ALPHAVIRUS EVASION OF TYPE I INTERFERONS

Reed S. Shabman

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Approved by:

Advisor: Mark T. Heise, Ph.D.
Reader: Stephen L. Bachenheimer, Ph.D.
Reader: Aravinda M. de Silva, Ph.D.
Reader: Dirk P. Dittmer, Ph.D.
Reader: Jon S. Serody, M.D., Ph.D.
Reader: Barbara Sherry, Ph.D.
Alphaviruses are mosquito transmitted viruses that cause either severe arthritis or encephalitis. A critical stage that determines whether or not alphavirus infection will result in disease is the early interaction of the virus with the host following delivery from the mosquito vector. Of particular importance are interactions between mosquito derived virions and myeloid dendritic cells (mDC’s) within the skin and draining lymph node. Therefore a focus of these studies was to evaluate if alphaviruses derived from either mosquito or mammalian cells interacted differently with mDC’s. Our results revealed mosquito-cell-derived Ross River virus and Venezuelan Equine Encephalitis virus (mos-RRV and mos-VEE) more efficiently infected mDC’s when compared to the same viruses grown in mammalian cells (mam-RRV and mam-VEE). However, mos-RRV and mos-VEE infection poorly induced type I interferons (IFN-αβ) when compared to mam-RRV and mam-VEE, suggesting that mosquito-cell-derived alphaviruses could either avoid or inhibit antiviral responses from infected mDC’s. A major difference between mammalian and mosquito grown viruses is that mosquito-cell-derived virus particles exclusively incorporate terminal mannose N-linked glycans onto their glycoproteins, while mammalian-cell-derived viral glycoproteins incorporate complex, hybrid, and terminal mannose oligosaccharides. Differential IFN-αβ induction was linked to glycosylation since mDC’s infected with viruses grown in mammalian cells that produce virions with only mannose glycans induced less IFN-αβ than mDC’s infected with
viruses grown in mammalian cells with complex glycans. Additional studies suggested that mos-RRV did not actively suppress mDC IFN-αβ production, since co-infection of mos-RRV with mam-RRV did not inhibit IFN-αβ responses. We next generated a panel of RRV glycan deficient viruses to identify the role for each N-linked glycan in IFN-αβ production. The panel revealed that E2 glycans on mam-RRV, but not mos-RRV, were required for robust IFN-αβ responses following mDC infection. These data suggest that poor IFN-αβ responses from mDC’s following mos-RRV infection is attributed to a lack of complex glycans on the virion envelope. In summary, these data provide new insight into how mosquito-borne viruses evade IFN-αβ responses which may aid in their ability to establish infection.
To my family,

Leonard, Janet and Mark,

For their unconditional support in all of my endeavors

And to my fiancé Tracy,

For her understanding and commitment over the last six years
ACKNOWLEDGEMENTS

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<th>Full Form</th>
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<tr>
<td>AST</td>
<td>Average survival time</td>
</tr>
<tr>
<td>BFV</td>
<td>Barmah Forest virus</td>
</tr>
<tr>
<td>BMDC</td>
<td>Bone marrow dendritic cell</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CHIKV</td>
<td>Chikungunya virus</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster ovary</td>
</tr>
<tr>
<td>CLR</td>
<td>C-type lectin receptor</td>
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<tr>
<td>CSE</td>
<td>Conserved sequence element</td>
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<tr>
<td>DC</td>
<td>Dendritic cell</td>
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<tr>
<td>DI</td>
<td>Defective interfering particle</td>
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<tr>
<td>DLN</td>
<td>Draining lymph node</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>dsRNA</td>
<td>Double stranded RNA</td>
</tr>
<tr>
<td>DV</td>
<td>Dengue virus</td>
</tr>
<tr>
<td>EEEV</td>
<td>Eastern Equine Encephalitis Virus</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence-activated cell sorting</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>GlcNAc</td>
<td>N-Acetylglucosamine</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte monocyte-colony stimulating factor</td>
</tr>
<tr>
<td>hpi</td>
<td>Hours post infection</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
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</tr>
<tr>
<td>HS</td>
<td>Heparin Sulfate</td>
</tr>
<tr>
<td>IFN-αβ</td>
<td>Interferon-αβ or type I interferon</td>
</tr>
<tr>
<td>IFN RKO</td>
<td>Interferon αβ receptor knockout</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IRF-3</td>
<td>Interferon regulatory factor-3</td>
</tr>
<tr>
<td>ISG</td>
<td>Interferon stimulated gene</td>
</tr>
<tr>
<td>ISRE</td>
<td>Interferon stimulated response element</td>
</tr>
<tr>
<td>IU</td>
<td>International units</td>
</tr>
<tr>
<td>Jak</td>
<td>Janus tyrosine kinase</td>
</tr>
<tr>
<td>LOD</td>
<td>Limit of detection</td>
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<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>mam-virus</td>
<td>Mammalian-cell-derived virus</td>
</tr>
<tr>
<td>Mda-5</td>
<td>Melanoma differentiation associated protein-5</td>
</tr>
<tr>
<td>mDC</td>
<td>Myeloid dendritic cell</td>
</tr>
<tr>
<td>MOI</td>
<td>Multiplicity of infection</td>
</tr>
<tr>
<td>mos-virus</td>
<td>Mosquito-cell-derived virus</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>MTT</td>
<td>3-[4,5-Dimethylthiazol-2-yl]-2,5-Diphenyltetrazolium</td>
</tr>
<tr>
<td>Mx</td>
<td>Myxovirus resistance protein</td>
</tr>
<tr>
<td>Myd88</td>
<td>Myeloid differentiation primary response gene 88</td>
</tr>
<tr>
<td>NFkB</td>
<td>Nuclear factor kappa beta</td>
</tr>
<tr>
<td>nsP</td>
<td>Non structural protein</td>
</tr>
<tr>
<td>OAS</td>
<td>2’-5’-oligoadenylate synthetase</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
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<tr>
<td>ONNV</td>
<td>O’nyoyn’nyong virus</td>
</tr>
<tr>
<td>pDC</td>
<td>Plasmacytoid dendritic cell</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen associated molecular pattern</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cell</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PFU</td>
<td>Plaque forming unit</td>
</tr>
<tr>
<td>PKR</td>
<td>Protein kinase-R</td>
</tr>
<tr>
<td>RIG-I</td>
<td>Retinoic acid inducible gene-I</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RRV</td>
<td>Ross River virus</td>
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<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SFV</td>
<td>Semliki Forest virus</td>
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<tr>
<td>SINV</td>
<td>Sindbis virus</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducer and activator of transcription</td>
</tr>
<tr>
<td>TGEV</td>
<td>Transmissible gastroenteritis virus</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated region</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet light</td>
</tr>
<tr>
<td>VEEV</td>
<td>Venezulean Equine Encephalitis virus</td>
</tr>
<tr>
<td>WEEV</td>
<td>Western Equine Encephalitis virus</td>
</tr>
<tr>
<td>WNV</td>
<td>West Nile virus</td>
</tr>
<tr>
<td>VLP</td>
<td>Virus like particle</td>
</tr>
<tr>
<td>VRP</td>
<td>Alphavirus replicon particle</td>
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<tr>
<td>VSV</td>
<td>Vesticular Stomatits Virus</td>
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CHAPTER 1

INTRODUCTION
MOSQUITO BORNE VIRUSES AND HUMAN DISEASE

Epidemiology of mosquito borne viruses

Mosquito-borne viruses consist of members from the families *Flaviviridae*, *Bunyaviridae*, and *Togaviridae*. Together these viruses represent serious emerging disease threats and are responsible for thousands to millions of cases of human disease each year. For example, the *Flaviviridae* member West Nile virus (WNV) has caused significant morbidity and mortality following its introduction into the United States in 1999 (57), while the related family member Dengue virus (DV) is responsible for an estimated 50 million cases of Dengue fever and 50,000 cases of dengue hemorrhagic fever each year (76). Members of the *Bunyaviridae* are also a significant emerging disease threat and have the potential to impact both humans and livestock (28).

The studies in this report focus on members of the *Togaviridae* family within the genus alphavirus. The alphavirus genus includes more than 20 members present on every continent with the exception of Antarctica (36, 100). In nature, alphaviruses are maintained in an enzootic cycle in nature between an animal reservoir and mosquitoes (17). Occasionally this cycle is interrupted by larger animals including horses and humans, which can result in severe disease in both species (36, 112). Alphavirus disease in humans generally manifests as either a severe arthritis/myositis or encephalitis. Severe arthritis and myositis are predominantly associated with old world alphaviruses which are endemic to Africa, Asia, Australia and the South Pacific (64, 75, 102). Old world alphaviruses include Sindbis (SINV) and the Sindbis groups viruses, Ross River virus (RRV), Barmah Forest virus (BFV), O’nyoyn’nyong (ONNV), and Chikungunya (CHIKV). New world
alphaviruses include Venezuelan, Eastern, and Western equine encephalitis (VEEV, EEEV, and WEEV, respectively) and are predominately located in North, Central, and South America (45, 112). Human infection by a new world alphavirus can result in encephalitis which is occasionally fatal (45). Both old and new world alphaviruses are a major emerging disease threat with the potential to cause serious outbreaks in the United States and around the world.

Old world alphaviruses have been historically responsible for massive epidemics that have affected millions of people. ONNV virus outbreaks caused approximately 2 million cases of arthritis in Africa around 1959, and an isolate similar in sequence to the 1959 strain reemerged in Uganda in 1996 (60). CHIKV has also caused several large scale epidemics since it was first described during an epidemic in Tanzania in the early 1950’s (15). Since 2005 CHIKV has caused a massive epidemic which initiated on the east coast of Africa and Reunion island in the Indian ocean before spreading into India and Sri Lanka. Currently there are estimates of up to 5 million afflicted individuals (15, 29) with the outbreak recently spreading into regions of northern Italy (84).

