Measure and Countermeasure: Type I IFN (IFN- α/β) Antiviral Response against West Nile Virus

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Abstract

As a first line of defense after viral infection, host cells develop an intrinsic immune response to control virus dissemination and protect against serious infection. Recent experiments have shown a dominant role of the IFN- α/β response in protection against lethal West Nile virus (WNV) by limiting the cellular and tissue tropism of infection. This review will focus on advances in identifying the host sensors that detect WNV and the adaptor molecules and signaling pathways that regulate the induction of IFN- α/β defenses that limit WNV replication, spread and pathogenesis.

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Introduction

West Nile virus (WNV) is a single-stranded RNA flavivirus in the Flaviviridae family and is closely related to other pathogenic flaviviruses in humans including Dengue, yellow fever, Japanese encephalitis and tick-borne encephalitis viruses. WNV is maintained in an enzootic cycle between mosquitoes and birds with some mammalian species, primarily horses and humans, becoming incidentally infected and developing severe WNV disease. Most human infections remain asymptomatic or result in a mild febrile illness. However, a subset (1/150) progress to severe neurological syndromes that include meningitis, acute flaccid paralysis and encephalitis. Although vaccines are available for horses, there is currently no approved vaccine or therapy for WNV infection in humans.

The 11-kb positive-sense WNV genome is translated as a polyprotein and subsequently cleaved into 3 structural proteins and 7 nonstructural proteins by host cell and viral proteases. The structural proteins include a capsid protein (C) that binds viral RNA, a pre-membrane

protein that blocks premature viral fusion, and an envelope (E) protein that mediates viral attachment, membrane fusion and virion assembly. The nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A NS4B and NS5) regulate viral translation, transcription and replication and also attenuate host antiviral responses.

Upon infection by a pathogen, the host must rapidly recognize the invasion and develop an effective inhibitory response to insure survival. In mammalian cells and animals, this response is characterized by the production of inhibitory and immunomodulatory cytokines after recognition of non-self pathogen-associated molecular patterns. Those motifs are detected by specific host sensors (described below), which trigger signaling cascades that induce antiviral effector proteins and maturation of an adaptive immune responses [1]. The IFN signaling pathway induces a broad and potent antiviral response against most mammalian viruses. IFNs are a large family of cytokines consisting of several members with IFN- α , β and γ the most thoroughly studied [2]. To date, 13 IFN- α genes and 1 IFN- β gene have been identified in humans and mice [3]. Type I IFN signaling is mediated through a common receptor, the IFN- α/β receptor (IFNAR), which is composed of a heterodimer of IFNAR1 and IFNAR2 subunits [4]. Signal transduction after IFNAR ligation occurs through JAK-STAT molecules and results in translocation into the nucleus of the transcription factor complex ISGF3 that induces hundreds of different interferon-stimulated genes (ISG). The ISG encode for distinct proteins with diverse biological effects that block viral replication and limit cell-to-cell spread [5–8].

Recent studies using genetically inbred mice have established the role of type I IFN in controlling WNV infection. Mice lacking IFN- α/β R are highly vulnerable to WNV with 100% mortality within 4 days, even after infection with 1 plaque forming unit of virus [9]. IFN- α/β R^{-/-} mice show uncontrolled WNV replication in peripheral and central nervous system (CNS) tissues and altered tissue and cellular tropism, thus establishing a primary role for IFN- α/β as a barrier to control the earliest steps of WNV pathogenesis. Correspondingly, pretreatment of cells in culture with type I IFN strongly inhibits WNV replication in vitro [9, 10] and prophylaxis of rodents with IFN- α protects against lethal WNV infection [11]. Overall, the containment of WNV infection requires a rapid IFN response by the host. Indeed, resistance to IFN by certain WNV strains is associated with increased virulence and pathogenicity [12].

