

**The essential, nonredundant roles of RIG-I and MDA5 in detecting and controlling West Nile virus infection**

John Errett

A dissertation

submitted in partial fulfillment of the  
requirements for the degree of

Doctor of Philosophy

University of Washington

2013

Reading Committee:

Michael Gale, Jr. (Chair)

Dan Stetson

Andrew Oberst

Program Authorized to Offer Degree:

Immunology

©Copyright 2013

John Errett

University of Washington

**Abstract**

The essential, nonredundant roles of RIG-I and MDA5 in detecting and controlling West Nile virus infection

John Errett

Chair of the Supervisory Committee:

Professor Michael Gale, Jr.

Departments of Immunology, Microbiology, and Global Health

Virus recognition and response by the innate immune system are critical components of host defense against infection. Activation of cell-intrinsic immunity and optimal priming of adaptive immunity against West Nile virus (WNV), an emerging vector-borne virus, depends on recognition by RIG-I and MDA5, cytosolic pattern recognition receptors (PRRs) of the RIG-I-like receptor (RLR) protein family that recognize viral RNA and activate defense programs that suppress infection. We evaluated the individual functions of RIG-I and MDA5 both *in vitro* and *in vivo* in pathogen recognition and control of WNV. Lack of RIG-I or MDA5 alone results in decreased innate immune signaling and virus control in primary cells *in vitro* and increased mortality in mice. We also generated *RIG-I*<sup>-/-</sup> x *MDA5*<sup>-/-</sup> double knockout mice and found that lack of both RLRs results in a complete absence of innate immune gene induction in target cells of WNV infection and a severe pathogenesis during infection *in vivo*, similar to animals lacking

MAVS, the central adaptor molecule for RLR signaling. We also found that RNA products from WNV infected cells but not incoming virion RNA display at least two distinct pathogen associated molecular patterns (PAMPs) containing 5' triphosphate and double-stranded RNA that are temporally distributed and sensed by RIG-I and MDA5 during infection. In addition to biochemical characterization of stimulatory PAMPs contained within WNV infected cells, we have also defined sequence identity of RNAs associated with RIG-I and MDA5 immunoprecipitated from infected cells. Thus, RIG-I and MDA5 are essential PRRs that recognize distinct PAMPs that accumulate during WNV replication. This study highlights the necessity and function of multiple related, cytoplasmic host sensors in orchestrating an effective immune response against an acute viral infection.

## **Acknowledgments**

I wish to thank all the individuals I have interacted with during my experiences in scientific research both before and during graduate school. These interactions with friends, colleagues and mentors has inspired me to pursue and eventually complete the work I have been able to accomplish as a graduate student in immunology. In particular I would like to thank Michael Gale, Jr. for being an excellent mentor and source of support. Mike has fostered my scientific curiosity and been unfailingly encouraging. The entire Gale, Jr. lab has provided an environment that allowed me to learn and grow as a scientist. Many members of the lab contributed significantly in mentorship and experimental expertise, but I would especially like to thank Mehul Suthar for his helpful efforts. I thank my advisory committee for being generous with their time and providing productive insight. I would also like to thank Michael Katze and Matthew Thomas for their contributions to the RNA sequencing work.

My family and friends have also been essential in my success. My parents and siblings have always encouraged my interests in science while providing a much needed outlet of unconditional emotional support. My girlfriend Bethany Craig has allowed me to achieve balance in my life and been incredibly patient during my graduate studies. Thank you to all my loved ones.

# TABLE OF CONTENTS

<b>I. INTRODUCTION .....</b>	<b>1</b>
MAMMALIAN DEFENSE AGAINST INFECTION.....	1
INITIATION OF IMMUNITY TO VIRUSES .....	2
TLRs .....	3
NLRs.....	4
ALRs.....	5
RLRs.....	6
‘OTHER’ VIRAL SENSORS .....	10
CONSEQUENCES OF INNATE IMMUNE RECOGNITION OF VIRUSES.....	12
WEST NILE VIRUS.....	14
WNV ECOLOGY AND EPIDEMIOLOGY.....	14
WNV PATHOLOGY AND THE IMMUNE RESPONSE .....	17
<b>II. MATERIALS AND METHODS.....</b>	<b>21</b>
Mouse studies .....	21
Cells and viruses.....	21
Immunoblot analysis.....	23
IFN- $\beta$ ELISA.....	23

Dual luciferase reporter assay.....	23
Density gradient sedimentation. ....	23
Co-immunoprecipitations of MDA5 and 14-3-3 proteins .....	24
Gel-shift assay. ....	24
RNA extraction and analysis. ....	25
RNA preparation.....	25
RLR PAMP-pulldown. ....	26
RNA deep sequencing. ....	26
Strand-specific WNV RT-qPCR. ....	27
Statistical analysis. ....	27
<b>III. RLR DETECTION AND CONTROL OF WNV <i>IN VITRO</i> AND <i>IN VIVO</i> .....</b>	<b>28</b>
INTRODUCTION.....	28
RESULTS.....	29
DISCUSSION .....	36
<b>IV. DEFINING THE WEST NILE VIRUS PAMP .....</b>	<b>49</b>
INTRODUCTION.....	49
RESULTS.....	50
DISCUSSION .....	57
<b>V. FINAL DISCUSSION .....</b>	<b>69</b>
Future Directions.....	75

VI. VI. REFERENCES.....	77
-------------------------	----

## Table of Figures

Figure I-1. Schematic representation of the RLR domains. ....	7
Figure I-2. Cell intrinsic response to RNA virus infection.....	10
Figure III-1. <i>In vivo</i> pathogenesis of RLR KO mice infected with WNV.. ....	40
Figure III-2. Figure III-2. Innate immune gene expression and viral RNA accumulation over a WNV-infection timecourse. ....	42
Figure III-3. MDA5 is essential for viral replication control and innate immune induction in primary myeloid cells. ....	43
Figure III-4. Innate immune gene expression and viral RNA accumulation in RLR ko MEFs infected with Japanese encephalitis virus.....	45
Figure III-5. Human RLR induction of innate immune signaling in response to WNV infection.....	46
Figure III-6. RLR relocalization following virus infection.. ....	47
Figure III-7. MDA5 interacts with 14-3-3 family members during WNV infection.....	48
Figure IV-1. RNA binding/gel-shift analysis of purified RIG-I with HCV poly-U/UC with a 5'ppp, WNV 5'NTR RNA with a 5'ppp, 7mGpppN (Cap 0), or 7mGpppNm (Cap 1) structure, and poly (I:C) .....	61
Figure IV-2. PAMP properties of WNV infection. ....	62
Figure IV-3. Distinct RIG-I and MDA5-dependent PAMPs accumulate with differential kinetics during WNV infection. ....	63

<b>Figure IV-4. WNV PAMP-pulldown.....</b>	<b>64</b>
<b>Figure IV-5. Viral genomic coverage from RNA isolated from immunoprecipitated RLRs during WNV infection.. ..</b>	<b>65</b>
<b>Figure IV-6. Non-virus, non-ribosomal RNA most enriched in RLR immunoprecipitation samples. ....</b>	<b>67</b>
<b>Figure IV-7. Strand specific WNV qRT-PCR of RNA isolated from immunoprecipitated RLRs.....</b>	<b>68</b>
<b>Figure V-1: Final model of RLR-mediated protection against WNV infection.....</b>	<b>73</b>

## TABLE OF TABLES

<b>Table I-1. WNV incidence and disease in the United States. ....</b>	<b>17</b>
<b>Table III-1. RLR-induced innate immune genes.. ....</b>	<b>39</b>
<b>Table IV-1. Summary of depth of coverage from deep sequencing of RLR-associated RNAs. .....</b>	<b>60</b>

## ABBREVIATIONS

WNV – West Nile virus

PRR – pattern recognition receptors

PAMP – pathogen associated molecular pattern

TLR – toll-like receptor

NLR – NOD -like receptor

ALR – AIM2-like receptors

RIG-I – retinoic acid-inducible gene I

RLR – RIG-I-like receptors

PKR – protein kinase R

DNA – deoxyribonucleic acid

RNA – ribonucleic acid

LRR – leucine-rich repeats

TIR – Toll/IL-1 receptor

NF $\kappa$ B – factor  $\kappa$ -light-chain-enhancer of activated B cells

IRF – interferon regulatory factor

IFN – interferon

DC – dendritic cell

pDC – plasmacytoid dendritic cells

ss – single-stranded

ds – double-stranded

NOD – nucleotide-binding, oligomerization domain

NBD – nucleotide binding domain

DAMP – danger-associated molecular patterns

IL – interleukin

MDA5 – melanoma differentiation-associated factor 5

LGP2 – laboratory of genetics and physiology 2

CARD – caspase activation and recruitment domains

RD – repressor domain

TBK1 – TANK-binding kinase 1

IKK $\epsilon$  – inhibitor of nuclear factor kappa-B kinase subunit epsilon

MAM – mitochondrial-associated membranes

HCV – hepatitis C virus

EMCV – encephalomyocarditis virus

JEV – Japanese encephalitis virus

LCMV – lymphocytic choriomeningitis virus

kb – kilobases

Pol III – polymerase III

eIF2  $\alpha$  –  $\alpha$  subunit of eukaryotic initiation factor 2

OAS – 2'-5'-oligoadenylate synthetase

cGAS – cGAMP synthase

cGAMP – cyclic dinucleotide GMP-AMP

HMGB – high-mobility group box

SR-A – class A scavenger receptors

TNF- $\alpha$  – tumor necrosis factor- $\alpha$

NK – natural killer cells

TCR – T cell receptor

BCR – B cell receptor

APC – antigen presenting cells

PFU – plaque-forming units

CNS – central nervous system

KO – gene-knockout

DKO – double gene-knockout

MOI – multiplicity of infection

icRNA – infected cell RNA

mcRNA – mock-infected cell RNA

AP – Antarctic phosphatase

CHX – cycloheximide

hpi – hours post infection

NTR – non-translated region

# I. INTRODUCTION

## MAMMALIAN DEFENSE AGAINST INFECTION

The field of immunology and its relevance to human health and disease largely came to light following the experiments of Edward Jenner and his successful attempts to vaccinate human patients against the smallpox virus (1, 2). These seminal experiments, that occurred more than 200 years ago, were not based on a deep, mechanistic understanding of the immune system but instead upon insightful, empirical observation. Nevertheless, the demonstration that in the right context the immune system can protect against an otherwise deadly disease was a very powerful one that helped lead to the development of immunology as one of the most important fields of scientific research today.

The following decades of research in immunology revealed an astounding degree of complexity in the mammalian immune system due to the evolutionary pressure to resist death and disease caused by an enormous array of pathogens (3). For many years the majority of research effort was focused on trying to understand the mechanisms that regulate the immune system's ability to respond to a variety of pathogens through highly specialized T cells, B cells and antibodies (4-7). These responses, termed adaptive immunity, are normally the result of a days long process requiring the expression of receptors encoded by DNA that has undergone gene rearrangement and hypermutation to bind specific antigens. However, in the late 1980's Charles Janeway hypothesized there must be another aspect to the immune system that allowed animals to respond more generally to pathogens and initiate immune responses (8). Indeed, shortly after this hypothesis was put forward, several germline encoded pattern recognition

receptors (PRRs) were identified that respond to evolutionarily conserved pathogen associated molecular patterns (PAMPs) present on invading microbes (9-12). PRR:PAMP interactions now form the basis of what is called the innate immune system although other important aspects of innate immunity have been described both before and after Janeway's original hypothesis (13-18).

One observation that led to Janeway's hypothesis was that even vaccines, which exploit the exquisite specificity of the adaptive immune system, require an adjuvant stimulation of the what would come to be called the innate immune system to jump-start the activity of the immune system as a whole (8). Ongoing work has helped highlight that the innate and adaptive immune systems are not separate, but actually highly interconnected components of a single program of vertebrate defense against infection. Immune defense against virus infection has been one of the best studied areas of immunology due to its importance to human health and disease and the body of research in this area highlights well the combined roles of innate and adaptive immunity. This introduction will review our current understanding of immunity to viruses with a focus on innate immune responses and the insights of host-pathogen interactions revealed by research on the human neurotropic West Nile virus.

## **INITIATION OF IMMUNITY TO VIRUSES**

Initiation of immunity to viruses is most commonly thought to begin with binding of a host PRR with its cognate viral PAMP. Many distinct families of PRRs have been shown to play a role in detecting viral infection including toll-like receptors (TLRs), nucleotide-binding, oligomerization domain (NOD)-like receptors (NLRs), AIM2-like receptors (ALRs) and retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) (19-22). In addition, several other PRRs

have also been shown to respond to viral infection such as protein kinase R (PKR), 2'-5'-oligoadenylate synthetase (OAS), cGAMP synthase (cGAS), non-RLR helicases and class A scavenger receptors (SR-As) (23-27). A common principle of most of the PRRs is their ability to discriminate between self vs. non-self during a viral infection despite the fact that viruses are obligate intracellular parasites and derived entirely from host cell components. This discrimination occurs due to either a unique identity of the viral PAMP or its aberrant location within an animal. With a few notable exceptions, the viral PAMP is usually comprised of the nucleic acid genome, or nucleic acid genome products, be they deoxyribonucleic acid (DNA) or ribonucleic acid (RNA) (28). Following detection of a viral PAMP, most PRRs initiate a signaling cascade that results in transcriptional activation and gene expression of a variety of antiviral effectors, cell pathway modulators, pro-inflammatory cytokines and chemokines and initiation of adaptive immune programs.

## **TLRs**

The toll-like receptors (TLRs) are a family of PRRs consisting of 12 individual members sharing a structural homology that was originally identified in the Toll gene of fruit flies (reviewed in (19, 29)). Each of the individual members have been shown to recognize specific components of pathogens and initiate innate immune signaling through a pair of TLRs assembled as a homo or heterodimer. The trans-membrane TLRs consist of an extracellular leucine-rich repeats (LRR) domain that engages the microbial PAMP and a cytoplasmic Toll/IL-1 receptor (TIR) domain that initiates downstream signaling cascades. TLR3 and TLR4 signal through the TRIF/TRAM pathway resulting in activation of the transcription factors factor  $\kappa$ -light-chain-enhancer of activated B cells (NF $\kappa$ B) and interferon regulatory factor 3 (IRF3) and the expression of pro-inflammatory genes as well as type I interferon (IFN). Type I IFN (IFN- $\beta$  and

IFN-  $\alpha$ ) is a central cytokine of antiviral defense and can signal in both an autocrine and paracrine manner to induce an antiviral state through the expression of hundreds of genes (interferon stimulated genes (ISGs)) as well as promote antigen presentation to initiate adaptive immunity (30). TLR4 and the rest of the family members signal through the TIRAP/MyD88 pathway resulting in activation of NF $\kappa$ B and AP1 (exceptions occur as discussed below). Despite the convergence of these receptors on common signaling pathways, engagement of different single or multiple TLRs ultimately results in highly disparate immune responses due to strength of signaling, cell-specific expression and other incompletely understood mechanisms.

The TLRs are primarily expressed on the cell surface or in endosomes of specialized cells of the immune system including plasmacytoid dendritic cells (pDCs), dendritic cells (DCs), macrophages, B cells and other immune cells. The TLRs collectively coordinate immunity to fungi, parasites, bacteria and viruses through the recognition of lipids, lipoproteins, proteins, and nucleic acids. TLRs 3, 7, 8, and 9 are all endosomally expressed and are the members most known to participate in defense against viruses as well as be involved in autoimmune disease. TLR3 recognizes double-stranded (ds)RNA (31-33). TLRs 7 and 8 recognize single-stranded RNA (ssRNA) (34, 35). TLR9 recognizes hypomethylated DNA (36, 37). TLRs 7 and 9 are especially highly expressed in pDCs and efficiently induce the expression of type I IFN through a transcriptional pathway unique to these cells (34).

## **NLRs**

Nucleotide-binding, oligomerization domain (NOD)-like receptors (NLRs) are a large family of widely expressed intracellular PRRs that respond to a variety of stimuli to affect a diverse array of biological outcomes (reviewed in (20)). Despite diverse structure and sequence, all NLRs share a nucleotide binding domain (NBD) and LRR domain and are cytosolically

expressed with the exception of membrane bound NLRX1. The natural ligands of all NLRs shown to be involved in antiviral immunity are still unknown, but some appear to respond to danger-associated molecular patterns (DAMPs) instead of PAMPs. NOD2 is involved in sensing ssRNA during viral infection to induce type I IFN through activation of NF $\kappa$ B and IRF3 (38). The NLRP3 activated inflammasome plays an important role in immunity against a number of viruses although the exact mechanism of activation is unclear in all contexts (39-41). NLRP3 is a pyrin domain containing NLR that interacts with and activates the cytosolic protease caspase-1 which drives interleukin (IL)-1 $\beta$  and IL-18 processing and release or even cell death. Activation of the NLRP3 inflammasome during influenza infection was shown to occur following virus induced ion flux and NLRP3 may therefore represent a sensor of DAMPS in the context of virus infection (42). NLRX1 has also been shown to act in both a pro-inflammatory and anti-inflammatory manner in response to viral infection but its precise role remains controversial (43-45).

### **ALRs**

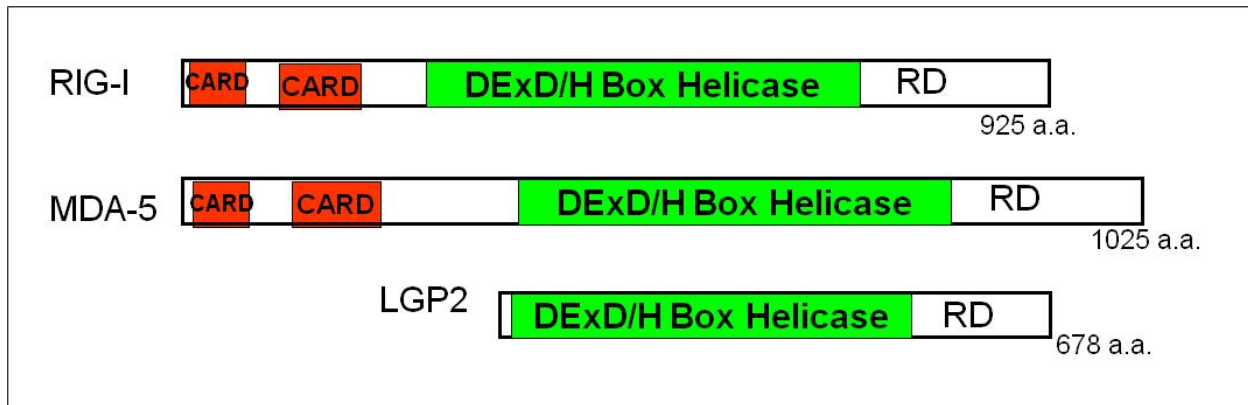
AIM2-like receptors (ALRs) (also known as the PYHIN family) are widely expressed intracellular receptors containing a pyrin domain mediating protein:protein interactions and a HIN200 domain mediating DNA binding (reviewed in (46)). Despite considerable evolutionary divergence between mice and humans, accumulating evidence has shown a clear role for the ALRs in both species in initiating innate immunity in defense against both virus and bacterial infection. Absent in melanoma 2 (AIM2) functions in both human and murine cells to sense intracellular DNA and initiate an apoptosis-associated speck-like protein containing a CARD (ASC)-dependent inflammasome response. Similar to NLRP3 inflammasome activation, ASC inflammasome activation leads to activation of caspase 1 and production of mature IL-1 $\beta$ , IL-18,

and potential cell death (22, 47, 48). Human IFI16 and several murine ALRs including PYR-A, PYHIN-A, and IFI203 all induce a stimulator of interferon genes (STING)-dependent IRF3 driven type I IFN response following intracellular DNA stimulation (22, 49). Unlike the NLRs which can drive inflammasome activation and type I IFN expression in response DNA and RNA, the ALRs appear to only respond to intracellular DNA.

## **RLRs**

The RIG-I-like receptors (RLRs) are three cytosolic PRRs that recognize RNA as PAMPs during viral infection; RIG-I, melanoma differentiation-associated factor 5 (MDA5) and laboratory of genetics and physiology 2 (LGP2) (reviewed in (21, 50)). These three receptors are widely expressed by most cells and are highly inducible in both a cell extrinsic (by type I IFN signaling) and/or cell intrinsic manner (direct IRF3-driven gene expression) (51). Given their ubiquitous expression, high inducibility and surprising degree of cross-talk with other innate and adaptive immune pathways (discussed below), RLRs have emerged as one of the most important PRR families in immunity to viruses.

The three RLRs are ATPase dependent DExD/H RNA helicases and share homology throughout their central helicase domains as well as the C-terminal domain that has been described as a repressor domain (RD) (52-54). RIG-I and MDA5 possess two N-terminal caspase activation and recruitment domains (CARDs) and have been shown to recognize cytosolic RNA during infection to activate innate immune programs. LGP2 lacks the N-terminal CARD domains and is thought to function as a regulator of RIG-I and MDA5 signaling, although it may play other roles in regulating immune responses during virus infection (see ref. (55) and discussion below for other possible roles of LGP2) (54-56). **Figure I-1** illustrates the basic organization of the RLR domains.



**Figure I-1. Schematic representation of the RLR domains.**

RIG-I and MDA5 function as PRRs by surveillance of the intracellular environment for stimulatory RNA PAMPs and undergo a conformational change upon binding RNA to expose their N-terminal CARD domains. The exposed CARD domains facilitate a homotypic protein:protein interaction with the CARD containing, membrane bound, common adaptor repressor domain (RD) (also known as IPS-1, CARDIF and VISA) (57-59). The assembly of the RLR/MAVS complex recruits further members of the signal transduction cascade (including TANK-binding kinase 1 (TBK1) and inhibitor of nuclear factor kappa-B kinase subunit epsilon (IKK $\epsilon$ )) ultimately resulting in the nuclear translocation of NF $\kappa$ B and IRF3 and the induction of pro-inflammatory genes and type I IFN. A combined model of RIG-I activation proposes that RIG-I actively surveys RNAs as an ATPase-dependent translocase (60). Following encounter with specific PAMP ligand properties (discussed in detail below), RIG-I undergoes a conformational change and associates into homo-oligomers that are brought to the intracellular surface of mitochondria, mitochondrial-associated membranes (MAMs) and peroxisomes by molecular chaperones such as TRIM25 and 14-3-3 $\epsilon$  (54, 61-63). Less is known about the specific events regulating MDA5 activation following RNA binding, but accumulating evidence suggests

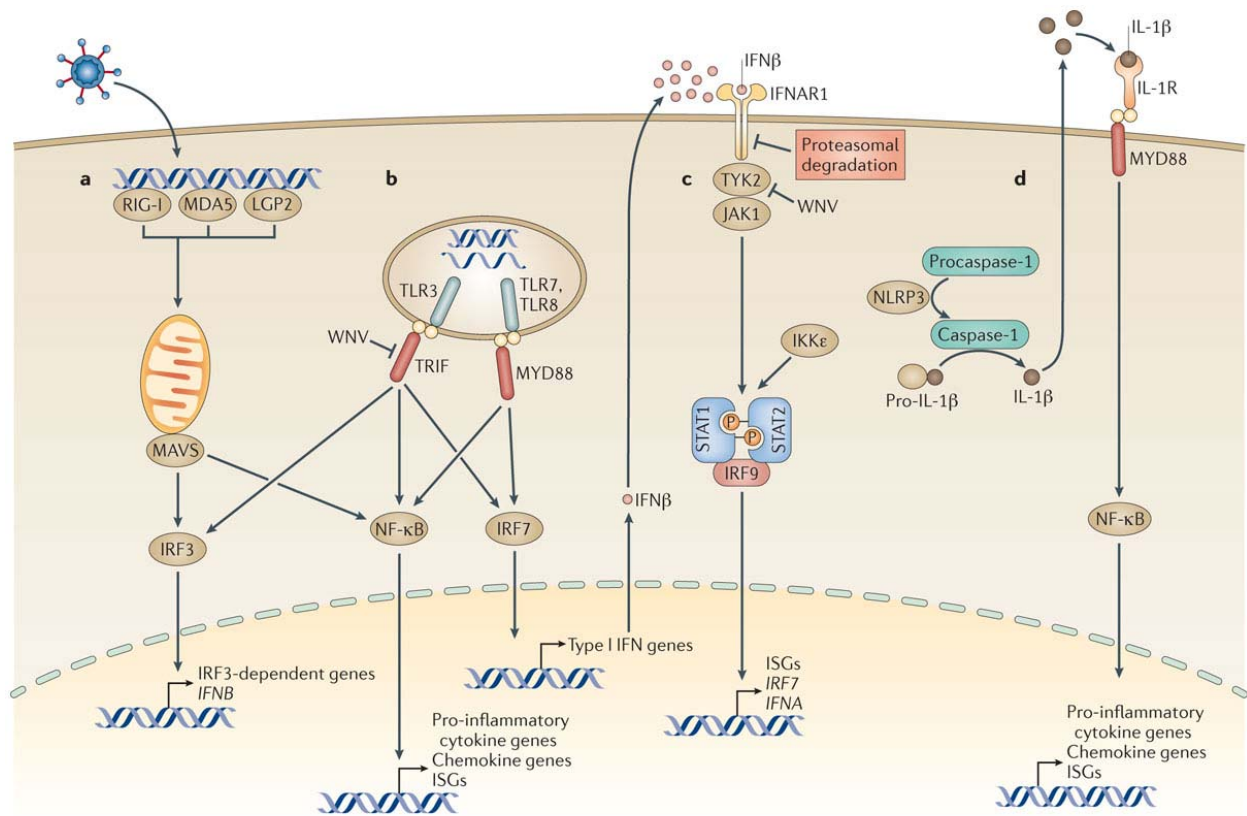
the signaling competent form of MDA5 is a large oligomer of multiple MDA5 molecules arranged along ligand RNA so that the CARD domains are exposed for interaction with MAVS (64, 65).

RIG-I and MDA5 have been reported to detect both distinct and overlapping groups of viruses (66-69). Briefly, RIG-I has been shown to be required for response to viruses including Paramyxoviridae family members, influenza and hepatitis C virus (HCV). MDA5 is essential for response to Picornaviridae family members such as encephalomyocarditis virus (EMCV). WNV, Dengue virus, reovirus and lymphocytic choriomeningitis virus (LCMV) have been reported to be detected by both MDA5 and RIG-I (66, 70, 71). However, subsequent work has shown that Sendai virus (originally thought to be a RIG-I dependent virus) specifically antagonizes MDA5 signaling and EMCV (originally thought to be a MDA5 dependent virus) targets RIG-I for degradation, raising the possibility that both RLRs may be able to detect a wider array of viruses than initially appreciated (72, 73). The importance of the RLRs in immunity against viruses is highlighted by the multiple ways these viruses have evolved to directly antagonize RLR signaling. The NS1 protein of influenza binds RIG-I and the V proteins of Paramyxoviridae family members efficiently bind to both MDA5 and LGP2 to prevent RLR signaling (68, 74). The HCV NS3/4A protein disrupts RLR signaling by proteolytically cleaving MAVS from the surface of intracellular membranes (57).

