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Gamma Interferon Plays a Crucial Early Antiviral Role in Protection against West Nile Virus Infection

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West Nile virus (WNV) causes a severe central nervous system (CNS) infection in humans, primarily in the elderly and immunocompromised. Prior studies have established an essential protective role of several innate immune response elements, including alpha/beta interferon (IFN-α/β), immunoglobulin M, γδ T cells, and complement against WNV infection. In this study, we demonstrate that a lack of IFN-γ production or signaling results in increased vulnerability to lethal WNV infection by a subcutaneous route in mice, with a rise in mortality from 30% (wild-type mice) to 90% (IFN-$\gamma^{-/-}$ or IFN-$\gamma R^{-/-}$ mice) and a decrease in the average survival time. This survival pattern in IFN-$\gamma^{-/-}$ and IFN-$\gamma R^{-/-}$ mice correlated with higher viremia and greater viral replication in lymphoid tissues. The increase in peripheral infection led to early CNS seeding since infectious WNV was detected several days earlier in the brains and spinal cords of IFN-$\gamma^{-/-}$ or IFN-$\gamma R^{-/-}$ mice. Bone marrow reconstitution experiments showed that γδ T cells require IFN-γ to limit dissemination by WNV. Moreover, treatment of primary dendritic cells with IFN-γ reduced WNV production by 130-fold. Collectively, our experiments suggest that the dominant protective role of IFN-γ against WNV is antiviral in nature, occurs in peripheral lymphoid tissues, and prevents viral dissemination to the CNS.

West Nile virus (WNV) is a mosquito-borne, single-stranded RNA flavivirus that is related to other viruses, including dengue virus (DENV), yellow fever virus (YFV), and Japanese virus (JEV), St. Louis virus, and tick-borne encephalitis virus, that cause human disease. WNV is endemic in mosquitoes and birds in parts of Africa, Europe, the Middle East, and Asia and has established its presence in North America. Humans develop a febrile illness with a subset of cases progressing to meningitis, encephalitis, or an acute flaccid paralysis syndrome (1, 16, 34, 42, 43).

After mosquito inoculation, initial WNV replication is believed to occur in the skin in Langerhans dendritic cells (DCs), which migrate to draining lymph nodes (11, 37), where viral amplification occurs (32, 38, 45, 49, 71). Subsequently, WNV enters the bloodstream and spreads to other peripheral organs, including the spleen. Within days of initial infection, WNV disseminates to the brain and spinal cord (19, 72) and in many animals infects a variety of neurons in the hippocampus, cerebellum, brain stem, cerebral cortex, and anterior horn of the spinal cord (19, 22, 63, 72).

In animals and humans the integrity of the immune system response correlates with resistance to WNV infection (1, 20, 22, 23, 28, 29, 31, 34, 42, 43, 50, 53, 64, 70, 73). Recent experiments with mice have eluicidated how the innate and adaptive immune responses protect against WNV dissemination (20, 66). Type I interferon (interferon alpha/beta [IFN-α/β]) controls initial WNV infection and restricts tropism in extraneural sites, thus limiting viremia and dissemination to the central nervous system (CNS) (55). The induction of WNV-specific immunoglobulin M (IgM) coincides with the clearance of WNV from the bloodstream (21), and WNV-specific IgG limits its dissemination and promotes clearance of virus from infected cells (4, 19, 24, 51). Changes in blood-brain barrier permeability associated with cytokine production and inflammation at peripheral sites facilitate WNV entry into the CNS (17, 68). Finally, $CD8^+$ T cells contribute to the eradication of WNV from infected cells since CD8-deficient or depleted mice fail to efficiently clear WNV from the CNS, resulting in increased morbidity and viral persistence (60, 69).

At present, less is known about the function of type II IFN (IFN-γ) in the control of WNV infection. IFN-γ is produced by natural killer (NK) cells, γδ T cells, and $CD8^+$ T cells and generally inhibits virus propagation by several mechanisms, including direct antiviral inhibitory activity, polarization and activation of T-helper-cell responses, enhanced class I major histocompatibility antigen expression and presentation, and activation of phagocytic myeloid cells (14, 30, 58). A recent study showed that γδ T cells, which produce IFN-γ in response to WNV infection, function to limit early dissemination (67). That study, however, did not directly address whether γδ T cells required IFN-γ to mediate their protective effects. Studies with other flaviviruses have provided some insight as to the potential protective function of IFN-γ. The pretreatment of cells with IFN-γ in vitro reduces the production of DENV (18) and JEV (46). In mice that were genetically deficient in IFN-γ, however, small or no changes in mortality were observed after

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infection with DENV (35, 59) or YFV (47), and only a moderate increase in mortality was seen with Murray Valley encephalitis virus (MVEV) (48).

Studies with neurotropic viruses from other families have suggested that IFN-γ plays a more critical role in controlling infection in the CNS (8, 12, 25, 33, 44, 54). Based on experiments with Sindbis and measles viruses, IFN-γ is believed to contribute directly to the noncytolytic clearance of virus from neuronal cells and thus minimize host-mediated tissue damage (7, 8, 52). However, $CD8^+$ T cells do not require IFN-γ to clear WNV infection since the adoptive transfer of IFN-γ-deficient $CD8^+$ T cells into CD8-deficient mice fully controlled peripheral and CNS infection (61). In the present study, using mice that are deficient in either IFN-γ or IFN-γ receptor (IFN-γR), we evaluated the protective effect of IFN-γ against a virulent North American WNV strain. An absence of IFN-γ function resulted in an increased viral burden in peripheral tissues early during the course of infection, leading to rapid spread to the CNS and early death.

MATERIALS AND METHODS

Virus propagation and titration. The lineage I WNV strain (3000.0259) isolated in New York in 2000 was used as described previously (60). The stock virus (2×10^8 PFU/ml) was propagated once in C6/36 cells and used for all in vivo and in vitro studies. The lineage II Madagascar strain was isolated in 1978 (DakAnMg798) as described previously (3) and was amplified once in Vero cells (4×10^7 PFU/ml). BHK21 baby hamster kidney fibroblasts and Vero cells were cultivated in Dulbecco modified Eagle medium (DMEM) as described previously and were used to determine viral burden in the infected mouse cells or tissues by plaque assay (18).

DC infections and IFN treatment. Bone marrow-derived dendritic cells (DCs) were generated according to a published protocol (65). Bone marrow cells were harvested from the femurs of wild-type C57BL/6 mice and cultured in six-well plates at a density of 10^5 cells per well in RPMI supplemented with 10% defined fetal calf serum (HyClone, Logan, Utah), granulocyte-macrophage colony-stimulating factor, sodium pyruvate, kanamycin sulfate, glutamine, and nonessential amino acids for 7 days. The nonadherent DC fraction was transferred to 12-well plates at a density of 1.5×10^5 cells per well. The purity of the DC population was confirmed by staining with phycoerythrin-conjugated rat anti-CD11c antibody by flow cytometry (BD Biosciences Pharmingen). DCs were treated with 100 IU of mouse IFN-α, IFN-β, or IFN-γ (PBL Laboratories, Madison, WI)/ml 24 h prior to infection with WNV (multiplicity of infection [MOI] of 0.2). At 1 h after infection, free virus was removed by washing, and DCs were incubated for 24 h at 37°C in 1 ml of medium. Supernatants were then harvested, and infectious virus was measured by plaque assay on Vero cell monolayers.

