MAVS IS ESSENTIAL FOR REGULATION OF INNATE IMMUNE SIGNALING DURING RIFT VALLEY FEVER VIRUS INFECTION

by

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List of Abbreviations

AIM2: absent in melanoma 2
ALT: alanine transaminase
ASC: apoptosis-associated speck-like protein containing a C-terminal caspase recruitment domain
ATP: adenosine triphosphate
BMDMs: bone marrow-derived macrophages
CARD: caspase recruitment domain
Cardif: caspase recruitment domain adaptor-inducing IFN-β
cDCs: conventional dendritic cells
CLEC: C-type lectin domain family member
CLR: C-type lectin receptor
CTD: C-terminal RNA binding domain
DC: dendritic cell
DCIR: dendritic cell immunoreceptor
DC-SIGN: dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin
DNA: deoxyribonucleic acid
dsRNA: double-stranded RNA
DTT: dithiothreitol
EDTA: ethylenediaminetetraacetic acid
eIF: eukaryotic translation initiation factor
ELISA: enzyme-linked immunosorbent assay
EMCV: encephalomyocarditis virus
ER: endoplasmic reticulum
ERK: extracellular signal-regulated protein kinase
FACS: fluorescence-activated cell sorting
FBS: fetal bovine serum
G-CSF: granulocyte colony-stimulating factor
GM-CSF: granulocyte-macrophage colony-stimulating factor
HCV: hepatitis C virus
HEK: human embryonic kidney
IFN: interferon
IFNAR: interferon (alpha and beta) receptor 1
IFNGR: interferon gamma receptor 1
Ig: immunoglobulin
IKK: IkappaB kinase
IL: interleukin
IL-1RA: IL-1 receptor antagonist
IP-10: IFN-γ-inducible protein 10
IPS-1: interferon-beta promoter stimulator 1
IRF: interferon regulatory factor
ISGs: IFN-stimulated genes
ISRE: interferon-sensitive response element
JEV: Japanese encephalitis virus
KC: keratinocyte-derived chemokine
kD: kilodalton
KO: knockout
LEW: Lewis
LGP2: laboratory of genetics and physiology 2
LIX: lipopolysaccharide-induced chemokine
LRR: leucine-rich repeat
L-SIGN: liver/lymph node-specific intercellular adhesion molecule 3-grabbing nonintegrin
MAL: MyD88 adaptor-like protein
MAVS: mitochondrial antiviral signaling protein
M-CSF: macrophage colony-stimulating factor
MDA5: melanoma differentiation-associated gene-5
MGL: macrophage galactose lectin
MIG: mitogen-inducible gene
MIP: macrophage inflammatory protein
MNDAL: myeloid nuclear differentiation antigen-like
MOI: multiplicity of infection
mRNA: messenger ribonucleic acid
MyD88: myeloid differentiation factor 88
NBD: nucleotide-binding domain
NEMO: nuclear factor kappa B essential modifier
NF-κB: nuclear factor kappa B
NLR: Nod-like receptor
NLRC: nucleotide-binding oligomerization domain, leucine rich repeat, and caspase recruitment domain-containing protein
NLRP: nucleotide-binding oligomerization domain, leucine rich repeat, and pyrin domain containing protein
NO: nitric oxide
NOD: nucleotide-binding oligomerization domain
NOS2: nitric oxide synthase 2
ODN: oligodeoxynucleotides
PAMP(s): pattern associated molecular pattern(s)
PBS: phosphate-buffered saline
pDCs: plasmacytoid dendritic cells
PKC: protein kinase C
PKR: double-stranded RNA-dependent protein kinase
Poly(I:C): polyinosinic-polycytidylic acid
Poly(ICLC): polyriboinosinic-polyribocytidylic acid complexed with poly-L-lysine and carboxymethylcellulose
PRR(s): pattern recognition receptor(s)
PVDF: polyvinylidene difluoride
RANTES: regulated upon activation normal T cell expressed and presumably secreted
RdRp: RNA-dependent RNA polymerase
RIG-I: retinoic acid-inducible gene I
RIPA: radioimmunoprecipitation assay buffer
RLR: RIG-I-like receptor
RNA: ribonucleic acid
RNAi: ribonucleic acid interference
ROS: reactive oxygen species
RSV: respiratory syncytial virus
RVF: Rift Valley fever
RVFV: Rift Valley fever virus
SAP30: Sin3A-associated protein, 30 kDa
SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis
shRNA: short hairpin ribonucleic acid
siRNA: small interfering ribonucleic acid
SNP: single nucleotide polymorphism
ssRNA: single-stranded ribonucleic acid
SV: Sendai virus
TAK: transforming growth factor-β activated kinase
TBK: TANK binding kinase
TCA: trichloroacetic acid
TFIIH: general transcription factor IIH
TIR: Toll-interleukin (IL)-1 receptor
TLR: Toll-like receptor
TNF-α: tumor necrosis factor alpha
TRAF: tumor necrosis factor receptor-associated factor
TRAM: TRIF-related adaptor molecule
TRIF: TIR domain-containing adaptor inducing IFN-β
TRIM: tri-partite motif

UTR: untranslated region

UV: ultraviolet

VEGF: vascular endothelial growth factor

VISA: virus-induced signaling adaptor

VSV: vesicular stomatitis virus

WF: Wistar-Furth

WT: wild-type
MAVS is Essential for Regulation of Innate Immune Signaling during Rift Valley Fever Virus Infection

Abstract

by

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Rift Valley fever virus (RVFV) is a Category A pathogen that impacts both humans and animals. Once the virus enters the cells of its host, innate immune receptors can recognize viral patterns and initiate an anti-viral signaling cascade leading to type I interferon or inflammatory cytokine response. While type I interferon has been demonstrated to be protective against morbidity and mortality during RVFV infection, studies delineating how type I interferon is generated in response to the virus are lacking. The studies contained within this thesis present a mechanism for pathways leading to type I interferon production and inflammasome activation using BSL-2 attenuated strains of RVFV. Initial importance of retinoic acid-inducible gene I (RIG-I) in mediating interferon signaling was established using human embryonic kidney 293 cells that were transfected with luciferase reporters and dominant-negative constructs. Findings were confirmed using bone marrow-derived macrophages and dendritic cells from wild-type and immune receptor knockout mice. These studies established that mitochondrial antiviral signaling protein (MAVS), an adaptor protein utilized by RIG-I-like receptors during signaling, was key for type I interferon production. Upstream receptors RIG-I and melanoma differentiation-associated gene-5 (MDA5) contributed to interferon signaling.
Interestingly, infection of macrophages and dendritic cells with RVFV only resulted in minor amplification of the virus. MAVS was also shown to be crucial for interleukin (IL)-1β processing during inflammasome activation. RVFV activates the nucleotide-binding oligomerization domain, leucine rich repeat, and pyrin domain containing protein 3 (NLRP3) inflammasome through the adaptor apoptosis-associated speck-like protein containing a C-terminal caspase recruitment domain (ASC) and caspase-1 in bone marrow-derived dendritic cells. This is the first evidence that RVFV can activate the NLRP3 inflammasome during infection. The importance of MAVS during infection was assessed using intranasal infection of mice with the attenuated RVFV strain rMP-12. MAVS was protective against mortality and morbidity as assessed by viral burden and serum alanine transaminase levels. We have demonstrated that MAVS is crucial for type I interferon production and protection during infection. We have also highlighted the importance of MAVS during NLRP3 activation by RVFV and bring to attention the pleiotropic effects of this protein during host immune response.
Chapter 1 Introduction

Rift Valley fever virus as a human and agricultural disease

Rift Valley fever virus (RVFV) is in the family \textit{Bunyaviridae}, genus \textit{Phlebovirus}, and is an emerging RNA virus of great importance for social and agricultural reasons. In humans, the virus typically causes febrile illness; however, a subset of patients experience more severe manifestations such as encephalitis, retinitis, hepatic necrosis, ocular symptoms, hemorrhagic fever or death [1]. While general risk factors for exposure to the virus have been identified, it is unknown why some patients experience more mild symptoms, while others ultimately succumb to RVFV infection. Factors such as route of exposure, viral dose, and host genetics may impact these outcomes and require further investigation.

During outbreaks, the major routes for RVFV transmission to humans are primarily through infected mosquitoes or virus aerosol exposure. Transmission of RVFV is persistent even between epidemics, as is evident with seropositive children born after recorded outbreaks [2]. RVFV can be maintained between outbreaks through mosquito and wildlife populations. Intermittent cycles of prolonged flooding alternating with periods of drought aid in the propagation of RVFV via floodwater Aedes species, a group of mosquitoes that can pass RVFV from generation to generation in their eggs in a process called transovarial transmission. This process requires that eggs go through a dessication cycle [1], followed by rapid local flooding events that favor mosquito
propagation [3]. The emergence of floodwater Aedes species following heavy rains thus allows reintroduction of RVFV into local vertebrate fauna, which can initiate local zoonotics and epidemics. When flooding has occurred, *Culex* mosquitoes deposit egg rafts on the water that further amplify mosquito populations and enhance virus spread upon hatching. Semi-arid areas of Africa that experience periodic flooding during El Niño/Southern Oscillation events are particularly prone to RVF outbreaks.

In addition to being an important human pathogen, RVFV also wreaks havoc among domesticated livestock. The virus induces high mortality in young livestock and causes pregnant animals to miscarry regardless of pregnancy stage [1]. Processing of infected animals or handling of aborted animal material can lead to aerosol exposure in humans. Given that many human risk factors for RVFV exposure include interactions with animals, the link between animal infection status and human health is very strong. Besides immediate effects on an individual’s health and ownership of livestock herds, RVFV outbreaks have far reaching economic implications, affecting producers and stakeholders and can lead to trade bans on live animals from afflicted countries [4]. One example of the devastating economic impacts resulting from an RVFV outbreak comes from estimates using a social accounting matrix based on 2003 data, in which it was estimated that the Kenyan economy may have lost up to Ksh 2.1 billion (or US $32 million) during the 2007 RVFV outbreak [4].

RVFV can also be transmitted through less common routes. Two case reports suggest that RVFV can be transmitted vertically in human patients. The first case was a 2 day old boy
who presented with fever, difficulty breathing, and poor feeding and was referred to a second hospital under the presumed diagnosis of sepsis [5]. Family history indicated that six of nine family members were ill with febrile illness and one family member died of confirmed Rift Valley fever (RVF). Two weeks after family members presented with illness, the mother developed fever, headache, dizziness, and malaise and the child was delivered four days later. On his fifth day of life, the child was brought to the second hospital and was jaundiced, lethargic, and had continued difficulty breathing. The child’s serum tested positive for RVF-immunoglobulin (Ig)M and the mother’s serum was positive for RVF-IgG. Assessing IgM levels in the infant allows for differentiation from any maternal antibodies since IgG is the only antibody which can cross the placenta in significant amounts; thus, any IgM present would have been generated by the infant [6]. Two days after admission, the child exhibited anemia and disseminated intravascular coagulation and required repeated transfusions. Six days after admission, the child had hepatomegaly and ecchymoses and died from infection. The second report was of a Sudanese mother and newborn child who tested positive for RVF-IgM [7]. The mother had been ill with fever, dizziness, and general malaise for 10 days while pregnant. She had originally been admitted as a case of malaria, but was resistant to artemether treatment. The newborn child had a palpable liver and spleen, a skin rash, and developed jaundice within a matter of days.

Many risk factors are associated with exposure to RVFV. A survey conducted in Kenya during the 2007 outbreak determined that severe disease in humans was associated with contact with or herding of animals, caring for animals during birthing, handling an
aborted animal fetus, or being a herdsperson [8]. The only factor assessed that was associated with human death was consuming or handling animal products from sick animals. Interestingly, factors affecting mosquito exposure were not associated with severe disease [8]. In a separate study, researchers examined risk factors associated with being seropositive for RVFV between outbreaks in Kenya. Of the factors studied, older age, male gender, rural location, disposing of an aborted animal fetus, and presence of eye disease were strongly associated with RVFV exposure [2]. Nearly four times as many individuals were seropositive in the rural region of Gumarey compared with individuals within the town area of Sogan-Godud; this result is surprising given that geographically these regions are only 500 meters apart. The authors concluded that the differences in disease distribution were attributed to more widespread mosquito and animal exposure in the rural village [2].

Epidemiology and public health threat

Rift Valley fever virus was first isolated in Kenya in the early 1930’s and has since been found throughout continental Africa (Figure 1.1). In 1977-1979, a large outbreak of RVFV occurred in Egypt and this was the first time the virus had been detected north of the Sahara Desert [1]. In 1979, RVFV was first isolated off the mainland in Madagascar [9]. The first time RVFV was found outside of Africa was in 2000 when it crossed continents to the Arabian Peninsula with cases reported in Saudi Arabia and Yemen [10]. Severe outbreaks of RVFV occur periodically and are heavily influenced by weather patterns. El Niño/Southern Oscillation phenomenon and rainfall are factors used in
prediction of RVFV outbreaks as flooding increases mosquito breeding grounds and thus viral amplification [11,12].

![Figure 1.1 Distribution map of Rift Valley fever virus](image)

*Countries with endemic disease and substantial outbreaks of RVF:*
- Gambia, Senegal, Mauritania, Namibia, South Africa, Mozambique, Zimbabwe, Zambia, Kenya, Sudan, Egypt, Madagascar, Saudi Arabia, Yemen

*Countries known to have some cases, periodic isolation of virus, or serologic evidence of RVF:*
- Botswana, Angola, Democratic Republic of the Congo, Congo, Gabon, Cameroon, Nigeria, Central African Republic, Chad, Niger, Burkina Faso, Mali, Guinea, Tanzania, Malawi, Uganda, Ethiopia, Somalia

*Figure 1.1 Distribution map of Rift Valley fever virus*
Locations of RVFV outbreaks and regions where the virus has been isolated. Obtained from Center for Disease Control website with permission ([www.cdc.gov](http://www.cdc.gov)).

The most notable recent RVFV outbreak occurred between 2006 and 2008 and affected many countries including Somalia, Tanzania, Kenya, South Africa, and Sudan [12]. Additionally, Madagascar experienced a large outbreak in 2008-2009. Based on viral isolate sequences, this outbreak was likely an extension of the 2006-2007 outbreak that affected many East African countries during this period [13]. The unprecedented outbreak of RVFV that occurred in Mauritania in 2010 occurred after uncharacteristically high rainfall [14]. During this outbreak, camels were the index case and were thought to
be major amplifiers of the virus. Spread of the virus eventually resulted in human cases and deaths. It is thought that heavy rain allowed for amplification of competent vectors and allowed RVFV infection to gain a foothold within animal populations. The virus may have been introduced to new geographical areas when infected animals were transported by truck in search of areas for grazing. Geographical expansion of the virus through animal populations further emphasizes the link between animal and human health and RVFV disease.

Besides having evident and persistent impact on endemic regions, RVFV is considered a select agent by both the US Department of Health and Human Services and Department of Agriculture. Select agents have potential to harm animal or plant health or products or can be a danger to society. RVFV also falls into the highest classified threat level for a biological, Category A, by the National Institute of Health and National Institute of Allergy and Infectious Disease for its impact on public health and ease in dissemination or transmittance. Although RVFV is not currently found in the United States, competent mosquito vectors are present and could easily perpetuate RVFV dissemination upon introduction. North American mosquitoes that have high vector potential include many Culex and Aedes species [15].

**RVFV structure and replication cycle**

Similar to other hemorrhagic fever viruses, RVFV has an RNA (ribonucleic acid) genome. The genome of RVFV is considered to be in the negative sense and is tri-partite,
meaning three independent RNA segments are packaged within each virion. The three genomic segments are termed small (S), medium (M), and large (L) as seen in Figure 1.2. The S segment is ambisense and codes for the nucleocapsid in anti-viral sense (anti-genome orientation) and non-structural protein NSs in the viral sense (genome orientation). The M segment encodes for NSm, the carboxy-terminal glycoprotein (Gc) and amino-terminal glycoprotein (Gn), and a 78 kilodalton (kD) protein of unknown function. The L segment encodes for the RNA-dependent RNA polymerase (RdRp) that is necessary for transcribing positive-sense RNA from the negative-sense genome and vice versa. Nucleic acid within the RVFV virion is packaged with nucleocapsid and RdRp proteins to form the ribonucleoprotein complex that is needed for transcription [16]. The nucleocapsid protein takes on a hexameric form when it binds RNA [16].

![Diagram of RVFV genomic segments and protein products.](image)

**Figure 1.2 RVFV genomic segments and protein products.**

RVF V has a small (S), medium (M), and large (L) RNA segment packaged within each virion. Depicted above in white are the locations of genes within each segment. Protein products from each segment are denoted in blue. The S segment is ambisense and codes for N in the anti-viral sense while NSs is in the viral sense. M and L segments code in the negative sense. The M protein is first processed into Gc and a 78 kDa protein of unknown function. The 78 kDa protein is further processed into NSm and Gn. The L protein encodes the RNA-dependent RNA polymerase used in replication.
Each virion of RVFV is spherical and enveloped with spikes of glycoproteins Gn and Gc embedded in the lipid bilayer. The glycoproteins form a capsomer with a $T = 12$ icosahedral lattice [17]. The most likely model for arrangement suggests that the Gn ectodomain forms the majority of the protruding side while the Gc proteins form the capsomer base (Figure 1.3) [18].

![Figure 1.3](image)

*Figure 1.3 Arrangement of glycoproteins in RVFV virion.*
A) Glycoprotein shell composed of Gn and Gc with $T=12$ icosahedral arrangement. B) Cartoon of Gn (yellow) and Gc (multicolor) arrangement from a representative triangular face of the 20 faces present on the virion shell. Figure reproduced with permission from Rusu et al [18].

Entry is the first aspect of viral infection and is mediated by interactions between viral glycoproteins and host receptors. Entry mechanisms and subsequent steps of RVFV replication are detailed in Figure 1.4. Recently, C-type lectin receptor (CLR) family member dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin (DC-SIGN) was found to serve as an entry receptor for many members of the family *Bunyaviridae*, including RVFV [19]. DC-SIGN is expressed on tissue macrophages and interstitial dendritic cells [20]. RVFV entry is also enhanced by the presence of the
glycosaminoglycan heparin sulfate, although entry can still occur in absence of this molecule [21]. The exact mechanism of RVFV entry into cells is unknown and is controversial within the literature. Studies suggest the possibility for calveolin or clathrin mediated uptake into cells (both of which would depend upon dynamin II). However the route of entry identified has varied depending on the cell types investigated and the inhibitory methods that were utilized [22,23]. Another report suggests that entry can be affected by protein kinase C (PKC)ε, an enzyme used in macropinocytosis, implicating yet another route that can be utilized by the virus [24]. After RVFV enters the host cell, the virus begins its uncoating process within the endosome. The acidic environment of the endosome leads to rearrangement of Gc [22] and presumably allows for separation of RVFV from DC-SIGN, as is observed with the phlebovirus Uukuniemi virus [19].

