a consequence of the 5-fold higher viral replication at this time point (Fig. 2G) ($P < 0.001$). Thus, in contrast to that seen with IRF-7, signaling through MyD88 had a less impressive effect on regulating WNV replication or IFN production in specific myeloid cell subsets.

Effects of MyD88 on B- and T-cell responses after WNV infection. TLR stimulation and MyD88 signaling in some cases is required for optimal antigen-specific antibody responses (29, 42) and T-cell activation (10, 52). As a depressed antiviral antibody response can promote dissemination and replication of WNV in the brain (15), we evaluated whether a deficiency of MyD88 modulated humoral immune responses. Notably, similar or higher levels of WNV-specific IgM and IgG were detected in *MyD88*^{-/-} mice at three time points after infection (Fig. 3A), and no defect in the development of neutralizing antibodies was observed (data not shown). Thus, the virologic phenotype observed in *MyD88*^{-/-} mice was not due to a defect in B-cell function.

As CD8⁺ T cells are also required for the control and clearance of WNV in the CNS (53), we evaluated whether a deficiency in MyD88 affected priming of antigen-specific CD8⁺ T cells. Splenocytes from WNV-infected wild-type or *MyD88*^{-/-} mice were harvested at day 8 after infection and restimulated with a D^b-restricted immunodominant NS4B peptide (8, 45). Activation was measured by intracellular staining of IFN- γ and TNF- α in CD8⁺ T cells by using flow cytometry. Restimulation with a WNV-specific peptide resulted in a similar percentage and number of splenic CD8⁺ T cells expressing IFN- γ or

TNF- α in wild-type and *MyD88*^{-/-} mice (Fig. 3B and C) ($P > 0.8$). Thus, the absence of MyD88 did not significantly affect WNV-specific CD8⁺ T-cell activation, and the higher viral burden in the brain of *MyD88*^{-/-} mice was not a consequence of inadequate priming of adaptive immune responses in peripheral tissues.

***MyD88*^{-/-} mice show a defect in accumulation of macrophages and T cells in the CNS.** A previous study with *TLR7*^{-/-} mice suggested that uncontrolled replication in the CNS after WNV infection was due to poor migration of CD45⁺ leukocytes to the brain because of depressed systemic production of IL-12 and IL-23 (61). Inefficient migration of leukocytes in the CNS results in enhanced vulnerability to lethal WNV infection as observed with chemokine-deficient and chemokine receptor-deficient mice (26, 31, 66). To determine whether this mechanism in part explained the WNV phenotype in *MyD88*^{-/-} mice, we evaluated leukocyte trafficking and accumulation in the brains of these mice. Leukocytes were recovered from the brains of wild-type and *MyD88*^{-/-} mice at day 9 after extensive perfusion and analyzed by flow cytometry. Based on the classification of CNS myeloid cells of Ford et al. (18), equivalent percentages and numbers of CD45^{low}/CD11b^{high} activated microglia were observed in the brains of WNV-infected wild-type and *MyD88*^{-/-} mice (Fig. 4A and B). In comparison, lower percentages and total numbers of CD45^{high}/CD11b^{high} monocyte-derived macrophages were detected in the brains of *MyD88*^{-/-} mice ($P < 0.02$), despite the higher viral burden. As observed previously in WNV-infected mice (54), few if any of the parenchymal brain leukocytes

FIG. 3. Peripheral humoral and CD8⁺ T-cell responses after WNV infection in *MyD88*^{-/-} mice are intact. (A) Wild-type and *MyD88*^{-/-} mice were inoculated with 10² PFU of WNV by footpad injection, and serum samples collected on days 6, 8, and 10 were assayed by ELISA for WNV E-specific IgM and IgG. Titers are expressed as the reciprocal serum dilution that was 3 standard deviations above background. IgM levels in *MyD88*^{-/-} mice were higher at days 8 and 10 ($P < 0.006$). All other IgM and IgG titers were not statistically significantly different from wild-type mice ($P > 0.1$). (B and C) Wild-type and *MyD88*^{-/-} mice were inoculated with 10² PFU of WNV by footpad injection, and spleens were harvested on day 8. Leukocytes were stimulated *ex vivo* with D^b-restricted NS4B peptide, stained for CD3 and CD8 and intracellular IFN-γ or TNF-α, and analyzed by flow cytometry. The primary data are shown as contour plots of gated CD3⁺ CD8⁺ cells (B), and a summary is shown of the total number of CD3⁺ CD8⁺ T cells (left) and the percentage CD3⁺ CD8⁺ T cells positive for intracellular IFN-γ or TNF-α after peptide restimulation (C). Differences were not statistically significant ($P > 0.8$), and data represent the averages of three independent experiments, each with three to five mice.