Mouse experiments and tissue preparation. C57BL/6J strain (*H-2*^{b}) inbred wild-type mice were obtained from a commercial vendor. The congenic IFN-$\gamma^{-/-}$ and IFN-$\gamma R^{-/-}$ mice, a gift from H. Virgin and R. Schreiber (Washington University School of Medicine, St. Louis, MO), were backcrossed onto the C57BL/6 background for 10 generations. All mice were genotyped and bred under pathogen-free conditions in the animal facilities of Washington University School of Medicine. Experiments were performed with approval and under the guidelines of the Washington University Animal Studies Committee. Eight- to twelve-week-old mice were inoculated with 10^2 PFU of WNV (New York) via a footpad route or 10^1 PFU of WNV (Madagascar) via an intracranial (i.c.) route.

Quantitation of viral burden in mouse tissues. To analyze the kinetics of virus replication in different tissues, groups of wild-type, IFN-$\gamma^{-/-}$, or IFN-$\gamma R^{-/-}$ mice were infected with 10^2 PFU of WNV and euthanized on day 1, 2, 3, 4, 5, 6, or 8 after infection. Before organs were harvested, blood was collected from the axillary vein of each animal by phlebotomy and centrifuged and the serum was isolated and stored at −80°C. Organs were removed and homogenized using a Bead-Beater apparatus, and titers were determined on BHK21 cells as previously described (19). Viral RNA was extracted from thawed aliquots of serum samples (50 μl) by using a Qia-Amp viral RNA recovery kit (QIAGEN, Palo Alto, CA) and quantitated by real-time fluorogenic reverse transcription-PCR (RT-PCR) with an ABI 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) (55). Viral RNA levels from the tissues of infected mice were determined as previously described (19) using an RNeasy kit (QIAGEN, Valencia, CA) and real-time fluorogenic RT-PCR. To normalize the levels of WNV RNA in the samples, eukaryotic 18S rRNA was analyzed in parallel by real-time RT-PCR.

WNV-specific antibody production. An enzyme linked-immunosorbent assay (ELISA) was used to measure the development of WNV-specific IgG and IgM with some modifications of a prior protocol (19, 60). Briefly, purified WNV envelope protein that was generated in baculovirus-infected Hi-5 insect cells (51) was coated on Maxi-Sorp microtiter plates (Nalge Nunc International, Rochester, NY) overnight at 4°C. In parallel, plates were also coated with bovine serum albumin as a control antigen. After the saturation of nonspecific sites with blocking buffer (phosphate-buffered saline, 0.05% Tween 20, 3% bovine serum albumin, and 3% horse serum), plates were incubated with serial dilutions of heat-inactivated serum from infected mice for 2 h at room temperature. After extensive washing, plates were incubated with biotin-conjugated goat anti-mouse IgG or IgM (Sigma Chemical Co., St. Louis, MO), followed by incubation with horseradish peroxidase-conjugated streptavidin (Sigma Chemical). A positive signal was detected with tetramethylbenzidine substrate (Dako, Carpinteria, CA), and the reaction was stopped with H_2SO_4. Plates were read in a GENios ELISA plate reader (Tecan US, Inc., Durham, NC) at 450 nm. The background optical density at 450 nm (OD_{450}) value for binding to bovine serum albumin alone was subtracted from the E protein wells to obtain an adjusted OD_{450} for each sample. Titers represent the serum dilution, yielding an OD_{450} value equivalent to three standard deviations above the background of the assay.

PRNT assay. The titer of neutralizing antibodies was determined by using a previously published protocol (19). Briefly, serum samples were diluted and incubated with 8×10^1 PFU of WNV at 37°C for 1 h. WNV-serum complexes were added to monolayers of BHK21 cells in six-well plates, rocked for 5 min, and incubated at 37°C for 1 h. Subsequently, alpha minimal essential medium supplemented with 4% fetal calf serum and 1% low-melting-point agarose was added, and the plates were incubated at 37°C for 3 days. After fixation with 10% formaldehyde and staining with a 1% crystal violet solution, the plaques were counted and plotted and the plaque reduction neutralization titer for 50% inhibition ($PRNT_{50}$) was calculated.

Measurement of IFN activity. The levels of biologically active type I IFN in serum of wild-type and IFN-$\gamma^{-/-}$ mice on days 1, 2, 3, 4, and 5 after WNV infection were determined by using a previously developed L929 cell bioassay (2). Briefly, serum samples were harvested from infected animals, diluted serially in DMEM containing 10% fetal bovine serum, and incubated in duplicate with adherent L929 cells (1.8×10^4/well) for 14 h at 37°C in a 96-well plate. The supernatants were removed and replaced with DMEM containing encephalomyocarditis virus (MOI of 7). Seven hours later, cells were inspected visually for cytopathic effect, and IFN-mediated protection was measured by colorimetric assay using a CellTiter 96 Aqueous Cell Proliferation Assay according to the manufacturer's instructions (Promega Corp., Madison, WI).

Cytokine detection by flow cytometric bead array assay. Inflammatory cytokine levels were measured in serum from wild-type and IFN-$\gamma^{-/-}$ mice by using the mouse inflammation cytometric bead array (CBA) kit (BD Biosciences, San Diego, CA). Six different inflammatory cytokines were measured: interleukin-6 (IL-6), IL-10, monocyte chemo-attractant protein (MCP-1), IFN-γ, tumor necrosis factor alpha (TNF-α), and IL-12p70. For this analysis, serum was collected at days 0, 1, 2, 3, 4, 6, and 8 postinfection from wild-type and IFN-$\gamma^{-/-}$ mice, mixed with 50 μl of cytokine detection beads for 2 h at room temperature, washed, and analyzed by flow cytometry according to the manufacturer's instructions using CBA software (BD Pharmingen). The concentration of a given cytokine in serum was determined after comparison with a standard curve that was generated as part of the kit.

Bone marrow chimeras and quantitation of WNV after infection. Bone marrow chimeras were prepared according to a previously described protocol (26). In brief, recipients were irradiated twice with 500 rads, with a 30-min interval between doses. Donor bone marrow cells (mixed equally from two donors, see Table 1 for details) were injected intravenously into recipients (1.4×10^7 cells/mouse). Antibiotics (sulfamethoxazole and trimethoprim oral pediatric suspension; Hi-Tech Pharmacal Co., Amityville, NY) were added to the drinking water for the first 7 weeks after reconstitution. At 11 weeks after reconstitution, mice were infected with 100 PFU of WNV by footpad inoculation. On days 2, 4, and 6 after infection, blood, spleens, and brains were harvested, and WNV RNA was analyzed by quantitative fluorogenic RT-PCR as described previously (67).

Data analysis. For survival analysis, Kaplan-Meier survival curves were plotted by using Prism software (GraphPad, San Diego, CA). Mortality curves were analyzed by the log-rank test. The viral burden and antibody titer experiments were evaluated by the Mann-Whitney test. Differences in cytokine production were analyzed by using an unpaired *t* test.

