Deficient IFN signaling by myeloid cells leads to MAVS-dependent virus-induced sepsis

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Deficient IFN Signaling by Myeloid Cells Leads to MAVS-Dependent Virus-Induced Sepsis

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Abstract

The type I interferon (IFN) signaling response limits infection of many RNA and DNA viruses. To define key cell types that require type I IFN signaling to orchestrate immunity against West Nile virus (WNV), we infected mice with conditional deletions of the type I IFN receptor (IFNAR) gene. Deletion of the Ifnar gene in subsets of myeloid cells resulted in uncontrolled WNV replication, vasoactive cytokine production, sepsis, organ damage, and death that were remarkably similar to infection of Ifnar2/−/− mice completely lacking type I IFN signaling. In Mavs−/−/Ifnar2/−/− myeloid cells and mice lacking both Ifnar and the RIG-I-like receptor adaptor gene Mavs, cytokine production was muted despite high levels of WNV infection. Thus, in myeloid cells, viral infection triggers signaling through MAVS to induce proinflammatory cytokines that can result in sepsis and organ damage. Viral pathogenesis was caused in part by massive complement activation, as liver damage was minimized in animals lacking complement components C3 or factor B or treated with neutralizing anti-CS antibodies. Disease in Ifnar2/−/− and CD11c Cre+Ifnar2/−/− mice was also facilitated by the proinflammatory cytokine TNF-α, as blocking antibodies diminished complement activation and prolonged survival without altering viral burden. Collectively, our findings establish the dominant role of type I IFN signaling in myeloid cells in restricting virus infection and controlling pathological inflammation and tissue injury.


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Introduction

Type I interferons (IFN) are a family of antiviral cytokines that are produced early in response to viral infection [1]. RNA intermediates of viral replication are recognized by cytosolic and endosomal pattern recognition receptors (PRR), such as RIG-I-like receptors (RLR) or Toll-like receptors (TLR), which signal through adaptor molecules (e.g., MAVS, TRIF, and MyD88) and transcription factors (e.g., IRF-3 and IRF-7) to induce type I IFN expression and secretion. Type I IFNs bind to a heterodimeric receptor (IFNAR) and mediate pleiotropic effects downstream of a canonical Janus kinase (JAK)-Signal transducers and activators of transcription (STAT) signaling pathway. This results in the induction of antiviral IFN-stimulated genes (ISGs), activation of antigen-presenting cells, and regulation of cytokine and chemokine production (reviewed in [2]). Although IFNAR is expressed on all nucleated cells, individual cell types may respond differently to signaling by type I IFN, as evidenced by overlapping yet distinct transcriptional programs [3,4].

CD11c+ cells are integral members of the mammalian innate and adaptive immune response. In the mouse, CD11c+ are highly on dendritic cell (DC) subsets (CD14+, CD8α+, and CD103+), some macrophage (MO) populations, and on selected CD8+ T cell subsets [5–7]. CD11c+ cells are professional antigen presenting cells that respond to viral infection through a number of PRR, including the RLRs. CD11c+ DCs process and present antigens, express co-stimulatory molecules, and secrete cytokines and chemokines that regulate cell migration, leukocyte recruitment, and activation of adaptive immunity [7].

West Nile virus (WNV) is a member of the Flaviviridae family of positive-stranded enveloped RNA viruses and causes neuroinvasive disease in humans and other vertebrate animals [8]. Mosquitoes transmit WNV and in the skin CD11c+ cells and keratinocytes are believed to be initial targets of infection [9,10]. Infected DCs migrate to the draining lymph node (LN) where viral replication ensues, resulting in viremia and spread to other peripheral organs [11]. Viral replication in the skin and LN induces a local and systemic type I IFN response, which is critical for limiting WNV replication and preventing dissemination to the brain and spinal cord. Indeed, subcutaneous WNV infection of Ifnar2/−/− mice results in a rapidly fatal infection, which is
Author Summary

Although it is well established that the interferon (IFN) signaling pathway restricts infection by many viruses, the key cell types in vivo that contribute to this process remain poorly characterized. To address this question in the context of West Nile virus (WNV) pathogenesis, we infected mice that specifically delete the type I IFN receptor gene (Ifnar) in subsets of myeloid cells, including dendritic cells and macrophages. Remarkably, mice lacking Ifnar expression only in myeloid cell subsets rapidly developed a sepsis-like syndrome that was characterized by enhanced WNV infection and visceral organ injury and caused by massive proinflammatory cytokine production and complement activation. By using additional gene targeted deletion mice, we show that WNV infection triggered signaling through the RIG-I like receptor adaptor protein MAVS to cause complement activation, sepsis, and tissue damage. Indeed, liver damage was minimized in animals lacking specific complement components, or treated with neutralizing anti-complement or anti-TNF-α antibodies. Our results establish how type I IFN signaling in dendritic cells and macrophages restricts infection, controls inflammatory cascades, and prevents pathogenesis in vivo.

associated with high viremia and altered tissue and cellular tropism compared to wild-type (WT) mice [12,13]. Analogously, Mavs−/− mice, which lack RLR signaling, exhibit increased susceptibility to WNV infection in many tissues [14]. Recent reports also have observed that the control and regulation of WNV infection requires IL-1ß production [15–17], and that inflammasome activation synergizes with type I IFN signaling to suppress WNV replication.

The significance of cell type-specific responses to IFN in vivo in the context of the control of viral pathogenesis is not well understood. Prior experiments with WNV infection suggested that type I IFN signaling has distinct temporal functions in the development of adaptive immunity [18]. The generation of cell type-specific conditional deletions of Ifnar has allowed its role on specific cell types to be analyzed in the context of infectious, inflammatory, or neoplastic disease [19]. Studies have shown an enhanced susceptibility to mouse hepatitis (MHV) and norovirus (MNoV) infection in animals with conditional Ifnar deletions, and this was associated with greater viral burden and decreased survival compared to WT animals [20–22].

Here, we evaluated WNV pathogenesis in mice lacking Ifnar expression in CD11c+ cells (CD11c CreIfnarf/f mice) or MO/myelocytes/granulocytes (LysM CreIfnarf/f mice). Remarkably, deletion of Ifnar in either CD11c+ or MO/myelocyte/granulocyte cells resulted in severe WNV disease that essentially copied the phenotype of the complete Ifnar−/− mice. Thus, the dominant antiviral effects in vivo of type I IFN signaling against WNV occur in myeloid cell types. Analysis of WNV-infected mice revealed preferential infection of Ifnar−/− myeloid cells, and this resulted in a syndrome of “cytokine storm”, which was associated with liver and kidney damage, and rapid death. Immunopathogenesis in WNV-infected Ifnar−/− and CD11c CreIfnarf/f mice animals was mitigated by exogenous administration of TNF-α blocking antibodies, and the sepsis syndrome was associated with massive alternative pathway complement activation as tissue damage was improved in mice lacking the complement components C3 or factor B. Our experiments suggest that high levels of viral replication in WNV-infected Ifnar−/− myeloid cells can trigger uncontrolled production of proinflammatory cytokines and pathological complement induction and activation, which together contribute to a sepsis-like syndrome.

Results

CD11c CreIfnarf/f, LysM CreIfnarf/f, and Ifnar−/− mice are vulnerable to WNV infection

In vivo, WNV preferentially infects myeloid cells in peripheral tissues and neurons in the brain and spinal cord [23–25]. Given that DCs are targets of WNV infection, produce antiviral cytokines, and shape adaptive immunity, we hypothesized that type I IFN signaling in these cells orchestrates protection against WNV. To assess the role of type I IFN receptor signaling on DCs, we utilized CD11c CreIfnarf/f mice, in which IFNAR expression is markedly decreased on CD4+ and CD8αα DCs but maintained on other hematopoietic cells, including neutrophils, natural killer, T and B cells [26] and data not shown).

