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# Beta Interferon Controls West Nile Virus Infection and Pathogenesis in Mice[▽]

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**Studies with mice lacking the common plasma membrane receptor for type I interferon (*IFN*-αβ$R^{-/-}$) have revealed that IFN signaling restricts tropism, dissemination, and lethality after infection with West Nile virus (WNV) or several other pathogenic viruses. However, the specific functions of individual IFN subtypes remain uncertain. Here, using *IFN*-β$^{-/-}$ mice, we defined the antiviral and immunomodulatory function of this IFN subtype in restricting viral infection. *IFN*-β$^{-/-}$ mice were more vulnerable to WNV infection than wild-type mice, succumbing more quickly and with greater overall mortality, although the phenotype was less severe than that of *IFN*-αβ$R^{-/-}$ mice. The increased susceptibility of *IFN*-β$^{-/-}$ mice was accompanied by enhanced viral replication in different tissues. Consistent with a direct role for IFN-β in control of WNV replication, viral titers in *ex vivo* cultures of macrophages, dendritic cells, fibroblasts, and cerebellar granule cell neurons, but not cortical neurons, from *IFN*-β$^{-/-}$ mice were greater than in wild-type cells. Although detailed immunological analysis revealed no major deficits in the quality or quantity of WNV-specific antibodies or CD8$^+$ T cells, we observed an altered CD4$^+$ CD25$^+$ FoxP3$^+$ regulatory T cell response, with greater numbers after infection. Collectively, these results suggest that IFN-β controls WNV pathogenesis by restricting infection in key cell types and by modulating T cell regulatory networks.**

Type I interferons (IFN) comprise a family of related cytokines that were identified originally for their ability to render cells resistant to virus infection (35). In mice, there are at least 14 IFN-α isoforms and one IFN-β isoform, in addition to multiple other subtypes (71). Production of type I IFN after viral infection is triggered by recognition of pathogen-associated molecular patterns (PAMP) on viral proteins or nucleic acids by host sensors known as pattern recognition receptors (PRR) (reviewed in references 36 and 53). PRR for RNA viruses recognize single- or double-stranded RNA motifs on the cell surface, in endosomes (Toll-like receptor 3 [TLR3], TLR7, and TLR8), or in the cytoplasm (MDA5, RIG-I, and LGP2) and activate the downstream transcriptional factors IFN regulatory factor 3 (IRF-3) and IRF-7 to induce IFN gene transcription and protein production (reviewed in references 11 and 33). In many cell types, the immediate response to infection is characterized by PRR signaling of IFN-β and IFN-α4 expression (48) through an IRF-3-dependent pathway. These events lead to local IFN production, which signals in autocrine and paracrine manners to promote expression of IFN-stimulated genes (ISG) with antiviral effector and transcriptional activities, the latter group including IRF-7 (48, 59, 60). Activation of IRF-7 then amplifies the IFN response via a positive feedback loop, resulting in the production of a wider array of IFN-α subtypes. The physiologic relevance of the apparent redundancy of type I IFN subtypes still remains uncertain, although many of these are conserved among animal species, suggesting an important function in host response and defense.

Innate immune responses are required for the control of West Nile virus (WNV) infection (reviewed in references 17 and 54). Type I IFN induction is essential to the host response against WNV, as mice lacking the IFN-α/β receptor (*IFN*-αβ$R^{-/-}$) are vulnerable to WNV infection, with expanded tissue tropism, uncontrolled viral replication, and rapidly uniform death (55). WNV triggers type I IFN production in different cell types through processes involving RIG-I, MDA5 (27), and IPS-1 (17, 65), with subordinate contributions from protein kinase R (PKR) (29), TLR3 (15, 72), TLR7 (70), and MyD88 (66).

Despite the apparent functional redundancy of the type I IFN response and the early induction of the IFN-α4 subtype after viral infection or TLR stimulation (31, 48), IFN-β likely functions to initiate and amplify innate immune signaling. IFN-β binds to the IFN-α/β receptor with higher affinity and triggers more potent and sustained signaling than IFN-α subtypes (6, 20). Although studies of *IFN*-β$^{-/-}$ fibroblasts confirmed it as an immediate-early cytokine and that production of IFN-α was dependent on IFN-β signaling through the IFN-αβR (3, 18, 24, 58), this phenotype has not been consistently observed *in vivo*. IFN-α was produced in *IFN*-β$^{-/-}$ mice in serum or tissue with normal kinetics and magnitude after infection with vesicular stomatitis virus (3), Sindbis virus (10), or Friend virus (28). In comparison, the ISG response in cardiac tissue after coxsackie B3 virus infection was depressed in *IFN*-β$^{-/-}$ mice (19). Through mechanisms that have not been fully

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characterized, IFN-β restricts pathogenesis of several viruses in mice. Increased lethality or viral burden was observed in *IFN*-$\beta^{-/-}$ mice after infection with vaccinia virus (18), influenza A virus (39, 46), coxsackie B virus (19), Sindbis virus (10), and Friend virus (28) but not with La Crosse virus (5).

Apart from phenotyping the number of immune cells in the spleen (28), no study has evaluated the impact of a deficiency of IFN-β on adaptive antiviral immune responses. This is especially surprising given the number of priming functions that have been ascribed to type I IFN (42, 44, 45) and the diminished effector and memory $CD8^+$ T cell responses in *IFN*-$\alpha\beta R^{-/-}$ mice after infection with vaccinia, vesicular stomatitis, or lymphocytic choriomeningitis viruses (40, 69). Although vaccination of *IFN*-$\alpha\beta R^{-/-}$ mice with DNA plasmids against Rift Valley fever or bluetongue viruses induced neutralizing antibody and protected against lethal virus challenge (26, 47), immunization or infection of complete or conditional *IFN*-$\alpha\beta R^{-/-}$ mice with vesicular stomatitis virus-like particles or virus failed to promote efficient immunoglobulin class switching, plasma cell induction, or germinal center reactions (2, 25).

Herein, we evaluated the antiviral and immunomodulatory function of IFN-β in restricting WNV infection. *IFN*-$\beta^{-/-}$ mice were more vulnerable to WNV infection, although the phenotype was less severe than in *IFN*-$\alpha\beta R^{-/-}$ mice. The increased susceptibility of *IFN*-$\beta^{-/-}$ mice was accompanied by enhanced viral replication in subsets of tissues and primary cell types. Although detailed analysis revealed only minor deficits in the quality or quantity of WNV-specific antibodies or $CD8^+$ T cells, we observed an altered $CD4^+$ $CD25^+$ $FoxP3^+$ regulatory T cell response. Overall, our results suggest that IFN-β restricts WNV in a cell- and tissue-specific manner, with remarkably few nonredundant effects on induction of adaptive immune responses.