Replication of all Bunyaviruses occurs exclusively in cytoplasm. Interestingly, it is believed that negative-strand RNA viruses produce less double-stranded RNA (dsRNA) intermediates than positive-strand RNA viruses, dsRNA viruses, or deoxyribonucleic acid (DNA) viruses during their replication cycle [25]. Double-stranded RNA is an important immunologically recognized viral pattern that leads to host anti-viral response through Toll-like receptors (TLRs) as well as through the RIG-I-like receptor (RLR) family member melanoma differentiation-associated gene-5 (MDA5). While it is not uncommon for RNA viruses to exhibit high mutation rates due to lack of proofreading mechanisms by the RdRp protein, RVFV exhibits a relatively low mutation rate, although there is some evidence for RNA segment reassortment [26,27]. One explanation for a lower mutation rate may be that the virus must retain the ability to infect both vertebrate
and invertebrate hosts. *In vitro* evidence supports this theory, as virus serially passed in either just mammalian or insect cells eventually accumulated attenuating mutations, while virus passaged in alternating cell types was genetically stable [28].

Host mRNAs possess a 5’ 7-methylguanosine cap that functions to enhance recognition of RNA during translation, prevent RNA degradation by 5’ exonucleases, and direct pre-mRNA splicing and export of RNA from the nucleus [29]. Some viruses are able to initiate translation without acquisition of a cap structure, such as members of the picornavirus family or calcivirus family. These viruses instead recruit a translation initiation complex independently of a cap, or utilize a virus encoded protein to directly interact with translation initiation factor eIF4E, respectively [29]. In order to protect their mRNA as well as ensure efficient translation, many viruses have adapted strategies that also utilize cap structures. Some viruses are able to synthesize new caps de novo, either using host encoded RNA pol II or virus encoded proteins. An alternative mechanism that is utilized by members of the families *Bunyaviridae*, *Arenaviridae*, or *Orthomyxoviridae* is to instead obtain pre-synthesized cap structures from host mRNAs.

Bunyaviruses, including RVFV, are unable to synthesize a messenger (m)RNA cap. Instead, they have evolved a mechanism termed “cap snatching” in which endonuclease activity on the RNA-dependent RNA polymerase can remove the 5’ 7-methylguanosine end of eukaryotic mRNA and use this as a primer for synthesis of viral mRNA [29]. Addition of a 5’ 7-methylguanosine cap to viral RNA may also have the evolutionary advantage of masking the 5’ triphosphate structure that can be added by RNA
polymerases during replication. The triphosphate moiety in combination with dsRNA is strongly recognized by cytoplasmic host receptor retinoic acid-inducible gene I (RIG-I) and can lead to downstream anti-viral signaling cascades [30,31]. Despite this evasion mechanism, detection of the triphosphate moiety can still occur on viral genomic segments that lack a cap. For instance, the genome of RVFV is believed to have an exposed 5′-triphosphate moiety that, upon recognition by RIG-I, can induce interferon (IFN)-β signaling [32].

**Figure 1.4 RVFV replication cycle.**
The first step in RVFV entry into a cell is adsorption, or attachment. Entry mechanisms are debated and may include clathrin or caveolin-mediated endocytosis or macropinocytosis. A pH change in the endosome allows for uncoating and release of viral contents into the cytoplasm. RVFV replication occurs in the cytoplasm. Endonuclease activity from the RNA-dependent RNA polymerase mediates cap-snatching during transcription. RVFV matures at the golgi and gains its envelope by budding into the lumen of the golgi. RVFV lyses the cell before moving on to a new target.
RVFV maturation occurs at the golgi. Glycoproteins G\(n\) and G\(c\) are first recruited to the golgi, followed by other RVFV proteins and the viral genome. The golgi localization signal for RVFV and other Bunyavirus family members was determined to reside in G\(n\). Glycoprotein G\(c\) expressed alone localizes to the endoplasmic reticulum (ER) and not the golgi [33,34]. G\(n\) (G2) and G\(c\) (G1) of RVFV relative Punta Toro virus were found to be linked by disulfide bonds and rapidly formed G\(n\)-G\(c\) heterodimers, or G\(n\) (G2) homodimers [35]. RVFV genomic segments S, M, and L each contain packaging signals. S and M genomic segments are able to become packaged without aid from other segments [36]. RNA produced from the M segment may contain a signal in the 5’ untranslated region (UTR) that allows for co-packaging of M and S segments, as deletion of this region abolished co-packaging [36]. The full-length L segment is not packaged effectively compared to shorter deletion mutants, suggesting that RNA compaction of the L segment may be necessary during assembly [37]. G\(n\) is necessary for packaging of the nucleocapsid and RdRp proteins [38]. After assembly, RVFV buds into the lumen of the golgi and lyses the cell during escape. The nucleocapsid protein and RVFV genome are required for effective release [38].

**Innate immunity and RVFV pathogenesis**

The RVFV genome encodes two major virulence factors, NS\(s\) and NS\(m\). NS\(m\) suppresses host apoptosis that would normally be mediated through caspase-8 [39]. Alternatively, virulence factor NS\(s\) has a multitude of functions. NS\(s\) specifically interferes with IFN-\(\beta\) production by associating with SAP30 and co-repressors on the IFN-\(\beta\) promoter to prevent transcription [40]. In addition, NS\(s\) prevents assembly of the general transcription
factor IIH (TFIIH) by sequestering p44 and XPB subunits and thus limits host RNA synthesis and production of host anti-viral cytokines in general [41]. This block of host transcription can have a substantial impact as early as 8 hours after infection and continues to decline over time. Furthermore, NSs down regulates the p62 subunit of TFIIH after translation [42]. This process is mediated by the proteasome and can occur in the cell nucleus. The effects of NSs on patient outcome have been observed in nature as well. Clone 13 is a naturally occurring isolate of RVFV from a mild patient case that has a major deletion in the NSs gene coding region. Without functional NSs protein, clone 13 was rendered avirulent in hamsters and mice [43]. Taken together, these data indicate ways in which viral virulence factors NSs and NSm can subvert the host immune response during infection.

The NSs protein was discovered to be the RVFV produced factor that induces pregnant animals to abort. This has major implications for vaccine design as an issue with previous vaccines was their potential for also causing abortion in pregnant animals and hesitancy to vaccinate for this reason [44,45,46]. Filamentous nuclear NSs was shown to interact with pericentromeric γ-satellite sequences of cellular DNA [47]. This interaction was dependent upon the region of NSs that binds to SAP30 [47]. Interactions between NSs and cellular DNA in vitro lead to chromosomal cohesion and segregation defects. This interaction is believed to be the mechanism behind fetal defects and abortion caused during RVFV infection of pregnant animals. NSs is dispensable for the viral life cycle and development of vaccines lacking NSs and NSm genes are underway [48].
Double-stranded RNA-dependent protein kinase (PKR) is a serine-threonine kinase present in latent form in cells and is upregulated in response to type I IFN. During viral infection, PKR is activated by viral RNA containing a triphosphate moiety and short stem-loop structure and shuts down translation by phosphorylating the α subunit of eukaryotic translation initiation factor (eIF)-2 [49]. NSs inhibits cellular anti-viral activity by initiating degradation of PKR via the proteasome, and thus prevents phosphorylation of eIF2-α and translation inhibition [49,50].

Type I interferon and RVFV infection

Type I IFN has been shown to be protective against pathogenesis during RVFV infection. Known interferon inducer polyriboinosinic-polyribocytidylic acid in a complex with poly-L-lysine and carboxymethylcellulose (poly[ICLC]) protected both mice and hamsters against challenge with virulent ZH501 RVFV [51]. It is likely that protection occurred due to activation of IFN signaling rather than general cell activation as administration of glucan, an activator of macrophages, extended time until death but did not significantly affect mouse survival [51]. RVFV was also shown to be sensitive to type I IFN in vitro [51]. The effects of IFN on RVFV pathogenesis were more directly assessed when wild-type (WT) and interferon (alpha and beta) receptor 1 (IFNAR) deficient mice were infected with virulent ZH548 as well as attenuated strains of RVFV. WT mice infected with ZH548 had delayed mortality compared to IFNAR −/− mice, although all mice eventually succumbed to infection [52]. Even more striking, attenuated RVFV strains lacking virulence in WT mice (up to 21 days) caused 100% mortality of all
IFNAR−/− mice within 2-3 days of infection. In contrast, all interferon gamma receptor 1 (IFNGR) deficient mice survived challenge with attenuated RVFV strains [52]. In conjunction with increased virulence, RVFV strain ZH548 was a poor inducer of type I IFN in mice in comparison with attenuated RVFV strains [52]. These studies emphasize the protective effects of type I IFN signaling during infection.

Induction of type I IFN signaling can induce Mx protein activity. Mx proteins are guanosine triphosphatases that elicit anti-viral activity. Rat Mx2 protein has been found to interfere with RVFV replication [53]. Mx2 is found within the cytoplasm of the cell where RVFV replication occurs. Rats have one nuclear Mx protein (ratMx1) and two cytoplasmic Mx proteins (ratMx2 and ratMx3). Similarly, mice possess nuclear MuMx1 proteins and cytoplasmic MuMx2 proteins that interfere with replication of many viruses including Hantaan virus (also in the family Bunyaviridae). Caution should be taken when interpreting effects of interferon stimulated genes in mice as MuMx2 is not functional within many inbred mouse strains due to an open reading frame mutation.

**RVFV induction of NF-κB signaling**

Nuclear factor-κB (NF-κB) is a key transcription factor that regulates induction of many inflammatory genes and can become active during viral infection as part of the anti-viral response. During resting state, NF-κB family members p65 (RelA), RelB, c-Rel, p50/p105 (NF-κB-1), and p52/p100 (NF-κB-2) form homo- or heterodimers that are bound by IκB proteins and are retained in the cytoplasm [54]. Activation occurs when the
IKK kinase (IKK) triumvirate, formed by IKK-α, IKK-β, and IKK-γ (also known as NF-κB essential modifier or NEMO), phosphorylates IκB. This induces ubiquitination and degradation of IκB, followed by exposure of the nuclear localization signal on NF-κB, allowing for translocation to the nucleus and initiation of transcription [54].

RVFV is known to activate NF-κB signaling that can lead to production of inflammatory cytokines. Both ZH501 and attenuated MP-12 strains have been demonstrated to induce NF-κB phosphorylation of p65 at serine residue 536 [55,56]. Induction of NF-κB by MP-12 occurs rapidly, with phosphorylation detected as early as one hour post infection [56]. Although the IKKs usually serve to phosphorylate IκB during infection to lead to activation of NF-κB signaling, IKK-β can have an alternative function of phosphorylating the NSs protein of RVFV. Interestingly, curcumin (also known as turmeric) has been shown to interfere with RVFV replication by preventing phosphorylation of NSs protein by IKK-β2 (a low molecular weight form of IKK-β present in infected cells) and altering the cell cycle [56]. Thus, while IKK can initiate anti-viral NF-κB mediated signaling, active IKK can also lead to enhanced RVFV replication.

Pattern recognition receptors and danger recognition receptors

While viruses have evolved proteins with virulence functions to shut down the host immune response and allow for perpetuation of virus production, host cells also have mechanisms to recognize broad pathogen patterns and elicit protective measures to prevent viral replication and spread to new target cells. Pattern recognition receptors (PRRs) are expressed on many different cell types including immune and non-immune
cells and recognize pathogen associated molecular patterns (PAMPs) [57]. The benefit of recognizing broad patterns is that the same receptors can recognize a multitude of pathogens and are recognizing substrates that cannot easily be discarded by the invading organism to avoid detection. Examples of PRRs that are important for viral recognition include the Toll-like receptors (TLRs), RLRs, C-type lectin receptors (CLRs), and nucleotide-binding oligomerization domain (Nod)-like receptors (NLRs). Activation of PRR signaling can lead to downstream anti-viral responses.

**Toll-like receptors**

One family of PRRs that is important for responses against viruses as well as many other pathogens is the Toll-like receptor family. Toll-like receptors (TLRs) are mammalian homologues of the protein Toll, a receptor originally characterized in *Drosophila melanogaster*, which facilitates anti-fungal responses [58]. TLRs are type I transmembrane receptors and are comprised of a leucine-rich repeat domain (LRR) that recognizes PAMPs, a transmembrane helix, and the Toll-interleukin (IL)-1 receptor (TIR) domain that executes signaling [59,60]. Approximately 13 TLRs have been identified in mammalian cells. TLRs 1 through 9 are conserved in both humans and mice, while TLR10 has been identified in humans and TLRs 11 through 13 have been found in mice [57].

TLRs can be found on the cell surface or within the endosome of the cell. TLRs that have potential to recognize viral nucleic acid within the endosome of the cell include TLRs 3,
7, 8, 9 and require endosomal acidification and maturation for signaling to occur [59]. TLRs 3, 7, and 8 can recognize single-stranded RNA (ssRNA) viruses while TLR9 recognizes CpG DNA motifs found in DNA viruses and bacteria. Extracellular TLRs typically recognize extracellular pathogens such as bacteria or fungus. Recent evidence suggests certain surface TLRs can also recognize viral patterns. In addition to being located on the cell surface of fibroblasts and epithelial cells, TLR3 is also expressed within intracellular vesicles in immune cells [61]. The first report of surface TLR4 involvement during virus infection detailed activation of TLR4 by the fusion protein of respiratory syncytial virus (RSV) [62]. Since then, TLR4 has also been demonstrated to recognize viral glycoproteins of Ebola and to signal in response to HCMV [63,64]. Surface TLR2 activation can be induced in response to HSV-1 glycoprotein {Cai, 2013 #278]. Signaling cascades initiated by TLRs that respond to viral patterns are detailed in Figure 1.5.

TLR3 recognizes many forms of double-stranded RNA (dsRNA), including synthetic dsRNA ligand polyinosinic-polycytidylic acid (poly(I:C)), genomic material from dsRNA viruses, and dsRNA replication intermediates from ssRNA viruses [61]. Innate signaling experiments in which siRNA (small interfering RNA) is utilized should be interpreted with caution as there are several reports demonstrating siRNA can activate TLR3 signaling through TIR domain-containing adaptor inducing IFN-β (TRIF) in a non-sequence dependent manner [65,66]. While TLR3 utilizes the adaptor molecule TRIF for either NF-κB mediated signaling or production of type I IFN, these pathways are differentially activated. TLR3 can activate NF-κB mediated signaling through two routes
involving interactions with downstream proteins and its C or N terminal domains [61,67].

To generate type I IFN, TRIF alternatively binds tumor necrosis factor receptor-associated factor (TRAF)3 and TANK binding kinase (TBK)-1 for activation of interferon regulatory factor (IRF)-3 to lead to a viral interfering state [61].

While TLR3 signals through the adaptor protein TRIF, all other TLRs signal through adaptor molecule myeloid differentiation factor 88 (MyD88) via TIR/TIR domain interactions [60]. TLR4 can signal either through MyD88 (in conjunction with MyD88 adaptor-like protein (Mal)) or TRIF (in concert with TRIF-related adaptor molecule (TRAM)) pathways depending upon the ligand that is being sensed [61,68].

Human TLR7 and TLR8 and mouse TLR7 are able to recognize ssRNA and imidazoquinoline compounds. In contrast to human TLR8, mouse TLR8 was originally believed to be unresponsive, but has subsequently been determined to activate NF-κB signaling in response to a combination of polyT oligodeoxynucleotides (ODN) and imidazoquinoline immune response modifiers [69]. TLR7 and TLR8 signal through the adaptor molecule MyD88. MyD88 interacts with the TLRs through its TIR domain and also contains a death domain. Signaling through the death domain recruits members of the IRAK family and leads to a signaling cascade that activates NF-κB signaling [61]. Alternatively, TLR7 or 8 signaling through MyD88 and IRAK family members can also lead to phosphorylation of IRF7, leading to its dissociation from the signaling complex and translocation to the nucleus to activate type I IFN production [61].
**Figure 1.5 TLR mediated sensing of viruses.**

Toll-like receptor family members TLR2 and TLR4 are found on the cell surface and become activated through interactions with glycoproteins of select viruses. In certain cell types, TLR3 can also be expressed on the cell surface and recognizes dsRNA patterns. Within the endosome of the cell, TLRs 7, 8, and 3 can detect viral RNA patterns while TLR9 recognizes CpG DNA motifs. Signaling through these pathways can lead to activation of NF-κB mediated cytokine production or type I interferon through IRFs. Above: (...) represents that this pathway has already been detailed in another part of the figure. Adapted from Jensen and Thomsen [70], Kawai and Akira [61].

**RIG-I-like receptors**

Members of the RLR family include RIG-I, MDA5, and laboratory of genetics and physiology 2 (LGP2) as shown in Figure 1.6. These proteins are similar structurally and contain a DExD/H box helicase domain and a C-terminal RNA binding domain (CTD). RIG-I and MDA5 contain two consecutive caspase recruitment domain (CARD) motifs
that can interact with CARD domains on adaptor proteins to perpetuate signaling [71]. LGP2 lacks CARD domains necessary for signaling and is believed to instead regulate RIG-I and MDA5 responses [72]. The CTD of RIG-I and LGP2 binds 5’ tri-phosphate single-stranded RNA as well as dsRNA, while the CTD of MDA5 binds dsRNA with reduced affinity [73]. RIG-I exhibits helicase activity that is dependent upon adenosine triphosphate (ATP) and is capable of unwinding RNA with at least a five nucleotide long 3’ overhang [74].

RIG-I and MDA5 signaling occurs when their CARD domains bind K63 polyubiquitin chains, leading to IRF3 activation and induction of IFN-β [71]. For RIG-I, this process is dependent upon ATP and 5’-triphosphate RNA [75] and is mediated by tripartite motif (TRIM)25 E3 ubiquitin ligase [76]. Polyubiquitin binding initiates formation of a RIG-I tetramer complex with four ubiquitin chains that initiates potent anti-viral signaling [71]. MDA5 binds RNA as a dimer with a footprint of 16-18 base pairs [77].

RIG-I and MDA5 can bind to signaling adaptor protein mitochondrial antiviral signaling protein (MAVS), also known as interferon-beta promoter stimulator 1 (IPS-1), during viral infection to activate interferon pathways or NF-κB signaling. MAVS was shown to form high molecular weight aggregates in response to viral infection downstream of both RIG-I and MDA5 signaling [78]. Interestingly, these functional aggregates convert native MAVS into its active form through a prion-like mechanism and lead to IRF3 mediated induction of IFN [78]. This conformational change among MAVS is able to better amplify downstream signaling.
Figure 1.6 RLR mediated sensing of viruses.