Infection of Ifnar−/− mice with WNV resulted in 100% mortality with a mean survival time of 3 days, as reported previously [12]. Unexpectedly, infection of CD11c CreIfnarf/f mice resulted in essentially the same phenotype (Fig. 1A) with no difference in the mean survival time compared to Ifnar−/− mice. Although WNV-infected Ifnar−/− and CD11c CreIfnarf/f mice were not apparent. Ifnar−/− and CD11c CreIfnarf/f mice were more vulnerable to WNV infection than CreIfnarf/f littermate controls or CD19 CreIfnarf/f mice, which lack IFNAR expression only on B cells. The decreased expression of IFNAR on CD11c CreIfnarf/f cells was confirmed in mice following WNV infection (Fig. 1B). Thus, the loss of IFNAR expression on CD11c+ cells resulted in a clinical phenotype after WNV infection that was nearly identical to a deletion of IFNAR on all cells. When we repeated a subset of experiments with LysM CreIfnarf/f mice, which delete IFNAR expression on MO, monocytes, and granulocytes (data not shown), a similar rapid death phenotype was observed after WNV infection (Fig. 1A), with a minimally longer survival time (mean of 3.5 days) compared to the Ifnar−/− or CD11c CreIfnarf/f mice.

To determine whether the susceptibility phenotype of the CD11c CreIfnarf/f mice could be generalized to other arthropod-borne viruses, we infected animals with chikungunya virus (CHIKV), an unrelated arthritogenic alphavirus (Fig. 1C). CHIKV preferentially targets myoblasts, fibroblasts, and some MO populations but is not reported to infect DCs [27]. As seen previously [28], Ifnar−/− mice infected with CHIKV succumbed to infection within 4 to 5 days. In contrast, the CD11c CreIfnarf/f, LysM CreIfnarf/f, or the CreIfnarf/f littermate control mice failed to develop lethal CHIKV infection. Thus, selective deletion of IFNAR expression on CD11c+ or other myeloid cells did not make mice vulnerable to other viruses, even if a complete gene deletion did; these results are consistent with studies with the coronavirus MHV, which showed a partial lethality phenotype in CD11c CreIfnarf/f mice [20].

We next assessed whether the loss of IFNAR expression on CD11c+ cells impacted tissue viral burden to the same extent as that of Ifnar−/− mice. High titers (106 to 109 FFU/mg) of WNV were detected in the spleen, liver, lung, kidney, brain, and heart of Ifnar−/− and CD11c CreIfnarf/f mice at 48 hours post infection (Fig. 2A–F). As expected, greater infection was observed in Ifnar−/− and CD11c CreIfnarf/f mice compared to CreIfnarf/f littermate controls. Similar trends in WNV infection were observed in the serum of Ifnar−/−, CD11c CreIfnarf/f, and LysM CreIfnarf/f mice at 48 hours (Fig. 2G). Flow cytometric analysis
of immune cells from the blood of infected Ifnar−/− mice revealed intracellular WNV antigen in CD11c+ CD8+ cells, CD11c+ CD4+ cells, CD11b+ cells, and some CD19+ cells (Fig. 2H), consistent with an earlier report [12]. In infected CD11c Cre Ifnar+/f mice, WNV antigen was detectable in ~3 to 6 percent of CD11c+ cells, and in ~8 to 15 percent of CD11b+ cells that co-expressed varying amounts of CD11c. In WNV-infected Cre−/− Ifnar−/− mice, viral antigen in CD11c+ cells from the blood was near the threshold of detection at 48 hours after infection. Thus, a loss of IFNAR signaling in subsets of CD11c+ cells created a more permissive intracellular environment for WNV replication.

**Pathological analysis of tissues from WNV-infected CD11c Cre−/Ifnar−/− mice**

Necropsy at 48 hours post infection revealed gross macroscopic tissue damage including hemorrhages, infarcts, and edema in the liver, spleen, and LN of Ifnar−/− and CD11c Cre−/Ifnar+/f mice that was not evident in WNV-infected Cre−/Ifnar+/f mice (data not shown). Histological analysis of visceral organs from Ifnar−/− and CD11c Cre−/Ifnar+/f mice showed increased cellular infiltrates, coagulation necrosis, and tissue destruction after WNV infection of the liver, spleen, and draining LN when compared to the Cre−/− Ifnar−/− mice. However, little to no cellular infiltrate or tissue destruction was observed in the brains of any of the mice at this early time point (Fig. 3A-D). Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) analysis confirmed greater numbers of dead cells in the liver, spleen, and LN but not in the brains of Ifnar−/− and CD11c Cre−/Ifnar+/f mice (Fig. 3E-H). Immunohistochemistry (IHC) was performed to determine whether these phenotypes correlated with cellular infection. Despite the high titer (10^8 FFU/mg) of infectious virus in liver homogenates (Fig. 2B), we observed relatively low levels of WNV antigen staining in hepatic sections from Ifnar−/− and CD11c Cre−/Ifnar+/f mice (Fig. 3J). We also failed to observe any obvious brain pathology in mice at 48 hours and this correlated with a paucity of viral antigen in tissue sections. In comparison, WNV antigen staining was more apparent in the draining LN and the spleen of Ifnar−/− and CD11c Cre−/Ifnar+/f mice compared to Cre−/Ifnar+/f mice (Fig. 3J and K). Combined with the flow cytometry data on cell subsets (Fig. 2H), our analysis suggests that the enhanced viral burden in Ifnar−/− and CD11c Cre−/Ifnar+/f mice was attributable to preferential infection of Ifnar−/− CD11c+ cells. The high viral titers in some organs (e.g., liver and brain) showing little WNV antigen staining by IHC might be due to infected CD11c+Cre−/Ifnar+/f cells that were retained in blood vessels; these animals showed a deteriorating clinical phenotype and were difficult to perfuse despite large volumes of saline administration. Overall, the histology revealed a high degree of tissue injury in lymphoid compartments of WNV-infected Ifnar−/− and CD11c Cre−/Ifnar+/f mice that reflected enhanced infection of cells lacking IFNAR expression. Tissue destruction in some organs, however, did not appear commensurate with the level of viral antigen present in the tissue parenchyma.

**Blood chemistry reveals extensive liver and kidney injury**

To understand why Ifnar−/− and CD11c Cre−/Ifnar+/f mice succumbed so rapidly to WNV infection, we analyzed their blood chemistries. At 48 hours after WNV inoculation we observed...
increased levels of blood urea nitrogen (BUN) in Ifnar$^{-/-}$ and CD11c Cre$^{+}$Ifnar$^{+/f}$ mice (22.8 mg/dL and 32.4 mg/dL respectively, versus 15.5 mg/dL, $P$, 0.02; Fig. 4A), serum creatinine (4.5 mg/L and 4.9 mg/L respectively, versus 1.5 mg/L, P=0.02; Fig. 4B), alanine aminotransferase (ALT) (2,495 u/L and 1,110 u/L respectively, versus 334 u/L, $P$, 0.02; Fig. 4C), and aspartate aminotransferase (AST) (4,070 u/L and 1,676 u/L respectively, versus 382 u/L, $P$, 0.004; Fig. 4D) compared to Cre$^{+}$Ifnar$^{+/f}$ mice. The elevated serum BUN and creatinine values indicate that WNV-infected Ifnar$^{-/-}$ and CD11c Cre$^{+}$Ifnar$^{+/f}$ mice experienced acute renal injury. The elevated liver enzymes (ALT and AST) reflect acute hepatic injury, which could be due to direct viral infection (although not seen by IHC), immune-mediated injury, or ischemia. The observed hypoglycemia may be secondary to depleted glycogen stores, impaired gluconeogenesis, or increased peripheral glucose utilization that occurs during infection or sepsis [29]. Similarly elevated serum levels of AST and ALT and decreased levels of glucose were observed in WNV-infected LysM Cre$^{+}$Ifnar$^{+/f}$ mice, compatible with a syndrome of acute hepatic injury (Fig. 4C–E).