## MATERIALS AND METHODS

**Mouse experiments.** C57BL/6 wild-type inbred mice were commercially obtained (Jackson Laboratories, Bar Harbor, ME). The congenic, backcrossed *IFN*-$\beta^{-/-}$ mice (68) were the generous gift of R. Schreiber (St. Louis, MO) and T. Taniguchi (Tokyo, Japan) and contain a *LacZ* insert in place of the mouse IFN-β gene. *IFN*-$\alpha\beta R^{-/-}$ mice backcrossed onto a C57BL/6 background have been described previously (13). All mice were genotyped and bred in the animal facilities of the Washington University School of Medicine, and experiments were approved and performed in accordance with Washington University animal studies guidelines. Eight- to 12 week-old mice were used for all *in vivo* studies. For peripheral infection, $10^2$ PFU of WNV 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 μl.

**Viruses.** The WNV strain (3000.0259) was isolated in New York in 2000 and passaged once in C6/36 *Aedes albopictus* cells to generate a stock virus that was used in all experiments.

**Quantification of tissue viral burden and viremia.** To monitor viral spread *in vivo*, mice were infected with $10^2$ PFU of WNV by footpad inoculation and sacrificed at days 1, 2, 4, 6, and 8 after inoculation. After extensive cardiac perfusion with phosphate-buffered saline (PBS), organs were harvested, weighed, and homogenized, and virus titers were determined by standard plaque assay as previously described (23). Viral burden in serum and draining lymph nodes was measured by analyzing WNV genomic RNA levels using fluorogenic quantitative reverse transcription-PCR (qRT-PCR) as previously described (57).

**Quantification of IFN activity.** Levels of biologically active type I IFN in serum were determined using an encephalomyocarditis virus (EMCV) L929 cell cytopathic effect bioassay as described previously (14).

**Primary cell infections. (i) Macrophages and dendritic cells.** Macrophages and dendritic cells were generated as described previously (14) after cell isolation from the bone marrow of wild-type or *IFN*-$\beta^{-/-}$ mice and culturing for 7 days either in the presence of 40 ng/ml macrophage colony-stimulating factor (M-CSF; PeproTech Inc., Rocky Hill, NJ) to generate macrophages or with 20 ng/ml granulocyte-macrophage CSF (GM-CSF) and 20 ng/ml interleukin-4 (IL-4; PeproTech Inc., Rocky Hill, NJ) to generate dendritic cells. Multistep virus growth curves were performed after infection at a multiplicity of infection (MOI) of 0.01 for macrophages or 0.001 for dendritic cells. Supernatant titers were determined by plaque assay on BHK21 cells (23).

**(ii) MEF.** Murine embryonic fibroblasts (MEF) were generated from day 15 wild-type or *IFN*-$\beta^{-/-}$ embryos and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, L-glutamine, and nonessential amino acids as described previously (4). Multistep virus growth curves were performed after infection at an MOI of 0.01.

**(iii) Cortical neurons.** Cortical neurons were prepared from day 15 wild-type and *IFN*-$\beta^{-/-}$ mouse embryos as described previously (37, 56). Neurons were seeded in 24-well poly-D-lysine/laminin-coated plates and cultured for 4 days with Neurobasal medium containing B27, L-glutamine, and penicillin-streptomycin (Invitrogen). Multistep virus growth curves were generated after infection at an MOI of 0.001.

**(iv) Cerebellar granule neurons.** Primary cultures of purified granule cell neurons from 6-day-old wild-type and *IFN*-$\beta^{-/-}$ mice were prepared as described previously (38). Multistep virus growth curves were performed after infection at an MOI of 0.001.

**WNV-specific antibody and 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 (49). The amount of neutralizing antibody in serum was quantitated in a focus-forming reduction assay that was adapted for WNV from previous studies with dengue virus (7). For T cell analysis, splenocytes were harvested from wild-type or *IFN*-$\beta^{-/-}$ mice on day 7 after infection. Intracellular IFN-γ or tumor necrosis factor alpha (TNF-α) staining was performed using a previously identified $D^b$-restricted NS4B peptide in a restimulation assay with 1 μM peptide and 5 μg/ml of brefeldin A (Sigma) as described in reference 52. The number and percentage of $CD4^+$ $CD25^+$ $FoxP3^+$ regulatory T cells in the spleens of naïve or WNV-infected wild-type or *IFN*-$\beta^{-/-}$ mice were measured using the regulatory T cell staining kit (Ebioscience). Samples were processed by multicolor flow cytometry on an LSR II flow cytometer (Becton Dickinson) and analyzed with FlowJo software (Treestar).

**CNS leukocyte isolation and phenotyping.** Quantification of infiltrating central nervous system (CNS) lymphocytes was based on a published protocol (67). Briefly, wild-type and *IFN*-$\beta^{-/-}$ mouse brains were harvested on day 7 after infection, minced, and digested with 0.05% collagenase D, 0.1 μg/ml trypsin inhibitor *N*α-*p*-tosyl-L-lysine chloromethyl ketone, and 10 μg/ml DNase I in HBSS supplemented with 10 mM HEPES, pH 7.4 (Life Technologies). Cells were dispersed into single-cell suspensions with a cell strainer and pelleted through a 30% Percoll cushion for 30 min (850 × *g* at 4°C). Cells were counted and either stained for CD4, CD8, CD45, and CD11b with directly conjugated antibodies (BD Pharmingen) or for intracellular IFN-γ or TNF-α to identify the WNV-specific CD8 T cells with the $D^b$-restricted NS4B peptide in a restimulation assay with 1 μM peptide and 5 μg/ml of brefeldin A (Sigma) as described previously (52).

**Statistical analysis.** Viral growth curves were analyzed by using a 2-way analysis of variance (ANOVA) to determine statistically significant differences. For viral burden, serum bioassay, antibody, and lymphocyte analyses, differences were analyzed by using the Mann-Whitney test. Kaplan-Meier survival curves were analyzed by the log rank test. All data were analyzed using Prism software (GraphPad, San Diego, CA).

## RESULTS

**IFN-β is required for control of WNV infection *in vivo*.** Mice lacking IFN-α/β receptors are exquisitely vulnerable to WNV infection due to rapid and overwhelming viral replication and altered tissue tropism (55). Analogously, *IRF-7*$^{-/-}$ mice, which have strongly reduced IFN-α yet largely intact IFN-β responses (34), also show early mortality (16). To begin to understand the specific nonredundant function of IFN-β and its contribution to antiviral and adaptive immunity, we infected wild-type and *IFN*-$\beta^{-/-}$ C57BL/6 mice with a pathogenic strain of WNV. After footpad inoculation with $10^2$ PFU of WNV, *IFN*-$\beta^{-/-}$ mice showed an increased rate and severity of clinical signs of illness, including fur ruffling and reduced activity. Whereas wild-type mice showed an ~60% survival rate