The majority of the studies in this report focus on a virus related to CHIKV, RRV. RRV is indigenous to Australia and the South Pacific. The virus was first isolated in 1959 at the Ross River from an Aedes vigilax mosquito (24). RRV infected individuals develop debilitating but self limiting arthritis/arthralgia which was initially described in the mid 1800’s (51). However, chronic symptoms in some patients can last for months to years (64) and the disease represents a significant economic burden to Australia (51). Like CHIKV and ONNV, RRV has epidemic potential. For example, an RRV outbreak on the island of Fiji occurred in 1979 which was believed to be initiated by an infected tourist. An estimated
500,000 people were subsequently infected, and 50,000 developed polyarthritis (53). Today RRV is considered an emerging pathogen in Australia and is of concern given the recent problems with CHIKV.

Animal Models to study alphavirus-induced disease

Mouse models have greatly improved our understanding of alphavirus-induced disease. These models indicate that most alphaviruses have a biphasic replication pattern in the animal where the virus initially replicates in lymphoid cells before entering the blood and reaching target tissues (7, 67, 86, 88, 113). A general overview of alphavirus-induced disease is described below.

Alphavirus transmission to a vertebrate host occurs via the bite of an infected mosquito. The mosquito bite delivers an extravascular, subcutaneous injection of the virus under the skin of the infected host. However, there is evidence with WNV that some virus is directly injected into the blood stream (101). In the lab, a mosquito bite is mimicked by a subcutaneous needle inoculation. Following transmission from the mosquito, most alphaviruses infect resident dendritic cells (DCs), and/or DCs to the draining lymph node (DLN) (67, 114). Alphaviruses then replicate in the DLN and surrounding tissues allowing for virus and/or virus infected cells to spill into the circulatory system creating a systemic infection. A serum viremia is then established facilitating spread to target tissues/organs in the body.

Several old world alphaviruses target to the periosteum and endosteum surrounding the knee and ankle joints, the synovial cavity, and muscle tissue of infected mice (39). RRV infected mice also have detectable viral replication directly in the joint and in surrounding
tissues of the hind limb (39, 75). Additional evidence has shown that mouse muscle fibers are disrupted following infection with RRV with the destruction related to macrophages migrating to the sites of the infected tissue (64, 68). Furthermore, mice treated with macrophage-toxic agents showed reduced disease signs implicating a critical role for macrophages as a mediator of RRV-induced disease (62, 63, 75). Additional studies using the RRV mouse model propose a role for complement activation in RRV-induced disease (74). Interestingly, the disease signs observed within the RRV mouse model closely recapitulates disease pathology seen in humans following RRV infection (64, 75). Both RRV antigen and monocyte infiltrates are found in the joints of people with the disease, and it is believed that both factors contribute to alphavirus-induced disease in afflicted patients (104). Taken together, both viral replication and an aberrant host immune response contribute to RRV-induced disease in humans and the established mouse model.

Mouse models have also highlighted the critical role for an intact type I interferon (IFN-αβ) pathway to control alphavirus replication (18, 86, 113). Removal of the IFN-αβ receptor changes the disease outcome from a self-limiting nonlethal disease to a lethal infection within days post inoculation. These data combined with in vitro studies implicate IFN-αβ signaling as a critical innate immune response that serves to control early alphavirus replication. The studies in this thesis focus on the early IFN-αβ responses following alphavirus infection and a more detailed review of the type I IFN pathway is discussed later in the chapter.

MOLECULAR ASPECTS OF ALPHAVIRUSES

*Alphavirus Structure/cellular attachment and entry*
Alphavirus genomes are encapsulated in an icosahedral shaped capsid (T=4 symmetry) surrounded by a lipid bilayer derived from the host cell plasma membrane (100). The E1 and E2 envelope glycoproteins are located within the bilayer and make up the exterior of the virus particle. The E1 and E2 glycoproteins are assembled as heterodimers which then form trimers. There are 80 trimers per alphavirus particle and each trimer creates a spike around the surface of the virion. The primary role of the envelope spikes is to mediate attachment and entry into the host cell (36, 100). Studies with SINV indicate that E2 is primarily involved in attachment to the cellular receptor and that the E1 glycoprotein is primarily involved in fusion of the glycoprotein with the cell membrane (100). Currently, there are two theories of how alphaviruses enter host cells. One line of evidence indicates that alphaviruses enter host cells via receptor-mediated endocytosis. Following virus binding to a host-cell receptor, the virion is internalized into an endosome and a decrease in pH causes a conformational change in the structural proteins leading to membrane fusion (22). Another line of data suggests that alphaviruses directly fuse at the plasma membrane to form a pore complex so that RNA is directly injected into host cell cytoplasm (110). It is likely that both of these models are correct, and that receptor mediated endocytosis versus direct fusion with the host cell envelope is cell-type specific.

*Genome Organization and Replication of Alphaviruses*

Members of the family *Togaviridae* contain a positive sense RNA genome with the nonstructural proteins (nsP’s) 1,2,3 and 4 encoded at the 5’ end of the genome. The capsid and glycoprotein structural genes are encoded at the 3’ end of the genome and are transcribed by a 26s subgenomic promoter at a 5 to 10 fold molar excess of genomic RNA (100). The
3’ untranslated region (UTR) of the alphavirus genome consists of a polyadenylated tail (pA) preceded by a 19 nucleotide conserved sequence element (3’CSE) (106). Following virus binding and entry, replication occurs exclusively in the cytoplasm of the host cell. While some nsP’s can traffic to the nucleus, enucleated mammalian cells are still capable of producing progeny virions to the same titers as nucleated cells (31).

The alphavirus genome is recognized as a messenger RNA (mRNA) upon entry into the host cell cytoplasm and the nsP’s are immediately translated. The nsP’s are initially translated as either a 123 and 1234 polyprotein, and viral replication steps are dictated based on cleavage of the nonstructural polyprotein (36, 100). First, nsp4 is cleaved to produce a 123 intermediate which together initiate negative strand synthesis. The 3’CSE serves as a promoter for the minus strand synthesis during alphavirus genome replication (106).

Next, cis-cleavage of nsP1 followed by cleavage of the nsP23 polyprotein results in a fully processed replication complex. This mature nsP1-4 complex associates with minus strand genomic RNA to replicate genome length, capped positive sense RNA as well as capped 26s subgenomic RNA. Translation of the 26s RNA results in a second polyprotein consisting of the capsid, envelope (E3, E2, and E1) and the 6k proteins. This polyprotein is then cleaved into the capsid protein a E3/E2 complex (called PE2) and E1. Post translational processing, which consists of folding and glycosylation of the envelope proteins occurs in the Endoplasmic Reticulum (ER) and the Golgi. Following exit from the Golgi, a furin protease cleaves PE2 into E3 and E2, and only the mature E2 and E1 glycoproteins are incorporated into the mature virus particle. Newly synthesized envelope glycoproteins then associate with the capsid and viral RNA to bud, incorporating the host cell plasma membrane upon egress (36).
**Alphavirus glycosylation**

N-linked glycosylation of the virus particle is a common feature among viruses that incorporate envelope proteins in their virion. Envelope N-linked glycosylation is critical for several different aspects of the virus replication cycle. Oligosaccharide modifications aid in proper folding of the envelope during virion assembly (37) and are critical for virus-host cell receptor interactions on specific cell types (54, 71). Additionally, recent evidence has demonstrated that both the presence and type of virion envelope glycosylation can modulate the host immune response following infection, and these studies are described in more detail later in the chapter.

The majority of alphaviruses contain multiple N-linked glycosylation sites on the E2 and E1 envelope glycoproteins. SINV envelope glycosylation is the best characterized and has 2 glycosylation sites on both the E2 and E1 glycoproteins. RRV contains three N-linked glycosylation sites on its envelope glycoproteins. Two are located on the E2 glycoprotein at amino acids 200 and 262, while the other is located on the E1 glycoprotein at amino acid 141. N-linked envelope glycosylation of alphaviruses grown in mammalian cells initiates immediately following translation at an asparagine residue by the addition of an N-Acetylglucosamine (GlcNac) linked to a high mannose glycan structure (a structure with up to 9 mannoses attached including a tri-mannose core) in the ER (65). From here, oligosaccharide trimming of mannose sugars followed by addition of complex sugars in the Golgi body results in a hybrid intermediate sugar (containing both high mannose and complex sugars). Further enzymatic processing occurs in the Golgi complex where mannose glycans are removed and are replaced by a combination of complex sugars. At this point the
mature viral glycoprotein is transported via the Trans-Golgi complex to the plasma membrane (65, 109).

It is important to note that factors in mammalian cells (including the duration of glycoprotein transport through the ER and Golgi as well as the accessibility of sugars on glycoproteins during processing) result in both the incomplete removal of high mannose sugars and reduce the amount of complex sugar additions. In this event, N-linked glycans on the viral envelope will contain a mixture of complex, hybrid, and high mannose N-linked glycans. Studies with SINV virus demonstrate that proteins PE2 and E1 heterodimerize through non convolent interactions (4) and subsequently form the trimer spike while still in the ER before moving to the Golgi. PE2 is cleaved into its mature E2 protein in the Trans-Golgi by a host cell enzyme (90, 111). These events likely result in incomplete oligosaccharide processing since studies with SINV reveal that a proportion of the glycans on the mature virion contain mannose, hybrid, as well as fully processed complex sugars (47, 48).

The type of oligosaccharide chain added to each glycosylation site on the virus envelope is also governed by the cell source of the virus, since insect cells producing alphaviruses lack enzymes that add complex carbohydrates to the surface of a virion, while the same viruses produced in mammalian cells contain complex sugars (46). Therefore, insect-cell-derived viruses will contain different species of mannose sugars ranging from high mannose (man-4 to man-9) to pauci-mannose (man-3) N-linked oligosaccharides (10, 42, 46). This is of particular importance with mosquito borne viruses since virions are produced from both species and properties of the virion differ depending on the cell source.
Introduced mutations into the viral genome that ablate N-linked glycan sites disrupt optimal particle production and infectivity, highlighting their importance. Pletnev et al. demonstrated point mutations in multiple SINV glycosylation sites severely impaired these viruses for growth and ablation of two or more glycan sites reduced viral titers by 2 logs or more (79). Similar results have been reported with other mosquito borne viruses. The Dengue virus E protein glycosylation site at amino acid 67 is essential for particle formation in mammalian cells and the 153 glycosylation site is required for optimal infectivity in vitro. However, neither site is required for replication in mosquitoes (9, 73). WNV E protein glycosylation at amino acid 153 is essential for particle formation and infection of C6/36 cells, but is dispensable for infection of BHK-21 cells (37).

