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The Interferon-stimulated gene Ifi27l2a restricts West Nile virus infection and pathogenesis in a cell-type and region-specific manner

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Running title: The ISG Ifi27l2a restricts WNV infection in the CNS

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ABSTRACT

The mammalian host responds to viral infections by inducing expression of hundreds of interferon-stimulated genes (ISGs). While the functional significance of many ISGs has yet to be determined, their cell-type and temporal nature of expression suggests unique activities against specific pathogens. Using a combination of ectopic expression and gene silencing approaches in cell culture, we previously identified Ifi27l2a as a candidate antiviral ISG within neuronal subsets of the central nervous system (CNS) that restricts West Nile virus (WNV) infection, an encephalitic flavivirus of global concern. To investigate the physiological relevance of Ifi27l2a in the context of viral infection, we generated Ifi27l2a−/− mice. Although adult mice lacking Ifi27l2a were more vulnerable to lethal WNV infection, viral burden was greater only within the CNS, particularly in the brain stem, cerebellum, and spinal cord. Within neurons of the cerebellum and brain stem, in the context of WNV infection, a deficiency of Ifi27l2a was associated with less cell death, which likely contributed to sustained viral replication and higher titers in these regions. Infection studies in primary cell culture revealed that Ifi27l2a−/− cerebellar granule cell neurons and macrophages but not cerebral cortical neurons, embryonic fibroblasts, or dendritic cells sustained higher WNV infection compared to wild-type cells, and this difference was greater under conditions of IFN-β pretreatment. Collectively, these findings suggest that Ifi27l2a has an antiviral phenotype in subsets of cells, and that at least some ISGs have specific inhibitory functions in restricted tissues.
IMPORTANCE STATEMENT

The interferon-stimulated gene, Ifi27l2a, is expressed differentially within the central nervous system upon interferon stimulation or viral infection. Prior studies in cell culture suggested an antiviral role for Ifi27l2a during infection by West Nile virus (WNV). To characterize its antiviral activity in vivo, we generated mice with a targeted gene deletion of Ifi27l2a. Based on extensive virological analyses, we determined that Ifi27l2a protects mice from WNV-induced mortality by contributing to the control of infection of the hindbrain and spinal cord, possibly by regulating cell death of neurons. This antiviral activity was validated in granule cell neurons derived from the cerebellum and in macrophages but was not observed in other cell types. Collectively, these data suggest Ifi27l2a contributes to innate immune restriction of WNV in a cell-type and tissue-specific manner.
INTRODUCTION

West Nile virus (WNV) is a positive-stranded, enveloped RNA virus that belongs to the Flavivirus genus of the *Flaviviridae* family. WNV and related flaviviruses typically are transmitted by arthropod vectors and include members that cause encephalitis (e.g., Japanese encephalitis virus (JEV), Saint Louis encephalitis virus (SLEV), and tick-borne encephalitis virus (TBEV)) or systemic and/or visceral disease (e.g., Dengue virus (DENV) and yellow fever virus (YFV)). WNV transmission occurs between *Culex* species mosquitoes and selected avian hosts, with incidental, dead-end infection of horses, humans, and other vertebrate animals. Humans can develop severe disease following WNV infection, as the virus can invade the central nervous system (CNS) and cause flaccid paralysis, meningitis, or encephalitis, often leading to long-term neurological sequelae or death (1). In the CNS, WNV replicates principally in neurons, and infection may lead to focal lesions, cell injury, and cell death within the brain and spinal cord (2-4). Factors governing WNV entry into and replication within the CNS are complex, and include age of the host, genetic background (5-8), quality of the immune response, and integrity of the blood-brain barrier (for review, 9, 10-12).

In response to viral infections, most mammalian cells secrete type I interferon (IFN), which promotes an antiviral state in an autocrine and paracrine manner by inducing expression of hundreds of interferon-stimulated genes (ISGs). The gene signature and inhibitory activity promoted by type I IFNs vary depending on the cell type, specific viral pathogen, and possible pathogen-induced immune evasion mechanisms. Within the CNS, the innate immune response must balance the need to restrict virus infection while simultaneously protecting non-renewable neurons. Indeed, selected regions of the brain and CNS have evolved distinct antiviral programs and mechanisms to restrict infection of different RNA and DNA viruses (13-18). Neurons derived from the cerebral cortex are more permissive to infection by multiple viruses, with IFN-β pretreatment only minimally reducing infection of several viruses (14). In comparison, granule cell neurons (GCN) derived from the cerebellum are less permissive to viral infection at baseline.
state and produce a stronger antiviral response following IFN-β pretreatment. Microarray analysis revealed differences in the basal and induced expression of ISGs in GCN compared to cortical neurons (CN) (14). As an example, *Ifi27l2a* is an ISG expressed at higher levels in GCN compared to CN under basal conditions, after IFN-β pretreatment, or following WNV infection. Ectopic expression of *Ifi27l2a* in CN suppressed infection of a neurotropic flavivirus (WNV) and coronavirus (murine hepatitis virus (MHV)) but not an alphavirus (Venezuelan equine encephalitis virus (VEEV)). Reciprocally, gene silencing of *Ifi27l2a* in GCN resulted in enhanced WNV infection (14).

*Ifi27l2a* (also termed ISG12b) is a 7.9 kDa protein belonging to a larger family of genes that include related *Ifi27/IFI27* genes, and the human *IFI6-16* gene (19), which are distinguished by an “ISG12” motif of unknown function (20). Family members are small, highly hydrophobic and may be localized to either mitochondrial (21, 22) or nuclear membranes (23, 24), although the exact localization has not been fully elucidated. Several *Ifi27* genes are IFN-inducible (19) yet others are not, and among the family members, some orthologs are not conserved across species. As an example, *IFI6-16* is an *IFI27* human gene family member that inhibits infection of YFV, WNV, and hepatitis C virus (HCV) (25-28), but does not have an apparent ortholog in mice. Although *Ifi27l2a* is induced broadly in peripheral organs after IFN stimulation, in the brain it is expressed in selected regions during development in an age-dependent manner (29) with high levels within the hippocampus (30). Cell culture studies have suggested that some *Ifi27* gene orthologs (e.g., ISG12a) promote apoptosis and cell death (21, 31, 32).

*Ifi27l2a* and its closest orthologs have been evaluated as candidate antiviral genes. Despite the strong upregulation of *Ifi27l2a* mRNA in lung tissues, largely by infiltrating immune cells, *Ifi27l2a*−/− mice were not more susceptible to influenza A virus (IAV) infection than wild-type (WT) mice (33). In contrast, ectopic expression of human *IFI27* inhibited HCV and Newcastle disease virus (NDV) infection in hepatocellular carcinoma cells; reciprocally, gene silencing of *IFI27* resulted in increased HCV and NDV infection (28, 34). Apart from its possible antiviral
activity, Ifi27 genes may regulate inflammation, as mice lacking Ifi27l2a sustained less vascular injury (24) and exhibited less septic shock after administration of endotoxin (35).