Specific Pattern Recognition Receptors Triggering IFN Response against WNV

Rapid pathogen recognition is among the first critical steps that a host must achieve to protect against infection. Multiple families of cellular pattern recognition receptors (PRRs) that detect various pathogen-associated molecular pattern ligands have been identified in invertebrates and vertebrates. In the mammalian antiviral response, PRRs induce immunity by triggering downstream signaling pathways that lead to the production of inflammatory cytokines including type I IFN. Three classes of PRRs have been identified in mammalian cells: the Toll-like receptors (TLRs), the retinoic-acid-inducible gene I (RIG-I)-like receptors and the nucleotide-binding oligomerization domain-like receptors [13–16]. PRR sense RNA viruses, such as WNV, by recognizing nucleic acid PAMP associated with their viral genome: single-stranded RNA (ssRNA) or double-stranded RNA (dsRNA). Members of the RIG-I-like receptor family (RIG-I and melanoma differentiation-associated gene 5, MDA5) and TLR family (TLR3, TLR7, and TLR8) recognize ssRNA or dsRNA and, therefore, are considered the dominant innate host sensors of WNV infection.

The Cytosolic PRRs, RIG-I and MDA5

RIG-I and MDA5 are structurally related RNA helicases that belong to the RIG-I-like receptor family and mediate antiviral activity by binding non-self RNA ligands in the cytoplasm and inducing a type I IFN response. Both RIG-I and MDA5 are composed of a DEXD/H helicase domain at the C-terminus and 2 caspase-recruitment domains at the N-terminus. RIG-I, but not MDA5, possess a C-terminal repressor domain, which regulates its activity [17]. A third member of the RIG-I-like receptor family has been identified and named LGP2 (laboratory of genetics and physiology 2). Its structure is similar to RIG-I and MDA5 but lacks the N-terminal caspase recruitment domains and is thought to regulate RIG-I activity [18]. RIG-I and MDA5 recognize unique structures and sequence motifs of viral RNA [19, 20]. ssRNA containing a 5' triphosphate, short dsRNA and uridine- or adenosine-rich viral RNA motifs have been identified as RIG-I ligands [21, 22]. The minimum RNA length for RIG-I recognition is 21 nucleotides, whereas MDA5 is believed to recognize longer dsRNA [19].

The mechanisms by which RIG-I and MDA5 bind RNA pathogen-associated molecular patterns are not completely understood. Recent studies indicate that RNA binding by RIG-I is mediated through pathogen-associ-

ated molecular pattern recognition by the repressor domain and involves RNA translocation via an ATPase activity that is stimulated by dsRNA binding [17, 21, 23]. Binding of non-self RNA ligands by RIG-I and MDA5 triggers type I IFN gene expression after interactions between caspase-recruitment domains with the mitochondria-associated IFN- β promoter stimulator-1 (IPS-1, also called MAVS, VISA or CARDIF) [24–26]. The importance of IPS-1 is highlighted by experiments with IPS-1^{-/-} cells and mice in which the type I IFN response was completely abolished [27, 28] for viruses that are recognized by RIG-I or MDA5. Activated IPS-1 leads to recruitment and activation of the latent transcription factors IRF-3 and IRF-7 through interactions with TRAF3, TBK1 and IKK ϵ , and activation of NF- κ B through interactions with NEMO. The recruitment of this ‘signosome’ by IPS-1 promotes IRF-3, IRF-7 and NF- κ B nuclear translocation, binding to the IFN- β gene promoter, and initiation of the host response.

Both RIG-I and MDA5 are required to activate antiviral responses against WNV. RIG-I^{-/-} murine embryonic fibroblasts (MEFs) support increased WNV replication and this was associated with reduced IRF-3 activation and a delayed IFN and ISG response [29, 30]. In contrast, MDA5^{-/-} MEF alone did not show enhanced viral replication following WNV infection [Daffis and Diamond, unpubl. data]. Nonetheless, other experiments suggest that RIG-I and MDA5 cooperatively induce an optimal IFN antiviral response against WNV: RIG-I primes the early response whereas both RIG-I and MDA5 together regulate the amplified response at later time points. Consistent with this, IPS-1^{-/-} MEFs, dendritic cells (DC) and macrophages (M Φ) sustain higher viral titers and fail to induce IFN [31; Suthar and Gale, unpubl. data].