"Cytokine storm" in WNV-infected CD11c Cre$^{+}$Ifnar$^{+/f}$, LysM Cre$^{+}$Ifnar$^{+/f}$, and Ifnar$^{-/-}$ mice

To understand further the basis of organ damage in Ifnar$^{-/-}$, LysM Cre$^{+}$Ifnar$^{+/f}$, and CD11c Cre$^{+}$Ifnar$^{+/f}$ mice after WNV infection, we measured serum levels of 23 pro- and anti-inflammatory cytokines and chemokines using a Bioplex assay (Fig. 5A and Table 1). At 24 hours after infection, we detected no difference in cytokine levels among Cre$^{+}$Ifnar$^{+/f}$, Ifnar$^{-/-}$, and CD11c Cre$^{+}$Ifnar$^{+/f}$ mice. However, one day later, we observed increased levels of most proinflammatory cytokines in the Ifnar$^{-/-}$ and CD11c Cre$^{+}$Ifnar$^{+/f}$ mice when compared to the Cre$^{+}$Ifnar$^{+/f}$
mice; 22 of the 23 cytokines in the panel were elevated in mice lacking IFNAR expression on CD11c+ cells (Table 1). Examination of the cytokine profile in the serum of LysM Cre+ Ifnarfl/fl mice 48 hours after WNV infection also revealed an increase in 20 of the 23 measured cytokines and chemokines (Fig. 5A and Table 1). We detected 2 to 1,600 fold increases (P<0.0001) in levels of individual proinflammatory cytokines after WNV infection, which included vasoactive (e.g., TNF-α) and inflammation-generated (e.g., IL-1β) cytokines. The massive increase in multiple proinflammatory cytokines after WNV infection in Ifnar−/− and CD11c or LysM Cre+ Ifnarfl/fl mice coupled with renal and hepatic injury and low viral antigen staining in tissues...
suggested a picture of sepsis due to “cytokine storm”. In comparison, relatively small increases in only six of the cytokines were observed in CHIKV infected Ifnar<sup>-/-</sup> and Cre<sup>+</sup> Ifnar<sup>fl/fl</sup> mice (Fig. S1 and Table S1).

We hypothesized that Ifnar<sup>-/-</sup> myeloid cells produced the high levels of proinflammatory cytokines in response to extensive viral replication and RNA pathogen-associated molecular pattern (PAMP) generation. To demonstrate that CD11c<sup>+</sup> cells in vivo induced pro-inflammatory cytokines, we performed qRT-PCR analysis of CD11c<sup>+</sup> cells isolated by antibody-coated magnetic bead separation from the spleens of Ifnar<sup>-/-</sup> mice; these cells had higher levels of IL-1β, IL-6 and TNF-α mRNA and viral RNA compared to those isolated from Cre<sup>-</sup> Ifnar<sup>fl/fl</sup> mice (Fig. 5B). MAVS-dependent induction of “cytokine storm”

WNV-infected Ifnar<sup>-/-</sup> CD11c<sup>+</sup> cells produced proinflammatory cytokines, which suggests a linkage between uncontrolled virus replication and “cytokine storm”. A recent study suggested that in the context of LPS-priming, MAVS provides a second signal for inflammasome activation [30]. Consistent with our hypothesis that excessive levels of viral RNA in Ifnar<sup>-/-</sup> myeloid cells results in MAVS signaling and inflammasome activation, WNV-infected Mavs<sup>-/-</sup> × Ifnar<sup>-/-</sup> double knockout (DKO) mice succumbed at a later time point compared to Ifnar<sup>-/-</sup> mice (4.3 days compared to 2.7 days, [13]) despite the absence of pathways that both sense and control WNV infection [13,31–37]. Based on these results, we predicted that the absence of MAVS signaling in WNV-infected Mavs<sup>-/-</sup> × Ifnar<sup>-/-</sup> DKO mice would limit cytokine induction despite high levels of infection and viral RNA generation in permissive myeloid cells.

To test this hypothesis, we infected Ifnar<sup>-/-</sup>, Mavs<sup>-/-</sup>, Mavs<sup>-/-</sup> × Ifnar<sup>-/-</sup> DKO, and WT mice with WNV and harvested serum at 48 hours. Analysis of viremia revealed 10<sup>6</sup> to 10<sup>9</sup>-fold higher levels in Ifnar<sup>-/-</sup> (1.6 × 10<sup>6</sup> FFU/ml, P = 0.03), Mavs<sup>-/-</sup> (4.4 × 10<sup>5</sup> FFU/ml, P = 0.03), and Mavs<sup>-/-</sup> × Ifnar<sup>-/-</sup> DKO (2.0 × 10<sup>5</sup> FFU/ml, P = 0.03) mice compared to WT (2.3 × 10<sup>5</sup> FFU/ml) mice (Fig. 6A). Among CD11c<sup>+</sup> cells isolated from the spleen, a similar percentage of cells were infected with WNV in Ifnar<sup>-/-</sup>, Mavs<sup>-/-</sup>, and Mavs<sup>-/-</sup> × Ifnar<sup>-/-</sup> DKO mice compared to WT mice (Fig. 6B). We next measured cytokine levels at 48 hours after WNV infection of Ifnar<sup>-/-</sup>, Mavs<sup>-/-</sup>, Mavs<sup>-/-</sup> × Ifnar<sup>-/-</sup> DKO, and WT mice (Fig. 6C and Table 2). Only serum from the Ifnar<sup>-/-</sup> mice revealed the profile of elevated cytokine levels. Microarray analysis from the spleens of WNV-infected Ifnar<sup>-/-</sup>, Mavs<sup>-/-</sup>, Mavs<sup>-/-</sup> × Ifnar<sup>-/-</sup> DKO, and WT mice (Fig. 6D) corroborated these findings. mRNA levels of cytokines associated with NF-κB and inflammasome activation (e.g., TNF-α, IL-6, IL-1β, and IL-33) were elevated in the spleens of Ifnar<sup>-/-</sup> mice compared to Mavs<sup>-/-</sup>, Mavs<sup>-/-</sup> × Ifnar<sup>-/-</sup> DKO, or WT mice. To further define the linkage between MAVS and cytokine production, we infected bone marrow derived DCs (BMDCs) from Ifnar<sup>-/-</sup>, Mavs<sup>-/-</sup>, Mavs<sup>-/-</sup> × Ifnar<sup>-/-</sup> DKO, and WT mice (Fig. 6F). We next measured cytokine levels at 48 hours after WNV infection of Ifnar<sup>-/-</sup>, Mavs<sup>-/-</sup>, Mavs<sup>-/-</sup> × Ifnar<sup>-/-</sup> DKO, and WT mice (Fig. 6B).
was not unexpected; even though IFIT genes are ISGs, their expression can be induced directly by IRF-3 [38,39]. In Ifnar−/− BMDCs, the increase in WNV infection results in enhanced RNA PAMP generation, MAVS signaling, and IRF-3 nuclear translocation, which induces IFIT genes independently of the IFN signaling pathway.

Inflammasome activation in Ifnar−/− CD11c+ cells after WNV infection

We observed higher levels of IL-1β in the serum of Ifnar−/− mice, which were absent in Mavs−/−×Ifnar−/− DKO mice (Fig. 6B). This observation suggested that PRR signaling through MAVS was required for inflammasome activation. Prior studies had established that NLRP3 is the key Nod-like receptor inducing IL-1β and restricting WNV infection in mice [17]. To test whether the increase in IL-1β levels observed in WNV-infected Ifnar−/− mice was due to excess triggering of the NLRP3 inflammasome, we used a MAb (MAR1-5A3) to block IFNAR signaling in WT or Nlrp3−/− BMDCs and mice. At 48 hours after WNV infection, Western blot analysis revealed reduced amounts of cleaved IL-1β (Fig. S3A) that were absent in Mavs−/− and Cre2−/−/Ifnar−/− mice which inhibited both soluble and cell surface-associated forms (Fig. 4A). This observation suggested that NLRP3 is the key inflammasome inducer of IL-1β in response to WNV infection.

To assess whether the inflammatory response contributed to the multi-organ injury and lethality, we neutralized TNF-α blockade prolongs survival after WNV infection in CD11c Cre2+Ifnar−/− mice

To assess whether the inflammatory response contributed to the multi-organ injury and lethality, we neutralized TNF-α (Fig. 7A). Administration of a TNF-α blocking MAb (200 µg), which inhibited both soluble and cell surface-associated forms [40], one day prior to infection increased the median survival time of Ifnar−/− (from 3 to 5 days, P=0.002) and CD11c Cre2+Ifnar−/− (from 3 to 8 days, P=0.0009) mice. To support of our hypothesis that the multi-organ failure induced by “cytokine storm” contributed to disease in the Ifnar−/− and CD11c Cre2+Ifnar−/− mice, we measured viral titers in the serum of anti-TNF-α and isotype control MAb-treated mice (Fig. 7B). Viremia at 48 hours post infection was not different suggesting that the beneficial effect of anti-TNF-α therapy was not due to effects on viral replication. Analysis of blood chemistries at 48 hours after infection of the CD11c Cre2+Ifnar−/− and Ifnar−/− mice following anti-TNF-α treatment revealed improved AST, ALT, and glucose levels that were not different from WNV-infected Cre2+Ifnar−/− control mice.