resembled neutrophils in morphology (data not shown). We also observed a decrease in the percentage and number of CD45⁺ CD11b⁻ leukocytes in *MyD88*^{-/-} mice (Fig. 4B and C and data not shown). Multicolor flow cytometric analysis revealed that *MyD88*^{-/-} mice had fewer CD4⁺ and CD8⁺ T cells in the brain at this time point (Fig. 4C) ($P < 0.05$). Although

there was a trend toward lower numbers of IFN-γ⁺ or TNF-α⁺ WNV-specific CD8⁺ T cells in the brains of WNV-infected *MyD88*^{-/-} mice, this did not attain statistical significance (Fig. 4D and E) ($P > 0.1$). Thus, although a deficiency of MyD88 reduced recruitment of macrophages and total numbers of CD3⁺ T cells, it did not significantly affect accumulation of antigen-specific CD8⁺ T cells or microglia. Given a hypothesized role for macrophage subsets in restricting WNV infection in the brain (55), these results likely explain, in part, the enhanced viral load in the CNS of *MyD88*^{-/-} mice.

WNV infection of the brain is associated with the early expression of the leukocyte chemoattractants CXCL10 and CCL5 within specific neuron and inflammatory cells of the brain (26, 31). Given the enhanced viral burden and decreased macrophage and total CD3⁺ T cell accumulation in the brains of WNV-infected *MyD88*^{-/-} mice, we hypothesized an absence of MyD88 signaling in neurons might cause defects in chemokine production that facilitate immune cell recruitment, retention, and control of infection. To assess this, we measured chemokine levels in the cerebral cortex of WNV-infected wild type and *MyD88*^{-/-} mice at a time point immediately prior to the influx of inflammatory cells (53). Notably, in *MyD88*^{-/-} mice we observed decreased mRNA levels of particular chemokines (CCL2, CCL5, CXCL9, and CXCL10) that regulate trafficking of CD11b⁺ and T cells into the brain (26, 31) (Fig. 5A to D) ($P < 0.04$). These results were corroborated at the protein level, as lower levels of CXCL10 were observed in the cerebral cortex based on confocal microscopic analysis of tissue sections (Fig. 5G); in contrast, the pattern of expression was more heterogeneous in the cerebellum, as CXCL10 was present in Purkinje neurons but absent in granular cell neurons in *MyD88*^{-/-} mice (data not shown). As chemokines are IFN-inducible genes, we also assessed the relative levels of IFN-β and IFN-γ in the cerebral cortex of WNV-infected wild-type and *MyD88*^{-/-} mice. Notably, lower levels of IFN-γ but not IFN-β were present in the tissue samples from *MyD88*^{-/-} mice (Fig. 5E and F). Thus, induction of chemokines that stimulate leukocyte recruitment to the CNS after WNV infection was blunted in *MyD88*^{-/-} mice in a regional manner, correlated with defects in IFN-γ expression, and associated with a failure of specific immune cell subsets to accumulate in the brain and control disease in a timely manner.

To confirm the effect of MyD88 on leukocyte accumulation patterns in the CNS, immunohistochemical analysis was performed. In the cerebral cortex, the number of CD11b⁺ macrophages (yellow arrows) but not microglia (red arrows) appeared diminished in the *MyD88*^{-/-} mice compared to wild-type mice (Fig. 6A, B, and E, left panel). One limitation of this analysis is that the morphologies of CD11b⁺ microglia and macrophages can vary during inflammation, making it difficult to absolutely distinguish these populations. Consistent with the flow cytometry data, a small yet statistically significant difference ($P = 0.01$) in the total number of CD3⁺ lymphocytes was apparent in the cerebral cortex (Fig. 6C, D, and E, right panel) in *MyD88*^{-/-} mice; this appeared to largely reflect the pool of CD3⁺ T cells that had migrated into the parenchyma.

