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New Insights into Innate Immune Restriction of West Nile Virus Infection

Helen M. Lazear^a and Michael S. Diamond^{a,b,c,d,*}

^aDepartment of Medicine, Washington University School of Medicine, St Louis, MO 63110, USA

^bDepartment of Molecular Microbiology, Washington University School of Medicine, St Louis, MO 63110, USA

^cDepartment of Pathology and Immunology, Washington University School of Medicine, St Louis, MO 63110, USA

^dCenter for Human Immunology and Immunotherapy Programs, Washington University School of Medicine, St Louis, MO 63110, USA

Abstract

West Nile virus (WNV) is an encephalitic flavivirus that has provided a valuable experimental system for studying viral pathogenesis and immunity. Although *in vitro* approaches and mouse models of infection have identified pattern recognition receptor and interferon pathways that control WNV infection, our appreciation of specific antiviral effectors has been more limited. In this review, we highlight recent advances in our understanding of the host factors that restrict WNV infection in mammals and insects, especially those resulting from large-scale screening approaches.

Introduction

West Nile virus (WNV) is an encephalitic flavivirus that is maintained in an enzootic cycle between *Culex* mosquitoes and birds [1,2], and is transmitted to humans and other vertebrates as incidental, dead-end hosts [3]. This biology has interesting implications for virus-host interactions and human disease: since humans do not transmit WNV to mosquitoes, selective pressure from the human immune response does not drive WNV evolution or impact virus transmission in nature. WNV infection provides an excellent model for understanding virus-host-vector interactions, due to its high level of infectivity in mammalian and insect cells in culture, the availability of mouse models that mimic features of human disease, and its importance as a zoonotic agent of human and animal disease.

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*Corresponding Author: 660 S. Euclid Ave., Box 8051, St. Louis, MO 63110, USA, Phone: 314-362-2842, MSD: diamond@borcim.wustl.edu; HML: hlazear@dom.wustl.edu.

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After its initial introduction into New York City in 1999, WNV rapidly spread across the continental United States and by 2004 had become endemic in most parts of the country [4]. Its importance as an ongoing public health concern is demonstrated by recent outbreaks of WNV encephalitis, including a large outbreak in Texas in 2012 [5], the spread of a lineage 2 strain across Europe since 2008 [6,7], and the emergence in Australia in 2011 of encephalitis caused by the Kunjin strain of WNV [8]. The latter two are examples of human disease caused by WNV strains that had rarely been associated with neuroinvasive disease in the past. Although the basis for the emergence of human disease from previously attenuated or avirulent WNV strains remains unclear, these outbreaks highlight the continued importance of understanding the biology of WNV pathogenesis and its interaction with its hosts.

WNV infection paradoxically is restricted by the DNA sensor cGAS

Several studies have shown that WNV infection is detected by pattern recognition receptors (PRRs) in the Toll-like receptor (TLR) and RIG-I-like receptor (RLR) families of RNA sensors [9–17] (Figure 1). However, recent studies have revealed that cGAS [18] and its downstream signaling molecule, STING [19] also restrict WNV infection. These observations were unexpected because cGAS is a DNA-sensing PRR, and had not been reported to control RNA virus infection [20]. Nonetheless, ectopic expression of cGAS inhibited WNV replication and cGAS^{-/-} mice exhibited increased lethality after WNV infection [18]. cGAS signals through the adaptor molecule STING to induce type I interferon (IFN- α/β) production [20]. Consistent with a possible role for cGAS in controlling WNV infection, STING^{-/-} mice also exhibited increased lethality after WNV infection [19], although this could be explained by cross-talk between STING and RLR signaling pathways [21]. The mechanism by which cGAS becomes activated following WNV infection remains unknown, but possibilities include: viral RNA-binding activity of cGAS, production of cDNA copies of viral sequences by cellular reverse transcriptases [22], or generation of host cell-derived DNA damage-associated molecular patterns (DAMPs) in response to cytopathic effects of virus infection [23].

Genetic screens identify IFN-induced antiviral effector molecules against WNV

Similar to many other RNA and DNA viruses, IFN- α/β is critical for controlling WNV infection and limiting pathogenesis (Figure 1). Early studies used *Ifnar*^{-/-} mice to demonstrate an essential role for IFN- α/β in controlling WNV [24,25], but the specific mechanisms through which this antiviral effect was exerted remained unknown. IFN- α/β signaling induces the expression of hundreds of IFN-stimulated genes (ISGs), and while these presumably encode the effector molecules of an antiviral response, until recently inhibitory mechanisms had been described for only a few ISGs (e.g. Mx1, PKR, RNase L, and OAS) [26,27]. One of the key recent advances in our understanding of host factors that restrict WNV (as well as many other viruses) has been the application of large-scale ectopic expression [28,29] or gene silencing [30,31] screens to identify sets of genes that control viral replication *in vitro*. Such approaches have detected previously described components of the antiviral response (e.g. MDA5, RIG-I, TLR-3, MAVS, IRF-3, JAK2, STAT-2, IRF-9, and PKR) but also have revealed many novel antiviral ISGs, thereby identifying promising