RLRs RIG-I, MDA5, and LGP2 are found within the cytoplasm of the cell. RIG-I recognizes the 5’ triphosphate moiety on viral RNA whereas MDA5 is responsive towards long dsRNA intermediates. RIG-I and MDA5 signal through adaptor protein MAVS (also known as IPS-1) to initiate NF-κB and type I interferon signaling. LGP2 is not involved directly in signaling but instead modulates responses through its interactions with RIG-I and MDA5 via its CARD domain. Figure adapted from Kawai and Akira [61].

The role of RNA helicase family member LGP2 during viral infection is highly controversial. While it was initially believed that LGP2 negatively regulated RIG-I induction of type I IFN through sequestering of PAMPS [72], subsequent work has demonstrated that LGP2 is required for RIG-I and MDA5 antiviral responses in vitro [79]. Another study utilized synthetic ligand poly(I:C) as a tool for studying innate pathway activation. This group demonstrated that when levels of transfected poly(I:C) were kept low, LGP2 could enhance IFN-β induction [80]. LGP2 did not heighten RIG-I mediated response to poly(I:C), but instead negatively regulated this signaling at higher concentrations. The role of LGP2 during viral infection in vivo is also poorly understood.
When mice or human cells were infected with H3N2 non-attenuated influenza A virus strains, LGP2 could mediate reduction in type I IFN response. However, no role for LGP2 was observed during infection with H1N1 strains that do not induce IRF3 mediated signaling [81]. The role of LGP2 during viral infection is cryptic and its impact upon RLR signaling may vary depending upon the virus being utilized and the amount of virus used in challenge.

**C-type lectin receptors**

C-type lectin receptors (CLRs) are a family of proteins that lack enzymatic activity and require calcium to bind carbohydrate patterns through their carbohydrate recognition domain. CLRs can either be membrane bound or soluble and can bind mannose (type I) or galactose-like products (type II) [82]. Membrane bound CLRs that have been demonstrated to bind viruses include DC-SIGN, liver/lymph node-specific intercellular adhesion molecule 3-grabbing nonintegrin (L-SIGN), Langerin, macrophage galactose lectin (MGL), C-type lectin domain family member (CLEC)5A, CLEC2, CD206, and DC immunoreceptor (DCIR) [83]. Collectins are soluble forms of CLRs that have a collagenous domain and that aid in host defense [82]. Under normal cellular conditions, CLRs have many functions such as mediating endocytosis, adhesion of cells, and serum glycoprotein turnover. During disease state, CLRs can recognize pathogen patterns and yield protective or harmful effects in the host [82].
CLR can facilitate entry of many viruses [84,85,86]. An interesting case of CLR impact on virus infectivity has been demonstrated with dengue virus. Dengue virus produced in primary dendritic cells (DC) contains complex glycans and has a low affinity for DC-SIGN, rendering it unable to infect other dendritic cells. However, virus generated in DCs was still capable of infecting L-SIGN positive cells. Virus produced in insect cell line C6/36 was able to infect DC- or L-SIGN positive cells [87]. Thus, the source of the virus affected its glycosylation pattern which ultimately affected entry.

In other instances, CLR do not act as functional receptors for the virus, but instead mediate overall infection of cells. For example, mutated glycoproteins from filoviruses can still bind CLR on cells but infectivity is impaired, indicating other cellular factors were necessary for mediating steps subsequent to attachment, such as internalization or membrane fusion, during viral replication [88]. CLR serve as immune modulators during infection with RSV. RSV can bind DC-SIGN and L-SIGN but these CLR are not required for entry. Instead, binding of RSV glycoproteins to DC- or L-SIGN dampens dendritic cell activation, as evidenced by higher levels of IFN-α, macrophage inflammatory protein (MIP)-1α, and MIP-1β upon neutralization of DC- and L-SIGN. In addition, binding RSV glycoproteins to DC- and L-SIGN leads to phosphorylation of extracellular signal-regulated protein kinase (ERK)1 and ERK2 [89]. In this case, viral interaction with CLR suppresses host immune response.
**Inflammasomes**

Inflammasomes are multi-protein complexes that can be activated during viral infection and lead to processing of interleukin (IL)-1β and IL-18. Inflammasomes that assemble in response to viruses include AIM2 (absent in melanoma 2), NLRP3, and RIG-I (Figure 1.7). AIM2 has been shown to function as an inflammasome in response to DNA viruses, such as vaccinia virus and mouse cytomegalovirus, leading to activation of caspase-1 in an ASC (apoptosis-associated speck-like protein containing a CARD) dependent manner [90].

Inflammasome complexes are also capable of responding to RNA viruses. The NLRP3 inflammasome becomes activated in response to many RNA viruses, including respiratory syncytial virus, EMCV, influenza, HCV, and VSV [91,92,93,94,95,96]. RIG-I has been demonstrated to act as an inflammasome in response to the RNA virus VSV; however, this report is controversial as another group showed that VSV instead activated the NLRP3 inflammasome in primed cells [96,97].

Viruses can activate inflammasome signaling through different mechanisms. The first “signal”, or initiation of NF-κB mediated signaling, can occur in response to TLR signaling or through RLRs as pattern recognition receptors [91,97]. Viral RNA can serve as a pattern that activates NLRP3 signaling. For example, synthetic RNA analogs poly(I:C) and ssRNA40 have been shown to induce NLRP3 activation [94]. Additionally, viral proteins such as Viroporin 2B produced by EMCV and the influenza protein M2, a proton-selective ion channel, have also been demonstrated to activate NLRP3 [92,93].
Figure 1.7 Inflammasomes that are activated during viral infection.

Inflammasome activation by viruses requires two signals. The first signal is typically driven through pattern recognition receptors such as TLRs or RLRs or can be initiated through TNF-α signaling through its receptor. The first “signal” results in activation of NF-κB signaling and accumulation of pro-IL-1β and pro-IL-18 and inflammasome components. The second signal provided during viral infection is more elusive but can include reactive oxygen species (ROS) production or potassium efflux. Three inflammasomes have been identified to be active during virus infection and include AIM2, NLRP3, and RIG-I and recognize the difference panel of viruses listed above. In addition, the RIG-I inflammasome is also activated by 5’ triphosphate double strand RNA (5’-ppp-dsRNA). Activation of these inflammasomes through adaptor protein ASC leads to activation of caspase-1 and cleavage of pro-IL-1β and pro-IL-18 into their mature forms.

Increase in reactive oxygen species production and potassium efflux can act as a second signal to activate caspase-1 during viral infection [91]. While active viral infection usually drives inflammasome activation, IL-1β production can sometimes occur in the absence of replication. HCV mediated activation of IL-1β production is not dependent upon replication and instead occurs in response to live or UV-inactivated virus that has
been phagocytosed by macrophages [95]. Mechanisms leading to IL-1β production vary between viruses and each virus may produce a different protein or pattern that is crucial for inflammasome activation.

Production of IL-1β during viral infection can be protective or harmful. For example, during West Nile virus infection protective IL-1β responses resulted in decreased viral load in the central nervous system and higher quality CD8+ T cell responses. Mice deficient in NLRP3 and lacking IL-1β signaling experienced increased inflammation that corresponded with an increase in viral burden compared to WT mice [98]. In contrast, infection with Epstein-Barr virus or cytomegalovirus infection can lead to macrophage activation syndrome that causes bone marrow suppression, hepatitis, and death. Effects of the disease can be reversed with an IL-1 blockade indicating the pathogenic effects of IL-1 signaling [99]. The need for balance between excessive and insufficient IL-1 responses is illuminated with a virus-induced model of multiple sclerosis in mice. Mice infected with Theiler’s murine encephalomyelitis virus experience demyelinating disease similar to multiple sclerosis in humans. In these studies, high levels of IL-1 resulted in pathogenic Th17 responses. However, a lack of IL-1 signaling resulted in poor T cell activation and residual viral infection in the spinal cord of infected mice. In this case, deviation from moderate IL-1 responses leads to progression of the demyelinating disease [100]. Thus, downstream effects of IL-1 signaling need to be carefully assessed for each system.
Nod-like receptors and innate signaling

Many NLRs can influence the outcome of viral infection through mechanisms outside of inflammasome involvement. They can serve to act as pattern recognition receptors or immune modulators of other innate pathways that become activated. During infection, nucleotide-binding oligomerization domain containing (NOD)2 can act as a PRR to initiate downstream antiviral signaling by activating IRF3 and IFN-β through interactions with adaptor molecule MAVS. This interaction relies upon the LRR and nucleotide-binding domain (NBD) regions of NOD2. NOD2 has been demonstrated to activate type I IFN signaling in response to synthetic ssRNA and RSV [101].

NOD-, LRR-, and CARD- containing protein (NLRC)5 has been shown to dampen innate immune responses during viral infection by preventing IKKα and IKKβ phosphorylation and NF-κB activation. Interestingly, expression of NLRC5 was also shown to be dependent on the NF-κB pathway [102]. In addition, NLRC5 suppresses type I IFN signaling through interaction with RIG-I and MDA5, but not the adaptor MAVS or TBK1. This effect is mediated through binding of the NLRC5 CARD domain to the CARD domain of RIG-I [102].

NLRX1 (also known as NOD5 or NOD9) is a cytoplasmic NLR that is homologous to NOD3 [103]. NLRX1 is found at the outer mitochondrial membrane and blocks IFN signaling through RIG-I (and possibly MDA5) via interactions with MAVS [104,105]. During viral infection, NLRX1 serves to reduce inflammation by preventing TRAF6
mediated activation of NF-κB [105]. Infection of \textit{Nlrx1} \textsuperscript{-/-} mice with influenza virus results in an increased level of inflammatory cytokines and increased morbidity, but can also lead to faster time until viral clearance compared to infection in WT mice [105].

\textbf{Natural resistance against RVFV mediated pathogenesis}

While route of exposure and viral properties may alter patient outcome during RVFV infection, host genetics may also play a role in resistance to RVFV. Variable responses to RVFV infection have occurred in rat and gerbil models [106]. Evidence suggests that a discrete factor for resistance against pathogenesis caused by RVFV can be inherited as a Mendelian dominant gene [106,107]. Susceptible rats exhibit morbidity such as liver and vascular involvement. While susceptible Wistar-Furth (WF) and resistant Lewis (LEW) rats used in this study were shown to have very different serum and organ titers within 24 hours of RVFV injection, evidence suggests that this is due to immune response controlling replication and not due to differences in viral entry [106]. This finding also hints that immune control of the virus occurs rapidly and may be due to innate rather than adaptive mechanisms.

The existence of a discrete genetic resistance factor that offers protection in response to RVFV became even more apparent as two scientific groups used the same strains of rats obtained from different vendors and observed drastically different results. Peters et al. investigated pathogenesis of the virulent ZH510 strain of RVFV in LEW, MAXX, and WF rats that were obtained from Microbiological Associates, Inc. in Walkersville, MD
In these studies, all inoculated WF rats succumbed rapidly to infection while MAXX and LEW rats exhibited initial protection. However, delayed viral growth in MAXX and LEW rats may have ultimately led to development of encephalitis in some rats. Another group also utilized WF and LEW rats (obtained from M&B (previously known as Mollegard Breeding & Research Center, Ry, Denmark) and obtained disparate results. In this case, rats were infected with virulent RVFV strain ZH548 and LEW rats developed hepatitis and died while all WF rats survived [107]. Cross-breeding of the rats allowed for transfer of resistance. The resistance phenotype was cell type specific as hepatocytes obtained from resistant rats had reduced RVFV titers compared to hepatocytes from susceptible rats. However, glial cells isolated from both susceptible and protected mice had similarly high titers [107]. Results from rat breeding experiments suggest that the gene of interest is not related to the major histocompatibility complex [106].

While type I IFN has a prominent role in protection against RVFV, studies suggest that the protective gene may not be downstream of IFN signaling. When hepatocytes isolated from protected and susceptible rats were exogenously stimulated with rat type I IFN, similar levels of RVFV replication were observed [107]. Thus, influence of the protective factor was not enhanced with additional type I IFN compared to susceptible cells as we would expect if it was an interferon stimulated gene.

While the gene that confers resistance to RVFV has yet to be identified, these studies highlight the importance of determining key innate signaling pathways that are activated...
during RVFV infection. Once pathways are further delineated, polymorphisms in genes that play an important role in altering RVFV infection could be further examined for their potential in mediating protection.
Chapter 2 RNA helicase pathway is critical for type I interferon production in response to RVFV

Abstract

RVFV is an emerging RNA virus with devastating economic and social consequences. Clinically, RVFV induces a gamut of symptoms ranging from febrile illness to retinitis, hepatic necrosis, hemorrhagic fever, and death. It is known that type I IFN responses can be protective against severe pathology; however, it is unknown which innate immune receptor pathways are crucial for mounting this response. Using both in vitro assays and in vivo mucosal mouse challenge, we demonstrate here that RNA helicases are critical for IFN production by immune cells and that signaling through the helicase adaptor molecule MAVS is protective against mortality and more subtle pathology during RVFV infection. In addition, we demonstrate that Toll-like receptor-mediated signaling is not involved in IFN production, further emphasizing the importance of the RNA cellular helicases in type I IFN responses to RVFV. Findings in this chapter are published in Journal of Virology [109] and are reproduced with permission. ©American Society for Microbiology [J. Virol. 87, 2013, 4846-4860, DOI 10.1128/JVI.01997-12].

Introduction

RVFV is a negative-strand RNA virus of the genus Phlebovirus (family Bunyaviridae) [1]. Recurrent outbreaks of Rift Valley fever virus have been documented throughout
Africa, with first reports in Kenya in 1930 [110]. Recently, the virus was established outside of continental Africa in Madagascar [13] and the Arabian Peninsula [10]. RVFV is virulent in young livestock and induces spontaneous abortion in pregnant animals. In humans, infection results in febrile illness and a subset of patients experience retinitis, hepatitis, encephalitis, hemorrhagic fever, or death. High case fatality rates were reported for the 2006-2008 outbreaks in several countries for patients presenting with severe symptoms suggestive of RVFV [12].

Although it is unknown which host factors determine whether an RVFV-infected patient experiences mild or severe disease manifestations, it is known that type I interferon (IFN) responses can be protective. Administration of the type I IFN inducer poly(I:C) stabilized with polylysine and carboxymethyl cellulose was shown to protect rodents from mortality associated with RVFV infection [51]. More directly, type I IFN has been shown to guard against pathology when recombinant or human-derived IFN-α was administered to rhesus macaques prior to, or shortly after, challenge with virulent RVFV [111]. In addition, mice lacking type I IFN receptor (IFNAR) were more susceptible to clinical isolate and lab-attenuated strains of RVFV than WT mice [52].

The innate immune receptors that recognize RVFV and induce a signaling cascade leading to type I IFN production have not been well characterized. Major classes of innate PRRs include CLR, TLR, NLR, and RLR, also known as cellular RNA helicases. These classic PRRs recognize PAMPs and initiate signaling that can lead to an inflammatory or viral replication-interfering state within the host [61]. Transmembrane-
bound CLRs recognize carbohydrate patterns and can be utilized for viral entry or can regulate IFN responses during viral infection [85,112,113]. The NLR family includes more than 20 proteins in humans. Recent work has demonstrated that NLR member NOD2 is capable of recognizing ssRNA viral genomes and can initiate IFN signaling through the adaptor molecule MAVS (also known as IPS-1, CARD adaptor-inducing IFN-β [Cardif], and virus-induced signaling adaptor [VISA]) [101]. In addition, many inflammasomes can be activated by viral nucleic acid [90,91,94,114].

Endosomal TLRs (TLR3, TLR7/8, TLR9) recognize nucleic acids and target recognition of intracellular pathogens, such as viruses [61]. TLR3 recognizes viral genomic double-stranded RNA (dsRNA) or replication intermediates and the synthetic dsRNA ligand poly(I:C) [61]. TLR7 and human TLR8 recognize viral ssRNA [115]. In contrast, murine TLR8 does not recognize ssRNA motifs and was originally thought to be nonfunctional; however, subsequent work has shown that stimulation of murine TLR8 and downstream NF-κB activation can be achieved with immune response modifiers used in combination with poly(T) oligodeoxynucleotide [69]. The potential role for TLR8 recognition of vaccinia virus and its DNA genome is under debate [116,117]. Because RVFV is a negative-sense ssRNA virus, TLR3, TLR7, or TLR8 could likely contribute to recognition and antiviral signaling.

Intracellular cytoplasmic DExD/H box helicase family members, including RIG-I, MDA5, and LGP2 [118], can also sense viral PAMPs. RIG-I recognizes shorter blunt-end dsRNA intermediates [119] with a 5’-triphosphate moiety [30] that are detected during
viral replication of negative-strand RNA viruses. MDA5 induces IFN-β production in response to the synthetic dsRNA ligand poly(I:C) and to picornaviruses [120]. It was generally assumed that MDA5 recognized longer dsRNA intermediates, although further analysis suggests that more complex structures, such as branched RNA, may be needed to initiate responses [121]. Both RIG-I and MDA5 have two caspase activation and recruitment domains (CARD) [122] and signal through the adaptor molecule MAVS to induce NF-κB and IFN signaling [123]. Although LGP2 lacks a CARD signaling motif, it has been shown to be required for RIG-I and MDA5 antiviral signaling [79] in contrast to a previous report suggesting that it may have function as a negative regulator [72].

Previous studies using RVFV clone 13 (a naturally attenuated strain lacking most of the NSs gene) demonstrated that shRNA targeting RIG-I abrogated IFN-β promoter activation in HEK (human embryonic kidney) 293T cells in response to isolated RVFV RNA, whereas shRNA targeting of MDA5 did not hinder IFN responses [32]. In addition, treatment of isolated clone 13 RNA with shrimp alkaline phosphatase greatly reduced IFN-β stimulation [32], suggesting that the 5’ triphosphate moiety was responsible for activation through RIG-I. These studies were an important first look into innate recognition of RVFV; however, interpretation of these studies is limited as HEK cells lack expression of TLR family members that potentially could contribute to antiviral responses [124,125,126]. It is likely that innate immune cells, such as dendritic cells and macrophages, will be the major sources of IFN during active RVFV infection, and these cells could potentially recognize and respond to RVFV using different receptor pathways than non-immune cells. In the present study, we investigated the role of RLR and TLR
signaling in the induction of IFN responses to RVFV, and the role that these innate pathways play in protection from mucosal challenge with RVFV.

**Materials and Methods**

**Mice.** C57BL/6 mice were purchased from Jackson Laboratories. Mice deficient in TLR3, MyD88, and TRIF were generated by Shizuo Akira (Osaka University, Osaka, Japan). RIG-I knockout mice were provided by Michael Gale, Jr. (University of Washington). MDA5-deficient mice were provided by Marco Colonna (Washington University). MAVS-deficient mice were generated by Zhijian Chen (University of Texas Southwestern). Wild-type controls from the same generation were used in MAVS animal experiments, since this mouse strain is not fully backcrossed onto C57BL/6. Mice were maintained in filter-top microisolator cages in ventilated racks. Animal experiments were carried out under conditions approved by the Institutional Animal Care and Use Committee at Case Western Reserve University and Saint Louis University.