To characterize further the antiviral effects of Ifi27l2a, we generated Ifi27l2a−/− mice directly in a C57BL/6 background. Ifi27l2a−/− GCN from the cerebellum and bone marrow-derived macrophages (Mφ) supported higher levels of WNV infection. Following infection with WNV in vivo, Ifi27l2a−/− mice exhibited increased mortality and higher viral burden in the cerebellum, brain stem, and spinal cord. The enhanced viral burden in the cerebellum and brain stem of Ifi27l2a−/− mice was associated with less death of neurons at early stages of CNS infection. Our findings suggest that Ifi27l2a contributes to an antiviral state against WNV within the CNS, and protects subsets of cell types and regions of the brain against infection.
MATERIALS AND METHODS

Virus. A WNV-New York (WNV-NY) stock was generated in C6/36 Aedes albopictus cells (ATCC) from a single passage of strain 3000.0259 isolated from a mosquito in New York in 2000 (36). The WNV Madagascar strain (WNV-MAD) stock was generated by passaging virus in Vero or C6/36 cells as described previously (37). WNV titers were assessed by plaque assay on BHK21-15 cells (38, 39). All virus stocks were stored at -80°C.

Mice. WT C57BL/6 mice were obtained from Jackson Laboratory (Bar Harbor, ME). Ifi27l2a−/− (ISG12b; 76933) mice were generated at Washington University after receiving heterozygous sperm from C57BL/6 containing a deleted gene (Ifi27l2a<sup>tm1(KOMP)Wlg</sup>) from the Knockout Mouse Project Repository (KOMP, University of California, Davis). Sperm was used for in vitro fertilization of eggs from C57BL/6 recipient female mice. Heterozygous Ifi27l2a<sup>+/−</sup> mice were backcrossed to establish the Ifi27l2a<sup>−/−</sup> colony. Ifi27l2a<sup>−/−</sup> mice produced normal litter sizes of expected Mendelian ratios, with all progeny appearing healthy. All animals were maintained in the pathogen-free animal facility of Washington University School of Medicine.

Mouse infection experiments. The experimental protocols were approved by the Institutional Animal Care and Use Committee at the Washington University School of Medicine (Assurance Number: A3381-01). Studies were performed on sex- and age-matched mice between 11 and 12 weeks of age. Peripheral infection was performed by subcutaneous inoculation into the footpad with 10<sup>2</sup> PFU of virus diluted in 50 μl Hank’s Balance Salt Solution (HBSS) with 1% heat-inactivated fetal bovine serum (HI-FBS). Survival analysis was followed for 30 days. For viral burden studies after subcutaneous infection, mice were sacrificed at days 2, 4, 6, 8, 10, and 14 and peripheral organ and CNS tissues were collected following extensive perfusion with PBS, and stored at -80°C. Serum was collected after cardiac puncture according to standard procedures. Intracranial infection was performed by injecting 10<sup>1</sup> PFU of WNV-NY or WNV-MAD in 10 μl HBSS supplemented with 1% HI-FBS. For analysis of viral burden after
intracranial infection, brain and spinal cord tissues were collected at 3 and 5 days and
processed as described for tissues from peripheral infection. Brains were divided by dissection
into brain stem, cerebellum, olfactory bulb, grey matter (cerebral cortex) and subcortex (corpus
callosum, hippocampus, thalamus, and hypothalamus). Plaque assays were performed as
previously described with Vero cells (38). Levels of WNV RNA in serum were measured by
quantitative reverse transcription-PCR (qRT-PCR) as described (38, 40).

**Generation and infection of primary cell cultures.** All primary cell culture preparation
and virus infection studies were performed as described below. In some experiments, cells were
pretreated with indicated doses of mouse IFN-β prior to infection.

(a) Murine embryonic fibroblasts. Murine embryonic fibroblasts (MEFs) were
generated from embryonic day 14 WT and *Ifl27l2a*<sup>−/−</sup> mice. Embryos were decapitated, livers
were removed and remaining minced tissue was digested in 0.25% (w/v) trypsin for 10 min at
37°C with periodic, gentle agitation and mechanical disassociation. Following trypsin
neutralization with FBS, cells were cultured in DMEM supplemented with 20% HI-FBS, 1%
HEPES, 1% Glutamax (Life Technologies), 100 U/ml penicillin and streptomycin (Gibco) and 1%
non-essential amino acids (Gibco). Cells were infected at a multiplicity of infection (MOI) of 0.01.
A subset of cells was pretreated with 5 U/ml of mouse IFN-β (*E. coli*-derived, PBL Assay
Science) for 12 h prior to infection with WNV.

(b) Macrophages and dendritic cells. Mφ and dendritic cells (DCs) were generated as
previously described (41) from bone marrow of WT or *Ifl27l2a*<sup>−/−</sup> mice. Mφ and DCs were
stimulated in culture for 8 days with either 40 ng/ml recombinant murine M-CSF (Peprotech) or
20 ng/ml recombinant murine IL-4 and recombinant murine GM-CSF (Peprotech), respectively.
Mφ and DCs were infected with WNV-NY at an MOI of 0.01 and 0.001, respectively.

(c) Cortical neurons. CN were prepared from the cerebral cortex of embryonic day 15
WT and *Ifl27l2a*<sup>−/−</sup> mice as described (42). Tissues were dissociated at 37°C in 1 ml 0.25% (w/v)
trypsin, 0.25 mg DNase I (Sigma) in HBSS for 20 min. Trypsin was neutralized with 10% HI-FBS in DMEM and cells were filtered through a 70 μm filter and seeded at 5 x 10^5 cells/well on poly-D lysine and laminin (10 μg/ml)-coated 24-well cell-culture treated plates. CN cells were cultured in Neurobasal medium (Life Technologies) supplemented with 2% B-27 (Gibco), 1% Glutamax and 100 U/ml penicillin and streptomycin.

(d) Granule cell neurons. GCN were prepared from the cerebellum of 7 day-old pups and dissociated using the same protocol as for CN. These cells were cultured in the same medium as CNs with the addition of 40 mM KCl. Medium changes (50% of starting volume) were performed every 2 to 3 days, and neurons were maintained for 21 days in culture. In some experiments, neurons were pretreated for 24 h with 100 U/ml of IFN-β. Neurons were infected for 1.5 h at 37°C, rinsed with HBSS twice and cultured in their respective complete neuronal medium. GCN were infected with WNV-NY or WNV-MAD at an MOI of 0.01 or 0.1, respectively. In some experiments, GCN were pretreated with IFN-β (150 or 100 IU/ml, for WNV-NY or WNV-MAD, respectively) for 24 h. Viral titer was determined by focus-forming assay, as previously described (43).

The purity of cultured neuron populations was defined by immunofluorescence microscopy analysis after incubating with antibodies to S100-β (1:200 dilution, Abcam 52642), NeuN (1:100 dilution, Milipore MAB377B), or Ibal (1:500 dilution, WAKO 019-19741) to identify astrocytes, neurons or microglia, respectively. Secondary Alexa fluor conjugated dyes 488 or 555 were used (1:400 dilution, Invitrogen) for detection. Samples were imaged with a Nuance FX multiplex biomarker imaging system (Perkin Elmer). Using this analysis, our GCN cultures were comprised of 85% neurons and 12% astrocytes.