MyD88 signaling directly controls WNV replication in the CNS. Although poor trafficking of leukocyte subsets to the brain could explain sustained WNV replication, because of

FIG. 4. Leukocyte accumulation in the CNS of *MyD88*^{-/-} mice after WNV infection. Wild-type and *MyD88*^{-/-} mice were inoculated with 10² PFU of WNV by footpad injection, brains were harvested on day 9, and leukocytes were isolated after Percoll gradient centrifugation. (A) Numbers of activated macrophages (CD11b^{high} CD45^{high}) and microglia (CD11b^{high} CD45^{low}) were evaluated. (B) Representative flow cytometry profiles are shown of CD11b and CD45 staining of brain leukocytes from wild-type and *MyD88*^{-/-} mice. (C) Total number of specific subsets of CD45⁺ CD11b⁻ leukocytes in the brain of WNV-infected wild-type and *MyD88*^{-/-} mice. Cells were stained with MABs against CD3, CD4, and CD8 and analyzed after gating on CD45⁺ CD11b⁻ cells. (D) Representative flow cytometry profiles of CD8 (after CD3⁺ gating) IFN-γ and TNF-α staining of brain leukocytes from wild-type and *MyD88*^{-/-} mice after restimulation *ex vivo* with an immunodominant D^b-restricted NS4B WNV peptide. (E) The total number of WNV-specific brain CD8⁺ T cells was determined after peptide restimulation. Cells were stained for CD3 and CD8 on their surface, permeabilized, stained intracellularly for IFN-γ and TNF-α, and analyzed by flow cytometry. For panels A and E, data are the averages of several experiments with a total of 8 wild-type and 10 *MyD88*^{-/-} mice. In panel E, the differences were not statistically different (*P* > 0.09), whereas asterisks in panels A and C indicate differences that were significant (*P* < 0.05) between cells of wild-type and *MyD88*^{-/-} mice.

our previous studies with *IRF-7*^{-/-} neurons in which enhanced WNV replication was observed in culture (13), we hypothesized that MyD88 signaling could independently contribute to higher WNV titers in the brain through direct inhibitory effects on neuronal infection. To test this, wild-type and *MyD88*^{-/-} mice were infected with 10¹ PFU of WNV directly into the cerebral cortex via an intracranial route, and viral burden in the cerebral cortex, white matter, brain stem, cerebellum, and spinal cord were measured on days 2, 4, and 6 after infection (Fig. 7A to E). Whereas no differences were observed at day 2 in any of the brain regions, by day 4, *MyD88*^{-/-} mice showed 9- to 17-fold in-

creases in viral titers in the cerebral cortex, white matter, and spinal cord (*P* ≤ 0.04); this time point is significant, as it occurs in the absence of inflammatory leukocytes in the brain in this model (Fig. 7F). By day 6, 6- to 72-fold-higher (*P* ≤ 0.03) levels of infectious WNV were detected in the cerebral cortex, white matter, brain stem, cerebellum, and spinal cord of *MyD88*^{-/-} mice. Immunohistochemical analysis at days 4 and 6 corroborated these findings and revealed increased numbers of WNV-infected neurons in different regions of the brains of *MyD88*^{-/-} mice (Fig. 7H to W). Consistent with our studies in peripheral tissues, the increase in viral replication in the brains of *MyD88*^{-/-} mice

FIG. 5. Chemokine and cytokine levels in the brains of WNV-infected wild-type and *MyD88*^{-/-} mice. C57BL/6 mice were inoculated with 10² PFU of WNV by footpad injection and sacrificed on day 6 after infection. Brains were recovered after cardiac perfusion with PBS. (A to F) CCL5 (A), CXCL9 (B), CXCL10 (C), CCL2 (D), IFN- γ (E), and IFN- β (F) mRNA levels in cerebral cortex samples analyzed via quantitative RT-PCR. Data were normalized to levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (A to E) or 18s rRNA (F). Data are averages of results for at least six mice and reflect two independent experiments. Statistical significance of decreased chemokine expression in WNV-infected *MyD88*^{-/-} mice (**, $P < 0.05$) was determined in comparison with infected wild-type mice. (G) Analysis of CXCL10 expression in wild-type and *MyD88*^{-/-} mice based on confocal microscopy. Representative microscopic images of NeuN (green), CXCL10 (red), and ToPro-3 nuclear staining (blue) of the cerebral cortex of wild-type (left) and *MyD88*^{-/-} (right) mice are shown. The data are representative of tissue sections from three independent mice.

was not associated with decreased levels of IFN- α/β mRNA (Fig. 7G). These results suggest that MyD88-dependent signaling also induces a response that directly limits WNV infection in the brain and spinal cord independently of the effects on trafficking leukocytes.