**Cells, viruses, and reagents.** Attenuated RVFV strains rMP-12 and NSs del were a gift from Shinji Makino (University of Texas Medical Branch, Galveston, TX). RVFV rMP-12 strain (recovered from cells using reverse genetics and containing a XhoI site) was derived from the MP-12 strain, initially made by passaging patient isolate ZH548 twelve times in the presence of 5-fluorouracil [127]. The NSs del strain was derived from rMP-12 and lacks virulence factor NSs [128]. These samples were handled under biosafety
level 2 conditions unless previously inactivated. Samples containing virus were inactivated by cross-linking RNA with 2 joules of ultraviolet (UV) light (Stratagene).

Immune cells were derived from the bone marrow of wild-type and knockout mice using standard protocols [129,130]. For generation of conventional dendritic cells (cDCs), bone marrow was isolated from femurs and tibias and was cultured in DC media composed of RPMI 1640 media with L-glutamine, 10% fetal bovine serum (FBS) (Atlanta Biologicals), 50 μM 2-mercaptoethanol (Sigma-Aldrich), 10 mM sodium pyruvate, 10 mM penicillin-streptomycin, and 1/30 total volume addition of J558L supernatant that contains granulocyte-macrophage colony-stimulating factor (GM-CSF) [131]. Cells were supplemented on days 3 and 6 with a half volume of fresh medium and J558L supernatant. Semi-adherent cells were harvested between days 8 to 10. More than 80% of the cDC population was CD11b+ and CD11c+ as determined by fluorescence-activated cell sorting (FACS) analysis. To generate mixed plasmacytoid dendritic cells (pDCs) and cDCs, DC media was used with the addition of 1 μg of FLT-3 ligand fusion protein (Bioexpress)/ml. Cells were fed on days 3 and 6 and were collected for use between days 8 to 10. The percent pDC/cDC populations were evaluated by FACS analysis. Bone marrow-derived macrophages (BMDMs) were generated by culturing total bone marrow in high glucose Dulbecco’s modified Eagle medium supplemented with 10% FBS, 10mM penicillin-streptomycin, 10mM sodium pyruvate, 10 mM HEPES, 50 μM 2-mercaptoethanol, with 20% L929 cell conditioned supernatant containing macrophage colony-stimulating factor (M-CSF). Cells were cultured for 5 days, after which the media and non-adherent cells were removed, and fresh media were added every other day until
harvest between days 7 and 10. Typically, BMDMs were > 95% positive for CD11b and F4/80 as determined by FACS analysis. Differentiated cells were counted, plated, and stimulated at 10^5 cells in 200 μl (total volume) per well in a 96-well plate. For infection studies with multiple MOI of virus, cells were stimulated with media only control, poly(I:C) (Imgenex), Pam3Cysk4 (InvivoGen), 5’-triphosphate RNA (InvivoGen), Gardiquimod (InvivoGen), R848 (InvivoGen), Sendai virus (Charles River Laboratories), or Rift Valley fever virus for 24 h. Combined supernatant and lysate samples were harvested for further analysis via enzyme-linked immunosorbent assay (ELISA) and plaque assay unless otherwise designated. For time course studies of IFN-α and viral production, cells were stimulated with RVFV NSs del at multiplicity of infection (MOI) of 1 for 2 h to allow for adsorption, after which the inoculum was removed and replaced with medium. Supernatant was harvested at 6, 12, and 24 h, and IFN-α levels were determined by ELISA. The viral load in the supernatant was determined via plaque assay.

**FACS analysis of cell surface markers.** The purity of bone marrow-derived dendritic cells and macrophages was confirmed with flow cytometry. J558L-derived cDCs and FLT3L-derived mixed pDCs/cDCs were stained with fluorochrome-linked antibodies for CD11c and CD11b (eBioscience). The purity of macrophages was confirmed with F4/80 and CD11b antibodies (eBioscience). Cells were incubated on ice for 15 min with FBS to block Fc receptors and then with primary antibodies for 30 min on ice. Cells were fixed in 2% formaldehyde and were analyzed using a BD LSRII flow cytometer and FlowJo software (Tree Star, Inc.).
**Stimulation of transfected cell lines.** HEK293XL cell lines stably expressing TLR7 and TLR8 were purchased from InvivoGen. A total of 40,000 cells were plated per well in a 96-well plate and transfected several hours later using PolyJet (Signagen). HEK293XL cells and HEK293XL cells stably transfected with TLR7 or TLR8 were transiently transfected with a luciferase reporter plasmid for IFN-β (promoter region) or NF-κB and constitutively active Renilla. The dominant-negative constructs RIG-I Dn (RIG-I helicase domain) and MyD88 Dn (TIR domain only) were transfected at the quantities described previously [72,132]. The total DNA in each well was adjusted to 140 ng with pcDNA 3.1. The cells were stimulated with media, RVFV, or control ligands SV, Gardiquimod (InvivoGen), R848 (InvivoGen), or 400 ng of poly(I:C) (Amersham) transfected into cells with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol for 18 h. Luminescence was assessed using a Promega Glomax 96 microplate luminometer. Luciferase values were normalized to Renilla and a medium-only control.

**Western blotting.** For Western blot analysis, 10^6 HEK293XL cells were transfected using the following conditions: (i) untransfected cells, (ii) pcDNA3.1, Renilla, and IFN-β reporter plasmid, (iii) Renilla, IFN-β reporter, and dominant-negative construct, or (iv) dominant-negative construct alone. The total amounts of DNA per transfection condition were equal. Cell lysates were prepared in radioimmunoprecipitation assay (RIPA) buffer with 1 mM dithiothreitol (DTT) and a protease inhibitor cocktail (Thermo Scientific). Samples were boiled for 5 min in Laemmli buffer. Protein samples were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 4% bisacrylamide stacking and 15% bisacrylamide resolving gels. Proteins were transferred
to polyvinylidene difluoride (PVDF) membrane and were blotted with anti-Flag (Sigma) or anti-AU-1 (Novus) in 4% nonfat dried milk. Blots were stripped with Western blot stripping buffer (Thermo Scientific) and were re-probed with anti-β-actin antibody A-15 (Santa Cruz).

**Experimental infection of mice.** For subcutaneous and intranasal challenges of mice, the animals were infected with $3.5 \times 10^3$ or $3.5 \times 10^4$ PFU of RVFV rMP-12. Intranasal challenges were performed by administering 10 μl of virus into the noses of anesthetized animals. Mortality was recorded throughout the experiment. Surviving mice were weighed daily (to 21 days), and all experiments were terminated on day 28.

To assess cytokine production, liver damage, and viral load throughout infection, a separate study was conducted in which mice were randomized based on sex and sacrificed on days 0, 2, 4, 6, 8, and 10 after intranasal infections or when humane sacrifice was deemed necessary. At the time of death, blood was collected using cardiac puncture, and serum was isolated and used for assays. Livers and lungs were harvested and homogenized in a 10% (wt/vol) solution of phosphate-buffered saline (PBS).

**Cytokine responses.** A cell-free sample from $10^5$ cells per well was harvested and inactivated, and the cytokine levels were assessed using ELISA. Sandwich ELISA for murine IFN-α was performed as previously described [133]. Serum samples from WT and knockout (KO) mice were analyzed for 32 cytokines simultaneously using a Milliplex array (Millipore). The following cytokines and chemokines were measured:
Eotaxin (CCL11), granulocyte colony-stimulating factor (G-CSF), GM-CSF, IFN-γ, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12p40, IL-12p70, IL-13, IL-15, IL-17, IFN-γ-inducible protein (IP)-10 (CXCL10), keratinocyte-derived chemokine (KC/ CXCL1), LIF, lipopolysaccharide (LPS)-induced chemokine (LIX/CXCL5), MCP-1 (CCL2), M-CSF, mitogen-inducible gene (MIG/ CXCL9), MIP-1α (CCL3), MIP-1β (CCL4), MIP-2 (CXCL2), RANTES (CCL5), tumor necrosis factor alpha (TNF-α), and vascular endothelial growth factor (VEGF). Samples that were out of range for LPS-induced chemokine (LIX/CXCL5) from multiplex analysis were determined by ELISA (R&D).

**Plaque assay.** Vero E6 cells were plated in 6- or 12-well plates 1 day prior to infection. Viral dilutions were prepared in 1x αMEM (Sigma Chemicals) with sodium bicarbonate and 2% FBS (Atlanta Biologicals). After 1 h of adsorption, viral dilutions were carefully removed and replaced with a 1:1 dilution of 1% agarose (Promega) and 2x αMEM with 4% FBS. Infected cells were incubated for 3 days at 37°C, fixed with 10% formaldehyde in PBS, and stained with 1% crystal violet–20% ethanol solution. The plaques were counted, and the titer of the original sample was determined.

**Liver function test.** Sera from WT and KO mice were assessed for alanine transaminase (ALT) levels for each mouse on the day of harvest using a color endpoint assay (Xpress Bio Life Science Products).
**NanoString gene expression analysis.** BMDMs or cDCs were plated at 10^6 cells per well in six-well plates and then mock infected or infected with rMP-12 or NSs del at MOIs of 1, 2, or 5 as indicated. Total RNA was isolated at 6 h post-infection using an RNeasy minikit (Qiagen). A total of 100 ng of total RNA was hybridized to a custom mouse gene expression CodeSet (consisting of a panel of inflammatory cytokines and IFN-stimulated genes [ISGs]) and analyzed on an nCounter digital analyzer (NanoString Technologies). Counts were normalized to internal spike-in and endogenous housekeeping controls. The results from the NanoString experiment were normalized according to the manufacturer’s protocol. A pseudocount was added to all values, such that the smallest value in the data set was equal to 1. All values were log transformed and, in the case of data obtained with cDCs, a heat map was generated using the ggplot package within the open source R software environment.

**Statistical analysis.** Data were analyzed using commercial software (GraphPad). ELISA and cultured macrophage and DC virus load were analyzed using a Student independent t test. Cytokine levels were assessed by multiplex and ALT levels were analyzed by using a non-parametric Mann-Whitney test. Comparison of survival curves was performed using a log-rank test. P values are presented when statistical significance was observed (significance was set at P ≤ 0.05).
Results

**RVFV-induced activation of IFN-β is dependent on RIG-I.** Previously, isolated RVFV RNA was shown to activate an IFN-β promoter in HEK293T cells and was dependent on RIG-I and not on MDA5 [32]. To confirm whether whole RVFV particles can activate the IFN-β promoter during the course of infection, HEK293XL cells (transfected with a luciferase reporter construct for IFN-β) were stimulated with rMP-12 and NSs del strains at MOIs of 1 and 5. We observed negligible activation of the IFN-β luciferase reporter by rMP-12, in contrast to the NSs del strain, which produced strong induction of the IFN-β reporter (Figure 2.1A). This was not unexpected, since rMP-12 expresses the virulence factor NSs which has been shown by others to specifically inhibit IFN-β transcription [40]. To determine whether activation was RIG-I specific, HEK293XL cells were transiently co-transfected with the IFN-β reporter and increasing concentrations of an inhibitory plasmid RIG-I Dn (helicase domain only). We show that inhibition of RIG-I resulted in a dose dependent reduction in IFN-β activation after stimulation with NSs del. This effect was specific, since MDA5-driven IFN-β activation by transfected poly(I:C) was not affected by the addition of the RIG-I dominant-negative construct, whereas RIG-I-mediated IFN-β activation by Sendai virus was reduced (Figure 2.1B) [134]. Expression of the RIG-I Dn construct was confirmed by Western blotting (Figure 2.1E).

**RVFV-induced activation of IFN-β is independent of endosomal TLRs.** In order to determine whether TLRs contribute to type I IFN promoter activation and production in response to RVFV, HEK293XL cells stably over-expressing TLR7 or TLR8 were transfected with the IFN-β luciferase reporter and then infected with rMP12 or NSs del
strains. As observed in HEK293XL null cells (i.e., not transfected with TLRs), rMP-12 did not induce IFN-β promoter activation (data not shown). The NSs del strain induced IFN-β promoter activation in TLR7 (Figure 2.1C) and TLR8 (Figure 2.1D) infected cells, but at a similar level to the HEK293XL null cells. MyD88 is an adaptor molecule that can be utilized by all TLRs except for TLR3 during signaling [61]. Activation of the IFN-β reporter was not affected by the addition of dominant-negative mutant MyD88 Dn (TIR domain only), suggesting that TLR7 and -8 do not contribute to IFN-β promoter activation by RVFV. As a control, the overexpression of MyD88 Dn did reduce the activation by Gardiquimod in TLR7 cells (Figure 2.1C) and by R848 in TLR8 cells (Figure 2.1D). The expression of the MyD88 Dn construct was demonstrated by Western blotting (Figure 2.1E).
Figure 2.1 RVFV-induced IFN-β responses are dependent on cytoplasmic RIG-I and are independent of TLRs.

(A) HEK293XL cells were transfected with a luciferase construct for the IFN-β promoter and stimulated with medium only (M) or RVFV rMP-12 or NSs del strains at an MOI of 1 or 5 for 18 h. (B) IFN-β activation in HEK293XL cells transfected with 0, 10, 50, or 100 ng of RIG-I dominant-negative construct (RIG-I Dn) and stimulated with medium (M) or NSs del at an MOI of 5 for 18 h. Cells were stimulated with control ligands Sendai virus (SV) and 400 ng of transfected poly(I:C) [Trans poly(I:C)] for 18 h with or without the addition of RIG-I dominant-negative construct. (C and D) HEK293XL cells were stably transfected with TLR7 (C) or TLR8 (D) and transiently transfected with MyD88 dominant-negative construct (MyD88 Dn) at 0, 10, 50, or 100 ng. The cells were transfected with IFN-β reporter construct and stimulated with medium (M) or NSs del strain at an MOI of 5. Controls were performed using the NF-κB luciferase reporter and the TLR7-specific ligand Gardiquimod (C) or the TLR7/8 ligand R848 (D) for 18 h. The data represent mean values ± the standard deviations based on triplicate wells from a representative experiment. Each experiment was performed at least three times. Significance: ***, P ≤ 0.001; **, P ≤ 0.01. (E) Western blot confirming the expression of RIG-I Dn in HEK293XL cells and of MyD88 Dn in TLR7 and TLR8 cells. The cells were either (i) not transfected, (ii) transfected with IFN-β luciferase reporter, Renilla, and pcDNA 3.1 (plasmids), (iii) transfected with the dominant-negative construct, reporter, and Renilla (MyD88 Dn + plasmids), or (iv) the dominant-negative construct alone (MyD88 Dn). Figure reproduced from Ermler et al. with permission [109].
**Induction of type I IFN is dependent on MAVS signaling in primary immune cells.**

Data from our transfection studies suggest that RIG-I is an important mediator of type I IFN production in response to infectious RVFV. In order to determine which innate immune receptors contribute to type I IFN production in primary immune cells, bone marrow cells from WT mice and mice lacking specific innate immune receptor and adaptor proteins were differentiated into macrophages, cDCs, or FLT3L-derived mixed pDCs/cDCs. Cells were stimulated with RVFV strains rMP-12 and NSs del at a range of MOIs for 24 h. The absence of MAVS (common adaptor for RIG-I and MDA5) led to a significant reduction in RVFV-induced type I IFN production by cDCs (Figure 2.2A) and macrophages (Figure 2.2B). This decrease was most notable in response to NSs del strain but could also be observed with rMP-12 strain. The absence of MAVS signaling also reduced IFN-α levels to below the level of detection when mixed pDCs/cDCs were stimulated with NSs del at an MOI of 1 (Figure 2.2C).
Figure 2.2 IFN production by primary immune cells challenged with RVFV is dependent on MAVS.

The dependence of type I IFN production on innate adaptor molecule MAVS was assessed in cDCs (A), macrophages (B), and FLT3L-derived mixed pDCs and cDCs (C). IFN-α levels were determined via ELISA from cell samples harvested at 24 h. The data represent mean values ± the standard deviations based on triplicate wells from a representative experiment. Each experiment was performed at least three times. Significance: ***, $P \leq 0.001$; **, $P \leq 0.01$; not significant (NS), $P > 0.05$. BLD is below the limit of detection. Figure reproduced from Ermler et al. with permission [109].

Although most of the type I IFN response was dependent on RNA helicase signaling, this did not exclude the possibility that TLRs could contribute to minor amounts of IFN in response to RVFV. The role of MyD88 was assessed in cDCs and macrophages using cells derived from $Myd88^{-/-}$ mice that were stimulated with virus for 24 h. The absence of MyD88 did not impact robust type I IFN production in cDCs (Figure 2.3A) or in macrophages (Figure 2.3B) in response to RVFV rMP-12 or NSs del.

TLR3 can recognize dsRNA intermediates from viral replication and signal through adaptor molecule TRIF to lead to type I IFN production [115,135]. Recent evidence
suggests that the amount of dsRNA intermediates generated by negative-strand RNA viruses is negligible compared to the dsRNA intermediates produced during positive-strand RNA viral replication [25]. In addition, TRIF or MyD88 can serve as an adaptor for TLR4 signaling [115]. TLR4 has been shown to be involved in the recognition of viral glycoproteins [62,63,136]. BMDMs and cDCs were generated from TLR3 and corresponding adaptor TRIF-deficient mice and were infected with RVFV rMP-12 and NSs del for 24 h. Neither the adaptor molecule TRIF nor the TLR3 contributed significantly to type I IFN production in cDCs (Figure 2.3C and E) or macrophages (Figure 2.3D and F). These studies demonstrate that RVFV-induced type I IFN production is primarily dependent on RNA helicase adaptor MAVS in immune cells and that TLR signaling does not contribute to type I IFN responses.
Figure 2.3 IFN-α production by immune cells is independent of TLR signaling.

The impact of TLR adaptor molecules MyD88 (A and B) and TRIF (C and D) or of TLR3 (E and F) on type I IFN production was determined in cDCs and macrophages. The IFN-α levels were determined via ELISA from a cell sample harvested at 24 h. The data represent mean values ± the standard deviations based on triplicate wells from a representative experiment. Each experiment was performed at least three times. NS (not significant), P > 0.05. BLD is below the limit of detection. Figure reproduced from Ermler et al. with permission [109].

The absence of MAVS results in increased viral load in cDCs. We hypothesized that RVFV-infected cells lacking robust type I IFN responses would have an increased viral burden compared to cells with competent IFN production. The total viral load (from supernatant and lysate) was assessed in WT and Mavs⁻/⁻ macrophages or cDCs by using a
plaque assay after 24 h of infection. As expected, in correlation with reduced IFN responses after infection, cDCs from $Mavs^{-/-}$ mice showed a significantly increased viral burden when infected with RVFV rMP-12 (Figure 2.4A) or NSs del (Figure 2.4B) compared to cDCs derived from WT mice. Interestingly, macrophages from $Mavs^{-/-}$ mice did not show a significant difference in viral load after infection with either RVFV rMP-12 (Figure 2.4C) or NSs del (Figure 2.4D) compared to WT cells. Macrophages derived from $Myd88^{-/-}$ mice also did not have a significant difference in total viral load of rMP-12 or NSs del compared to WT (Figure 2.4E and F).
Figure 2.4 MAVS-dependent signaling controls viral load in cDCs.