**ALPHAVIRUS INTERACTIONS WITH THE INNATE IMMUNE SYSTEM**

*Type I Interferon*

Type I interferons (IFN-αβ) are critical molecules produced by the host cell to control viral replication (8). The antiviral properties of IFN-αβ were first described in 1957 by Isaacs and Lindenmann (49). Since this discovery, the properties of IFN-αβ have been intensely studied and demonstrated to be one of the first responses of a host cell involved in controlling virus replication. As mentioned previously, an intact IFN-αβ system is extremely critical to protect against alphavirus infection. The IFN-αβ response following virus infection is divided into two phases: 1) IFN-αβ induction following the sensing of virus infection and 2) IFN-αβ amplification of both IFN-αβ genes as well as hundreds of interferon responsive genes (ISG’s).
The IFN-αβ induction phase senses a viral infection that then triggers host cell signaling events resulting in the production IFN-αβ. Following infection, viral RNA is recognized by cytoplasmic RNA helicases including Retinoic acid inducible gene-I (RIG-I), Melanoma differentiation associated protein-5 (Mda-5), and the protein kinase R (PKR) proteins. Activation of these proteins leads to nuclear factor kappa B (NFκB) and interferon regulatory factor-3 (IRF-3) signaling pathways that drive the transcription of the early type I IFN genes IFN-β1 and IFN-α4 (82).

Additionally, toll-like receptors (TLR’s) recognize pathogen associated molecular patterns (PAMP’s) which then drive production of IFN-αβ. Viral PAMPs and their TLR’s include single-stranded RNA (TLR-7 and -8) or double stranded RNA (TLR-3), and (TLR-4) (105). While primarily described to respond to bacterial components such as liposaccharide (LPS), TLR-4 also recognizes respiratory syncitial virus, mouse murine leukemia virus, and vesicular stomatitis virus glycoproteins (33, 58, 83, 91). Furthermore, there is evidence that C-type lectin receptors (CLRs) can interact with viral glycoproteins to induce or suppress IFN-αβ (2, 50, 97). While some lectins in these pathways have been described, the details of how many lectins interact with viral glycoproteins to induce IFN-αβ is not well described.

Following IFN-αβ induction, IFN amplification occurs. The initially produced IFN-αβ binds to the type I IFN receptors (IFN-αβR) on either the same cell (autocrine) or neighboring (paracrine) cells. Following ligation of the IFN-αβR receptor, janus tyrosine kinase phosphorylates signal transducers and activators of transcription (STAT’s) signaling pathways. Upon phosphorylation, STAT-1 and STAT-2 form a heterodimer and associate with IRF-9 to form the ISGF3 complex. This complex translocates to the nucleus where it binds to interferon stimulated response elements (ISRE’s) to turn on transcription of all the
IFN-α genes as well as hundreds of interferon stimulated genes (ISG’s) (92). IFN-αβ amplification is critical for the host to control virus infection, since mice deficient in the IFNαβR or STAT-1 are extremely sensitive to infection when compared to wild type mice (86-88, 113). This is especially true with alphavirus infections that are self limiting in wild type mice but lethal in mice deficient in an intact IFN signaling pathway.

IFN-αβ signaling exerts its antiviral effects by specifically inducing genes that inhibit virus transcription and translation, as well as by causing host cell growth arrest and apoptosis. Three major antiviral pathways include PKR, the coupled 2’-5’ oligoadenylate synthetase (OAS)/RNaseL, and the Myxovirus resistance protein (Mx) pathways. All three pathways are upregulated following IFN-αβ stimulation. Upon IFN-αβ stimulation, PKR and the 2’-5’ OAS pathways are activated in response to dsRNA. PKR activation inhibits translation of viral RNA (89) while the 2’-5’ OAS pathway degrades both viral and host cell RNA preventing virus replication (13). The Mx proteins have potent antiviral activity (3) and can associate with viral nucleocapsids to prevent viral replication (55). Both mice and cell lines deficient in these pathways are inherently more susceptible to viral infections highlighting their role to control virus infection.

The importance of an intact IFN-αβ signaling pathway in limiting viral replication is also evident by the fact that almost every virus studied can block specific steps in the IFN-αβ signaling pathway through virally encoded IFN-αβ antagonists. For example, the VP35 protein of Ebola and the NS1 protein of influenza block RIG-I signaling to shut down IFN-αβ induction (12). The capsid of EEEV virus can nonspecifically shut down host protein synthesis to prevent IFN-αβ transcription (1). The Vesicular Stomatitis virus M protein provides an example of a block on IFN-αβ amplification which prevents STAT
phosphorylation and translocation to the nucleus (103). The non structural proteins of the mosquito borne virus DV also blocks STAT activation in infected cells (43, 77). Furthermore, a common theme among viruses is that a virulent virus often has a greater number and/or more potent IFN antagonists than a less virulent virus (82).

Recent studies have revealed that in addition to viral encoded proteins, mosquito saliva can suppress the immune response in a localized area which enhances viral replication (93). Furthermore, both our group and others have shown that N-linked glycans on the virus envelope play a role in either suppressing or promoting IFN-αβ induction during infection of DCs and macrophages (2, 95-97, 99). These data are the focus of this thesis and indicate that arboviruses derived from mosquito cells are more infectious, but induce lower levels of IFN-αβ in specific cell types. More detailed examples are described later in the chapter. Taken together, viruses activate the IFN-αβ response as well as block the IFN-αβ pathway at several different steps.

**Dendritic cells (DCs)**

DCs are central players in the immune system that detect and orchestrate the removal of viral pathogens. DCs are resident in almost every tissue and are subdivided into several different tissue specific subsets (98). One DC subset, known as plasmacytoid DCs (pDCs) are derived from a B-cell-like progenitor (26). While sporadic reports indicate that they can function as antigen presenting cells (78), their main function is to produce IFN-α to high levels in response to interactions with viruses (30). pDCs generally circulate in the blood, however, they also reside in tissues.
Another subset of DCs, myeloid DCs (mDCs) engulf both self and non-self antigens and present peptides of these antigens to T and B lymphocytes in the context of MHC class I and class II (98). In addition to antigen presentation, mDCs also elicit strong innate immune responses through the production of proinflammatory cytokines and IFN-αβ (23). These potent innate immune responses do not require productive viral replication since ultraviolet (UV) inactivated New Castle Disease virus, Herpes Simplex virus, and alphaviruses are still capable of inducing IFN-αβ to similar levels to virus infection in the absence of UV inactivation (41, 81, 95, 115). This data suggests that while intracellular RNA helicases (such as RIG-I, Mda-5, and PKR) recognize viral RNA in mDCs subsets (52), additional proteins may sense viral infection that could contribute to IFN-αβ induction. mDCs can be further subdivided into five tissue-specific subsets. Extensive studies in mice have demonstrated that these five classes of mDCs reside in tissues including the skin, lymph nodes, spleen and thymus (98). Specifically, mDCs in the skin and DLN are believed to be important in mosquito-borne virus infection since these are the initially infected tissues following virus delivery from the mosquito vector (44, 54, 66, 86, 114). mDC’s in the skin are termed Langerhans or dermal DCs. Langerhan DCs express high levels of langerin and the surface marker CD205 (40). Dermal mDCs express lower levels of CD205 but are CD11c+ and CD11b+, which are present on other mDC subsets. mDCs in other tissues including lymph nodes not in the skin or in the spleen express CD11c and CD11b but do not express CD205 (98). While these cell surface markers help to distinguish these different DC subsets, their specific functions are still not fully understood. Identifying the specific skin DC subsets that are infected by mosquito transmitted alphaviruses remains unclear, but infected CD205+ cells have been identified in the DLN following VEEV infection (67).
Altogether, it is likely that both pDCs and mDCs play a role in virus infection. The studies in this dissertation predominately examine the role of mDCs derived from the bone marrow of mice (BMDCs). While BMDCs are not identical to skin DCs, we are using these cells as a representative mDCs. BMDCs have the fundamental properties and surface markers of mDCs and protocols exist to generate BMDCs in large enough numbers for detailed studies (94).

**The role for C-type Lectin Receptors (CLR)s on DCs**

In addition to the well described virus sensors (e.g. RIG-I, Mda-5, and TLR’s) present in DCs and other cell types, CLR\s are a family of receptors that can bind and internalize pathogens and trigger signaling events to regulate innate immune response genes (85). These receptors are defined by having a carbohydrate recognition domain, and are located on most DC and macrophage subsets (25). CLR\s are also well documented in their ability to bind host-derived molecules (e.g. DC-SIGN mediates DC interactions with T cells and endothelial cells by interacting with ICAM-3 and ICAM-2, respectively (107, 108)). While the presence of CLR\s on immune cells is conserved across vertebrate species, the specificity for pathogen recognition appears to differ between mice and humans (34, 56). Both viruses and bacteria interact with CLR\s through interactions between the glycosylated surface of the microbe and the cellular receptor (20, 54, 71, 97). Both human DC-SIGN and the mannose receptor on DCs and macrophages recognize mannose glycans on HIV, mycobacteria, and Dengue virus to enhance infection (32, 71, 97). Other CLR\s including human macrophage galactose (hMGL) and DC-SIGNR recognize complex glycans to mediate virus entry (20, 72, 80).
addition, CLRs bind complex sugars on parainfluenza and influenza viruses which are critical for infection of host cells (70, 115).

Several reports indicate that viral N-linked glycans on mosquito borne viruses mediate DC tropism through interactions with CLRs. The presence of high mannose N-linked glycans on mosquito-cell-derived SIN and WNV infect mDCs more efficiently than mammalian-cell-derived SIN and WNV due to specific interactions with DC-SIGN (20, 54). In addition to the type of glycan, the positions of N-linked glycans are critical for WNV to interact with DC-SIGN expressing cells (19). Therefore, CLRs also serve as attachment receptors to enhance mosquito borne virus infection of DCs and macrophages, though it is likely that other molecules act as entry receptors.