Additional studies point to a subsidiary role for MDA5 in restricting WNV infection. MDA5^{-/-} M Φ sustained elevated WNV titers and an altered IFN response, pointing a cell type-specific utilization of RIG-I and MDA5 as sensors of WNV infection. Moreover, an earlier WNV spread into the brain was observed in MDA5^{-/-} mice [Daffis and Diamond, unpubl. data]. These effects, however, did not translate into increased disease, as MDA5^{-/-} mice infected with WNV or the closely related Japanese encephalitis virus [32] showed no increase in mortality. In contrast, experiments with RIG-I^{-/-} mice revealed enhanced mortality after WNV infection, although some infected animals survived. The essential role of the RIG-I–MDA5–IPS-1 pathway was corroborated in experiments with IPS-1^{-/-} mice, which show 100% mortality within 9 days of infection [Suthar and Gale,

unpubl. data]. Correspondingly, IPS-1^{-/-} MEF, conventional DC, M Φ and cortical neurons have an ablated IFN- α / β response after WNV infection. However, in vivo, the IPS-1 pathway is not the only one involved in detecting WNV as the IPS-1^{-/-} mice produced higher levels of systemic type I IFN than wild-type controls.

Protein Kinase R

The dsRNA-dependent protein kinase R (PKR) is both a cytosolic PRR that detects dsRNA and an ISG capable of controlling viral replication by blocking translation of both viral and cellular mRNA [33–35]. Gilfoy and Mason [36] initially proposed a role of PKR in inducing IFN production in response to WNV. In MEF or human cell lines in which PKR expression was genetically or chemically knocked down, the type I IFN response to WNV was reduced. Our recent experiments with WNV-infected PKR^{-/-} MEF also show a reduced but not abolished early IFN- α and IFN- β response [Daffis and Diamond, unpubl. data]. PKR may prime and amplify the type I IFN amplification loop rather than initially sensing WNV, which is likely to depend primarily on RIG-I and IPS-1. Consistent with an important role for PKR in the early immune response against WNV, mice lacking both PKR and RNase L showed increased viral dissemination and enhanced mortality after infection [8]. The specific role of PKR in controlling WNV in vivo was inferred as RNase L^{-/-} mice exhibited an intermediate phenotype compared to wild-type or PKR^{-/-} $\times$ RNase L^{-/-} mice [8]. However, PKR^{-/-} $\times$ RNase L^{-/-} mice show no defect in type I IFN response in blood, suggesting a limited contribution of PKR in systemic IFN induction, although tissue-specific responses were not examined. PKR and RNase L appeared to contribute directly to antiviral effector functions as IFN- β pretreated PKR^{-/-} $\times$ RNase L^{-/-} macrophages and cortical neurons were more susceptible to WNV than wild-type cells [8].

Toll-Like Receptors

The TLRs that sense viral RNA are localized on the cell surface or in endosomal compartments. Recognition of WNV by TLR is mediated by TLR3, which likely binds to viral dsRNA, and TLR7/8, which bind ssRNA, including uridine-rich RNA motifs. Activation of both TLR3 and TLR7/8 in response to viral infection has been shown to induce production of type I IFN [37, 38]. However, the signaling pathways that TLR3 and TLR7/8 utilize differ. TLR7/8 recruits the adaptor protein MyD88, which forms a complex with TRAF3, TRAF6, IRAK1 and IRAK4. This complex recruits TAK1, a kinase that activates NF-

κ B, or TBK1 and IKK ϵ , kinases that activate IRF-3 and/or IRF-7. TLR3, and unlike most other TLR-dependent pathways, does not recruit MyD88 as an adaptor protein but rather uses TRIF. TRIF stimulates the IRF-3/IRF-7-dependent induction of type I IFN genes via its interaction with TRAF3, TBK1 and IKK ϵ .