Bone marrow-derived cDCs and macrophages from WT and Mavs−/− mice were infected with rMP-12 (A, C, and E) and NSs del (B, D, and F) at various MOIs. The total viral load was determined by plaque assay after 24 h of infection in cDCs (A and B) and macrophages (C, D, E, and F). (A to D) The data represent mean values ± the standard deviations based on triplicate wells from a representative experiment. Each experiment was performed at least three times. (E and F) The mean PFU is shown for an MOI of 1. The mean PFU ± the standard deviation is shown for MOIs of 0.01 and 0.1 (n = 2). Significance: *, P ≤ 0.05. Figure reproduced from Ermler et al. with permission [109].

Although our findings show that MAVS is crucial for type I IFN response to RVFV at 24 h, we sought to determine whether other receptors, including TLRs, could have an impact on IFN production earlier during infection. cDCs were generated from WT and Mavs−/−
or Myd88/Trif\(^{-/-}\) mice (lacking all TLR signaling) and were stimulated with rMP-12 and NSs del at an MOI of 1. IFN responses were assessed at 6, 12, or 24 h. A significant decrease in IFN-\(\alpha\) production in response to NSs del virus by cDCs from Mavs\(^{-/-}\) compared to WT mice was detected at all of the measured time points (Figure 2.5A). The absence of TLR signaling in cDCs did not impact IFN-\(\alpha\) production at early or late time points in response to rMP-12 (when detectable) or NSs del compared to WT cells (Figure 2.5B). We examined the degree to which cDCs and macrophages could amplify RVFV over time. Cells were infected with rMP-12 or NSs del at an MOI of 1, and virus was removed after 2 h of adsorption to ensure viral load measured in the supernatant at early time points was due to productive infection versus lack of entry. Supernatants were collected after 6, 12, or 24 h of infection, and the virus titers were determined. Viral release was minimal in cDCs and macrophages, with no detectable difference between WT and Mavs\(^{-/-}\) (Figure 2.5C and E) or Myd88/Trif\(^{-/-}\) cDCs and macrophages (Figure 2.5D and F).
Figure 2.5 Time course of IFN responses to RVFV.
Bone marrow-derived cDCs or macrophages from wild-type mice or MAVS- or MyD88/TRIF-deficient mice were infected with rMP-12 or NSs del at an MOI of 1 for 6, 12, or 24 h. The IFN-α responses of cDCs from wild-type mice (A and B) MAVS (A)- or MyD88/TRIF (B)-deficient mice were measured by ELISA. The virus titer was determined in supernatant collected from infected cDCs (C and D) or macrophages (E and F) by plaque assay. The mean levels of IFN-α ± the standard deviations from three experiments are shown. Significance: ***, \( P \leq 0.001 \); **, \( P \leq 0.01 \); *, \( P \leq 0.05 \). Figure reproduced from Ermler et al. with permission [109].

Having established that MAVS is a central regulator of the type I IFN response to RVFV infection in macrophages and cDCs, we next used a multiplex gene expression analysis platform (NanoString nCounter) to examine the role of MAVS in transcriptional regulation of a panel of type I IFN-inducible genes in RVFV infected cells. BMDMs and cDCs from WT mice infected with the rMP-12 strain showed upregulated expression of a
panel of 42 genes that included Ifnb, Ifna4, and ISGs such as Adar (adenosine deaminase, RNA-specific), Ddx58 (RIG-I), Dhx58 (Lgp2), Rsad2 (Viperin), Stat1, Mndal (myeloid nuclear differentiation antigen-like), and Ifi204 (Figure 2.6A). Gene expression profiles in WT cDCs were compared after infection with rMP-12 or NSs del virus and were normalized to mock-infected controls. Gene expression in cDCs showed similar patterns of regulation in cells infected with either viral strain (Figure 2.6B). We also assessed the requirement for RNA helicase signaling by comparing gene expression between WT and Mavs−/− cells (Figure 2.6C). In most cases, the induction of these genes in response to RVFV was dependent on MAVS. These observations suggest that MAVS is a central regulator of the transcriptional response to RVFV infection in DCs.
Figure 2.6 MAVS-dependent gene expression induced by RVFV.

(A) Multiplexed NanoString analysis was performed on BMDMs and cDCs 6 h after infection with rMP-12 at an MOI of 1. The data are shown as the log₁₀ fold change in infected cells compared to uninfected cells.

(B) Normalized mRNA levels for a panel of innate genes in WT cDCs infected for 6 h with rMP-12 or NSs del at an MOI of 2. (C) Innate gene responses of WT and Mavs⁻⁻ cDCs 6 h after infection with rMP-12 at an MOI of 5. The data are shown as log-transformed normalized counts. RNA isolation and hybridization for NanoString were performed by Stefan Schattgen. Analysis was performed by Adam Wespiser and Daniel Caffrey (University of Massachusetts). Figure reproduced from Ermler et al. with permission [109].
MAVS is protective against mucosal challenge with RVFV in mice. RVFV can infect humans and animals in a natural setting through multiple mechanisms, such as bites from mosquitoes harboring the virus or through mucosal exposure to aerosols and droplets [2]. Infectious droplet and/or aerosol exposure can occur during the slaughter or processing of infected livestock or the handling of aborted fetuses when pregnant animals become infected [2]. In order to compare the impact of route of infection on mortality, mice were challenged either intranasally or subcutaneously to mimic these natural routes of exposure. C57BL/6 mice (7 to 9 weeks of age) were infected via subcutaneous injection or intranasal droplet with different doses of rMP-12. All mice infected via either route with 3.5 x 10³ PFU of virus survived challenge. Mice infected with 3.5 x 10⁴ PFU of virus intranasally experienced higher mortality compared to mice infected subcutaneously with the same dose of rMP-12 (Figure 2.7A). Therefore, during subsequent in vivo challenges, 3.5 x 10⁴ PFU of virus was administered via the intranasal route.

Our in vitro studies have demonstrated a clear role for MAVS and RIG-I in RVFV-induced type I IFN responses; however, the role of these molecules in clinical infection is unclear. To determine the role of MAVS in susceptibility to RVFV infection in vivo, intranasal inoculation with rMP-12 was performed using WT and Mavs⁻/⁻ mice, and survival was monitored for 28 days. After mucosal challenge, mice lacking MAVS experienced significantly more mortality over time compared to WT mice (Figure 2.7B).
Figure 2.7 Survival following RVFV infection is dependent on the route of infection and MAVS.

(A) Mice were infected either subcutaneously or intranasally and were monitored daily for death or severe morbidity (five mice per group). (B) WT (n = 12) or Mavs−/− (n = 13) and (C) WT (n = 8) or Myd88/Trif−/− (n = 10) mice were challenged intranasally and monitored daily for 28 days. All infections were performed with 3.5 × 10⁴ PFU of rMP-12. Significance: **, P ≤ 0.01. In vivo challenge and recording of mortality in this figure was performed by Jill Schriewer (Saint Louis University). Figure 2.7 A and B reproduced from Ermler et al. with permission [109].

In order to determine whether MAVS had an impact on early innate immune responses and morbidity, intranasally infected WT and Mavs−/− mice were sacrificed in groups every other day out to 10 days. Spontaneous death in Mavs−/− mice began occurring on day 5 of infection, whereas Mavs+/+ mice began to succumb to infection on day 8. All euthanized mice underwent necropsy and were photographed. Mavs−/− mice sacrificed on day 8 had a pale ischemic liver and necrotic bowel compared to uninfected control mice (data not shown). We also noted that mice requiring humane sacrifice deteriorated quickly, transitioning from apparently healthy to moribund within a matter of hours. No neurological symptoms in infected mice were observed during the course of infection.
Wild-type mice infected intranasally with RVFV rMP-12 had undetectable viral loads in the serum, liver, and lungs at early (day 0 to 2), middle (day 4 to 6), and late (day 8 to 10) time points during the study (Figure 2.8A to C). In contrast, a subset of \( Mavs^{-/-} \) mice exhibited elevated viral loads in serum (Figure 2.8A) during the middle (days 4 to 6) to late (days 8 to 10) stages of infection; however, the majority of the mice had undetectable viral loads. The mean serum viral loads for the positive mice were \( 1.3 \times 10^5 \) PFU/ml in the middle period and \( 1.2 \times 10^3 \) PFU/ml in the late period.

\( Mavs^{-/-} \) mice also exhibited higher viral loads compared to WT mice in the liver (Figure 2.8B) during the middle to late periods of infection. The mean liver titer during the middle period of infection in MAVS-deficient mice was \( 8.5 \times 10^5 \) PFU/g. During the late period of infection, the mean virus titer was \( 6 \times 10^2 \) PFU/g. In correlation with elevated viral loads in the liver, \( Mavs^{-/-} \) mice exhibited elevated ALT levels, a marker for liver damage, compared to WT mice with levels peaking during the middle period of infection (days 4 to 6) (Figure 2.8D).

Viral load was higher in the lungs of \( Mavs^{-/-} \) mice compared to WT mice during the middle period of infection, with a mean titer of \( 3.8 \times 10^4 \) PFU/g in viremic mice (Figure 2.8C). Similar to WT mice, the MAVS-deficient mice showed undetectable levels of virus in the lungs during the late period of infection.
Figure 2.8 Increased viral burden and organ damage in Mavs -/- mice compared to WT mice after mucosal RVFV exposure.

The viral burden was determined by plaque assay in serum (A), liver (B), and lung (C) in Mavs -/- mice (○) compared to WT mice (●) during intranasal infection with 3.5x10^4 PFU of rMP-12. The dotted line signifies the lower limit of detection. (D) Serum ALT levels in Mavs -/- mice (○) compared to WT mice (●). Significance: *, P ≤ 0.05. ND is not determined. Figure reproduced from Ermler et al. with permission [109].

Despite a deficiency in type I IFN and ISG production observed in vitro, Mavs -/- mice exhibited a robust inflammatory response throughout infection as measured in serum (Figure 2.9). In mice humanely sacrificed on day 5, cytokines with the overall highest induction in Mavs -/- mice included IL-6, G-CSF, and MIG (Figure 2.9).
Figure 2.9 Cytokines measured by Bioplex in serum of WT and MAVS deficient mice.

Serum cytokines from WT mice are depicted by —●—. Serum cytokines from Mavs <sup>−/−</sup> mice are depicted by ---○---.

- MIP-1β
- IL-2
- MIP-1α
- MCP-1
- IL-17
- GM-CSF
- IL-10
- IL-1β
Serum obtained from WT and Mavs $^{−/−}$ mice on day 8 post infection was analyzed for inflammatory cytokine levels. These values were listed in Table 2.A and are also included within the complete data set graphed in Figure 2.9. Day 8 was chosen for comparison as WT mice had begun to succumb to infection and a second wave of cytokine production was apparent in Mavs $^{−/−}$ mice at this time. Cytokines IL-6, IL-10, MCP-1, and MIG were significantly increased in Mavs $^{−/−}$ mice compared to wild-type on day 8 of infection (Table 2.A). Other cytokines that had a notable (> 4- fold) induction in Mavs $^{−/−}$ compared to WT mice included GM-CSF, IFN-γ, IL-1β, IL-2, IL-4, IL-12p70, IL-17, MIP-1α, MIP-1β, and TNF-α. Cytokines with <4-fold induction between WT and Mavs $^{−/−}$ on day 8, when wild-type mice began to succumb to infection, included Eotaxin, G-CSF, IL-1α, IL-5, IL-9, IL-13, IL-15, IP-10, KC, LIF, LIX, M-CSF, MIP-2, RANTES, and VEGF. The levels of IL-3 and IL-12p40 were below the limit of detection in serum samples from both mouse groups on day 8 (data not shown). Interestingly, of the 32 cytokines and chemokines tested, CXCL5 (LIX) was the only protein measured that was higher in WT mice and decreased in Mavs $^{−/−}$ mice on all days measured (Figure 2.9). LIX protein levels tended to decrease in WT and Mavs $^{−/−}$ mice over time compared to uninfected mice.
### Table 2.A Serum cytokine responses at peak of RVFV rMP-12 infection.

<table>
<thead>
<tr>
<th>Cytokine or chemokine</th>
<th>Avg level (pg/ml) ± SD(^a)</th>
<th>Mavs(^{-/-}) mice</th>
<th>Δ</th>
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<tbody>
<tr>
<td></td>
<td>WT mice</td>
<td></td>
<td></td>
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<tr>
<td>MIP-1β</td>
<td>4 ± 8</td>
<td>264 ± 229</td>
<td>66.0</td>
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<tr>
<td>IL-2</td>
<td>3 ± 6</td>
<td>164 ± 260</td>
<td>54.7</td>
</tr>
<tr>
<td>MIP-1α</td>
<td>39 ± 62</td>
<td>1,369 ± 1,673</td>
<td>35.1</td>
</tr>
<tr>
<td>MCP-1</td>
<td>25 ± 34</td>
<td>687 ± 539*</td>
<td>27.5</td>
</tr>
<tr>
<td>IL-17</td>
<td>3 ± 6</td>
<td>63 ± 90</td>
<td>21.0</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>21 ± 36</td>
<td>368 ± 567</td>
<td>17.5</td>
</tr>
<tr>
<td>IL-10</td>
<td>18 ± 15</td>
<td>305 ± 296*</td>
<td>16.9</td>
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<tr>
<td>IL-1β</td>
<td>5 ± 12</td>
<td>80 ± 91</td>
<td>16.0</td>
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<tr>
<td>IL-12p70</td>
<td>16 ± 24</td>
<td>253 ± 422</td>
<td>15.8</td>
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<tr>
<td>MIG</td>
<td>909 ± 912</td>
<td>11,091 ± 12,506*</td>
<td>12.2</td>
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<tr>
<td>TNF-α</td>
<td>3 ± 7</td>
<td>30 ± 26</td>
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<tr>
<td>IL-4</td>
<td>4 ± 9</td>
<td>37 ± 65</td>
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<tr>
<td>IFN-γ</td>
<td>21 ± 31</td>
<td>193 ± 322</td>
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<tr>
<td>IL-6</td>
<td>31 ± 38</td>
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<td>G-CSF</td>
<td>720 ± 760</td>
<td>2,775 ± 3,542</td>
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<td>7 ± 9</td>
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<tr>
<td>IL-13</td>
<td>413 ± 125</td>
<td>1,457 ± 754</td>
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<tr>
<td>IL-5</td>
<td>35 ± 20</td>
<td>91 ± 115</td>
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<td>IL-7</td>
<td>13 ± 12</td>
<td>19 ± 6</td>
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<tr>
<td>IL-9</td>
<td>931 ± 528</td>
<td>1,401 ± 944</td>
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<td>76 ± 92</td>
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<td>882 ± 333</td>
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<td>43 ± 29</td>
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<td>1,141 ± 412</td>
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<td>IL-1α</td>
<td>508 ± 332</td>
<td>536 ± 323</td>
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<tr>
<td>VEGF</td>
<td>7 ± 9</td>
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<td>IL-15</td>
<td>237 ± 295</td>
<td>244 ± 139</td>
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<tr>
<td>LIF</td>
<td>18 ± 25</td>
<td>17 ± 5</td>
<td>0.9</td>
</tr>
<tr>
<td>KC</td>
<td>93 ± 82</td>
<td>85 ± 54</td>
<td>0.9</td>
</tr>
<tr>
<td>LIX</td>
<td>13,676 ± 13,160</td>
<td>9,369 ± 4,434</td>
<td>0.7</td>
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</table>

\(a\) The average cytokine levels were measured in serum samples from infected animals in Mavs\(^{-/-}\) mice compared to wild-type mice on day 8. Δ, fold change in Mavs\(^{-/-}\) cytokine levels compared to WT cytokine levels; *, \(P \leq 0.05\). Figure reproduced from Ermler et al. with permission [109].

**RIG-I and MDA5 mediate type I IFN response through MAVS adaptor molecule.**

After demonstrating a clear role for MAVS in vitro and in vivo, we further delineated which upstream receptors were recognizing RVFV and signaling through this adaptor
molecule. IFN-α responses of cDCs generated from WT and \textit{Rig-I}^{−/−} and \textit{Mda5}^{−/−} mice were measured after infection with rMP-12 or NSs del for 6, 12, or 24 h. Negligible IFN-α was induced by rMP-12 in cDCs from WT, \textit{Rig-I}^{−/−}, and \textit{Mda5}^{−/−} mice at all time points. A significant reduction in IFN-α production was observed in NSs del-infected cDCs from \textit{Rig-I}^{−/−} mice compared to WT mice at 12 and 24 h (Figure 2.9A). cDCs from \textit{Mda5}^{−/−} mice also showed significantly reduced levels of IFN-α at 6 and 12 h compared to cDCs from WT mice. However, by 24 h IFN-α responses from \textit{Mda5}^{−/−} cells were comparable to those of the WT mice (Figure 2.9B).

\textbf{Figure 2.10} Type I IFN production is mediated through both \textit{RIG-I} and MDA5.
cDCs from wild-type or mice deficient in \textit{RIG-I} (A) or MDA5 (B) were infected with rMP-12 or NSs del at an MOI of 1 for 6, 12, or 24 h. IFN-α levels in supernatants were measured by ELISA. All results are means ± the standard deviations ($n = 3$). Significance: *, $P \leq 0.05$. Figure reproduced from Ermler et al. with permission [109].
Discussion

The data presented here demonstrate for the first time that the RNA helicase adaptor MAVS is required for type I IFN production in primary immune cells and is protective against mortality and morbidity during live RVFV mucosal challenge. We show that RIG-I is the predominant helicase responsible for type I IFN responses, although MDA5 may also play a role at the earliest time points of viral entry. Our studies demonstrate that TLRs do not play a role in RVFV induced type I IFN production, either in a human cell line or in murine immune cells. This is an important finding, since viruses can potentially enter the endosome after binding to a receptor on the cell surface, and thus genomic material could then be sensed by endogenous TLRs during infection [123]. Viral nucleic acid can also be taken up into the endosome during autophagy [123], which would provide another potential mechanism for endosomal receptor sensing.