While CLRs clearly play a role in infection for mosquito borne viruses, there are other cellular proteins that function as entry receptors. Alphaviruses replicate in multiple tissues in both vertebrate and invertebrate cells, and it is likely that RRV enters different cell types through a variety of receptors. Previous studies identify the importance of the α1β1 integrin for RRV entry into HeLa and mouse embryonic fibroblasts (59), but other receptors are not yet defined. Other studies have indicated that VEEV can use the laminin receptor for entry in Vero and 293 cells (35). One reason it is difficult to identify cellular receptors in vitro is because alphaviruses can rapidly adapt to binding the glycosaminoglycan heparin sulfate (HS) in vitro, and this may not reflect the true receptor in vivo. However, HS binding by RRV is not thought to occur unless a mutation at amino acid 218 of the E2 glycoprotein is introduced (38). Similar mutations in VEEV can confer enhanced HS binding (7). However, wild type SINV and SFV are able to bind HS in cell culture (7, 117). Altogether, it is likely
that RRV and other alphaviruses interact with both CLRs and other cellular receptors for entry into a variety of cell types.

The role for viral envelope glycosylation in mediating IFN-αβ responses on different host cells.

For many RNA viruses, glycosylation of the virion is required for many of their pathogenic properties (109). However, the studies in this report predominately focus on the role of alphavirus glycosylation during infection of DCs in vitro. Examples described in the above section demonstrate the requirement of N-linked glycans on the virus particle for infection. As introduced previously, interactions between virus glycosylation with CLRs on DCs and macrophages can also both promote and suppress antiviral responses.

Suppression of the innate immune response following CLR ligation

Ligation of a CLR receptor often results in suppression of the innate immune response following infection. For example, ligation of a CLR with an antibody in the absence of a pathogen can suppress IFN and proinflammatory cytokine secretion (11, 16, 27). Human DC-SIGN and the mannose receptor have been shown to recognize mannose glycans on mycobacteria and DV which both enhance infection and elicit an anti-inflammatory/Th2 response following ligation (32, 71, 97). There are also examples of high mannose N-linked glycans on arboviruses derived from mosquito cells that either avoid or suppress IFN-αβ responses on DCs and macrophages following infection when compared to mammalian-cell-derived viruses which have complex glycans. Our studies have demonstrated that mosquito-cell-derived alphaviruses are poor inducers of IFN-αβ in mDCs compared to mammalian-
cell-derived alphaviruses (95, 96). A similar observation was reported with mosquito-cell-derived WNV infection of pDCs (99). Another report indicated mosquito-cell-derived WNV actively suppressed poly I:C induced IFN-αβ responses in macrophages, and this was attributed to a specific type of insect-cell glycosylation (2). Virus inhibition of the innate immune response is not limited to arthropod borne viruses, since high mannose N-linked glycans on the gp120 molecule of HIV suppress mDC responses following DC-SIGN binding (97).

Ligation of C-type lectin receptors by pathogens promotes innate immune responses

While a growing body of evidence suggests that high mannose N-linked glycans negatively regulate antiviral responses, other studies indicate that viral glycoproteins, either assembled as virus-like particles (VLP’s) or glycoproteins fixed on the surface of infected cells, can stimulate type I IFN through interactions with lectins (50). Previous studies indicate that in addition to viral RNA, viral glycoproteins from Corona, Influenza, and paramyxoviruses are capable of inducing IFN-αβ responses (6, 14, 21, 61, 70, 116). Charley et al. demonstrated that complex, but not high mannose, N-linked glycans on the transmissible gastroenteritis virus (TGEV) M protein were responsible for IFN induction in porcine peripheral blood mononuclear cells (14). TGEV mutants lacking N-linked glycan sites were poor inducers of IFN-α (61). Additional studies demonstrated that both the highly ordered structure of an assembled virus-like-particle (5, 6) and the glycan linkage (N-linked but not O-linked glycans) were critical for robust IFN-αβ production (21). Similar results obtained with influenza virus demonstrated a requirement for interactions between sialic acid
on the virus particle and mouse spleen cells for IFN-αβ production (70). Finally, Ebola VLP stimulation of human mDCs requires the heavily glycosylated mucin domain (69).

Induction of IFN-αβ by viral glycoproteins is hypothesized to occur as a result of interactions between viral glycoproteins and CLRs (50), however the mechanisms of how CLRs signal to induce antiviral responses is still not completely understood. Taken together, these data suggest that interactions between lectin receptors and viral N-linked glycans are capable of both suppressing and activating antiviral responses in IFN producing cells. Furthermore, the specific glycan species on the virus particle dictates whether or not the cell will trigger a proinflammatory or anti-inflammatory immune response.

**DISSERTATION OBJECTIVES**

This dissertation investigates differences between mammalian-cell-derived and mosquito-cell-derived alphaviruses. These studies are important since alphaviruses are mosquito transmitted viruses that have different structural features depending on the host cell from which they are derived. Since a virus from a mosquito cell would only retain the properties of that cell for an initial round of infection, we hypothesized that mosquito- and mammalian-cell-derived virions would interact differently with cells that are initially infected in the vertebrate host. Previous reports have indicated these first infected cells are DCs. We have used bone marrow dendritic cells (BMDCs) to model the initial virus-host cell interactions and designed experiments to address the following questions:

1. Do mosquito- and mammalian-cell-derived alphaviruses differentially interact with myeloid dendritic cells?

2. Are there roles for N-linked glycans on RRV to either suppress or promote IFN-αβ induction in myeloid dendritic cells?
3. Do 1) mosquito and mammalian-cell-derived RRV differ in their ability replicate and establish disease in a mouse model and 2) are the presence of N-linked glycans on RRV required for severe disease in a mouse model?
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CHAPTER TWO

DIFFERENTIAL INDUCTION OF TYPE I INTERFERON RESPONSES IN MYELOID DENDRITIC CELLS BY MOSQUITO AND MAMMALIAN-CELL-DERIVED ALPHAVIRUSES

Reed S Shabman\textsuperscript{1,2,3}, Thomas E Morrison\textsuperscript{1,2,3}, Christopher Moore\textsuperscript{4}, Laura White\textsuperscript{2,3}, Mehul S Suthar\textsuperscript{1,2,3}, Linda Hueston\textsuperscript{7}, Nestor Rulli\textsuperscript{5}, Brett Lidbury\textsuperscript{5}, Jenny P-Y Ting\textsuperscript{4}, Suresh Mahalingam\textsuperscript{5}, and Mark T Heise\textsuperscript{1,2,3*}

\textsuperscript{1} The Department of Genetics, \textsuperscript{2} The Department of Microbiology and Immunology, \textsuperscript{3} The Carolina Vaccine Institute, \textsuperscript{4} The Lineberger Cancer Center, The University of North Carolina-Chapel Hill, Chapel Hill, NC 27599; \textsuperscript{5} University of Canberra, Canberra, Australia

Corresponding Author. The Carolina Vaccine Institute, The University of North Carolina, Chapel Hill, NC 27599. Phone: 919-843-1492, Fax: 919-843-6924, Email: heisem@med.unc.edu

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ABSTRACT

Dendritic cells (DCs) are an important early target cell for many mosquito-borne viruses, and in many cases mosquito-cell derived arboviruses more efficiently infect DCs than viruses derived from mammalian cells. However, whether mosquito-cell derived viruses differ from mammalian-cell derived viruses in their ability to induce antiviral responses in the infected dendritic cell has not been evaluated. In this report, alphaviruses, which are mosquito-borne viruses that cause diseases ranging from encephalitis to arthritis, were used to determine whether viruses grown in mosquito-cells differed from mammalian-cell-derived viruses in their ability to induce type I interferon (IFN) responses in infected primary dendritic cells. Consistent with previous results, mosquito-cell derived Ross River virus (mos-RRV) and Venezuelan Equine Encephalitis (mos-VEE) virus exhibited enhanced infection of primary myeloid dendritic (mDCs) cells as compared to mammalian-cell derived virus preparations. However, unlike the mammalian-cell derived viruses, which induced high levels of type I IFN in the infected mDC cultures, mos-RRV and mos-VEE were poor IFN inducers. Furthermore, the poor IFN induction by mos-RRV contributed to the enhanced infection of mDCs by mos-RRV. These results suggest that the viruses initially delivered by the mosquito vector differ from those generated in subsequent rounds of replication in the host not just with respect to their ability to infect dendritic cells, but also in their ability to induce or inhibit antiviral type I IFN responses. This difference may have an important impact on the mosquito-borne virus’s ability to successfully make the transition from the arthropod vector to the vertebrate host.
INTRODUCTION

Mosquito-borne viruses, which include a wide range of human pathogens, including flaviviruses, alphaviruses, and bunyaviruses, cause diseases ranging from arthritis to hemorrhagic fever or encephalitis (10, 31, 38, 42, 43). A number of mosquito-borne viruses are thought to initially interact with and replicate in myeloid dendritic cells (mDCs) in the skin following delivery from the mosquito vector (26, 36, 46) and it is likely that early interactions with mDCs play an important role in determining whether the virus successfully establishes infection and ultimately causes disease.

One of the most important obstacles that an invading virus has to overcome is the host type I interferon (IFN) system, which plays an essential role in the early control of many viral infections, modulates downstream aspects of other components of the innate immune response, and helps orchestrate the adaptive immune response (reviewed in (3)). This may be particularly important for mosquito-borne pathogens that initially replicate in dendritic cells of myeloid origin, since mDCs are capable of mounting robust type I interferon responses (7). Several arthropod-borne viruses, including members of the Flaviviridae and Bunyaviridae encode antagonists of the type I IFN system that can block multiple components in the type I IFN pathway (33, 44). However, mDCs are different from many other cell types, such as fibroblasts, since they can rapidly respond to the incoming virus and do not require viral replication to initiate a type I IFN response (15), which may make these cells less susceptible to virally encoded interferon antagonists than other cell types. Therefore, other factors such as immune suppressive components of mosquito saliva may also promote transmission to the vertebrate host (23, 24, 37). However, whether the virus derived from the mosquito differs from virus derived from
mammalian cells in its ability to induce an antiviral response in mDCs or other cell types has not been evaluated.