Toll-Like Receptor 3. TLR3 was initially identified as mediator of host response after recognition of synthetic dsRNA (polyI:C) or purified dsRNA from reovirus [37]. Despite several in vitro studies showing that ligation of TLR3 by dsRNA in vitro regulates IFN and other cytokine responses, its role in inducing IFN and protecting against viral infection in vivo remains less clear (reviewed in [39, 40]). Similarly, conflicting results have been observed for WNV. Two independent studies using same TLR3^{-/-} mice reported somewhat opposing phenotypes: Wang et al. [41] and colleagues showed a detrimental role of TLR3 as deficient mice had improved survival rates after WNV infection. In this study, TLR3^{-/-} mice showed a mildly increased WNV burden in peripheral tissues with a decreased cytokine response (TNF- α , IL-6). This diminished inflammatory response reduced blood-brain barrier permeability and entry of WNV into the brain [41]. A second recent study showed a protective role with decreased survival of TLR3^{-/-} mice after WNV infection, mildly elevated viral titers in peripheral tissues, and early viral entry in the CNS [42]. At present, it remains unclear why the results are discordant although the disparate route of inoculation and passage history of the virus could impact differential cytokine responses. Regardless of its net effect on pathogenesis, ex vivo studies showed a dispensable role of TLR3 in regulating the IFN response and controlling WNV replication in MEF, DC and M Φ . Instead, TLR3 appears to have a more significant role in the CNS, potentially by restricting WNV replication in neurons. TLR3^{-/-} cortical neurons sustained enhanced WNV viral replication, although type I IFN responses were normal. TLR3^{-/-} microglia and astrocytes showed reduced activation and production of proinflammatory cytokines (TNF- α , IL-6 and IL-12 p40) after poly (I:C) challenge [43, 44]. Thus, the exact contribution of TLR3 for WNV protection requires further study but likely involves both cell autonomous and non-autonomous effects in the CNS.

Toll-Like Receptors 7 and 8. TLR7 and TLR8 were initially identified as triggers of the IFN- α response after exposure to synthetic ssRNA and genomic ssRNA from influenza or other viruses [38, 45–47]. TLR7 was also characterized as the primary PRR responsible for systemic IFN production by plasmacytoid DC (pDC) through a

MyD88-dependent pathway [48, 49]. Recently, the contribution of TLR7 in protecting against WNV infection in vivo was examined [50]. TLR7^{-/-} mice were more vulnerable to WNV infection and sustained increased viremia after infection. These mice showed a defect of immune cell homing to WNV-infected tissues via a novel IL-23-dependent mechanism. Interestingly, systemic levels of proinflammatory cytokines (IL-6, TNF- α and IL-12) and type I IFN were higher in TLR7^{-/-} mice when compared to wild-type animals. This result suggests that abrogation of the TLR7 pathway, which according to current dogma should eliminate IFN production in pDC, has little systemic impact on IFN production after WNV infection. Studies have yet to be conducted to evaluate the specific role of TLR8 in detection of WNV and induction of the host inflammatory response.

Transcriptional Regulation of the Type I IFN Response after WNV Infection

The interferon regulatory factors (IRF) family is composed of 9 closely related members; these proteins were initially characterized as transcription factors that regulated cytokine genes, including IFN [reviewed in 51, 52]. Subsequent studies have demonstrated a pleiotropic function for IRF in both innate and adaptive immunity, cell growth, apoptosis and hematopoietic cell development. IRF-3 and IRF-7 have been identified as the key transcriptional regulators of the type I IFN genes following viral infection [53, 54]. IRF-3 and IRF-7 are structurally homologous, present in an inactive state in the cytosol, and translocate to the nucleus after phosphorylation and dimerization to induce type I IFN gene transcription. IRF-3 is constitutively expressed in most of the cell types whereas IRF-7 is itself an IFN-inducible gene. Triggering of PRR pathways by RNA viruses through TLR3, TLR7/8, RIG-I, and MDA5 converge on the activation of IRF-3 and IRF-7.