MyD88 controls WNV replication subsets of primary neurons. To evaluate directly whether MyD88-dependent signals limit WNV replication in a key CNS target cell, we cultured

wild-type and *MyD88*^{-/-} cortical and cerebellar granule neurons (98 to 99% purity) derived from embryonic or neonatal mice and performed multistep growth curve analysis. Although no difference in WNV infection was observed between wild-type and *MyD88*^{-/-} primary cortical neurons (Fig. 8A) ($P > 0.2$), we did observe a small yet statistically significant 3- to 4-fold increase in WNV infection in the *MyD88*^{-/-} cerebellar granule neurons at 24, 48, and 72 h (Fig. 8B) ($P < 0.04$). The

FIG. 6. MyD88 regulates leukocyte accumulation in the cerebral cortex. Wild-type and *MyD88*^{-/-} mice were infected with 10² PFU of WNV by the subcutaneous route, and 8 days later brains were harvested for immunohistochemical analysis. (A and B) Representative confocal microscopic images of CD11b (green) and ToPro-3 nuclear staining (blue) and showing microglia (red arrows) and macrophages (yellow arrows) in the cerebral cortex of wild-type (A) and *MyD88*^{-/-} (B) mice. (C and D) Confocal microscopic images of CD3⁺ lymphocytes (red), CD31⁺ endothelial cells (green), and ToPro-3 nuclear staining (blue) from the cerebral cortex of wild-type (C) and *MyD88*^{-/-} (D) mice. The data are representative of results from at least three independent mice. Bar, ~20 μ m. (E) Quantification of confocal microscopic images for CD11b⁺ macrophages and microglia or CD3⁺ T cells (perivascular versus parenchymal) in the cerebral cortex of WNV-infected wild-type and *MyD88*^{-/-} mice. The results were quantified from 5 to 10 high-power fields per brain region per mouse for at least three mice per group. Asterisks (*, $P < 0.05$; **, $P < 0.005$) indicate values that are statistically different.

cell-type-specific effect was not that surprising, given the differences in antiviral effects of IFN that have been observed in different neuronal subtypes (48, 49). In both neuronal cell types, microarray analysis showed that MyD88 was basally expressed and induced by either IFN- β or WNV infection. Additionally, no difference in induced IFN- β mRNA expression was observed after WNV infection in *MyD88*^{-/-} cortical or cerebellar granule neurons compared to wild-type cells (data not shown). These experiments, combined with the intracranial inoculation studies, suggest that MyD88-dependent signaling directly restricts WNV infection in specific neuronal subsets.

DISCUSSION

Host control of viral infections within a cell requires an intrinsic response after recognition of nonself ligands, such as PAMPs. Recognition of viral RNA triggers a signaling cascade that induces type I IFN- α/β and a panoply of antiviral effector molecules that inhibit key steps in the virus life cycle. IFN- α/β also have cell nonautonomous functions, including priming of uninfected host cells to resist infection and modulating other features of innate and adaptive immunity. Here, we characterized the function of MyD88, a key adaptor molecule that transmits signals from endosomal TLRs, which recognize single-stranded viral RNA, against the encephalitic flavivirus WNV *in vivo* and in cell culture. We demonstrated that MyD88 is essential for control of WNV infection, as its absence resulted in increased lethal infection with higher viral burden and poorly controlled replication in the CNS. Interestingly, despite its documented importance in the induction of IFN responses after ligation of TLR7 in pDC, a deficiency of MyD88 was not associated with blunted IFN responses in the context of WNV *in vitro* or *in vivo*. Our results are most consistent with a model in which MyD88 prevents WNV encephalitis by both direct antiviral effects on replication in neuronal subsets and indirect immunomodulatory effects on leukocyte trafficking.