Cytokine and chemokine profiling. Cytokines and chemokines were profiled from serum at days 4 and 6 after peripheral WNV-NY infection. Protein levels were assayed with Bio-Plex Pro Cytokine Assay per the manufacturer’s protocol.
**Immune cell analysis.** Splenocytes and peripheral blood mononuclear cells were harvested from WT and *Ifi27l2a<sup>-/-</sup>* mice at day 8 after subcutaneous infection with WNV. Cells were stained for the following surface antigens following a 10 min preincubation with Fc-block (1:25 dilution; eBioscience): CD3 (1:25 dilution; BD Horizon, 500A2), CD4 (1:100 dilution; BioLegend, RM4-5), CD8<sub>α</sub> (1:100 dilution; Biolegend, 53-6.7), and CD19 (1:100 dilution; BioLegend, 6D5). Dead cells were excluded from analysis using Viability Dye eFluor (1:300 dilution; eBioscience). Cells were washed, fixed, permeabilized and stained for granzyme B (1:50 dilution; Invitrogen, GB11) and the APC-conjugated D<sup>α</sup>-restricted NS4B peptide (SSVWNATTAl) tetramer (1:300 dilution, NIH Tetramer Facility (Atlanta, GA)). Blood monocytes were detected after staining with Gr-1 (1:100 dilution; BioLegend, RB6-8C5), CD115 (1:100 dilution; eBioscience AFS98), CD8<sub>α</sub> (1:100 dilution), and F4/80 (1:100 dilution; Serotec, CI:A3-1) antibodies. CD8<sup>-</sup> CD115<sup>+</sup> F4/80<sup>+</sup> cells were designated as monocytes after extensive gating analysis (44). The monocytes in blood include circulating CD115<sup>+</sup> F4/80<sup>+</sup> Gr-1<sup>+</sup> monocytes that likely do not become resident within tissues. Circulating CD115<sup>+</sup> F4/80<sup>+</sup> Gr-1<sup>+</sup> monocytes may enter tissue during inflammation (“inflammatory monocytes”) and differentiate into macrophages (44-47). All samples were processed on a LSR Fortessa and data were analyzed by FlowJo software (Tree-Star).

CNS leukocytes were isolated according to a published method (48). Briefly, eight days following subcutaneous WNV infection, mice were perfused extensively with PBS. Brain tissue was minced, and digested in HBSS supplemented with 0.05% collagenase D (Sigma), 0.1 μg/ml trypsin inhibitor TLCK (N-α-p-tosyl-L-lysine chloromethyl ketone), 10 μg/ml DNase I (Sigma), and 10 mM HEPES, pH 7.3 for 30 min. CNS cells were strained with a 70 μm filter and subjected to Percoll gradient (30% v/v) purification (1,200 x g, 30 min). Cells were washed, incubated with Fc-block, and stained for CD8<sub>α</sub> (1:100 dilution), CD11b (1:100 dilution), CD19 (1:100 dilution), CD45 (1:100 dilution), Viability Dye eFluor (1:300 dilution) for 1 h at 4°C, then
rinsed, and incubated with commercial cell fixative (eBioscience). Samples were processed on an LSR Fortessa flow cytometer and analyzed with FlowJo commercial software (Tree-Star).

Neutrophils (CD11b\textsuperscript{high} CD45\textsuperscript{high}) were identified by their unique high side scatter profile. M\textrm{φ} (CD11b\textsuperscript{high} CD45\textsuperscript{high}) and microglial (CD11b\textsuperscript{high} CD45\textsuperscript{low}) populations were identified by their relative expression of CD11b and C45 and their low to medium side scatter profiles.

The T\textsubscript{FH} and germinal center B cell responses were measured in the draining lymph node (DLN) 8 days post infection with WNV-NY. Cells were stained as previously described with fluorochrome or biotin-conjugated antibodies purchased from BD Biosciences, Biolegend, and eBioscience: CD3\textgreek{e} (145-2C11), CD4 (RM4-5), CD19 (1D3), PD1 (29F.1A12), FAS (Jo2), GL7 and CXCR5 (2G8) (49).

**Serum antibody analysis.** WNV-specific IgG and IgM dilution endpoint titers were determined by ELISA against purified WNV E protein, as previously described (50). Focus reduction neutralization (FRNT50) assays were performed as previously described on Vero cells following serial dilution of serum with 100 FFU of WNV (39, 49).

**TUNEL staining.** Brain tissue was harvested from mice nine days after subcutaneous WNV infection. Mice were perfused with 30 ml PBS and half of the brain was retained for viral titer analysis, while the other brain half was fixed in 4% paraformaldehyde (PFA) in PBS overnight. This was followed by a 6 h incubation of brains in 20% sucrose solution and overnight incubation in 30% sucrose solution, all at 4°C. Selected brains (from WT or Ifi27l2\textgreek{a} mice) with equivalent viral titers (1 to 8 \times 10\textsuperscript{5} PFU/g) were embedded in Optimal Cutting Temperature medium (OTC; Tissue-Tek), frozen at -80°C, and sectioned in 10 \mu m slices on a Microm HM505N Cryostat on positively charged slides (Globe Scientific, 1358W). TUNEL staining was performed with the Roche In Situ Cell Death Detection Kit-TMR red per the manufacturer’s instructions. Neurons were co-stained with anti-NeuN (1:100 dilution; Millipore, A60), secondary Alexa Fluor 488 (1:400 dilution) and nuclei were visualized with DAPI. Slides were mounted with
Prolong Gold Diamond anti-fade mounting media (Invitrogen). Tissues were imaged on a Zeiss LSM880, AxioObserver confocal microscope at the Washington University Microscopy Core Facility, with a Plan-Apochromat 40x/1.4 oil DIC M27 objective. DAPI, Alexa Fluor 488, and TMR-red were detected with the respected wavelength channels: 415-470, 491-553, and 553-624. The image, approximately 637.64 μm x 637.64 μm, was comprised by automated tiling of 9 panels (Zeiss Zen), with the central panel being selected for TUNEL positive cells in the same region of the brain tissue in each animal. Four mice were imaged per genotype with two tissue slices per mouse and two images per tissue slice for both brain stem and cerebellum. TUNEL positive events were counted within the 9-tiled composite image.

qRT-PCR assays. WNV, Oas1a and Ifit1 mRNA was analyzed from RNA extracted from GCN following treatment with either IFN-β (100 U/ml), poly(I:C) (50 μg/ml) (InvivoGen), WNV-NY (MOI, 5) or WNV-MAD (MOI, 5) for 8 h prior to collection in lysis buffer. qRT-PCR was performed as previously described for WNV, Oas1a and Ifit1 and gene expression was normalized to Gapdh (51). Commercially available Ifi27l2a primer-probe assay was purchased from IDT. TaqMan RNA-to-CT 1-Step Kit was used for qRT-PCR.

Blood-brain barrier permeability. Blood-brain barrier (BBB) studies were performed as previously described (52). Briefly, 4 days after subcutaneous infection with WNV-NY, WT and Ifi27l2a−/− mice were injected via an intraperitoneal route with 100 μl of a 100 mg/ml fluorescein (Sigma) in PBS. Dye was allowed to circulate for 45 min, serum was collected as a normalization control, mice were perfused with 20 ml PBS, and brain regions were collected for analysis as previously described (52).