TABLE 1. Generation of bone marrow chimeras[a]

Group	Donor 1	Donor 2	Recipient	Phenotype
1	TCRδ$^{-/-}$	IFN-γ$^{-/-}$	Wild type	γδ T cells do not produce IFN-γ; 50% of other cells (αβ T cells, NK cells) produce IFN-γ
2	TCRδ$^{+/+}$	IFN-γ$^{-/-}$	Wild type	50% of γδ T cells produce IFN-γ; 50% of other cells (αβ T cells and NK cells) produce IFN-γ

[a] Bone marrow chimeras were prepared as described in Materials and Methods. Bone marrow cells from IFN-γ$^{-/-}$ mice were mixed with bone marrow cells from TCRδ$^{-/-}$ mice (normal αβ T cells without γδ T cells) or from TCRδ$^{+/+}$ mice (with γδ T cells) in a 1:1 ratio and injected intravenously into irradiated wild-type mice. At 11 weeks after reconstitution, mice were phenotyped and used for infection experiments.

RESULTS

Mice that lack IFN-γ function are more susceptible to WNV infection after peripheral infection. Previous studies have suggested that IFN-γ plays a critical role in controlling infection of some (8, 12, 25, 44), but not all, neurotropic viruses (41). The role of IFN-γ in vivo in modulating flavivirus infection is less certain since studies with DENV and YFV (35, 47, 59) showed little or no increased mortality in IFN-γ$^{-/-}$ mice. To assess the effect of IFN-γ on WNV infection and disease, we compared survival rates in wild-type and congenic IFN-γ$^{-/-}$ or IFN-γR$^{-/-}$ C57BL/6 mice. After subcutaneous inoculation with 10^2 PFU of a virulent New York strain of WNV, survival was markedly lower in mice that lacked IFN-γ function: IFN-γ$^{-/-}$ or IFN-γR$^{-/-}$ mice had a 5 to 10% survival rate compared to 70% in wild-type mice (Fig. 1A, $P \leq 0.0001$). Mortality occurred earlier in the IFN-γ$^{-/-}$ and IFN-γR$^{-/-}$ mice since the mean time to death was shorter (IFN-γ$^{-/-}$ mice, 9.2 ± 0.8 days; IFN-γR$^{-/-}$ mice, 9.7 ± 0.7 days; wild-type mice, 10.9 ± 1.3 days; $P \leq 0.002$). Thus, an absence of IFN-γ function caused a more fulminant WNV infection with increased mortality after peripheral infection.

WNV burden in IFN-γ$^{-/-}$ mice. To understand how an absence of IFN-γ function increased the susceptibility of mice to WNV infection, the levels of WNV RNA and infectious virus in peripheral and CNS tissues were determined by fluorogenic RT-PCR and plaque assay in BHK21 cells, respectively. Wild-type and IFN-γ$^{-/-}$ mice were infected with 10^2 PFU and viral burden was measured at days 1, 2, 3, 4, 6, and 8 after infection in serum, lymph nodes, spleens, brains, and spinal cords.

(i) Viremia. A higher and more prolonged viremia was observed in IFN-γ$^{-/-}$ mice (Fig. 2A). WNV RNA was detected in the serum by fluorogenic RT-PCR from day 1 after infection in wild-type and IFN-γ$^{-/-}$ mice. At days 2 and 4 after infection approximately 10- to 50-fold-higher levels of WNV RNA were detected in IFN-γ$^{-/-}$ mice ($P \leq 0.05$). At day 6 after infection, viral RNA was cleared from wild-type serum, and yet significant levels were still detected in IFN-γ$^{-/-}$ mice ($P < 0.05$). By day 8, WNV RNA was undetectable in almost all serum samples from IFN-γ$^{-/-}$ mice. These results were confirmed by viral plaque assays in which higher levels of infectious virus were detected in IFN-γ$^{-/-}$ mice through day 6 after infection (data not shown).

(ii) Draining lymph node. Because viremia was elevated early after infection, we evaluated the effect of IFN-γ on viral replication in the draining lymph nodes. Throughout the time course, WNV replication in individual ipsilateral inguinal lymph nodes from wild-type and IFN-γ$^{-/-}$ mice was below the limit of detection of our plaque assays. However, when viral RNA was measured by fluorogenic RT-PCR assay, significant differences were observed (Fig. 2B). Although viral RNA was detected between days 1 and 4 after infection in both wild-type and IFN-γ$^{-/-}$ mice, higher levels (ca. 20- to 60-fold, $P \leq 0.005$) were observed in the lymph nodes of IFN-γ$^{-/-}$ mice.

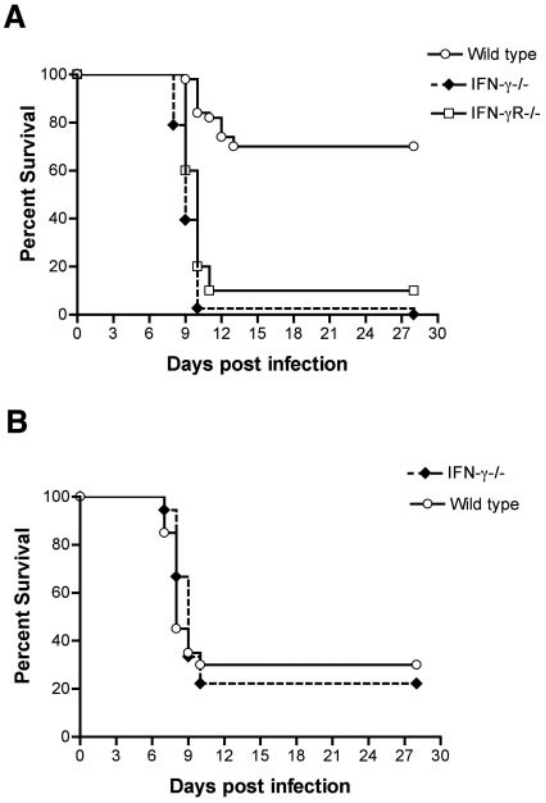

FIG. 1. Survival analysis of wild-type, IFN-γ$^{-/-}$, and IFN-γR$^{-/-}$ mice after WNV infection. (A) Wild-type, IFN-γ$^{-/-}$, and IFN-γR$^{-/-}$ mice were infected subcutaneously with 10^2 PFU of WNV (New York) and monitored for 28 days for morbidity and mortality. The survival curves were constructed using data from three to five independent experiments. The survival differences between wild-type, IFN-γ$^{-/-}$, and IFN-γR$^{-/-}$ mice were statistically significant ($P < 0.0001$). The numbers of animals were $n = 50$ for wild-type, $n = 37$ for IFN-γ$^{-/-}$, and $n = 29$ for IFN-γR$^{-/-}$ mice. (B) Wild-type and IFN-γ$^{-/-}$ mice were infected i.c. with 10^1 PFU of WNV (Madagascar) and monitored for 28 days for morbidity and mortality. The survival curves were constructed with data from two independent experiments. The survival differences between wild-type and IFN-γ$^{-/-}$ mice were not statistically significant ($P = 0.7$). The numbers of animals were $n = 20$ for wild-type mice and $n = 20$ for IFN-γ$^{-/-}$ mice.