Alphaviruses are single-stranded, positive sense RNA viruses that are transmitted by mosquitoes and cause human diseases ranging from infectious arthritis to lethal encephalitis (reviewed in (42)). Alphaviruses are extremely amenable to genetic manipulation and in addition, well established cell culture and animal pathogenesis models exist for several of these viruses (31, 42, 43, 45). Furthermore, several alphaviruses target to dendritic cells in vivo (26, 36). Additionally, mosquito-cell derived Sindbis virus has been shown to preferentially infect human mDCs due to interactions between the dendritic cell specific C-type lectin DC-SIGN and high mannose glycans on the mosquito-cell derived virion (18). Therefore, alphaviruses represent an extremely useful set of viruses for studying how mosquito-borne viruses interact with mDCs.

In this study, Ross River virus (RRV) and other alphaviruses were grown in either mosquito or mammalian cell cultures (mam- or mos-RRV) and evaluated for their ability to infect murine or human mDCs and induce an antiviral type I interferon response. These studies demonstrate that for several alphaviruses, the mosquito-cell derived virus was able to efficiently infect the mDCs, but was an extremely poor inducer of type I IFN, while the same virus derived from mammalian cells was a potent IFN inducer. These results strongly suggest that alphaviruses delivered from mosquito vectors are able to infect mDCs while simultaneously avoiding the type I IFN response.
MATERIALS AND METHODS

Virus stocks, and cell lines. Ross River virus (T48 strain) was produced from the pRR64 infectious clone (generously provided by Dr. Richard Kuhn, Purdue University), which includes the full length T48 cDNA clone (21) originally isolated from *Aedes vigilax* mosquitoes in Queensland, Australia (8). The RR64-GFP virus, which expresses green fluorescent protein from a second viral subgenomic promoter, was constructed as described previously (31). Briefly, genome length viral RNA was transcribed using mMessage mMachine SP6 in vitro transcription kits (Ambion) and the RNA electroporated into baby hamster kidney-21 cells (BHK-21; ATCC CCL-10). Twenty four hours post electroporation, supernatants were harvested, and the virus pelleted through a 20% (wt/vol) sucrose cushion by ultracentrifugation at 72,000 x g, and the virus was resuspended in PBS. Venezuelan equine encephalitis virus (VEE), which had been engineered to express GFP from a second subgenomic promoter was generated from clone dpV3000-GFP in a similar manner as described previously (45). To generate mos-RRV or mos-VEE, virus was passaged a single time at a multiplicity of infection of 0.1 in *C6/36 Aedes albopictus* and input virus was washed off after a one hour infection. Alternatively, C6/36 cells were electroporated with in vitro transcribed RR64 RNA using the same protocol to generate BHK cell derived virus. Both passaged and electroporated mos-RRV showed equivalent levels of IFN induction (data not shown). At 18-24 hours, supernatant was collected and concentrated. RRV generated from Chinese Hamster Ovary (CHO) cells was obtained by a single passage of mam-RRV through either the parental Pro-5 (ATCC CRL-1781) or mutant Lec-1 (ATCC CRL-1735) CHO cells. Virus stocks were titrated on BHK-21 cells by standard plaque assay. Barmah Forest
virus (BFV) (strains 10E101SC and BH2193) (22) were grown in BHK-21 cells to generate mam-BFV. To generate mos-BFV, virus was passaged a single time in C6/36 Aedes albopictus cells for 18-24 hours and supernatant was collected and concentrated by ultracentrifugation the same way as mam-BFV. Both virus stocks were reconstituted in phosphate buffered saline and titered on BHK-21 cells by standard plaque assay.

BHK-21 cells were grown in Alpha Minimal Essential Medium (Gibco) with 10% fetal bovine serum (FBS), 10% tryptose phosphate broth and 0.29 mg of L-glutamine per ml. C6/36 cells were grown in Minimal Essential Medium with Earl’s Salts and supplemented with 5% FBS, non essential amino acids, penicillin and streptomycin.

**Bone Marrow Dendritic cells cultures and virus infection conditions.** Murine bone marrow derived dendritic cells were generated from 129 Sv/Ev mice and 129 Sv/Ev αβ RKO bone marrow as described previously (39). Bone marrow was harvested from the femur and tibia of 1-3 month old mice, red blood cells were lysed, and cells were resuspended in RPMI 1640, 10% FBS, penicillin, streptomycin, and 2-mercaptoethanol. Cells were grown in suspension in the presence of recombinant GM-CSF and IL-4 for seven days at 20ng/ml (day 0-3), 10ng/ml (day3-5), 5ng/ml (day 5-7) in ultra low cluster 6-well Costar plates (Costar) to obtain an immature dendritic cell phenotype, which was confirmed by staining with anti-CD11b, CD11c, B220, CD80, CD86 and CD40 (eBioscience). At day 7 post bone marrow harvest, cells were infected with either RRV or VEE for two hours in a total volume of 0.2 ml. Following infection, 0.5 mls of media was added back to the culture. At various times post infection, supernatants were removed and assayed for type I IFN levels, while the cells were fixed in 4%
paraformaldehyde and analyzed by flow cytometry for GFP expression to quantify the percentage of infected cells within the culture.

**Generation of Human monocyte derived DC’s.** Peripheral blood was obtained from healthy volunteers and diluted 1/2 in PBS. The PBMC’s were isolated following separation over lymphocyte separation medium (ICN Biomedicals), washed in PBS, and resuspended in RPMI 1640 media as described in murine DC’s. Cells were plated in culture flasks and allowed to adhere for 2 hours, at which time non adherent PBMC’s were removed and discarded. Cells were cultured in the presence of 800 IU/ml GM-CSF and 500 IU/ml IL-4 (Peprotech) for 6 days with fresh cytokines added every 48-72 hours. On day 6, cells were resuspended, phenotyped by flow cytometry staining with anti-CD11c, HLA-DR, DC-SIGN, and CD14 (BD Biosciences Pharmingen) and infected in the same manner as the murine mDCs. Experiments were performed on blood from two separate donors.

**IFN Bioassay.** Type I interferon levels in cell culture supernatants were measured by interferon bioassay as described previously (2, 45). Briefly, L929 mouse fibroblasts (ATCC CCL-1), human lung carcinoma A549 (CCL-185) or Vero cells (CRL-1586) were seeded in 96 well plates. All samples were acidified to a pH of 2.0 for 24 hours and then were neutralized to pH 7.4 and further inactivated by UV light for 10 minutes prior to titration by two fold dilutions down the plate. Twenty four hours after the addition of the supernatant, the interferon sensitive viruses encephalomyocarditis virus (EMCV) or Sindbis virus were added to each plate. At 18-24 hours post infection, 3-[4,5-Dimethylthiazol-2yl]-2,5-Diphenyltetrazolium bromide (MTT) (Sigma), an indicator of viable cells, was added to each well in the murine bioassay only. The MTT product
was then dissolved in isopropanol/0.4% HCl and absorbance was read on a microplate reader at 570 nm. Alternatively, human bioassay plates were scored for 50% cytopathic effect as an indicator of IFN concentrations. Each plate contained an IFN standard (Chemicon or R&D systems) to determine the international units (IU/ml) of type I IFN in each culture.

**Real Time PCR and semi-quantitative PCR assays.** Total cellular RNA was isolated with Ultraspec RNA isolation reagent (Biotex) and total RNA was reverse transcribed with a cDNA archive kit (Applied Biosystems). Taq-Man Real Time PCR was performed with primer probe sets for specific genes of interest (Applied Biosystems) and analyzed on the Prism 7000 machine (Applied Biosystems). For all samples, an equivalent amount of RNA was reverse transcribed and an internal reference control of 18s ribosomal RNA or GAPDH was included. For studies with Barmah Forest virus, total RNA was isolated from cells by standard methods using RNAwiz (Ambion). Reverse-transcriptase PCR (RT-PCR) was then carried out as previously described (28). Primer sequences for HPRT and IFN-β have been described previously (27). For RRV genome analysis, primers for sequences specific to the NSP1 and NSP4 region of RRV were used. The primer sequences for NSP1 were (Forward 5’-AGAGTGCGGAAGACCCAGAG-3’ and Reverse 5’-CCGTGATCTTTACCAGGACACA-3’) and for NSP4 (Forward 5’-ACCCGACAGTGGCTAGTTAC-3’ and Reverse 5’ CGGTTG GTGGTAAAGCATGAT-3’). Purified virions were isolated with the MagMax viral RNA extraction kit (Ambion), reverse transcribed using the cDNA archive kit (Applied Biosystems) and cDNA was used for quantitative Real Time PCR using Sybrgreen
(Applied Biosystems). A standard curve was generated to ensure optimal primer efficiency and relative differences between virus stocks were calculated.

Specific infectivity assays. The specific infectivity of RRV produced in BHK-21 cells or C6/36 was performed as previously described (19). In brief, at 4 hpi culture media was replaced with methonine free medium. At 8 hpi, virus growth media was radiolabeled with $[^{35}\text{S}]$ methionine at 10 µCi/ml. At 24 hpi, virus was harvested and banded twice over two 20/60% discontinuous sucrose gradients in PBS and pelleted over a 20% sucrose cushion at 70,000 x g. Virus was reconstituted in PBS, total counts per minute were measured (CPM), and equal CPM were used for a standard plaque assay on BHK-21 cells. The CPM to plaque forming unit ratio was then calculated to determine particle to PFU ratios for each virus preparation.

**Western Blot.** Purified RRV virions were analyzed on a 10% SDS-PAGE gel and western blots were performed using an anti-RRV mouse hyperimmune ascites fluid. Total fluorescence was detected by ECL Plus immunofluorescence (Amersham) and fluorescence was measured on a phosphoimager (Storm Scanner) and quantified on Image Quant 5.0.

**Statistics.** All groups were compared using a two-tailed unpaired student T-test or a one way ANOVA where indicated using GraphPad InStat software.