Studies that characterize PAMP ligands of RIG-I have demonstrated that non-self recognition depends on several properties of viral RNA including PAMP motif length, structure, modification, and composition (75-78). The most consistent observation from these studies is that RIG-I ligand RNA requires a 5'ppp in conjunction with at least a small amount of secondary dsRNA structure. Importantly though, some RNAs that contain both 5'ppp and dsRNA do not

stimulate RIG-I-dependent signaling, suggesting that sequence composition is another incompletely understood determinant of RIG-I PAMP specificity (77, 79). The 5'ppp physically interacts with the C-terminal RD of RIG-I while the helicase domain makes contact with dsRNA (53, 80). The precise nature of the viral ligand for MDA5 has not been well described. MDA5 ligand specificity has been reported to be for either long dsRNA (>2 kilobases (kb)), or that ligand RNA not only requires long dsRNA but “higher order” molecular complexes of RNA containing both dsRNA and ssRNA (67, 81). The question of how MDA5 discriminates between long dsRNAs, short dsRNAs, and other higher order complexes is interesting and it is likely that there are several unidentified factors that define the viral ligand of MDA5 just as there are for RIG-I. An overall illustration of innate immune recognition of RNA virus infection and initial signaling events is depicted in Figure I-2.

An unexpected role for the RLR pathway was initially discovered in 2007 and further elucidated in 2009 when it was demonstrated that the dsDNA poly(dA-dT)-poly(dA-dT) (poly(dA-dT)) and several DNA viruses can induce an IFN response through the RLR pathway (82, 83). Poly(dA-dT) or other viral substrate RNA is sensed by the DNA-dependent RNA polymerase III (Pol III) and transcribed into a RIG-I stimulatory, 5' phosphate containing RNA ligand. Other non-canonical roles for the RLR pathway have also been described as RIG-I and MAVS may play a direct role in activating inflammasome activity and apoptosis(84, 85).



Nature Reviews | Microbiology

**Figure I-2. Cell intrinsic response to RNA virus infection (Figure 3 from(86)). RLR, TLR and NLRP3 inflammasome activation and signaling in the context of an RNA virus infection is depicted.** The RLRs sense intracellular RNA PAMPs to initiate innate immune signaling. The TLRs sense dsRNA or ssRNA located within endosomes to drive TRIF or MYD88-dependent signaling. Type I IFN production following RLR or TLR recognition of PAMPs leads to the transcription of hundreds of ISGs. Upregulation of immune genes allows cells to activate the inflammasome pathway and produce more pro-inflammatory cytokines.

## **‘OTHER’ VIRAL SENSORS**

Several sensors that do not fit into the above families have been shown to play an important role in the innate immune response to virus infection. PKR is a dsRNA activated, cytoplasmic, serine/threonine kinase that has been shown to have at least two major roles as a PRR during viral infection (reviewed in (23, 87)). Following recognition of dsRNA, PKR phosphorylates the  $\alpha$  subunit of eukaryotic initiation factor 2 (eIF2  $\alpha$ ) to shut down host cell translation. PKR also plays a role in type I IFN induction by activating NF $\kappa$ B and IRF3 transcription factors in a MAVS-dependent manner (88). 2'-5'-oligoadenylate synthetase (OAS) is another cytoplasmic dsRNA sensor and following activation leads to the synthesis of 2'-5' oligoadenylates that then proceed to activate the ribonuclease RNase L (24). Activation of RNase L leads to processing of both host and viral RNA and may lead to the production of additional RNA ligands for the RLR pathway, thus amplifying type I IFN signaling (89). cGAMP synthase (cGAS) is a recently identified PRR of cytosolic DNA that shares structural and functional homology with OAS and binds cytoplasmic DNA. Following recognition of PAMP DNA, cGAS is activated to generate the cyclic dinucleotide GMP-AMP (cGAMP) (25). cGAMP then functions as an endogenous second messenger to bind and activate the adaptor molecule STING, leading to activation of IRF3 and production of type I IFN (90). The high-mobility group box (HMGB) family and class A scavenger receptors (SR-As) have both been proposed to play a role as accessory or sentinel PRRs to potentiate the more well known PRR pathways discussed above. SR-As are expressed on the cell surface and bind extracellular dsRNA and help shuttle the stimulatory RNA into the cytosol or endosomes where they can be sensed by TLRs and RLRs to initiate innate immune signaling cascades (26). HMGBs perform a similar role but aid in the detection of non-self RNA and DNA, but the exact role they play as a chaperone or

cooperative binding partner remains unclear (91). Non-RLR helicases are another emerging family of PRRs that have been shown to be involved in innate immunity to viruses (reviewed in (27)). Interestingly, these putative RNA helicases have been shown to detect and initiate innate immune signaling in response to DNA as well as RNA (92, 93). An opposing role of these RNA helicases has also been shown as DDX3 has been identified as an essential host factor to support viral replication (94, 95).

## **CONSEQUENCES OF INNATE IMMUNE RECOGNITION OF VIRUSES**

PRR recognition of invading pathogens initiates a complex set of events that can lead to local clearance of infection or recruitment and activation of additional immune cells and eventually adaptive immunity. The specific innate and adaptive immune responses to WNV will be further detailed below, but a generalized sequence of events to viral infection will be discussed here. Following local infection of cells, PRRs induce prototypic cytokines such as tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), type I IFN and IL-1 $\beta$  (96). Release of these cytokines can cause recruitment of immune cells like macrophages, neutrophils, natural killer (NK) cells and DCs. Recruitment and activation of these innate cells can result in either decreased or increased severity of disease during a viral infection by effectively acting in a direct antiviral role or increasing inflammatory pathology, respectively (97, 98).

Whereas cell-intrinsic and cell-mediated innate immunity can respond within minutes or hours of infection, the highly specific responses of the adaptive immune system can take days or weeks to develop. T and B cell responses are dependent on a random genetic rearrangement of the sequences encoding their receptors during a positive and negative selection process that results in a T cell receptor (TCR) or B cell receptor (BCR) with appropriate affinity for microbial

antigens but not self ones. CD8 T cell, CD4 T cell and B cell development and function are reviewed in the following references, respectively (99-101). The inflammatory milieu present during infection helps drive maturation and antigen presentation by antigen presenting cells (APCs). CD8 $\alpha$ <sup>+</sup> DCs (either by infection or uptake of antigen) are especially efficient at presenting viral antigen to naive T cells to initiate adaptive immune responses (102). Primary antigen presentation and activation of T cells usually occurs within lymphoid organs by APCs and consists of three signals delivered to the T cells; (1) inflammatory milieu, (2) TCR ligation by antigen presented as a MHC-antigen complex on APCs and (3) co-stimulation by cell surface protein interaction between the APC and T cell. Successful activation of a T cell leads to clonal expansion, differentiation, effector function and memory. CD8 T cells migrate to sites of infection and have direct antiviral action through both cytolytic and non-cytolytic actions on infected cells via interactions with their TCR and viral antigens presented on MHC-I. CD4 T cells can also be directly antiviral through release of cytokine and effector molecules, but their main function is to provide help to CD8 T cells and B cells during an infection (103). CD4 T cell help aids in proliferation and functional specialization of CD8 T cells and B cells (104, 105). B cells differentiate into antibody secreting plasma cells following binding of antigen to their BCR and CD4 T cell help in the form of cytokine stimulation and cell:cell interactions. B cell produced antibody can be antiviral by coating viruses to prevent cell entry or by binding to viral antigens on cells (106, 107). A hallmark of the adaptive immune system is that following activation, clonal expansion and effector function differentiation, a population of CD8 T cells, CD4 T cells and B cells persist as a long-lived memory population capable of responding quickly to infection up to decades later (108).

## WEST NILE VIRUS

### WNV ECOLOGY AND EPIDEMIOLOGY

West Nile virus (WNV) has recently emerged as a primary cause of viral encephalitis in the Western hemisphere (109). To date, WNV has now been identified in more than 80 countries throughout North America, Europe, Australia, Asia and Africa where it cycles in nature between birds and mosquitoes (110). More than 60 species of mosquito and hundreds of species of birds have been found to carry WNV, but *Culex pipiens* and *Passeriformes* birds are the predominant vector and host in the United States (111, 112). Infection of *Culex pipiens* and *Passeriformes* birds, such as crows and jays, results in viremias high enough to complete the enzoonotic life cycle of WNV. Other vertebrates can become infected by feeding mosquitoes or even from consuming infected birds, however they rarely support levels of replication sufficient to complete the viral life cycle. An infected mosquito can deliver up to  $10^6$  plaque-forming units (PFUs) during a single feeding on a host and WNV can replicate to titers of  $10^{11}$  PFU/ml in highly susceptible bird species (113, 114). An interesting aspect of the natural biology of WNV is the extreme promiscuity of species found to be infected. Well over 30 species of vertebrates have been shown to be infected by WNV including; alligators, baboons, bats, dogs, horses, iguanas, raccoons, reindeers, and turtles (112). WNV infection was even identified as the cause of fatal encephalitis in a captive orca (115).

As with other mammals, humans typically become infected with WNV as incidental hosts following virus exposure from a feeding, infected mosquito. Since its emergence into North America in 1999, there have been over 39,000 cases of human WNV infection in the United States including the second highest annual peak of over 5,000 cases in 2012 alone (see **Table I-1** (116, 117)). Especially in North America, infection of humans follows a highly

seasonal pattern with the number of infections peaking in late summer when populations of mosquitoes are high and female mosquitoes change feeding habits to focus on mammals instead of birds as the primary source of blood meals (118). Over 39,000 cases of human WNV infections have been confirmed as clinical diagnoses, but serological studies of humans in the United States in regions of previous outbreaks suggest that the majority of infections are undiagnosed or asymptomatic (119). Using the estimates from Hayes et al. in reference (119), the current calculation reveals that more than 2.3 million Americans have likely been productively infected by WNV. The high level of exposure to WNV is concerning as dramatic shifts in the dominant genetic strains of WNV have been observed to occur over relatively short time periods. Following the introduction of WNV to New York in 1999, the virus quickly spread throughout North America and the original genetic strain of virus was displaced by a newly dominant genetic variant that was first identified in Texas in 2002 (120). Recent molecular epidemiological studies show that there is significant continued genetic evolution of WNV occurring throughout North America and that new genetic variants may be responsible for seasonal shifts in the observed number of human cases and clinical severity (121).

WNV has a large global distribution where it exists as an endemic, emerging and re-emerging viral disease of humans. The virus is endemic to portions of Africa where it was originally identified from a febrile patient in Uganda in 1937 (122). Seroprevalence in certain regions in Africa has shown that 75-98% of the population had been infected with WNV (123). Despite such high prevalence, incidence of disease was remarkably low until the 1990s when several outbreaks of disease occurred in Africa and Europe (110). WNV has since been classified into two major lineages that represent the low virulence strains (lineage 2) that circulate endemically in Africa and parts of Asia and the emergent, more highly virulent strains (lineage

1) associated with outbreaks in North America, Europe, northern Africa, Asia and Australia (124). The determinants of virulence are incompletely understood, but include the ability to antagonize the immune system and overall rates of viral replication. Lineage 1 strains have greatly increased their global distribution over the last 30 years and there remains a potential for newly emerging pathogenic WNV strains to cause additional and possibly increasingly severe human outbreaks (125, 126).

Infection with WNV is characterized by an acute febrile episode that can progress to neuroinvasive disease including encephalitis, meningitis, and flaccid paralysis. No effective therapies are currently available to directly treat WNV infection in humans and intervention is limited to palliative and supportive care. A majority of symptomatic patients will experience a resolving disease course known as WNV fever that includes symptoms of fever, fatigue, myalgia and gastrointestinal distress. The greatest risk for severe disease is in the elderly and immunocompromised (127). In human WNV cases with neuroinvasive disease, death occurs in approximately 10% of patients (109, 116, 119, 128). A largely under-appreciated aspect of WNV disease is the potential for long-term persistence of the virus and chronic symptoms. Evidence in humans and animal models has shown that infectious virus and/or viral genetic material are detectable for months to years following acute infection (129). Chronic symptoms of WNV disease include fatigue, myalgia and neurological complications such as difficulty with memory and concentration (130, 131).

Year	Neuroinvasive disease	Total	Deaths
1999	59	62	7
2000	19	21	2
2001	64	66	9
2002	2946	4156	284
2003	2886	9862	264
2004	1148	2539	100
2005	1309	3000	119
2006	1495	4269	177
2007	1227	3630	124
2008	687	1356	44
2009	373	720	32
2010	629	1021	57
2011	486	712	43
2012	2873	5674	286
2013 (preliminary)	1140	2271	100
total	17341	39359	1648

**Table I-1. WNV incidence and disease in the United States.** Data compiled from:

<http://www.cdc.gov/westnile/statsMaps/> [Accessed November 2014].

## **WNV PATHOLOGY AND THE IMMUNE RESPONSE**

Many aspects of WNV infection, immunity, and clinical disease in humans are recapitulated in mouse challenge models. Subcutaneous inoculation of WNV in mice results in a pathogenesis sequence that includes local infection in cells (keratinocytes, Langerhans cells) of the skin, migration of infected DCs to the draining lymph node, and the development of viremia, which ultimately facilitates crossing of the blood-brain-barrier and infection of neurons in the

central nervous system (CNS) (128, 132). The mouse model of WNV infection has revealed many key host-pathogen interactions that control the outcome of WNV infection and immunity (reviewed in (86, 128)). Components of the innate and adaptive immune system are essential for protection from WNV infection. Innate immune programs involved in pathogen recognition, signal transduction, and effector function are required for effective antiviral immunity against WNV infection and disease (133-141), whereas antibody and cell mediated immunity are necessary for limiting virus dissemination, clearance of WNV from target cells in the periphery and CNS, and preventing disease after secondary exposure (142-144). An underlying concept extending from many studies of WNV infection in gene-knockout (KO) mice is that compromise of innate antiviral immunity can ultimately result in enhancement of virus entry or replication in the CNS, leading to neurological disease and mortality (86).

During WNV infection the innate immune response serves to control tissue tropism of infection and restrict virus entry into the CNS (86, 145, 146). Prior studies in cell culture have shown that flavivirus recognition during acute infection is mediated by both RIG-I and MDA5 (70). For WNV, the RLR pathway is required for induction of type I IFN and innate antiviral responses, with RIG-I proposed to induce gene expression early in infection and MDA5 signaling occurring at a later stage (134, 136, 147, 148). The common adaptor molecule MAVS has clearly been shown to be essential for controlling WNV infection and immunity *in vitro* and *in vivo* (136). MAVS deficiency *in vitro* in key target cells of infection leads to a complete loss of innate immune signaling and increase in viral replication while MAVS deficiency *in vivo* results in an increase in viral replication and inflammation. MAVS-dependent RLR signaling activates the transcription factors IRF3, IRF-5, IRF-7, and NF- $\kappa$ B, to promote gene expression

programs that limit WNV replication and spread, and modulate adaptive immunity (57, 138, 139, 147, 149, 150).

The initiation of innate immune signaling listed above leads to expression of hundreds of genes and some of the consequent mechanisms of cell-intrinsic virus inhibition have been attributed to individual effector genes. Viperin is an innate immune stimulated gene that does not affect the early stages of viral binding or cell entry, but instead blocks efficient replication of virus and is essential for *in vivo* protection (151, 152). IFITM proteins are ISGs that have been shown to block early events of cell binding/entry by WNV (151, 153). IFIT protein family members have been proposed to play a role in PAMP sensing, but also exhibit effector function against WNV by inhibiting viral translation (154, 155).

In addition to inducing effector genes that are antiviral on a cell-intrinsic level, the central role of the innate immune response in programming an effective adaptive response in the context of WNV is highlighted by several key studies. As previously noted, MAVS deficiency *in vivo* results in lethality and increased viral replication, but also a dysregulated adaptive response. Despite an increase in most inflammatory pathways and immune cellularity, anti-WNV antibodies had reduced function and regulatory T cells were reduced in number (136). Interestingly, deficiency in the RLR family member LGP2 did not greatly affect observed innate immune gene induction, but instead lead to an increased lethality in mice that was attributed to a CD8 T cell-intrinsic defect (55). Loss of inflammasome signaling also lead to an increased lethality in mice that was linked to a lack of CD8 T cell function in the CNS (40). Innate immune signaling thus regulates both cell intrinsic immune responses as well as adaptive immunity to program successful defense against WNV.

In the work presented below, we assessed the individual and combined roles of RIG-I and MDA5 in pathogen recognition and immunity to WNV infection using WT and RLR KO mice and cells. We show that each RLR individually contributes to pathogen recognition and immune protection against WNV *in vivo* and *in vitro*, and establish that RIG-I and MDA5 detect distinct PAMPs with differential kinetics during the course of WNV replication to mediate complementary, nonredundant roles in viral detection and innate immune gene induction.

## II. MATERIALS AND METHODS

**Mouse studies.** *RIG-I*<sup>-/-</sup> and their wild type (WT) littermate control mice have been published (66, 156) and were obtained as a generous gift from S. Akira (Osaka University). *MDA5*<sup>-/-</sup> mice were kindly provided by M. Colonna (Washington University St Louis). *MAVS*<sup>-/-</sup> mice were previously described (55). For production of double knockout (DKO) mice lacking both *RIG-I* and *MDA5*, *RIG-I*<sup>-/-</sup> and *MDA5*<sup>-/-</sup> mice were intercrossed, and the resulting DKO offspring were backcrossed into a C57BL/6 background through the F3 generation. The resulting DKO line used in this study is approximately 94% C57BL/6 genetic background, as determined by microsatellite DNA analysis. All mice were genotyped and bred under pathogen-free conditions in the animal facility at the University of Washington. Experiments were performed with approval from the University of Washington Institutional Animal Care and Use Committee. Age-matched six to twelve week old mice were inoculated subcutaneously (s.c.) in the left rear footpad with 100 PFU of WNV-TX in a 10 µl inoculum diluted in Hanks balanced salt solution (HBSS) supplemented with 1% heat-inactivated FBS. Mice were monitored daily for morbidity and mortality.

**Cells and viruses.** Working stocks of WNV isolate TX 2002-HC (WNV-TX) were generated by propagation on Vero E6 cells and titered by standard plaque assay on BHK-21 cells as previously described (136). WNV infections were performed by incubating virus at the indicated multiplicity of infection (MOI) with cells in serum free media for 1 hour followed by removal of the virus and addition of DMEM supplemented with 10% FBS, 1 mM sodium

pyruvate, 2 mM L-glutamine, 1x HEPES pH 7.3, antibiotic-antimycotic solution, and 1x non-essential amino acids (complete DMEM).

Primary mouse embryonic fibroblasts (MEFs) were generated from *RIG-I*<sup>-/-</sup>, *MDA5*<sup>-/-</sup>, *MAVS*<sup>-/-</sup>, *RIG-I*<sup>-/-</sup> x *MDA5*<sup>-/-</sup> *DKO*, and WT control mice as previously described (139). Briefly, 13-14 day embryos were harvested from pregnant mice. The head and developing organs from the stomach cavity were removed and the remaining tissues were minced. The tissue was then treated with 0.25% trypsin-EDTA and incubated for 5 minutes at room temperature. Cells were recovered and grown in complete DMEM. To facilitate direct comparison of signaling in our PAMP characterization studies, we also generated primary MEFs from *RIG-I*<sup>-/-</sup> embryos of the F3 generation backcrossed onto the C57BL/6 background and used these cells to compare directly to WT, *MDA5*<sup>-/-</sup>, and *DKO* MEFs.

Primary bone-marrow derived macrophages (MΦ) and dendritic cells (DCs) were generated as described (139). Briefly, bone marrow cells were collected from age-matched adult mice by flushing hind limbs with media. Red blood cells (RBC) were lysed using RBC lysis buffer (Sigma). Remaining cells were cultured in complete DMEM or complete RPMI supplemented with MCSF or GM-CSF and IL-4 (Peprotech) for MΦ and DCs, respectively. Following 6-8 days in culture, cells were harvested, counted and plated for further experiments.

293T, Huh 7 and Huh 7.5 cells were cultured in complete DMEM; DMEM supplemented with 10% FBS, 1 mM sodium pyruvate, 2 mM L-glutamine, 1x HEPES pH 7.3, antibiotic-antimycotic solution, and 1x non-essential amino acids.

Sendai virus Cantell strain was purchased from Charles River. Cells were infected with 100 HA units per ml of Sendai virus and harvested at 24 hours post infection (hpi). Japanese

encephalitis virus (JEV), vaccine strain (SA 14-14-2) was a gift of M. Diamond (Washington University, St. Louis). Encephalomyocarditis virus (EMCV) was a gift of R. Silverman (Cleveland Clinic) and cells were infected at a MOI of 5 and harvested at 24 hpi.

**Immunoblot analysis.** Protein extracts were prepared as previously described (157) and 20  $\mu$ g of protein lysate was analyzed by SDS-polyacrylamide gel electrophoresis followed by immunoblotting. The following primary antibodies were used;  $\alpha$ -murine IFIT2 (gift of G. Sen, Cleveland Clinic); goat  $\alpha$ -WNV NS3 (R&D Systems); mouse  $\alpha$ -tubulin (Sigma); mouse  $\alpha$ -GAPDH (Santa Cruz); mouse  $\alpha$ -myc 9E10 (Pierce); and mouse  $\alpha$ -FLAG M2 (SIGMA). All secondary antibodies were purchased from Jackson ImmunoResearch. Immunoreactive bands were detected with Amersham ECL Plus Reagent (GE Healthcare).

**IFN- $\beta$  ELISA.** IFN- $\beta$  in cell culture supernatants was measured by using the mouse-specific IFN- $\beta$  ELISA kit following the manufacturer's protocol (PBL Biomedical Laboratories).

**Dual luciferase reporter assay.** Human pFLAG-RIG-I, pFLAG-MDA5 and expression controls were a kind gift from Takashi Fujita (Kyoto University, Japan). Huh7.5 cells were plated at a density of  $1.5 \times 10^4$  cells per well in a 48 well plate. Cells were then transfected with Fugene 6 reagent (Roche) with RLR expression construct, IFN- $\beta$ -luciferase (firefly) and pRL-CMV (renilla). 24 hours later cells were infected with WNV as described above at an MOI=5. At the given time after infection cells were harvested for dual luciferase reporter assay (Promega).

**Density gradient sedimentation.** Density gradient sedimentation was performed as previously described (61). Briefly, cells were lysed in buffer (10 mM Tris (pH 7.5), 10 mM KCl, 5 mM MgCl<sub>2</sub>) and passed through a 25-gauge needle 20 times. Lysates were cleared of nuclei by centrifugation at 1000g for 5 min. Lysates were then mixed with a 72% sucrose containing buffer

and overlaid with 55 and 10% containing sucrose buffer successively. Following centrifugation for 14 hrs at 38,000 rpm in a Beckman SW41 Ti rotor, 7 successive fractions were removed and concentrated by Centricon filter units (Millipore). Concentrated fractions were then analyzed by immunoblot for presence of RLR-associated proteins.

**Co-immunoprecipitations of MDA5 and 14-3-3 proteins.** 293T cells were transfected with Fugene 6 with pFLAG-MDA5 expression and myc-tagged 14-3-3 proteins or empty vector. 24 hours later the cells were infected or mock. The cells were next lysed in ice-cold RIPA buffer (50 mM Tris-Cl pH 7.5, 150 mM NaCl, 5mM EDTA, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) in the presence of Protease Inhibitor Cocktail (Roche). Lysates were clarified by centrifugation and incubated with  $\alpha$ -FLAG M2 agarose beads (Sigma) for 2 hours. Beads were washed 3 times with RIPA buffer and resuspended in SDS sample buffer for analysis by immunoblot.

**Gel-shift assay.** Gel-shift assay was performed as previously described(77). Briefly, 0-60 pmol of purified RIG-I was incubated with 6 pmol of RNA for 15 min at 37°C. RIG-I/RNA complexes were resolved on an agarose gel stained with SYBR© Gold (Invitrogen) and formation of stable complexes was visualized by a shift/disappearance of RNA from its original migration pattern. HCV poly U/UC was prepared as previously described (77). Poly (I:C) was from Sigma. The first 190 nt of the WNV 5'UTR RNA were *in vitro* transcribed as previously described (158). Following phenol/chloroform extraction and ethanol precipitation, WNV RNA was left with a 5'ppp or capped to contain a Cap 0 structure or Cap 1 structure using kits from Epicentre© Biotechnologies (cat. # SCCE0610 and SCMT0610) according to the manufacturer's protocol.

**RNA extraction and analysis.** Total cellular RNA from cultured cells was collected for RT-quantitative PCR (RT-qPCR) using the RNeasy kit (Qiagen), and reverse transcribed using the iScript select cDNA synthesis kit using both oligo (dT) and random primers (Biorad). Cellular mRNA and viral RNA expression levels were determined by SYBR Green RT-qPCR using gene- or virus-specific primers. Specific primer sets are as follows:

mGAPDH forward: CAACTACATGGTCTACATGTTC, mGAPDH reverse: CTCGCTCCTGGAAGATG; mIFN $\beta$  forward: GGAGATGACGGAGAAGATGC mIFN $\beta$  reverse: CCCAGTGCTGGAGAAATTGT; mL6 forward: GTTCTCTGGGAAATCGTGGA, mL6 reverse: TGTACTCCAGGTAGCTATGG; WNV forward: CGCCTGTGTGAGCTGACAAAC, WNV reverse: CATAGCCCTCTTCAGTCC; mIFN-a2a: purchased from SABiosciences; mIRF-7 forward: CCCATCTTCGACTTCAGCAC, mIRF-7 reverse: TGTAGTGTGGTGACCCTTGC; mIFIT2 forward: CTGGGGAAACTATGCTTGGGT, mIFIT2 reverse: ACTCTCTCGTTTTGGTTCTTGG.