Statistical analyses. All data were analyzed with Prism software (GraphPad Prism, San Diego, CA). qRT-PCR with more than two comparisons between groups was analyzed by one-way ANOVA with Tukey’s HSD post hoc analysis. qRT-PCR data with two group comparisons was analyzed by Student’s t-test with correction for multiple comparisons Holm-Sidak method.
Serum cytokine levels were analyzed by Student’s t-test with correction for multiple comparisons Holm-Sidak method. Kaplan-Meier survival curves were analyzed by the Mantel-Cox Log-rank test. Viral burden in tissues was analyzed by the Mann-Whitney test. Serum antibody titers were analyzed by Student’s t-test. For viral growth kinetics in cell culture, the log transformed viral titer was analyzed by Student’s t-test. Flow cytometry based assays, where total cell count or percent total cell count was measured, also was analyzed by a Student’s t-test.
RESULTS

A deficiency of Ifi27l2a increases susceptibility to WNV infection. The ISG Ifi27l2a is differentially upregulated in selected neurons of the brain after WNV infection, and ectopic expression of Ifi27l2a in cultured cortical neurons inhibited infection by WNV (14). To explore an antiviral role in vivo for this relatively poorly characterized ISG, we generated Ifi27l2a−/− mice using a targeted gene deletion strategy (Fig 1A); deletion of Ifi27l2a was validated by PCR (Fig 1B). In WT mice, basal Ifi27l2a mRNA expression was observed in lymph node, heart, lung and testes, and to a lesser extent in the kidney and spleen. Following WNV infection, Ifi27l2a mRNA expression was induced in the brain and spinal cord (Fig 1C). To quantify Ifi27l2a mRNA expression in response to viral infection, we analyzed selected tissues at successive time points following peripheral inoculation (Fig 1D). At 4 and 8 days post infection, Ifi27l2a mRNA expression was enhanced in the brain (2.0- and 3.3-fold, respectively; P < 0.005) and the spinal cord (2.1- and 2.0-fold, respectively; P < 0.005). Within the spleen, higher levels of Ifi27l2a mRNA were observed 4 days after infection (12-fold, P < 0.05). We also analyzed Ifi27l2a−/− and wild-type (WT) mice for possible differences in immune cells subsets in the spleen and blood. Although the numbers of CD4+ and CD8+ T cells and CD19+ B cells were similar, Ifi27l2a−/− mice had slightly greater numbers of splenic NK cells (NK1.1+) than WT mice (Table 1); while noteworthy, this phenotype may less important in the context of WNV infection, as NK cell depletion does not impact WNV pathogenesis in mice (53, 54). Within the peripheral blood leukocyte compartment, we observed similar numbers of myeloid cells, monocytes, and subsets of granulocytes (Table 2).

We next infected WT and Ifi27l2a−/− congenic mice with a pathogenic isolate of WNV (strain 3000.0259, WNV-NY). After subcutaneous infection with 10^2 PFU of WNV-NY, Ifi27l2a−/− mice exhibited a decreased survival rate compared to WT animals (29% versus 63%, P < 0.05) although the mean time to death was similar between the two groups (Fig 2A).
WNV burden in the CNS of Ifi27l2a−/− mice. To understand why an absence of Ifi27l2a resulted in enhanced pathogenicity of WNV-NY, viral burden was examined at different days (2, 4, 6, 8, 10, or 14) in serum, peripheral organs (spleen and kidney), and CNS tissues (brain and spinal cord). WNV viremia at days 2, 4, and 6 was similar between WT and Ifi27l2a−/− mice (Fig 2B). At all time points tested, viral burden in the spleen also was similar between WT and Ifi27l2a−/− mice (Fig 2C). Moreover, a deficiency in Ifi27l2a did not result in productive infection of the kidney (Fig 2D), an organ that is typically resistant to WNV-NY infection in WT mice yet permissive in animals with defects in type I IFN induction, signaling, or effector functions (55-59). However, within the CNS at day 8 after infection, WNV-NY burden increased in the brain (2.4-fold, P < 0.05) and spinal cord (170-fold increase, P < 0.005) of Ifi27l2a−/− mice (Fig 2E and F). This difference in viral titer was not apparent at later time points, and by day 14, infectious virus was not detectable within the CNS or peripheral tissues in surviving animals from both genotypes, suggesting that Ifi27l2a−/− animals did not have a defect in the clearance phase of WNV, which requires CD8+ effector T cells (36).

To corroborate these findings, we performed plaque assays on tissue homogenates isolated from specific regions of the CNS. WT and Ifi27l2a−/− mice were infected with WNV-NY via a subcutaneous route and viral burden in the brain stem, cerebellum, cerebral cortex, sub-cortex (defined in Methods), and olfactory bulb was measured at day 8 after infection (Fig 2G-K). Although we observed no differences in WNV titers in the cerebral cortex, sub-cortex, or olfactory bulb, higher levels of infection were observed in the cerebellum and brain stem (590-fold, P < 0.05; 5,200-fold, P < 0.05, respectively) from Ifi27l2a−/− mice. These data suggest that Ifi27l2a has a protective, antiviral role in selected regions of the CNS.

WNV infection after direct intracranial inoculation. As Ifi27l2a−/− mice exhibited higher viral titers in the brain stem and cerebellum, we postulated that Ifi27l2a might protect specific regions when virus was administered directly into the CNS. Unexpectedly, following intracranial inoculation of WNV-NY into the cerebral cortex, we observed no differences in viral titers within
different regions of the CNS at either 3 or 5 days after infection (Fig 3A-F). Because WNV-NY
strain is highly virulent, we repeated intracranial infection studies with the attenuated WNV
Madagascar strain (WNV-MAD), which is more sensitive to the antiviral effects of type I IFN (14,
37, 60). We observed a modest, (17-fold, \( P < 0.05 \)) yet statistically significant phenotype, with
greater WNV-MAD infection at day 3 after infection in the brain stem of \( \text{Ifi27l2a}^{-/-} \) mice (Fig 3G-
L). Thus, Ifi27l2a had an antiviral effect when virus was introduced directly into the CNS,
although its magnitude was limited and only apparent with an attenuated, more IFN-sensitive
strain.

Ifi27l2a does not alter cellular or humoral immune responses during acute WNV
infection. As depressed antiviral CD8\(^+\) T cell or antibody responses can facilitate dissemination
to and replication of WNV within the CNS (reviewed in, 12), we investigated whether an
absence of Ifi27l2a influenced the development of cellular and adaptive immunity during WNV-
NY infection. Initially, we examined the effects of Ifi27l2a on lymphocyte numbers in the
peripheral tissues. At baseline, normal numbers and percentages of B cells, CD4\(^+\) T cells, and
CD8\(^+\) T cells were present in the blood and spleen. Because a previous study suggested that
Ifi27l2a modulates inflammation, possibly through regulation of M\(\Phi\) differentiation (24, 35), we
profiled monocytes in blood during WNV infection (Fig 4A). At day 8 after infection, \( \text{Ifi27l2a}^{-/-} \)
and WT mice had similar percentages and numbers of circulating and inflammatory blood
monocytes based on differential expression of the surface markers F4/80, CD115, and Gr-1
(Ly6C and Ly6G) (Fig 4B-C and Methods).