**RESULTS**

**Mosquito and mammalian-cell derived RRV differ in their ability to induce type I interferon in bone marrow derived dendritic cells (mDCs).**

In order to assess the ability of mammalian and mosquito-cell derived virions to infect murine mDCs, RRV expressing green fluorescent protein (GFP) was grown in
either BHK-21 cells (mam-RRV) or C6/36 *Aedes Albopictus* cells (mos-RRV) and GFP was measured as an indicator of infection. Consistent with previous reports with Sindbis virus and West Nile virus infection of human dendritic cells (6, 18), mos-RRV infected a higher percentage of mDCs than mam-RRV (Figure 2.1a and 2.1b) at 12 hours post infection (hpi). However, although mos-RRV infected a higher percentage of the mDCs, mos-RRV infected cultures produced little type I interferon (IFN), while mam-RRV induced a robust IFN response (Figure 2.1c) as measured by a type I IFN bioassay. Similar results were obtained when RRV without the GFP transgene was used (data not shown). These results were confirmed by quantitative RT-PCR to measure IFN-β mRNA levels in the virally infected cultures. Consistent with the bioassay results, mam-RRV induced an approximately 13 fold increase in IFN-β message over mock-infected cultures, compared to approximately a 2 fold induction by mos-RRV (Figure 2.1d). These results strongly suggest that mos-RRV, but not mam-RRV, was able to suppress or avoid the induction of type I IFN following infection of mDCs.

To determine if type I IFN differences between the mammalian and mosquito-cell derived viruses extended beyond murine DC’s, human monocyte-derived DC’s were infected with either mam- or mos-RRV and assessed for infectivity and IFN production. When these cells were infected with mam-RRV or mos-RRV, little productive infection was observed with either virus as determined by GFP expression (data not shown). However, when type I IFN levels in these cultures were assessed by bioassay, significantly higher levels of type I IFN were observed in supernatants from mam-RRV infected DC’s as compared to mos-RRV infected cells (Figure 2.1e). These results suggest that the differences in type I IFN induction between the mosquito and
mammalian-cell derived viruses also extends to human DCs, which further underscores the potential relevance of this phenotype to alphavirus-induced disease in humans.

Another important question was whether type I IFN induction by the mammalian derived virus was replication dependent, since UV inactivated Semliki Forest virus (SFV) is a potent inducer of type I IFN in mDC’s. Consistent with the SFV findings, UV inactivated RRV was able to induce type I IFN (figure 2.1f). However, heat inactivation of the virus ablated type I IFN induction and IFN induction co-purified with the viral particle (data not shown). This data suggests that an intact particle, but not productive replication was required for IFN induction by mam-RRV. This also suggests that endotoxin which previously has been shown to be heat stable (29) and is a potent inducer of type I IFN (17), did not contribute to the type I IFN induction by the mammalian-cell derived virus. Similar to infectious mos-RRV, UV inactivated mos-RRV did not induce type I IFN suggesting that the lack of type I IFN induction by mos-RRV was not dependent on viral replication, which indicated that a virally encoded de novo synthesized factor was not required for this effect.

The differences in type I IFN induction between mam- and mos-RRV might simply reflect a difference in the kinetics of type I IFN induction. Therefore, mDC cultures were infected with mam-RRV or mos-RRV and both the percentage of infected cells and the levels of type I IFN produced in the cultures were evaluated at several times post infection. As shown in Figure 2, mos-RRV infected a higher percentage of mDCs than mam-RRV at all time points with peak infection at 12 hpi (Figure 2.2a). However, the levels of type I IFN in the supernatants were significantly lower from the mosquito virus infected cultures compared to the mammalian virus infected cultures at the 9, 12,
and 20 hour time points (Figure 2.2b). Only at 20 hpi was IFN detectable by bioassay following mos-RRV infection, however, this response was approximately 10% of the IFN induction in mam-RRV infected cultures (242 IU/ml compared to 2138 IU/ml in mam-RRV infected cultures) in a representative study. The low level of IFN in mos-RRV cultures at 20 hpi may reflect secondary infection in the culture where virus produced by the infected mDCs would have properties of mammalian-cell derived virus.

**Particle differences do not explain the differential type I IFN induction by mam- and mos-RRV.**

Since alphavirus defective interfering (DI) particles have been shown to induce type I IFN (11, 12), and inactivated RRV and SFV particles induce type I IFN responses in mDCs (figure 2.1f and (15)), particle to PFU ratios for mam- and mos-RRV were calculated. If the mam-RRV preparations contained a larger number of particles per plaque forming unit (PFU) than mos-RRV, excess particles might be responsible for the difference in type I IFN induction between the two RRV preparations. However, when the ratio of viral particles per PFU was determined for mam- and mos-RRV stocks, mos-RRV had 6.6 to 11 fold more particles/pfu than the mammalian-cell derived virus stocks (figure 2.3). To confirm these results, two additional assays were used to evaluate the ratio of particles or genomes per PFU for both viruses.

Quantitative realtime PCR was used to measure the relative numbers RRV genomes between mam- and mos-RRV. This assay indicated that there were 2-4 fold more genomes/pfu of mam-RRV compared to mos-RRV (Figure 2.3). Additionally, semi-quantitative western blots were performed to measure relative glycoprotein and capsid content of both mam- and mos-RRV (Figure 2.3c and 2.3d). This assay, where
equivalent PFU of each stock were analyzed by western blot with polyclonal antiserum recognizing the viral E2 glycoprotein and capsid, showed some variation, where matched mam- and mos-RRV virus preparations ranged from equivalent levels of glycoprotein and capsid signal to either virus stock having up to 2-6 fold more glycoprotein and capsid specific signal than the corresponding virus stock from the other cell type. However, we saw no differences in the type I IFN induction phenotype for mam- and mos-RRV virus stocks, regardless of whether the virus stocks had equivalent capsid/glycoprotein signal, or if mos-RRV had more or less glycoprotein specific signal than the matched mam-RRV stock. This suggests that higher particle/pfu ratios of mam-RRV does not explain the difference in type I IFN induction. However, the higher ratio of genomes/pfu of mam-RRV compared to mos-RRV detected by the real time PCR assay (Figure 2.3b) could result in enhanced IFN induction. Therefore, we performed dose response experiments to determine whether small fold differences in genome to PFU ratios between the mammalian and mosquito derived viruses could contribute to the differential IFN induction. Myeloid DCs derived from wild type 129 Sv/Ev mice were infected with mam-RRV or mos-RRV over a range of MOI (0.8 to 100.0 plaque forming units (PFU)/cell) and the percent of infected cells and levels of type I IFN were determined at 12 hpi. As shown in Figure 2.4a, mos-RRV infected a higher percentage of cells than mam-RRV at all MOI tested; however, mam-RRV infection induced higher levels of type I IFN at MOIs from 4 to 100 (Figure 2.4b). The fact that mam-RRV induced higher IFN levels than mos-RRV over a 25 fold range of input virus, where mam-RRV induced more type I IFN at an MOI of 4.0 than mos-RRV at an MOI of 100.0, supports the idea that
differences in particle number between the virus stocks is not solely responsible for the differences in IFN induction.

**Differential type I IFN induction enhances mDC infection by mosquito-cell derived RRV.**

The observation that mos-RRV infected a higher percentage of mDCs than mam-RRV, but was a poor inducer of type I IFN compared to mam-RRV raised the question of whether enhanced DC infection by mos-RRV was due to the viral effects on type I IFN induction. Therefore, mDCs were generated from mice lacking a functional type I IFN receptor (32) and infected with mosquito or mammalian-cell derived RRV at the same range of MOI utilized in wild type mDCs (see Figure 2.4). In contrast to the wild type 129 mDCs, where mos-RRV showed enhanced infection, the type I IFN receptor deficient mDCs showed equivalent levels of infection by viruses derived from either mammalian or mosquito cells (Figure 2.5a). This result strongly suggests that the ability of mos-RRV to avoid or suppress the induction of the type I IFN response in the infected cultures enhances that virus’s ability to infect wildtype mDCs.

Another important question was whether the differences in type I IFN induction between mos- and mam-RRV were due to a block of the initial IFN induction or an inhibition of amplification of the IFN response within the cultures. In most cells, including mDCs, the type I IFN response is an amplification loop, where a subset of early IFN genes (e.g. IFN-β and IFN-α4) are induced early in the virus infection, and these proteins then signal through the type I IFN receptor in an autocrine or paracrine manner to induce much higher levels of their own expression as well as the expression of late interferon genes (reviewed in (3)). The absence of signaling from the receptor
significantly reduces the levels of IFN induced both at the protein and mRNA level. The quantitative RT-PCR results, which showed significant differences in IFN-β mRNA induction between mos- and mam-RRV infected cultures at 2 hpi (Figure 2.1d), strongly suggested that the differences in type I IFN production between the mammalian and mosquito virus infected cultures was at the level of the initial IFN induction. However, to test this further, IFN levels were measured in mDC cultures derived from the type I IFN receptor deficient mice, as these cells are able to induce early IFN gene expression, but the lack of type I IFN receptor signaling in these cells prevents amplification of the response (32). As shown in Figure 2.5b, mam-RRV was able to induce type I IFN in the interferon receptor deficient mDCs, while mos-RRV failed to induce detectable type I IFN responses as measured by bioassay. These results indicate that the mosquito and mammalian-cell derived viruses differ in their ability to induce type I IFN, though an effect on the amplification step cannot be ruled out.

**A single passage of Mos-RRV through BHK-21 cells restores the ability of the virus to induce type I IFN in mDC cultures.**

It is possible that genetic differences between mam- and mos-RRV explain the enhanced infection and decreased IFN production on mDCs. To test the stability of the mos-RRV phenotype, the mosquito-cell derived virus, which was a poor type I IFN inducer, was passaged once at a MOI of 1 through BHK-21 cells and the resulting mam-RRV virions were placed on mDCs (Figure 2.6). A single passage of mos-RRV in mammalian-cells resulted in a reduction of infection efficiency (Figure 2.6a) on mDC cultures as well as an increase in type I IFN induction (Figure 2.6b). This suggests that the lack of IFN induction by mos-RRV is likely not due to a coding change(s) in the viral
genome as genetic variation would not be predicted to consistently occur after just a single passage. Furthermore, these results strongly suggest that evasion of type I IFN induction by mos-RRV in the vertebrate host would only apply to the first infected cells, since progeny virions would have the properties of mammalian-cell-derived virus.