**Figure 5. Serum cytokine levels in WNV-infected mice. A.** Ifnar−/−, CD11c Cre2+Ifnar−/−, LysM Cre2+Ifnar−/−, and Cre2−Ifnar−/− mice (n = 13 for each group) were infected 102 PFU of WNV. Forty-eight hours later, serum was collected and the concentration of IL-1β, IL-6, and TNF-α was determined. Mean values ± SD are shown. B. Forty-eight hours after WNV infection splenic CD11c+ cells were isolated from Ifnar−/−, CD11c Cre2+Ifnar−/−, and Cre2−Ifnar−/− mice by positive selection with antibody-coated magnetic beads (n = 3 for each group). Total RNA was prepared and qRT-PCR was used to determine the amount of IL-18, IL-6, TNF-α, and WNV E RNA. The mean values ± SD are shown. The data is expressed as fold-increase in mRNA relative to samples from WNV-infected Cre2−Ifnar−/− mice.

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proinflammatory cytokines, and this correlated with Cre blockade can reduce induction of most but not all of the Cre (CCL5), and MIP1-ß (CCL4) that were decreased in CD11c treatment, five cytokines (IL-2, IL-12p40, GM-CSF, Rantes IL-6 were observed in Ifnar and hypothesized that complement activation and production of [41–43]. Prior studies also have established that TNF- develops vascular leakage and sepsis syndrome and have evidence

ifnar−−/−, CD11c Cre+ ifnar+/+ LysM Cre+ ifnar+/+ and Cre− ifnar+/+ mice during WNV infection.

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<td>GM-CSF</td>
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<td>492</td>
<td>288</td>
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<tr>
<td>RANTES</td>
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<td>TNF-α</td>
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<td>241</td>
<td>2144</td>
<td>1397</td>
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</tbody>
</table>

Ifnar−−/−, CD11c LysM Cre+ ifnar+/+, and Cre− ifnar+/+ mice (n = 13 for each group) were infected with 10^{5} PFU of WNV. Forty-eight hours later serum was collected and cytokines were analyzed by bioplex assay. Mean (pg/ml) and standard deviations (SD) are shown and P values are compared to Cre− ifnar+ + mice. Data are pooled from four independent experiments. N/a indicates results that are not statistically different compared to infected Cre− ifnar+ + mice.

doi:10.1371/journal.ppat.1004086.t001

Alternative pathway of complement activation contributes to disease pathogenesis

Humans infected with Dengue virus, a closely related flavivirus, develop vascular leakage and sepsis syndrome and have evidence of extensive complement activation in their plasma and tissues [41–43]. Prior studies also have established that TNF-α regulates expression of complement genes in myeloid cells [44]. We hypothesized that complement activation and production of anaphylatoxins (e.g., C3a and C5a) might be induced by TNF-α and contribute to disease pathogenesis in WNV-infected CD11c-Cre+ ifnar+/+ mice. To evaluate this hypothesis, we first assessed mRNA levels of individual complement components in the spleen and liver of WNV-infected WT, Mavs−−/−, Ifnar−−/−, and Mavs−−/− × Ifnar−−/− DKO mice. Notably, C3 and factor B (Fb) levels were greater in WNV-infected Ifnar−−/− mice compared to the other genotypes (Fig. 8A) suggesting that expression of key complement components was regulated via a MAVS-dependent pathway. We next examined complement activation in serum by assessing levels of complement proteins and split products by Western blot and ELISA. At 24 to 48 hours after WNV infection, levels of C3, Bb, and their cleaved products of (C3-a and Ba) were higher in Ifnar−−/− and CD11c-Cre+ ifnar+/+ mice compared to WT mice (Fig. 8B). These results confirm the induction of key complement proteins and activation of the complement cascade in vivo. In contrast, C3 and Bb cleavage products were decreased or absent in the serum of WNV-infected Mavs−−/− or Ifnar−−/− × Mavs−−/− DKO mice at 48 hours (Fig. 8C), indicating that RLR signaling through MAVS was required for complement induction and activation. Consistent with a role for complement contributing to pathogenesis, liver injury at 48 hours was minimized in WNV-infected C3−−/− and Fb−−/− mice given the IFNAR-blocking MAb MAR1-5A3 compared to similarly-treated WT mice. Analysis of blood chemistries after WNV infection of MAR1-5A3 treated C3−−/− and Fb−−/− mice revealed normal AST and ALT values, compared to MAR1-5A3 treated C57BL/6 control mice (Fig. 8D), even though viremia and cytokine levels remained elevated (Fig. 8E and F). While liver injury persisted in MAR1-5A3-treated
Figure 6. Effect of MAVS signaling on cytokine induction. A–C. WT, Ifnar<sup>−/−</sup>, Mavs<sup>−/−</sup>, or Mavs<sup>−/−</sup> x Ifnar<sup>−/−</sup> DKO mice (n = 4 for each group) were infected with 10<sup>2</sup> PFU of WNV and 48 hours later serum was collected. A. Viremia was determined using focus-forming assays. Data are shown as FFU per ml of serum. The dotted line represents the limit of sensitivity of the assay. Error bars indicate the SD. B. At 48 hours after WNV inoculation, splenocytes were gated on CD19<sup>+</sup> CD11c<sup>+</sup> CD11b<sup>lo</sup> followed by intracellular staining for WNV antigen. C. The concentration of IL-1β, IL-6 and TNF-α cytokine levels were equal between Mavs<sup>−/−</sup>, Mavs<sup>−/−</sup> x Ifnar<sup>−/−</sup> and WT C57BL/6 control mice. D. A microarray was performed on RNA isolated from spleen of mock-infected mice (n = 2) and WNV-infected WT (n = 3), Mavs<sup>−/−</sup> (n = 3), Ifnar<sup>−/−</sup> (n = 3), and Mavs<sup>−/−</sup> x Ifnar<sup>−/−</sup> DKO (n = 3) mice. A student’s t-test (P<0.01) was performed.
performed to determine the genes that had different expression levels with infection compared to levels in mock infections for each of the four mouse strains (1.5 fold change cut-off). Quantitative analysis of pro-inflammatory cytokines and chemokines is shown on the right. E, WT, Ifnar\(-/-\), Mavs\(-/-\), Ifnar\(-/-\)×Mavs\(-/-\) DKO BMDCs were infected with WNV and 0, 24 or 48 hours later viral burden in the supernatant was measured. The data are the mean of three independent experiments. Error bars indicate SD. Asterisks denote statistical significance relative to WT cells (*, \(P<0.05\); **, \(P<0.01\)). F, Western blot showing phospho-p65 and total (relative) p65 staining in WT, Mavs\(-/-\), Ifnar\(-/-\), Ifnar\(-/-\)×Mavs\(-/-\) DKO BMDCs at 0 (M, mock), 24 or 48 after WNV infection. β-actin staining is included as a loading control. The results are representative of two independent experiments. doi:10.1371/journal.ppat.1004086.g006

\[\text{Ifnar}\]-deficient mice, indicating that disease was induced primarily by the alternative and not classical or lectin pathways of complement activation, glycermia was restored to normal levels. A partial phenotype also was observed in MAR1-5A3-treated normal levels. A partial phenotype also was observed in MAR1-Cre\(-/-\) mice, which delete the receptor for the complement anaphylatoxin C3a: Cytokine (IL-1β, IL-6, and TNF-α) and liver enzymes levels remained elevated in the serum but glucose levels normalized (Fig. 8A). In comparison, treatment of CD11c-Cre\(^{+/-}\) Ifnar\(^{WT}\) mice with neutralizing MAbs against C5 reduced liver injury and prevented hypoglycemia compared to animals administered isotype control MAbs (Fig. 8G). To link our findings showing TNF-α signaling and complement activation contribute to WNV-induced disease and sepsis, we assessed complement activation in WNV-infected CD11c-Cre\(^{+/-}\) Ifnar\(^{WT}\) mice treated with blocking anti-TNF-α MAbs. Lower levels of C3 and factor B split-products (C3a and Bb) were observed in the serum and plasma of mice administered blocking TNF-α compared to isotype control MAbs (Fig. 8H and I). These experiments place pathologic complement activation downstream of viral infection, MAVS signaling, and TNF-α signaling and indicate that it contributes to the sepsis-like syndrome.