**RNA preparation.** Infected cell RNA (icRNA) or mock-infected cell RNA (mcRNA) was extracted using Trizol LS reagent according to the manufacturer's protocol (Invitrogen). WNV virus particle RNA (virion RNA) was purified by layering pre-cleared infected cell supernatants over a 20% sucrose cushion followed by centrifugation for 4 hours at 70,000 g in a Beckman Coulter SW 40 Ti rotor. RNA was extracted from sedimented virions using Trizol LS. *In vitro* transcribed 5' and 3' non-translated regions (NTR) of WNV were generated as previously described (159, 160). RNA concentrations were determined by absorbance using a Nanodrop spectrophotometer. icRNA was treated with Antarctic phosphatase to remove phosphate moieties

or RNase III to digest dsRNA followed by ethanol and sodium acetate precipitation (New England Biolabs). Transfections of RNA were performed with TransIT-mRNA transfection kit according to the manufacturer's protocol (Mirus Bio) into cells pretreated with for 30 minutes with 20  $\mu$ g/ml of cycloheximide (CHX) in complete DMEM. Total cellular RNA from transfected cells was collected for RT-qPCR 8-10 hours post transfection using an RNeasy kit.

**RLR PAMP-pulldown.** FLAG-tagged RLRs or vector control were transfected into 293T cells as described above. Cells were mock infected or infected with WNV for the indicated times at an MOI=5. Cells were lysed in ice-cold RLR lysis buffer (137 mM NaCl, 20 mM Tris-Cl pH 7.5, 0.5 % NP40) supplemented with Protease Inhibitor Cocktail (Roche) and SUPERase RNase inhibitor (Ambion). Lysates were clarified by centrifugation and incubated with  $\alpha$ -FLAG M2 agarose beads (Sigma) for 1 hour. Beads were washed three times with RLR lysis buffer and RNA and protein were extracted by Trizol LS according to the manufacturer's protocol (Invitrogen).

**RNA deep sequencing.** RLR-associated RNA was purified by Trizol LS as described above. RNA integrity was analyzed by Bioanalyzer (Agilent) and all samples had an RNA integrity number of greater than 8. Prior to library generation the RNA samples were subjected to ribosomal RNA depletion with the RiboZero kit (Epicentre) as even after RLR-immunoprecipitation and RNA extraction the majority of all RNA was of ribosomal origin before depletion. cDNA libraries were then prepared using the mRNA Seq Sample Preparation v1.5 kit from Illumina according to the manufacturer's protocols. All samples were sequenced on the Illumina Genome Analyzer II platform and small RNA sequences were submitted to Geospiza (Perkin Elmer) for initial analysis. We obtained greater than 20 million reads per sample that mapped to either the human genome or WNV (WNV reference genome ((161))).

Mapped WNV RNA sequences were visualized using Integrative Genomics Viewer (Broad Institute).

**Strand-specific WNV RT-qPCR.** Strand-specific WNV RT-qPCR was performed as previously described(140). Briefly, strand specificity was achieved through use of a T7-tagged strand specific primer as the primer for reverse transcription, followed by TaqMan based qPCR using primers for the T7 tag and WNV E protein sequence. Each reaction was performed with an equivalent fraction of RNA from each RLR-associated RNA sample. Positive strand primer (T7E1229R: GCGTAATACGACTCACTATAGGGTCAGCACGTTTGTTCATTG) or negative-strand primer (T7E1160F: GCGTAATACGACTCACTATATCAGCGATCTCTCCACCAAAG) was incubated with 2  $\mu$ l RNA, 12.5  $\mu$ l 2 $\times$  RT-PCR mixture and 0.625  $\mu$ l 40 $\times$  Multiscribe (Applied Biosystems). Reaction mixtures were incubated for 30 min at 55 $^{\circ}$ C and 95 $^{\circ}$ C for 10 min, followed by the addition of 5 pmol of probe (FAM-CAACCTCACCTACAGGGCGGACTTCAAG-TAMRA) and 20 pmol each of primers (T7: GCGTAATACGACTCACTATA) and E1160F to positive-strand reaction mixtures and primers T7 and E1229R to negative-strand reaction mixtures. The thermal cycling reaction was then allowed to proceed for 40 cycles.

**Statistical analysis.** *In vivo* Kaplan-Meier curves were analyzed by log-rank test. All *in vitro* statistics were performed by unpaired, two-tailed student *t*-test. A *P*-value < 0.05 was considered significant. All data were analyzed using Prism software (GraphPad Prism).

### **III. RLR DETECTION AND CONTROL OF WNV IN *VITRO AND IN VIVO***

#### **INTRODUCTION**

The mouse model of WNV infection has greatly enhanced our understanding of the immune mechanisms that regulate protection and pathogenesis in response to virus infection. Few other *in vivo* model systems have been as informative in defining the numerous components of the immune system that contribute to protection against a virus that causes significant global health burden in humans. For example, mice deficient in the chemokine receptor CCR5 were found to uniformly succumb to WNV infection and the increased lethality was shown to be due to a lack of recruitment of immune cells into the CNS (141). Human patients with a genetic deletion in CCR5 were then shown to be at increased risk of more severe WNV disease once infected(162). The knowledge gained from the mouse studies has identified an at risk population and raised the possibility of targeted therapeutic intervention in these patients. Previous work from our lab and others has clearly implicated the RLR pathway in immune protection against WNV *in vivo*. Animals deficient in MAVS, the central RLR adaptor, have enhanced mortality, increased viral burden and dysregulated adaptive immune responses (136). Likewise, mice deficient in any number of genes involved in propagating RLR signaling have also been identified to have increased mortality and pathology (135, 138, 139, 147, 149, 157). However, previous work has not addressed the individual or combined roles of RIG-I and MDA5 in providing protection against WNV *in vivo*.

*In vitro* data has shown that cells lacking RIG-I have an abrogated innate immune response, while cells lacking MAVS, the central adaptor protein for RLR signaling, have a near complete absence of innate immune activation. Further evidence to suggest additional PRR signaling through the RLR pathway was that *RIG-I*<sup>-/-</sup> cells infected with a virus known to antagonize MDA5-mediated signaling, and then subsequently infected with WNV, fail to induce nuclear translocation of IRF3 (134, 136). These results lead us to hypothesize that the second PRR known to transduce signals through MAVS, MDA5, plays an important role in detecting WNV infection and activating innate immune programs. In addition to an unknown role of MDA5 in detecting WNV, it is also unknown if loss of MDA5 results in a similar or distinct lack of innate immune induction compared to RIG-I. Our goal was to clearly establish a role for RIG-I and MDA5 in detecting West Nile virus and initiating innate immune responses by analyzing *in vitro* models of key target cells of infection. We assessed the ability of RLR KO cells to control virus replication and characterized the signaling response initiated by each RLR.

## RESULTS

**RIG-I and MDA5 are essential for protection against WNV infection *in vivo*.** To determine the individual roles of RIG-I and MDA5 in WNV infection and immunity, we assessed WNV pathogenesis in WT mice and in *RIG-I*<sup>-/-</sup> or *MDA5*<sup>-/-</sup> mice. An in-depth, direct analysis of the respective roles of the individual RLRs has been hampered by the embryonic lethality of *RIG-I*<sup>-/-</sup> mice in a complete C57BL/6 background, while *MDA5*<sup>-/-</sup> mice are fully viable on several genetic backgrounds (66). To circumvent embryonic lethality, the *RIG-I*<sup>+/+</sup> and

*RIG-I*<sup>-/-</sup> mice were generated on a mixed ICR × 129Sv × C57BL/6 genetic background (66). *MDA5*<sup>-/-</sup> mice, in comparison, were generated originally on a 129Sv background without any noted developmental defects (163) and were backcrossed subsequently to a 99% pure C57BL/6 background to facilitate WNV pathogenesis studies. Each RLR single knockout mouse line was evaluated in comparison to its own individual wild type (WT) control. We infected mice subcutaneously with 10<sup>2</sup> plaque forming units (PFU) of WNV-TX and monitored morbidity and mortality. *RIG-I*<sup>-/-</sup> mice were more susceptible to WNV infection compared to their WT controls, with enhanced lethality (50% vs. 17%, *P* < 0.05) observed over the 20-day monitoring period of infection (**Fig. III-1a**). Similar results were obtained when we compared *MDA5*<sup>-/-</sup> mice with their respective WT controls, with significantly more (70% vs. 30%, *P* < 0.02) *MDA5*<sup>-/-</sup> mice dying from WNV infection (**Fig. III-1b**). We also intercrossed *RIG-I*<sup>-/-</sup> × *MDA5*<sup>-/-</sup> double knockout (DKO) mice lacking expression of both RIG-I and MDA5, and this line was backcrossed into a C57BL/6 background through the F3 generation to yield a strain of ~94% C57BL/6, as defined by microsatellite DNA analysis (data not shown). DKO mice exhibited markedly increased susceptibility to WNV infection compared to WT C57BL/6 control mice or *RIG-I*<sup>-/-</sup> or *MDA5*<sup>-/-</sup> mice, and had a more rapid mean time to death of ~ 8 days (100% vs. 50%, *P* < 0.0001) (**Fig. III-1c**). The susceptibility of DKO mice to WNV infection was remarkably similar to that of *MAVS*<sup>-/-</sup> mice, which were generated on a pure C57BL/6 background (**Fig. III-1c**) (136). These results demonstrate that RLR signaling from both RIG-I and MDA5 is required for protection against WNV infection *in vivo*.

**RIG-I and MDA5 are both required for innate immune gene induction and control of WNV replication.** To determine how RIG-I and MDA5 individually regulate innate immune gene expression and control of WNV replication, we performed a detailed time-course analysis

of gene expression and virus replication within low passage, primary MEFs from *RIG-I*<sup>+/+</sup> WT, *RIG-I*<sup>-/-</sup>, C57BL/6 WT, *MDA5*<sup>-/-</sup>, and DKO mice. For each analysis, we measured viral RNA and compared innate immune gene expression in mock-infected cells with WNV-infected cells at a high multiplicity of infection (MOI=5), thus determining the fold-change in RNA expression levels (**Fig. III-2**). Whereas WNV RNA replicated to higher levels (4-fold difference,  $P < 0.003$  at 48 hpi) throughout the 48 hr time-course in *RIG-I*<sup>-/-</sup> compared to cognate WT MEFs (**Fig III-2a**), no appreciable differences in viral infection were observed between WT and *MDA5*<sup>-/-</sup> cells (**Fig III-2b**,  $P > 0.05$  at 48 hpi). IFN- $\beta$  is an acute innate immune response gene that is induced early after virus infection (86). The increase in WNV replication in *RIG-I*<sup>-/-</sup> MEFs corresponded with an early deficit (14 and 13-fold difference,  $P < 0.03$  at 8 and 10 hpi) of IFN- $\beta$  mRNA induction, with expression reaching WT levels subsequently (**Fig. III-2c**). In comparison, IFN- $\beta$  expression was induced equivalently in WNV infected WT and *MDA5*<sup>-/-</sup> cells throughout the time-course (**Fig. III-2d**,  $P > 0.05$  at 10 and 48 hpi). We also examined the WNV-induced expression of IFN- $\alpha$ 2a, a comparably late-expressed innate immune response gene and cytokine that amplifies and diversifies innate immune gene expression. IFN- $\alpha$ 2a is induced after IFN- $\beta$  signaling, as it requires IRF-7 induction and activation (139, 164). We failed to detect a major difference in IFN- $\alpha$ 2a mRNA expression over a course of 22-48 hpi between WT and *RIG-I*<sup>-/-</sup> cells (**Fig. III-2e**). Remarkably, *MDA5*<sup>-/-</sup> cells showed a significant deficit in IFN- $\alpha$ 2a expression (2 and 3.8 fold difference,  $P < 0.003$  at 34 and 42 hpi respectively) compared to WT controls at late time points of infection (**Fig III-2f**). These results verify that RIG-I is essential for early innate immune gene induction and virus control (134) and reveal a specific role for MDA5 in the later, amplification phase of the innate immune response after WNV infection. We note that the early deficit in IFN- $\beta$  production resulted in increased viral replication in infected cells during

time points before WNV can efficiently antagonize IFN signaling, whereas the late defect in IFN- $\alpha$ 2a production did not result in increased WNV replication in MEFs under single-step growth kinetic conditions, possibly because at these points accumulated viral non-structural proteins can antagonize IFN signaling (161, 165).

We next assessed the combined roles of RIG-I and MDA5 in controlling WNV replication and promoting innate immune gene induction in MEFs. DKO cells were mock-infected or infected with WNV over a 72 hr time-course. As expected, WNV RNA accumulated to much higher levels (>60 fold difference at 72 hpi) in DKO compared to WT cells (**Fig. III-2g**). We also performed real-time qRT-PCR analysis to examine the expression of a subset of RLR-responsive innate immune genes previously identified from transcriptional profiling studies of WNV infected cells (134). Remarkably, while these genes were induced highly in WT cells, none were significantly induced in DKO cells after WNV infection (**Table III-1**). These observations are consistent with recent results demonstrating that in target cells of WNV infection, MAVS-dependent signaling is the predominant pathway through which viral RNA is sensed for host defense gene induction (149). Our results now demonstrate that RIG-I and MDA5 are the two essential PRRs that sense WNV infection and induce the antiviral response through MAVS-dependent signaling.

**MDA5 is required to control virus replication in myeloid cells.** Our results suggest that RIG-I mediates early/initial PAMP recognition and signaling while MDA5 mediates late signaling to amplify innate immune actions during WNV infection. While previous studies support a role for RIG-I in initial triggering of innate immune defenses against WNV infection, the role of MDA5 in this process has not been evaluated (134, 148, 166). To assess the role of MDA5 in innate immune signaling in bona fide *in vivo* target cells of WNV infection, we

performed time-course analysis to evaluate the levels of infectious WNV production and gene induction in primary bone marrow-derived macrophages (MΦ) and dendritic cells (DCs) prepared from WT and *MDA5*<sup>-/-</sup> mice. MΦ and DCs from *MDA5*<sup>-/-</sup> mice supported increased virus growth when compared to WT cells (**Fig. III-3a and b**, 4-fold difference,  $P < 0.04$  at 36 hours; 2- and 3-fold difference,  $P < 0.008$  and  $P < 0.02$  respectively). Immunoblot analysis demonstrated that WNV proteins accumulated to higher levels in *MDA5*<sup>-/-</sup> MΦ and DCs compared to WT cells (especially at 36 hours), whereas DCs exhibited a concomitant reduction in the virus-induced expression of IFIT2, an ISG that is downstream of RLR signaling and restricts WNV infection (**Fig. III-c and d**) (154, 157). We also found that *MDA5*<sup>-/-</sup> MΦ and DCs produced lower levels of IFN-β post-infection than WT cells (**Fig. III-3e and f**), despite the increased viral burden. As controls, we also assessed IFN-β production in response to Sendai virus (a RIG-I-specific stimulus (66)) and EMCV; an MDA5-specific stimulus (163)) respectively in WT and *MDA5*<sup>-/-</sup> MΦ and DCs. Although Sendai virus induced robust production of IFN-β, EMCV infection failed to induce IFN-β production in *MDA5*<sup>-/-</sup> DCs. These results demonstrate that MDA5 is essential for optimal control of WNV replication and induction of antiviral host defense genes in cells that are targets of infection *in vivo*.

To assess the combined roles of RIG-I and MDA5 in detecting and controlling WNV infection in myeloid cells we generated DKO DCs and compared their response with WT and *MAVS*<sup>-/-</sup> cells. WNV replicated to increased levels (18-fold higher) in DKO cells compared to WT cells, similar to that observed in parallel cultures of *MAVS*<sup>-/-</sup> DCs (17-fold higher) (**Fig. III-3g**). Moreover, and in contrast to *MDA5*<sup>-/-</sup> cells, there was no detectable induction of IFN-β production or innate immune gene expression (compare **Fig. III-3a-d** with **Fig. III -3h and i**) by DKO or *MAVS*<sup>-/-</sup> DCs after WNV infection. Thus, while MDA5 is required for optimal innate

immune gene induction and control of WNV infection, in myeloid cells the combination of RIG-I and MDA5 and subsequent signaling through MAVS is essential for WNV detection and innate immune gene induction.

**Dual RLR recognition of flaviviruses can be extended to other related viruses.** We next wanted to assess if the dual recognition of flaviviruses could be extended to another related virus, Japanese encephalitis virus (JEV), that is a leading cause of virus induced encephalitis in eastern and southern Asia. Japanese encephalitis virus (JEV) is a neurotropic virus maintained in a similar enzoonotic cycle as WNV and exists as a threat to become an emerging virus in other parts of the world including North America (167). WT, *RIG-I*<sup>-/-</sup>, *MDA5*<sup>-/-</sup> and DKO MEFs were infected at a high MOI (MOI=5) with JEV for a 48 hour time-course and measured for viral RNA accumulation and IFN- $\beta$  and IFN- $\alpha$ 2a mRNA induction. Consistent with our observations with WNV infection, JEV infected cells lacking either RIG-I or MDA5 can be seen to have a reduction in innate immune gene induction at some time point, but the loss of both RLRs results in a complete lack of measured innate immune gene induction (**Fig. III-4b and c**). Again, lack of RIG-I or MDA5 lead to an intermediate increase in viral RNA accumulation, but lack of both RLRs lead to a dramatic increase in viral RNA replication (**Fig. III-4a**).

**Human RLR recognition of WNV infection.** The above results clearly demonstrate a contribution of mouse RIG-I and MDA5 in detecting WNV infection and activating innate immune genes. We also wanted to test if human RIG-I and MDA5 can detect WNV infection. To do this we over-expressed FLAG-tagged human constructs of RIG-I and MDA5 in the human hepatoma cell line Huh 7.5 and subsequently infected with WNV. Huh 7.5 cells lack a functional endogenous RIG-I receptor and have been used extensively to study intracellular innate immune signaling (168, 169). In addition to transfecting the RLR expression constructs, we also

transfected in dual luciferase reporters to assess IFN- $\beta$  promoter activity following WNV infection. We achieved roughly equivalent expression of human RIG-I and MDA5 (**Fig. III-5a**). Expression of either RLR alone is capable of inducing IFN- $\beta$  promoter activity in response to WNV infection, defining dual RLR recognition of WNV as a common principle of both mouse and human (**Fig. III-5b**).

**Regulation of RLR-induced signaling in response to WNV.** Previous results from the our lab utilizing density gradient sedimentation have demonstrated that RIG-I relocates from the cytoplasm to intracellular membranes following RIG-I stimulation (61). RIG-I relocation was found to be dependent on several host factors including the ubiquitin ligase TRIM25 and physical interaction with members of the molecular chaperone family called 14-3-3 proteins. We therefore performed experiments to determine if MDA5 is subject to similar recruitment to intracellular membranes and if 14-3-3 proteins associate with MDA5 during virus infection. To determine if MDA5 is recruited to intracellular membranes during virus infection we infected Huh 7 cells with Sendai virus and harvested uninfected cells or cells infected for 4 and 24 hours. Sendai virus was originally identified as a virus specifically recognized by RIG-I, but it was subsequently shown that defective interfering particles of Sendai virus are detected by MDA5(170). Cell lysates were subjected to density gradient sedimentation to isolate various cytoplasmic and membrane associated fractions. Confirming previous results, RIG-I was recruited from cytosolic fractions (fractions 6 and 7) to a membrane associated fraction (fraction 2) at 4 and 24 hpi (**Fig. III-6**). MDA5 was also recruited to this membrane-associated fraction at 24 hpi suggesting that MDA5 is subject to similar intracellular recruitment mechanisms as RIG-I.

To determine if any 14-3-3 proteins associate with MDA5 during WNV infection we co-expressed 6 separate isoforms of myc-tagged 14-3-3 proteins with FLAG-tagged MDA5 and infected with WNV. Uninfected and infected cells were lysed at 36 hpi and subjected to FLAG immunoprecipitation to determine association between 14-3-3 and MDA5 with 14-3-3 proteins serving as the target and MDA5 as the bait. All isoforms of 14-3-3 were detectable in samples from uninfected cells following immunoprecipitation of MDA5, but 14-3-3 $\zeta$  and  $\beta$  increased in abundance following WNV infection suggesting that MDA5 interacts with and co-immunoprecipitates with MDA5 during WNV infection (**Fig. III-7**).

## DISCUSSION

The central role of MAVS-dependent signaling in controlling WNV infection and pathogenesis has been established and implicates an essential role of RLRs in immunity against WNV (55, 134, 136, 149). Herein, we delineated the role of RIG-I and MDA5 as individual PRRs in contributing to the control of WNV and induction of innate immune genes *in vitro* and *in vivo*. We found that each PRR is essential for full immune protection against WNV. In addition, the susceptibility of mice to WNV infection lacking both PRRs recapitulates the phenotype of *MAVS*<sup>-/-</sup> mice, confirming the essential nature of RLR signaling over other innate immune induction pathways (136). The susceptibility phenotype in DKO mice occurred despite a full complement and expression of TLRs and NLRs in these mice. Thus, RIG-I and MDA5 are essential PRRs of WNV recognition, with each serving to transduce innate immune signaling through MAVS in response to infection of key target cells. The loss of RIG-I or MDA5 can be

compensated for partially by the other, but loss of both genes results in a severe loss of innate immunity to infection.

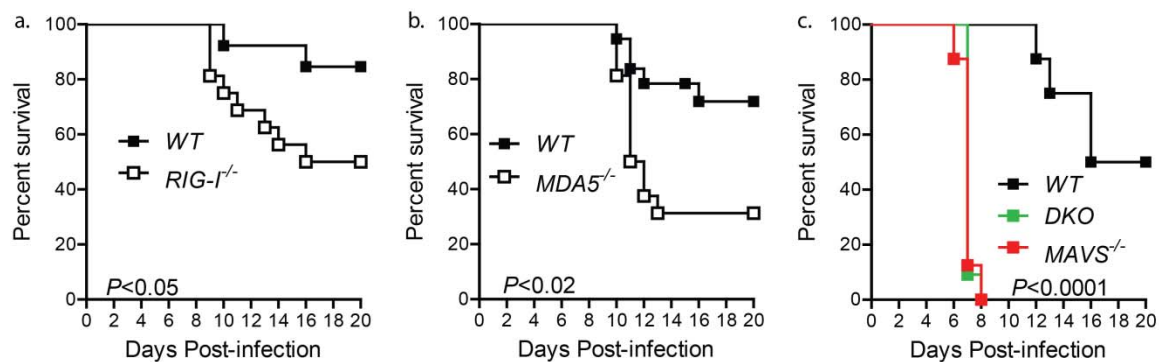
Efficient and early PAMP recognition is required to induce an IRF-3-dependent gene expression signature that controls viral replication in a cell-intrinsic manner (138). In cells lacking RIG-I expression, WNV replication proceeds at a higher rate. In contrast, in *MDA5*<sup>-/-</sup> MEFs, a deficit of innate immune signaling amplification and diversification is marked by a deficiency in IRF-7-driven IFN- $\alpha$ 2a expression without an increase in viral replication. This phenotype may reflect the ability of pathogenic WNV to block type I IFN signaling at later times in infection when MDA5 PAMPs accumulate (70, 139, 161, 165, 171). It has recently been proposed the signaling-active form is a large MDA5 oligomer bound to RNA (64). In the context of WNV, MDA5 might require long dsRNA replication intermediates for such oligomer assembly, which accumulate at later times when replication peaks.

Immune responses in mice and humans can be highly divergent and mouse models of human disease do not always translate well to interpretation of immune mechanisms in humans (172). As previously noted, the mouse model of WNV pathology closely recapitulates the known course of human disease. Dengue virus is a related flavivirus with a large global health burden (173). Considerable work and insight in basic research on Dengue virus has been achieved, but the necessity of complicated animal models (severely immunocompromised mice and mouse/human chimeras) has hindered the pace of progress (174). The systematic dissection of immune mechanisms using mice with specific genetic ablations, but otherwise possessing a fully functional immune response, to understand WNV pathology therefore represents one of the most powerful tools to understand immunity to flavivirus infection in animal and human disease. In that regard, we were able to expand our observation of dual RLR recognition of WNV infection

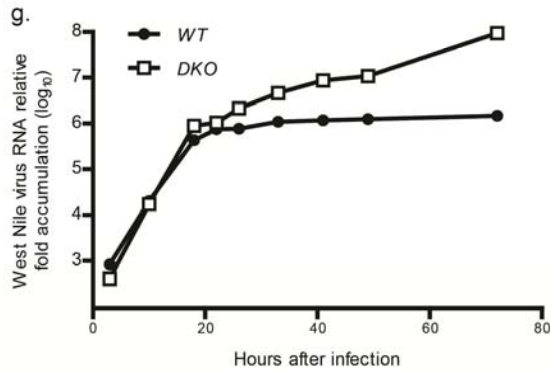
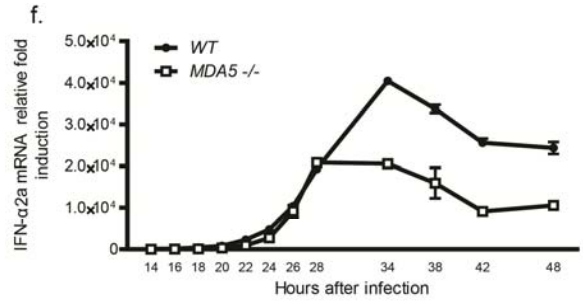
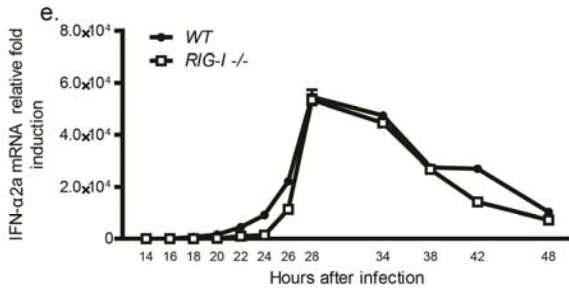
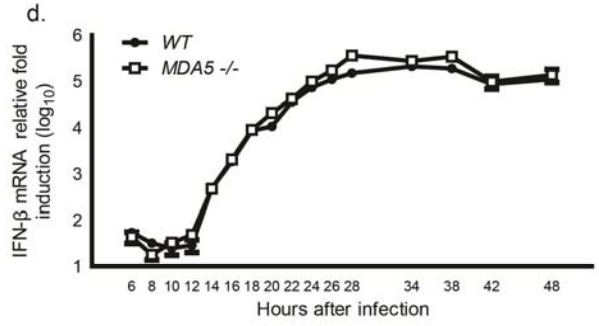
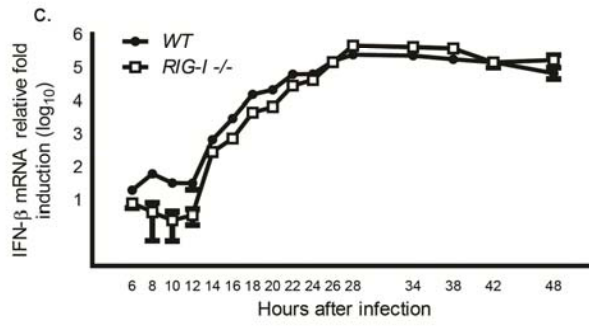
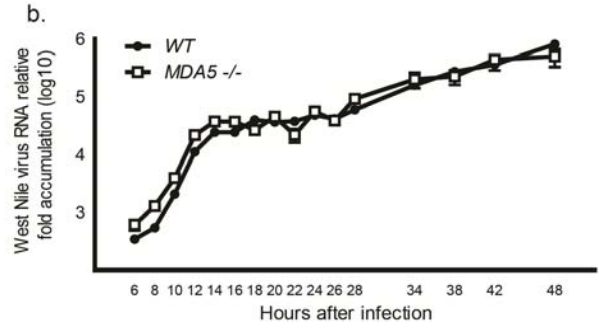
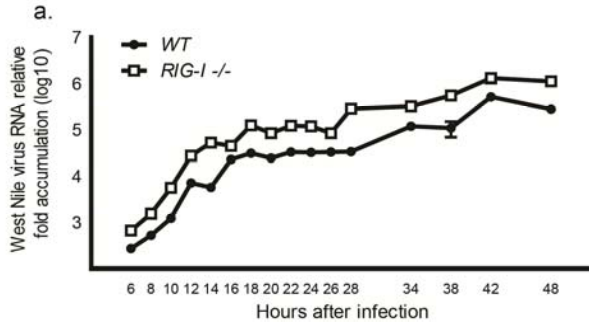
in mouse cells and demonstrate that the same phenomenon occurs in human cells as well. Likewise, dual RLR recognition of flavivirus infection was extended from our initial observations with WNV to another closely related virus causing human disease: JEV. Future experiments will seek to further define principles regulating innate immune recognition of JEV and WNV in human cells. Our preliminary experiments suggest an important component of innate immune activation by virus infection in humans is the recruitment of RLRs to intracellular membranes by chaperone proteins including members of the 14-3-3 protein family. Findings from future *in vitro* experiments defining these requirements for RLR activation can then be validated by subsequent experiments to define their contribution to *in vivo* pathology.