**Differential type I interferon responses between mosquito and mammalian-cell derived viruses occur with other alphaviruses.**

To evaluate whether type I IFN differences on primary mDCs was specific to RRV or applied to other alphaviruses, similar experiments were performed with Venezuelan Equine Encephalitis Virus (VEE) and Barmah Forest virus (BFV). Previous studies have demonstrated that VEE infects human dendritic cells in vitro (30) as well as murine mDCs in vitro (Moran, Pressley and Johnston, unpublished data) and targets murine dendritic cells in vivo (26). Consistent with previous data, VEE was able to infect mDCs in vitro and similar to RRV, mos-VEE infected more mDCs at 12 hpi compared to mam-VEE at 12 hpi (Figure 2.7a), yet mos-VEE induced less type I IFN than mam-VEE (Figure 2.7b). Similar results were observed following infection of mDC’s with mosquito and mammalian-cell derived Barmah Forest virus (strain 10E101SC) in mDCs at 12hpi by IFN bioassay (Figure 2.7c). These results suggest that the differential type I IFN induction by the mosquito and mammalian-cell derived viruses is not specific to RRV, and in fact may be a general trait of alphaviruses.

**Differential N-linked glycosylation contributes to the differences in type I IFN induction by mosquito and mammalian cell derived alphaviruses.**

The 10E101SC strain of BFV initially used in these studies differs from most BFV strains in that it contains N-linked glycans on its E2 glycoprotein (S. Mahalingam,
unpublished). The IFN bioassay results shown in Figure 2.7c were confirmed using a semiquantitative RT-PCR assay for IFN-β transcripts (Figure 2.8a) and we next assessed whether classical strains of BFV, which do not have N-linked glycans on the E2 glycoprotein (22), also exhibited the same phenotype. When the BH2193 strain of BFV, which lacks N-linked E2 glycans was assessed for type I IFN induction, there were no significant differences in type I IFN responses between mam- and mos-BFV by semi-quantitative RT-PCR (Figure 2.8b). While there are likely to be other differences between the two virus strains, these results strongly suggest that the N-linked glycans on the E2 glycoprotein contribute to the differential type I IFN induction by the mosquito and mammalian-cell derived viruses.

We therefore tested whether the presence of high mannose N-linked glycans on mos-RRV were contributing to the poor IFN induction phenotype. RRV stocks were generated in parental Chinese hamster ovary (CHO) cells (Pro-5) or mutant CHO cells (Lec-1). Lec-1 CHO cells lack GlcNAc glycosyl transferase and glycans are blocked at the Man5-GlcNAC2-Asn intermediate. Therefore, viruses generated in Lec-1 cells have high mannose, but no complex glycans on the virion (similar to mos-RRV) while the parental Pro-5 RRV contains complex glycans (similar to mam-RRV) (40, 41). Like mos-RRV generated from C6/36 cells, Lec-1 RRV was sensitive to EndoH digestion, which is indicative of high mannose glycans on the virion while virus grown in BHK and Pro-5 cells was EndoH resistant (data not shown). Furthermore, virus derived from Pro-5 cells (complex glycans) induced type I IFN in infected mDC cultures, while IFN levels in cultures infected with Lec-1 derived virus (high mannose) were below the limit of detection (Figure 2.8c). Taken together, this data indicates that the presence of complex
versus high mannose glycans on the mammalian and mosquito derived virus contributes to their differential ability to induce type I IFN responses in infected dendritic cells.

**DISCUSSION**

The transition from the arthropod vector to the vertebrate host is a critical step that is likely to play a major role in determining whether arboviruses successfully establish infection and disseminate throughout the vertebrate host. Therefore, mechanisms that would enable the newly delivered arbovirus to subvert the type I IFN response in mDCs may provide an advantage to the virus as it establishes infection in the vertebrate host. A number of factors, including virally encoded interferon antagonists, (1, 4, 33, 44) and immunosuppressive components of the arthropod saliva (23, 24, 37), are thought to modulate the host antiviral response against arthropod-borne viruses. However, the question of whether viruses delivered by the mosquito vector differ from mammalian-cell derived viruses with respect to their ability to induce type I interferon responses has not previously been addressed.

In this report, we demonstrate that mammalian-cell derived alphaviruses are potent inducers of type I IFN in mDCs while mosquito-cell derived viruses are poor inducers of type I IFN. This suggests that the virus initially delivered by the mosquito vector is able to suppress or avoid the induction of type I IFN in the initially infected dendritic cell. It is possible that this intrinsic ability of the mosquito-cell derived virus to avoid the induction of a type I IFN response acts in concert with immuno-suppressive factors present in arthropod saliva, and/or virally encoded interferon antagonists to promote the successful transition of the virus from the arthropod vector to the vertebrate host.
Recent work with several mosquito-borne viruses, including dengue virus and VEE have demonstrated that dendritic cells and/or Langerhans cells are an early target of viral replication following delivery into the skin (6, 26, 35, 46). Furthermore, the presence of high mannose glycans on mosquito derived Sindbis and West Nile virions has been shown to enhance viral infection of dendritic cells via interactions with the dendritic cell specific mannose binding lectins DC-SIGN (CD209) (6, 18). The findings presented in this paper indicate that mosquito derived viruses not only have an enhanced ability to infect murine dendritic cells, as compared to mammalian-cell derived viruses, but that these viruses also suppress or avoid the induction of type I IFN responses in the infected dendritic cell cultures. Furthermore, this effect on interferon induction is likely to contribute to the enhanced infection efficiency of mDCs by the mosquito derived virus, as evidenced by the decrease in infection differences between mam- and mos- RRV in type I IFN receptor deficient DC’s compared to wild type DC’s (Figure 2.5b).

Myeloid DCs are capable of quickly responding to the incoming virus by mounting a very rapid antiviral response. This response has been shown to be independent of MyD88 for Semliki forest virus, another alphavirus (15), and preliminary experiments with RRV and VEE indicate type I IFN induction in mDCs is MyD88 and TLR3 independent (Shabman, Morrison, and Heise unpublished results). Furthermore, several studies have shown that mDCs rely on the RIG-I/Mda5 pathway to sense incoming viruses and induce type I IFN responses (16). Although a growing body of evidence indicates that mDCs can rapidly respond to viral infections and that they play a central role in regulating the host immune response to incoming pathogens, these cell types are also an early target for arbovirus replication. Our results suggest that the virus
initially delivered by the mosquito vector differs from the virus subsequently produced in mammalian-cells, and this difference may allow the mosquito derived virus to avoid induction of an antiviral response in the initially infected DC.

One of the major differences between the mosquito and mammalian-cell derived viruses is the exclusive presence of mannose glycans (high mannose and paucimannose) on the mosquito derived viruses and complex/hybrid/high mannose glycans on the mammalian-cell derived virus (25). A role for N-linked glycans in mediating the differential type I IFN induction by the mammalian and mosquito derived viruses is supported by the finding that the 10E101SC strain of Barmah Forest virus, which has N-linked glycans on its E2 glycoprotein, exhibits the differential type I IFN induction, while the BH2193 strain, which lacks N-linked glycans on its E2 glycoprotein, showed no difference in type I IFN induction between the mammalian and mosquito derived virus preparations (Figure 2.8a and 2.8b). Furthermore, RRV generated in Pro-5 cells with complex glycan additions induced more type I IFN on mDC’s than RRV generated in Lec-1 cells which contain exclusively mannose glycans (Figure 2.8c). Therefore, additional studies are needed to directly assess the role of N-linked glycans in this phenotype, since it is possible that high mannose glycans on the mosquito-derived virus interact with C-type lectins on dendritic cells resulting in an inhibition of type I IFN from the mDC cultures. This is intriguing since previous data indicates that ligation of DC-SIGN, the mannose receptor, or other C-type lectin receptors suppresses or modifies Toll-like receptor (TLR) signaling in human mDCs and pDCs (5, 9, 14, 20). Therefore, additional studies on the role of high mannose glycans on the mosquito-cell-derived virus in blocking type I IFN induction by the virus are required.
Experiments presented here were designed to explore several potential mechanisms other than the presence of high mannose species on the mosquito-cell derived viruses, including non viral factors, differences in the number of defective interfering particles, which are known to be potent inducers of type I IFN (11, 12), or genetic differences in the viruses derived from the mammalian versus mosquito cells.

Several lines of evidence argue against non viral factors being responsible for the differing type I IFN induction between the mosquito and mammalian-cell derived viruses. First, the results are highly reproducible between multiple virus preparations and with several different alphaviruses. Secondly, the type I IFN induction by mam-RRV appears to be virus specific, since the IFN inductive capacity separated with the ultracentrifuged virus pellet. Boiling, which would denature the virus, but not LPS, which can also induce type I IFN (13), also abolished the ability of the virus preparation to induce type I IFN.

The finding that multiple assays comparing mos- and mam-RRV preparations only vary in their particle to PFU ratio by 2-6 fold, while the difference in type I IFN induction in mDCs occurs over a 25 fold range of input virus argues against a role for defective interfering particles being responsible for the type I IFN induction differences. A role for genetic differences between the mosquito and mammalian derived viruses cannot be ruled out. Strong selection can rapidly select for adaptive mutations in alphaviruess (19, 34), however, the fact that a single pass through mosquito or mammalian cells reproducibly results in a switch in the interferon inductive phenotype argues against this possibility or suggests that an extremely strong selection event is occurring that selects for a genetic change leading to poor type I IFN induction. Finally, it is possible that in addition to differential glycosylation, differences in viral cholesterol
content or modifications to the viral nucleic acid in a cell type specific manner could affect the ability of the virus to induce type I IFN responses in mDCs, but not other cell types. Therefore, additional studies are needed to address these possibilities.