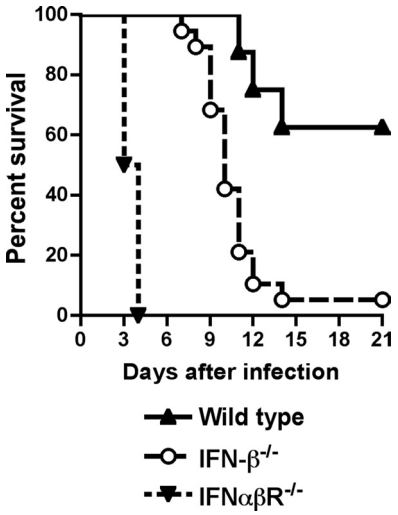


FIG. 1. Survival analysis of wild-type, *IFN*-αβ$R^{-/-}$, and *IFN*-$\beta^{-/-}$ C57BL/6 mice. Eight- to 12-week-old age-matched wild-type ($n = 8$), *IFN*-αβ$R^{-/-}$ ($n = 10$), and *IFN*-$\beta^{-/-}$ ($n = 19$) mice were inoculated subcutaneously with $10^2$ PFU of WNV, and mortality was observed for 21 days. Survival differences were tested for statistical significance by the log rank test ($P < 0.001$).

and a mean time of death of 12.3 days after WNV infection, *IFN*-$\beta^{-/-}$ mice were more vulnerable, with an ~5% survival rate and a mean time of death of 10.2 days ($P < 0.0001$) (Fig. 1). A similar increase in lethality was observed after WNV infection when mice were pretreated with a neutralizing anti-IFN-β monoclonal antibody (MAb) but not isotype control MAb (data not shown). Although a genetic or acquired absence of IFN-β conferred increased vulnerability to WNV infection in mice, it caused a less severe phenotype than that observed in animals lacking IRF-7 (16) or IFN-αβR (Fig. 1).

**WNV replicates at enhanced levels in selected tissues of IFN-$\beta^{-/-}$ mice.** As IFN-β induces an antiflavivirus response during the initial phases of infection in cell culture (22), we hypothesized that the increased lethality of *IFN*-$\beta^{-/-}$ mice would correlate with higher viral burdens in tissue. To evaluate this, *IFN*-$\beta^{-/-}$ and wild-type mice were infected with $10^2$ PFU of WNV, and viral burden was measured by fluorogenic quantitative RT-PCR or viral plaque assay at days 2, 4, 5, 6, and 8 after infection in serum, peripheral organs (draining lymph nodes, spleen, and kidney) and the CNS (brain and spinal cord).

**(i) Peripheral tissues.** Within 2 days of WNV infection, higher levels (~75-fold; $P < 0.02$) of viral RNA were detected in the lymph nodes of *IFN*-$\beta^{-/-}$ mice. Increased RNA levels (375-fold; $P < 0.001$) persisted in *IFN*-$\beta^{-/-}$ mice through day 6 (Fig. 2A). In serum, although small increases in viral RNA were observed at days 2 and 4 after infection in *IFN*-$\beta^{-/-}$ mice, these did not attain statistical significance (Fig. 2B) ($P > 0.05$). However, by day 5 after infection, elevated levels were observed (13-fold; $P < 0.009$) compared to wild-type mice, suggesting sustained replication or a delay in the clearance phase of WNV from serum. Based on this analysis, IFN-β appears to restrict WNV replication more significantly at early times after infection in the draining lymph nodes than in the intravascular compartment.

Consistent with earlier studies (14, 57), WNV was not detected in the spleen by plaque assay at day 2 after infection in wild-type mice. Similarly, no *IFN*-$\beta^{-/-}$ mice had measurable infection at day 2 (Fig. 2C). By day 4, which corresponds to the peak of spleen infection in wild-type animals, a trend toward higher WNV titers was observed in *IFN*-$\beta^{-/-}$ mice was observed (mean titer, $10^{4.2}$ PFU/g for *IFN*-$\beta^{-/-}$ versus $10^{3.7}$ PFU/g for wild type), although this did not reach significance ($P > 0.1$). Overall, there were few differences in the kinetics of clearance or magnitude of viral burden in the spleens of *IFN*-$\beta^{-/-}$ and wild-type mice. The kidneys in wild-type C57BL/6 mice were relatively resistant to WNV infection, as virus is not usually detected in this organ. In contrast, in mice lacking cellular intrinsic immunity (e.g., *IFN*-α/β$R^{-/-}$, *IRF-7*$^{-/-}$, or *IRF-3*$^{-/-}$ mice [14, 16, 55]), WNV replication is detected in the kidneys, suggesting that type I IFN responses restrict WNV tissue tropism. Notably, in *IFN*-$\beta^{-/-}$ mice WNV replication was observed (day 6, $10^{2.5}$ PFU/g [$P < 0.05$]; day 8, $10^{2.6}$ PFU/g [$P > 0.1$]) (Fig. 2D), but the phenotype was subtle compared to that seen with other mice lacking intact type I IFN responses. Thus, an absence of IFN-β results in limited increases of WNV replication in visceral organs, likely because other type I IFN subtypes (e.g., IFN-α) provide redundant restrictive effects on infection.

**(ii) CNS tissues.** We observed no difference in the time of onset of WNV replication in the brain in *IFN*-$\beta^{-/-}$ mice. Infectious virus was first detected in the brains of wild-type and deficient mice at day 6 (Fig. 2E). However, once WNV entered the brain, it accumulated to higher levels in *IFN*-$\beta^{-/-}$ mice. By day 8, we observed 15-fold-higher levels of WNV in the brains of all *IFN*-$\beta^{-/-}$ mice ($10^{4.7}$ PFU/g for *IFN*-$\beta^{-/-}$ versus $10^{3.5}$ PFU/g for wild type; $P < 0.05$). A similar pattern was observed in the spinal cord, although there were some differences. We did detect earlier spinal cord infection at day 6 in a subset (5 of 12) of *IFN*-$\beta^{-/-}$ mice, although this did not achieve statistical significance ($P > 0.1$). Consistent with the results in the brain, increased replication in the spinal cord was observed at day 8 in *IFN*-$\beta^{-/-}$ mice ($10^{3.7}$ PFU/g for *IFN*-$\beta^{-/-}$ versus $10^{2.6}$ PFU/g for wild type; $P = 0.04$). These data suggest that an absence of IFN-β signaling more dominantly affected WNV replication within the CNS rather than spread to the CNS.

**Levels of type I IFN in circulation of *IRF*-$\beta^{-/-}$ mice.** To assess whether induction of the other type I IFN subtypes might compensate for the absence of IFN-β, mice were infected with WNV, and the level of biologically active type I IFN in serum was monitored in a validated EMCV L929 cell bioassay (1). Notably, we observed no decrease in type I IFN levels in sera of *IFN*-$\beta^{-/-}$ mice relative to the wild-type mice; instead, as has been observed in *IPS-1*$^{-/-}$, *IRF-3*$^{-/-}$, *TLR7*$^{-/-}$, or *MyD88*$^{-/-}$ mice after WNV infection (14, 65, 66, 70), significantly higher levels (3- to 50-fold; $P < 0.02$) of type I IFN activity were observed at several time points, possibly due to enhanced viral replication in the immunodeficient mice (Fig. 3).