We next evaluated T cell responses in peripheral organs by characterizing the relative
levels of CD4\(^+\) and CD8\(^+\) T cells (Fig 5A). At day 8 after WNV-NY infection, equivalent
percentages and numbers of CD4\(^+\) and CD8\(^+\) cells were observed in the spleen (Fig 5B-C).
Furthermore, no difference in granzyme B\(^+\) NS4B tetramer\(^+\) antigen-specific CD8\(^+\) T cells was
observed in the spleens of WT and \( \text{Ifi27l2a}^{-/-} \) mice. We assessed whether leukocyte
accumulation in the CNS was altered, which independently could affect disease outcome.
Leukocytes were isolated from brains of WT and Ifi27l2a−/− mice at day 8 and analyzed by flow cytometry (Fig 5D). We observed similar percentages and numbers of CD4+ and CD8+ T cells or granzyme B+ NS4B tetramer+ CD8+ T cells within the brain (Fig 5E-F). Microglia and infiltrating Mφ were characterized by CD45 and CD11b surface expression (Fig 5G). We also observed no differences in the percentage or numbers of activated microglia (CD11bhigh CD45low) or Mφ (CD11bhigh CD45high) (Fig 5H-I) in the brains of WT and Ifi27l2a−/− mice after WNV-NY infection. Thus, a deficiency of Ifi27l2a did not affect priming, the recruitment, or activation of antigen-specific or innate immune cells in the CNS of WNV-infected mice.

To assess the effect of Ifi27l2a on WNV-specific antibody responses, we analyzed serum from Ifi27l2a−/− and WT mice on day 8 after infection for binding to the WNV E protein. We observed elevated IgG titers (3.2-fold, P < 0.0005) in Ifi27l2a−/− mice compared to WT mice (Fig 6A), but no difference in IgM titers (Fig 6B). However, neutralization assays detected no difference in the ability of serum-derived antibody from WT and Ifi27l2a−/− mice to neutralize WNV-NY infection (Fig 6C).

Because of the increased IgG titers in Ifi27l2a−/− mice, we next characterized whether there were differences in the T cell-dependent germinal center response in DLN of WNV-NY infected mice at 8 days post infection. T follicular helper cells were characterized as CD4+, PD1+, and CXCR5+ (Tfh, Fig 6D) and germinal center B cells were classified as CD19+, Fas+, and GL7+ (GC B, Fig 6E). As the total numbers and percentages of Tfh and GC B cells were similar in WNV-infected WT and Ifi27l2a−/− mice, a deficiency in Ifi27l2a did not appear to alter the germinal center response within the DLN.

Cytokine and chemokine expression profiles in serum of WNV-infected Ifi27l2a−/− mice. Because specific vasoactive cytokines (e.g., TNF-α, IL-1β, and IL-6) can alter the blood-brain barrier (BBB) and affect transit of WNV into the brain parenchyma and early replication (reviewed in 10, 11), we measured whether a deficiency of Ifi27l2a affected systemic production
of cytokines and chemokines at 4 or 6 days after WNV-NY infection. In WNV infected mice, we observed greater expression of IL-1β and eotaxin in WT mice compared to Ifi27l2a<sup>−/−</sup> at 4 days after infection (3.0-fold, \( P < 0.05 \); 1.2-fold, \( P < 0.05 \)), but not other cytokines and chemokines (Table 3). Consistent with this small variation in cytokine expression in serum, we did not observe differences in blood-brain barrier permeability between WT and Ifi27l2a<sup>−/−</sup> mice (data not shown). To assess whether this small variation in cytokine expression in serum impacted BBB permeability and possibly virus entry into the CNS, we injected the small molecule sodium fluorescein via an intraperitoneal route into WT and Ifi27l2a<sup>−/−</sup> at 4 days after WNV infection and then measured extravasation into different regions of the CNS. Notably, similar levels of sodium fluorescein accumulated in the cerebral cortex, cerebellum, brain stem, and spinal cord (data not shown). Thus, the small increases in serum cytokine levels in the absence of Ifi27l2a did not substantively impact BBB permeability.

**Ifi27l2a exhibits antiviral effects against WNV in Mϕ but not DCs or MEFs.** Although Ifi27l2a is expressed after WNV infection in primary and secondary lymphoid tissues, we did not observe greater viral burden in peripheral organs. We speculated that the antiviral effect of Ifi27l2a against WNV infection might not occur in non-neuronal cell types. To evaluate this hypothesis, we generated bone marrow-derived Mϕ and DCs and primary MEFs from WT and Ifi27l2a<sup>−/−</sup> mice. Cells were either pretreated with IFN-β or not treated and were then subsequently infected at a low MOI with WNV-NY. We observed increased WNV-NY replication in untreated or IFN-β treated Mϕ at later time points (\( P < 0.05 \), Fig 7A). However, we observed no difference in viral infection at any time point in DC or MEF cultures generated from WT and Ifi27l2a<sup>−/−</sup> mice (Fig 7B-C).

**Subsets of primary neurons from Ifi27l2a<sup>−/−</sup> mice exhibit enhanced WNV infection.** Given that the virologic phenotype occurred in selected brain regions of Ifi27l2a<sup>−/−</sup> mice, we investigated whether Ifi27l2a had differential antiviral activity in different neuron populations. We
prepared primary neurons from the cerebral cortex (CN) and cerebellum (GCN) of WT and Ifi27l2a-/- mice, pretreated select cells with IFN-β, and measured viral growth kinetics after infection with WNV-NY. We detected no differences in replication kinetics in CN, with only mild suppression of infection with IFN-β pretreatment (Fig 8A), as seen previously (14). Somewhat unexpectedly, we observed similar viral growth kinetics in GCN from WT and Ifi27l2a-/- mice for WNV-NY, with replication being suppressed to a greater degree following IFN-β pretreatment (Fig 8B), also as reported previously (14). We reassessed viral growth kinetics with the more IFN-sensitive strain, WNV-MAD. Ifi27l2a-/- GCN supported higher levels of WNV-MAD infection and this effect was more pronounced when cells were pretreated with IFN-β (18-fold, \( P < 0.05 \), Fig 8C) and a difference in viral replication was present in non-IFN-β treated cells (55-fold). Consistent with this data, by 72 hours after infection, a greater proportion of WNV-MAD infected GCN was observed in Ifi27l2a-/- versus WT GCN (Fig 8D). Because we observed differences in WNV restriction in WT and Ifi27l2a-/- GCN, we considered whether a deficiency of Ifi27l2a altered the general ISG response in GCN. We treated cells with known ISG inducers (IFN-β, Poly(I:C), WNV-NY and WNV-MAD) and assessed expression of Oas1a and Ifit1 mRNA. As Oas1a and Ifit1 induction was similar in WT and Ifi27l2a-/- GCN (Fig 8E-F), a deficiency of Ifi27l2a did not broadly impact expression of other antiviral ISGs.