RLR-induced innate immune genes			
		WT (fold induction)	DKO (fold induction)
RIGI dependent early/ MDA5 dependent late	IFIT2	146.1	1.7
	IL-6	36.6	0.6
	IFN- $\alpha$ 2a	467.6	.9
	IRF7	804.5	2.0
	IFN- $\beta$	32294.5	2.5

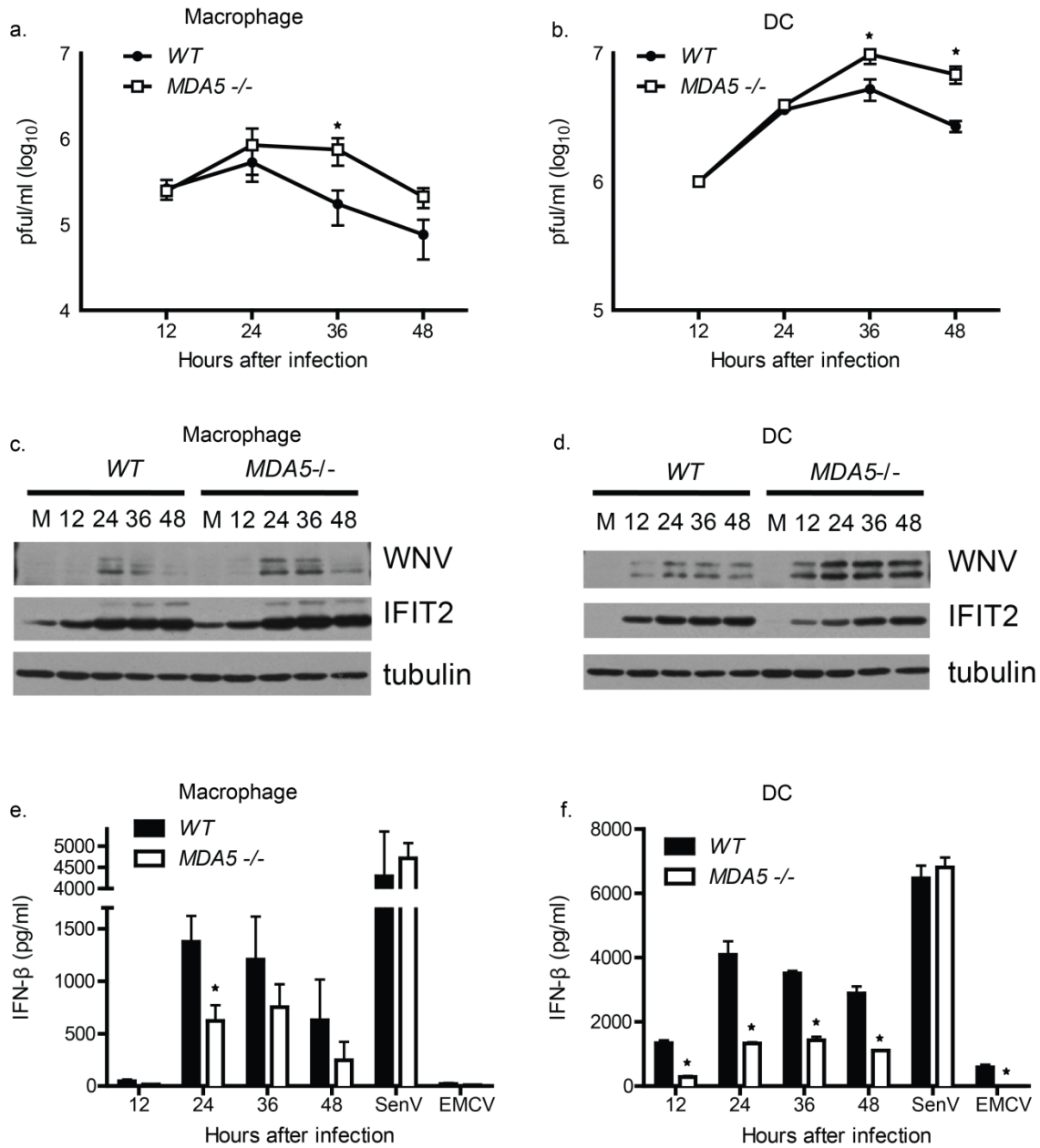
**Table III-1. RLR-induced innate immune genes.** A summary of the innate immune gene induction from WT and DKO cells infected with WNV and plotted in Fig. III-2g. Values represent the fold induction at the peak of the innate immune response from the time-course infection in **Fig. III-2g**.



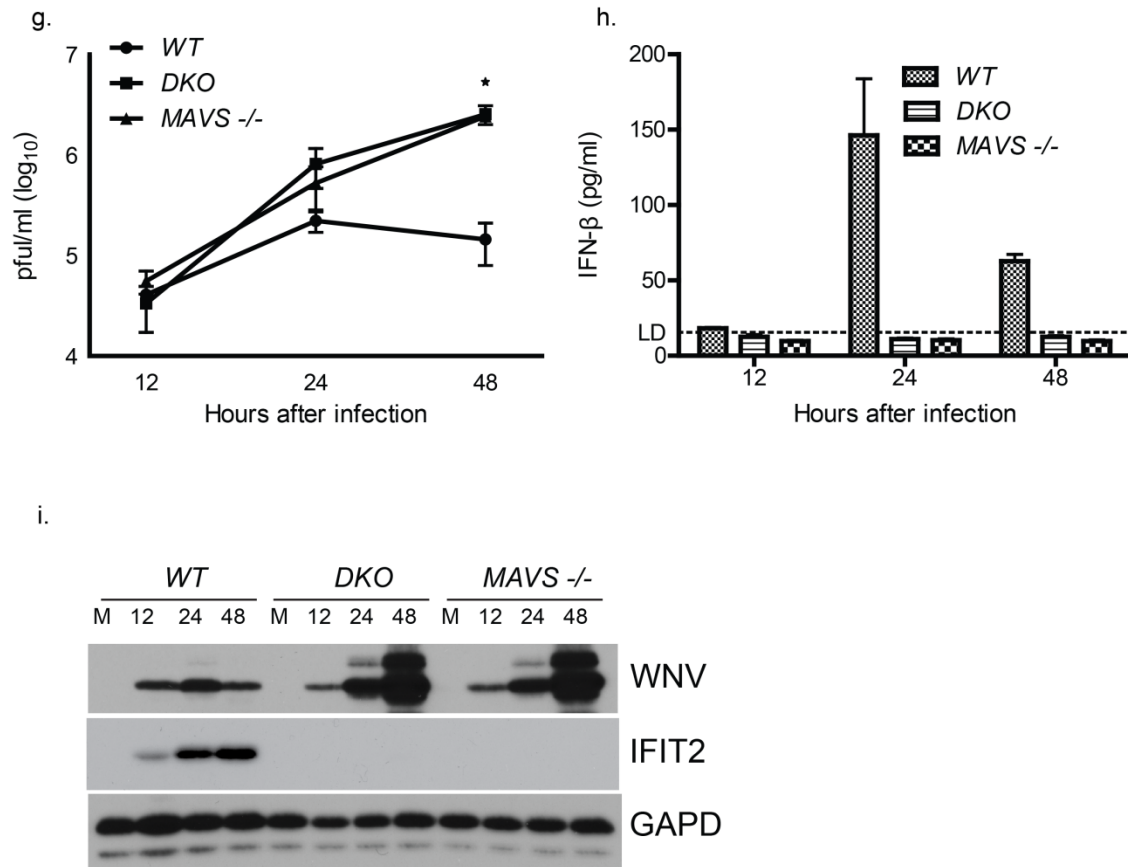
**Figure III-1. *In vivo* pathogenesis of RLR KO mice infected with WNV.** Adult RLR KO mice and WT controls were infected with  $10^2$  PFU of WNV and monitored for survival. (a) *RIG-I*<sup>+/+</sup> mixed background mice (n=13) and *RIG-I*<sup>-/-</sup> mixed background mice (n=16), (b) C57BL/6 WT (n=37) and *MDA5*<sup>-/-</sup> mice (n=16), and (c) and *RIG-I*<sup>-/-</sup> × *MDA5*<sup>-/-</sup> (n=11), *RIG-I*<sup>+/+</sup> × *MDA5*<sup>+/+</sup> littermate controls (n=8) and *MAVS*<sup>-/-</sup> (n=8) mice all exhibit significantly greater mortality compared to their respective controls ( $P < 0.05$ ,  $P < 0.02$ ,  $P < 0.0001$ ).



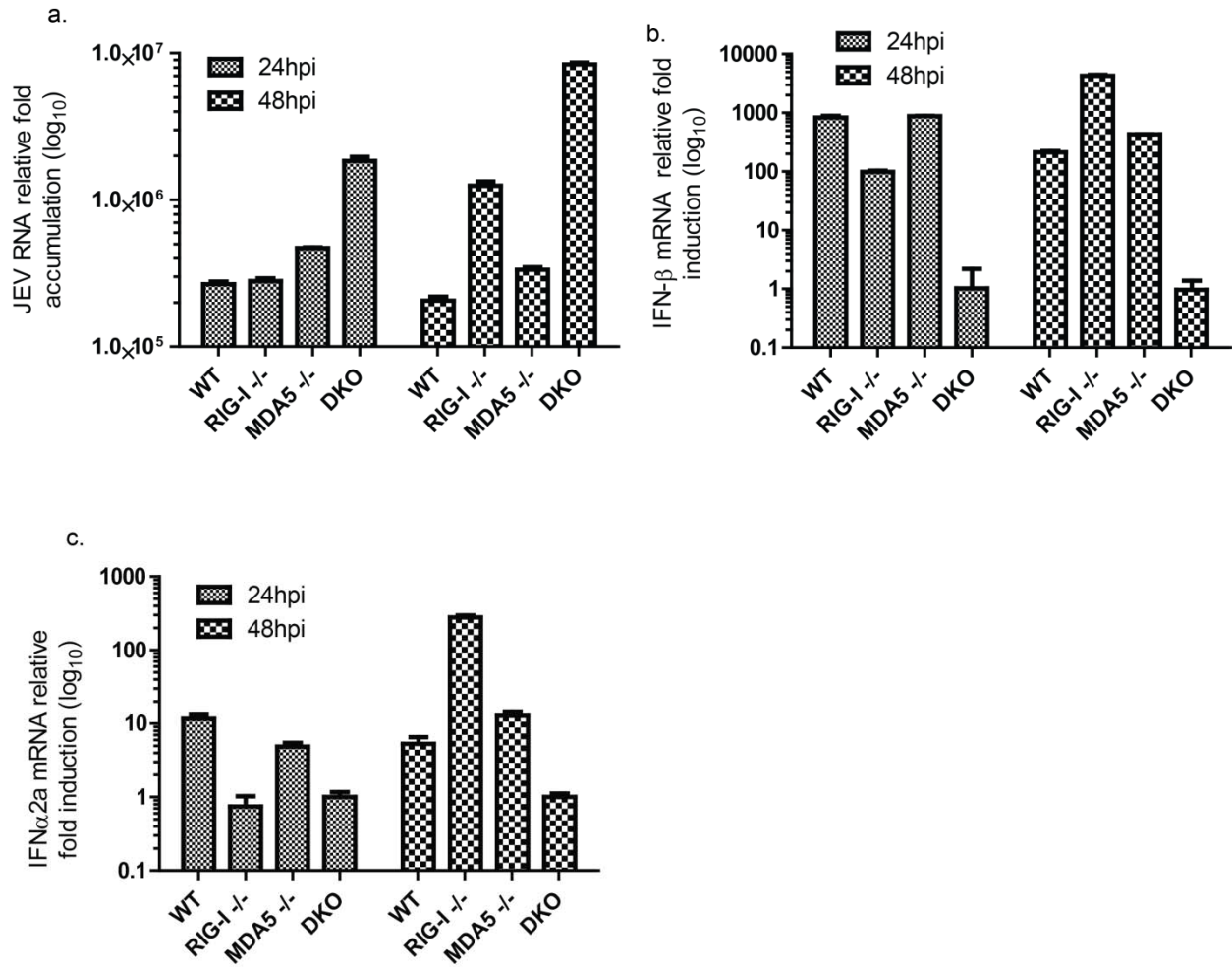
**Figure III-2. Innate immune gene expression and viral RNA accumulation over a WNV-infection timecourse.** Primary MEFs were infected with WNV or mock-infected and innate immune gene induction or viral RNA accumulation in each cell line was determined by qRT-PCR and plotted as relative fold induction compared to mock cells and normalized to GAPDH expression. Cell lines used for (a-f) were mixed background *RIG-I*<sup>+/+</sup> or *RIG-I*<sup>-/-</sup> compared to each other or C57BL/6 WT and *MDA5*<sup>-/-</sup> compared to each other. (a,b), (c,d), and (e,f) represent WNV RNA, IFN-β mRNA, and IFN-α2a mRNA accumulation over time in infected MEFs. The graphs show the mean ± standard deviation (SD) from triplicate analyses and are representative of three independent experiments. (g) WNV RNA accumulation in WT versus *RIG-I*<sup>-/-</sup> × *MDA5*<sup>-/-</sup> MEFs with a summary of innate immune gene induction shown in **Table III-1.**



**Figure III-3. MDA5 is essential for viral replication control and innate immune induction in primary myeloid cells.** Primary bone-marrow derived MΦ and DCs were generated from control and RLR KO mice and infected with WNV. Supernatants and cellular protein lysates were collected over a time-course to analyze viral replication and innate immune gene induction.

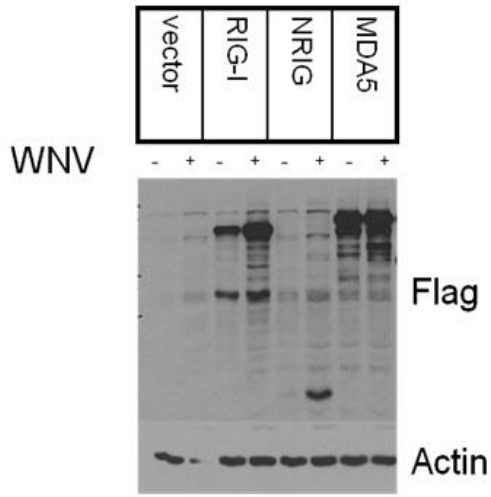


**(Figure III-3 continued).** (a-b) Supernatants from infected M $\Phi$  and DCs were assayed for viral load by PFU assay. (c-d) Immunoblot analysis of protein abundance of WNV, IFIT2, and tubulin (control) in M $\Phi$  and DCs. (e-f) IFN- $\beta$  ELISA from WNV infected cells or cells infected with Sendai virus or EMCV. (g-i) Represents DCs generated from WT, *RIG-I*<sup>-/-</sup>  $\times$  *MDA5*<sup>-/-</sup> or *MAVS*<sup>-/-</sup> mice and

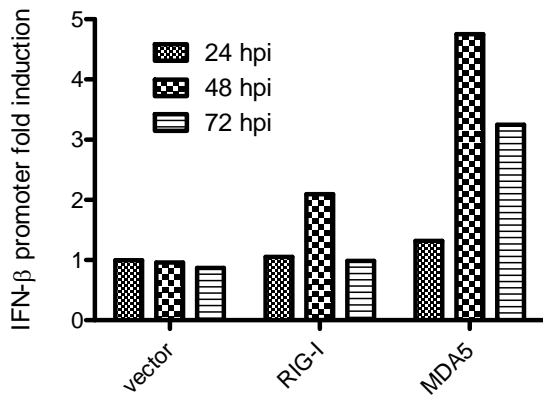


**Figure III-4. Innate immune gene expression and viral RNA accumulation in RLR ko MEFs infected with Japanese encephalitis virus.** Primary MEFs were infected with JEV or mock-infected and innate immune gene induction or viral RNA accumulation in each cell line was determined by qRT-PCR and plotted as relative fold induction compared to mock cells and normalized to GAPDH expression. (a) JEV RNA, (b) IFN-β mRNA and (c) IFN-α2a mRNA accumulation over time in infected MEFs. The graphs show the mean ± standard deviation (SD) from triplicate analyses.

a.

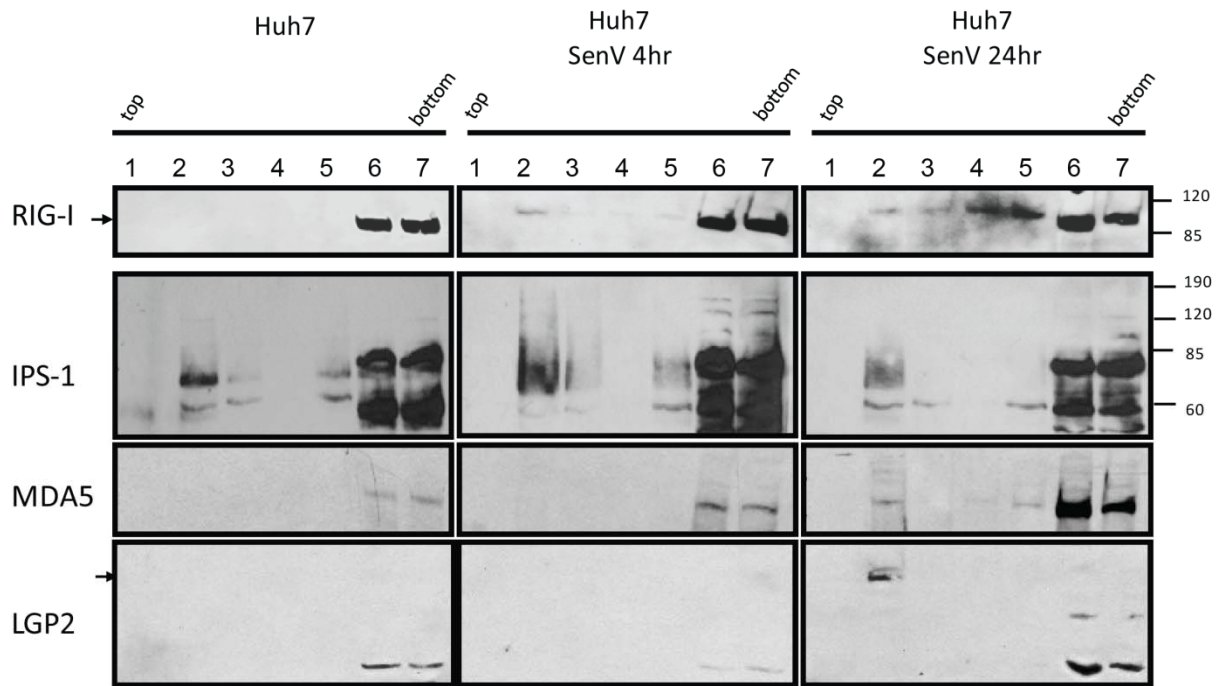


b.

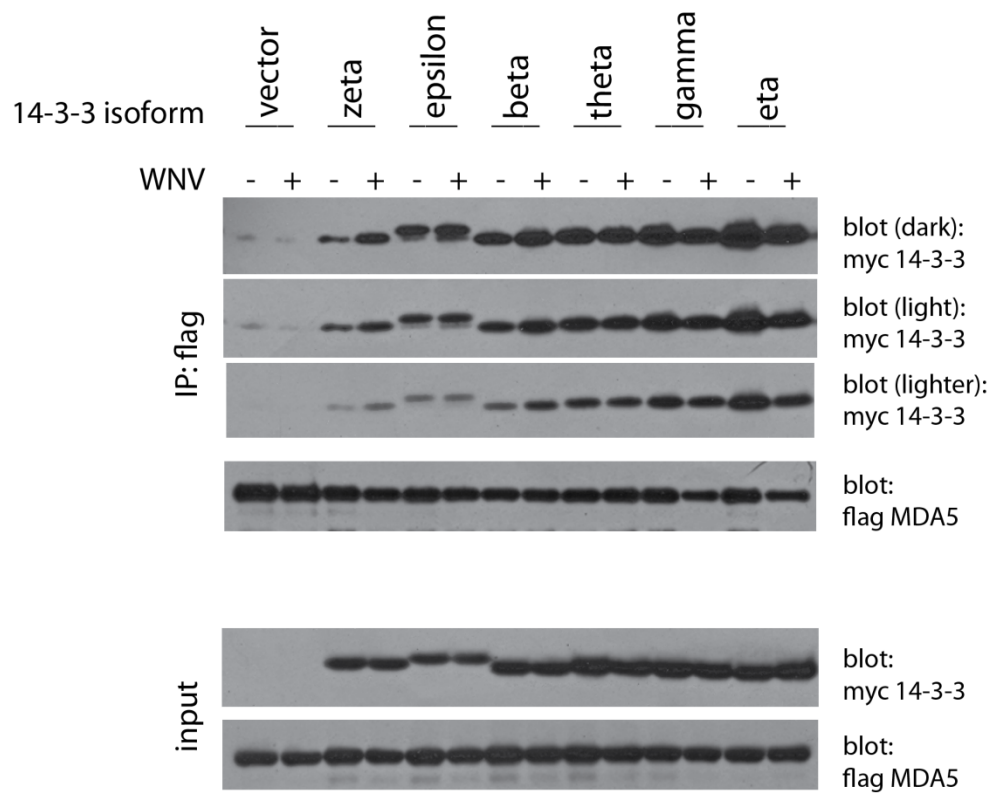


**Figure III-5. Human RLR induction of innate immune signaling in response to WNV**

**infection.** Huh 7.5 cells were transfected with expression constructs for FLAG-tagged vector, RIG-I or MDA5 and infected with WNV. (a) Immunoblot of FLAG-tagged RLRs. (b) IFN- $\beta$  promoter activity following WNV infection over a 72 hour time-course plotted as fold induction over mock infection.



**Figure III-6. RLR relocation following virus infection.** Acute Sendai virus infection in Huh 7 cells leads to relocation of RIG-I (at 4 and 24 hpi) and MDA5 (at 24 hpi) from an exclusively cytosolic distribution to include RLR membrane-association. Fractions 6-7 are cytosolic. Fractions 2-3 are membrane-associated.



**Figure III-7. MDA5 interacts with 14-3-3 family members during WNV infection.** FLAG-tagged MDA5 was co-expressed with individual myc-14-3-3 isoforms in 293T cells. Cells were infected or not and subjected to FLAG immunoprecipitation. Products were analyzed by immunoblot for myc-tagged 14-3-3 protein co-immunoprecipitation with MDA5.

## IV. DEFINING THE WEST NILE VIRUS PAMP

### INTRODUCTION

RIG-I is believed to bind to WNV RNA to initiate innate immune signaling, but the specific determinants of recognition of flavivirus RNA by RIG-I remain unknown (166). Moreover, the role of MDA5 in WNV recognition is not well-defined nor have the combinatorial and distinct functions of RIG-I and MDA5 in pathogen recognition and host defense been revealed. As previously noted in the introduction, studies that characterize pathogen-associated molecular pattern (PAMP) ligands of RIG-I have demonstrated that non-self recognition depends on several properties of viral RNA including PAMP motif length, structure, modification, and composition (75-78). Multiple studies show that RNA ligands of RIG-I require an exposed 5'-triphosphate (5'-ppp) for recognition and binding (75, 77, 175). Studies to characterize the features of an MDA5-specific PAMP ligand show that long, stable dsRNA and/or "higher order" RNA complexes containing both dsRNA and ssRNA are preferred PAMP ligands, though the nature of such RNA complexes is unknown (67, 81). The ~11 kb ssRNA WNV genome contains a 5' Cap-1 structure that is expected to mask the 5'-ppp necessary for RIG-I recognition. The dsRNA replication intermediates or highly structured subgenomic fragments that accumulate within cells during infection could serve as possible MDA5 ligands (128, 160), although such PAMPs may be sequestered in membrane-derived replication 'packets' with limited accessibility to RLRs (176, 177). As flaviviruses share a common replication program (178), assessment of WNV interactions with RIG-I and MDA5 will provide a general understanding of how the host

recognizes cellular infection by flaviviruses, which include a family of related pathogens of global public health concern such as dengue virus (70), Japanese encephalitis virus (66), and yellow fever virus (179). WNV therefore presents an attractive and interesting model to assess the precise properties of viral RNA that allow discrimination between self RNA, RIG-I substrate RNA, and MDA5 substrate RNA.

We employed two complementary strategies to define the WNV PAMP. The first strategy was to assess the biochemical requirements for PAMP RNA isolated from WNV infected cells. The second strategy allowed us to identify sequences of RNA associated with the RLRs during WNV infection. New insights into the biochemical and immunological aspects of host pathogen interactions of RNA viruses and mammalian innate immunity will be revealed by these experiments.