**An absence of IFN-β results in increased WNV infection in most but not all primary cell types.** To begin to investigate the cell-specific function of IFN-β in restricting WNV infectivity, we compared multistep growth kinetics in several different wild-type and *IFN*-$\beta^{-/-}$ primary cells, including macrophages, dendritic cells, fibroblasts, and two types of neurons, after

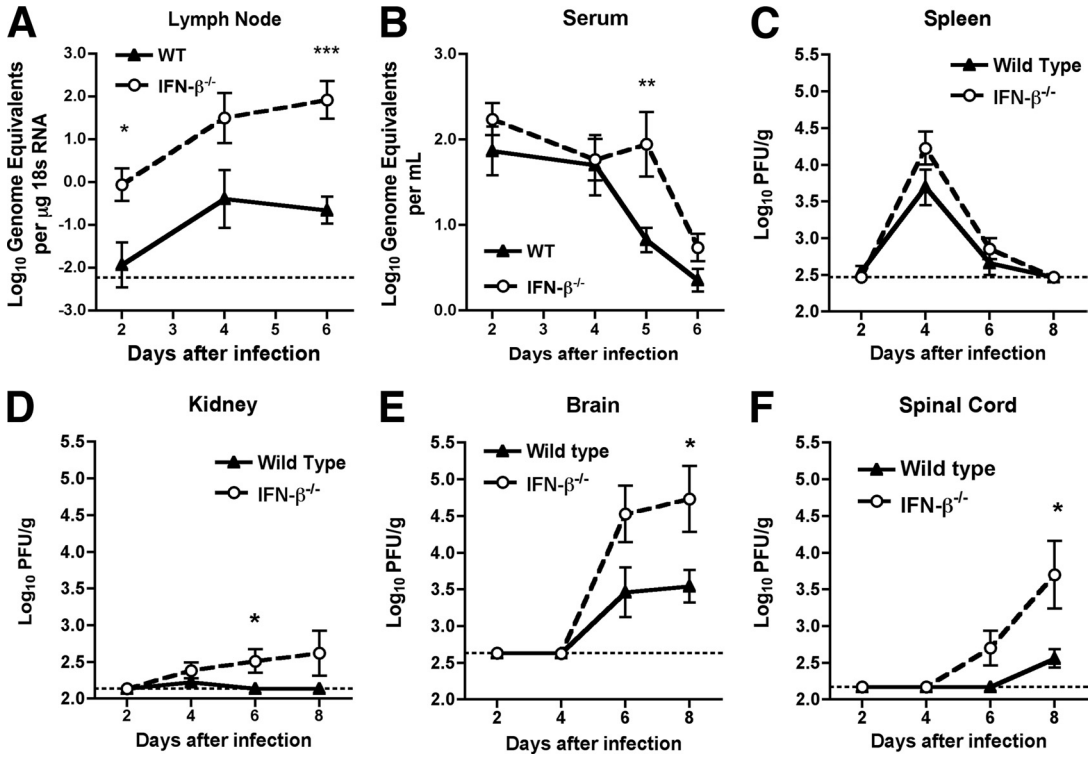


FIG. 2. Viral burden in peripheral and central nervous system tissues after subcutaneous WNV infection. Wild-type and *IFN*-$\beta^{-/-}$ C57BL/6 mice were infected subcutaneously with $10^2$ PFU of WNV, and viral burden was measured by qRT-PCR (A and B) or plaque assay in BHK21 cells (C to F). For WNV RNA measurements, viral burdens on days 2 through 6 are expressed as genome equivalents per μg 18S RNA (lymph node) or per ml (serum). Infectious virus in the spleen (C), kidney (D), brain (E), or spinal cord (F) was measured on days 2, 4, 6, and 8 after infection and is expressed as PFU per gram of tissue. Lines represent the means ± standard errors of 8 to 12 mice per time point, and the dotted line represents the limit of sensitivity of the assay. Asterisks indicate values that were statistically significant: *, $P < 0.05$; **, $P < 0.01$, ***, $P < 0.001$, compared to wild-type mice, based on the Mann-Whitney test.

WNV infection at a low MOI (0.001 to 0.01). In myeloid cells an absence of IFN-β resulted in enhanced WNV replication. For example, 8- to 30-fold ($P < 0.05$) increased WNV titers were observed in *IFN*-$\beta^{-/-}$ bone marrow-derived macrophages and dendritic cells between 24 and 72 h after infection (Fig. 4A and B). Analogous results were observed in macrophages pretreated with a neutralizing IFN-β MAb prior to infection (data not shown); thus, *IFN*-$\beta^{-/-}$ cells do not have intrinsic antiviral defects associated with development in the absence of IFN-β in mice. Somewhat smaller (3- to 7-fold) yet still significant ($P < 0.05$) increases also were detected in *IFN*-$\beta^{-/-}$ MEF and cerebellar granule cell neurons (Fig. 4C and D). However, no change in WNV replication was observed in *IFN*-$\beta^{-/-}$ cortical neurons compared to wild-type cells ($P > 0.05$) (Fig. 4E). Notably, these data contrast with results from cortical neurons lacking IRF-7 (16) or IFN-αβR (55), both of which showed ~10-fold increases in replication. Thus, an absence of IFN-β showed a cell-specific effect on altered WNV replication, with lesser effects in neurons.

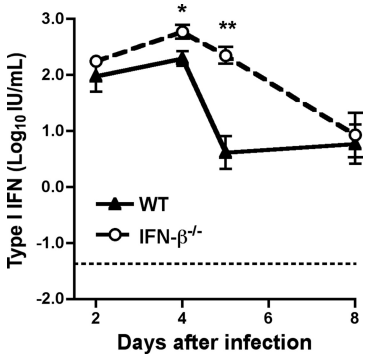


FIG. 3. Type I IFN levels in serum of wild-type and *IFN*-$\beta^{-/-}$ mice after infection with WNV. Mice were inoculated subcutaneously with $10^2$ PFU of WNV, and serum was collected on days 2 to 8 after infection. Type I IFN activity was determined in an EMCV bioassay in L929 cells, and IFN levels were calculated from an IFN-α standard curve. Data represent the means ± standard errors of 8 to 12 mice per time point, and the dotted line represents the limit of sensitivity of the assay. Asterisks indicate values that were statistically significant: *, $P < 0.05$; **, $P < 0.01$, compared to wild-type mice, based on the Mann-Whitney test.