Using *in vitro* reporter assays, we demonstrate that intact RIG-I signaling is necessary for IFN-β promoter activation by RVFV. HEK293 cells are a useful model system for TLR activation since the basal expression of most TLRs is negligible. Cells stably transfected with TLR7 or TLR8 that have potential for the recognition of single-stranded viral genomic RNA did not have enhanced IFN-β signaling compared to basal HEK293 cells, indicating that the endosomal TLRs do not contribute substantively to IFN induction by RVFV. The addition of a dominant-negative construct targeting MyD88 (a common adaptor molecule for these receptors) did not impact IFN-β promoter activation, indicating that TLR7 or TLR8 activation and signaling via MyD88 is dispensable for production of RVFV-induced IFN-β.
Initial studies performed in HEK293 cells to determine key PRRs were confirmed using primary immune cells from WT and genetically deficient mice. These studies reveal for the first time important innate receptor recognition utilization by macrophages and DCs during RVFV infection. RNA cellular helicases were confirmed to be key receptors used by primary immune cells for recognition of RVFV, leading to the induction of type I IFN throughout infection. We were surprised at the overall low level of IFN produced by FLT3L-induced DCs compared to GM-CSF-induced cDCs or L929-derived macrophages, since pDCs have been thought to be the main producers of type I IFN in response to viral infection [137]. Although pDCs are known to express RLRs, literature suggests that pDCs rely mainly on the TLR system for viral sensing [138,139]. FLT3L induces a mixed population of cDCs and pDCs; however, these cDCs may be functionally different from cDCs induced by GM-CSF since these populations are known to contain DC subsets that express different PRRs [140].

The RNA helicase adaptor molecule MAVS was necessary for control of total viral load in conventional DCs at 24 h for higher MOIs. It is likely that viral load was suppressed by IFN generated through MAVS signaling. In contrast, lack of MAVS signaling or MyD88-dependent TLR signaling did not alter viral replication in macrophages under these same conditions. This was an unexpected finding, since MAVS was shown to be necessary for type I IFN production in primary immune cells, including macrophages, and type I IFN is known to hinder replication of other viruses. There are several reasons why this discrepancy could occur. For example, our studies have shown that cDCs
produce higher levels of type I IFN compared to macrophages, and a more robust IFN response may be needed to have an effect on RVFV replication. It is also possible that macrophages are utilizing other antiviral defenses besides type I IFN, such as the generation of reactive oxygen species or nitric oxide (NO), phagocytosis, or other inflammatory pathways. For example, multiplexed analysis of gene regulation in rMP-12-infected macrophages revealed that Nos2 (nitric oxide synthase 2) was upregulated 44-fold compared to uninfected cells. In comparison, GM-CSF-derived cDCs showed only 5-fold upregulation after infection (data not shown). Another explanation may be that the potential for viral entry may differ between macrophages and cDCs due to differential expression of surface receptors. Recently, DC-SIGN was determined to be a receptor used in RVFV cell entry [19]. However, not all cells that are infected with RVFV express DC-SIGN, suggesting there may be other critical receptors for viral entry.

Time course studies demonstrate that both macrophages and cDCs allow for very minimal viral amplification since similar viral burdens in the cell supernatant were observed over time. In these experiments, virus was removed after 2 h adsorption so that only virus released from productively infected cells could be assessed in the supernatant. The shorter adsorption time and analysis of only virus present in the supernatant versus the total viral load of cells may be why a difference in WT and Mavs \(^{-/-}\) cDC viral burden could no longer be observed at 24 h for these studies.

We utilized two strains of attenuated RVFV for our \textit{in vitro} studies. The NSs del strain offers insight into which receptors can be activated by the virus, since virulent and rMP-
12 strains of RVFV would normally suppress type I IFN responses due to the ability of NSs protein to specifically inhibit this pathway [40,41,49,50]. It is important to note that some type I IFN was generated in response to the rMP-12 strain in primary immune cells, indicating that the inhibition of the host response is not complete.

Our studies are the first to demonstrate that MAVS is protective against mortality during in vivo RVFV infection. We observed increased amounts of type I IFN protein and ISG mRNA in cells from WT compared to Mavs−/− mice, confirming the critical role for type I IFN in RVFV infection. It had previously been established that Ifnar−/− mice have more rapid mortality with virulent RVFV strain ZH548 compared to WT mice. Also, Ifnar−/− mice succumbed to infection with MP-12 and clone 13 strain infections compared to no mortality observed with WT mice inoculated with those strains [52]. We show that MAVS-deficient mice have increased viral burden and more liver damage, as assessed by the ALT level after in vivo challenge. It is interesting that even the WT mouse requiring humane sacrifice on day 8 had a lower ALT level compared to non-moribund Mavs−/− mice sacrificed at this time. High mortality in Mavs−/− mice after intranasal challenge with rMP-12 was expected because of the deficit seen in the IFN production of immune cells from these mice in vitro and supports previously published studies showing high mortality in Ifnar−/− mice [52]. Although this group reported a lack of virulence in WT mice infected intraperitoneally with MP-12, we observed >50% mortality with intranasal administration of rMP-12 in WT mice (Fig. 7A). We have observed that the route of infection largely impacts virulence, with significantly less virulence observed with subcutaneous challenge than with intranasal challenge (Fig. 7A). This may be due to a
difference in which cells and organs first come into contact with the virus based on the route of virus introduction, leading to different profiles or magnitudes of host inflammatory responses. Further studies, beyond the scope of the present study, may also show a differential susceptibility in animal models challenged with virulent RVFV depending on the route of exposure. This could have potential implications for human RVFV infections, wherein a range of clinical severity and symptoms have been observed, and where risk factors for infection include both mosquito and mucosal routes of exposure [2,141].

Interestingly, in vivo, a variety of inflammatory cytokine responses were not hindered in the absence of MAVS signaling. Many of the Mavs−/− mice that were humanely sacrificed due to moribund appearance exhibited a cytokine storm. The inflammatory proteins abundant in overwhelming amounts on day 5 in two mice requiring humane sacrifice included IL-6, G-CSF, and MIG (data not shown). IL-6 is known to be fever inducing and has been shown to be associated with other hemorrhagic fever virus infections [142,143]. G-CSF has many functions, including reducing cellular apoptosis and quelling inflammation associated with neurodegenerative diseases [144]. G-CSF has also been shown to promote the accumulation of Ly6G+ granulocytes during influenza virus or Sendai virus infection to aid in viral clearance and maintain survival [145]. MIG (CXCL9) has been shown to reduce coronavirus-induced liver and brain pathology [146]. The cytokines IL-10 and MCP-1 were shown to be significantly different between WT and MAVS-deficient mice. IL-10 is known to be an immunomodulator and can inhibit antigen presentation and the production of inflammatory cytokines [147]. IL-10 has been
shown to decrease inflammation and liver damage without altering viral load in a model of murine cytomegalovirus infection [148]. MCP-1 alters the migration of monocytes and macrophages that are important for combating viral infection [149].

RLRs upstream of MAVS are important for type I IFN production in response to RVFV infection. As expected, the absence of RIG-I significantly reduced IFN-α production by cDCs when infected with NSs del, strengthening our findings from in vitro studies using HEK cells in which RIG-I Dn negatively impacted activation of the IFN-β promoter. In bone marrow-derived cDCs, MDA5 also appeared to influence early IFN-α production in response to RVFV, which likely accounts for the residual responses seen in the Rig-I−/− cells at 24 h. Rig-I and Mda5 genes were similarly activated during rMP-12 and NSs del infection of WT cDCs. RIG-I and MDA5 recognize different viral nucleic acid patterns; RIG-I recognizes the 5’-triprophosphate end of ssRNA generated by viral polymerases, whereas MDA5 recognizes dsRNA and has been shown to be critical for recognizing members of the picornavirus family [134]. Despite recognizing distinct substrates, both RIG-I and MDA5 have also been shown to contribute to recognition of West Nile virus and dengue virus [150,151]. Here, we also demonstrate a redundant role for these molecules in the induction of type I IFN responses by RVFV.

Although these studies have identified the initial receptor dependence in IFN production in mice, receptor preference in human cells should be verified. In a clinical setting, RVFV infected patients exhibit a wide range of symptoms, from minor febrile illness to much more severe manifestations such as hemorrhagic fever and death. It is unknown
why such a range of variability exists between patients. It is likely that genetic factors may contribute to this diversity. Studies with rats have confirmed that resistance against severe RVFV-induced pathology can be inherited as a dominant gene [106] and that subtle differences between rats of the same strain from different facilities can alter disease outcomes [107,108]. Ultimately, polymorphisms in crucial innate immune receptors in human populations could be identified and screened in conjunction with monitoring the gamut of patient disease progression. Polymorphisms in receptors that bolster early and robust type I IFN responses could hold the key to unlocking the source of diversity between severe and mild clinical outcomes in patients infected with Rift Valley fever virus.
Chapter 3 Rift Valley fever virus infection drives NLRP3 inflammasome activation

Abstract

Inflammasome activation is gaining recognition as an important mechanism for protection during viral infection. Here, we investigate whether Rift Valley fever virus, a negative strand RNA virus, can induce inflammasome responses and IL-1β processing in immune cells. We have determined that RVFV induces NLRP3 inflammasome activation in murine dendritic cells, and that this process is dependent upon ASC and caspase-1. Furthermore, absence of the cellular RNA helicase adaptor protein MAVS/IPS-1 significantly reduces extracellular IL-1β during infection.

Introduction

The inflammasome is a large multi-protein complex that can assemble in response to viral, fungal, or bacterial pathogens. The active inflammasome leads to auto-catalytic cleavage of cysteine protease caspase-1, which in turn processes pro-IL-1β and pro-IL-18 into their mature biologically active forms [152,153,154]. In addition to processing its specific substrates, caspase-1 also has a role in the export of pro-IL-1α and in mediating cell death through pyroptosis [155,156]. Mature IL-18 is secreted as a result of inflammasome activation and results in elevated NK and NK-T cell cytotoxic activity as well as IFN-γ secretion by T cells [157,158]. Secreted mature IL-1α and IL-1β act as
pyrogens to induce fever [159]. While inflammasome activation during infection is often thought of as protective, disregulated IL-1β production in the absence of pathogens can be harmful. For instance, a hallmark of autoinflammatory disease is the potential for resolution with an IL-1β blockade [160,161,162]. Thus, activation of inflammasome signaling is carefully regulated.

Activation of the inflammasome is a complex process requiring two signals. The first step involves initiation of NF-κB mediated signaling through pattern recognition receptors, such as Toll-like receptors (TLRs), and results in accumulation of pro-IL-1β and increased expression of inflammasome components [163]. TNF-α has also been shown to serve as a first signal through mechanisms independent of TLRs [164]. The second signal leads to assembly of the inflammasome in the cytoplasm and activation of caspase-1. Even after activation of the inflammasome has occurred, further regulation of IL-1β and IL-1α signaling can be achieved by secretion of the IL-1 receptor antagonist (IL-1RA) which prevents binding of IL-1α and IL-1β to their shared receptor, IL-1R1 [160].

Classical inflammasomes are found within the NOD-like receptor (NLR) family and include NLRPs (containing leucine rich repeat and pyrin domain containing proteins) and NLRCs (containing leucine rich repeat and caspase recruitment domain containing proteins). RNA viruses such as respiratory syncytial virus, encephalomyocarditis virus, influenza, and rabies virus have been shown to induce activation of the NLRP3 inflammasome [91,92,93,165]. Additionally, inflammasomes can be found outside of the NLR family. Absent in melanoma 2 (AIM2) can serve as a pattern recognition receptor
and an inflammasome. The AIM2 protein, like other members of the Hin200 family, contains a DNA binding domain [166]. Viruses with DNA genomes such as vaccinia and mouse cytomegalovirus have been shown to activate the AIM2 inflammasome leading to caspase-1 activation [90]. Retinoic acid-inducible gene 1 (RIG-I) has been suggested to act as an inflammasome in concert with the adaptor protein ASC (apoptosis-associated speck-like protein containing C-terminal caspase recruitment domain) in response to vesicular stomatitis virus (VSV) [97]. However, this report is controversial as another group demonstrated that IL-1β was not present without priming during VSV infection and that the NLRP3 inflammasome was primarily responsible for IL-1β processing [96].

Rift Valley fever virus (RVFV) is a negative strand RNA virus of the family Bunyaviridae. The virus causes high mortality in young livestock and will induce infected pregnant livestock to abort. In humans, RVFV can cause a multitude of disease manifestations ranging from febrile illness to hemorrhagic fever and death. In our previous studies, we observed a low level of IL-1β in the serum of mice that had been infected with attenuated RVFV strain rMP-12 via the intranasal route [109]. In addition, goats infected subcutaneously with the virulent ZH501 strain of RVFV are reported to have elevated IL-1β in their serum [167]. Human serum collected from patients during the 2000-2001 RVFV outbreak in Saudi Arabia was analyzed for pro-inflammatory and suppressive cytokines, including inflammasome related cytokines. Levels of IL-1RA were elevated overall and relative to IL-1α levels in fatal versus non-fatal cases [168]. These studies led us to question whether RVFV could induce inflammasome activation during infection.
Materials and Methods

Mice. C57BL/6 mice were obtained from Jackson Laboratories. \textit{Myd88}^{−/−} or \textit{Trif}^{−/−} mice were generated by Shizuo Akira (Osaka University, Osaka, Japan) and were subsequently used to produce \textit{Myd88}/\textit{Trif} double knockout mice. \textit{Nlrp3}^{−/−}, \textit{Asc}^{−/−} and \textit{Nlrc4}^{−/−} mice were generated by Millenium Pharmaceuticals. \textit{Casp1}^{−/−} mice were generated by R. Flavell (Yale University). \textit{Rig-I}^{−/−} mice were provided by Michael Gale, Jr. (University of Washington). \textit{Mda5}^{−/−} mice were provided by Marco Colonna (Washington University). \textit{Mavs}^{−/−} mice were generated by Zhijian Chen (University of Texas Southwestern). Since the MAVS mouse strain is not fully backcrossed onto C57BL/6, wild-type controls from the same generation were used in these experiments. Mice were housed in filter-top micro-isolator cages in ventilated racks. Conditions for animal experiments were approved by the Institutional Animal Care and Use Committee at Case Western Reserve University.

Cells, viruses, reagents. Attenuated rMP-12 and NSs del strains of RVFV were a gift from Shinji Makino (UTMB, Galveston, TX) and were previously described [109]. Viral infection of cells was carried out under BSL-2 conditions. Samples were inactivated with 2-4J UV light to cross-link RNA (Stratagene).

Bone marrow-derived cDCs were generated as previously described [109]. Cells were harvested between days 8-10 of maturation. For ELISA analysis of cytokines, \textit{10}^{5} cells were plated per well in a 96 well plate. Cells were stimulated with tri-phosphate (ppp).
RNA (Invivogen) for 8 hours, 1 ug Ultrapure *E. coli* K12 LPS (Invivogen) for 4.5 hours, rMP-12 and NSs del at MOI 1 or as otherwise designated for 6 hours. Primed cells were first stimulated with LPS for 4 hrs after which the supernatant was removed and replaced with 5 mM ATP (Sigma Aldrich) for 30 minutes, or rMP-12 and NSs del strains for 6 hours.

For western blot analysis, cDCs were plated in 6 well plates with 4 million cells per well and were stimulated with control ligands or infected with virus as described above. For these samples, LPS was administered at 500 ng/mL.

**Cytokine responses.** R&D Systems DuoSet ELISA kit was used to assess IL-1β present in cell supernatant (DY401). The assay was run according to the manufacturer’s protocol.

**Plaque assay.** Vero E6 cells were plated in 6 well plates and were near 90% confluency at the time of infection. Live or UV inactivated virus sample was diluted in 1X αMEM media (Sigma Chemicals) with sodium bicarbonate and 2% FBS (Atlanta Biologicals). After 1 hr adsorption, the inoculum was removed and replaced with 1:1 dilution of 1% agarose (Promega) and 2X αMEM with 4% FBS. After incubating for 3 days at 37°C, cells were fixed with 10% formaldehyde in PBS and stained with 1% crystal violet 20% ethanol solution.

**Western blotting.** Cell lysates were prepared on ice in RIPA buffer with 1 mM DTT and a protease inhibitor cocktail with ethylenediaminetetraacetic acid (EDTA) (Thermo
Scientific). Supernatants were precipitated using 10% sodium deoxycholate and 100% trichloroacetic acid (TCA). Samples were boiled for 5 min in Laemmli buffer and were subjected to SDS-PAGE using 4% bis-acrylamide stacking and 15% resolving gels. Proteins were transferred to PVDF membrane and 4% non-fat dried milk was used for blocking and antibody dilutions. Blots were stripped with Restore stripping buffer (Thermo Scientific) and were re-probed with anti-β-actin antibody A-15 (Santa Cruz sc-69879) and detected with secondary goat anti-mouse IgG –HRP (sc2005). Mouse IL-1β antibody was obtained from R&D (BAF410) and was detected with bovine anti-goat IgG-HRP (sc-2378). Pro- and mature caspase-1 p10 (M-20) was purchased from Santa Cruz (sc-514) and were detected with secondary antibody goat anti-rabbit IgG-HRP (sc2004).

NanoString gene expression analysis. Cells were plated at $10^6$ cells per well in 6 well plates and were mock infected or infected with rMP-12 at MOI 5. After 6 hrs, total RNA was extracted using an RNeasy Mini kit (Qiagen). 100 ng of total RNA was hybridized to a custom mouse-gene expression CodeSet and analyzed on an nCounter Digital Analyzer (NanoString Technologies). Counts were normalized to internal spike-in and endogenous housekeeping controls according to the manufacturer’s protocol.

Statistical analysis. Data were analyzed using commercial software (GraphPad). ELISA data from at least three independent experiments were averaged and graphed as means ± standard deviation. Significance was determined using Student’s independent t-test and is defined as *** $P \leq 0.001$, ** $P \leq 0.01$, * $P \leq 0.05$. 
Results

**IL-1β production in response to RVFV is dose dependent and requires viral replication.** In order to assess whether RVFV can induce IL-1β responses, conventional dendritic cells (cDCs) were generated from the bone marrow of WT mice and were infected with attenuated strains rMP-12 and NSs del of RVFV at varying MOI. The NSs del strain was generated on the rMP-12 backbone and lacks the gene encoding the NSs virulence factor [128]. Dendritic cells had previously been identified to be potent producers of IL-1β in response to many viruses [96,165]. While macrophages have also been shown to support inflammasome activation in response to viruses, we chose to focus our efforts on dendritic cells [91,96]. Cells were infected under both unprimed and LPS-primed conditions. When administered alone, LPS is known to induce production of pro-IL-1β. In the absence of a secondary signal, pro-IL-1β accumulates within the cell and cannot be processed by caspase-1 into its 17 kDa biologically active form. Addition of ATP to LPS-primed cells is a known inducer of NLRP3 inflammasome activation and served as a positive control for inflammasome activation and IL-1β processing. LPS priming of cells has been a useful tool for other virus infection studies in which ample amounts of pro-IL-1β are not produced in response to the virus alone *in vitro*, yet inflammasome activation and IL-1β processing clearly occurs when enough substrate is present [92].