Ifi27l2a-/- mice exhibit less neuronal death in the cerebellum and brain stem after WNV infection. To provide a mechanistic link between our in vitro and in vivo phenotypes with Ifi27l2a-/- cells and mice, we prepared brain tissue sections for histological and immunohistochemical analyses. Although historically we have detected WNV antigen staining in neurons of different brain regions at day 9 after infection in younger (e.g., 5 and 8 week-old mice) (61, 62), despite multiple attempts, the viral antigen staining in 11 to 12 week-old WT or Ifi27l2a-/- mice was inconclusive. The levels of viral antigen in the CNS were at the threshold of detection, with only sporadic staining of infected neurons in different brain regions of a subset of the mice (data not shown). Because of this, and prior reports suggesting that some Ifi27 family
members (e.g., ISG12a) were required for IFN-induced cellular apoptosis (21, 31, 32), we evaluated neuronal cell death in WT and Ifi27l2a−/− mice that had equivalent WNV titers in the brain at 9 days after infection. We noted significantly more cell death in the cerebellum (5-fold, \( P < 0.05 \)) (Fig 9A and C) and brain stem (4-fold, \( P < 0.005 \)) (Fig 9B and C) of WT mice, whereas TUNEL positive neurons were largely absent in the hindbrain regions of Ifi27l2a−/− mice.
DISCUSSION

Viral replication and the subsequent immune response within the CNS can result in significant morbidity and mortality. Because neurons are largely non-renewable, it is imperative that the host clears viral infection while protecting cells from direct or collateral immune-mediated damage. We previously identified Ifi27l2a as a putative inhibitory ISG that was expressed preferentially within neurons of the cerebellum compared to those from the cerebral cortex (14). Here, we established a protective antiviral role for Ifi27l2a in vivo against WNV. Ifi27l2a−/− mice were more susceptible to WNV-induced mortality, and sustained higher viral titers in the cerebellum, brain stem, and spinal cord. Remarkably, at day 9 after infection, Ifi27l2a−/− mice had less cell death in neurons of the cerebellum and brain stem. Ifi27l2a−/− mice showed no apparent defects in their ability to generate peripheral or CNS cellular immune response, and WNV replication was equivalent in several other cell types that lacked or expressed Ifi27l2a.

Several members of the Ifi27 family have been studied in the context of viral infections. Many viruses induce expression of Ifi27 family members, including influenza A virus, Sindbis virus, WNV, JEV, and human immunodeficiency virus-1 (14, 63-66). Our prior study reported that ectopic expression of Ifi27l2a in CN reduced WNV infection whereas siRNA mediated gene silencing in GCN resulted in enhanced viral replication (14). Work by others has shown that ectopic expression of human IFI27 (ISG12A) inhibited replication of HCV in Huh-7.5 cells, and reciprocally siRNA mediated silencing of human IFI27 enhanced HCV replication (67). High levels of expression of human IFI27 also inhibited Newcastle Disease virus (NDV) replication and oncolytic activity in Huh7 cells (34).

Ifi27l2a−/− mice have been investigated in other contexts. Although Ifi27l2a was identified as an upregulated ISG in lung tissue following influenza virus infection (65), Ifi27l2a−/− C57BL/6 mice did not sustain higher viral burden or altered pathology in the lungs of infected animals (33). In another Ifi27l2a−/− mouse of mixed genetic background, gene-deficient animals were protected against caecal ligation induced-sepsis, LPS endotoxemia, or vascular injury after arterial ligation.
In contrast, our Ifi27l2a−/− C57BL/6 mice succumbed to LPS administration at a rate similar to WT mice (T. Lucas and M. Diamond, unpublished observations). Although Ifi27l2a has a postulated role in regulating inflammation, at least in the context of WNV infection, we failed to observe hypercytokinemia, changes in the infiltrating immune cells, or altered adaptive immunity in Ifi27l2a−/− mice.

The unique cell-type expression, sub-cellular localization, and induction patterns of Ifi27 family genes suggest possible modular functions. Humans have four IFI27 members, of which only IFI27 and IFI6-16 (IFI6) are IFN-inducible. Mice have three gene paralogs, Ifi27 (Ifi27l11), Ifi27l2a, and Ifi27l2b, all of which are IFN-inducible, with Ifi27l2a exhibiting the greatest induction after type I IFN treatment (19). IFI27 family members have been suggested to localize to the mitochondria (21, 22) or to the nuclear membrane (23, 24); in the latter case, IFI27 interacts with and sequesters the nuclear receptor NR4A1, which regulates expression of anti-inflammatory genes (24). IFI27 family members also appear to have pro-apoptotic effects (21, 32, 34, 68, 69), possibly through stabilization of the mitochondrial membrane and regulation of caspase activity (21, 68). Perhaps because of these proposed pleotropic activities, IFI27 family members have been associated with over-expression in certain cancers (69, 70), promotion of skin keratinocyte replication (71), and DNA-damage induced apoptosis and cytochrome c release (21). IFI6-16 (IFI6) is an ISG12-motif containing family member that may share some functions with Ifi27l2a. In the context of DENV infection, a deficiency of IFI6-16 was associated with increased caspase levels and decreased Bcl-2 expression and mitochondrial membrane stabilization (68). Additionally, ectopic expression of IFI6-16 has been shown to suppress infection of YFV virus (72). Our in vivo data is most consistent with a model in which Ifi27l2a is induced by type I IFN in response after WNV infection, and in a cell-type specific manner (for reasons that still remain uncertain), promotes cell death. In its absence (Ifi27l2a−/− mice), subsets of virally infected neurons (e.g., in the cerebellum, brain stem, and possibly spinal cord) live longer, which allows greater yields of virus to accumulate, at least during the early phase of
CNS infection. We speculate that the increased rate of death of WNV-infected Ifi27l2a−/− mice ultimately is caused by virus-induced injury of neurons in regions of the CNS that control key or autonomic function. Our study, along with the work of others, suggest multiple possible functions for different Ifi27 family members, with some of the antiviral properties being linked to cell death phenotypes in infected cells.

The predominant effect of Ifi27l2a in the CNS suggests a specialization of the host antiviral immune response. Analogously, preferential antiviral roles in the CNS for other ISGs (Ifit2 and Rsad2 (viperin)) have been reported. In Ifit2−/− mice, higher WNV and VSV titers were observed in the olfactory bulb, brain stem, and cerebellum (13, 73). In Rsad2−/− mice, an increase in WNV infection was observed in the cerebral cortex, white matter, and spinal cord (55). We observed differences in the regional restriction of WNV in the CNS mostly in the context of peripheral viral but not intracranial infection, with the exception of the brain stem. Viral infection of peripheral immune tissues (e.g., lymph node or spleen) may induce systemic accumulation of type I IFN that primes the antiviral response in the brain either earlier or prior to viral entry into the CNS, whereas direct administration of virus to the CNS may permit rapid replication of WNV in neurons in the context of a less robust type I IFN response. Although future studies are needed to determine why region-specific antiviral effects of individual ISGs occur, we speculate that these genes do not function in isolation, and partner proteins that are expressed differentially may regulate their activity. While our findings suggest an antiviral function within select neurons and Mφ, the precise cellular mechanism of action of Ifi27l2a warrants further investigation.