The current paradigm for activation of the type I IFN response after viral infection is a 2-step amplification sequence (fig. 1). In the first phase, viral sensing by PRR triggers phosphorylation and translocation of constitutively expressed IRF-3 with subsequent production of IFN- β and IFN- α 4 by the infected cell. In the second step, the newly synthesized IFN- β and IFN- α 4 signals infected and uninfected cells by binding to IFNAR and activating a JAK-STAT signaling cascade [54]. This results in an 'antiviral state' by inducing expression of hundreds of different ISG, including IRF-7. Activation of

Fig. 1. Detection of WNV and activation of IFN- α/β genes and ISG according to the 2-step model. Infection by WNV produces dsRNA intermediates in the cytosol that are detected as non-self by the host. RIG-I acts as the main sensor for WNV during the early steps of infection. RIG-I activation promotes interaction with IPS-1 that leads to the recruitment of TRAF3, TBK1 and IKK ϵ , which phosphorylate and activate IRF-3. Very small amounts of constitutively produced IRF-7 may be activated via this pathway. IRF-3 then translocates into the nucleus, binds the IFN- β gene promoter and promotes transcription and translation. Secretion of IFN- β by the infected cells results in paracrine type I IFN signaling through the IFNAR receptor. Activation of IFNAR induces phosphorylation of JAK1 and Tyk2, which can promote the formation of the heterotrimer IFN-stimulated gene factor-3 formed by STAT1, STAT2

and IRF-9. Ultimately, translocation into the nucleus of IFN-stimulated gene factor-3 induces hundreds of ISGs, including IRF-7. During late phases of infection, detection of WNV also relies on MDA5. Induction of the IFN- α and IFN- β genes then occurs mainly via the transcriptional activity of IRF-7. Detection of WNV during the late phase may also involve PKR, which induces the IFN- α and IFN- β genes via an unknown mechanism (potentially via NF- κ B activation and/or TRAF3, TBK1 and IKK ϵ recruitment). In M Φ and mDC, IFN- β is induced at least partially through IPS-1-dependent yet IRF-3 and IRF-7-independent pathways. In M Φ , IRF-3 regulates the basal expression of host defense genes as described in the text. X = Sites in the recognition and signaling cascade at which WNV has developed specific mechanisms to counteract IFN. These are described in detail in the text.

IRF-7 amplifies expression of IFN- β and all IFN- α subtypes, effectively creating a type I IFN-positive feedback loop. This model has been questioned as studies show that in IRF-7 $^{-/-}$ MEF IFN- α/β gene induction was severely altered after infection with vesicular stomatitis and encephalomyocarditis viruses [53]. As a result, it has been hypothesized that small amounts of constitutively expressed IRF-7 may prime the IFN response [52]. More recent studies suggest that the IFN response is cell-type specific as individual cells utilize a distinct repertoire of PRR and IRF to induce IFN. The next section will focus on how IRF-3 and IRF-7 regulate the type I IFN response in different cells after WNV and the effect of these molecules on WNV pathogenesis in vivo.

Effect of Deficiencies of IRF-3 and/or IRF-7 on WNV Pathogenesis

Mice with genetic deficiencies of IRF-3 and/or IRF-7 have provided insight into the regulation of type I IFN after viral infection. Given their central role in translating signals from PRR to the nucleus, it was not entirely surprising that IRF-3 $^{-/-}$ and IRF-7 $^{-/-}$ mice showed increased susceptibility to infection by several RNA and DNA viruses [52, 54]. Although systemic production of type I IFN was strongly reduced in IRF-7 $^{-/-}$ mice, it was relatively preserved in IRF-3 $^{-/-}$ mice, suggesting a more dominant role of IRF-7 in regulating this response, at least in blood. These experiments were consistent with studies with pDC, which are hypothesized as a primary producer of type I IFN in vivo, using an axis of TLRs 7–9, MyD88 and IRF-7 to regulate IFN- α and IFN- β gene induction.

IRF-3 $^{-/-}$ or IRF-7 $^{-/-}$ mice have increased mortality after WNV infection, with IRF-7 $^{-/-}$ mice exhibiting a more severe phenotype [55, 56]. In both deficient mice, enhanced viral burden was observed at peripheral sites (spleen, viremia, lymph node), which led to earlier and more sustained replication in the brain and spinal cord. Altered tissue tropism was also observed with viral replication seen in kidneys, an organ that is usually not infected by WNV in wild-type mice. Analysis of serum from IRF-3 $^{-/-}$ mice infected with WNV confirmed the dispensable role of IRF-3 in regulating the systemic production of type I IFN [55]. Analogously, IRF-3 $^{-/-}$ mice inoculated with WNV-virus-like particles had a normal systemic IFN response [57]. In contrast, WNV-infected IRF-7 $^{-/-}$ mice showed reduced but not ablated systemic IFN responses, clearly confirming the importance of the IRF-7 axis in generating IFN in blood [56]. The IRF-7-dependent production of IFN in blood appears indepen-

dent of TLR7 as levels were not decreased in TLR7 $^{-/-}$ mice after WNV infection [50]. At present, it remains uncertain as to which cell type or how IRF-7 regulates systemic IFN production after WNV infection.