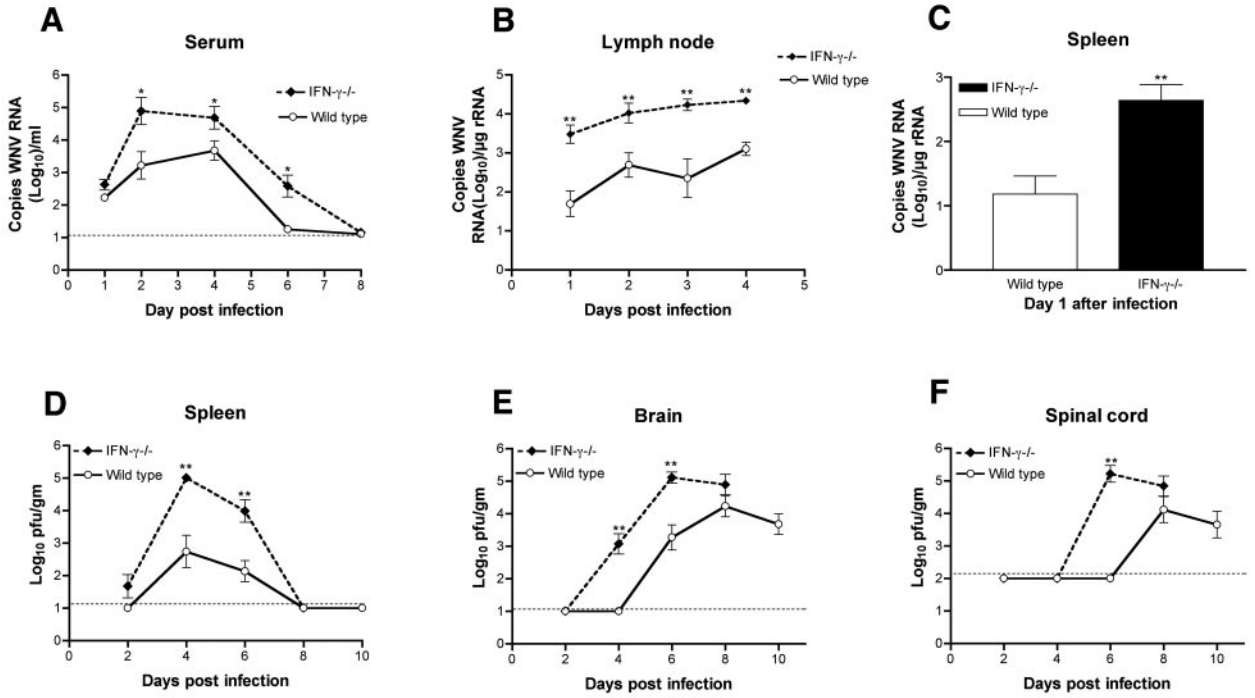

FIG. 2. WNV burden in peripheral and CNS tissues of wild-type and IFN-$\gamma^{-/-}$ mice. (A) Levels of viral RNA in serum detected by fluorogenic real-time RT-PCR. The kinetics and level of viral RNA were determined from the sera of wild-type and IFN-$\gamma^{-/-}$ mice after WNV (New York) infection at the indicated time point by fluorogenic real-time RT-PCR. The data are expressed as number of copies of WNV RNA per milliliter of serum ($n = 10$ mice per time point). (B) Levels of viral RNA in inguinal lymph nodes. Viral RNA was extracted from the draining inguinal lymph nodes of wild-type and IFN-$\gamma^{-/-}$ mice at the indicated time points after WNV infection and quantitated by fluorogenic real-time RT-PCR. The data are expressed as the number of copies of WNV RNA per microgram of rRNA after normalization for tissue content ($n = 6$ mice per time point). (C) Levels of viral RNA in the spleen on day 1 postinfection. Viral RNA was extracted from wild-type or IFN-$\gamma^{-/-}$ spleens at day 1 postinfection and quantitated by fluorogenic real-time RT-PCR. The data are expressed as the number of copies of WNV RNA per microgram of rRNA after normalization for tissue content ($n = 6$ mice per group). (D to F) Levels of infectious virus in tissues. Infectious WNV levels were measured in the spleen (D), brain (E), and spinal cord (F) by using a viral plaque assay in BHK21 cells after tissues were harvested and homogenized at the indicated time points after WNV infection. The data are shown as the average PFU per gram of tissue ($n = 10$ mice per time point). The dotted line represents the limit of sensitivity of the assay (*, $P \leq 0.05$; **, $P \leq 0.005$ [compared to wild-type mice]).

(iii) Spleen. WNV infection was detected at higher levels in the spleens of IFN-$\gamma^{-/-}$ mice. Although infectious WNV was not detected 1 day after infection, ~30-fold higher levels of viral RNA were measured in spleens from IFN-$\gamma^{-/-}$ mice compared to wild-type mice at this time (Fig. 2C, $P = 0.001$). By day 2 after infection, infectious WNV (~10^2 PFU/g, 3 of 10 mice) was detected in the spleen of IFN-$\gamma^{-/-}$ mice, whereas virus was not detected in any of the wild-type mice (Fig. 2D). On days 4 and 6 after infection, ~100-fold-higher levels of WNV were observed in IFN-$\gamma^{-/-}$ mice ($P \leq 0.005$). Nonetheless, by day 8 all IFN-$\gamma^{-/-}$ and wild-type mice had completely cleared infectious virus from the spleen. Overall, a lack of IFN-γ activity resulted in a failure to control infection in the spleen during the initial stages of infection.

(iv) CNS tissues. (a) Brain. WNV spread earlier into the brain of IFN-$\gamma^{-/-}$ mice (Fig. 2E). At day 4 after infection, 90% (9 of 10 mice) of IFN-$\gamma^{-/-}$ mice had detectable WNV in the brain (10^3 to 10^4 PFU/g), whereas no infectious virus was present in any of the wild-type mice at this time. By day 6, ~100-fold-higher levels of infectious WNV were observed in the brains of IFN-$\gamma^{-/-}$ mice ($P = 0.0004$). Interestingly, by day 8, the levels of WNV declined in IFN-$\gamma^{-/-}$ mice such that no significant difference was observed compared to wild-type mice ($P = 0.15$).

(iv) CNS tissues. (b) Spinal cord. In the spinal cord, a pattern was observed that is similar to that seen in the brain (Fig. 2F). Earlier entry into the spinal cord was observed in IFN-$\gamma^{-/-}$ mice. At day 6 after infection, 100% of IFN-$\gamma^{-/-}$ mice had significant viral infection (~10^4 to 10^7 PFU/g), whereas virus was not measurable in the spinal cords of wild-type mice. By day 8, viral burden in the IFN-$\gamma^{-/-}$ mice declined slightly such that no statistical difference was observed compared to the wild-type mice ($P = 0.14$). Since there was higher and earlier WNV replication in the CNS of IFN-$\gamma^{-/-}$ mice, we evaluated whether there was a difference in viral tropism. Similar to findings seen with several other immunodeficient mice (19, 60, 68, 69), immunohistochemical analysis revealed an increase in the number of neurons that expressed WNV antigen in the CNS of IFN-$\gamma^{-/-}$ mice (data not shown).

Clearance of WNV from the CNS of surviving IFN-$\gamma^{-/-}$ mice. A lack of $CD8^+$ T cells or perforin molecules results in WNV persistence in the CNS up to 5 weeks after infection (60, 61). Because IFN-γ participates in the clearance of other neurotropic viruses from the CNS (7, 8), we analyzed viral persis-

tence in IFN-$\gamma^{-/-}$ mice at later time points. The infection of IFN-$\gamma^{-/-}$ mice resulted in a small (~5 to 10%) number of surviving animals. In surviving wild-type or IFN-$\gamma^{-/-}$ mice, infectious virus was not detected in the brain after 21 days (data not shown). Thus, a deficiency of IFN-γ did not appear to affect the kinetics of viral clearance from the CNS, results that agree with adoptive transfer experiments that showed that IFN-γ-deficient $CD8^+$ T cells efficiently cleared WNV from the CNS (61).