In the absence of LPS priming, minimal IL-1β was detected in the cell supernatant (Fig. 3.1A). With priming, IL-1β was detected after infection with both the parent rMP-12 and
NSs del strains. The level of extracellular IL-1β from NSs del infected cells was higher than that produced by rMP-12 infected cells and also positively correlated with increasing dose of virus. The elevated production of IL-1β from NSs del infected cells compared to rMP-12 infected cells was expected since the NSs protein of RVFV limits host transcription during infection by preventing assembly of general transcription factor TFIIH [41]. The requirement for live viral infection to induce IL-1β production was assessed by infecting or stimulating cDCs with live or UV-inactivated virus. Extracellular levels of IL-1β were significantly reduced in response to UV-inactivated virus (Fig. 3.1B). Complete inactivation of the virus was verified using plaque assay (Fig. 3.1C).

**Figure 3.1 RVFV infection induces IL-1β release from cDCs.**
Bone marrow-derived cDCs were infected with rMP-12 or NSs del RVFV strains for 6 h. In some cases, cells were first primed with LPS for 4 h prior to infection (as designated). A) cDCs were infected with rMP-12 or NSs del at an MOI of 0.3, 1, or 3 and IL-1β in the supernatant was detected by ELISA. B) Dependence of IL-1β production on viral replication was assessed by treating cDCs with NSs del or UV inactivated NSs del at an MOI of 1. A and B) The mean level of IL-1β ± the standard deviation from three experiments is shown. Significance: *, $P \leq 0.05$. C) Efficiency of UV inactivation was verified by plaque assay. Inactivation was verified for each sample of UV inactivated NSs del virus that was used in panel B.
**IL-1β production is dependent upon MAVS signaling during RVFV infection.** The pro form of IL-1β is typically generated after an innate immune receptor recognizes the virus and signals through the adaptors MyD88/TRIF (for TLRs) or MAVS (for RNA helicases) resulting in NF-κB activation and induction of the IL-1β gene. In order to determine the impact of TLR signaling through MyD88 and TRIF on RVFV induced IL-1β, we examined pro-IL-1β in the lysate of unprimed RVFV infected cells. We did not use priming for these studies, since LPS would drive production of pro-IL-1β through the TLR pathway, thus confounding interpretation. We observed that even in the absence of priming, pro-IL-1β could be detected in NSs del infected cDCs from *Myd88/Trif* −/− mice (Figure 3.2).

**Figure 3.2 Lack of TLR signaling does not hinder production of pro-IL-1β.** cDCs from WT and *Myd88/Trif* −/− mice were stimulated with LPS, LPS/ATP, or rMP-12 and NSs del (6 h). Presence of pro-IL-1β was determined via western blot assay. Data shown is representative of three independent experiments.

Next, we examined the dependence of a number of IL-1 and inflammasome related responses in RVFV infected cDC on MAVS vs. MyD88/TRIF signaling pathways by using NanoString technology. Upregulation of many of the examined genes was dependent upon the MAVS pathway, including *Il-1β, Il-1α, Il-1ra, caspase-1, Nf-kb1, Nf-...*
$kb_2$, $Nlrp3$, $Nlrp12$, $Aim2$, and $Nos2$ (Figure 3.3). In contrast, $Il-18$, $Nlrc4$ and $Unc93b$ were similarly and only modestly induced in WT, $Myd88/Trif^{-/-}$ and $Mavs^{-/-}$ cells. None of the responses examined was found to be strongly dependent on MyD88/TRIF, indicating a minimal role for this pathway in RVFV induced inflammation.

**Figure 3.3 Dependence of inflammasome related genes on MAVS or MyD88/TRIF pathways.**

**Dependence of inflammasome related genes on MAVS or MyD88/TRIF pathways.** Bone marrow-derived cDCs from WT, $Mavs^{-/-}$, or $Myd88/Trif^{-/-}$ mice were infected with rMP-12 for 6 h as single samples. Total RNA was isolated and analyzed using NanoString technology. Fold change is relative to uninfected cells of the same strain and normalized to endogenous housekeeping controls.

The impact of MAVS on IL-1$\beta$ protein production in RVFV infected cDCs was then determined via ELISA. WT and $Mavs^{-/-}$ cDCs were infected with rMP-12 or NSs del with or without LPS priming. In the absence of priming, negligible IL-1$\beta$ responses were observed; however, with LPS priming followed by RVFV infection, IL-1$\beta$ responses were detected from WT cells but were significantly reduced in the absence of MAVS (Figure 3.4A).
Figure 3.4 IL-1β responses to RVFV are dependent on MAVS.
Differentiated cDCs from WT and A) Mavs−/−, or B) Rig-I−/− or Mda5−/− mice were primed with LPS for 4 hrs, or were infected without priming with rMP-12 and NSs del strains for 6 hours. Supernatant was analyzed for IL-1β protein by ELISA. The mean level of IL-1β ± the standard deviation from three experiments is shown. Significance: *, P ≤ 0.05. NS: not significant, P > 0.05. C) Mature IL-1β and caspase-1 p10 protein were detected in the supernatant of cDCs from WT, Rig-I−/− or Mda5−/− mice. Pro-IL-1β and pro-caspase-1 were detected in the lysate. Data shown is representative of three independent experiments.

RIG-I and MDA5 have potential to recognize RNA viruses and to signal through adaptor protein MAVS, leading to NF-κB activation and IL-1β production. Additionally, RIG-I has been shown to function as an inflammasome during viral infection [97]. We examined whether RIG-I or MDA5 were required for IL-1β production and processing. Primed cDCs from WT, Rig-I−/−, or Mda5−/− mice secreted similar levels of IL-1β in response to rMP-12 or NSs del virus as measured by ELISA (Figure 3.4B). Mature IL-1β protein was also present in supernatants of primed cDCs from Rig-I−/− and Mda5−/− mice that were
infected with NSs del (Figure 3.4C). Finally, caspase-1 activation occurred in the absence of RIG-I or MDA5 in primed cells as indicated by presence of the p10 subunit in the supernatant (Figure 3.4C).

**Secretion of IL-1β is dependent upon NLRP3, ASC, and caspase-1.** Several RNA viruses can initiate formation of the NLRP3 inflammasome complex, which results in caspase-1 activation and cleavage of IL-1β into its mature form [114,165,169]. We examined whether NLRP3 and adaptor ASC were required for release of IL-1β by cDCs during RVFV infection. Absence of NLRP3 or ASC resulted in negligible amounts of IL-1β in the supernatant from RVFV infected cells (Figure 3.5A). Cells from caspase-1 deficient mice (which have also been shown to lack caspase-11) [170] also showed reduced IL-1β responses compared to WT cells.

We next verified whether NLRP3 was necessary for processing of pro-IL-1β into mature IL-1β in response to RVFV by Western blot analysis. Levels of pro-IL-1β were clearly detected in the lysate of WT and Nlrp3−/− cDCs after RVFV infection. However, mature IL-1β (17 kD) was only detected in the extracellular fraction of WT cDCs that had been infected with RVFV compared to cDCs from Nlrp3−/− mice (Figure 3.5B).
Figure 3.5 IL-1β production is dependent upon NLRP3, ASC, and caspase-1.
A) Bone marrow-derived cDCs from WT, Nlrp3−/−, ASC−/−, or caspase-1−/− mice were stimulated with rMP-12 and NSs del with and without LPS priming. IL-1β from the supernatant was quantified by ELISA. The mean level of IL-1β ± the standard deviation from three experiments is shown. Significance: **, \( P \leq 0.01 \). BLD denotes the value was below the limit of detection. B) Supernatant and lysates from stimulated or infected cDCs was subjected to western blot. Mature IL-1β was detected in the supernatant, and pro-IL-1β and β actin levels were determined in cell lysate. Data shown is representative of three independent experiments.

Discussion

These studies show that IL-1β is produced in response to RVFV and that processing of this cytokine is dependent upon inflammasome NLRP3, as well as adaptor ASC and caspase-1 activation. This is the first report of inflammasome activation by RVFV. Viral
replication was required for IL-1β production. This is important to assess as cDCs are capable of phagocytosis and other viruses have been shown to induce IL-1β through replication independent as well as dependent mechanisms [95]. It is important to note that Casp-1^-/- mice used in the ELISA studies are functionally lacking both caspase-1 and caspase-11 (also known as caspase-4) [170] and caspase-11 has been shown to be necessary for caspase-1 activation [171]. However, the mature caspase-1 p10 subunit was detected by western blot in NSs del infected cells, verifying a role for caspase-1 in IL-1β processing.

In the absence of priming, pro-IL-1β was produced in response to RVFV in cDCs from Myd88/TRIF^-/- mice. This emphasizes that production of pro-IL-1β is TLR independent, and may result from activation through other signaling pathways such as the helicases. Upregulation of RNA from many of the inflammasome related genes was dependent upon MAVS, and not MyD88/TRIF, as determined by NanoString analysis. Indeed, in cDCs that were infected with RVFV strains, MAVS had a profound effect on secreted IL-1β responses.

Despite the pronounced decrease of IL-1β in the absence of MAVS, potential upstream receptors RIG-I and MDA5 did not have as dramatic of an impact on secreted IL-1β when examined individually. There are several reasons why this may occur. As we had previously seen with type I IFN production, RIG-I and MDA5 may compensate for each other, as type I IFN responses were not completely dependent upon either receptor [109]. A similar scenario may occur for IL-1β production given the partial decrease in mature
IL-1β produced by primed RIG-I or MDA5 deficient cDCs that were infected with RVFV. NOD2 has been shown to signal through adaptor MAVS to lead to type I IFN production in response to RSV [101]. Whether NOD2/MAVS signaling could also lead to accumulation of pro-IL-1β in cell lysates has yet to be determined. MAVS may instead have effects on IL-1β release that are independent of generation of pro-IL-1β. Recently, MAVS was shown to directly interact with NLRP3 and to influence IL-1β secretion for many known activators of NLRP3 [172]. This is an interesting finding that may have relevance for our studies, although in absence of MAVS we did not see a significant difference in IL-1β produced in response to LPS/ATP, which would also signal through NLRP3. It is worth noting that we had used different kinetics for ATP stimulation compared to those used by Subramanian et al.

Although RIG-I has been shown to have potential to serve as an inflammasome during viral infection, we did not see dependence upon RIG-I for IL-1β processing in primed cDC. We also did not observe residual IL-1β in cell supernatant of NLRP3 deficient cells, indicating IL-1β processing is primarily driven through the NLRP3 inflammasome. In this study, we have demonstrated that RVFV induces NLRP3 inflammasome activation leading to the processing and release of IL-1β. The impact of inflammasome activation during RVFV infection should be further investigated using in vivo studies to determine whether this will protect against severe morbidity and mortality.
Chapter 4 Discussion and Future Directions

Innate signaling can offer early protection against invading pathogens and mediate disease outcomes. Previous literature suggests that type I IFN can be protective during infection with RVFV. Through \textit{in vitro} and \textit{in vivo} assays, this thesis delineates pathways leading up to activation and production of type I IFN. The inflammasome has also been shown to elicit protection during challenge with many RNA viruses. The work contained in this thesis examines the mechanism behind IL-1\(\beta\) processing and inflammasome activation in response to RVFV.

Unexplored avenues for TLR involvement

In Chapter 2 of this thesis, a model is presented for steps leading to type I IFN production by immune cells in response to RVFV. In pDCs, cDCs, and macrophages, signaling through MAVS was critical for IFN-\(\alpha\) production. It was determined that this signaling occurred primarily through RIG-I, but also through MDA5 early on. Mortality was significantly higher between WT and MAVS deficient mice that were infected intranasally with rMP-12 (Figure 2.7). Further highlighting the importance of RLR signaling over TLRs during RVFV infection, no significant difference in mortality was observed between B6 and MyD88 deficient mice (Figure 2.7). While RLRs are capable of recognizing a variety of RNA viruses, it is curious that TLRs did not appear to contribute to signaling leading to type I IFN production. TLR3 is capable of recognizing dsRNA intermediates that can be produced during replication. While evidence exists that
negative-strand RNA viruses may produce less of these intermediates than positive-strand RNA viruses [25], it is still notable that other endosomal TLRs also were not apparently involved in IFN signaling.

Although TLR8 did not appear to play a role in our mouse studies since absence of its signaling adaptor MyD88 did not affect IFN production or mouse survival, human TLR8 requires further investigation as TLR8 functions differently in mice than in humans [69]. While we did not see a role for human TLR8 in IFN-β promoter activation in HEK293T cells, signaling pathways utilized for viral recognition can differ between phagocytic immune cells and non-immune cells, thus TLR8 may yet have a role for IFN production or inflammatory cytokine production in human immune cell populations.

One approach to investigate a potential role for TLR8 in human immune cells could be to generate human mDCs from monocytes isolated from PBMCs. Human mDCs are known to express TLR8, in contrast to human pDCs which preferentially express TLR7 [173]. Protocols for this procedure have been established by the laboratory of Thomas Nutman (NIH, NIAID) [174]. A population of mDCs is generated by supplementing monocytes with GM-CSF and IL-4. Cell purity will be assessed by flow cytometry; DCs should be CD1a+, HLA-DR+, CD86+, CD40+, CD3-, CD14-/low, CD19-, and CD56-. Generated mDCs should be transduced with shRNA against TLR8 during the differentiation process using lentiviral vectors as has been previously described [175]. While this method is technically challenging, it may allow for more efficient and stable knockdown of TLR8. RVFV could then be added to cells that have received scrambled or TLR8 shRNA.
Differences in type I IFN or inflammatory cytokine production by these cells would be determined via ELISA. Donors for PBMCs should be genotyped to determine which human TLR8 single nucleotide polymorphism (SNP) is encoded. Differences in which SNP is expressed could alter immune response to RVFV. Comparative studies with rMP-12 and WT RVFV should also be conducted to ensure that any attenuating mutations present within rMP-12 are not hindering immune recognition or response towards RVFV.

TLR7 was a likely candidate for RVFV recognition and signaling as it would be capable of recognizing the ssRNA genome and mRNA of RVFV during replication. RVFV would be contained within endosomes subsequent to receptor mediated entry but is ineffective in activating endosomal TLR signaling for type I IFN production. Given the apparent lack of involvement for multiple endosomal TLRs, it is tempting to hypothesize that a more general mechanism may be preventing RVFV recognition.

TLR activation during viral infection is a dynamic process. In the absence of infection, endosomal TLRs 3, 7, 8, and 9 are expressed on the ER. Subsequent to PAMP stimulation, these TLRs traffic to the endosome where they can recognize nucleic acid patterns [176]. Trafficking of TLRs 7 and 9 to the endosome is governed by host protein UNC93B [177]. UNC93B has also been shown to bind the transmembrane domains of TLR3, TLR7, and TLR9 and this physical interaction has been shown to be critical for downstream signaling [178]. In Chapter 3, NanoString data demonstrates that Unc93b mRNA was not upregulated in response to rMP-12 infection of cDCs (Figure 3.3). This
suggests that RVFV may be actively inhibiting downstream TLR signaling by preventing their trafficking to the endosome via UNC93B.

If RVFV did specifically interfere with UNC93B, this would likely occur at a transcriptional rather than translational or post-translational level as NanoString did not detect a change in Unc93b mRNA after DCs were infected with RVFV (Figure 3.3). Studies could be conducted to determine influence of RVFV on the Unc93b promoter. HEK293 cells stably transfected with TLR3, TLR7, or TLR8 would be transfected with a luciferase reporter for Unc93b and Renilla. Activation of the Unc93b promoter would be assessed with and without addition of appropriate TLR ligands. If an increase in promoter activation is observed with TLR ligand addition as a positive control, the next step is to determine whether RVFV also leads to Unc93b promoter activation or inhibits this process. Because RVFV will lead to an overall reduction in transcription due to NSs interaction with TFIIH, it becomes more complicated to try and determine specific targets of the virus. Isolated RVFV NSs del RNA could be added or transfected into the cell to determine whether RVFV RNA could serve as a ligand for the upregulation of Unc93b in the absence of any virulence proteins that might otherwise inhibit activation. RNA would need to be isolated from RVFV lacking the NSs gene since NSs is in the viral sense orientation and thus can be directly translated (i.e. produced even from transfected RNA). If induction of the Unc93b promoter can be observed with isolated NSs del RNA (in absence of RVFV proteins and virulence factors), this is a good indication that RVFV may be specifically inhibiting UNC93B mediated interactions with endosomal TLRs and downstream signaling.
The next step is to determine which RVFV virulence factor may be inhibiting Unc93b induction. Our laboratory has obtained NSs and NSm mutant strains of RVFV from Shinji Makino at UTMB. TLR-transfected HEK293 cells would be transfected with the Unc93b luciferase promoter and infected with rMP-12, NSs del (on rMP-12 background), arMP-12 (similar to rMP-12) or arMP-12 NSm deletion mutant viruses. Activation of the Unc93b luciferase promoter would be determined by reading luciferase activity on a luminometer. If either NSs or NSm deletion mutants lead to a noticeable defect in luciferase production, this may indicate whether a particular virulence factor is involved in preventing Unc93b promoter activation. In order to test whether the defect in Unc93b promoter activation is specific toward that virulence factor and is not a byproduct of other processes being affected such as overall transcription, a chromatin immunoprecipitation assay could be performed in order to determine whether NSs or NSm can bind to the Unc93b promoter. NSs or NSm would be likely contributors to virulence functions given their previously discovered roles in suppressing host response [39,40,41,42,49,50]. The 78 kDa protein of unknown function would also be an interesting protein to screen for virulence function. Given the elegance of RVFV’s ability to utilize few genes for effective infection and persistence, it is likely the 78kDa protein has an important function that has yet to be characterized.

It is likely that immune and non-immune cells would be able to sense virus infection through different mechanisms, as immune cells can take up infected apoptotic cells through phagocytosis and thus can activate the TLR pathway even in the absence of
intrinsic infection [179]. It would be interesting to determine whether different PRRs are utilized in non-immune cells compared to predominant activation of the RLR pathway that we have observed in immune cells during RVFV infection. Differential activation of PRRs in immune vs. non-immune cells is known to occur during Junín virus infection. An attenuated strain of Junín was demonstrated to induce type I IFN and TNF-α cytokine response through TLR2 in mouse macrophages whereas type I IFN production by human A549 lung epithelial cells was mainly driven through RIG-I signaling during infection[180,181]. Cells of interest would include hepatocytes which are an established target of RVFV infection. Lung epithelial cells and other target tissues may also be of interest. While these cells may not be major contributors of type I IFN, they may contribute to inflammatory cytokines that are present during infection. The overwhelming burden of inflammatory cytokines present in rMP-12 infected Mavs −/− mice (Table 2A) suggest that pathways not mediated by the RLRs may be involved in this response. All cytokine data obtained by Bioplex has been graphed to illustrate patterns throughout infection (Appendix 2). This data set includes samples from Table 2A. Liver cell lines HepG2 and KC13-2 (the immortalized Kupffer cell line that our lab has obtained) as well as lung cell line A549 could be transfected with siRNA against TLRs or RLRs of interest before infection with attenuated RVFV strains. Knockdown of the TLR/RLR would be confirmed via western blot and impact of innate immune receptor depletion would be determined with ELISA for IFN-α or for NF-κB mediated inflammatory cytokines such as IL-6 or IL-8.
TLRs and RLRs can have non-redundant roles during viral infection. For example, during LCMV infection RLRs are critical for type I IFN production and enhancing CD8+ T cell responses while TLRs are dispensable. In contrast, TLRs are needed for robust anti-viral antibody responses for viral clearance [182]. Despite being dispensable for type I IFN production by immune cells, TLRs may still have important roles during RVFV infection that have yet to be defined, such as inducing inflammatory or adaptive responses.