Transcriptional Regulation of IFN- α and IFN- β Genes in Primary Cells after WNV Infection

Fibroblasts. MEF have been extensively studied as a model primary cell type for understanding host immune responses against viruses. In these cells, IFN induction follows the 2-step amplification loop model described above. Our recent work with WNV and IRF-7 $^{-/-}$ MEF confirmed this as the IFN- α response, and the positive feedback loop was virtually abolished in these cells. The IFN- β response in IRF-7 $^{-/-}$ MEF, however, remained intact, possibly through the redundant activity of IRF-3 and IRF-7. Experiments with deficient MEF showed that IRF-3 plays an important role in controlling cell-to-cell spread of WNV as the loss of IRF-3 caused significant reductions in the early IFN- α and IFN- β responses [29]. However, at later time points the levels of IFN- α and IFN- β mRNA accumulated to near normal levels [Daffis and Diamond, unpubl. data]. The results confirm that the transcriptional activity of IRF-3 is required to efficiently activate IFN- α/β genes during the initial phase of WNV infection and that sensing of WNV in MEF (by RIG-I-dependent actions) signals primarily through IRF-3-mediated pathways. The existence of a residual IFN- α/β response was hypothesized to be IRF-7-dependent, and is probably sufficient to sustain the positive feedback loop in MEF. Experiments in MEF lacking both transcriptional regulators (IRF-3 $^{-/-}$ $\times$ IRF-7 $^{-/-}$ double knockout MEF) confirmed this, as the IFN- α and IFN- β responses were virtually abolished. Thus, regulation of the type I IFN in response to WNV in MEF almost entirely depends on IRF-3 and IRF-7, following the classical 2-step model.

Macrophages. M Φ are susceptible to WNV infection and are even more permissive in the absence of IFN signaling [9]. Bone-marrow-derived M Φ generated from IRF-3 $^{-/-}$ mice show a distinct pattern of IFN- α/β gene regulation compared to MEF with normal or even higher levels of IFN- α and IFN- β mRNA. Thus, the contribution of IRF-3 in the IFN response differs between MEF and M Φ . In M Φ , IRF-3 was identified as an essential regulator of the basal expression of host defense molecules including ISG54, ISG56, RIG-I and MDA5, potentially controlling the permissiveness of this cell type for WNV. These findings are consistent with prior studies that showed that IRF-3 could regulate ISG expression in an

IFN-independent manner [58]. The contribution of IRF-3 and IRF-7 in MΦ was more fully established in studies with IRF-7^{-/-} or double knockout cells. A deficiency of IRF-7 completely abrogated the IFN-α response, confirming the essential role of IRF-7 in the IFN amplification loop. However, no effect on IFN-β gene induction was observed in these cells. Analysis of the IFN-β response in double knockout MΦ revealed an unexpected finding, as this response was completely abolished at early time points after WNV infection but was not substantially altered in the absence of both IRF-3 and IRF-7 at late time points. This result was confirmed by ISG analysis, which showed delayed induction of ISG proteins in MΦ lacking both IRF-3 and IRF-7. Thus, IRF-3 and IRF-7 only partially regulate the IFN-α/β gene and ISG expression in MΦ.

Which proteins are involved in inducing the IFN-β gene in primary MΦ? The question remains open as MΦ lacking the expression of other IRF (e.g. IRF-1) that putatively activate IFN responses in distinct cell types with other viruses [59–61] did not manifest altered IFN-β gene induction in response to WNV [Daffis and Diamond, unpubl. data]. IRF-5-deficient MΦ, however, showed a small decrease in IFN-α/β mRNA induction at early time points. The significance of this result is currently under investigation but suggests a role of IRF-5 in regulating the IFN-α/β response, as has been reported in with other viruses and/or TLR ligands [62, 63].