targets for subsequent mechanistic studies and possible drug development. ISGs that displayed significant antiviral activity against WNV *in vitro* include: C6orf150, DDX24, HPSE, IFI44L, IFI6, IFITM2, IFITM3, IFRD1, IL13RA1, ISG20, MAFK, NAMPT, PAK3, PHF15, SAMD9L, SC4MOL, and viperin. The antiviral mechanisms of some well-described ISGs have been reviewed [27,32], but the mechanisms by which these novel ISGs restrict WNV replication and their roles in controlling WNV pathogenesis remain to be determined. ISGs for which antiviral activity against WNV has been demonstrated *in vivo* include PKR, RNase L, viperin, and IFIT2 [33–36], several of which control WNV infection specifically in neurons of the central nervous system (CNS). Systems biology approaches will be critical for developing testable hypotheses from these complex datasets [37]. Indeed, a recent systems biology analysis of WNV infection *in vivo* revealed novel cross-talk between the RLR and IFN signaling pathways and the induction of inflammatory cytokines, as well as an unexpected role for natural killer cells in restricting WNV tropism [38].

There are several limitations to consider when interpreting large-scale ISG screens (Figure 2), particularly given the apparent lack of overlap in the sets of antiviral genes identified by different investigators [28–31]. First, as ISGs normally are co-induced by similar stimuli, a single ISG expressed in isolation (e.g. ectopic expression) may lack interactions with and regulation by other ISGs that are present in the context of a viral infection. Some ISGs may be active only under particular circumstances (cell type, stage of virus infection, expression of other infection-induced factors) and the effect any individual ISG may be small. The modest antiviral effects observed for most ISGs suggest that these molecules act in concert to produce a cellular environment that is refractory to viral replication. Second, large-scale screens have been conducted in cell lines with defective antiviral responses, (e.g. Huh7, HeLa, or STAT1^{-/-} fibroblasts). The dominant antiviral effector genes in these cells may not reflect those controlling viral replication in immune competent cells and tissues. Of particular relevance to WNV are antiviral effector molecules that are active in myeloid cells or neurons, the primary targets for WNV *in vivo* [1]. This issue is of great interest given recent studies demonstrating that the cerebral cortex and cerebellum have different basal levels of ISG expression and differential IFN responsiveness [39]. Specifically, tissue-specific epigenetic modifications result in relatively higher basal levels of ISG expression in neurons of the cerebellum compared to the cortex, although the upregulation of ISG expression is more evident in the cortex. The end-result is that cerebellar neurons are more resistant to infection by WNV and other RNA viruses [39]. These findings highlight cell- and tissue-specific differences in the antiviral response, which are critical for understanding viral tropism and the antiviral response *in vivo*. Third, ISG screens generally have been conducted using virulent strains of WNV. Since viral pathogenesis likely reflects an ability to overcome key immune restrictions, virulent strains may antagonize or avoid the antiviral effects of some ISGs *in vitro*. In fact, critical antiviral restriction factors might be missed by this approach, since stronger antiviral effects produce greater selective pressures for viral immune evasion. Therefore, attenuated virus strains may be useful as probes to identify novel antiviral restriction factors. As an example, a virulent strain of WNV commonly used for pathogenesis studies exhibited no phenotype in IFIT1^{-/-} primary cells or mice; the role of IFIT1 as an antiviral effector was revealed by an attenuated genetic mutant virus that lacked 2'-O methylation of its viral RNA cap structure [34,40].

IFNs limit WNV neuroinvasion by tightening the blood-brain barrier

IFN- α/β has been thought to control WNV and other viruses by two primary mechanisms: i) the induction of cell-intrinsic antiviral effectors that restrict different steps in virus replication and ii) modulation of the adaptive immune response. Recent work suggests that IFN- α/β may act by an additional, novel mechanism: controlling blood-brain barrier (BBB) permeability [41]. The BBB is a physical barrier formed by tight junctions between endothelial cells in the CNS microvasculature, with input from astrocyte foot processes. The BBB protects vulnerable CNS tissues from harmful substances in the peripheral circulation, including viruses [42]. Following inoculation by a mosquito, WNV infects keratinocytes and resident dendritic cells in the skin [43], traffics to draining lymph nodes, and establishes infection in other myeloid cells, which results in viremia [1]. WNV and other encephalitic flaviviruses cause CNS disease when they breach the BBB and infect neurons in the brain and spinal cord. The mechanisms by which WNV crosses the BBB remain unclear, but could include transit of free virions across a compromised barrier, trafficking of infected leukocytes from the periphery, or direct infection of the endothelial cells that comprise the BBB [44]. One study found that inflammatory cytokine production downstream of TLR3 signaling promoted BBB opening and WNV neuroinvasion [45], although a subsequent paper identified a protective role for TLR3 in limiting WNV pathogenesis [9]. Recent work has used *in vivo* and *in vitro* models to demonstrate that IFN- α/β signaling induces BBB tightening, thereby restricting WNV neuroinvasion. This tightening effect is rapid and involves rearrangement of endothelial cell junction proteins via Rho-Rac signaling pathways [41].