In summary, we have identified a protective role for Ifi27l2a in the CNS following WNV infection. Ifi27l2a mediated restriction of WNV was greatest in the cerebellum, brain stem and spinal cord, and correlated with an antiviral and cell survival effect in subsets of neurons and myeloid cells. These findings suggest that Ifi27l2a may have a discrete antiviral activity within selected regions of the CNS.
ACKNOWLEDGEMENTS

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Figure 1. Generation of *Ifi27l2a*<sup>-/-</sup> mice and tissue expression of *Ifi27l2a*. Scheme of *Ifi27l2a* locus with targeting cassette (A). Exons are noted in grey and target location is noted with black arrow. *Ifi27l2a* gene deletion was verified by PCR. Genotyping was verified with a positive control plasmid containing wild-type *Ifi27l2a* and negative controls with a null plasmid control. *Ifi27l2a* deletion was confirmed by the presence of 408 bp band, whereas WT *Ifi27l2a* manifests as a 612 bp band (B). The *Ifi27l2a* RT-PCR product was screened for in brain, spinal cord, lymph node (LN), spleen, kidney, lung, liver, white fat, and testes (C). Selected mice were infected subcutaneously with WNV-NY and tissues were collected 4 days after infection and compared to mock-infected animals. The results are representative of 2 to 3 mice per treatment group. Following peripheral infection by WNV-NY, selected tissues were collected at 4 and 8 days after infection and expression of *Ifi27l2a* mRNA was compared to mock infected animals (D). *Ifi27l2a* mRNA was measured in brain, spinal cord, spleen, and lymph node, and normalized to Gapdh by qRT-PCR. Means were compared between mock and infected groups using one-way ANOVA followed by Tukey’s HSD Post Hoc analysis (*, P < 0.05, **, P < 0.005).

Bars are mean ± SEM.

Figure 2. Survival and viral burden analysis for WT and *Ifi27l2a*<sup>-/-</sup> mice infected with WNV. (A) Survival analysis of 11 to 12 week-old WT or *Ifi27l2a*<sup>-/-</sup> mice after inoculation with 10<sup>2</sup> PFU of WNV-NY by subcutaneous injection in the footpad. In (A) WT (n = 39) and *Ifi27l2a*<sup>-/-</sup> (n = 34) mice were used for survival curves. Asterisks indicate differences that were statistically significant compared to WT mice (Mantel-Cox log rank test analysis; P < 0.05). Viral burden after WNV-NY infection of WT or *Ifi27l2a*<sup>-/-</sup> mice was measured by qRT-PCR (B) or plaque assay (C-G) in samples from serum (B), Spleen (C), kidney (D), brain (E), and spinal cord (F) at the indicated time points after infection. (G-K) Selected brain regions were assayed for viral burden at 8 days post subcutaneous infection with WNV-NY. Data points represent individual mice. Bars indicate median values and were obtained from 16 to 17 mice per tissue. Asterisks
indicate statistical significance as determined by the Mann-Whitney test (*, P < 0.05, **, P < 0.005). Dotted line indicates the limit of detection for each tissue. Data are pooled from at least three independent experiments.

Figure 3. Viral titers in the brain after intracranial infection of WT and Ifi27l2a<sup>−/−</sup> mice. Mice were infected with either 10<sup>1</sup> PFU WNV-NY (A-F) or WNV-MAD (G-L) via an intracranial route and selected CNS regions were harvested and viral burden was determined by plaque assay. Data points represent individual mice. Bars indicate median values and were obtained from 4 to 10 mice per time point per tissue. Dotted lines represent the limit of detection of the assay. Asterisks indicate statistical significance as determined by the Mann-Whitney test (*, P < 0.05).

Figure 4. Circulating monocytes isolated from the blood of WT and Ifi27l2a<sup>−/−</sup> mice. Circulating blood monocytes were gated as CD8<sup>+</sup> F4/80<sup>+</sup> CD115<sup>+</sup> and analyzed for expression of additional surface markers including Gr-1 (Ly6C and Ly6G) (A). WT and Ifi27l2a<sup>−/−</sup> monocytes were present at similar levels at 8 days post infection in the blood. Specific monocyte populations of Gr-1<sup>high</sup> and Gr-1<sup>low</sup> cells were phenotyped according to prior studies (45, 74) and presented as either percent (B) or total number (C) of cells per ml of blood from WT and Ifi27l2a<sup>−/−</sup> mice. For each group, a Student’s t-test was used to compare cells from WT to Ifi27l2a<sup>−/−</sup> mice. Bars indicate mean values for 8 to 9 mice for each genotype from three independent experiments.

Figure 5. Splenic T cell and brain-specific immune response to WNV infection in WT and Ifi27l2a<sup>−/−</sup> mice. (A) T cells were identified by inclusion of CD19<sup>−</sup> and CD3<sup>+</sup> cells, and analyzed by CD4 and CD8. CD8<sup>+</sup> cells were additionally analyzed as granzyme B<sup>+</sup> and WNV-specific NS4B tetramer<sup>+</sup>. At 8 days after subcutaneous infection with WNV-NY, splenocytes were harvested. Similar percentages (B) and absolute cell numbers (C) were observed for CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cells or WNV specific granzyme B<sup>+</sup> NS4B tetramer<sup>+</sup> CD8<sup>+</sup> T cell (n = 11). For each group, Student’s t-test was used to compare values from WT to Ifi27l2a<sup>−/−</sup> mice (P < 0.05).
Bars indicate mean values. Brain cells were purified by Percoll gradient centrifugation from brains of mice at 8 days post infection. (D) Cells were gated as CD8+ positive, and WNV-specific CD8+ T cells were identified by co-staining for granzyme B and with WNV-specific NS4B tetramer. The percentages of CD4+ and CD8+ T cells, as well as NS4B-specific cells, were similar between WT and Ifi27l2a−/− mice (E). No difference in absolute number of infiltrating CD4+ and CD8+ T cells, or the WNV specificity of CD8+ T cells was observed (F). Brains were analyzed for numbers of macrophages (CD45high CD11b+) and microglia (CD45low and CD11b+) (G). The percentages and number (H-I) of macrophages and microglia were similar between WT and Ifi27l2a−/− mice. WT and Ifi27l2a−/− samples percentages and absolute numbers for three independent experiments were compared with a Student’s t-test (n = 7 to 11 mice, *, P < 0.05).

Figure 6. Antibody responses in WT and Ifi27l2a−/− mice after WNV infection. Serum was obtained from WNV-infected WT and Ifi27l2a−/− mice and IgG levels (A) or IgM levels (B) at 8 days after infection and measured by ELISA for reactivity with WNV E protein. Neutralizing antibody titers were determined by a focus-reduction assay from serum at day 8 (C). Results are shown as a scatter plot and represent samples from 7 to 10 mice per group. Data are plotted as the log10 endpoint neutralization titer or log10 focus reduction neutralizing titer 50 (FRNT50). A Student’s t-test was used to compare data from WT and Ifi27l2a−/− mice (***, P < 0.0005). D-G. TFH cells (PD1+ and CXCR5+) (D) and GC B cells (Fas+ and GL7+) (E) populations were identified in the DLN at 8 days post WNV-NY infection. Total numbers of TFH cells and GC B cells (F) and percentages of TFH cells of total CD4+ cells and GC B cells of total CD19+ cells (G) were similar between WT and Ifi27l2a−/− mice. Bars indicate mean values.