Myeloid Dendritic Cells (mDC). DCs have primary roles in regulating innate and adaptive immune responses against viruses. These cells are hypothesized as early targets for infection in vivo by flaviviruses, including WNV [64–66]. Although pDC are potent producers of type I IFN after viral infection [reviewed in 49], including WNV [67], other DC subsets, particularly mDC, are now recognized as major sources of type I IFN in response to viral infection [68]. Indeed, mDC, which are permissive for infection, secrete large amounts of IFN-α and IFN-β in response after incubation with WNV. [56, 67]. The IRF-3- and IRF-7-dependent regulation of IFN-α and IFN-β gene induction in mDC also strongly differed compared to that observed with MEF. Whereas the IFN-α response was entirely regulated by IRF-7, the IFN-β response was largely unaffected by the absence of IRF-3, IRF-7 or both [56]. Pharmacological inhibition of NF-κB activity in double knockout mDC also failed to reduce the IFN-β response after WNV infection [Daffis and Diamond, unpubl. data]. Similar to MΦ, preliminary experiments with IRF-1^{-/-}, IRF-5^{-/-} and IRF8^{-/-} mDC also showed only small effects on the IFN-β response upon

WNV infection. These results suggest a novel cell-type-specific transcriptional pathway that induces IFN-β gene expression, which is initiated by IPS-1-dependent signals.

Neurons. As WNV is a neurotropic virus, the study of the innate immune response in CNS cells is relevant for understanding pathogenesis. Neurons, once thought of as passive cells in the immune response, actively produce type I IFN and chemokines after infection by RNA viruses, including WNV [55, 69, 70]. Studies with deficient cells and WNV showed a primary regulatory role for IRF-3 and IRF-7 on the IFN response [55, 56]. IRF-3 regulates the early phase of IFN-α/β gene induction whereas IRF-7 controls the IFN-α response. Moreover, as observed with MEF, the type I IFN response and ISG gene expression was completely abolished in the absence of both IRF-3 and IRF-7. Thus, type I IFN regulation in neurons occurs through IRF-3 and IRF-7-dependent pathways and adheres to the 2-step amplification model.

Evasion of the Type I IFN Response by WNV

To replicate and spread rapidly, WNV has evolved countermeasures to evade or attenuate the IFN antiviral response. Indeed, in cell culture WNV becomes resistant to the antiviral effects of IFN once infection is established [10]. This may explain in part, the relatively modest therapeutic window for IFN-α administration that has been observed clinically in humans infected with WNV [71].

Inhibition of IFN-β Gene Induction

IFN-β Gene Transcription. Studies with Kunjin virus, a less pathogenic lineage I WNV isolate from Australia, have identified the nonstructural protein NS2A as an inhibitor of IFN-β gene transcription [72, 73]. Transgenic expression of NS2A was sufficient to suppress IFN-β transcription in Semliki Forest virus-infected cells. Incorporation of an A30P mutation of NS2A into a Kunjin virus genome resulted in a virus that elicits more rapid and sustained synthesis of type I IFN. Accordingly, infection of this mutant virus was highly attenuated. The exact cellular target of NS2A and its mechanism of inhibition remain unknown.

Avoiding PRR Detection. Highly pathogenic WNV strains evade IRF-3-dependent recognition pathways without actively antagonizing the host defense signaling pathways [29]. Replication of WNV-TX-02 (isolated in Texas in 2002) did not alter the ability of Sendai virus to

activate IRF-3. Thus, virulent WNV strains appear to delay activation of PRR, such as RIG-I, through uncertain mechanisms to provide the virus with a kinetic advantage to elude host detection during replication at early times after infection. In contrast, less pathogenic lineage 2 strains of WNV induced greater levels of IFN at early time points [12].