**Discussion**

Although the contribution of type I IFN signaling to the control of viral infection \textit{in vivo} is established, its importance in specific cell subsets remains less well understood. We evaluated the function of type I IFN signaling in myeloid cells \textit{in vivo} against WNV infection. We targeted these cells because prior studies had indicated that WNV replicated preferentially in CD11b\(^+\) and CD11c\(^+\) cells [12,45]. A striking phenotype was observed after WNV infection of CD11c or LysM Cre\(^{+/-}\) Ifnar\(^{WT}\) mice, as these animals succumbed with a similar rate and kinetics compared to the complete gene deletion Ifnar\(-/-\) mice. In contrast, deletion of IFNAR expression only on B cells (CD19 Cre\(^{+/-}\) Ifnar\(^{WT}\)) showed a phenotype after WNV infection that resembled the Cre\(-/-\) Ifnar\(^{WT}\) mice. Viral burden in several organs of CD11c Cre\(^{+/-}\) Ifnar\(^{WT}\) mice was markedly elevated and approached that seen in Ifnar\(-/-\) mice.

### Table 2. Cytokine and chemokine levels in serum of WNV-infected WT, Mavs\(-/-\), Ifnar\(-/-\), and Mavs\(-/-\)×Ifnar\(-/-\) DKO mice.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>WT pg/ml</th>
<th>SD</th>
<th>Mavs(-/-) pg/ml</th>
<th>SD</th>
<th>P value</th>
<th>Ifnar(-/-) pg/ml</th>
<th>SD</th>
<th>P value</th>
<th>Mavs(-/-)×Ifnar(-/-) DKO pg/ml</th>
<th>SD</th>
<th>P value</th>
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</thead>
<tbody>
<tr>
<td>IL-1α</td>
<td>12.8</td>
<td>3.64</td>
<td>15.7</td>
<td>0.97</td>
<td>ns</td>
<td>54.9</td>
<td>3.05</td>
<td>0.029</td>
<td>13.13</td>
<td>3.42</td>
<td>ns</td>
</tr>
<tr>
<td>IL-1β</td>
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<td>163</td>
<td>38.8</td>
<td>ns</td>
<td>381</td>
<td>38.8</td>
<td>0.029</td>
<td>142</td>
<td>12.6</td>
<td>ns</td>
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<tr>
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<td>23.04</td>
<td>5.93</td>
<td>ns</td>
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<td>0.029</td>
<td>14.8</td>
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<td>ns</td>
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<td>0.26</td>
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<td>ND</td>
<td>ND</td>
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<td>ns</td>
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<td>79.8</td>
<td>8.53</td>
<td>ns</td>
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<td>ns</td>
<td>12743</td>
<td>1143</td>
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<td>402</td>
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<td>ns</td>
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<td>1.25</td>
<td>ns</td>
<td>71.5</td>
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<td>TNF-α</td>
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<td>1245</td>
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<td>2636</td>
<td>562</td>
<td>ns</td>
<td>661</td>
<td>188</td>
<td>ns</td>
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WT, Ifnar\(-/-\), Mavs\(-/-\), Ifnar\(-/-\)×Mavs\(-/-\) DKO (n = 4 for each group) were infected with 10\(^2\) PFU of WNV. After 48 hours, serum was collected and the concentration of cytokines was determined. Mean values, SD, and P values are compared to WT mice. Not detected (ND) indicates samples that were below the limit of detection for the assay. ns, indicates results that are not statistically different from WT mice. doi:10.1371/journal.ppat.1004086.t002
Our experiments revealed a dominant antiviral effect in vivo of type I IFN signaling in myeloid cells, either CD11c+ cells or MØ/monocytes/granulocytes. Pathological analyses showed preferential WNV infection of Ifnar2/2 myeloid cells, and this resulted in "cytokine storm", which was associated with liver and kidney damage, and rapid death. High levels of WNV RNA in Ifnar2/2 myeloid cells triggered uncontrolled production of proinflammatory cytokines, pathological complement induction and activation, which together caused a sepsis-like syndrome. This phenotype did not occur universally: whereas 100% of Ifnar2/2 mice succumbed to infection with the arthritogenic CHIKV alphavirus, no disease or lethality was observed in CD11c or LysM Cre Ifnar2/2 mice.

The phenotype after WNV infection of CD11c or LysM Cre Ifnar2/2 mice varies from that observed after infection with MHV [20] or MNoV [22]. In the MHV study, increased vulnerability was observed in LysM Cre Ifnar2/2 mice but it did not recapitulate that seen in Ifnar2/2 mice. In comparison, deletion on Ifnar on CD11c cells resulted in only a subset (~30%) of animals dying, and this occurred almost a week later. In the MNoV study, IFNAR-dependent responses in MØ and DCs limited viral...
infection but were dispensable for preventing lethal infection, as all MNoV-infected CD11c or LysM Cre<sup>Ifnar<sup>f/f</sup></sup> animals survived in contrast to Ifnar<sup>2/2</sup> mice. Systemic cytokine levels were not measured in either study. Related to these findings, cell type-restricted deletion of Stat1 in MØ, monocytes, and granulocytes (LysM Cre<sup>Stat1<sup>f/f</sup></sup>) but not DCs (CD11c Cre<sup>Stat1<sup>f/f</sup></sup>) resulted in enhanced lethality after <i>Listeria monocytogenes</i> infection [20,21]. Higher levels of cytokines and chemokines were observed in <i>Listeria</i>-infected LysM Cre<sup>Stat1<sup>f/f</sup></sup> mice, although they were lower than in infected Stat1<sup>2/2</sup> mice. Analogous to our studies, <i>Listeria</i>-infected mice lacking IFNAR expression on MØ also sustained liver damage. Thus, loss of IFNAR expression in distinct

### Table 3. Blood chemistry in MAb treated WNV-infected CD11c Cre<sup>+</sup> Ifnar<sup>f/f</sup> and Ifnar<sup>−/−</sup> mice.

<table>
<thead>
<tr>
<th></th>
<th>AST Conc. (u/L)</th>
<th>SD</th>
<th>P value</th>
<th>ALT Conc. (u/L)</th>
<th>SD</th>
<th>P value</th>
<th>Glucose Conc. (mg/dL)</th>
<th>SD</th>
<th>P value</th>
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<td>CD11c Cre&lt;sup&gt;+&lt;/sup&gt; anti-TNFα</td>
<td>199</td>
<td>91.1</td>
<td>0.032</td>
<td>194</td>
<td>93.6</td>
<td>0.004</td>
<td>314</td>
<td>130</td>
<td>0.008</td>
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<tr>
<td>CD11c Cre&lt;sup&gt;+&lt;/sup&gt; isotype</td>
<td>505</td>
<td>238</td>
<td>4422</td>
<td>913</td>
<td>50.8</td>
<td>23.4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ifnar&lt;sup&gt;−/−&lt;/sup&gt; anti-TNFα</td>
<td>83.7</td>
<td>43.8</td>
<td>0.001</td>
<td>208</td>
<td>89.2</td>
<td>0.001</td>
<td>280</td>
<td>87.2</td>
<td>0.001</td>
</tr>
<tr>
<td>Ifnar&lt;sup&gt;−/−&lt;/sup&gt; isotype</td>
<td>6317</td>
<td>1320</td>
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<td>1192</td>
<td>82</td>
<td>25.7</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

CD11c Cre<sup>+</sup> Ifnar<sup>f/f</sup> and Ifnar<sup>−/−</sup> mice were treated with either anti-TNF-α blocking or isotype control MAb (200 µg/mouse) one day prior to infection (<i>n</i> = 6 for each group). The mice were infected with 10<sup>5</sup> PFU of WNV. At 48 hours serum was collected and AST, ALT and glucose levels were measured. Mean values ± SD are shown. P values are compared to isotype control treated samples for each group. Data are pooled from two independent experiments. doi:10.1371/journal.ppat.1004086.t003

### Table 4. Cytokine levels in serum of Ifnar<sup>−/−</sup> and CD11c Cre<sup>+</sup> Ifnar<sup>f/f</sup> mice after treatment with anti-TNF-α MAb and WNV infection.