For WNV, recent studies have indicated a dominant role for RIG-I, MDA5, and IPS-1 in IFN- α/β induction and virus restriction through the transcription factors IRF-3 and IRF-7 (7, 11, 13, 14, 20). PKR also may serve as a pattern recognition receptor (PRR) for WNV and regulate IFN- α/β induction through NF- κ B, at least in some cell types, although the mechanism remains unclear (25). In contrast, TLR3, although it can act as a PRR by recognizing double-stranded viral RNA and induce IFN through IRF-3- and NF- κ B-dependent transcriptional activation (2), its role for WNV appears dispensable for IFN production both *in vitro* (19, 51) and *in vivo* (12). Our experiments showed that systemic IFN- α/β levels were not decreased in *MyD88*^{-/-} mice after WNV infection. This was surprising for two reasons. (i) MyD88 is the adaptor molecule downstream of TLR7, which is the major recognition molecule for induction of IFN in pDC in the circulation. Deletion of MyD88 in the context of lymphocytic choriomeningitis or vesicular stomatitis virus infection blunts systemic levels of IFN (30, 39). (ii) A deficiency of IRF-7, which is downstream of MyD88, reduced IFN- α/β levels in the circulation after WNV infection (13). Nonetheless, these results were consistent with our *in vitro* experiments, which showed no deficit in IFN- α or - β production in *MyD88*^{-/-} M ϕ or mDC after WNV infection, or *in vivo* studies with *TLR7*^{-/-} mice (61), which showed paradoxically elevated rather than decreased IFN- β levels in the blood after WNV infection. Collectively, these studies argue that a cooperative signal through multiple PRR (e.g., RIG-I, MDA5, PKR, TLR3, and TLR7), adaptors (e.g., IPS-1, MyD88, and TRIF), and transcription factors (e.g., IRF-3, IRF-5, IRF-7, and NF- κ B) in multiple cell types (e.g., pDC, mDC, and monocytes) accounts for the systemic accumulation of IFN- α/β . In support of this, whereas deletion of IPS-1 did not alter systemic production of IFN- α/β , the combined deficiency of IRF-3 and IRF-7 blunted and delayed the systemic IFN response after WNV infection, even in the setting of

FIG. 7. A deficiency of MyD88 facilitates spread of WNV in the brain after intracranial inoculation. Wild-type or *MyD88*^{-/-} mice were inoculated with 10¹ PFU of WNV by intracranial injection. Brains were harvested on days 2, 4, or 6. (A to E) Viral burden was determined by plaque assay from wild-type and *MyD88*^{-/-} mice, and the CNS tissues were separated into cerebral cortex (gray matter) (A) and white matter (B), brain stem (C), cerebellum (D), and spinal cord (E). Data are shown as PFU per gram of tissue for 8 to 10 mice per time point. The dotted line represents the limit of sensitivity of the assay. (F) Leukocyte accumulation in the CNS of *MyD88*^{-/-} mice after intracranial WNV infection. Wild-type and *MyD88*^{-/-} mice were inoculated with 10¹ PFU of WNV by intracranial injection, brains were harvested on day 4, and leukocytes were isolated. The numbers of activated macrophages (CD11b^{high} CD45^{high}) and microglia (CD11b^{high} CD45^{low}), resting microglia (CD11b^{low} CD45^{low}), and T cells (CD3⁺ CD8⁺) was determined. Results were compared to cell numbers from mock-infected mice (results indicated by dashed lines). Differences between wild-type and *MyD88*^{-/-} mice were not statistically significant ($P > 0.2$). No CD3⁺ CD8⁺ T cells were observed in any of the brains at day 4 after intracranial infection. (G) Levels of IFN-α and IFN-β mRNA in the cerebral cortex of wild-type and *MyD88*^{-/-} mice 6 days after WNV infection as determined by qRT-PCR. Data were not statistically different and reflect the averages of four to six mice per condition. (H to W) Fixed, frozen sections at day 4 (H, J, L, N, P, R, T, and V) or 6 (I, K, M, O, Q, S, U, and W) after intracranial infection from the cerebral cortex (H to K), hippocampus (L to O), cerebellum (P to S), and brain stem (T to W) of wild-type and *MyD88*^{-/-} mice were costained for WNV antigen (red), the neuronal marker MAP2 (green), and nuclei (blue). The data are representative of sections from four to six wild-type or *MyD88*^{-/-} mice at each time point. Asterisks (*, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$) indicate values that are statistically different.

higher viral burdens (14, 58). However, in other tissues (e.g., lymphoid) individual PRR may have dominant roles because of cell-type-specific basal or induced expression of key signal transduction intermediates (11). The fact that the triggering of different types of PRR (RLR or TLR) can result in common outcomes (IFN induction) is likely explained by the activation of shared downstream transcription factors (IRF family members, NF-κB, and ATF-2/c-Jun).