## RESULTS

**5' modifications of WNV RNA ablate RIG-I binding.** RIG-I has been shown to respond to ligand RNA in a 5' phosphate-dependent and independent manner (77, 81). WNV 5' modifications have been shown to be essential for virus replication and we hypothesized this might be due in part to differential recognition of 5' modified WNV RNA by RIG-I (159). WNV undergoes two sequential methylation modifications to its 5' genome; addition of a Cap 0 structure (7mGpppN) followed by another methylation event giving a Cap 1 structure (7mGpppNm) (159). We performed *in vitro* transcription of the 5' non-translated region (NTR) of WNV and left it uncapped or added a Cap 0 or 1 structure. We next performed an RNA binding/gel shift assay with purified RIG-I protein and the three differentially modified 5' NTR

WNV RNAs. As previously reported, addition of increasing amounts of RIG-I protein lead to efficient loss of unbound HCV derived poly U/UC and poly (I:C) (**Fig. IV-1**) (77). Addition of RIG-I protein led to a modest loss/shift of unbound 5'PPP WNV RNA, but addition of either a Cap 0 or 1 structure ablated RIG-I-dependent loss of unbound WNV RNA (**Fig. IV-1**). These results suggest that RIG-I can weakly bind unmodified 5' WNV RNA, but that 5' modification would be expected to eliminate RIG-I-mediated recognition of WNV.

**WNV PAMP characteristics for RLR detection.** To determine the properties of the PAMPs that trigger RLR signaling during WNV infection, we examined the signaling actions of RNA recovered from mock-infected control cells (mcRNA), cells infected with WNV for 24 hrs (icRNA), specific WNV RNA secondary structure motifs, and native virion RNA when transfected into primary MEFs. We first purified RNA from mock- and WNV-infected cells to generate control mcRNA and icRNA. mcRNA transfection induces only very low IFN- $\beta$  mRNA levels, and we thus normalized all data sets against the response to mcRNA (**Fig. IV-2a**). RNA also was obtained from WNV virions (vRNA) by extraction and purification after ultracentrifugation of infected cell supernatants. As the WNV genome 5' and 3' NTR contain dsRNA loop structures that might confer RLR recognition, we also prepared *in vitro* transcribed RNA fragments of the 5' and 3' NTR of the viral genome RNA. Each RNA preparation was transfected into WT MEFs in the presence of cycloheximide to prevent translation and *de novo* viral transcription, thus allowing us to assess the ability of the input RNA to stimulate innate immune signaling of IFN- $\beta$  mRNA expression. Notably, transfection of icRNA significantly induced IFN- $\beta$  mRNA expression in recipient cells as compared to control cells transfected with mcRNA (1,050 fold,  $P < 1 \times 10^{-5}$ ) (**Fig. IV-2b**). We treated icRNA with Antarctic phosphatase (AP) to remove 3' and 5' phosphate moieties or with RNase III to digest dsRNA. In parallel, we

recovered icRNA from WNV-infected *RNaseL*<sup>-/-</sup> cells, allowing us to assess innate immune signaling induced by possible RNA products of RNaseL cleavage (89). icRNA stimulation of innate immune signaling was reduced by ~50% following phosphatase treatment, and it was completely ablated following RNase III treatment (1050 fold vs. 550 fold, *P*<0.001, **Fig. IV-2b**). icRNA recovered from *RNaseL*<sup>-/-</sup> MEFs contained ~8 times more WNV RNA (not shown); as such, we adjusted the amount of input icRNA into recipient cells to equalize input RNA levels based on WNV genome equivalents. icRNA from *RNaseL*<sup>-/-</sup> cells induced comparable levels of innate immune signaling as icRNA recovered from WT cells. These results suggest that the WNV stimulatory PAMPs are generated independently of RNase L cleavage products. Neither vRNA nor the *in vitro* transcribed viral NTR RNA induced appreciable levels of IFN-β compared to icRNA. This outcome was despite the presence of a 5'-ppp on the viral NTR RNA, and that larger copy number of viral RNA were transfected into cells from the NTR RNA compared to icRNA because equivalent mass quantities of RNA were transfected into cells for each condition. Despite an absence of innate immune signaling induction, there were in fact greater than 470-fold more WNV genomes transfected from the vRNA compared to icRNA, as determined by qRT-PCR (see **Fig. IV-2b**). Thus, icRNA but not WNV virion RNA nor viral 5'ppp-NTR RNA motifs induce innate immune signaling. These results imply that RLR PAMPs are not carried within the incoming WNV genome RNA of the virion but instead are produced within infected cells, and that PAMP recognition of icRNA by the RLRs includes RNA components with phosphate moieties and dsRNA motifs.

#### **Differential kinetics of RIG-I and MDA5 PAMP production in WNV infected cells.**

To determine how RIG-I and MDA5 individually contribute to the recognition of icRNA PAMPs, we examined icRNA signaling of innate immune genes in WT, *RIG-I*<sup>-/-</sup>, and *MDA5*<sup>-/-</sup>

MEFs. We isolated mcRNA from WT mock-infected cells and icRNA from WNV-infected cells at 6, 10, 12 and 34 hrs after infection. Equal mass amounts of the recovered icRNA or mcRNA were transfected into WT, *RIG-I*<sup>-/-</sup>, and *MDA5*<sup>-/-</sup> cells in the presence of cycloheximide, and IFN-β mRNA levels were measured by RT-qPCR. icRNA recovered after 6 hr post-infection stimulated IFN-β mRNA induction in WT cells but this response was reduced in both *RIG-I*<sup>-/-</sup> or *MDA5*<sup>-/-</sup> cells (**Fig. IV-3a**). We observed a difference in early signaling between *RIG-I*<sup>-/-</sup> and *MDA5*<sup>-/-</sup> cells in response to icRNA harvested at 10 and 12 hr after infection but not at 34 hrs after infection. As shown in **Figure IV-3a**, the early signaling of IFN-β mRNA induction was impaired in *RIG-I*<sup>-/-</sup> cells in which signaling was mediated exclusively by MDA5. Signaling was comparable in both *RIG-I*<sup>-/-</sup> or *MDA5*<sup>-/-</sup> cells upon transfection of icRNA recovered from later times (34 hr) post-infection. Thus, icRNA from early time points of WNV infection contains PAMPs that were sensed preferentially by RIG-I, whereas icRNA from later time points of infection contains PAMPs that were detected by both RIG-I and MDA5.

As phosphatase treatment of 24 hr-derived icRNA reduced PAMP stimulation by ~50% (see **Fig. IV-2b**) we next assessed the requirement for RNA phosphate moieties to affect PAMP sensing by either RIG-I or MDA5. icRNA was recovered from cells 24 hrs after WNV infection and treated with phosphatase prior to transfection of WT, *RIG-I*<sup>-/-</sup>, or *MDA5*<sup>-/-</sup> MEFs.

Phosphatase treatment of icRNA had a minimal effect on reducing IFN-β signaling in *RIG-I*<sup>-/-</sup> MEFs, suggesting that the remaining MDA5 sensing of the WNV PAMP was not affected by loss of exposed phosphates on PAMP RNA. In contrast, phosphatase treatment of icRNA caused an almost complete loss of innate immune signaling in *MDA5*<sup>-/-</sup> MEFs such that less than 10% signaling remained compared to that observed for untreated icRNA transfected into *MDA5*<sup>-/-</sup> MEFs (**Fig. IV-3b**). As a control, these RNAs also were transfected into DKO and *MAVS*<sup>-/-</sup> cells;

we failed to see significant signaling in either cell type (**Fig IV-3b**). These results reveal a temporal distribution of PAMP detection by RIG-I and MDA5 during WNV infection. Recognition of PAMP RNA within WNV-infected cells at early times occurs in a RIG-I-predominant manner that depends on exposed phosphate moieties, whereas at later times PAMP recognition is performed cooperatively by RIG-I and MDA5. Additionally, our results suggest that WNV generates RIG-I and MDA5-specific PAMPs with differential kinetics over the course of viral replication, and that both PAMPs feature a component of dsRNA that imparts RLR recognition. Results from these RNA transfection experiments agree with infection data (see **Fig. II-2**) showing that the loss of RIG-I and MDA5 or MAVS abolishes innate immune signaling in response to cytosolic RNA.

**WNV PAMP-pulldown.** In order to understand the identity and dynamics of RNA ligands bound by the RLRs, we undertook experiments to isolate, characterize, and sequence RLR bound RNA during a WNV infection. These experiments utilize expression of human FLAG-tagged RIG-I, MDA5, and epitope tag vector alone in West Nile virus infected 293T cells. RIG-I, MDA5 and tag alone were immunoprecipitated from 293T cells mock infected or infected with WNV (MOI=5). The RLR-associated RNA was then isolated and analyzed by RNA deep sequencing. The goal of these experiments is to determine the sequence identity and relative abundance of RNA bound to RIG-I and MDA5 during a West Nile virus infection and how the individual contribution of the RLRs to detection of West Nile virus infection differs. We hypothesized that subgenomic regions of WNV may be enriched in the RLR-associated RNAs (180, 181). In addition, these experiments would also reveal if there was any enrichment of host RNAs bound to either RIG-I or MDA5 as a possible result of the activation of the RNase L

pathway or other RNA modifying enzymes (89). By using RNA deep sequencing we were able to map RLR-associated RNAs to both the West Nile virus genome as well as host genomic RNA.

A schematic representation of our PAMP-pulldown strategy is depicted in **Fig. IV-4a**. Expression and subsequent immunoprecipitation of the FLAG-tagged RLRs after infection was confirmed by immunoblot (**Fig. IV-4b**). RNA was extracted from the immunoprecipitated RLR samples and subjected to ribosomal RNA depletion prior to generating libraries for analysis on the Illumina Genome Analyzer II platform (see chapter 2 for more details). Analyzed sequences were mapped to the human and WNV genome (summary of coverage in **Table IV-1**). The total percentage of reads mapping to the WNV genome for each sample is shown in **Figure IV-4c**. We interpreted the percent of viral reads in the vector samples to be the background amount of WNV RNA that non-specifically associated with the immunoprecipitation complexes. Plotting the percent of total reads mapping to the WNV genome as a fold increase over vector, revealed that WNV RNA was only enriched in the RIG-I complexes to a significant degree over vector (approximately 8 and 3 fold at 24 and 48 hpi, respectively (**Fig. IV-4d**)).

We visualized genomic coverage of WNV by plotting a histogram where the y axis represents the number of reads mapped to a single nucleotide position and the x axis represents the nucleotide position. Setting the y axis to the same value for all samples ( $y \sim 113,000$ ) reveals that there are many more reads mapping to all sequences of the WNV genome from the RNA samples associated with RIG-I immunoprecipitation than vector or MDA5 over the first 5 kb of genome (**Fig. IV-5a**). However, by adjusting the y axis to depict the same height for the maximum value of each sample (Auto-scale), we can see there is no enrichment of specific viral RNA sequences in the RIG-I-associated RNA samples compared to vector or MDA5 (**Fig. IV-5a**). **Figure 5b** depicts the entire WNV genome and shows there is no enrichment of specific

sequences over the whole length of the genome. **Figure 5** shows histograms for RNAs associated with immunoprecipitation samples at 24 hpi and is representative of histograms showing genomic coverage at 48 hpi.

Non-ribosomal, non-WNV RNA made up the majority of all sequences mapped in every sample (**Table IV-1**). To investigate the identity of these RNAs and relative abundance across the samples we performed hierarchical clustering of relative expression/abundance values of all samples compared to vector mock. **Figure IV-6** is a heat map representation of the RNAs most highly upregulated compared to vector mock. Samples from RNA associated with MDA5 immunoprecipitations during WNV infection had the greatest increase in abundance compared to vector mock samples, although RIG-I-associated samples were also increased for many of these RNAs compared to mock. Many of the RNAs most highly enriched code for genes associated with innate immune signaling (top five upregulated RNAs code for; CCL5, IFIT1, OAS, IFIT2 and ISG15). We considered two, non-exclusive hypotheses to explain this observation. One possible explanation is that the RLRs, especially MDA5, are specifically binding host RNAs during WNV infection and this host-RNA binding may serve to regulate RLR signaling. The other hypothesis is that expression of the RLRs potentiates innate immune signaling during WNV infection and we are just mapping sequences of RNA that are non-specifically associating with the immunoprecipitations.

**Strand-specific determination of WNV RNA associated with RIG-I.** Mapping the immunoprecipitation-associated RNA sequences to the WNV genome revealed no specific enrichment of WNV RNA sequences in the RIG-I-associated immunoprecipitation samples even though there was significantly increased abundance. This result could be consistent with other reports demonstrating that RIG-I does not display any preference for viral subgenomic regions

during infection (182). Nevertheless, we were surprised by the lack of sequence specificity since we observed negligible innate immune stimulation when transfecting very large amounts of virion RNA into cells (see **Fig. IV-2b**). Flavivirus replication occurs through a series of RNA-dependent RNA polymerase (RdRP) genome amplifications (183). The incoming positive strand genomic RNA is amplified by viral RdRPs to produce a negative strand genome. The negative strand then serves as a template for the amplification of multiple new positive strand genomes by viral RdRPs. *In vitro* RdRP assays revealed a strict dependence on a 5' cap for efficient positive strand-templated amplification of negative strand genomes (184). The 5' cap on negative strand RNA was completely dispensable for efficient negative strand-templated amplification of positive strand genomes. Taken together with our results demonstrating negligible signaling by virion RNA, we performed a PAMP-pulldown experiment and subjected the RLR-associated RNA to TaqMan based strand-specific qRT-PCR assay. At 24 hpi, during peak enrichment of WNV RNA by immunoprecipitated RIG-I, we observed enrichment of positive and negative strand WNV RNA by RIG-I, but negative strand genomes were preferentially enriched (**Fig. IV-7**).

## **DISCUSSION**

During an extensive time-course infection of MEFs we observed a deficit in innate immune signaling at early time points in *RIG-I*<sup>-/-</sup> cells. This observation is consistent with our finding that viral PAMPs from early time points after WNV infection are preferentially sensed by RIG-I. Additionally, we demonstrated that the WNV PAMP recognized by RIG-I is almost completely dependent on phosphate moieties and dsRNA structure whereas the MDA5 PAMP is

largely independent of phosphate moieties but dependent on dsRNA structure. Prior studies have suggested that multiple regions of the WNV RNA present within both genome and anti-genome replication intermediates can be recognized by RIG-I as PAMP motifs (166). We too observed a modest binding to RIG-I and innate immune gene induction in response to WNV 5' NTR RNA (3-fold IFN- $\beta$  mRNA induction) but this stimulation was marginal compared to the PAMPs present in RNA generated in WNV-infected cells (icRNA; >1,000-fold induction of IFN- $\beta$  mRNA expression). The low level of innate immune stimulation by *in vitro* transcribed WNV RNA sequences containing highly structured motifs and 5'-ppp, coupled with the reduction of signaling observed from phosphatase-treated icRNA, indicates that WNV PAMPs of RIG-I recognition are comprised of multiple motifs. In this case, 5'-ppp is a component of this recognition that may also include specific sequence composition and certain RNA structures (77). Such a 5'-ppp PAMP unit would be present on viral RNA during virus replication but is not associated within the incoming virion RNA, due to the absence of negative strand RNA and presence of a 5' Cap 1 structure on the genomic RNA, which effectively blocks 5'-ppp recognition by RIG-I.

For MDA5, our observations reveal a strict dependence on dsRNA for signaling by icRNA. Moreover, our data indicate that MDA5 contributes to innate immune induction at later times after initial WNV infection. This response leads to an amplification of innate immune signaling to IFN- $\beta$ , and response diversification due to the induced expression (and activation) of IRF-7 and other innate immune signal transducers to drive the expression of IFN- $\alpha$ 2a and increased ISG and cytokine expression (139, 157). Consistent with this, collaborative studies reveal that MDA5 is essential for the optimal priming of effector T cells, and that this process occurs in a T cell-non-autonomous manner, with MDA5 function required for the priming

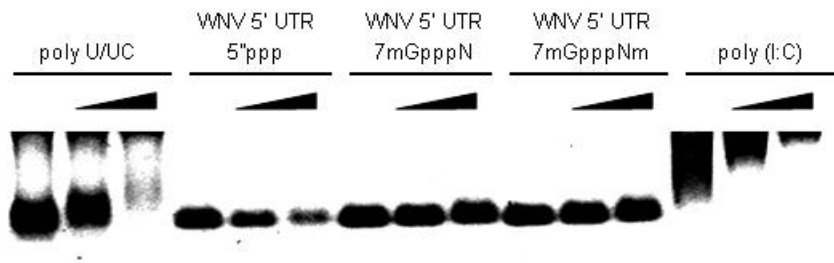
environment (171). Thus, PAMP recognition and signaling by MDA5, as WNV replication proceeds, mediates an innate immune response that restricts viral infection and produces mediators supporting T cell priming, which links innate to adaptive immunity through RLR signaling.

WNV PAMP-pulldown experiments have revealed that RIG-I binds and enriches WNV RNA, preferentially interacting with the negative sense genome. Initial experiments with RNA deep sequencing of MDA5-associated WNV RNA has not revealed a clear interaction between MDA5 and WNV RNA. Instead, we observed an increase in host sequences of RNA in the RLR-immunoprecipitation samples compared to vector. Unfortunately our current experiment design does not allow us to discriminate between the possibility of specific RLR binding to host RNAs or simply a potentiated innate immune response in samples over-expressing RIG-I and MDA5. The future directions section of chapter V will discuss strategies to determine if specific host RNAs are being bound by the RLRs as well as increase our sensitivity to assess MDA5 binding to WNV RNA.

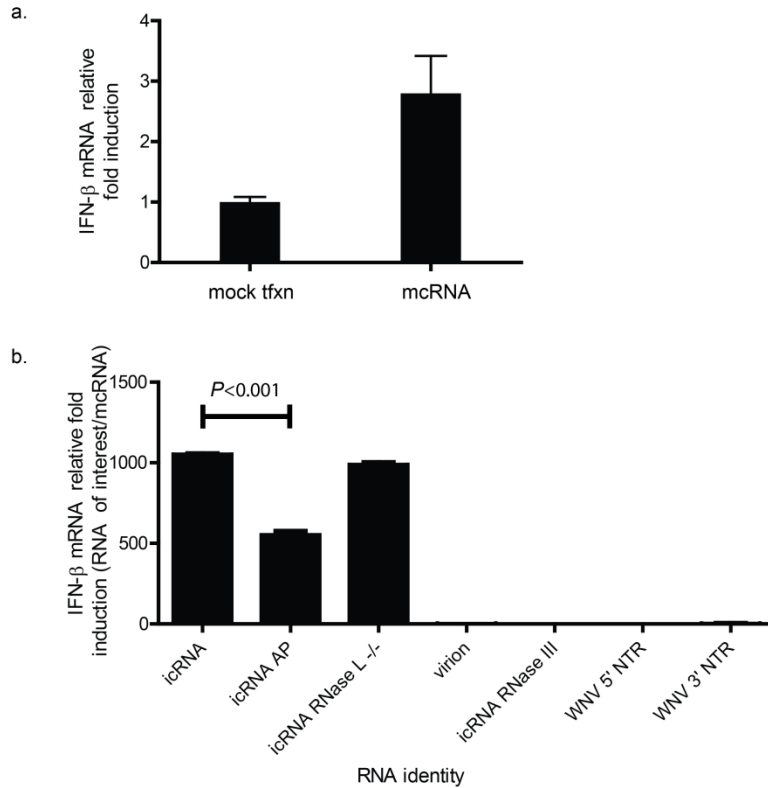
<b>IP sample</b>	<b>Total # of reads</b>	<b># viral reads</b>	<b>% Viral</b>	<b>% rRNA</b>
<b>Vector Mock</b>	<b>21560587</b>	<b>5642</b>	<b>0.0</b>	<b>1.92</b>
<b>Vector 24 hr</b>	<b>30464946</b>	<b>591460</b>	<b>1.9</b>	<b>2.7</b>
<b>Vector 48 hr</b>	<b>31920651</b>	<b>3788992</b>	<b>11.9</b>	<b>10.74</b>
<b>MDA5 Mock</b>	<b>23108125</b>	<b>4056</b>	<b>0.0</b>	<b>4.19</b>
<b>MDA5 24 hr</b>	<b>27616488</b>	<b>513702</b>	<b>1.9</b>	<b>10.82</b>
<b>MDA5 48 hr</b>	<b>39113305</b>	<b>5178774</b>	<b>13.2</b>	<b>9.92</b>
<b>RIG-1 Mock</b>	<b>26177140</b>	<b>2397</b>	<b>0.0</b>	<b>9.32</b>
<b>RIG-I 24 hr</b>	<b>38298886</b>	<b>5863391</b>	<b>15.3</b>	<b>6.88</b>
<b>RIG-I 48 hr</b>	<b>34366122</b>	<b>10967527</b>	<b>31.9</b>	<b>11.77</b>

**Table IV-1. Summary of depth of coverage from deep sequencing of RLR-associated RNAs.**

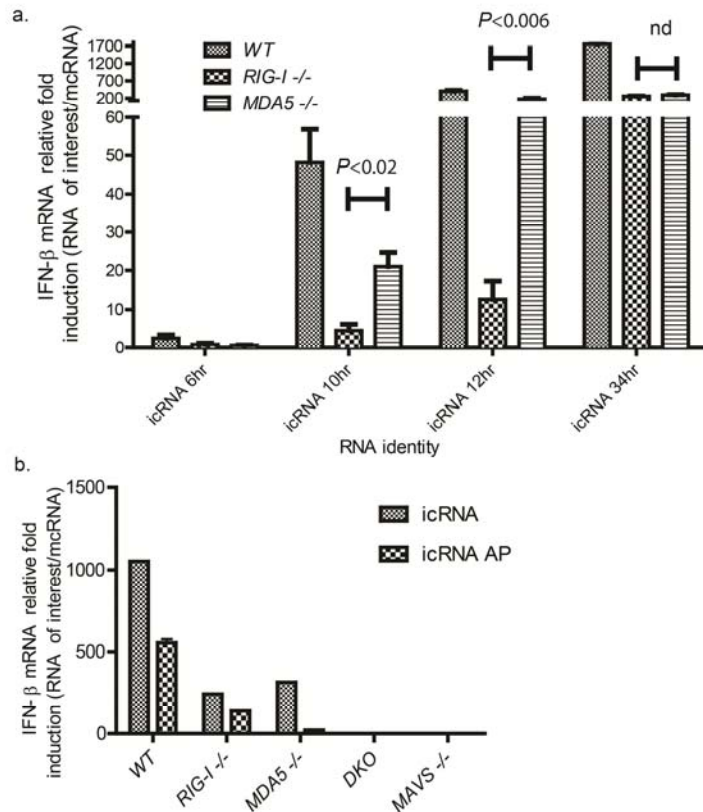
Greater than 20 million reads per sample were mapped to the human or WNV genome. A significant percentage of reads mapped to the viral genome in all infected samples, but only RIG-I-associated RNAs were enriched compared to vector pulldowns that were interpreted as background.



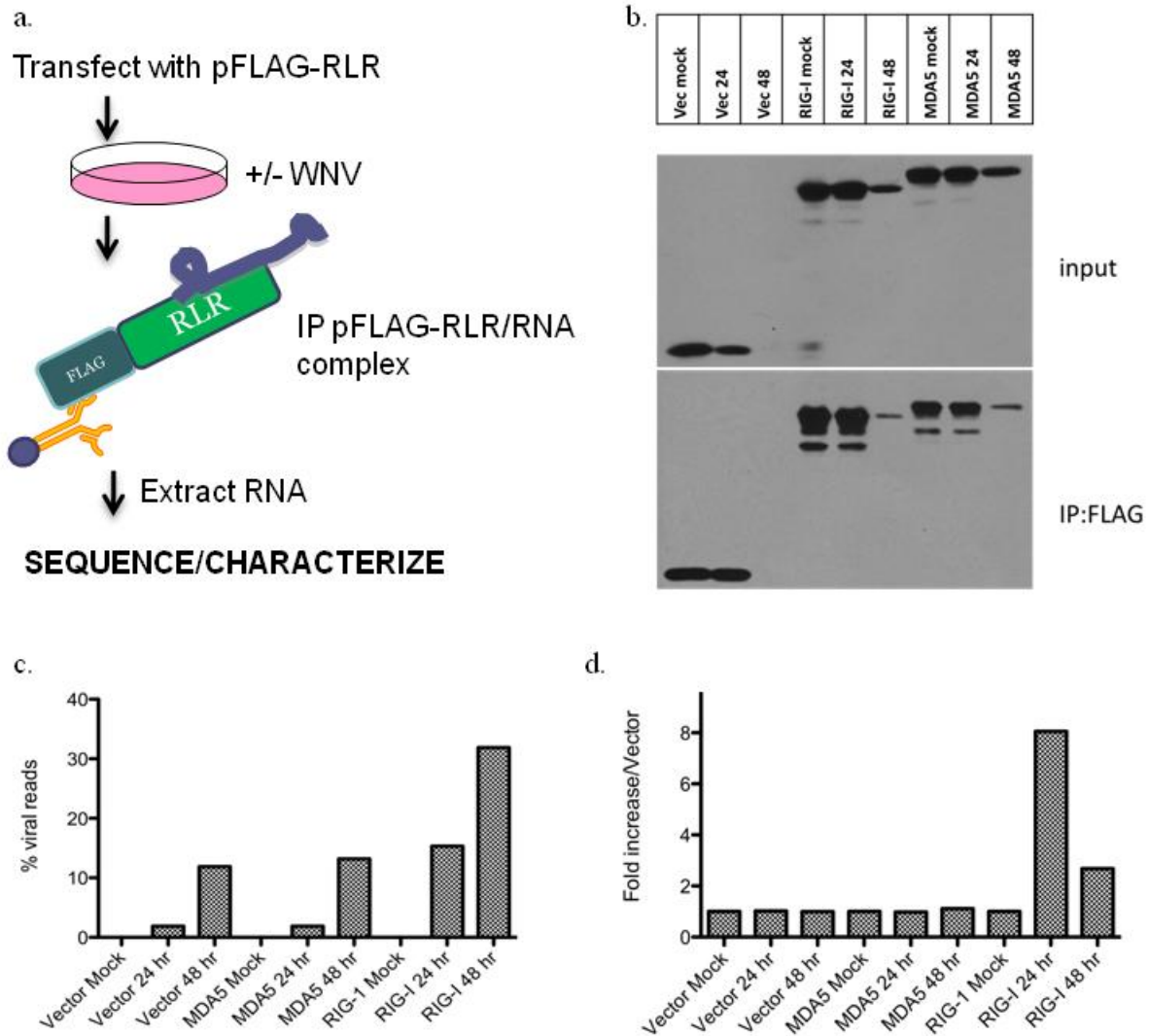
**Figure IV-1. RNA binding/gel-shift analysis of purified RIG-I with HCV poly-U/UC with a 5'ppp, WNV 5'NTR RNA with a 5'ppp, 7mGpppN (Cap 0), or 7mGpppNm (Cap 1) structure, and poly (I:C).** Purified RIG-I (0, 15, and 60 pmol) was incubated with 6 pmol of the respective RNA. RIG-I efficiently bound to HCV poly U/UC and poly (I:C) and weakly to the WNV 5'NTR with a 5'ppp. Addition of a 2'O methyl group to the Cap 0 structure showed no change in RIG-I binding to the WNV 5'NTR.