WNV burden in IFN-$\gamma R^{-/-}$ mice. To confirm the role of IFN-γ in controlling WNV infection, a subset of analogous virologic experiments were performed with IFN-$\gamma R^{-/-}$ mice at key time points (Fig. 3). Similar to IFN-$\gamma^{-/-}$ mice, infectious virus (~10^3 PFU/g) was detected by viral plaque assay in the serum at days 2 and 4 in 80% (four of five mice) of IFN-$\gamma R^{-/-}$ mice (data not shown). Compared to wild-type mice, IFN-$\gamma R^{-/-}$ mice had significantly enhanced levels of infection in the spleen at days 4 and 6 (Fig. 3A, $P \leq 0.05$); however, these levels were similar to those observed with IFN-$\gamma^{-/-}$ mice ($P \geq 0.1$). In addition, early entry and replication of WNV in the brain and spinal cord were observed with IFN-$\gamma R^{-/-}$ mice (Fig. 3B and C).

Effect of IFN-γ after i.c. infection. Since WNV is naturally acquired through peripheral inoculation and subsequently spreads to the CNS, an effective immune response to WNV controls viral replication both in the periphery and in the CNS compartments. Our experiments with a subcutaneous infection model show that IFN-γ is required to control peripheral viral replication and spread (see Fig. 2). To determine whether IFN-γ plays an independent role in the CNS after WNV infection, we inoculated wild-type and IFN-$\gamma^{-/-}$ mice i.c. with 10^1 PFU of an attenuated WNV strain (Madagascar [3]); the attenuated strain was used because i.c. inoculation of 10^1 PFU of the virulent New York WNV strain causes complete lethality in wild-type mice (55). As no difference in lethality after i.c. infection with the Madagascar strain was observed between wild-type and IFN-$\gamma^{-/-}$ mice (Fig. 1B, $P = 0.7$), IFN-γ does not appear to have an essential antiviral effect in the CNS. Importantly, at day 30, surviving wild-type and IFN-$\gamma^{-/-}$ mice all had high-titer WNV-specific antibodies, thus confirming productive infection with the Madagascar strain (data not shown).

WNV-specific IgM and IgG production in the absence of IFN-γ. IFN-γ is an immunomodulatory cytokine with specific roles in the priming of adaptive immune responses (58). Because a higher viremia was observed in mice that lacked IFN-γ function, we reasoned that this could be due to depressed WNV-specific antibody responses. Blunted WNV-specific antibody responses early during the course of infection correlate with higher viremia, earlier spread to the CNS, and increased mortality (19, 21). Interestingly, higher rather than lower titers of WNV-specific IgM and IgG were detected in IFN-$\gamma^{-/-}$ mice (Fig. 4A and B). Increased levels of WNV-specific IgM were detected after day 4 ($P \leq 0.05$) and throughout the time course compared to the wild-type mice (Fig. 4A). Although an earlier WNV-specific IgG response was detected in IFN-$\gamma^{-/-}$ mice at day 6 (Fig. 4B, $P < 0.05$), by day 8 no statistical difference was observed ($P = 0.12$). Despite the somewhat higher level of antibody production in IFN-$\gamma^{-/-}$ mice, similar neutralization titers were detected in both wild-type and IFN-$\gamma^{-/-}$ mice from

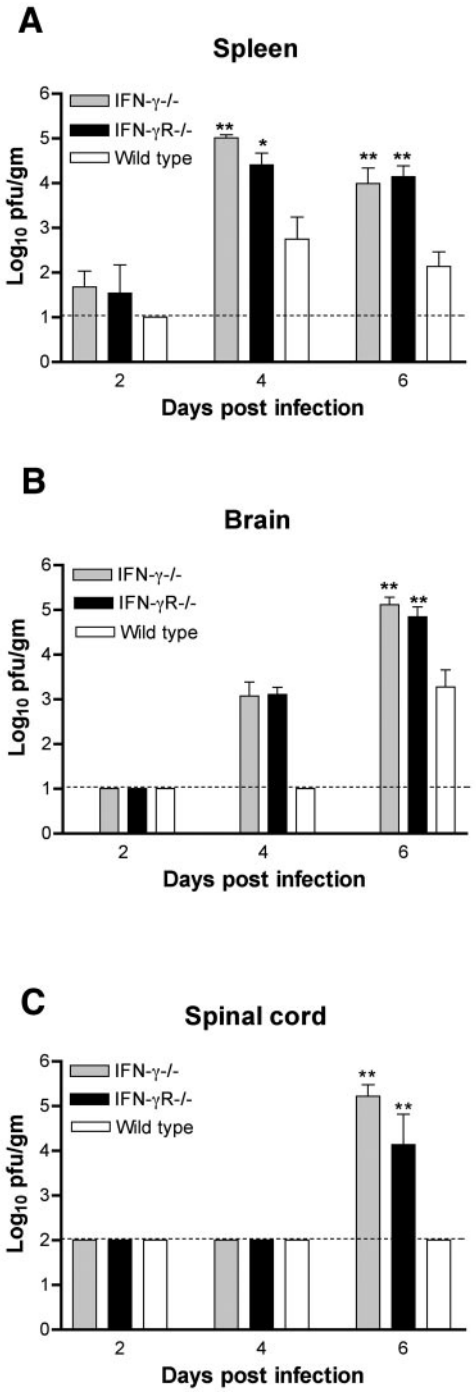

FIG. 3. Level of infectious WNV burden in the peripheral and CNS tissues of wild-type, IFN-$\gamma^{-/-}$, and IFN-$\gamma R^{-/-}$ mice. The level of infectious virus was measured from the spleens (A), brains (B), and spinal cords (C) of wild-type, IFN-$\gamma^{-/-}$, and IFN-$\gamma R^{-/-}$ mice by using a viral plaque assay in BHK21 cells at the indicated time points after WNV (New York) infection. The data are shown as the average PFU per gram of tissue ($n = 5$ to 10 mice per time point). The dotted line represents the limit of sensitivity of the assay (*, $P \leq 0.05$; **, $P \leq 0.005$ [compared to wild-type mice]).