**Effects of IFN-β on antibody responses after WNV infection.** Type I IFN has been reported to enhance isotype switching and humoral immune responses *in vivo* by stimulating dendritic cells (43, 44), as well as having direct effects on B cells (12, 25, 51). Remarkably, the specific nonredundant contribution of IFN-β to the antiviral humoral response *in vivo* remains unknown. Because of this, and the observation that a depressed antiviral antibody response independently facilitates WNV replication in the brain (23), we evaluated the virus-specific humoral immune responses in *IFN*-$\beta^{-/-}$ mice (Fig. 5). For WNV-specific IgM, we observed lower levels in *IFN*-$\beta^{-/-}$ mice

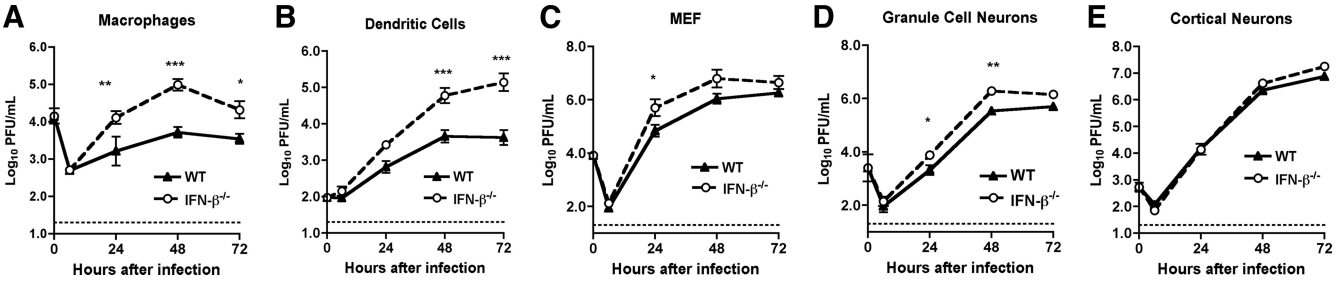


FIG. 4. Multistep growth curves in wild-type and *IFN*-$\beta^{-/-}$ primary cells. Primary cells from wild-type and *IFN*-$\beta^{-/-}$ mice were infected with WNV, and viral replication from 0 to 72 h was measured by plaque assay. (A) Bone marrow-derived macrophages (MOI, 0.01); (B) bone marrow-derived dendritic cells (MOI, 0.001); (C) mouse embryonic fibroblasts (MOI, 0.01); (D) cerebellar granule cell neurons (MOI, 0.001); (E) cortical neurons (MOI, 0.001). Data represent the means ± standard errors from two to three independent experiments performed in triplicate, and the dotted lines represent the limit of sensitivity of the plaque assay. Asterisks indicate values that were statistically significant: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, compared to wild-type cells, by a two-way ANOVA.

at day 4, which approached but did not quite attain statistical significance ($P = 0.053$; $n = 8$ to 12) (Fig. 5A). Despite the trend toward higher viral burden in lymphoid tissue at this time, only 1 of 12 *IFN*-$\beta^{-/-}$ mice, compared to 5 of 8 wild-type mice, had induced anti-WNV IgM titers above our cutoff of a 1/20 dilution of serum. At days 6 and 8, no appreciable differences in WNV IgM levels were observed between *IFN*-$\beta^{-/-}$ and wild-type mice. Similar levels of WNV-specific IgG also were detected in *IFN*-$\beta^{-/-}$ and wild-type mice at days 6 and 8 after infection ($P > 0.4$, $n = 7$ to 11) (Fig. 5B), and no defect in the development of neutralizing antibodies was observed ($P > 0.5$, $n = 7$ to 12) (Fig. 5C). Thus, although small differences were observed early in the humoral response, an absence of IFN-β had no significant impact on WNV-specific antibody levels. Thus, the virologic phenotype observed in *IFN*-$\beta^{-/-}$ mice likely was not due to major defects in B cell function.

**Effects of IFN-β on peripheral T cell responses after WNV infection.** As cytolytic $CD8^+$ T cells are also required for the control and clearance of WNV (61), we evaluated whether a deficiency of IFN-β affected priming of antigen-specific $CD8^+$ T cells. Because a prior study had reported some differences in the composition of splenocytes in *IFN*-$\beta^{-/-}$ mice (28), we initially evaluated the percentage and total number of $CD8^+$ T cells at day 7 after WNV infection. Notably, although a slightly lower percentage (9.5% compared to 12%; $P < 0.04$) of $CD8^+$ T cells was detected in the spleens of *IFN*-$\beta^{-/-}$ mice, this did not reflect a quantitative defect in number (Fig. 6A and B). To evaluate antigen-specific $CD8^+$ T cell responses, splenocytes from WNV-infected wild-type or *IFN*-$\beta^{-/-}$ mice were restimulated with a $D^b$-restricted immunodominant NS4B peptide (8, 52). Antigen specificity was inferred after intracellular staining of IFN-γ or TNF-α in $CD8^+$ T cells using flow cytometry. Notably, no statistical differences were observed in the number or percentage of IFN-$\gamma^+$ $CD8^+$ or TNF-$\alpha^+$ $CD8^+$ T cells in the spleen after WNV-specific peptide restimulation ($P > 0.3$) (Fig. 6C to F). We also analyzed $CD4^+$ T cell responses at day 7 after infection, as both effector and regulatory $CD4^+$ T cell responses protect against WNV infection in mice and humans (9, 41, 63). We observed no differences in the percentage or number of total or $CD4^+$ T cells in the spleens of *IFN*-$\beta^{-/-}$ mice compared to wild-type mice (Fig. 7A and B). However, we did detect an elevated percentage (14 versus 9%; $P < 0.002$) and number ($8.3 \times 10^5$ versus $3.1 \times 10^5$ cells; $P < 0.001$) of $CD4^+$ $CD25^+$ $FoxP3^+$ regulatory T cells in WNV-infected