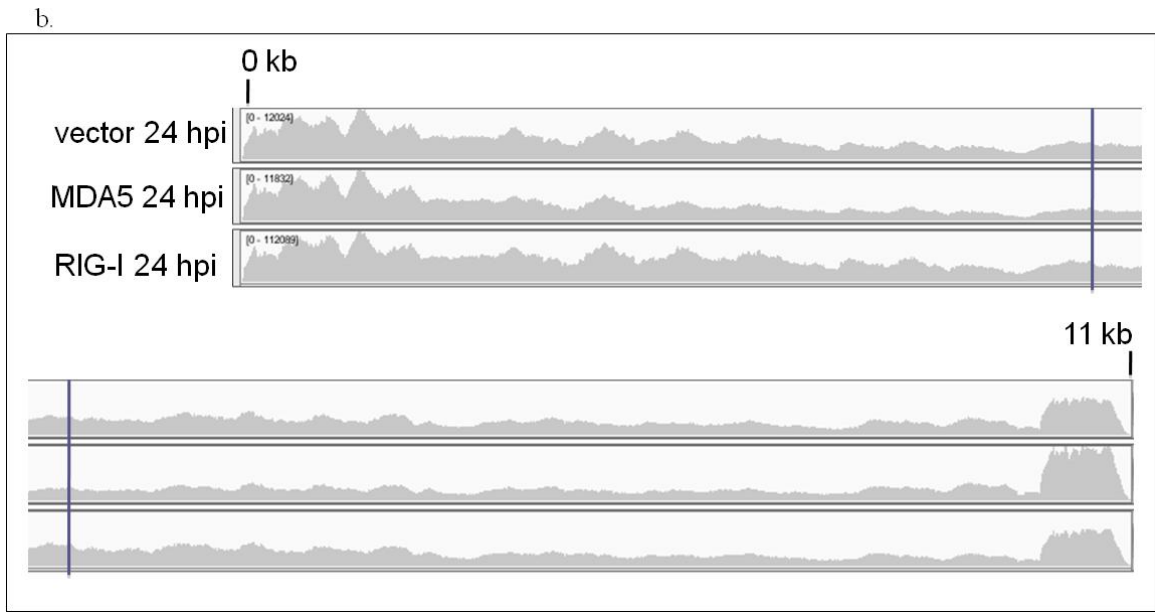
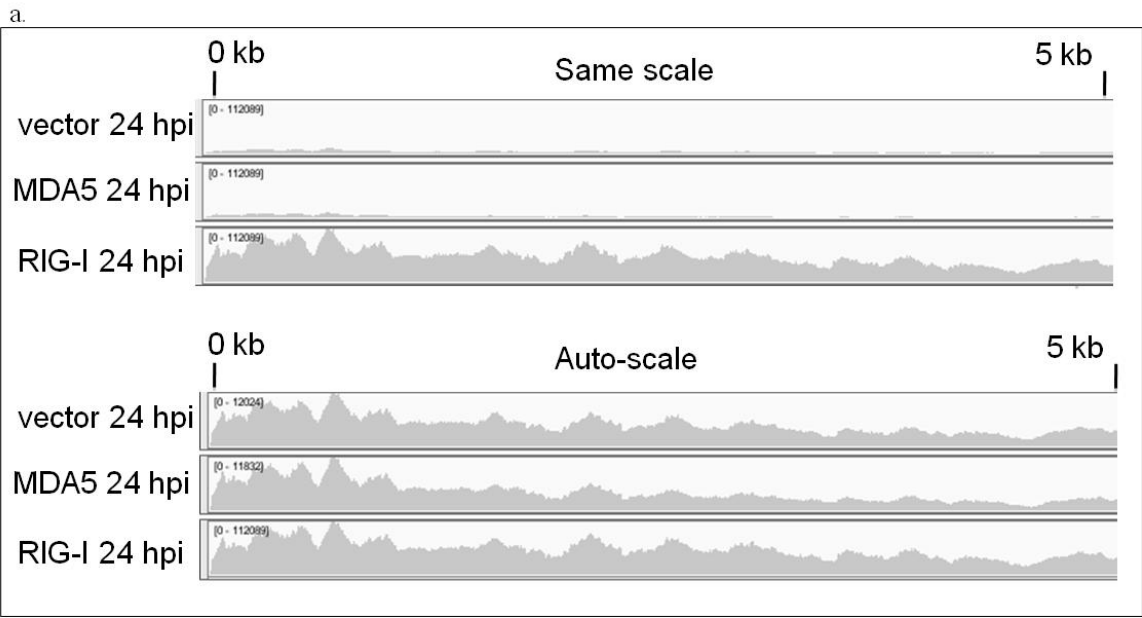
**Figure IV-2. PAMP properties of WNV infection.** (a) WT MEFs were transfected with reagent alone (mock tfxn) or with RNA isolated from uninfected cells (mcRNA). IFN-β mRNA was measured by qRT-PCR analysis and is shown relative to GAPDH. (b) RNA purified from cells infected with WNV for 24 h (icRNA), differentially modified icRNA (phosphatase-treated or RNase III-digested), *in vitro*-transcribed WNV RNA NTR motifs, and native virion RNA were purified, and equal mass quantities were transfected into WT MEFs in the presence of CHX. Innate immune gene induction was measured by qRT-PCR analysis for IFN-β mRNA, and relative fold induction normalized to GAPDH was determined compared to cells transfected with mock-infected cell RNA (mcRNA). Results are representative of three independent experiments.



**Figure IV-3. Distinct RIG-I and MDA5-dependent PAMPs accumulate with differential kinetics during WNV infection.** In (a) icRNA was isolated from WT cells infected with WNV after 6, 10, 12, and 34 hrs of infection and subsequently transfected into WT, *RIG-I*<sup>-/-</sup>, or *MDA5*<sup>-/-</sup> MEFs in the presence of CHX. Innate immune gene induction was measured by qRT-PCR analysis for IFN-β mRNA and relative fold induction after normalization to GAPDH was compared to cells transfected mcRNA. icRNA induced less IFN-β mRNA at all time points in RLR deficient recipient MEFs, but significantly less IFN-β mRNA only was induced in *RIG-I*<sup>-/-</sup> MEFs from icRNA collected at 10 and 12 hpi ( $P < 0.02$  and  $P < 0.006$  respectively). In (b) icRNA from 24 hpi was treated with phosphatase and transfected into WT, *RIG-I*<sup>-/-</sup>, and *MDA5*<sup>-/-</sup> MEFs in the presence of CHX and IFN-β mRNA induction was measured. Results are representative of three independent experiments.



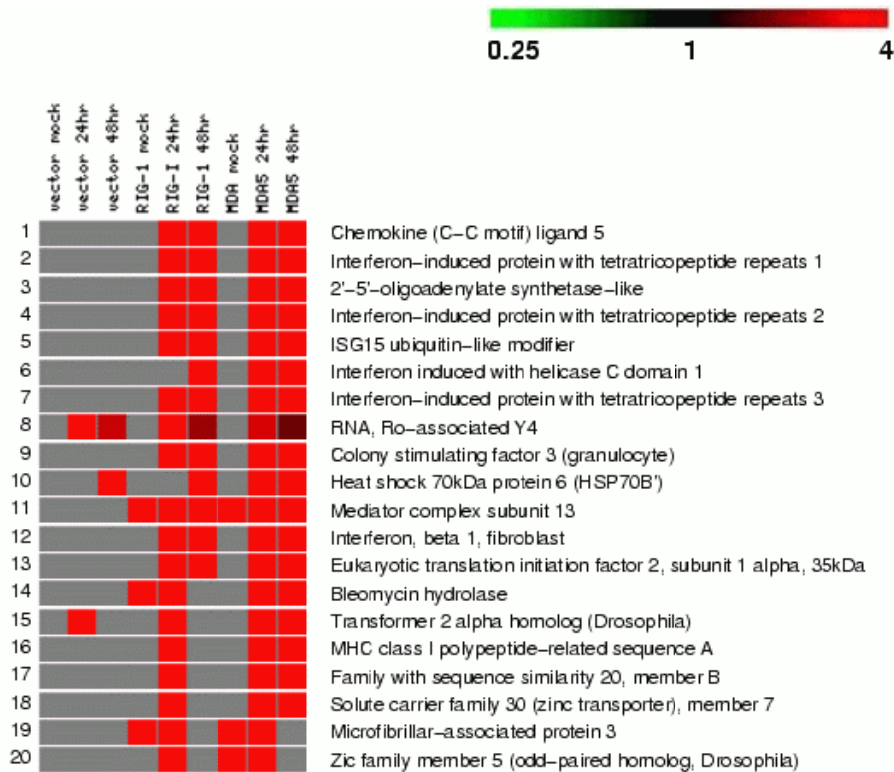
**Figure IV-4. WNV PAMP-pulldown.** (a) Schematic depiction of the WNV PAMP pulldown strategy. (b) Immunoblot staining for FLAG-tagged RLRs and vector from whole cell lysate (input) and following immunoprecipitation (IP:FLAG) from 293T cells mock infected or infected with WNV. (c) Percent of total reads mapping to the WNV genome following immunoprecipitation of each sample. (d) Same data as in (c) plotted as fold increase of WNV mapped reads compared to vector pulldowns.



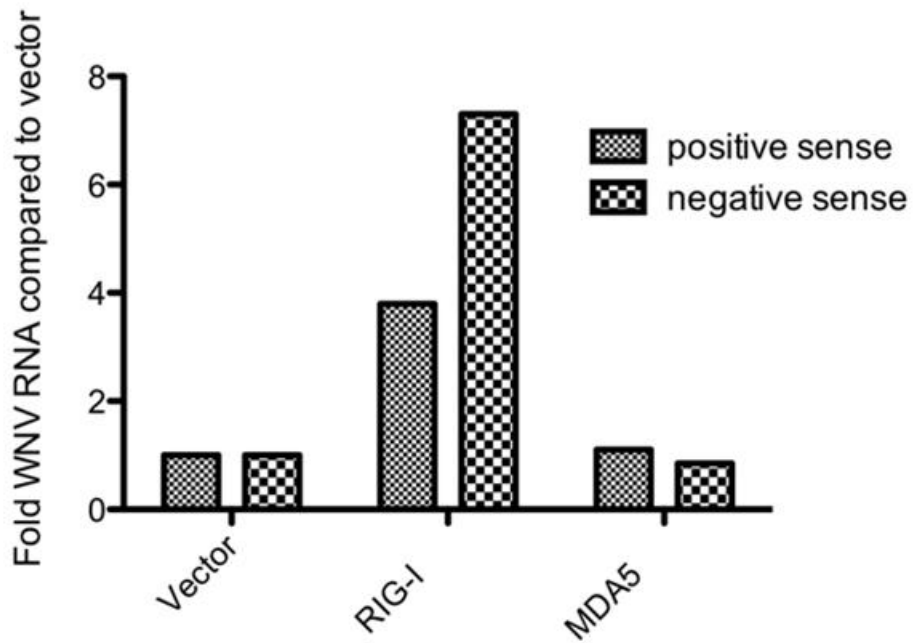
**Figure IV-5. Viral genomic coverage from RNA isolated from immunoprecipitated RLRs during WNV infection.** The y axis represents the number of reads mapped to a single nucleotide position. (a) The first 5 kb of the WNV genome are represented for each of the three samples at 24 hpi.

**(Fig. IV-5. continued).** The top set depicts all graphs set to the same maximum y value (Same scale). The bottom set depicts coverage when the maximum y value for each sample is set to the same height (Auto-scale). (b) The entire WNV genome is represented as a wrapped illustration with a break at approximately 5 kb for the three samples at 24 hpi. The scale is set to the same height for maximum y value for each sample.

All



**Figure IV-6. Non-virus, non-ribosomal RNA most enriched in RLR immunoprecipitation samples.** The heat map represents the hierarchical clustering analysis of RNAs exhibiting the highest enrichment compared to the vector mock sample. Colors on the heat map represent the log<sub>2</sub> ratios of expression (representing normalized read counts) of the all samples to the vector mock sample.



**Figure IV-7. Strand specific WNV qRT-PCR of RNA isolated from immunoprecipitated RLRs.** Values are represented as the fold enrichment of WNV RNA associated with vector, RIG-I, or MDA5 pulldowns at 24 hpi compared to vector.

## V. FINAL DISCUSSION

PRR signaling in response to microbial infection can have complex effects on the overall outcome of *in vivo* pathogenesis. Under certain experimental conditions, loss of TLR signaling was shown to decrease virulence in a mouse model of bacterial infection (185). The loss of a single PRR during WNV infection has been described to have a positive or negative effect on *in vivo* pathogenesis. One group of researchers demonstrated that animals lacking TLR3 had enhanced survival compared to WT controls and attributed this to a lack of maintenance of the blood brain barrier (31). Another report defined TLR3-mediated signaling to be protective against fatal WNV infection and suggested this could be due a loss of local type I IFN production in the CNS (133). We have now defined both RIG-I and MDA5 as essential PRRs that mediate *in vivo* protection against WNV.

Furthermore, we have utilized *in vitro* infections to show that loss of either RLR can result in a loss of innate immune signaling and an increase in viral replication. An increasing number of PRRs and adaptor molecules have been described to promote innate immune signaling in response to RNA PAMP stimulation. With the work described above we have defined RIG-I and MDA5 as the two PRRs responsible for initiating innate immune signaling in a MAVS-dependent manner in key target cells of infection. DDX3, the DDX1/DDX21/DHX36 complex and STING have all been implicated as intracellular PRRs or adaptor molecules that function independently of the RLR pathway to sense RNA PAMPs (93, 186, 187). We have found no evidence for this interpretation in our experimental models of infection of fibroblast and myeloid cells, but cannot exclude the possibility that these genes function as accessories to

RIG-I and MDA5-mediated signaling or play a role not revealed in our experiments. *MAVS*<sup>-/-</sup> animals actually have elevated levels of circulating IFN before they succumb to fatal WNV infection (136). Clearly other PRR pathways are capable of sensing WNV infection, but we hypothesize that the majority of IFN is likely due to TLR sensing on cells such as pDCs in the presence of elevated viremia.

Our study supports a model in which capped, incoming WNV RNA genomes are hidden from RLR detection, yet during viral RNA replication the accumulation of uncapped genomes with exposed phosphate moieties and secondary structure are initially sensed by RIG-I with MDA5 contributing to detection of highly structured PAMPs accumulating later. It remains a possibility that cleavage of viral or endogenous RNA by cellular enzymes may contribute to or amplify PAMP generation. In this respect, we note that icRNA contains both viral and cellular RNA, the latter might contribute to RLR signaling if specific PAMP motifs are generated and displayed. One scenario would involve RLR recognition of virus-induced cellular RNA species that can serve as endogenous PAMPs of innate immune signaling.

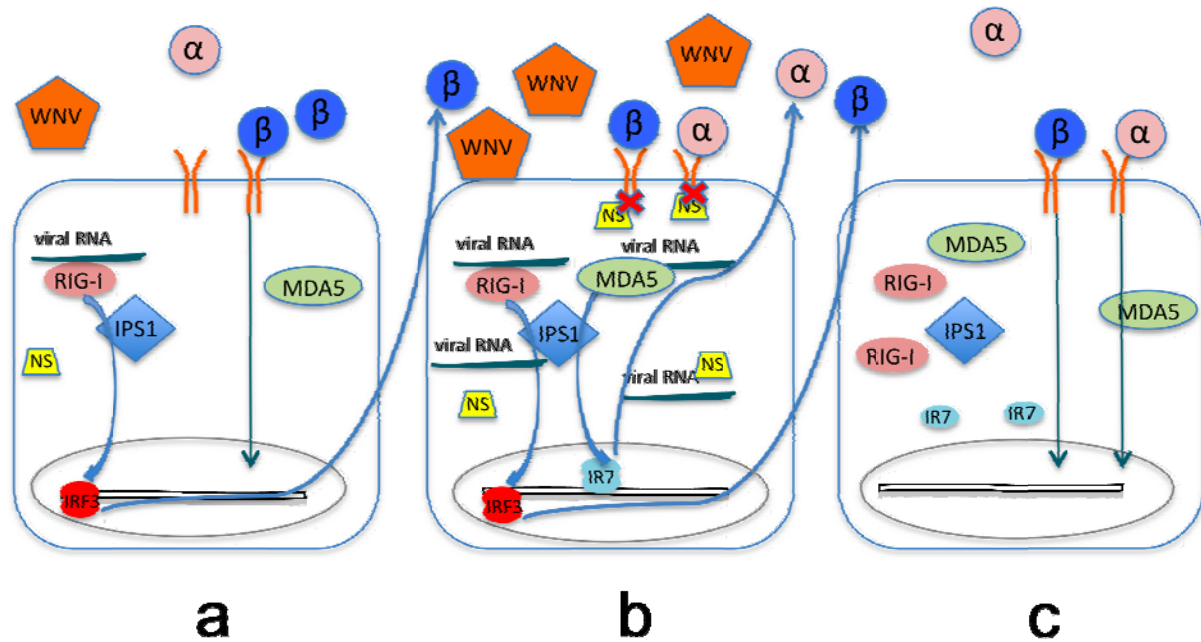
RNase L had previously been implicated in a role generating PAMP RNAs from cleavage of host RNA, but our results do not reveal a major role for RNase L in generating stimulatory RNAs during a WNV infection. Other host RNA modification enzymes may play a role such as XRN1 which has been shown to be active during WNV infection and modifies both viral and host mRNA stability (160, 188). Additional candidate RNA enzymes could also include DDX60, IRE1 and SKIV2L. Intriguingly, DDX60 was recently identified as an RNA helicase that enhances RLR-dependent signaling and shares sequence similarity with SKIV2L (189). SKIV2L is part of the mammalian RNA exosome, but the SKI2 gene in yeast was originally identified as being part of yeast antiviral defense (190). IRE1 plays an important role in immune defense

against infection by promoting adaptive immune cell responses, but has also been shown to play a role in cell-intrinsic immunity to HCV, an RNA flavivirus (191, 192). Adenosine deaminases acting on RNA (ADARs) have been reported to alter RNA composition to become hyperedited-inosine containing antagonist ligands of the RLR pathway (193). Alternative mechanisms may exist for how these putative RNA modification enzymes affect antiviral immunity, but they may be altering viral or host RNAs to generate additional RLR agonists and/or antagonists.

A relatively unexplored question is whether signaling through RIG-I vs. MDA5 has a functional difference in terms of downstream signaling cascades and innate immune activation. We now know that the RLRs are capable of signaling through at least three distinct pathways to activate transcription and gene expression; NF- $\kappa$ B activation, interferon production through IRF3/IRF7 activation, and inflammasome activation. The precise ability of RIG-I vs. MDA5 to signal to each of these pathways and their relative magnitude of contribution to each is unknown. Poly(I:C), a well established agonist of both RIG-I and MDA5 *in vitro*, has highly different RIG-I-dependent and MDA5-dependent effects when administered *in vivo* (163, 194, 195). Our results do demonstrate that infection with WNV results in slightly differential induction of innate immune genes in RLR ko MEFs. However, our data also support the conclusion that in the case of WNV infection this is likely due to differential kinetic accumulation of viral PAMPs. Our experiments to understand the mechanisms governing functional activation of MDA5 following infection with WNV may enable us to explain some of these differential responses. RIG-I primarily utilizes 14-3-3 $\epsilon$  as a molecular chaperone to assist in translocation to intracellular membranes, but our preliminary results suggest MDA5 interacts most strongly with 14-3-3 $\zeta$  and  $\beta$  during infection. Future experiments will reveal if these interactions are required for efficient

MDA5 signaling and may provide a mechanistic explanation for how RIG-I and MDA5 propagate different downstream signatures.

Our observations define the complementary and individually essential roles of RIG-I and MDA5 in detecting and controlling WNV infection. While each RLR can recognize WNV independently, signaling by both optimally restricts infection and protects against viral pathogenesis. In cells in which both RIG-I and MDA5 are present, the RLR signaling of each PRR could be enhanced by the other as both RIG-I and MDA5 are ISGs themselves. Indeed, RIG-I and MDA5 may both be able to detect PAMPs present in multiple RNA virus infections but effectively confer no innate immune signaling, due to direct viral antagonism of PRRs. The evolutionary forces that drive other viruses to directly antagonize RLR detection may not have played as strong a role for WNV since it initially evades detection of virion RNA and subsequently antagonizes type I IFN signaling (161). WNV therefore presents an attractive model to further our understanding of basic principles of self versus non-self detection during infection by other flaviviruses and RNA viruses in general due to the engagement of both RIG-I and MDA5 (see **Fig. V-1**).



**Figure V-1: Final model of RLR-mediated protection against WNV infection.** (a) RIG-I mediated control of viral replication by early production of IFN- $\beta$  occurs intrinsically (autocrine signaling) and extrinsically (paracrine signaling). (b) During later time points of infection, IFN signaling is antagonized and does not influence cell-intrinsic viral replication even though RIG-I and MDA5 are contributing to the type IFN production. (c) RIG-I and MDA5 contribute to viral replication control by inducing a full IFN- $\alpha/\beta$  response that signals to uninfected, or early infected cells. Processing of host or viral RNA at any step could modulate RLR recognition of PAMP RNA and immune signaling.

Given the central role of the RLRs in protection against virus infection and initiating innate immune responses, they can be considered as therapeutic targets to treat WNV or other diseases. There is currently no effective therapy against WNV infection in humans but several therapeutic agents and vaccines are actively being investigated (overview in (86)). The ability to selectively activate the appropriate PRR pathway as a direct antiviral is an attractive option. One

of the major targets of RLR activation, type I IFN, has been used as an effective antiviral agent for decades (196). However, therapy with other pharmaceutical agents that induce interferon have been shown to be effective when type I IFN fails (197). This suggests that stimulation of multiple pathways, including type IFN receptor signaling, can be an effective strategy. RLR stimulation has been shown to lead to type IFN production and signaling, NF- $\kappa$ B activation, and inflammasome activation. Such pleiotropic responses could be highly beneficial when a more limited therapy fails. IFN- $\lambda$  is one of the most promising new antiviral therapies and was recently shown to be regulated by a similar transcriptional machinery as type I IFN and indeed is induced by RLR activation (198).

RLR-targeted therapeutic strategies could include use as direct antivirals or as new adjuvants for vaccines. The innate/adaptive immune interface has been highlighted as key to effective immunity for many years now (199). The direct RLR/adaptive interface has already been highlighted (55, 136, 171, 195, 200). Approved adjuvants such as alum and monophosphoryl lipid A (MPL) may not accurately represent the appropriate innate immune stimulation for a natural course of infection for the pathogen being immunized against. An excellent example of an effective non-conventional adjuvant was revealed by work from the Pulendran lab. Systems biology approaches identified the gene signatures of an effective vaccine and this informed the creation of a new combinatorial vaccine adjuvant that was highly effective in eliciting an adaptive immune response, at least in part by targeting multiple PRRs (179, 201, 202). Our work here has demonstrated that multiple, highly related PRRs (RIG-I and MDA5) in the same pathway are essential for full immune protection against infection and suggest that the most efficacious adjuvants may be most effective if targeting of both RLRs is achieved.

Direct targeting of the RLRs for activation will require an increase in our understanding of the exact specificities of RIG-I and MDA5. The proposed experiments in the future directions section below seek to achieve this and will inform our understanding of how viral infections initiate non-self sensing of PAMPs. The knowledge gained could enable therapeutic intervention during virus infection or when aberrant RLR signaling leads to autoimmunity (203, 204).

## **FUTURE DIRECTIONS**

The research presented above leaves several interesting questions unanswered, but the primary focus of our future research is to further characterize the RNAs that are bound to RIG-I and MDA5 during a WNV infection. We clearly demonstrated that RIG-I binds to WNV RNA and preferentially interacts with the negative strand genome. Specific MDA5 interaction with WNV RNA was not observed, but this could be due to a reduced ligand affinity of MDA5 compared to RIG-I (53). RNAs associated with RIG-I and MDA5 immunoprecipitations were also enriched for many host RNAs compared to vector, but our initial experiments were not able to differentiate if this was specific binding or just a background of higher gene induction in the presence of over-expressed RLRs.

The next series of experiments will directly address the limitations of our first PAMP-pulldown efforts. Utilizing metallic beads instead of agarose gel as the platform for immunoprecipitation will decrease background and increase sensitivity. Preliminary results suggest that this reduces non-specific RNA binding by ~8 fold. Additionally, we will further investigate the relative binding of positive/negative strand genomes to RIG-I by preparing libraries for sequencing with Illumina's "Directional mRNA Seq Sample Preparation" guidelines. Directional deep sequencing will allow us to determine the relative binding of RIG-I

to the positive and negative strand genomes in a non-sequence biased manner. Most importantly, future experiments will also include directional deep sequencing of the whole transcriptome before any immunoprecipitation. Comparison of the ratio of RNAs in a single sample before and after immunoprecipitation will allow us to conclusively determine if there is enrichment for any specific RNA sequences. Novel identities of enriched RNA will inform further experiments to determine if or which host RNA modifying enzymes are contributing to RLR PAMP recognition and signaling regulation.