The insect antiviral response to WNV

While much attention has been given to the host response to WNV in humans and mice, there is a growing understanding of the insect antiviral response to arthropod-borne viruses, including WNV. The need to cycle between bird and mosquito hosts imposes evolutionary constraints on WNV, including selection for viruses that are non-pathogenic in mosquitoes [46, 47]. In addition to replicating in distinct cellular environments (e.g. temperature and lipid composition), WNV must evade both vertebrate and insect antiviral responses, including IFN and RNA interference (RNAi), respectively [48,49].

Drosophila systems have provided valuable models for understanding the insect response to viral infections, due to the wealth of genetic, developmental, and technical resources available [50]. Advantages of studying antiviral restriction factors in *Drosophila* include the ease with which cells in culture take up exogenous dsRNA, the availability of gene silencing reagents including whole-genome silencing screens, and the ability to validate *in vitro* findings in an animal model using existing mutant fly strains or rapidly generating new mutants of interest. A recent gene silencing screen in *Drosophila* DL1 cells identified putative WNV restriction factors; 86% of these have a human ortholog, suggesting commonalities between the antiviral pathways in insects and mammals [51]. In particular, the genes dRUVBL2, Tip60, and XPO1 shared antiviral activity in *Drosophila*, mosquitoes, human cells, and mouse primary neurons. The restriction factors identified in this approach surprisingly were enriched for nuclear functions such as RNA metabolism and transcription,

suggesting that *Drosophila*-based screens may reveal novel pathways in cell-intrinsic antiviral immunity.

There has been an increasing ability to translate findings from *Drosophila* into the mosquitoes that serve as WNV vectors. For example, the secreted peptide Vago has antiviral activity in *Drosophila* [52], and recently was shown to restrict WNV infection in mosquito cells [53,54]. The inhibitory activity of Vago requires JAK-STAT signaling [53]. Furthermore, Vago production is induced in response to pathogen-sensing by Dicer-2, a DExD/H-box helicase that also initiates the antiviral RNAi response in insects and is homologous to mammalian RLRs [52,55]. Thus, while insects do not possess an IFN system, Vago may serve as an antiviral functional analog.

While *Drosophila* models have identified many host factors that are conserved in other insects and even in mammals, other studies have revealed mosquito restriction factors that were not evident from *Drosophila*-based approaches. For example, aae-miR-2940 is a micro RNA (miRNA) that promotes WNV replication and is downregulated by mosquito cells in response to WNV infection [56]. This miRNA is found in *Aedes* and *Culex* mosquitoes, but has no counterpart in *Drosophila*. Validation of *Drosophila* factors in mosquitoes, as well as characterizing mosquito-specific factors, will provide new insights into the interactions of WNV with its biologically relevant insect vectors.

Conclusions

Our knowledge of the host factors that control WNV infection has expanded greatly in recent years, due in part to the results of large-scale genetic screens to identify antiviral factors. Ongoing work is needed to translate lists of possible antiviral genes into testable hypotheses and to understand the mechanisms by which these factors limit viral pathogenesis in insects and mammals.

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Highlights

- Genetic screens have identified host factors restricting WNV infection in cells
- WNV infection paradoxically is restricted by cGAS, a DNA sensor
- Interferon- α/β tightens the blood-brain barrier, which limits WNV neuroinvasion

Figure 1. The IFN-mediated antiviral response to WNV

WNV infection is sensed by pattern-recognition receptors (PRRs) including RIG-I-like receptors (RIG-I, MDA5) and Toll-like receptors (TLR3, TLR7). These signal to activate IRF-family transcription factors, which induce IFN-β transcription and production. New evidence suggests that a DNA sensor, cGAS, also activates the antiviral response after WNV infection. IFN-α/β signals in an autocrine and paracrine manner to induce the expression of hundreds of IFN-stimulated genes (ISGs) that inhibit viral replication by a variety of mechanisms. Many ISGs have been shown to restrict WNV replication *in vitro* (a selection of which are listed), but few of these have confirmed roles in controlling WNV pathogenesis *in vivo* (e.g., PKR, IFIT2, viperin, and RNase L). Virulent strains of WNV evade the antiviral activity of IFIT1; only mutant viruses that lack 2'-O methylation on the 5' cap structure of their viral RNA are restricted by IFIT1.

Figure 2. Limitations of genetic screens to identify antiviral interferon-stimulated genes (ISGs)
 Large-scale ectopic expression and gene silencing screens have provided new insights into the IFN-induced host factors that restrict infection by WNV and other viruses *in vitro*. However, the roles of these host factors in controlling WNV pathogenesis *in vivo* remain less clear, owing to several limitations of this approach. First, viral infection triggers global changes in cellular gene expression and produces an antiviral milieu that is absent in the context of ectopic expression of a single ISG. Second, the antiviral response in transformed or knockout cell lines used for genetic screens may not faithfully represent that found in differentiated and immune competent cells. Third, some restriction factors may remain undetected because their antiviral activities are antagonized by the virulent strain of virus used in the genetic screen.