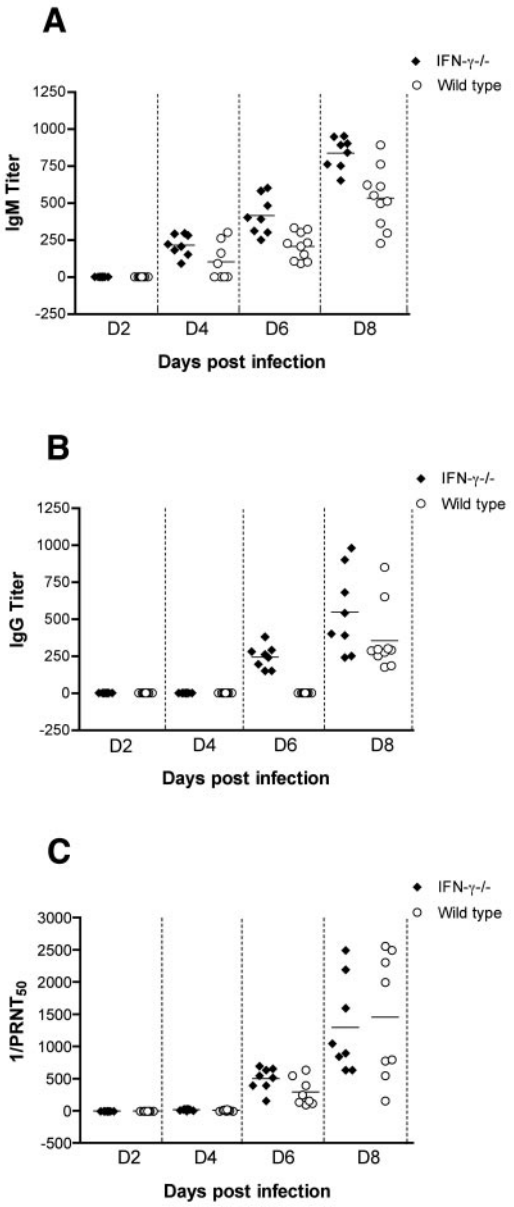

FIG. 4. WNV-specific antibody responses in wild-type and IFN-$\gamma^{-/-}$ mice. Wild-type and IFN-$\gamma^{-/-}$ mice were infected with WNV (New York), and sera were collected at the indicated time points. The development of specific IgM (A) or IgG (B) antibodies to WNV was determined by ELISA using purified WNV E protein. The data are the averages of at least eight mice per time point. (C) Neutralizing antibody titers. Neutralizing titers were determined by using a PRNT assay. All serum samples were performed in duplicate, and the data are expressed as reciprocal $PRNT_{50}$s (i.e., the antibody titers that reduced the number of plaques by 50%). The data are the average of eight mice per time point.

day 4 after infection (Fig. 4C). Because WNV-specific antibody responses are not blunted in IFN-$\gamma^{-/-}$ mice, the increased WNV infection in the periphery is not due to a compromised ability to prime B-cell responses.

Serum levels of IFN in the absence of IFN-γ. A lack of type I IFN receptor resulted in a higher WNV burden in peripheral and CNS tissues and uniform mortality in mice by day 4 after infection (55). Since we observed a similar increase in the peripheral replication of WNV in IFN-$\gamma^{-/-}$ mice, we hypothesized that IFN-γ could protect against WNV by amplifying the type I IFN response. To test this, we measured the levels of biologically active type I IFN in serum from wild-type and IFN-$\gamma^{-/-}$ mice after WNV infection using an encephalomyocarditis bioassay (see Materials and Methods). In both wild-type and IFN-$\gamma^{-/-}$ mice, type I IFN was detected on day 1 after infection and increased through day 5 (Fig. 5A). Importantly, a lack of IFN-γ resulted in no significant difference ($P \geq 0.1$) in the level of the type I IFN antiviral effect throughout the time course compared to wild-type mice. Thus, the early increased viral burden in peripheral lymphoid tissues in IFN-$\gamma^{-/-}$ mice was not due to a decreased type I IFN response.

Inflammatory cytokine profile after WNV infection in the absence of IFN-γ. A prior report suggested that Toll-like receptor-3-induced production of TNF-α in peripheral tissues could promote blood-brain barrier permeability changes and enhanced WNV entry into the CNS (68). To test whether the earlier WNV entry in IFN-$\gamma^{-/-}$ mice was due to a difference in inflammatory cytokine and chemokines, we measured the levels of TNF-α, IL-6, MCP-1, IL-12p70, IL-4, IFN-γ, and IL-10 in sera after WNV infection by using a flow cytometric bead array assay. Among the sera tested, significant levels of IFN-γ, MCP-1, IL-6, and TNF-α were detected. As expected, IFN-γ was measurable in sera only from wild-type mice and was present soon after infection (day 1, ~4 pg/ml), a finding consistent with a previous study (28). Significant increases in MCP-1 and IL-6 levels in the serum in IFN-$\gamma^{-/-}$ mice were transiently detected and only at day 6 after infection (Fig. 5B and C). Although increased TNF-α levels were present in the sera of IFN-$\gamma^{-/-}$ mice, statistically significant differences were not observed until 6 days after infection (Fig. 5D, ~8-fold increase, $P < 0.05$). Because of the disparity in kinetics between viral CNS entry and TNF-α levels, we hypothesized that the early dissemination in IFN-$\gamma^{-/-}$ mice was not due to differential TNF-α-dependent changes in CNS blood-brain barrier permeability. Indeed, no difference in blood-brain barrier permeability was observed by Evans blue staining in IFN-$\gamma^{-/-}$, IFN-$\gamma R^{-/-}$, or wild-type mice at days 2 to 4 after infection, and the administration of a neutralizing anti-TNF-α antibody did not affect the kinetics of viral entry into the CNS or survival in IFN-$\gamma^{-/-}$ mice (data not shown).

γδ T cells require IFN-γ to control early WNV infection. γδ T cells control the early replication and spread of WNV infection to the CNS (67). To directly assess the role of IFN-γ in γδ-T-cell-mediated control of WNV, bone marrow chimeras were generated. To create mice in which only the γδ T cells were deficient in IFN-γ, bone marrow cells from TCRδ$^{-/-}$ and IFN-$\gamma^{-/-}$ mice were mixed at a 1:1 ratio and transferred to lethally irradiated wild-type mice (see Table 1). Mice were generated in parallel with IFN-γ-sufficient γδ T cells by using a 1:1 mixture of TCRδ$^{+/+}$ and IFN-$\gamma^{-/-}$ donor cells. Six weeks later, after reconstitution phenotypes were confirmed, mice were infected with WNV, and viral burdens were analyzed in the sera, spleens, and brains by using fluorogenic quantitative RT-PCR. Mice reconstituted with IFN-γ-deficient γδ T cells had significantly higher levels of WNV RNA in the sera (Fig. 6A), spleens (Fig. 6B), and brains (Fig. 6C) throughout the time course compared to mice reconstituted with IFN-γ-sufficient γδ T cells