## VI. REFERENCES

1. **Barquet N, Domingo P.** 1997. Smallpox: the triumph over the most terrible of the ministers of death. *Annals of internal medicine* **127**:635-642.
2. **Jenner E.** 1863. On the Origin of the Vaccine Inoculation. G. Elsick.
3. **Hedrick SM.** 2004. The acquired immune system: a vantage from beneath. *Immunity* **21**:607-615.
4. **Bevan MJ.** 1976. Cross-priming for a secondary cytotoxic response to minor H antigens with H-2 congenic cells which do not cross-react in the cytotoxic assay. *J Exp Med* **143**:1283-1288.
5. **Burnet FM.** 1976. A modification of Jerne's theory of antibody production using the concept of clonal selection. *CA: a cancer journal for clinicians* **26**:119-121.
6. **Rajewsky K.** 1996. Clonal selection and learning in the antibody system. *Nature* **381**:751-758.
7. **Zinkernagel RM, Doherty PC.** 1974. Restriction of in vitro T cell-mediated cytotoxicity in lymphocytic choriomeningitis within a syngeneic or semiallogeneic system. *Nature* **248**:701-702.
8. **Janeway CA, Jr.** 1989. Approaching the asymptote? Evolution and revolution in immunology. *Cold Spring Harbor symposia on quantitative biology* **54 Pt 1**:1-13.
9. **Hemmi H, Takeuchi O, Kawai T, Kaisho T, Sato S, Sanjo H, Matsumoto M, Hoshino K, Wagner H, Takeda K, Akira S.** 2000. A Toll-like receptor recognizes bacterial DNA. *Nature* **408**:740-745.
10. **Hoffmann JA, Kafatos FC, Janeway CA, Ezekowitz RA.** 1999. Phylogenetic perspectives in innate immunity. *Science* **284**:1313-1318.
11. **Medzhitov R, Preston-Hurlburt P, Janeway CA, Jr.** 1997. A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. *Nature* **388**:394-397.
12. **Poltorak A, He X, Smirnova I, Liu MY, Van Huffel C, Du X, Birdwell D, Alejos E, Silva M, Galanos C, Freudenberg M, Ricciardi-Castagnoli P, Layton B, Beutler B.** 1998. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science* **282**:2085-2088.
13. **Matzinger P.** 1994. Tolerance, danger, and the extended family. *Annual review of immunology* **12**:991-1045.
14. **McCall CE, De Chatelet LR, Brown D, Lachmann P.** 1974. New biological activity following intravascular activation of the complement cascade. *Nature* **249**:841-843.
15. **Sancho D, Joffre OP, Keller AM, Rogers NC, Martinez D, Hernanz-Falcon P, Rosewell I, Reis e Sousa C.** 2009. Identification of a dendritic cell receptor that couples sensing of necrosis to immunity. *Nature* **458**:899-903.
16. **Shi Y, Evans JE, Rock KL.** 2003. Molecular identification of a danger signal that alerts the immune system to dying cells. *Nature* **425**:516-521.
17. **Isaacs A, Lindenmann J.** 1957. Virus interference. I. The interferon. *Proceedings of the Royal Society of London. Series B, Containing papers of a Biological character. Royal Society (Great Britain)* **147**:258-267.
18. **Ganz T.** 2003. Defensins: antimicrobial peptides of innate immunity. *Nature reviews. Immunology* **3**:710-720.
19. **Takeda K, Akira S.** 2004. TLR signaling pathways. *Semin Immunol* **16**:3-9.
20. **Elinav E, Strowig T, Henao-Mejia J, Flavell RA.** 2011. Regulation of the antimicrobial response by NLR proteins. *Immunity* **34**:665-679.
21. **Takeuchi O, Akira S.** 2008. MDA5/RIG-I and virus recognition. *Curr Opin Immunol* **20**:17-22.
22. **Brunette RL, Young JM, Whitley DG, Brodsky IE, Malik HS, Stetson DB.** 2012. Extensive evolutionary and functional diversity among mammalian AIM2-like receptors. *J Exp Med* **209**:1969-1983.

23. **Williams BR.** 2001. Signal integration via PKR. *Sci STKE* **2001**:re2.
24. **Sen GC.** 2000. Novel functions of interferon-induced proteins. *Semin Cancer Biol* **10**:93-101.
25. **Sun L, Wu J, Du F, Chen X, Chen ZJ.** 2013. Cyclic GMP-AMP synthase is a cytosolic DNA sensor that activates the type I interferon pathway. *Science* **339**:786-791.
26. **DeWitte-Orr SJ, Collins SE, Bauer CM, Bowdish DM, Mossman KL.** 2010. An accessory to the 'Trinity': SR-As are essential pathogen sensors of extracellular dsRNA, mediating entry and leading to subsequent type I IFN responses. *PLoS Pathog* **6**:e1000829.
27. **Fullam A, Schroder M.** 2013. DExD/H-box RNA helicases as mediators of anti-viral innate immunity and essential host factors for viral replication. *Biochim Biophys Acta* **1829**:854-865.
28. **Barbalat R, Lau L, Locksley RM, Barton GM.** 2009. Toll-like receptor 2 on inflammatory monocytes induces type I interferon in response to viral but not bacterial ligands. *Nat Immunol* **10**:1200-1207.
29. **Kawai T, Akira S.** 2010. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. *Nat Immunol* **11**:373-384.
30. **Stetson DB, Medzhitov R.** 2006. Type I interferons in host defense. *Immunity* **25**:373-381.
31. **Wang T, Town T, Alexopoulou L, Anderson JF, Fikrig E, Flavell RA.** 2004. Toll-like receptor 3 mediates West Nile virus entry into the brain causing lethal encephalitis. *Nat Med* **10**:1366-1373.
32. **Le Goffic R, Balloy V, Lagranderie M, Alexopoulou L, Escriou N, Flavell R, Chignard M, Si-Tahar M.** 2006. Detrimental contribution of the Toll-like receptor (TLR)3 to influenza A virus-induced acute pneumonia. *PLoS Pathog* **2**:e53.
33. **Touil T, Fitzgerald D, Zhang GX, Rostami A, Gran B.** 2006. Cutting Edge: TLR3 stimulation suppresses experimental autoimmune encephalomyelitis by inducing endogenous IFN-beta. *J Immunol* **177**:7505-7509.
34. **Diebold SS, Kaisho T, Hemmi H, Akira S, Reis e Sousa C.** 2004. Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. *Science* **303**:1529-1531.
35. **Giltiay NV, Chappell CP, Sun X, Kolhatkar N, Teal TH, Wiedeman AE, Kim J, Tanaka L, Buechler MB, Hamerman JA, Imanishi-Kari T, Clark EA, Elkon KB.** 2013. Overexpression of TLR7 promotes cell-intrinsic expansion and autoantibody production by transitional T1 B cells. *J Exp Med* **210**:2773-2789.
36. **Sato A, Linehan MM, Iwasaki A.** 2006. Dual recognition of herpes simplex viruses by TLR2 and TLR9 in dendritic cells. *Proc Natl Acad Sci U S A* **103**:17343-17348.
37. **Christensen SR, Shupe J, Nickerson K, Kashgarian M, Flavell RA, Shlomchik MJ.** 2006. Toll-like receptor 7 and TLR9 dictate autoantibody specificity and have opposing inflammatory and regulatory roles in a murine model of lupus. *Immunity* **25**:417-428.
38. **Sabbah A, Chang TH, Harnack R, Frohlich V, Tominaga K, Dube PH, Xiang Y, Bose S.** 2009. Activation of innate immune antiviral responses by Nod2. *Nat Immunol* **10**:1073-1080.
39. **Allen IC, Scull MA, Moore CB, Holl EK, McElvania-TeKippe E, Taxman DJ, Guthrie EH, Pickles RJ, Ting JP.** 2009. The NLRP3 inflammasome mediates in vivo innate immunity to influenza A virus through recognition of viral RNA. *Immunity* **30**:556-565.
40. **Ramos HJ, Lanteri MC, Blahnik G, Negash A, Suthar MS, Brassil MM, Sodhi K, Treuting PM, Busch MP, Norris PJ, Gale M, Jr.** 2012. IL-1beta signaling promotes CNS-intrinsic immune control of West Nile virus infection. *PLoS Pathog* **8**:e1003039.
41. **Negash AA, Ramos HJ, Crochet N, Lau DT, Doehle B, Papic N, Delker DA, Jo J, Bertoletti A, Hagedorn CH, Gale M, Jr.** 2013. IL-1beta production through the NLRP3 inflammasome by hepatic macrophages links hepatitis C virus infection with liver inflammation and disease. *PLoS Pathog* **9**:e1003330.

42. **Ichinohe T, Pang IK, Iwasaki A.** 2010. Influenza virus activates inflammasomes via its intracellular M2 ion channel. *Nat Immunol* **11**:404-410.
43. **Moore CB, Bergstralh DT, Duncan JA, Lei Y, Morrison TE, Zimmermann AG, Accavitti-Loper MA, Madden VJ, Sun L, Ye Z, Lich JD, Heise MT, Chen Z, Ting JP.** 2008. NLRX1 is a regulator of mitochondrial antiviral immunity. *Nature* **451**:573-577.
44. **Soares F, Tattoli I, Wortzman ME, Arnoult D, Philpott DJ, Girardin SE.** 2013. NLRX1 does not inhibit MAVS-dependent antiviral signalling. *Innate Immun* **19**:438-448.
45. **Tattoli I, Carneiro LA, Jehanno M, Magalhaes JG, Shu Y, Philpott DJ, Arnoult D, Girardin SE.** 2008. NLRX1 is a mitochondrial NOD-like receptor that amplifies NF-kappaB and JNK pathways by inducing reactive oxygen species production. *EMBO Rep* **9**:293-300.
46. **Schattgen SA, Fitzgerald KA.** 2011. The PYHIN protein family as mediators of host defenses. *Immunol Rev* **243**:109-118.
47. **Hornung V, Ablasser A, Charrel-Dennis M, Bauernfeind F, Horvath G, Caffrey DR, Latz E, Fitzgerald KA.** 2009. AIM2 recognizes cytosolic dsDNA and forms a caspase-1-activating inflammasome with ASC. *Nature* **458**:514-518.
48. **Rathinam VA, Jiang Z, Waggoner SN, Sharma S, Cole LE, Waggoner L, Vanaja SK, Monks BG, Ganesan S, Latz E, Hornung V, Vogel SN, Szomolanyi-Tsuda E, Fitzgerald KA.** 2010. The AIM2 inflammasome is essential for host defense against cytosolic bacteria and DNA viruses. *Nat Immunol* **11**:395-402.
49. **Unterholzner L, Keating SE, Baran M, Horan KA, Jensen SB, Sharma S, Sirois CM, Jin T, Latz E, Xiao TS, Fitzgerald KA, Paludan SR, Bowie AG.** 2010. IFI16 is an innate immune sensor for intracellular DNA. *Nat Immunol* **11**:997-1004.
50. **Loo YM, Gale M, Jr.** 2011. Immune signaling by RIG-I-like receptors. *Immunity* **34**:680-692.
51. **Yount JS, Moran TM, Lopez CB.** 2007. Cytokine-independent upregulation of MDA5 in viral infection. *J Virol* **81**:7316-7319.
52. **Takahashi K, Yoneyama M, Nishihori T, Hirai R, Kumeta H, Narita R, Gale M, Jr., Inagaki F, Fujita T.** 2008. Nonself RNA-sensing mechanism of RIG-I helicase and activation of antiviral immune responses. *Mol Cell* **29**:428-440.
53. **Cui S, Eisenacher K, Kirchhofer A, Brzozka K, Lammens A, Lammens K, Fujita T, Conzelmann KK, Krug A, Hopfner KP.** 2008. The C-terminal regulatory domain is the RNA 5'-triphosphate sensor of RIG-I. *Mol Cell* **29**:169-179.
54. **Saito T, Hirai R, Loo YM, Owen D, Johnson CL, Sinha SC, Akira S, Fujita T, Gale M, Jr.** 2007. Regulation of innate antiviral defenses through a shared repressor domain in RIG-I and LGP2. *Proc Natl Acad Sci U S A* **104**:582-587.
55. **Suthar MS, Ramos HJ, Brassil MM, Netland J, Chappell CP, Blahnik G, McMillan A, Diamond MS, Clark EA, Bevan MJ, Gale M, Jr.** 2012. The RIG-I-like Receptor LGP2 Controls CD8(+) T Cell Survival and Fitness. *Immunity*.
56. **Venkataraman T, Valdes M, Elsby R, Kakuta S, Caceres G, Saijo S, Iwakura Y, Barber GN.** 2007. Loss of DExD/H box RNA helicase LGP2 manifests disparate antiviral responses. *J Immunol* **178**:6444-6455.
57. **Meylan E, Curran J, Hofmann K, Moradpour D, Binder M, Bartenschlager R, Tschopp J.** 2005. Cardif is an adaptor protein in the RIG-I antiviral pathway and is targeted by hepatitis C virus. *Nature* **437**:1167-1172.
58. **Seth RB, Sun L, Ea CK, Chen ZJ.** 2005. Identification and characterization of MAVS, a mitochondrial antiviral signaling protein that activates NF-kappaB and IRF 3. *Cell* **122**:669-682.
59. **Xu LG, Wang YY, Han KJ, Li LY, Zhai Z, Shu HB.** 2005. VISA is an adapter protein required for virus-triggered IFN-beta signaling. *Mol Cell* **19**:727-740.

60. **Myong S, Cui S, Cornish PV, Kirchhofer A, Gack MU, Jung JU, Hopfner KP, Ha T.** 2009. Cytosolic viral sensor RIG-I is a 5'-triphosphate-dependent translocase on double-stranded RNA. *Science* **323**:1070-1074.
61. **Liu HM, Loo YM, Horner SM, Zornetzer GA, Katze MG, Gale M, Jr.** 2012. The mitochondrial targeting chaperone 14-3-3epsilon regulates a RIG-I translocon that mediates membrane association and innate antiviral immunity. *Cell Host Microbe* **11**:528-537.
62. **Horner SM, Liu HM, Park HS, Briley J, Gale M, Jr.** 2011. Mitochondrial-associated endoplasmic reticulum membranes (MAM) form innate immune synapses and are targeted by hepatitis C virus. *Proc Natl Acad Sci U S A* **108**:14590-14595.
63. **Dixit E, Boulant S, Zhang Y, Lee AS, Odendall C, Shum B, Hacohen N, Chen ZJ, Whelan SP, Fransen M, Nibert ML, Superti-Furga G, Kagan JC.** 2010. Peroxisomes are signaling platforms for antiviral innate immunity. *Cell* **141**:668-681.
64. **Berke IC, Modis Y.** 2012. MDA5 cooperatively forms dimers and ATP-sensitive filaments upon binding double-stranded RNA. *EMBO J* **31**:1714-1726.
65. **Peisley A, Lin C, Wu B, Orme-Johnson M, Liu M, Walz T, Hur S.** 2011. Cooperative assembly and dynamic disassembly of MDA5 filaments for viral dsRNA recognition. *Proc Natl Acad Sci U S A* **108**:21010-21015.
66. **Kato H, Takeuchi O, Sato S, Yoneyama M, Yamamoto M, Matsui K, Uematsu S, Jung A, Kawai T, Ishii KJ, Yamaguchi O, Otsu K, Tsujimura T, Koh CS, Reis e Sousa C, Matsuura Y, Fujita T, Akira S.** 2006. Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses. *Nature* **441**:101-105.
67. **Pichlmair A, Schulz O, Tan CP, Rehwinkel J, Kato H, Takeuchi O, Akira S, Way M, Schiavo G, Reis e Sousa C.** 2009. Activation of MDA5 requires higher-order RNA structures generated during virus infection. *J Virol* **83**:10761-10769.
68. **Mibayashi M, Martinez-Sobrido L, Loo YM, Cardenas WB, Gale M, Jr., Garcia-Sastre A.** 2007. Inhibition of retinoic acid-inducible gene I-mediated induction of beta interferon by the NS1 protein of influenza A virus. *J Virol* **81**:514-524.
69. **McCartney SA, Thackray LB, Gitlin L, Gilfillan S, Virgin HW, Colonna M.** 2008. MDA-5 recognition of a murine norovirus. *PLoS Pathog* **4**:e1000108.
70. **Loo YM, Fornek J, Crochet N, Bajwa G, Perwitasari O, Martinez-Sobrido L, Akira S, Gill MA, Garcia-Sastre A, Katze MG, Gale M, Jr.** 2008. Distinct RIG-I and MDA5 signaling by RNA viruses in innate immunity. *Journal of virology* **82**:335-345.
71. **Zhou S, Cerny AM, Zacharia A, Fitzgerald KA, Kurt-Jones EA, Finberg RW.** 2010. Induction and inhibition of type I interferon responses by distinct components of lymphocytic choriomeningitis virus. *J Virol* **84**:9452-9462.
72. **Andrejeva J, Childs KS, Young DF, Carlos TS, Stock N, Goodbourn S, Randall RE.** 2004. The V proteins of paramyxoviruses bind the IFN-inducible RNA helicase, mda-5, and inhibit its activation of the IFN-beta promoter. *Proc Natl Acad Sci U S A* **101**:17264-17269.
73. **Barral PM, Sarkar D, Fisher PB, Racaniello VR.** 2009. RIG-I is cleaved during picornavirus infection. *Virology* **391**:171-176.
74. **Parisien JP, Bamming D, Komuro A, Ramachandran A, Rodriguez JJ, Barber G, Wojahn RD, Horvath CM.** 2009. A shared interface mediates paramyxovirus interference with antiviral RNA helicases MDA5 and LGP2. *J Virol* **83**:7252-7260.
75. **Hornung V, Ellegast J, Kim S, Brzozka K, Jung A, Kato H, Poeck H, Akira S, Conzelmann KK, Schlee M, Endres S, Hartmann G.** 2006. 5'-Triphosphate RNA is the ligand for RIG-I. *Science* **314**:994-997.

76. **Marques JT, Devosse T, Wang D, Zamanian-Daryoush M, Serbinowski P, Hartmann R, Fujita T, Behlke MA, Williams BR.** 2006. A structural basis for discriminating between self and nonself double-stranded RNAs in mammalian cells. *Nature biotechnology* **24**:559-565.
77. **Saito T, Owen DM, Jiang F, Marcotrigiano J, Gale M, Jr.** 2008. Innate immunity induced by composition-dependent RIG-I recognition of hepatitis C virus RNA. *Nature* **454**:523-527.
78. **Schlee M, Roth A, Hornung V, Hagmann CA, Wimmenauer V, Barchet W, Coch C, Janke M, Mihailovic A, Wardle G, Juranek S, Kato H, Kawai T, Poeck H, Fitzgerald KA, Takeuchi O, Akira S, Tuschl T, Latz E, Ludwig J, Hartmann G.** 2009. Recognition of 5' triphosphate by RIG-I helicase requires short blunt double-stranded RNA as contained in panhandle of negative-strand virus. *Immunity* **31**:25-34.
79. **Schnell G, Loo YM, Marcotrigiano J, Gale M, Jr.** 2012. Uridine composition of the poly-U/UC tract of HCV RNA defines non-self recognition by RIG-I. *PLoS Pathog* **8**:e1002839.
80. **Jiang F, Ramanathan A, Miller MT, Tang GQ, Gale M, Jr., Patel SS, Marcotrigiano J.** 2011. Structural basis of RNA recognition and activation by innate immune receptor RIG-I. *Nature* **479**:423-427.
81. **Kato H, Takeuchi O, Mikamo-Satoh E, Hirai R, Kawai T, Matsushita K, Hiiragi A, Dermody TS, Fujita T, Akira S.** 2008. Length-dependent recognition of double-stranded ribonucleic acids by retinoic acid-inducible gene-I and melanoma differentiation-associated gene 5. *The Journal of experimental medicine* **205**:1601-1610.
82. **Cheng G, Zhong J, Chung J, Chisari FV.** 2007. Double-stranded DNA and double-stranded RNA induce a common antiviral signaling pathway in human cells. *Proc Natl Acad Sci U S A* **104**:9035-9040.
83. **Chiu YH, Macmillan JB, Chen ZJ.** 2009. RNA polymerase III detects cytosolic DNA and induces type I interferons through the RIG-I pathway. *Cell* **138**:576-591.
84. **Poeck H, Bscheider M, Gross O, Finger K, Roth S, Rebsamen M, Hanneschlager N, Schlee M, Rothenfusser S, Barchet W, Kato H, Akira S, Inoue S, Endres S, Peschel C, Hartmann G, Hornung V, Ruland J.** 2010. Recognition of RNA virus by RIG-I results in activation of CARD9 and inflammasome signaling for interleukin 1 beta production. *Nat Immunol* **11**:63-69.
85. **Guan K, Zheng Z, Song T, He X, Xu C, Zhang Y, Ma S, Wang Y, Xu Q, Cao Y, Li J, Yang X, Ge X, Wei C, Zhong H.** 2013. MAVS regulates apoptotic cell death by decreasing K48-linked ubiquitination of voltage-dependent anion channel 1. *Mol Cell Biol* **33**:3137-3149.
86. **Suthar MS, Diamond MS, Gale M, Jr.** 2013. West Nile virus infection and immunity. *Nat Rev Microbiol* **11**:115-128.
87. **Pfaller CK, Li Z, George CX, Samuel CE.** 2011. Protein kinase PKR and RNA adenosine deaminase ADAR1: new roles for old players as modulators of the interferon response. *Curr Opin Immunol* **23**:573-582.
88. **McAllister CS, Samuel CE.** 2009. The RNA-activated protein kinase enhances the induction of interferon-beta and apoptosis mediated by cytoplasmic RNA sensors. *J Biol Chem* **284**:1644-1651.
89. **Malathi K, Dong B, Gale M, Jr., Silverman RH.** 2007. Small self-RNA generated by RNase L amplifies antiviral innate immunity. *Nature* **448**:816-819.
90. **Wu J, Sun L, Chen X, Du F, Shi H, Chen C, Chen ZJ.** 2013. Cyclic GMP-AMP is an endogenous second messenger in innate immune signaling by cytosolic DNA. *Science* **339**:826-830.
91. **Yanai H, Ban T, Wang Z, Choi MK, Kawamura T, Negishi H, Nakasato M, Lu Y, Hangai S, Koshiba R, Savitsky D, Ronfani L, Akira S, Bianchi ME, Honda K, Tamura T, Kodama T, Taniguchi T.** 2009. HMGB proteins function as universal sentinels for nucleic-acid-mediated innate immune responses. *Nature* **462**:99-103.

92. **Zhang Z, Yuan B, Bao M, Lu N, Kim T, Liu YJ.** 2011. The helicase DDX41 senses intracellular DNA mediated by the adaptor STING in dendritic cells. *Nat Immunol* **12**:959-965.
93. **Zhang Z, Kim T, Bao M, Facchinetti V, Jung SY, Ghaffari AA, Qin J, Cheng G, Liu YJ.** 2011. DDX1, DDX21, and DHX36 helicases form a complex with the adaptor molecule TRIF to sense dsRNA in dendritic cells. *Immunity* **34**:866-878.
94. **Ariumi Y, Kuroki M, Abe K, Dansako H, Ikeda M, Wakita T, Kato N.** 2007. DDX3 DEAD-box RNA helicase is required for hepatitis C virus RNA replication. *J Virol* **81**:13922-13926.
95. **Yedavalli VS, Neuveut C, Chi YH, Kleiman L, Jeang KT.** 2004. Requirement of DDX3 DEAD box RNA helicase for HIV-1 Rev-RRE export function. *Cell* **119**:381-392.
96. **Barton GM, Medzhitov R.** 2002. Control of adaptive immune responses by Toll-like receptors. *Curr Opin Immunol* **14**:380-383.
97. **Lodoen M, Ogasawara K, Hamerman JA, Arase H, Houchins JP, Mocarski ES, Lanier LL.** 2003. NKG2D-mediated natural killer cell protection against cytomegalovirus is impaired by viral gp40 modulation of retinoic acid early inducible 1 gene molecules. *J Exp Med* **197**:1245-1253.
98. **Peterson KE, Errett JS, Wei T, Dimcheff DE, Ransohoff R, Kuziel WA, Evans L, Chesebro B.** 2004. MCP-1 and CCR2 contribute to non-lymphocyte-mediated brain disease induced by Fr98 polytropic retrovirus infection in mice: role for astrocytes in retroviral neuropathogenesis. *J Virol* **78**:6449-6458.
99. **Prlic M, Williams MA, Bevan MJ.** 2007. Requirements for CD8 T-cell priming, memory generation and maintenance. *Curr Opin Immunol* **19**:315-319.
100. **Zhou L, Chong MM, Littman DR.** 2009. Plasticity of CD4+ T cell lineage differentiation. *Immunity* **30**:646-655.
101. **McHeyzer-Williams M, Okitsu S, Wang N, McHeyzer-Williams L.** 2012. Molecular programming of B cell memory. *Nat Rev Immunol* **12**:24-34.
102. **Allan RS, Smith CM, Belz GT, van Lint AL, Wakim LM, Heath WR, Carbone FR.** 2003. Epidermal viral immunity induced by CD8alpha+ dendritic cells but not by Langerhans cells. *Science* **301**:1925-1928.
103. **Casazza JP, Betts MR, Price DA, Precopio ML, Ruff LE, Brenchley JM, Hill BJ, Roederer M, Douek DC, Koup RA.** 2006. Acquisition of direct antiviral effector functions by CMV-specific CD4+ T lymphocytes with cellular maturation. *J Exp Med* **203**:2865-2877.
104. **Koch MA, Tucker-Heard G, Perdue NR, Killebrew JR, Urdahl KB, Campbell DJ.** 2009. The transcription factor T-bet controls regulatory T cell homeostasis and function during type 1 inflammation. *Nat Immunol* **10**:595-602.
105. **Sun JC, Bevan MJ.** 2003. Defective CD8 T cell memory following acute infection without CD4 T cell help. *Science* **300**:339-342.
106. **Corti D, Voss J, Gamblin SJ, Codoni G, Macagno A, Jarrossay D, Vachieri SG, Pinna D, Minola A, Vanzetta F, Silacci C, Fernandez-Rodriguez BM, Agatic G, Bianchi S, Giacchetto-Sasselli I, Calder L, Sallusto F, Collins P, Haire LF, Temperton N, Langedijk JP, Skehel JJ, Lanzavecchia A.** 2011. A neutralizing antibody selected from plasma cells that binds to group 1 and group 2 influenza A hemagglutinins. *Science* **333**:850-856.
107. **Hessell AJ, Hangartner L, Hunter M, Havenith CE, Beurskens FJ, Bakker JM, Lanigan CM, Landucci G, Forthal DN, Parren PW, Marx PA, Burton DR.** 2007. Fc receptor but not complement binding is important in antibody protection against HIV. *Nature* **449**:101-104.
108. **Hammarlund E, Lewis MW, Hansen SG, Strelow LI, Nelson JA, Sexton GJ, Hanifin JM, Slifka MK.** 2003. Duration of antiviral immunity after smallpox vaccination. *Nat Med* **9**:1131-1137.
109. **Campbell GL, Marfin AA, Lanciotti RS, Gubler DJ.** 2002. West Nile virus. *The Lancet infectious diseases* **2**:519-529.