The results with *MyD88*^{-/-} mice, combined with data from prior studies with *TLR3*^{-/-} (12) and *IPS-1*^{-/-} (58) mice, provide further insight into the tissue and cell type specificity of PRR pathways in restricting WNV infection (Tables 1 and 2). TLR-dependent signals appear less important for control of WNV replication in peripheral tissues and myeloid cells, and accordingly, loss of individual TLR does not greatly impact IFN-α/β induction. Consistent with this, infection in less per-

FIG. 8. MyD88 restricts WNV infection in subsets of primary neurons. Primary cortical (A) or cerebellar granule (B) neurons generated from wild-type or *MyD88*^{−/−} mice were infected at an MOI of 0.01, and virus production was evaluated at the indicated times by plaque assay. Values are averages of triplicate samples generated from three independent experiments. Asterisks indicate values that were statistically significant (*, *P* < 0.05). The dotted line represents the limit of sensitivity of the assay.

missive tissues (e.g., kidney) was restricted in *TLR3*^{−/−} and *MyD88*^{−/−} mice, in contrast to the altered tropism observed in *IPS-1*^{−/−} and *IFN-αβR*^{−/−} mice (48). TLR3 and MyD88, instead, contribute directly to restricting infection in certain neuronal subtypes, even though significant differences in IFN induction were not observed. In contrast, IPS-1 has a more dominant phenotype in myeloid cells in regulating IFN induction, as its absence results in markedly enhanced replication and depressed levels of IFN-α/β.

The most pronounced virologic phenotype in the *MyD88*^{−/−} mice was an increase in WNV replication in the brain. This likely was not due to differentially altered blood-brain barrier permeability or earlier entry, as was reported with *TLR3*^{−/−} mice (64), since initial infection in the brain was observed on

the same day and at the same magnitude in wild-type and *MyD88*^{−/−} mice. Instead, we hypothesize two possible mechanisms to explain the virologic phenotype. (i) MyD88 had a direct and local effect on WNV replication in neurons. This effect could be due to altered host antiviral effector molecule expression, which would affect neuron-to-neuron spread of WNV infection. (ii) MyD88 had an immunomodulatory effect on B cells, T cells, or other leukocytes that regulated production of antibodies or trafficking and clearance by effector cells. Several experiments were performed to address this, including direct intracranial inoculation, *in vitro* infections of primary neurons, evaluation of B- and T-cell responses in the periphery, and quantitation of infiltrating leukocytes in the context of infection.

The intracranial inoculation studies demonstrated that within 4 days of infection, higher levels of WNV were measured in the cerebral cortex, white matter, and spinal cord of *MyD88*^{−/−} mice. This time point was noteworthy, as flow cytometric and immunohistochemical analyses showed that it preceded significant trafficking of leukocytes into the brain in response to infection. The *in vitro* infection studies with primary neurons supported a model for a direct effect of MyD88 on neuronal subsets, as an increase in viral replication was observed in *MyD88*^{−/−} cerebellar granule but not cortical neurons. The latter finding was not altogether surprising, as cortical neurons are relatively insensitive to the antiviral effects of IFN (49), whereas cerebellar neurons are strongly inhibited from WNV infection by IFN-β pretreatment (B. Zhang, H. Lazear, R. Klein, and M. Diamond, unpublished results). Nonetheless, these results differed from those obtained with *IRF-7*^{−/−} cortical neurons, in which a 12-fold increase in viral infection was observed at 48 h (13). These experiments suggest that MyD88 serves a protective role, in part, by restricting replication in subsets of neurons. It remains uncertain as to whether the antiviral effect of MyD88 in neurons occurs through IFN-independent antiviral gene induction that is stimulated by downstream targets of MyD88 that regulate transcription, such as IRF-5 or IRF-7.

The immunological analysis revealed no defect in induction of adaptive immune responses in the periphery. Equivalent levels and kinetics of WNV-specific IgM and IgG were measured in the blood of wild-type and *MyD88*^{−/−} mice. These results contrast with experiments with *Salmonella typhimurium* (6), *Borrelia hermsii* (3), and murine gammaherpesvirus 68 (22), in which blunted antibody responses and IgG class switching was observed in *MyD88*^{−/−} and chimeric mice. However,