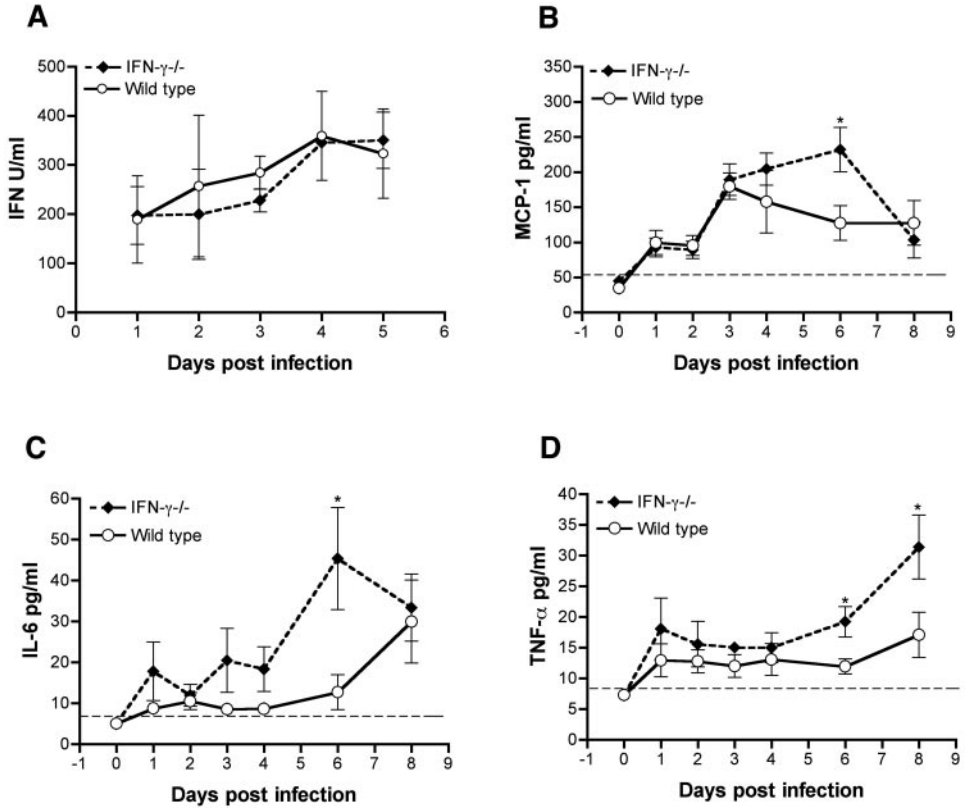

FIG. 5. Cytokine profiles in the sera of wild-type and IFN-$\gamma^{-/-}$ mice. Wild-type and IFN-$\gamma^{-/-}$ mice were infected with WNV (New York) via the footpad, and sera were collected at the indicated time points after infection. (A) Type I IFN activity was measured by bioassay using encephalomyocarditis virus and L929 cells. The data are an average of three to five mice per time point. (B to D) Other inflammatory cytokines and chemokines. The levels of MCP-1 (B), IL-6 (C) and TNF-α (D) in serum were determined by using a flow cytometric bead array. The data are expressed as an average of six mice per time point from two independent experiments. The dotted line represents the limit of sensitivity of the assay (*, $P \leq 0.05$ compared to wild-type mice).

($P \leq 0.05$). These experiments suggest that the early replication and dissemination of WNV observed in IFN-$\gamma^{-/-}$ mice is due in part to the inability of γδ T cells to produce IFN-γ in the early phases of infection.

Antiviral effect of IFN-γ in bone marrow-derived DCs. Our studies in vivo suggested that IFN-γ limits WNV replication in peripheral lymphoid tissues within days of infection prior to the development of an adaptive immune response. Based on this, we postulated that IFN-γ, produced by γδ T cells and perhaps by other immune cells, acts as a direct antiviral agent to control WNV infection in lymphoid compartments. To evaluate this, we assessed the antiviral activity of IFN-γ in vitro on cultured DCs derived from wild-type C57BL/6 mice. DCs, by virtue of their expression of DC-SIGN-like attachment molecules (15), are believed to be initial targets for WNV replication after skin inoculation (11, 37). $CD11c^+$ DCs were pretreated with physiologically relevant doses (100 U/ml) of IFN-α, IFN-β, or IFN-γ and then infected with WNV at an MOI of 0.2. One day later, supernatants were analyzed for the production of infectious virus by plaque assay. Pretreatment of DCs with any of the IFNs significantly reduced ($P \leq 0.0004$) virus titers in the supernatant (Fig. 7). Notably, compared to untreated DCs, there was a 130-fold reduction in WNV production in cells pretreated with IFN-γ.

DISCUSSION

Previously, we have shown that type I IFN (IFN-α/β) plays a key role in the innate defense against WNV infection by restricting the tropism of WNV infection in peripheral tissues (55). Nonetheless, the role of IFN-γ in WNV infection and other flavivirus infections has remained more uncertain since it has both antiviral and immunomodulatory functions against other viruses. Using multiple experimental approaches we investigated how IFN-γ controls WNV infection. C57BL/6 mice that lacked IFN-γ or the IFN-γR showed increased susceptibility to lethal WNV infection with a rise in mortality and a decrease in the average survival time compared to congenic wild-type mice. The increase in mortality in IFN-$\gamma^{-/-}$ or IFN-$\gamma R^{-/-}$ mice was associated with greater viral replication in lymphoid tissues and higher viremia, which led to a more rapid dissemination of WNV into the brain and spinal cord. These in vivo results, which suggested an early antiviral function of IFN-γ, were supported by in vitro infection experiments with primary DCs.

A previous study showed that WNV infection of mice deficient in γδ T cells resulted in elevated viral loads in the periphery, enhanced dissemination to the CNS, and increased lethality (67). Although the γδ T cells expanded rapidly in vivo

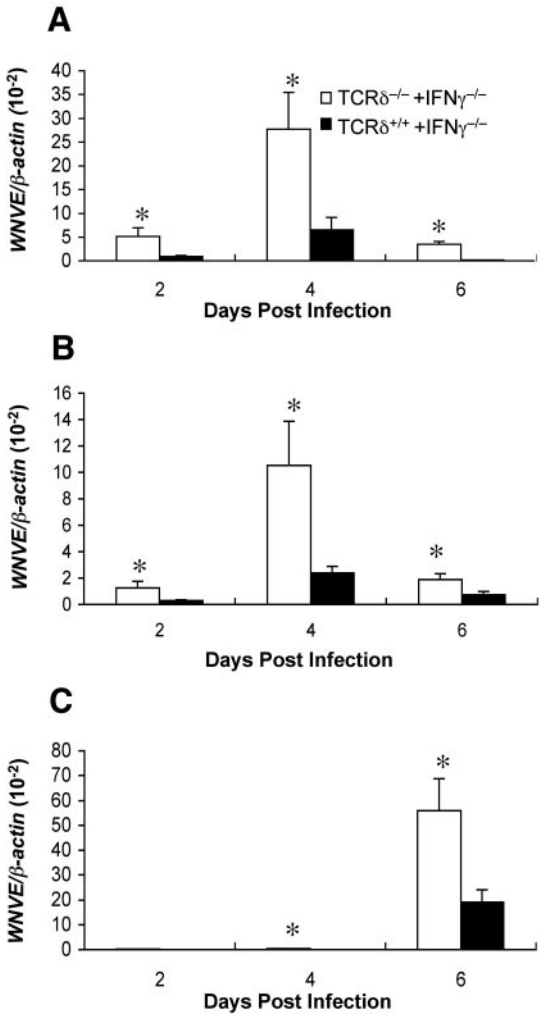

FIG. 6. WNV burden in peripheral and CNS tissues of bone marrow chimeras. Bone marrow chimeras were prepared as described in Materials and Methods using donors and recipients (see Table 1). Reconstituted mice were infected subcutaneously with 10^2 PFU of WNV (New York). Viral loads were measured at the indicated days in the blood (A), spleens (B), and brains (C) of mice by using quantitative RT-PCR. The *y* axis depicts the ratio of the amplified WNV-E cDNA to β-actin cDNA of each sample. At each time point, five mice per group were analyzed (✱, $P \leq 0.05$ indicates a significant difference in viral RNA levels between reconstituted groups).