110. **Dauphin G, Zientara S, Zeller H, Murgue B.** 2004. West Nile: worldwide current situation in animals and humans. *Comp Immunol Microbiol Infect Dis* **27**:343-355.
111. **Komar N, Langevin S, Hinten S, Nemeth N, Edwards E, Hettler D, Davis B, Bowen R, Bunning M.** 2003. Experimental infection of North American birds with the New York 1999 strain of West Nile virus. *Emerg Infect Dis* **9**:311-322.
112. **van der Meulen KM, Pensaert MB, Nauwynck HJ.** 2005. West Nile virus in the vertebrate world. *Arch Virol* **150**:637-657.
113. **Styer LM, Kent KA, Albright RG, Bennett CJ, Kramer LD, Bernard KA.** 2007. Mosquitoes inoculate high doses of West Nile virus as they probe and feed on live hosts. *PLoS Pathog* **3**:1262-1270.
114. **Kipp AM, Lehman JA, Bowen RA, Fox PE, Stephens MR, Klenk K, Komar N, Bunning ML.** 2006. West Nile virus quantification in feces of experimentally infected American and fish crows. *Am J Trop Med Hyg* **75**:688-690.
115. **St Leger J, Wu G, Anderson M, Dalton L, Nilson E, Wang D.** 2011. West Nile virus infection in killer whale, Texas, USA, 2007. *Emerg Infect Dis* **17**:1531-1533.
116. 2011. West Nile virus disease and other arboviral diseases--United States, 2010. *MMWR. Morbidity and mortality weekly report* **60**:1009-1013.
117. **cdc.gov** 2013, posting date. West Nile Virus; Statistics, Surveillance, and Control. Centers for Disease Control and Prevention. [Online.]
118. **Kilpatrick AM, Kramer LD, Jones MJ, Marra PP, Daszak P, Fonseca DM.** 2007. Genetic influences on mosquito feeding behavior and the emergence of zoonotic pathogens. *Am J Trop Med Hyg* **77**:667-671.
119. **Petersen LR, Hayes EB.** 2004. Westward ho?--The spread of West Nile virus. *N Engl J Med* **351**:2257-2259.
120. **Davis CT, Ebel GD, Lanciotti RS, Brault AC, Guzman H, Siirin M, Lambert A, Parsons RE, Beasley DW, Novak RJ, Elizondo-Quiroga D, Green EN, Young DS, Stark LM, Drebot MA, Artsob H, Tesh RB, Kramer LD, Barrett AD.** 2005. Phylogenetic analysis of North American West Nile virus isolates, 2001-2004: evidence for the emergence of a dominant genotype. *Virology* **342**:252-265.
121. **Mann BR, McMullen AR, Swetnam DM, Salvato V, Reyna M, Guzman H, Bueno R, Jr., Dennett JA, Tesh RB, Barrett AD.** 2013. Continued evolution of West Nile virus, Houston, Texas, USA, 2002-2012. *Emerg Infect Dis* **19**:1418-1427.
122. **Smithburn KC, Hughes TP, Burke AW, Paul JH.** 1940. A Neurotropic Virus Isolated from the Blood of a Native of Uganda. *American Journal of Tropical Medicine* **20**:471-472.
123. **Murgue B, Zeller H, Deubel V.** 2002. The ecology and epidemiology of West Nile virus in Africa, Europe and Asia. *Curr Top Microbiol Immunol* **267**:195-221.
124. **Scherret JH, Mackenzie JS, Hall RA, Deubel V, Gould EA.** 2002. Phylogeny and molecular epidemiology of West Nile and Kunjin viruses. *Curr Top Microbiol Immunol* **267**:373-390.
125. **Papa A, Xanthopoulou K, Gewehr S, Mourelatos S.** Detection of West Nile virus lineage 2 in mosquitoes during a human outbreak in Greece. *Clin Microbiol Infect* **17**:1176-1180.
126. **Savini G, Capelli G, Monaco F, Polci A, Russo F, Di Gennaro A, Marini V, Teodori L, Montarsi F, Pinoni C, Pisciella M, Terregino C, Marangon S, Capua I, Lelli R.** 2012. Evidence of West Nile virus lineage 2 circulation in Northern Italy. *Veterinary microbiology*.
127. **Hayes EB, Komar N, Nasci RS, Montgomery SP, O'Leary DR, Campbell GL.** 2005. Epidemiology and transmission dynamics of West Nile virus disease. *Emerging infectious diseases* **11**:1167-1173.
128. **Samuel MA, Diamond MS.** 2006. Pathogenesis of West Nile Virus infection: a balance between virulence, innate and adaptive immunity, and viral evasion. *Journal of virology* **80**:9349-9360.

129. **Murray K, Walker C, Herrington E, Lewis JA, McCormick J, Beasley DW, Tesh RB, Fisher-Hoch S.** 2010. Persistent infection with West Nile virus years after initial infection. *J Infect Dis* **201**:2-4.
130. **Carson PJ, Konewko P, Wold KS, Mariani P, Goli S, Bergloff P, Crosby RD.** 2006. Long-term clinical and neuropsychological outcomes of West Nile virus infection. *Clin Infect Dis* **43**:723-730.
131. **Cook RL, Xu X, Yablonsky EJ, Sakata N, Tripp JH, Hess R, Piazza P, Rinaldo CR.** 2010. Demographic and clinical factors associated with persistent symptoms after West Nile virus infection. *Am J Trop Med Hyg* **83**:1133-1136.
132. **Lim PY, Behr MJ, Chadwick CM, Shi PY, Bernard KA.** 2011. Keratinocytes are cell targets of West Nile virus in vivo. *Journal of virology* **85**:5197-5201.
133. **Daffis S, Samuel MA, Suthar MS, Gale M, Jr., Diamond MS.** 2008. Toll-like receptor 3 has a protective role against West Nile virus infection. *J Virol* **82**:10349-10358.
134. **Fredericksen BL, Keller BC, Fornek J, Katze MG, Gale M, Jr.** 2008. Establishment and maintenance of the innate antiviral response to West Nile Virus involves both RIG-I and MDA5 signaling through IPS-1. *Journal of virology* **82**:609-616.
135. **Fredericksen BL, Smith M, Katze MG, Shi PY, Gale M, Jr.** 2004. The host response to West Nile Virus infection limits viral spread through the activation of the interferon regulatory factor 3 pathway. *Journal of virology* **78**:7737-7747.
136. **Suthar MS, Ma DY, Thomas S, Lund JM, Zhang N, Daffis S, Rudensky AY, Bevan MJ, Clark EA, Kaja MK, Diamond MS, Gale M, Jr.** IPS-1 is essential for the control of West Nile virus infection and immunity. *PLoS Pathog* **6**:e1000757.
137. **Samuel MA, Whitby K, Keller BC, Marri A, Barchet W, Williams BR, Silverman RH, Gale M, Jr., Diamond MS.** 2006. PKR and RNase L contribute to protection against lethal West Nile Virus infection by controlling early viral spread in the periphery and replication in neurons. *Journal of virology* **80**:7009-7019.
138. **Daffis S, Samuel MA, Keller BC, Gale M, Jr., Diamond MS.** 2007. Cell-specific IRF-3 responses protect against West Nile virus infection by interferon-dependent and -independent mechanisms. *PLoS pathogens* **3**:e106.
139. **Daffis S, Samuel MA, Suthar MS, Keller BC, Gale M, Jr., Diamond MS.** 2008. Interferon regulatory factor IRF-7 induces the antiviral alpha interferon response and protects against lethal West Nile virus infection. *Journal of virology* **82**:8465-8475.
140. **Samuel MA, Diamond MS.** 2005. Alpha/beta interferon protects against lethal West Nile virus infection by restricting cellular tropism and enhancing neuronal survival. *Journal of virology* **79**:13350-13361.
141. **Glass WG, Lim JK, Cholera R, Pletnev AG, Gao JL, Murphy PM.** 2005. Chemokine receptor CCR5 promotes leukocyte trafficking to the brain and survival in West Nile virus infection. *The Journal of experimental medicine* **202**:1087-1098.
142. **Diamond MS, Shrestha B, Marri A, Mahan D, Engle M.** 2003. B cells and antibody play critical roles in the immediate defense of disseminated infection by West Nile encephalitis virus. *Journal of virology* **77**:2578-2586.
143. **Shrestha B, Diamond MS.** 2004. Role of CD8+ T cells in control of West Nile virus infection. *Journal of virology* **78**:8312-8321.
144. **Sitati EM, Diamond MS.** 2006. CD4+ T-cell responses are required for clearance of West Nile virus from the central nervous system. *Journal of virology* **80**:12060-12069.
145. **Cho H, Proll SC, Szretter KJ, Katze MG, Gale M, Jr., Diamond MS.** 2013. Differential innate immune response programs in neuronal subtypes determine susceptibility to infection in the brain by positive-stranded RNA viruses. *Nature medicine* **19**:458-464.

146. **Suthar MS, Brassil MM, Blahnik G, McMillan A, Ramos HJ, Proll SC, Belisle SE, Katze MG, Gale M, Jr.** 2013. A systems biology approach reveals that tissue tropism to West Nile virus is regulated by antiviral genes and innate immune cellular processes. *PLoS pathogens* **9**:e1003168.
147. **Daffis S, Suthar MS, Szretter KJ, Gale M, Jr., Diamond MS.** 2009. Induction of IFN-beta and the innate antiviral response in myeloid cells occurs through an IPS-1-dependent signal that does not require IRF-3 and IRF-7. *PLoS pathogens* **5**:e1000607.
148. **Fredericksen BL, Gale M, Jr.** 2006. West Nile virus evades activation of interferon regulatory factor 3 through RIG-I-dependent and -independent pathways without antagonizing host defense signaling. *Journal of virology* **80**:2913-2923.
149. **Lazear HM, Lancaster A, Wilkins C, Suthar MS, Huang A, Vick SC, Clepper L, Thackray L, Brassil MM, Virgin HW, Nikolich-Zugich J, Moses AV, Gale M, Jr., Fruh K, Diamond MS.** 2013. IRF-3, IRF-5, and IRF-7 Coordinately Regulate the Type I IFN Response in Myeloid Dendritic Cells Downstream of MAVS Signaling. *PLoS pathogens* **9**:e1003118.
150. **Paz S, Sun Q, Nakhaei P, Romieu-Mourez R, Goubau D, Julkunen I, Lin R, Hiscott J.** 2006. Induction of IRF-3 and IRF-7 phosphorylation following activation of the RIG-I pathway. *Cell Mol Biol (Noisy-le-grand)* **52**:17-28.
151. **Jiang D, Weidner JM, Qing M, Pan XB, Guo H, Xu C, Zhang X, Birk A, Chang J, Shi PY, Block TM, Guo JT.** 2010. Identification of five interferon-induced cellular proteins that inhibit west nile virus and dengue virus infections. *J Virol* **84**:8332-8341.
152. **Szretter KJ, Brien JD, Thackray LB, Virgin HW, Cresswell P, Diamond MS.** 2011. The interferon-inducible gene viperin restricts West Nile virus pathogenesis. *J Virol* **85**:11557-11566.
153. **Brass AL, Huang IC, Benita Y, John SP, Krishnan MN, Feeley EM, Ryan BJ, Weyer JL, van der Weyden L, Fikrig E, Adams DJ, Xavier RJ, Farzan M, Elledge SJ.** 2009. The IFITM proteins mediate cellular resistance to influenza A H1N1 virus, West Nile virus, and dengue virus. *Cell* **139**:1243-1254.
154. **Daffis S, Szretter KJ, Schriewer J, Li J, Youn S, Errett J, Lin TY, Schneller S, Züst R, Dong H, Thiel V, Sen GC, Fensterl V, Klimstra WB, Pierson TC, Buller RM, Gale M, Jr., Shi PY, Diamond MS.** 2'-O methylation of the viral mRNA cap evades host restriction by IFIT family members. *Nature* **468**:452-456.
155. **Szretter KJ, Daniels BP, Cho H, Gainey MD, Yokoyama WM, Gale M, Jr., Virgin HW, Klein RS, Sen GC, Diamond MS.** 2012. 2'-O methylation of the viral mRNA cap by West Nile virus evades ifit1-dependent and -independent mechanisms of host restriction in vivo. *PLoS Pathog* **8**:e1002698.
156. **Kawai T, Takahashi K, Sato S, Coban C, Kumar H, Kato H, Ishii KJ, Takeuchi O, Akira S.** 2005. IPS-1, an adaptor triggering RIG-I- and Mda5-mediated type I interferon induction. *Nature immunology* **6**:981-988.
157. **Perwitasari O, Cho H, Diamond MS, Gale M, Jr.** 2011. Inhibitor of kappaB kinase epsilon (IKK(epsilon)), STAT1, and IFIT2 proteins define novel innate immune effector pathway against West Nile virus infection. *The Journal of biological chemistry* **286**:44412-44423.
158. **Dong H, Ray D, Ren S, Zhang B, Puig-Basagoiti F, Takagi Y, Ho CK, Li H, Shi PY.** 2007. Distinct RNA elements confer specificity to flavivirus RNA cap methylation events. *J Virol* **81**:4412-4421.
159. **Dong H, Ren S, Zhang B, Zhou Y, Puig-Basagoiti F, Li H, Shi PY.** 2008. West Nile virus methyltransferase catalyzes two methylations of the viral RNA cap through a substrate-repositioning mechanism. *J Virol* **82**:4295-4307.
160. **Pijlman GP, Funk A, Kondratieva N, Leung J, Torres S, van der Aa L, Liu WJ, Palmenberg AC, Shi PY, Hall RA, Khromykh AA.** 2008. A highly structured, nuclease-resistant, noncoding RNA produced by flaviviruses is required for pathogenicity. *Cell host & microbe* **4**:579-591.

161. **Keller BC, Fredericksen BL, Samuel MA, Mock RE, Mason PW, Diamond MS, Gale M, Jr.** 2006. Resistance to alpha/beta interferon is a determinant of West Nile virus replication fitness and virulence. *J Virol* **80**:9424-9434.
162. **Glass WG, McDermott DH, Lim JK, Lekhong S, Yu SF, Frank WA, Pape J, Cheshier RC, Murphy PM.** 2006. CCR5 deficiency increases risk of symptomatic West Nile virus infection. *J Exp Med* **203**:35-40.
163. **Gitlin L, Barchet W, Gilfillan S, Cella M, Beutler B, Flavell RA, Diamond MS, Colonna M.** 2006. Essential role of mda-5 in type I IFN responses to polyriboinosinic:polyribocytidylic acid and encephalomyocarditis picornavirus. *Proceedings of the National Academy of Sciences of the United States of America* **103**:8459-8464.
164. **Sato M, Suemori H, Hata N, Asagiri M, Ogasawara K, Nakao K, Nakaya T, Katsuki M, Noguchi S, Tanaka N, Taniguchi T.** 2000. Distinct and essential roles of transcription factors IRF-3 and IRF-7 in response to viruses for IFN-alpha/beta gene induction. *Immunity* **13**:539-548.
165. **Laurent-Rolle M, Boer EF, Lubick KJ, Wolfinbarger JB, Carmody AB, Rockx B, Liu W, Ashour J, Shupert WL, Holbrook MR, Barrett AD, Mason PW, Bloom ME, Garcia-Sastre A, Khromykh AA, Best SM.** 2010. The NS5 protein of the virulent West Nile virus NY99 strain is a potent antagonist of type I interferon-mediated JAK-STAT signaling. *Journal of virology* **84**:3503-3515.
166. **Shipley JG, Vandergaast R, Deng L, Mariuzza RA, Fredericksen BL.** 2012. Identification of multiple RIG-I-specific pathogen associated molecular patterns within the West Nile virus genome and antigenome. *Virology* **432**:232-238.
167. **van den Hurk AF, Ritchie SA, Mackenzie JS.** 2009. Ecology and geographical expansion of Japanese encephalitis virus. *Annu Rev Entomol* **54**:17-35.
168. **Lindenbach BD, Evans MJ, Syder AJ, Wolk B, Tellinghuisen TL, Liu CC, Maruyama T, Hynes RO, Burton DR, McKeating JA, Rice CM.** 2005. Complete replication of hepatitis C virus in cell culture. *Science* **309**:623-626.
169. **Sumptner R, Jr., Loo YM, Foy E, Li K, Yoneyama M, Fujita T, Lemon SM, Gale M, Jr.** 2005. Regulating intracellular antiviral defense and permissiveness to hepatitis C virus RNA replication through a cellular RNA helicase, RIG-I. *J Virol* **79**:2689-2699.
170. **Yount JS, Gitlin L, Moran TM, Lopez CB.** 2008. MDA5 participates in the detection of paramyxovirus infection and is essential for the early activation of dendritic cells in response to Sendai Virus defective interfering particles. *J Immunol* **180**:4910-4918.
171. **Lazear HM, Pinto AK, Ramos HJ, Vick SC, Shrestha B, Suthar MS, Gale M, Jr., Diamond MS.** 2013. Pattern recognition receptor MDA5 modulates CD8+ T cell-dependent clearance of West Nile virus from the central nervous system. *J Virol* **87**:11401-11415.
172. **Seok J, Warren HS, Cuenca AG, Mindrinos MN, Baker HV, Xu W, Richards DR, McDonald-Smith GP, Gao H, Hennessy L, Finnerty CC, Lopez CM, Honari S, Moore EE, Minei JP, Cuschieri J, Bankey PE, Johnson JL, Sperry J, Nathens AB, Billiar TR, West MA, Jeschke MG, Klein MB, Gamelli RL, Gibran NS, Brownstein BH, Miller-Graziano C, Calvano SE, Mason PH, Cobb JP, Rahme LG, Lowry SF, Maier RV, Moldawer LL, Herndon DN, Davis RW, Xiao W, Tompkins RG.** 2013. Genomic responses in mouse models poorly mimic human inflammatory diseases. *Proc Natl Acad Sci U S A* **110**:3507-3512.
173. **Guzman MG, Halstead SB, Artsob H, Buchy P, Farrar J, Gubler DJ, Hunsperger E, Kroeger A, Margolis HS, Martinez E, Nathan MB, Pelegrino JL, Simmons C, Yoksan S, Peeling RW.** 2010. Dengue: a continuing global threat. *Nat Rev Microbiol* **8**:S7-16.
174. **Bente DA, Rico-Hesse R.** 2006. Models of dengue virus infection. *Drug Discov Today Dis Models* **3**:97-103.

175. **Pichlmair A, Schulz O, Tan CP, Naslund TI, Liljestrom P, Weber F, Reis e Sousa C.** 2006. RIG-I-mediated antiviral responses to single-stranded RNA bearing 5'-phosphates. *Science* **314**:997-1001.
176. **Gillespie LK, Hoenen A, Morgan G, Mackenzie JM.** 2010. The endoplasmic reticulum provides the membrane platform for biogenesis of the flavivirus replication complex. *Journal of virology* **84**:10438-10447.
177. **Hoenen A, Liu W, Kochs G, Khromykh AA, Mackenzie JM.** 2007. West Nile virus-induced cytoplasmic membrane structures provide partial protection against the interferon-induced antiviral MxA protein. *The Journal of general virology* **88**:3013-3017.
178. **Lindenbach BDR, C., M.** 2001. Flaviviridae: The Viruses and Their Replication, p. 991-1041. *In* Knipe D, M.; Howley, P., M. (ed.), *Fields Virology*, 4 ed, vol. 1. Lippincott Williams and Wilkins, Philadelphia, PA, USA.
179. **Querec TD, Akondy RS, Lee EK, Cao W, Nakaya HI, Teuwen D, Pirani A, Gernert K, Deng J, Marzolf B, Kennedy K, Wu H, Bennouna S, Oluoch H, Miller J, Vencio RZ, Mulligan M, Aderem A, Ahmed R, Pulendran B.** 2009. Systems biology approach predicts immunogenicity of the yellow fever vaccine in humans. *Nature immunology* **10**:116-125.
180. **Malathi K, Saito T, Crochet N, Barton DJ, Gale M, Jr., Silverman RH.** 2010. RNase L releases a small RNA from HCV RNA that refolds into a potent PAMP. *Rna* **16**:2108-2119.
181. **Baum A, Sachidanandam R, Garcia-Sastre A.** 2010. Preference of RIG-I for short viral RNA molecules in infected cells revealed by next-generation sequencing. *Proc Natl Acad Sci U S A* **107**:16303-16308.
182. **Rehwinkel J, Tan CP, Goubau D, Schulz O, Pichlmair A, Bier K, Robb N, Vreede F, Barclay W, Fodor E, Reis e Sousa C.** 2010. RIG-I detects viral genomic RNA during negative-strand RNA virus infection. *Cell* **140**:397-408.
183. **Westaway EG, Mackenzie JM, Khromykh AA.** 2002. Replication and gene function in Kunjin virus. *Curr Top Microbiol Immunol* **267**:323-351.
184. **Nomaguchi M, Teramoto T, Yu L, Markoff L, Padmanabhan R.** 2004. Requirements for West Nile virus (-) and (+)-strand subgenomic RNA synthesis in vitro by the viral RNA-dependent RNA polymerase expressed in *Escherichia coli*. *J Biol Chem* **279**:12141-12151.
185. **Arpaia N, Godec J, Lau L, Sivick KE, McLaughlin LM, Jones MB, Dracheva T, Peterson SN, Monack DM, Barton GM.** 2011. TLR signaling is required for *Salmonella typhimurium* virulence. *Cell* **144**:675-688.
186. **Oshiumi H, Sakai K, Matsumoto M, Seya T.** 2010. DEAD/H BOX 3 (DDX3) helicase binds the RIG-I adaptor IPS-1 to up-regulate IFN-beta-inducing potential. *Eur J Immunol* **40**:940-948.
187. **Ishikawa H, Barber GN.** 2008. STING is an endoplasmic reticulum adaptor that facilitates innate immune signalling. *Nature* **455**:674-678.
188. **Moon SL, Anderson JR, Kumagai Y, Wilusz CJ, Akira S, Khromykh AA, Wilusz J.** 2012. A noncoding RNA produced by arthropod-borne flaviviruses inhibits the cellular exoribonuclease XRN1 and alters host mRNA stability. *Rna* **18**:2029-2040.
189. **Miyashita M, Oshiumi H, Matsumoto M, Seya T.** 2011. DDX60, a DEXD/H box helicase, is a novel antiviral factor promoting RIG-I-like receptor-mediated signaling. *Mol Cell Biol* **31**:3802-3819.
190. **Widner WR, Wickner RB.** 1993. Evidence that the SKI antiviral system of *Saccharomyces cerevisiae* acts by blocking expression of viral mRNA. *Mol Cell Biol* **13**:4331-4341.
191. **Reimold AM, Iwakoshi NN, Manis J, Vallabhajosyula P, Szomolanyi-Tsuda E, Gravallesse EM, Friend D, Grusby MJ, Alt F, Glimcher LH.** 2001. Plasma cell differentiation requires the transcription factor XBP-1. *Nature* **412**:300-307.

192. **Tardif KD, Mori K, Kaufman RJ, Siddiqui A.** 2004. Hepatitis C virus suppresses the IRE1-XBP1 pathway of the unfolded protein response. *J Biol Chem* **279**:17158-17164.
193. **Vitali P, Scadden AD.** 2010. Double-stranded RNAs containing multiple IU pairs are sufficient to suppress interferon induction and apoptosis. *Nat Struct Mol Biol* **17**:1043-1050.
194. **Wang Y, Cella M, Gilfillan S, Colonna M.** 2010. Cutting edge: polyinosinic:polycytidylic acid boosts the generation of memory CD8 T cells through melanoma differentiation-associated protein 5 expressed in stromal cells. *J Immunol* **184**:2751-2755.
195. **McCartney S, Vermi W, Gilfillan S, Cella M, Murphy TL, Schreiber RD, Murphy KM, Colonna M.** 2009. Distinct and complementary functions of MDA5 and TLR3 in poly(I:C)-mediated activation of mouse NK cells. *J Exp Med* **206**:2967-2976.
196. **Lange CM, Jacobson IM, Rice CM, Zeuzem S.** 2013. Emerging therapies for the treatment of hepatitis C. *EMBO Mol Med*.
197. **Ferenci P, Scherzer TM, Kerschner H, Rutter K, Beinhardt S, Hofer H, Schoniger-Hekele M, Holzmann H, Steindl-Munda P.** 2008. Silibinin is a potent antiviral agent in patients with chronic hepatitis C not responding to pegylated interferon/ribavirin therapy. *Gastroenterology* **135**:1561-1567.
198. **Onoguchi K, Yoneyama M, Takemura A, Akira S, Taniguchi T, Namiki H, Fujita T.** 2007. Viral infections activate types I and III interferon genes through a common mechanism. *J Biol Chem* **282**:7576-7581.
199. **Iwasaki A, Medzhitov R.** 2010. Regulation of adaptive immunity by the innate immune system. *Science* **327**:291-295.
200. **Longhi MP, Trumpfheller C, Idoyaga J, Caskey M, Matos I, Kluger C, Salazar AM, Colonna M, Steinman RM.** 2009. Dendritic cells require a systemic type I interferon response to mature and induce CD4+ Th1 immunity with poly IC as adjuvant. *J Exp Med* **206**:1589-1602.
201. **Kasturi SP, Skountzou I, Albrecht RA, Koutsonanos D, Hua T, Nakaya HI, Ravindran R, Stewart S, Alam M, Kwissa M, Villinger F, Murthy N, Steel J, Jacob J, Hogan RJ, Garcia-Sastre A, Compans R, Pulendran B.** 2011. Programming the magnitude and persistence of antibody responses with innate immunity. *Nature* **470**:543-547.
202. **Querec T, Bennouna S, Alkan S, Laouar Y, Gorden K, Flavell R, Akira S, Ahmed R, Pulendran B.** 2006. Yellow fever vaccine YF-17D activates multiple dendritic cell subsets via TLR2, 7, 8, and 9 to stimulate polyvalent immunity. *J Exp Med* **203**:413-424.
203. **Nejentsev S, Walker N, Riches D, Egholm M, Todd JA.** 2009. Rare variants of IFIH1, a gene implicated in antiviral responses, protect against type 1 diabetes. *Science* **324**:387-389.
204. **Funke B, Lasitschka F, Roth W, Penzel R, Meuer S, Saile M, Gretz N, Sido B, Schirmacher P, Autschbach F.** 2011. Selective downregulation of retinoic acid-inducible gene I within the intestinal epithelial compartment in Crohn's disease. *Inflamm Bowel Dis* **17**:1943-1954.