<table>
<thead>
<tr>
<th></th>
<th>CD11c Cre&lt;sup&gt;+&lt;/sup&gt; Anti-TNFα MAb</th>
<th>CD11c Cre&lt;sup&gt;+&lt;/sup&gt; Ifnar&lt;sup&gt;f/f&lt;/sup&gt; MAb</th>
<th>Ifnar&lt;sup&gt;−/−&lt;/sup&gt; Anti-TNFα MAb</th>
<th>Ifnar&lt;sup&gt;−/−&lt;/sup&gt; Ifnar&lt;sup&gt;f/f&lt;/sup&gt; MAb</th>
</tr>
</thead>
<tbody>
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<td>pg/ml SD</td>
<td>pg/ml SD</td>
<td>pg/ml SD</td>
<td>pg/ml SD</td>
</tr>
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<td>IL-1α</td>
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<td>132.9 28.06</td>
<td>48.79 27.63</td>
</tr>
<tr>
<td>IL-1β</td>
<td>450.9 105.8</td>
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<td>44.74 289</td>
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CD11c Cre<sup>+</sup> Ifnar<sup>f/f</sup> and Ifnar<sup>−/−</sup> mice were treated with either anti-TNF-α blocking or isotype control MAb (200 µg/mouse) one day prior to infection (<i>n</i> = 6 for each group). The mice were infected with 10<sup>5</sup> PFU of WNV and at 48 hours serum was collected and the concentration of cytokines was determined. Mean values ± SD are shown. P values are compared to the isotype control MAb treated samples for each group. Data are pooled from three independent experiments. ns indicates results that are not statistically different compared to mice treated with isotype control MAb. Not detected (ND) indicates samples that were below the limit of detection for the assay. doi:10.1371/journal.ppat.1004086.t004
Figure 8. Complement activation contributes to liver injury after WNV infection. A. Microarray analysis of complement genes was performed on RNA isolated from spleen and liver of WNV-infected WT, *Ifnar*−/−, and Mavs−/−×*Ifnar*−/− mice. Genes that showed statistically significant increases (P<0.05) compared to WT are colored in yellow. Red arrows denote C3 as well as factor B relative mRNA levels. B-C. Analysis of C3 (left) and factor B (right) levels and split-products (labeled as C3–a, C3–b, and Ba) in the serum of Cre−*Ifnar*−/−, CD11c− and Cre−*Ifnar*−/− mice at 24, 36, and 48 hours after WNV infection (B) as determined by Western blotting. Similar experiments were performed on serum samples at 48 hours after WNV infection from *Ifnar*−/−, Mavs−/−, and Mavs−/−×*Ifnar*−/− mouse controls (C). The results are representative of samples from different mice. D. Serum levels of glucose, AST, and ALT in WT, C3−/− and factor B−/− mice after treatment with the IFNAR blocking MAb (MAR1-5A3) and infection with WNV. The serum was harvested 72 hours after infection. For the WT mice, a comparison is made with treatment with the isotype control MAb (GIR-208). The results are the average of two independent experiments with a total of 8 mice per group, and asterisks indicate statistically significant differences (**, P<0.01). E-F. Viremia and serum cytokines (IL-18, IL-6, and TNF-α) at 72 hours in WT, C3−/− and factor B−/− mice after treatment with the IFNAR blocking MAb (MAR1-5A3) and infection with WNV. For the WT mice, a comparison is made with treatment with an isotype control MAb (GIR-208) (**, P<0.05). G. Effect of treatment with C5 blocking MAb on liver injury in WNV-infected CD11c−*Ifnar*−/−. CD11c−Cre−*Ifnar*−/− and Cre−*Ifnar*−/− mice were treated with 1.25 mg (50 mg/kg) of BBS.1 anti-C5 antibody or isotype control (GIR-208) (at days −1 and +2) and infected with 104 PFU of WNV on day 0. At 48 hours, serum was harvested and glucose, ALT, and AST were measured. The results are the mean of two independent experiments with n = 7 or 8 mice in total (**, P<0.03). (***, P<0.001). H. Western blotting analysis of C3 (left) and factor B (right) split-products (labeled as C3–a, C3–b and Ba) in the serum of Cre−*Ifnar*−/− and *Ifnar*−/− mice at 48 hours after WNV infection in animals treated with isotype or anti-TNF-α MAb. The results are representative of two independent experiments. I. ELISA showing C3a levels in plasma of CD11c−Cre−*Ifnar*−/− and *Ifnar*−/− mice at 48 hours after WNV infection in animals treated with isotype or anti-TNF-α MAb. The results are the mean of two independent experiments with a total of n = 6 mice and asterisks indicate significant differences (**, P<0.01).

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populations of cells can enhance pathogenesis of viral and bacterial pathogens, although the level of mortality and cytokine production can vary.

Our IHC and flow cytometric analysis of tissues and blood revealed that a demonstrable fraction of infected cells in CD11c Cre−*Ifnar*−/− mice belonged to the CD11c− subset. It remains unclear why only a fraction (~5 to 10%) of CD11c− cells were targeted; this could reflect a difference in vulnerability of DC subsets or a stochastic process. A similar phenomenon was observed in vivo after WNV infection of *Ifnar*−/− BMDCs, in which a maximum of 10 to 20% of CD11c− cells were infected even at high multiplicities of infection [35]. In comparison, CHIKV does not target myeloid cells for infection in mice [28,46], and thus may not gain the same replication advantage in CD11c Cre−*Ifnar*−/− or LysM Cre−*Ifnar*−/− animals.

Prior studies in *Ifnar*−/− mice with virulent or attenuated WNV strains revealed enhanced infection and lethality [12,32–34]. While the susceptibility phenotype is related to the higher levels of WNV replication, the contribution of cytokines was not assessed. Although we measured high viral titers in the serum, organs, and brains of CD11c Cre−*Ifnar*−/− and *Ifnar*−/− mice, IHC revealed less viral antigen staining in the brain parenchyma than anticipated by the viral titer data. This suggested to us that (a) the high levels of WNV in brain homogenates could be due to infected cells in the intravascular space that were not removed despite extensive perfusion; and/or (b) although CD11c Cre−*Ifnar*−/−, LysM Cre−*Ifnar*−/− and *Ifnar*−/− mice rapidly succumbed to WNV, it might not be due to infection in the brain. Indeed, high levels of TNF-α and IL-1β and other proinflammatory cytokines were present in the serum of CD11c or LysM Cre−*Ifnar*−/− and *Ifnar*−/− mice but not in infected Cre−*Ifnar*−/− mice at 48 hours. Vasoactive cytokines affect blood vessel permeability and vascular tone [47,48], and could have limited perfusion in the CD11c Cre−*Ifnar*−/− and *Ifnar*−/− mice. Our results are most consistent with a model in which cytokine-mediated changes to the vasculature limited organ perfusion and resulted in high titers that reflect viral burden in blood rather than replication within the parenchyma of some tissues.

“Cytokine storm” reflects excessive cytokine production that occurs following infection with gram-negative bacteria or pathogenic influenza A viruses [reviewed in [47,48]]. Excessive cytokine production with vascular permeability changes also has been described for flaviviruses in the context of severe dengue virus infection in humans or IFN-signaling-deficient mice [49–51]. Our studies in CD11c Cre−*Ifnar*−/− and *Ifnar*−/− mice revealed that serum levels of twenty different inflammatory cytokines were elevated at 48 hours after WNV inoculation. Loss of type I IFN signaling on the CD11c+ or other myeloid cells affected viral tropism and resulted in dysregulated cytokine responses after WNV infection. Combining this with the likely changes in vascular resistance that affected perfusion, our results are compatible with a clinical picture of “cytokine storm”.

WNV-infected CD11c or LysM Cre−*Ifnar*−/− and *Ifnar*−/− mice developed marked elevations in serum liver enzymes and profound hypoglycemia, consistent with hepatocellular injury. Histopathological analysis of the liver in CD11c Cre−*Ifnar*−/− and *Ifnar*−/− mice revealed zonal coagulative necrosis with apoptotic hepatocytes. Similarly, in the spleen and LN marked changes in the organ architecture and cell viability were apparent. While viral antigen staining was present in lymphoid organs, it was limited in the liver, and suggested that end-organ damage was secondary to ischemia and/or toxic effects of cytokines rather than direct virus-induced apoptosis.