**An unconventional role for pDCs during RVFV infection?**

One surprising finding was that pDCs did not appear to contribute large amounts of type I IFN and the little production that occurred appeared to be driven through a RLR-dependent mechanism rather than through TLRs. Literature suggests pDCs mainly signal through TLRs in response to viruses despite expressing RLRs [138,139]. If TLR signaling was somehow prevented, our results may well fit with the diminished amounts of IFN that were observed compared to what was expected. Studies by Gommet et al. examined the impact of immune cells during mouse infection with RVFV [183]. Clodronate was administered to the mice prior to infection in order to reduce populations of monocytes, macrophages, and dendritic cells. Interestingly, mouse treatment with clodronate resulted in decreased viral burden and slightly delayed time until mortality [183]. Although seemingly counterintuitive, it is likely that pDCs may contribute to increased viral spread and faster time until mortality given their low production of IFN *in vitro*. This would be in stark contrast to the current dogma that pDCs are major interferon producers and aid to control viral infection and needs to be further investigated assuming
pDCs are permissive to RVFV infection [61,184]. In order to clarify this point, pDCs should be sorted from the pDC/cDC mix generated using FLT3L and should be infected in vitro. Titer of the virus can be determined over time using a plaque assay.

To determine the impact of pDCs in vivo, mice could be subcutaneously injected with B16 melanoma cells expressing FLT3L. I have performed these preliminary experiments and saw a gross increase in spleen size from B16 melanoma injected mice compared to control mice (Appendix 3). An increase in pDCs to around 20% of spleen cells was also observed (Appendix 3) compared to 3% of CD11c+ (includes pDCs and cDCs) that are normally observed in the mouse spleen [185]. Mice would subsequently be infected intranasally with WT RVFV (ZH501 or ZH548) and viral titer in key organs such as liver, lung, spleen, and brain could be determined. While brain was not assessed in our previous studies, it would be an interesting organ to include as animals that survive hepatitis can sometimes progress to develop encephalitis [186]. Timing of infection with virus after B16 cell injection would need to be optimized because after 14 days mice have a large tumor size and would need to be sacrificed for humane reasons. For this reason, WT RVFV should be utilized rather than attenuated strains to speed up time to measurable viral burden and death. An alternative method would be to infect mice with luciferase or GFP tagged RVFV and perform live in vivo imaging to examine viral spread. If an increase in pDC specific populations exacerbates RVFV pathology and leads to increased viral burden and spread, this would be a very novel finding and would challenge the assumption that pDCs are beneficial during viral infection.
Implications for decrease in cytokine LIX throughout RVFV infection

An interesting result from our in vivo studies presented in Chapter 2 was that serum cytokine LIX was decreased in MAVS deficient mice compared to WT mice and decreased over time for all mouse populations after infection with RVFV. This was the only cytokine investigated that was consistently lower in the susceptible MAVS deficient mice compared to WT mice at all time points during infection. This could suggest that cells producing LIX are being destroyed throughout the course of infection, or could suggest that LIX was a protective cytokine and that this protection diminishes with time after RVFV exposure. LIX is a known neutrophil chemoattractant secreted by alveolar epithelial cells and has been shown to be mainly expressed by cells that were negative for virus in a coronavirus infected culture [187], further suggesting a protective effect. I had
assessed pooled serum and organ cytokine levels from B6 mice harvested at early time points during infection (PBS treated, day 2, 4, 6, 8, and 10 after infection). Interestingly, out of lung, liver, and spleen homogenates, splenic cells seemed to be major contributors of LIX compared to other organs examined, with lung samples only having notable amounts at day 10 (Figure 4.1). If a target cell population within the spleen can be determined, *in vitro* studies could characterize *Lix* mRNA and protein expression in that cell type before and after stimulation with RVFV as well as examining cell viability with an LDH assay. Alternatively, if LIX is believed to be a protective cytokine, exogenous LIX protein could be administered to mice just prior to infection with RVFV. Survival, viral burden in serum and organs, and liver damage could be assessed. Liver damage could be assessed using the previously utilized colorimetric assay for ALT.
Figure 4.2 LIX levels from select organs of B6 mice.

Levels of LIX were determined in organ homogenates by Bioplex from B6 mice on 0, 2, 4, 6, 8, and 10 days after intranasal infection with $3.5 \times 10^4$ PFU of rMP-12.

<table>
<thead>
<tr>
<th>Sample</th>
<th>LIX (pg/mL)</th>
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<tbody>
<tr>
<td>PBS treated animal liver</td>
<td>57.7</td>
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<tr>
<td>rMP-12 day 2 liver</td>
<td>BLD</td>
</tr>
<tr>
<td>rMP-12 day 4 liver</td>
<td>BLD</td>
</tr>
<tr>
<td>rMP-12 day 6 liver</td>
<td>BLD</td>
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<tr>
<td>rMP-12 day 8 liver</td>
<td>BLD</td>
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<tr>
<td>rMP-12 day 10 liver</td>
<td>BLD</td>
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<tr>
<td>PBS treated animal lung</td>
<td>BLD</td>
</tr>
<tr>
<td>rMP-12 day 2 lung</td>
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<td>rMP-12 day 4 lung</td>
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<td>rMP-12 day 10 lung</td>
<td>12980</td>
</tr>
<tr>
<td>PBS treated animal spleen</td>
<td>2570</td>
</tr>
<tr>
<td>rMP-12 day 2 spleen</td>
<td>4380</td>
</tr>
<tr>
<td>rMP-12 day 4 spleen</td>
<td>11450</td>
</tr>
<tr>
<td>rMP-12 day 6 spleen</td>
<td>2530</td>
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<tr>
<td>rMP-12 day 8 spleen</td>
<td>2750</td>
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<td>rMP-12 day 10 spleen</td>
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BLD denotes below the limit of detection.

The liver is a critical organ during RVFV infection

Liver damage is a hallmark of RVFV infection and can result in coagulopathy that is sometimes concomitant with hemorrhagic fever [188]. We had detected elevated liver damage in MAVS deficient mice infected intranasally with rMP-12 which was measured by a substantial increase in serum ALT (Figure 2.8). It is also known that animals that survive hepatitis can go on to develop delayed encephalitis during RVFV infection [186]. Given the importance of this key organ, it would be beneficial to determine specific roles of discrete cell populations within the liver during infection. One population that may be
of interest is liver resident macrophages, also known as Kupffer cells. During infection, hepatocytes become heavily burdened with virus and undergo apoptosis [189]. Kupffer cells can be found outside of the sinusoidal endothelial lumen in close proximity to hepatocytes and contain degraded cells within their lysosome [189]. It is also known that rats that recover from RVFV have Kupffer cell hyperplasia and hepatocellular regeneration [108]. Finally, during RVFV infection hepatic lesions are observed without evidence of infiltration by inflammatory cells, suggesting that resident cells may be responsible for hepatitis [190].

Kupffer cells have been shown to have a critical role during infection with other RNA viruses. For example, during HCV infection Kupffer cells were found to be primary sources of IL-1β, in contrast to a hepatocyte cell line [95]. While Kupffer cells were not productively infected with HCV, the virus instead activated TLR signaling after being taken up through phagocytic mechanisms. During influenza-induced hepatitis, Kupffer cells play a critical role by inducing liver damage. Depletion of Kupffer cells with clodronate liposomes prevented hepatitis, and it was believed that Kupffer cells mediated damage by inducing CD8+ T cell focus formation [191]. In this case, hepatitis did not correlate with viral load in the liver, suggesting that damage may be due to bystander effect and not directly from the virus.

In order to study Kupffer cell function during RVFV infection, preliminary studies could be performed on an immortalized Kupffer cell line KC13-2 that was generated by the Landmann-Suter laboratory in Switzerland (shown in Appendix 5A)[192]. Briefly, cells
from H-2Kb-tsA58 transgenic mice which stably express SV40 large tumor antigen under control of promoter H-2Kb grow at 33°C but not 37°C. Promoter stimulation and cell growth is initiated with addition of interferon-γ. One clone was selected for its ability to grow at 33°C and 37°C without addition of interferon-γ. These cells have been stably growing for eight years and have retained functional properties and markers typical of primary peritoneal large Kupffer cells. Initially, these cells could be tested for ability to produce IL-1β, IL-18, or other cytokines of interest in response to RVFV. Kupffer cells are known to secrete IL-18 [193], which can alter cytotoxicity of NK-T cells within the liver [157]. Alternatively, studies could be performed with primary isolated Kupffer cells. I have optimized methods to obtain primary Kupffer cells from livers of mice with near 72% purity (Appendix 5B). I have performed initial studies with primary Kupffer cells demonstrating that Kupffer cells can be infected by RVFV as virus was isolated from the lysate (Appendix 5C) and that Kupffer cells can secrete TNF-α in response to RVFV (Appendix 5D). TNF-α secreted by Kupffer cells has been shown to induce liver damage during other infections [194].
In brief, for isolation of primary Kupffer cells, mice were perfused through the sinus of the right atrium towards the inferior vena cava with cold HBSS 1% pen/strep solution and the hepatic portal vein was severed to allow for outflow. Next, the same region was perfused with a cold solution of 0.05% collagenase type IV and 0.5 mM calcium chloride in HBSS. The liver was removed from site and cells were strained and rinsed with PBS. The slurry was incubated at 37 °C for 10 minutes with constant swirling to allow for destruction of parenchymal cells. The liver suspension was diluted with equal volumes of ice cold HBSS (without calcium or magnesium) and was centrifuged at 50 x g (4 °C) for
10 minutes. The supernatant was subjected to further purification and the pellet contained primary hepatocytes. Supernatant was centrifuged at 500 x g (4 °C) for 9 minutes and the pellet was washed with HBSS and resuspended in 2.5 mL media mixed with 3.5 mL of 30% (wt/vol) metrizamide solution and overlaid with 1 mL PBS. The solution was centrifuged at 1400 x g (4 °C) for 15 minutes. Cells of interest (non-parenchymal cells) were collected from the interface and were washed with HBSS at 500 x g for 9 minutes (4 °C). Cells were resuspended in Williams medium E (10% FBS, 1% pen/strep, 10mM HEPES of pH 7.4, 2 mM L-glutamine) and were incubated for 1 hr at 37 °C. Supernatant and non-adherent cells were removed and fresh media was added. Adherent cells were harvested around 34 hrs after plating.

The role of Kupffer cells in vivo should also be assessed. While Kupffer cells have been depleted using clodronate in some studies, this method is not specific and would also lead to depletion of many phagocytic cell populations [183]. Therefore, an alternate method to study Kupffer cell function during in vivo challenge would involve treatment with gadolinium chloride [195,196]. Gadolinium chloride has been determined to inhibit Kupffer cell function while leaving alveolar and interstitial macrophage unaltered [197]. This is important if intranasal infection is utilized since the lung would be an early target organ for viral replication. Mice would be infected with virulent RVFV and would be monitored for mortality and morbidity. Serum cytokine levels would be determined via Bioplex. Liver, lung, spleen, and brain would be monitored for viral load and histology would be performed to determine damage and infiltrating cell populations. Viral load in
and histology of the brain would be particularly interesting in this scenario as mice that survive hepatitis can sometimes progress to developing encephalitis [186].

Without more preliminary data, it is difficult to predict whether Kupffer cells would have a protective or harmful role in RVFV progression. While Kupffer cells have been shown to mediate damage to other viruses, it is also possible they could play a protective role if they were major supporters of inflammasome activation. Kupffer cells can also play immunomodulatory roles and can dampen inflammation and T cell activation by creating a tolerant environment [193,198].

**Influence of virus source on infectivity and host immune response**

The innate immune studies in this thesis were performed using rMP-12 and NSs del attenuated RVFV strains that were generated by passaging our original virus stock in Vero E6 cells (green monkey kidney cells). Vero E6 cells were utilized because of their lack of interferon response [199]. This trait may allow viral titers to reach higher capacity and puts less selective pressure on RNA virus stocks that are being passaged. It also reduces type I IFN that would be contained within the viral preparation. One caveat of these studies is that although RVFV can be naturally transmitted between mammals from aerosol transmission, transmission can also occur between insect and mammalian hosts through mosquito vectors. By only using virus stocks generated in Vero E6 cells, we are potentially limiting variations in host immune response that might otherwise occur due to virus propagation in different phyla. Virus replication in insect or mammalian cells alters glycosylation patterns and recent literature suggests this ultimately affects both innate
and adaptive responses from the host. Type I IFN levels in response to mosquito derived Ross River virus are lower than levels induced by Ross River virus derived from mammalian cells [200]. Evidence suggests that lower IFN induction was due to a lack of complex carbohydrates, such as N-linked glycans, rather than ability of mosquito derived virus to suppress IFN production [200].

The source of the virus can also affect its ability to infect host cells. Sindbis virus is an arbovirus (arthropod-borne virus) that can be transmitted from mosquito to human. Researchers found that mosquito derived Sindbis virus is more efficient at infecting DCs compared to mammalian derived virus [112]. It is believed that Sindbis virus entry is facilitated through DC-SIGN when virus is produced under conditions that limit complex carbohydrate and leads to a higher composition of DC-SIGN ligand Man$_3$GlcNAc$_2$ [112]. Similar to Sindbis virus, RVFV is also an arbovirus that can naturally be transmitted between mosquitoes and humans and utilizes DC-SIGN as an entry receptor [19]. Thus, further investigation is required to determine whether viral source influences RVFV infectivity of immune cells.

It would also be interesting to investigate whether CLR preference changes as RVFV transfers from one cell type to another as occurs with dengue virus infection. Dengue produced in dendritic cells lost its ability to propagate in DC-SIGN positive cells, but retained ability to infect L-SIGN positive cells, in contrast to insect derived dengue that was able to infect DC- and L-SIGN positive cells [87]. If CLR preference does change during the course of RVFV infection depending on the source of the virus, this may in
part explain why we see poor virus production in immune cells such as macrophages and dendritic cells. Previous studies had demonstrated that RVFV antigen was present in the cytoplasm of macrophages and that clodronate-loaded liposome depletion of macrophages and dendritic cells lead to a decrease in overall viral load of infected mice [183]. Possible interpretations of this result include that dendritic cells and macrophages are major amplifiers of virus or that these cells instead help traffic virus to more permissive cell types. Our studies suggest that dendritic cells and macrophages are not easily infected by RVFV and are not potent amplifiers of the virus, although some infection does occur as we see some increase in viral production. A switch in virus tropism from DC-SIGN to L-SIGN might also help the virus leave circulating immune cells and would encourage active infection of the liver, which is the major target organ during RVFV infection.

Future studies could elucidate whether DCs are perpetuating RVFV spread through trans-infection to more permissive cell types. Trans-infection has been shown to be a mechanism for HIV transmission during which the virus is held within a pocket-like structure at the dendritic cell surface to later emerge and infect target T cells [201]. Trans-infection has also been demonstrated with spread of HIV among CD4+ T cell populations [202]. This route of infection is not specific to HIV and is also employed by measles virus to perpetuate spread from dendritic cells to T cells [203]. Furthermore, trans-infection is mediated differentially by L-SIGN alleles and allows HCV to cross from liver sinusoidal endothelial cells to hepatoma cells [204]. Epifluorescent
microscopy with GFP-tagged virus has proven to be a useful tool for observing this phenomenon.

**Inflammasome activation during RVFV infection**

Our studies indicate that the NLRP3 inflammasome can form in reaction to RVFV infection, and that this process is dependent upon ASC, caspase-1, and MAVS. The role for NLRP3 during *in vivo* RVFV infection has yet to be assessed and will be critical to understanding the importance of this pathway during infection. NLRP3 activation may have a protective effect on mice during RVFV infection as seen with influenza infection [94] or could lead to heightened liver inflammation and pathogenesis similar to HCV infection [95]. In addition to surveying for mortality in WT and NLRP3 deficient mice, more subtle pathology such as liver damage should also be assessed. If liver damage as well as mortality is reduced in the absence of inflammasome signaling, this would provide further rationale for examining liver resident cells during RVFV infection.

Kupffer cells have been identified as major sources of inflammation and IL-1β production during HCV infection [95] and could play similar roles during infection with RVFV since this virus also targets the liver.

It is interesting that receptors RIG-I or MDA5, which are upstream of MAVS, did not individually have the same effects as loss of MAVS on IL-1β production. This could occur if RIG-I and MDA5 were compensating for each other, as we had previously observed with type I IFN production. There is also the possibility that another receptor upstream of MAVS may be responsible for driving IL-1β production. NOD2 has been
shown to signal through MAVS to induce type I IFN production [101]. It is known that NOD2 can activate NF-κB independently of MAVS through RICK and TAK1 [205]. As of yet, no studies have demonstrated a role for NOD2/MAVS in IL-1β production through NF-κB. It would be interesting to determine whether NOD2 may have a MAVS dependent role in IL-1β production during RVFV infection. This could easily be investigated with bone marrow-derived dendritic cells obtained from NOD2 deficient mice.

**Genetic implications for innate pathways activated by RVFV**

Ultimately, identification of key players in innate signaling pathways that are activated during RVFV infection could provide a starting point for trying to dissect what host genetic factors may offer protection during infection. Studies currently underway by the MRCE funded RVFV group at Case Western Reserve University are investigating whether polymorphisms in some of these innate signaling pathways correlate with patient disease outcomes. While a clear role for genetic protection against RVFV has been demonstrated in rat studies, the identity of the protective gene is unknown. Although type I IFN clearly has a protective role during infection, the resistant gene of interest may not be part of the IFN pathway. This further emphasizes the need for studying alternative innate pathways that may be activated, such as the NLRP3 inflammasome. If NLRP3 offers protection in mice during challenge with RVFV, players in this pathway may also be worth investigating for polymorphisms and inherent genetic protection.
Summary

The research body encompassed in this thesis delineates signaling pathways necessary for type I IFN production and inflammasome activation during RVFV infection. By understanding which cascades are utilized for protection, future research can investigate these signaling proteins for polymorphisms that may contribute to the wide array of outcomes that RVFV infected patients exhibit. We have set the stage for future work investigating the role of the NLRP3 inflammasome during RVFV infection. If effects of inflammasome activation are pathogenic, IL-1β responses could be targeted therapeutically to alleviate inflammation and mediate improved patient outcomes.
Literature Cited


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