TABLE 1. Comparison of viral burden and IFN production in tissues from WNV-infected <i>MyD88</i> ^{−/−} , <i>TLR3</i> ^{−/−} , and <i>IPS-1</i> ^{−/−} mice									
Sample site	Viral burden or IFN production change on indicated day postinfection for indicated genotype ^a								
	<i>MyD88</i> ^{−/−}			<i>TLR3</i> ^{−/−}			<i>IPS-1</i> ^{−/−}		
	Day 2	Day 4	Day 8	Day 2	Day 4	Day 8	Day 2	Day 4	Day 8
Viremia	No Δ ^b	No Δ	No Δ	No Δ	No Δ	No Δ	120-fold	↑ 1,000-fold	No survivors
Spleen	No Δ	No Δ	No Δ	No Δ	↑ 6-fold	No Δ	↑ 20-fold	↑ 10,000-fold	No survivors
Kidney	No Δ	No Δ	No Δ	No Δ	↑ 3-fold	No Δ	↑ 4-fold	↑ 100-fold	No survivors
Brain	No Δ	No Δ	↑ 56-fold	No Δ	↑ 4-fold	↑ 10-fold	No Δ	↑ 500-fold	No survivors

^a Data are expressed as the fold increase (↑) or decrease (↓) in viral burden or IFN gene expression compared to wild-type tissues, as determined in this study or obtained from references 12, 14, and 58.
^b No difference compared to wild type.

TABLE 2. Comparison of viral burden and IFN production in *MyD88*^{-/-}, *TLR3*^{-/-}, and *IPS-1*^{-/-} cells infected with WNV *in vitro*

Cell type and parameter monitored	Viral burden or IFN production change on indicated day postinfection for indicated genotype ^a								
	<i>MyD88</i> ^{-/-}			<i>TLR3</i> ^{-/-}			<i>IPS-1</i> ^{-/-}		
	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h
Macrophages									
Virus production	No Δ ^b	↑ 5-fold	No Δ	No Δ	No Δ	No Δ	↑ 5-fold	↑ 20-fold	ND ^c
IFN-α mRNA or protein induction	↑ 1.5-fold	↑ 8-fold	ND	No Δ	No Δ	ND	ND	ND	ND
IFN-β mRNA or protein induction	↑ 1.5-fold	↑ 3.5-fold	ND	No Δ	No Δ	ND	↓ 40-fold	↓ 90-fold	ND
Bone marrow-derived DC									
Virus production	No Δ	No Δ	No Δ	No Δ	No Δ	No Δ	↑ 5-fold	↑ 50-fold	ND
IFN-α mRNA or protein induction	No Δ	No Δ	ND	No Δ	No Δ	ND	↓ 200-fold	↓ 500-fold	ND
IFN-β mRNA or protein induction	No Δ	No Δ	ND	No Δ	No Δ	ND	↓ 1,000-fold	↓ 3,000-fold	ND
Cortical neurons									
Virus production	↑ 1.7 fold	No Δ	No Δ	↑ 1.5-fold	↑ 3-fold	ND	↑ 3-fold	↑ 8-fold	ND
IFN-α mRNA induction	ND	ND	ND	No Δ	No Δ	No Δ	ND	ND	ND
IFN-β mRNA induction	ND	ND	ND	No Δ	↑ 3-fold	No Δ	↓ 5-fold	No Δ	ND

^a Data are expressed as the fold increase (↑) or decrease (↓) in viral burden or IFN gene or protein expression compared to wild-type cells as determined in this study paper or as reported in references 12, 14, and 58.

^b No difference compared to wild type.

^c ND, not determined.

the lack of a B-cell phenotype in *MyD88*^{-/-} mice after WNV infection is consistent with studies showing that T-cell-dependent (T_D) antibody responses do not require TLR or MyD88 (23). In mice, after day 4 the induction of WNV-specific antibodies is a T_D response and requires signals from CD4⁺ T cells (56) and the surface molecules CD40 (57) and CD21 (complement receptor 2) (41). The B-cell response in *MyD88*^{-/-} mice after WNV infection, although similar to that seen in *TLR3*^{-/-} mice (12), contrasts with that in *IPS-1*^{-/-} mice (58), in which substantial qualitative defects were observed.