The high levels of IL-1β in the serum of the WNV-infected CD11c Cre−*Ifnar*−/−, LysM Cre−*Ifnar*−/− and *Ifnar*−/− mice indicated significant inflammasome activation. WNV activates the NLRP3 inflammasome to produce mature IL-1β, which helps control infection in the brain through a pathway that synergizes with type 1 IFN signaling [15,17]. Inflammasome activation requires two signals: signal 1 induces transcription of IL-1β and IL-18 protein complex that activates caspase-1 to cleave IL-1β and IL-18 (reviewed in [52]) and signal 2 promotes assembly of a multi-protein complex that activates caspase-1 to cleave IL-1β and IL-18 to their mature forms [53]. The high amount of IL-1β detected in the serum of the *Ifnar*−/− and CD11c or LysM Cre−*Ifnar*−/− mice that indicates inflammasome activation during WNV infection occurs independently of IFNAR signaling in infected myeloid cells. The lower levels of cytokines in the serum of *Mavs*−/−×*Ifnar*−/−×DKO mice establish that inflammasome activation following WNV infection occurs in part, via a MAVS-dependent pathway.

Recent studies in the context of bacterial infection have suggested a role for IFNAR signaling in inflammasome activation [17,31,32,34,54–57] and production of vasoactive cytokines, including IL-1β. Whereas in the context of *Francisella tularensis* and *Listeria monocytogenes* infection IFNAR expression was required for inflammasome activation [58], in our study, MAVS and not IFNAR signaling played the dominant role. In animals lacking IFNAR expression on CD11c+ cells, enhanced WNV replication produces excessive viral RNA PAMPs that are recognized by the cytosolic PRR, RIG-I and MDA5 [59–62]. This sensing event signals through MAVS to induce proinflammatory cytokines.
through inflammasome-dependent (IL-1B) and -independent (e.g., NF-kB) pathways. Accordingly, the combined absence of IFNAR and MAVS resulted in enhanced infection without early “cytokine storm”, with DKO mice succumbing to infection days later likely due to massive virus infection in the central nervous system. The importance of the NF-kB pathway in cytokine induction is highlighted by WNV infection studies in Ifnar-/-, Irf7-/-, LysM-Cre+ and LysM-Cre-/-. These mice still showed evidence of liver injury (high serum AST and ALT) and over-exuberant cytokine production (A. K. Pinto and M. S. Diamond, unpublished results).

Consistent with our hypothesis that proinflammatory cytokines contributed to WNV-induced lethality in CD11c Cre+ Ifnar+/+ and Ifnar-/- mice, administration of a blocking TNF-α MAb prolonged survival but did not affect viral replication. Moreover, pre-treatment with anti-TNF-α MAb resulted in lower systemic cytokine levels and less end-organ tissue injury at 48 hours after infection. Somewhat surprisingly, disruption of inflammasome activation in Nlrp3-/-, Il1r-/-, or caspase-1/11-/- mice did not affect WNV-induced liver and renal injury when IFNAR signaling was blocked. Analogous results were observed in Ifnar-/- mice treated with neutralizing anti-IL-1 B and IL-6 MAb (A. K. Pinto and M. S. Diamond, unpublished results). Thus, even though there was marked inflammasome activation in the context of “cytokine storm”, these cytokines were not primarily responsible for the severe clinical phenotype.

So how did TNF-α promote the sepsis-like syndrome associated with enhanced WNV infection of myeloid cells? Although high levels of serum TNF-α can have vasoactive effects that alter endothelial cell barrier integrity [63–65], our experiments suggest alternative pathway complement activation and possibly resultant production of C5a anaphylatoxin contributed to the phenotype. Liver injury was minimized in WNV-infected C3-/-, Fb-/-, and C5-depleted mice lacking type I IFN signaling (A. Pinto and M. Diamond, unpublished results), likely because of the rapid spread of virus to the central nervous system due to an absence of the protective antiviral effects of complement [66] and type I IFN [67]. Associated with WNV infection of CD11c Cre+ Ifnar+/+ and Ifnar-/- mice, we observed massive activation of complement including accumulation of C3 and B complement products. The induction of C3, B, and other complement proteins in the spleen and liver occurred after WNV infection in a MAVS-dependent manner. Indeed, in the serum of mice lacking Mavs alone or MAVS and IFNAR, we failed to observe the accumulation of C3 and B cleavage products after WNV infection. These and other results place complement induction and/or activation downstream of TNF-α, possibly through direct actions on myeloid cells and/or hepatocytes. We propose a model...
for virus-induced sepsis-like syndrome (Fig. 9) in which infection in myeloid cells results in excessive viral RNA production, RIG-I and/or MDA5 recognition, MAVS signaling, NF-κB activation, proinflammatory cytokine production (including TNF-α), complement protein induction, alternative pathway activation, and complement anaphylatoxin production. Enhanced infection in myeloid cells could occur because of experimental blockade or genetic deficiency of IFNAR or through virus-induced immune evasion mechanisms; many human viruses target and disable key components of the type I IFN signaling cascade [68–71]. As recent studies with bacterial sepsis models also suggest key roles for TLR and complement peptides in mediating vascular leakage and hemodynamic instability [72–74], pharmacological blockade of C3a and C5a might mitigate the sepsis-like syndrome after viral infections, including the more globally relevant flavivirus, Dengue virus.

In summary, our data establishes that the selective loss of IFNAR expression on myeloid cell subsets results in an unanticipated rapid mortality following WNV infection, which was not observed when IFNAR was removed from CD19 cells or in other viral models, which still are restricted by IFNAR signaling pathways in other cells. An absence of IFNAR signaling in myeloid cells facilitated WNV infection and caused MAVS-dependent dysregulated cytokine responses that promoted induction and activation of the alternative complement pathway, which resulted in tissue injury. As different viruses antagonize IFNAR function by targeting distinct steps in the downstream signaling pathway including Jak1, Tyk2, Stat1, and Stat2 phosphorylation (reviewed in [75]), this model of infection and inflammation may be relevant in the context of other infections. Given these findings, targeted anti-cytokine and/or immunomodulatory agents may be a possible therapeutic option when sepsis is induced by massive viral infection in subsets of myeloid cells.

**Materials and Methods**

**Ethics statement**

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocols were approved by the Institutional Animal Care and Use Committee at the Washington University School of Medicine (Assurance Number: A3381-01). Dissections and footpad infections were performed under anesthesia that was induced and maintained with ketamine hydrochloride and xylazine, and all efforts were made to minimize suffering.

**Mice**

Wild type C57BL/6 mice were purchased commercially (Jackson Laboratories). *Ifnar<sup>−/−</sup>* mice were obtained from J. Sprent (Scripps Institute, San Diego CA) and backcrossed ten times onto the C57BL/6 background. CD11c Cre<sup>Ifna1<sup>−/−</sup></sup>, LysM Cre<sup>f/f</sup>, mice and *Ifnar<sup>−/−</sup>* mice were obtained from R. Schreiber (St. Louis, MO) and U. Kalinke (Hannover, Germany). The *Ifnar<sup>−/−</sup>* [76], CD19 Cre<sup>+</sup>, LysM Cre<sup>+</sup>, and CD11c Cre<sup>+</sup> mice (Jackson Laboratories) were backcrossed using speed congenic analysis so times onto the C57BL/6 background.

**Viruses and cells**

The WNV-NY strain 3000.0259 (WNV-NY) used was isolated in New York in 2000 [79] and passaged twice in C6/36 *Aedes albopictus* cells. WNV isolate, TX 2002-HC (WNV-TX02), was titered by a standard plaque assay on BHK21 cells and working stocks were generated as previously described [14]. The CHIKV strain was isolated from an infectious clone of CHIKV La Reunion 2006 OPY-1 (strain 142, CHIKV-LR, gift from S. Higgs (Manhattan, KS)) [80] and was passaged in C6/36 *Aedes albopictus* cells. BMDCs were generated as previously described [81].

**Analysis of IFNAR expression, cytokine production, and WNV infection**

Forty-eight hours after WNV infection, blood was obtained by intracardiac heart puncture and spleens were recovered. Live cells were stained with MAb specific for CD11c, CD8, Ly6G, CD11b, CD3, CD19, and IFNAR (Biogen) to define cell types and determine IFNAR expression. To determine which leukocytes were infected with WNV, after incubating with MAb against specific leucocyte surface markers, cells were fixed and permeabilized with Fixation and Permeabilization buffers (eBioscience), and stained with a combination of two mouse anti-WNV specific MAb (WNV E16 and WNV E18) and a MAb against IFN-γ (Biogen) [82]. All samples processed on an LSRII or LSRFortessa flow cytometer (BD Biosciences). The resulting data was analyzed using FlowJo (Treestar).