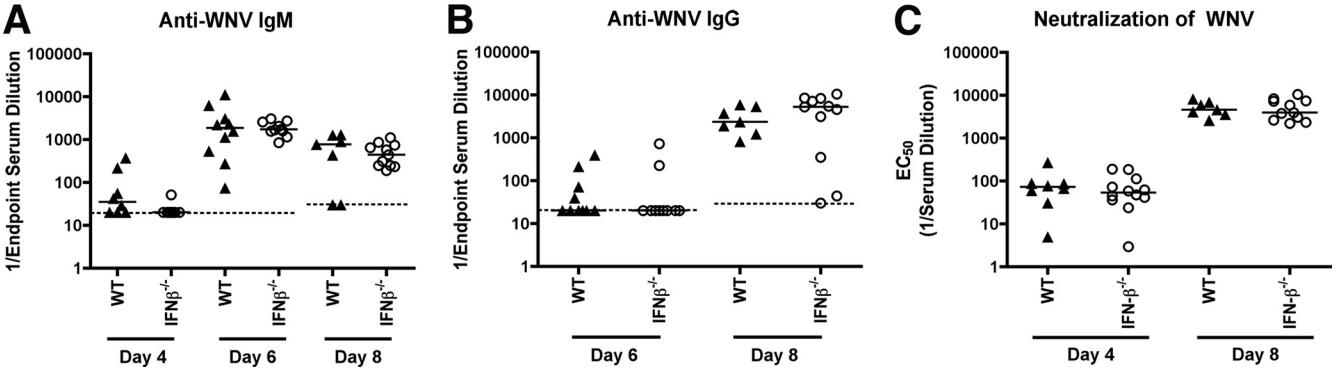


FIG. 5. Antibody responses after WNV infection in *IFN*-$\beta^{-/-}$ mice. (A) Wild-type and *IFN*-$\beta^{-/-}$ mice were inoculated with $10^2$ PFU of WNV by subcutaneous injection, and serum samples collected on days 4, 6, and 8 were assayed by ELISA for WNV-E-specific IgM (A) and IgG (B). Titers are expressed as the reciprocal serum dilution that was 3 standard deviations above background after subtraction of values for serum from a naïve mouse assayed on the same ELISA plate. Differences were not statistically significant ($P > 0.05$) as judged by the Mann-Whitney test. Data are from at least 10 mice per group. Dotted lines indicate the limit of detection of the assay. (C) Sera from the indicated days after infection also were tested for neutralizing activity in a focus reduction assay. Indicated data points represent serum dilutions that reduced focus formation by 50%. No differences were statistically significant as judged by the Mann-Whitney test. Data are from 7 to 12 mice per group.

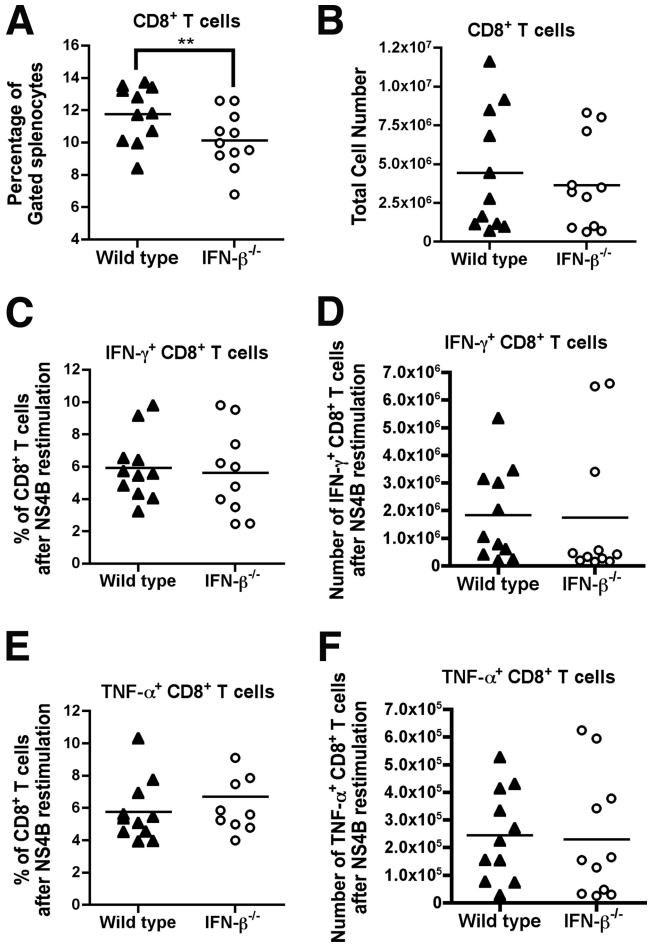


FIG. 6. Peripheral $CD8^+$ T cell responses in wild-type and *IFN*-$\beta^{-/-}$ mice. Wild-type and *IFN*-$\beta^{-/-}$ mice were inoculated subcutaneously with $10^2$ PFU of WNV, and spleens were harvested at day 7 after infection. (A and B) Total percentage and number of $CD3^+$ $CD8^+$ T cells. (C to F) Leukocytes were stimulated *ex vivo* with $D^b$-restricted NS4B peptide, stained for CD8β and intracellular IFN-γ or TNF-α, and analyzed by flow cytometry. Summaries of the percentage (C and E) and total number (D and F) of $CD8^+$ T cells that stained positive for intracellular IFN-γ (C and D) or TNF-α (E and F) after peptide restimulation are shown. Data represent results for 9 to 11 mice from three independent experiments. Asterisks indicate values that were statistically significant (**, $P < 0.05$) compared to wild-type mice by the Mann-Whitney test.

*IFN*-$\beta^{-/-}$ mice; part of this increase reflected a higher regulatory T cell tone in naive *IFN*-$\beta^{-/-}$ mice (Fig. 7C and D).

**Immune cell responses in the brains of IFN-$\beta^{-/-}$ mice after WNV infection.** The accumulation in the brain of inflammatory leukocytes, including $CD8^+$ T cells, can limit WNV infection and neuronal injury (61, 62, 73). To assess whether IFN-β modulated this, leukocytes were recovered from the brains of wild-type and *IFN*-$\beta^{-/-}$ mice at day 7 after infection. Equivalent percentages and numbers of $CD4^+$, $CD4^+$ $CD25^+$ $FoxP3^+$, $CD11b^+$, $CD11c^+$, and $B220^+$ cells were observed in the brains of WNV-infected wild-type and *IFN*-$\beta^{-/-}$ mice (Fig. 8A to E and data not shown). Similarly, no differences in the number or percentage of IFN-$\gamma^+$ $CD8^+$ or TNF-$\gamma^+$ $CD8^+$ T cells were detected in the brains of *IFN*-$\beta^{-/-}$ compared to wild-type mice after *ex vivo* restimulation with a $D^b$-restricted NS4B peptide (Fig. 8F and G and data not shown). Thus, a deficiency of IFN-β did not alter accumulation of immune effector cells in the brains of WNV-infected mice.

## DISCUSSION

Type I IFN signaling programs limit infectivity of a wide array of RNA and DNA viruses in cell culture and animals. Because there are at least 14 IFN-α and 1 IFN-β isoforms in addition to multiple other subtypes in mice (71), the physiologic relevance for host defense via any given type I IFN subtype has remained uncertain. IFN-β has been proposed to have a central regulatory function, because in many cell types the immediate-early type I IFN response is characterized by an early induction of IFN-β (74). Here, we demonstrated that IFN-β is an essential type I IFN subtype, serving nonredundant antiviral and immunomodulatory effects in the context of WNV infection. Mice lacking IFN-β had greater lethality and viral burden, with significantly increased WNV infection in several but not all tissues. The phenotype, however, was less severe than in *IFN*-αβ$R^{-/-}$ or *IRF-7*$^{-/-}$ mice, suggesting that IFN-α and perhaps other type I IFN subtypes (e.g., IFN-κ) also have regulatory properties *in vivo*. Although no major deficits in adaptive immunity were detected, *IFN*-$\beta^{-/-}$ mice manifested an enhanced peripheral $CD4^+$ $CD25^+$ $FoxP3^+$ regulatory T cell response after WNV infection.