Surprisingly, we observed normal expansion and secretion of IFN-γ by NS4B-specific CD8⁺ T cells in *MyD88*^{-/-} mice after WNV infection. This result varies with studies with vaccinia virus infection and DNA plasmid vaccination, which showed reduced antigen-specific CD8⁺ T-cell expansion and antiviral cytokine production in *MyD88*^{-/-} mice (43, 67). It also contrasts with data showing that *MyD88*^{-/-} mice have defects in the activation of antigen-specific T helper type 1 (T_H1) responses after immunization with ovalbumin (50) and with studies using a vaccine strain of yellow fever virus in *MyD88*^{-/-} mice, which showed trends toward lower levels of IFN-γ⁺ CD4⁺ and CD8⁺ T cells (46). Consistent with our finding of no altered phenotype of antigen-specific CD8⁺ T-cell priming, we observed relatively preserved accumulation and distribution of WNV-specific CD8⁺ T cells in the cerebral cortex of *MyD88*^{-/-} mice. Our result appears to differ from those of others, who observed a decreased percentage of bulk CD8⁺ T cells in the brain of WNV-infected *TLR7*^{-/-} and *MyD88*^{-/-} mice (61), although the flux of WNV-specific CD8⁺ T cells was not evaluated. Indeed, when we gated on total CD45⁺ CD3⁺ T cells in the brain, we also observed a decrease in the number of CD4⁺ and CD8⁺ T cells in *MyD88*^{-/-} mice. Thus, a deficiency of MyD88 appears to preferentially limit trafficking or accumulation of total but not antigen-specific CD8⁺ T cells in the brain. Antigen-specific T cells, which are activated initially in peripheral lymphoid tissues, may require fewer migratory cues for blood-brain barrier crossing and retention compared

to those of disparate specificity (35). We also detected a decrease in the percentage and number of CD45^{high} CD11b^{high} infiltrating monocyte-derived macrophages in the brains of *MyD88*^{-/-} mice early in the course of CNS infection; these results are consistent with those observed previously with WNV and *TLR7*^{-/-} and *MyD88*^{-/-} mice (61). Activated myeloid cells in the brain correlate with control of WNV pathogenesis (59).

We observed a defect in production and accumulation of specific leukocyte chemoattractants in the brains of *MyD88*^{-/-} mice. Significantly less CCL2, CCL5, CXCL9, and CXCL10 were present in the cerebral cortex of WNV-infected *MyD88*^{-/-} mice. This is relevant because WNV-infected neurons *in vitro* and *in vivo* produce these chemokines, resulting in enhanced trafficking across the blood-brain barrier of protective CXCR3⁺ or CCR5⁺ leukocytes (26, 31, 66). Moreover, neutralization of CCL2 reduced trafficking of inflammatory monocytes into the brain after WNV infection (24). Additionally, a genetic deficiency of the CCL5 ligand, CCR5, is associated with impaired monocyte-derived macrophage and lymphocyte trafficking in mice and an enhanced rate of symptomatic WNV disease in humans (27, 36, 37). The decreased chemokine levels in the cerebral cortex in *MyD88*^{-/-} mice after WNV infection was associated with reduced numbers of infiltrating macrophages and total T cells, results that are consistent with the data from WNV-infected *CCR5*^{-/-} mice (27). Given that antigen-specific CD8⁺ T-cell numbers were not grossly altered in *MyD88*^{-/-} mice after WNV infection, the chemokine trafficking requirements of these cells may be distinct; the blunted levels of CXCL10 and CCL5 in *MyD88*^{-/-} mice appear sufficient to preferentially reduce the flux or retention of antigen-nonspecific CD8⁺ T cells. Although we did not directly evaluate an independent role or function of IL-12 or IL-23 in *MyD88*^{-/-} mice, our results are more consistent with a model in which leukocyte trafficking to the CNS requires MyD88 signaling in WNV-infected cells to produce chemokines that direct recruitment of protective leu-

kocytes. The prior study did not directly examine chemokine levels in the brains of infected *TLR7*^{-/-} mice, although no difference was observed in cultured macrophages (61).

In summary, our results expand the understanding of how key innate immune response adaptor and signaling molecules function to limit viral infection. We show that MyD88 likely functions at multiple levels to prevent WNV encephalitis. These levels include antiviral effects on replication in neuronal subsets and immunomodulatory effects on chemokine production and leukocyte trafficking. A more clear understanding of the mechanisms of protection by the host may facilitate novel strategies for therapeutic intervention against viral pathogens. Possibly, new classes of drugs that modulate MyD88 signaling could reduce WNV replication in target cells and enhance clearance mechanisms through recruitment and expansion of infiltrating leukocytes.

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We report no conflicts of interest.

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