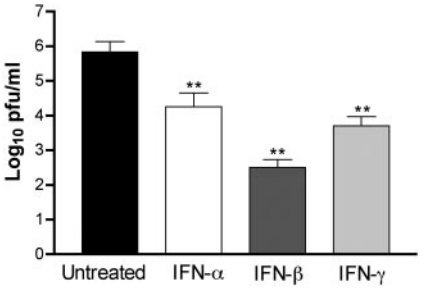

FIG. 7. Inhibition of WNV production from the bone marrow-derived DCs treated with IFN. DCs were generated from the bone marrow of wild-type mice. DCs were pretreated with 100 IU of IFN-α, IFN-β, or IFN-γ 24 h before infection. Cells were infected at an MOI of 0.2, and the production of infectious WNV (New York) was determined 1 day later by plaque assay in Vero cells. The data are expressed as an average of two independent experiments performed in triplicate (✱✱, $P \leq 0.005$ compared to untreated cells).

and produced IFN-γ in ex vivo restimulation assays, experiments did not directly identify the mechanism of protection. Here, using bone marrow chimeras, we demonstrate that γδ T cells require IFN-γ to limit dissemination by WNV. Mice reconstituted with IFN-γ-deficient γδ T cells had significantly higher levels of WNV RNA in the serum, spleen, and brain throughout the time course compared to mice reconstituted with IFN-γ-sufficient γδ T cells. These experiments suggest that the early replication of WNV observed in IFN-$\gamma^{-/-}$ mice is due in part to the inability of γδ T cells to produce IFN-γ. Interestingly, although NK cells also produce IFN-γ early during an immune response, antibody depletion studies suggest they do not have a significant role in the control of WNV infection in mice (61).

Our results are most consistent with a model in which IFN-γ has an early antiviral role, limiting the spread of WNV infection prior to activation and control by adaptive B and T-cell responses. The following data from our studies with IFN-$\gamma^{-/-}$ or IFN-$\gamma R^{-/-}$ mice support this model: (i) enhanced WNV infection was observed in lymphoid tissues (spleen and lymph node) by day 1 after infection, (ii) increased viremia was seen within 2 days of infection, and (iii) a more rapid spread of WNV to the brain and spinal cord was consistently observed in all mice lacking the IFN-γ signaling function. Moreover, ex vivo infection of DCs, the cell type that is believed to sustain early rounds of WNV replication (11, 15, 37, 38) was markedly decreased after exposure to physiologically relevant concentrations of IFN-γ. In contrast, we did not observe major deficits in the adaptive immune response or function in IFN-γ-deficient mice. IgM and IgG responses against WNV developed with normal kinetics in IFN-$\gamma^{-/-}$ mice. The clearance of WNV from the spleen was normal, a process that depends on priming and activation of $CD8^+$ T cells (60, 61). In addition, although most IFN-$\gamma^{-/-}$ mice succumbed rapidly to WNV infection, those that survived showed a relatively typical pattern of clearance of infectious WNV from the CNS. This was reflected by a decrease in viral burden between days 6 and 8 after infection and an absence of CNS viral persistence in any of the few surviving animals.

An absence of IFN-γ in C57BL/6 mice resulted in a marked increase in WNV infection and mortality, results that are somewhat distinct from that previously observed with other flaviviruses. No increase in mortality was observed in IFN-$\gamma^{-/-}$ C57BL/6 mice after primary YFV infection, although the levels of infectious virus in the brain were higher at day 4 (47). For DENV, moderately increased death was observed in IFN-$\gamma^{-/-}$ 129 Sv mice (59), with peak mortality reaching 30 and 43% for DENV-1 and DENV-2 infections, respectively. However, compared to wild-type mice, no increase in viral burden was observed by indirect plaque assay at day 3 or 7 after infection in lymphoid or CNS tissues. Notably, no mortality or illness was observed after DENV-2 infection of IFN-$\gamma^{-/-}$ mice on a BALB/c background (35). C57BL/6 mice deficient in IFN-γ showed a moderate (60% compared to 30% for wild-type mice) increase in mortality after infection with MVEV, a re-

lated encephalitic flavivirus (48). Increased MVEV burden was detected in the serum and spleen at day 4 after infection in IFN-$\gamma^{-/-}$ mice, and brain titers at day 8 were indistinguishable from wild-type mice, results that are consistent with our findings. Interestingly, higher mortality after MVEV infection was also observed in mice deficient in inducible nitric oxide synthase-2, an effector molecule downstream of IFN-γ that has been reported to affect outcome after infection with JEV (56, 57) and other RNA viruses (14, 40).

Surprisingly, our virologic data suggest that IFN-γ has a less significant antiviral role in the CNS. No difference in survival was observed after i.c. infection with the attenuated Madagascar strain of WNV. Although IFN-γ is induced in CNS tissues by day 8 after WNV infection (28, 39) and is capable of inhibiting infection ~10-fold in some primary neuron cultures (55), these effects did not modulate survival. In contrast to other neurotropic RNA viruses such as Sindbis virus (7–9), measles virus (52), mouse hepatitis virus (5, 6) or Theiler's encephalomyelitis virus (54), which clear infection through a noncytolytic, IFN-γ-dependent mechanism (13, 14), our data suggest that IFN-γ does not have a dominant inhibitory role against WNV in the CNS. Consistent with this, our recent adoptive-transfer studies showed efficient clearance of infection in the spleen and CNS by IFN-γ-deficient WNV-primed $CD8^+$ T cells (61).

Our experiments with IFN-$\gamma^{-/-}$ and IFN-$\gamma R^{-/-}$ mice show earlier dissemination of WNV to the CNS, with at least 3- to 5-$\log_{10}$-higher levels of viral replication in the brain and spinal cord at days 4 and 6 after infection, respectively. Earlier entry into the CNS by encephalitic flaviviruses was previously observed in mice that lack functional type I IFN-α and -β responses (48, 55). Notably, normal levels of type I IFN were observed in the sera of IFN-$\gamma^{-/-}$ mice; thus, IFN-γ does not appear to be essential for amplifying the type I IFN response after WNV infection. Early entry into the CNS by WNV correlates directly with the increased infection in peripheral lymphoid tissues and viremia. The direct mechanism for enhanced WNV neuroinvasiveness in IFN-$\gamma^{-/-}$ mice remains uncertain. Although the clearance of WNV from the bloodstream is linked to the development of neutralizing IgM antibodies (21), no significant differences in WNV-specific antibody responses were observed throughout the infection time course. A previous study showed that Toll-like receptor-3-dependent production of TNF-α at days 2 and 3 after infection enhanced blood-brain barrier permeability and invasion of the CNS by WNV (68). However, the early entry into the CNS by WNV in IFN-$\gamma^{-/-}$ mice appeared to be independent of TNF-α: (i) no difference in blood-brain barrier permeability, as judged by Evans' blue staining, was seen among wild-type and IFN-$\gamma^{-/-}$ mice, (ii) the levels of TNF-α in serum were not statistically different until day 8 after infection, and (iii) the administration of a neutralizing TNF-α antibody had no effect of the enhanced mortality observed in IFN-$\gamma^{-/-}$ mice. Although future studies are required to define precisely how IFN-γ regulates WNV neuroinvasion, myeloid cells have been hypothesized to carry viruses in the brain by a "Trojan horse" mechanism (10, 28, 36, 62). Indeed, a recent study shows that equine monocytes support productive WNV replication in blood (27). Based on our infection data with DCs, IFN-γ could limit myeloid cell infection in the intravascular compartment and the transport of infected cells across the blood-brain barrier.

In summary, our experiments demonstrate an essential protective role for IFN-γ in WNV infection. Innate immune responders, including γδ T cells, generate IFN-γ after WNV infection in peripheral lymphoid tissues. We show here that IFN-γ appears to function as an antiviral agent, possibly limiting infection in DCs and other myeloid cells. This decreased viral burden prevents early dissemination to the CNS and infection and allows the adaptive immune response to develop before significant irreversible damage to neurons has occurred.

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