Experiments in *IFN*-$\beta^{-/-}$ mice surprisingly showed no reduction in systemic levels of type I IFN after WNV infection; indeed, they were noticeably higher on several days. This result contrasts with previous studies with *IRF-7*$^{-/-}$ mice, in which the amplification loop was abolished and IFN-α response in circulation after viral infection was blunted (16, 34, 64). However, similarly elevated systemic type I IFN levels were observed after WNV infection in *TLR-7*$^{-/-}$, *MyD88*$^{-/-}$, *IPS-1*$^{-/-}$, and *IRF-3*$^{-/-}$ mice (14, 65, 66, 70). Despite the elevated systemic IFN responses in *IFN*-$\beta^{-/-}$ mice, viral burden was greater in several tissues; the most likely explanation for the increased IFN response in serum was that a deficiency of IFN-β in selected peripheral tissues (e.g., lymph nodes) supported enhanced WNV replication and type I IFN production via IFN-β-independent pathways, including IFN-α4 (48) and IRF-3 (30).

Despite the existence of several independent lines of *IFN*-$\beta^{-/-}$ mice generated by different groups (18, 24, 46, 68), no prior study has evaluated in detail the effect of IFN-β on adaptive immunity in the context of infection by any pathogen. This is noteworthy, given the antiviral priming functions ascribed to type I IFN with respect to cross-priming of $CD8^+$ T cells, enhancement of antibody responses, and maintenance of dendritic cells in a state competent for antigen presentation (42, 44, 45, 75). One study group evaluated lymphocyte expansion in *IFN*-$\beta^{-/-}$ mice in the context of Friend retrovirus infection; they observed no difference in the relative percentage of splenic $CD19^+$ B cells, $NK1.1^+$ natural killer cells, or $CD8^+$ T cells, although a decrease in $CD4^+$ T cells was reported (28). In comparison, immune functions in the context of a tumor eradication model have been evaluated. Decreased circulating myeloid cells ($CD11b^+$ or $Gr1^+$), altered splenic architecture, and an apparent defect in early B cell maturation

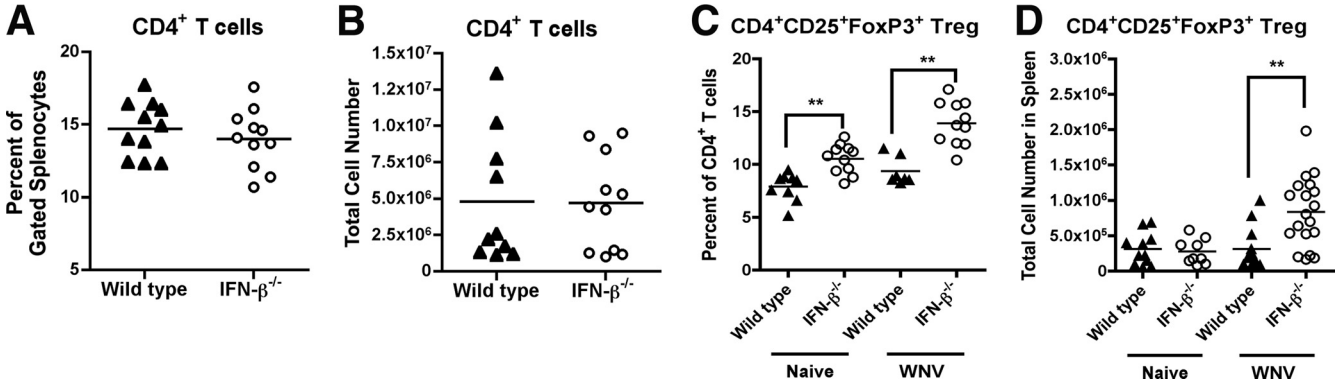


FIG. 7. Peripheral $CD4^+$ and regulatory T cell responses in wild-type and *IFN*-$\beta^{-/-}$ mice. Mice were inoculated subcutaneously with $10^2$ PFU of WNV, and spleens were harvested 7 days after infection. The total percentage (A and C) and number (B and D) of $CD4^+$ (A and B) or $CD4^+$ $CD25^+$ $FoxP3^+$ (C and D) T cells are shown. In addition, the percentage (C) and number (D) of $CD4^+$ $CD25^+$ $FoxP3^+$ cells were also assessed in naïve wild-type and *IFN*-$\beta^{-/-}$ mice as a comparison. Data represent results for at least 10 mice from three to four independent experiments. Asterisks indicate values that were statistically significant (**, $P < 0.01$) compared to wild type mice by the Mann-Whitney test.

($B220^+$ $CD43^+$ pro-B cells) were reported in *IFN*-$\beta^{-/-}$ mice (21). Additionally, *IFN*-$\beta^{-/-}$ bone marrow-derived macrophages or T cells exhibited blunted TNF-α production or hyperproliferative responses and reduced TNF-α levels after *ex vivo* activation with lipopolysaccharide or anti-CD3 and anti-CD28, respectively. These defects translated into more aggressive tumor growth in *IFN*-$\beta^{-/-}$ mice, although no differences in T cell or macrophage infiltration were observed (21).

Our immunological analysis revealed only small effects of IFN-β on the humoral response against WNV. Despite greater replication in peripheral lymphoid organs, there appeared to be a slight delay in the WNV-specific IgM response in the *IFN*-$\beta^{-/-}$ mice, as evidenced by fewer mice developing measurable levels at day 4 and lower mean titers, although the latter approached but did not reach statistical significance. At later time points (days 6 and 8), however, no difference was observed between wild-type and *IFN*-$\beta^{-/-}$ mice in the quality or quantity of virus-specific IgM or IgG responses. These re-