Figure 7. Ifi27l2a restricts WNV replication in Mφ, but not DC or MEFs. (A) Bone marrow derived Mφ were infected with WNV (MOI, 0.01) and viral replication kinetics were followed for 72 h. A subset of Mφ was pretreated with IFN-β (1 U/ml for 6 h) prior to infection. (B)
Bone marrow derived DCs were infected with WNV (MOI, 0.001) and viral kinetics were followed for 72 h. A subset of DCs was pretreated with IFN-β (10 U/ml for 6 h) prior to infection. (C) MEFs were infected with WNV (MOI, 0.01) and viral kinetics were followed for 72 h. A subset of MEFs was pretreated with IFN-β (10 U/ml for 6 h). The data analyzed by a Student’s t-test for each time point, between each treatment group and is expressed as the log_{10} median titers ± SEM as reflects pooled data from 3 to 4 independent experiments with three technical replicates per independent experiment for each cell type. Y-axes range is cell-type dependent.

Figure 8. Viral infection of WT and Ifi27l2a^-/- primary neurons. Primary neuron cultures were generated from WT and Ifi27l2a^-/- mice and infected with WNV-NY or WNV-MAD. Cell supernatants were harvested at the indicated time points and titrated by focus forming assay. CN (A) and GCN (B-C) were infected at the following MOI: WNV-NY, 0.01; WNV-MAD, 0.1. In some experiments, GCN and CN were pretreated with IFN-β for 24 h (CN: 150 U/ml for WNV-NY; GCN: 150 U/ml for WNV-NY or 100 U/ml WNV-MAD). (D) WT and Ifi27l2a^-/- GCN were infected with WNV-MAD for 72 h and infected neurons (MAP2^+) cells were counted by automated high throughput imaging. WT and Ifi27l2a^-/- GCN were analyzed by qRT-PCR for expression of Oas1 (E) and Ifit1 (F). Viral replication data was analyzed at each time point, for each treatment by a Student’s t-test for each treatment group (*, P < 0.05, n = 3 to independent replicates). GCN infection assay results were analyzed by Student’s t-test (two experimental replicates, 3 to 4 samples wells per replicate) (*, P < 0.05). For high throughput imaging, three wells per treatment group were analyzed for each biological replicate (n = 3). For each well, 60 computer-randomized images were collected and analyzed by GE IN Cell 2000 imager and IN Cell Software. qRT-PCR data was analyzed by a Student’s t-test with correction for multiple comparisons by Holm-Sidak method (*,P < 0.05). NT = Not treated.

Figure 9. Neuronal death within the cerebellum and brain stem of WNV infected mice. Nine days following subcutaneous infection with WNV-NY, brains of selected mice with
similar levels of virus were sectioned and stained for neurons (NeuN, green), cell death (as determined by TUNEL staining, red) and nuclei (DAPI, blue). Cerebellum (A) and brain stem (B) tissues were analyzed by confocal microscopy and fields of view containing TUNEL staining were quantitated for number of events per field of view (C) (n = 4 mice per genotype, 2 sections per mouse, 2 fields of view per section). The relative number of TUNEL+ cells in WT and Ifi27l2a−/− mice were analyzed by Student’s t-test, with bars indicating the means (*, P < 0.05; **, P < 0.005). Scale bars = 100 μm. Yellow arrows indicate examples TUNEL+ nuclei.
Table 1. Immunophenotyping of lymphocytes in the spleen of naive WT and Ifi27l2a⁻/⁻ mice.

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Mean (AVG) ± standard deviation (SD) for cells from WT and Ifi27l2a⁻/⁻ mice (n = 5 mice each, *, P < 0.05). The percentage of indicated populations are calculated as a proportion of the total parent population.
Table 2. Immunophenotyping of myeloid cells in peripheral blood of naïve WT and Ifi27l2a-/- mice.

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</tr>
<tr>
<td>Eosinophil</td>
<td>3.53E+05 1.30E+05 11.20 2.44</td>
<td>3.16E+05 9.53E+04 10.48 2.02</td>
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</tr>
<tr>
<td>-</td>
<td>3.07E+04 1.84E+04 0.96 0.41</td>
<td>3.02E+04 1.78E+04 0.99 0.47</td>
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</tr>
</tbody>
</table>

Number of cells per ml blood (AVG) + standard deviation (SD) from WT and Ifi27l2a-/- (n = 5) mice. The percentage of indicated populations are calculated as a proportion of the total parent population.
Table 3. Serum cytokine levels at days 4 and 6 after subcutaneous inoculation of WNV.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>4 dpi WT</th>
<th>MEAN SD</th>
<th>6 dpi WT</th>
<th>MEAN SD</th>
<th>4 dpi Ifi27l2a&lt;sup&gt;-/-&lt;/sup&gt;</th>
<th>MEAN SD</th>
<th>6 dpi Ifi27l2a&lt;sup&gt;-/-&lt;/sup&gt;</th>
<th>MEAN SD</th>
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<tr>
<td>IL-1α</td>
<td>4.66</td>
<td>6.4</td>
<td>4.7</td>
<td>4.6</td>
<td>0.8</td>
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<td>32.95</td>
<td>182.2*</td>
<td>88.2</td>
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<td>44.0</td>
<td>53.2</td>
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<td>27.0</td>
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<td>19.1</td>
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<td>2.6</td>
<td>2.8</td>
<td>1.4</td>
<td>2.9</td>
<td>1.0</td>
<td>1.9</td>
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<td>7.2</td>
<td>2.0</td>
<td>5.7</td>
<td>1.2</td>
<td>5.3</td>
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<td>IL-12(p40)</td>
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<td>IL-12(p70)</td>
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</table>

Cytokine levels were assayed via Bioplex Pro to determine relative differences between WT and Ifi27l2a<sup>-/-</sup> mice (n = 8 to 9 mice per genotype per time point) at 4 and 6 days after infection of 10<sup>2</sup> FFU by subcutaneous route. Data is the mean ± standard deviation (SD). Levels were compared between WT and Ifi27l2a<sup>-/-</sup> by Student’s t-test (P < 0.05), with correction for multiple comparisons with Holm-Sidak method. LOD = Limit of Detection for each cytokine based on manufacture’s protocol for establishing standard curves for each parameter measured.


A

Gate: Live

SSC-H

CD8

Gate: Live CD8−

CD115

F4/80

Gate: Live CD8− CD115− F4/80−

Cell Count

Gr-1

B

Monocytes

Gr-1 high

Gr-1 low

Percent Circulating

Percent Circulating Monocytes

WT Ifi27I2a+/+

WT Ifi27I2a−/−

WT Ifi27I2a−/−

C

Monocytes

Gr-1 high

Gr-1 low

Log10 Cells/ml blood

Log10 Cells/ml blood

Log10 Cells/ml blood

WT Ifi27I2a+/+

WT Ifi27I2a−/−

WT Ifi27I2a−/−