TLR3-Dependent Responses. Activation of IRF-3 and stimulation of IFN- β transcription in response to dsRNA [poly (I:C)] have been reported to be inhibited in HeLa cells infected with WNV or stably propagating a subgenomic replicon [74]. The viral NS1 protein may mediate this inhibitory effect as expression of WNV NS1 inhibited TLR3-induced transcriptional activation of the IFN- β and IL-6 transcription and NF- κ B promoter activity [75]. The high mannose carbohydrates on the viral E protein may independently block the production of IFN- β , IL-6 and TNF- α that is induced by dsRNA in M Φ . This effect was not directly dependent on TLR3 or its adaptor molecule TRIF but instead occurred downstream at the level of the signaling intermediate and NF- κ B activator, receptor-interacting protein-1 [76]. Based on studies with M Φ from different age cohorts, this E protein inhibitory pathway may be dysregulated in elderly humans, leading to a pathogenic cytokine response [77]. Although the mechanistic basis for how specific forms of the E protein alter antiviral signaling programs remains uncertain, glycosylated E proteins of other flaviviruses can bind and signal through multiple cell surface lectins, including the mannose receptor [78] and CLEC5a [79].

Antagonism of the Type I IFN Signaling

WNV can limit the efficacy of the IFN response by antagonizing the downstream signaling cascade. Dimerization of IFNAR1 and IFNAR2 upon IFN- α/β ligation induces phosphorylation of JAK1 and Tyk2, which in turn recruit and phosphorylate STAT1 and STAT2. Once activated, STAT1 and STAT2 heterodimerize and recruit IRF-9 to form the heterotrimeric IFN-stimulated gene factor-3 complex. This translocates into the nucleus where it induces expression of hundreds of ISG. The non-structural proteins (e.g. NS4B) of WNV appear to inhibit JAK1 and Tyk2 phosphorylation [80], thus preventing transcriptional activation of STAT1 and STAT2 and subsequent ISG expression. Mutagenesis studies have identified a sequence determinant on WNV NS4B (E22/K24) that controls IFN resistance in cells expressing subgenomic replicons, although in cells expressing infectious virus this NS4B determinant did not regulate the IFN re-

sponse, suggesting an independent role for structural genes [81]. A recent study by Mackenzie et al. [82] showed that WNV can promote redistribution of cellular cholesterol, which not only enhances replication but diminishes the formation of cholesterol-rich lipid rafts in the plasma membrane and attenuates the IFNAR signaling response. Taken together, these studies demonstrate that WNV, like many other highly pathogenic viruses, limits the antiviral effect IFN at multiple stages including induction and signaling. The exact biochemical and cellular mechanisms by which this occurs for WNV and other related flaviviruses, however, remain unanswered and undoubtedly will be avenues for further investigation by many groups.

Conclusions

The outcome of viral infection is determined by a competition between viral replication and host immune programs. The innate immune response of the host is programmed to rapidly control viral replication and limit virus spread by recognizing non-self nucleic acid as pathogen-associated molecular patterns and triggering an antiviral response. Conversely, to be successful, the viral pathogen manipulates, antagonizes and evades the host response in order to establish a productive infection and disseminate to new hosts. Although type I IFN was discovered more than 50 years ago, only recently have the mechanisms by which these molecules are induced, signal and produce an antiviral effect been delineated. The generation and distribution of targeted gene knockout mice have allowed scientists to dissect and evaluate the function of individual host defense genes in the context of infection by specific viruses. WNV is a zoonotic disease whose pathogenesis can be readily studied in mice, thus the use of knockout strains has afforded a unique perspective on the interface between the mammalian host and WNV.

The use of animal and cell culture models has fostered an improved understanding of the balance between WNV pathogenesis and immune control. IFN responses limit WNV replication and the virus has developed countermeasures to facilitate infection and transmission. In the last 5 years, the field has learned the identity of specific PRR that detect entry and infection by WNV and initiate a protective IFN response, and which viral proteins allow evasion of the response. As these basic mechanisms are further explored and characterized, the field undoubtedly will gain insight into fundamental cellular

responses as well as mechanisms of viral pathogenesis. This information may facilitate the design of novel vaccine or targeted therapeutic strategies and enhance our understanding of how pathogens of all types cause disease.

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