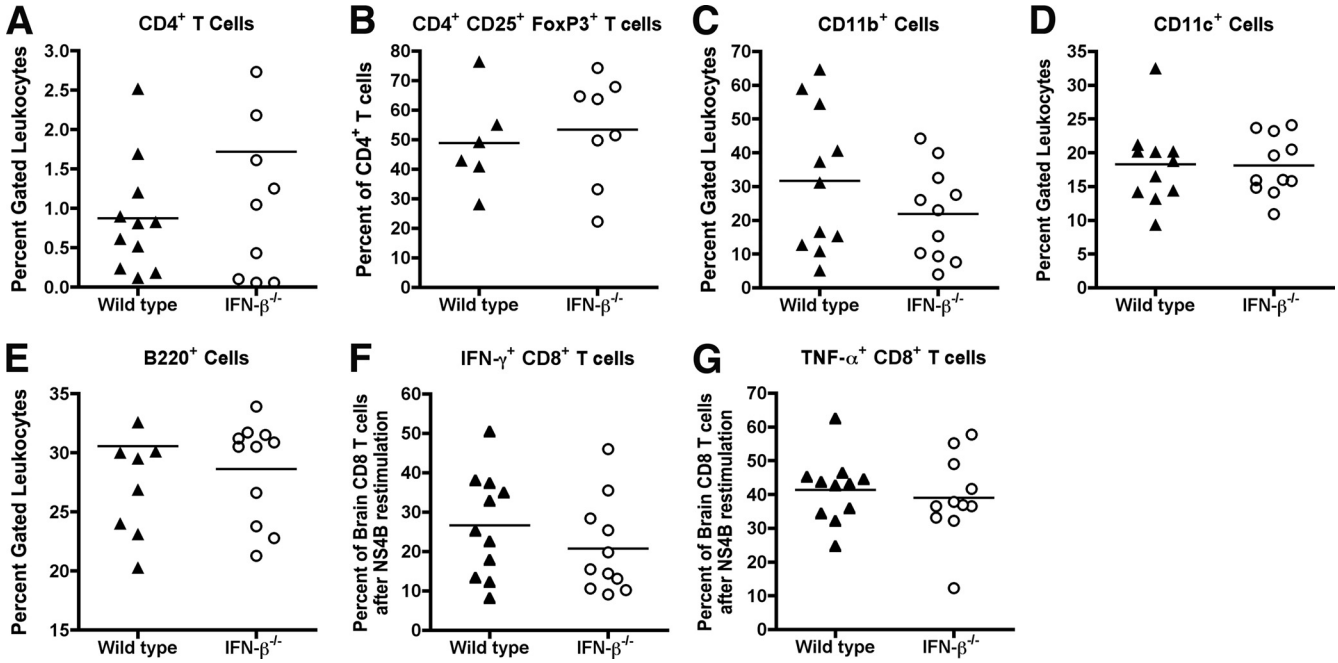


FIG. 8. Leukocyte accumulation in the CNS of *IFN*-$\beta^{-/-}$ mice after WNV infection. Wild-type and *IFN*-$\beta^{-/-}$ mice were infected with $10^2$ PFU of WNV by a subcutaneous route, and 7 days later brains were harvested and leukocytes were isolated after centrifugation through a Percoll cushion. No differences in the percentage of $CD4^+$ (A), $CD4^+$ $CD25^+$ $FoxP3^+$ (B), $CD11b^+$ (C), $CD11c^+$ (D), or $B220^+$ (E) cells were observed between wild-type and *IFN*-$\beta^{-/-}$ mice. Data are from three independent experiments with a total of 6 to 11 mice per group. (F and G) The percentage of WNV-specific brain $CD8^+$ T cells was determined after restimulation with an immunodominant $D^b$-restricted NS4B peptide. Cells were stained for CD8β on their surface, permeabilized, stained intracellularly for IFN-γ (F) or TNF-α (G), and analyzed by flow cytometry. Data are from three independent experiments with a total of 9 to 11 mice per group. The differences were not statistically different as judged by the Mann-Whitney test.

sults, while somewhat surprising given prior study results showing a direct effect of type I IFN on B cell function (44), nonetheless are consistent with results showing no quantitative decrease in WNV-specific IgM and IgG levels in mice deficient in pattern recognition receptors and their cognate adaptor molecules which induce IFN-β, including *TLR3*$^{-/-}$ (15), *MyD88*$^{-/-}$ (66), and *IPS-1*$^{-/-}$ (65) animals.

Although type I IFN promotes the priming and longevity of an adaptive CD8$^+$ T cell response, (42, 45), we did not observe an effect of IFN-β on WNV-specific responses. The number and magnitude of antigen-specific CD8$^+$ T cells secreting IFN-γ or TNF-α were similar in the spleens and brains of infected *IFN*-β$^{-/-}$ and wild-type mice. This observation is noteworthy, because type I IFN facilitates CD8$^+$ T cell activation and maturation through a cross-presentation mechanism, and optimal induction of WNV-specific CD8$^+$ T cells requires CD8α dendritic cells, the primary cell type that mediates antigen cross-presentation *in vivo* (32). Thus, the redundant effects of other type I IFNs (e.g., IFN-α) must be sufficient in WNV-infected *IFN*-β$^{-/-}$ mice to promote cross-presentation and expansion of effector CD8$^+$ T cells in the acute setting.

As part of our immunological analysis, we examined levels of CD4$^+$ CD25$^+$ FoxP3$^+$ regulatory T cells in *IFN*-β$^{-/-}$ mice. A recent study reported that regulatory T cells protect against WNV disease likely by mitigating immune injury. In mice, an acquired deficiency of regulatory T cells was associated with increased lethality, and in humans and mice, lower levels of regulatory T cells were associated with more symptomatic disease (41). Notably, in *IFN*-β$^{-/-}$ mice, we observed a significant increase in the percentage and number of regulatory T cells in the spleens after WNV infection. These findings appear to conflict with recent studies of WNV-infected *IPS-1*$^{-/-}$ mice, in which there was a reported lack of expansion of regulatory T cells (65). Although RIG-I is a dominant pathogen recognition sensor for WNV (27) and it signals through IPS-1, paradoxically, increased levels of type I IFN were observed in *IPS-1*$^{-/-}$ mice, secondary to the enhanced WNV replication in immune cells and peripheral tissues. Thus, higher type I IFN tone may inhibit regulatory T cell expansion. Consistent with this, *ex vivo* studies showed that IFN-α can decrease the suppressive activity and proliferation of regulatory T cells, in part through effects on antigen-presenting cells (50). Thus, although an absence of IFN-β resulted in increased T cell regulatory numbers, which likely reduced WNV immune pathogenesis, the effect was not sufficient to overcome the direct viral injury associated with increased replication in tissues.

Our experiments in cell culture showed an important role of IFN-β in restricting WNV infection. This observation was true in most, but not all, cell types examined, as enhanced viral replication was observed in primary *IFN*-β$^{-/-}$ macrophages, myeloid dendritic cells, MEF, and cerebellar granule cell neurons but not cortical neurons. The latter finding was not entirely surprising, as distinct neuronal subtypes show inherent differences in the ability of IFN-β pretreatment to limit WNV infection (55, 57). Our findings are consistent with increased replication of influenza A virus (39) in *IFN*-β$^{-/-}$ MEF, but they contrast with data for La Crosse virus, which showed no difference in infectivity (5).

In summary, despite the functional redundancies of the type I IFN response, IFN-β in particular is essential for restricting WNV infection *in vivo*. This analysis revealed cell- and tissue-type-specific effects on IFN-β on restricting viral infection and identified novel effects on the regulatory T cell responses. This study, combined with previous work in *IFN*-αβ*R*$^{-/-}$ and *IRF-7*$^{-/-}$ mice, clarifies how individual IFN subtypes orchestrate a protective immune response against WNV and, likely, other pathogenic acute viral infections. A greater understanding of the molecular mechanisms of innate antiviral immune control may foster the development of targeted strategies for therapeutic interventions against viral pathogens.

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