**Measurement of viral burden**

Forty-eight hours after WNV infection, serum was obtained by intracardiac heart puncture, followed by intracardiac perfusion (20 ml of PBS), and organ recovery. Organs were weighed, homogenized using a bead-beater apparatus, and titrated by focus-forming assay [83] on Vero cells. Infected cell foci were stained with a flavivirus-cross-reactive, chimeric mouse-human MAb (WNV E18, 1 µg/ml) [84] for one hour at 37°C and then washed. Foci were detected after the cells were incubated with a 1:2,000 dilution of horseradish peroxidase (HRP)-conjugated goat anti-human IgG (Sigma) for one hour. Staining was visualized by addition of TrueBlue detection reagent (KPL). Spots were analysis with a Biotec spot counter (Cellular Technology) using Immuncapture software.

**Histology, IHC, and TUNEL staining**

Eight to nine-week old *Ifnar<sup>−/−</sup>* , CD11c Cre<sup>+</sup> *Ifna1<sup>−/−</sup>* , or Cre<sup>−</sup> *Ifna1<sup>−/−</sup> Ifnar<sup>−/−</sup>* mice were infected with WNV. Forty-eight hours later, mice were perfused sequentially with 20 ml PBS and 20 ml 4% PFA in PBS, and tissues were harvested and fixed in 4% PFA in PBS overnight at 4°C. Staining (hematoxylin and eosin or for WNV antigen) of paraffin-embedded tissue sections was performed as previously described [85]. After blocking non-specific binding sites, sections were incubated overnight at 4°C with an anti-WNV hyperimmune rat sera [24]. Primary antibodies were detected with secondary HRP goat anti-mouse or rat IgG (Molecular Probes). Nuclei were counter-stained with To-Pro3 (Molecular Probes). For TUNEL staining, sections were deparaffinized and rehydrated by heating to 57°C for 5 minutes then incubated with xylene, 100% ethanol, 95% ethanol, 70% ethanol.
and distilled water. Cells were permeabilized in proteinase K (Roche) for 30 minutes. DNase (Sigma) treatment for 10 minutes was used to introduce nicks into DNA as a positive control. In Situ Cell Death Detection Kit, TMR Red (Roche) was used for Tdt-mediated dUTP nick end labeling (TUNEL) and the manufacturer’s protocol was followed. Cells were counter-stained with DAPI (Invitrogen) for five minutes. Slides were visualized using an Axioscope (Zeiss) microscope. Images were captured with an AxioCam MRm (Zeiss) and Axiovision Rel4.8 (Zeiss) software was used. The control and experimental images were collected and processed using the same instrument settings.

**Cytokine bioplex assay**

WT, *Ifnar*+/−, LysM Cre+/−, CD11c Cre+/−, *Ifnar*+/−, Cre− *Ifnar*+/−, Mavs−/−, Mavs+/− × *Ifnar*+/−, Mbl-a−/− × Mbl-c+/−, C7−/−, C3−/−, B2−/−, or C3αR−/− mice were infected with WNV, and at specified times blood was collected and serum was isolated. The BioPlex Pro Assay was performed according to the manufacturer’s protocol (BioRad). The cytokine screen included interleukin-12 (IL-12), interleukin-13 (IL-13), interleukin-17 (IL-17), eotaxin, granulocyte colony-stimulating factor (G-CSF), granulocyte macrophage colony-stimulating factor (GM-CSF), interferon-γ (IFN-γ), and tumor necrosis factor (TNF-α). Pretreatment did not impact chemistry results (data not shown). The control and experimental images were collected and processed using the same instrument settings.

**Microarray analysis**

Expression oligonucleotide arrays were performed on RNA isolated from spleen and liver tissues from strain and time-matched mock-infected mice and WNV-infected WT, *Mavs*−/−, *Ifnar*+/−, and *Mavs*−/− × *Ifnar*+/− DKO mice. RNA was prepared as previously described [13]. Raw data were loaded into a custom-designed laboratory information management system (LIMS). Data were warehoused in a Labkey system (Labkey, Inc., Seattle, WA) and analyzed using GeneData Analyst 2.2.1 software (GeneData Solutions In Silico, San Francisco, CA) and TIBCO Spotfire with Integromics. Raw microarray data have been deposited in NCBI’s Gene Expression Omnibus under GEO Series accession number GSE39259 and also are accessible via the Katze Laboratory website (www.virometics.washington.edu) in accordance with proposed Minimum Information About a Microarray Experiment (MIAME) standards. A student’s t-test was performed to determine the genes that had different expression levels after WNV infection compared to mock infection for each of the four mouse strains.

**Western blotting**

WT, *Mavs*−/−, *Ifnar*−/−, and *Mavs*−/− × *Ifnar*−/− DKO BMDCs were infected with WNV at an MOI of 2.5 and harvested and lysed 48 hours later. WT and *Mipp3−/−* BMDCs were treated for 30 minutes prior to infection with 25 μg/ml of MAR1-5A3 or isotype control MAb (GIR-208) one day prior to infection with 105 FFU of WNV- NY. Anti-TNF-α and the isotype control MAb treated mice were monitored for survival or serum and organs harvested at 48 hours for further analysis. To block IFNAR signaling, WT or specific KO mice were treated with a single 1 mg (40 mg/kg) dose of MAR1-5A3 or isotype control MAb (GIR-208) one day prior to infection, as described previously [18]. To block C5 function, CD11c Cre+ *Ifnar*+/− mice were treated with two 1.25 mg (50 mg/kg) doses of BBS.1 or isotype control MAb (GIR-208) at days −1 and +2 relative to WNV infection, as described previously [86].
were resolved by electrophoresis on 10% SDS-polyacrylamide gels. Following transfer of proteins, membranes were blocked with 5% non-fat dried milk and probed with the following panel of primary antibodies: anti-WNV NS3 (R&D Systems), anti-mouse tubulin (Sigma), anti-Nlrp3 (gift of Dr. G. Sen, Cleveland, OH.), anti-mouse STAT1 (Cell Signaling), anti-mouse IL-1β (pro and cleaved forms, Abcam), anti-phospho-p65 (Ser536; Cell Signaling), and anti-α-actin (Cell Signaling).

Data analysis
All data was analyzed using Prism software (GraphPad4, San Diego, CA). Kaplan-Meier survival curves were analyzed by the log rank test. Differences in viral burden, cytokine levels and blood chemistries were analyzed by the Mann-Whitney test.

Supporting Information

Figure S1 Serum cytokine levels in CHIKV-infected mice. ischem−/−, CD11c Cre+ Ifnar−/−, and Cre− Ifnar+/+ mice (n = 6 for each group) were infected with 10 PFU of CHIKV. Seventy-two hours later, serum was collected and the concentration of IL-1β, IL-6, and TNF-α was determined. Mean values and SD are shown. Asterisks indicate differences that are statistically significant (*, P<0.05).

Figure S2 Levels of cytokines, ISGs, and chemokines in WT and KO DCs after WNV infection. qRT-PCR of WT, Nlrp3−/−, Ifnar−/−, and Nlrp3−/−×Ifnar−/− DKO DCs 24 and 48 hours after WNV infection. Relative RNA levels of IL-1β, IL-6, CCL5, IFT1, IFT2, and IFT3 are shown and compared to uninfected cells. Mean values ± SD are shown. Asterisks indicate differences that are statistically significant (*, P<0.05; **, P<0.01).

Figure S3 Effects of the NLRP3 inflammasome on cytokine induction. BMDCs (n = 3) from C57BL/6 or Nlrp3−/− mice were pretreated with 25 μg/ml of the IFNAR receptor blocking antibody MAR1-5A3 or an isotype control MAb (GIR-208) prior to infection with WNV. At 48 hours, serum was collected and the concentration of cytokines was determined by bioplex assay. Mean (pg/ml) ± SD are shown and P values are compared to WT Nlrp3+/+ mice. Data are pooled from two independent experiments. ns indicates differences that are not statistically different.

References