The poor IFN induction by mosquito-cell derived viruses was observed for several different alphavirus family members. These included RRV, VEE, and BFV, suggesting that poor IFN induction by mosquito derived virus may be a general trait of alphaviruses. Whether this effect is specific to alphaviruses, or reflects a more general trait of arthropod-borne viruses is an important question that remains to be determined. However these studies demonstrate that at least a subset of mosquito derived viruses are able to infect dendritic cells without initiating a potent antiviral response. Though this effect is likely to only occur during the initial round of replication, since the subsequent mammalian-cell derived virus would be expected to induce an IFN response (Figure 6), this may be sufficient to give the incoming virus an advantage, to permit the establishment of infection. Therefore, further study of the mechanisms underlying the ability of the mosquito-cell derived alphaviruses to avoid or suppress type I IFN induction in mDCs is likely to significantly enhance our understanding of how mosquito borne viruses initially establish infection and interact with the host innate immune system.

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Figure 2.1. Mosquito-cell derived RRV (mos-RRV) infects myeloid bone marrow derived dendritic cells (mDCs) more efficiently than mammalian-cell derived RRV (mam-RRV) but induces less type I interferon. A. Mos-RRV infected a higher percentage of mDCs than mam-RRV as shown in representative histograms measuring GFP expression in infected mDCs at 12 hours post infection flow cytometry. B. Data from (A) displaying the mean and SEM of infection percentages from three independent samples. C. Type I IFN responses measured from the same cultures as in (B). Each bar represents the mean and SEM of three independent samples. D. Myeloid DCs were infected with either mam- or mos-RRV and RNA was isolated at 2 hpi and analyzed by real time RT-PCR for IFN-β message. Data is presented as fold induction over mock and normalized to 18S ribosomal RNA. E. Mam-RRV induces more type I IFN than mos-RRV in human monocyte derived dendritic cells. Immature human DC’s were generated and infected with either mam- or mos-RRV at an MOI of 20. At 18 hours post infection, type I IFN responses were measured by IFN bioassay. F. UV inactivated mam-RRV induces type I IFN while UV inactivated mos-RRV does not induce type I IFN. Mam- or mos-RRV was inactivated by UV light and type I IFN responses were measured from infected mDC’s by IFN bioassay. All data is representative of at least two to three independent experiments. Differences between mam- and mos-RRV were statistically significant (p< 0.05) for 1b, 1c, 1d and 1e determined by one way ANOVA. Mam- and mos-RRV corresponding time points in figure 1f were statistically different determined by students unpaired t-test.
Figure 2.2. Mosquito-cell derived RRV (mos-RRV) infects more myeloid DCs (mDCs) yet induces less type I IFN than mammalian derived RRV (mam-RRV) over time. A. Immature murine mDCs were infected with either mos- or mam-RRV at a multiplicity of infection of 20 and wells were harvested at the indicated times and assessed for GFP positive cells by flow cytometry. B. Supernatants from (A) were collected and type I IFN was measured by IFN bioassay. Solid lines represent mam-RRV and dotted lines represent mos-RRV. Each data point represents the mean and SEM of three samples. Data is representative of at least three independent experiments.
Figure 2.3. Comparison of particle to PFU ratios for mam-RRV and mos-RRV.

A. The total number of counts per minute to PFU for both mam- and mos-RRV were determined by $^{35}$S Methonine labeling of virus particles followed by a plaque assay. The data is represented as fold difference in particle to pfu ratio between mos-RRV and mam-RRV. B. The relative number of genomes for both mos- and mam-RRV was determined using a virus specific real time PCR assay. Data is presented as fold difference in genome to PFU ratio between mos- and mam-RRV. C. Equal PFU of mos- and mam-RRV contain similar amounts of glycoprotein. Western blot of serial two-fold dilutions of mam- and mos-RRV probed with a polyclonal anti-RRV antibody. D. Fluorescent signal quantified for capsid bands from either mos- or mam-RRV presented the same as figure 3C.
Figure 2.4. Mammalian-cell derived RRV (mam-RRV) induces more type I IFN than mosquito-cell derived RRV (mos-RRV) over a broad range of viral doses. A. Infection percentages of immature mDCs from 129 Sv/ev mice infected with either mam- or mos-RRV ranging from 100 to 0.8 at 12 hours post infection. B. Type I IFN responses measured from the same cultures as (A). Solid lines represent mam-RRV and dotted lines represent mos-RRV. Each point represents the mean and SEM of three samples. Differences between mam- and mos-RRV were statistically significant (p< 0.05) for all input doses except 0.8 as determined by a two-tailed unpaired students t-test.
Figure 2.5. Mammalian-cell derived RRV (mam-RRV) induces more type I IFN than mosquito-cell derived RRV (mos-RRV) in 129 αβ receptor deficient mDC’s. A. Infection percentages of immature mDCs from type I IFN receptor deficient mice infected with mam- or mos-RRV at 12 hours post infection. B. IFN responses measured from the same samples as in (A). Solid lines represent mam-RRV and dotted lines represent mos-RRV. Each point represents the mean and SEM of three samples. Data is representative of at least three independent experiments.
Figure 2.6. A single passage of mosquito-cell derived virus (Mos-RRV) through mammalian-cells (generating Mam-RRV) restores type I IFN induction in myeloid dendritic cells (mDCs). A. Myeloid DCs were infected with mam-RRV, mos-RRV and mos-RRV passaged a single time back through BHK-21 cells. Infection percentages were calculated for GFP positive cells by flow cytometry at 12 hours post infection. B. Supernatants from each of the corresponding samples were evaluated by type I IFN bioassay. Data are from a representative experiment and presented as the mean and SEM of three independent samples. Mam-RRV compared to mos-RRV and mos-RRV compared to mam-RRV → mos-RRV were statistically significant (p< 0.05) as determined by one-way ANOVA for both A and B. Mam-RRV compared to mos-RRV → mam-RRV was not statistically significant as determined by one-way ANOVA for both A and B.
Figure 2.7. Mosquito-cell-derived Venezuelan Equine Encephalitis (VEE) virus and Barmah Forest Virus (BFV) induce less type I IFN than mammalian-cell derived viruses on myeloid DCs (mDCs). A. Myeloid DCs were infected with mam- and mos-VEE at an MOI of 8.0 and infectivity was calculated by flow cytometry measuring GFP expression at 12 hours post infection. B. Supernatants from the same samples in (A) were analyzed by type I IFN bioassay. C. Mos-BFV (strain 10E101SC) with E2 N-linked glycans, induces less type I IFN than Mam-BFV at 12 hours post infection by IFN
bioassay. Data in A and B represent the mean and SEM of three independent samples and is representative of results from two independent experiments. Differences between mam- and mos-VEE were statistically significant ($p<0.05$) determined by one-way ANOVA for figure A and B.
Figure 2.8. Glycosylation differences between mammalian and mosquito-cell derived virions of Barmah Forest Virus (BFV) and Ross River Virus (RRV) contribute to differential type I IFN induction in myeloid DC’s. A. Analysis of IFN induction by mam- or mos-BFV (strain 10E101SC), which has N-linked glycans on the E2 glycoprotein. Mos-10E101SC BFV induced less type I IFN than mam-10E101SC BFV at 12 hours post infection as measured by RT-PCR for IFN-β transcripts. B. RT-PCR analysis for IFN-β message in mDCs following infection with BFV strain BH2193, which lacks glycosylation sites on the E2 glycoprotein. Both mos- and mam-BH2193 BFV induced IFN-β mRNA in infected mDCs. Each lane represents an independent sample. C. Virus derived from wild type Pro-5 CHO cells (complex carbohydrates), induced more type I IFN in infected mDC cultures than virus derived from mutant Lec-1 CHO cells (high mannose N-linked glycans) as measured by bioassay.
CHAPTER THREE

ROSS RIVER VIRUS ENVELOPE GLYCANS CONTRIBUTE TO TYPE I INTERFERON RESPONSES IN MYELOID DENDRITIC CELLS

Reed S Shabman\textsuperscript{1,2,3} and Mark T Heise\textsuperscript{1,2,3*}

\textsuperscript{1} The Department of Genetics, \textsuperscript{2}The Department of Microbiology and Immunology, \textsuperscript{3}The Carolina Vaccine Institute, The University of North Carolina-Chapel Hill, Chapel Hill, NC 27599

\textsuperscript{*}Corresponding Author. The Carolina Vaccine Institute, The University of North Carolina, Chapel Hill, NC 27599. Phone: 919-843-1492, Fax: 919-843-6924, Email: heisem@med.unc.edu

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ABSTRACT

Alphaviruses are mosquito transmitted viruses that cause significant human disease, and understanding how these pathogens successfully transition from the mosquito vector to the vertebrate host is an important area of research. Previous studies demonstrated that mosquito and mammalian-cell-derived alphaviruses differentially induce type I interferons (IFN-αβ) in myeloid dendritic cells (mDC’s), where the mosquito-cell-derived virus was a poor inducer of IFN-αβ compared to the mammalian-cell-derived virus. Furthermore, the reduced IFN-αβ induction by the mosquito-cell-derived virus was attributed to differential N-linked glycosylation (29). To further evaluate the role of viral envelope glycans in regulating the IFN-αβ response, studies were performed to assess whether the mosquito-cell-derived virus was actively inhibiting IFN-αβ induction or simply a poor inducer of IFN-αβ. Co-infection studies using mammalian- and mosquito-cell-derived Ross River virus (mam- and mos-RRV, respectively) indicated that mos-RRV was unable to suppress IFN-αβ induction by mam-RRV in mDC cultures. Additionally, a panel of mutant viruses lacking either individual or multiple N-linked glycosylation sites was used to demonstrate that N-linked glycans were essential for high level IFN-αβ induction by the mammalian-cell-derived virus. These results suggest that the failure of the mosquito-cell-derived virus to induce IFN-αβ is due to a lack of complex carbohydrates on the virion rather than active suppression of the DC antiviral response.
INTRODUCTION

Mosquito borne viruses, which include toga-, flavi-, and bunyaviruses, are a significant and emerging cause of human diseases ranging from hemorrhagic fever, to encephalitis or arthritis (8, 12, 24). Though the mechanisms by which these viruses cause disease are diverse, a common and essential step in the viral life cycle is successful transition from the mosquito vector to the vertebrate host. For many of these viruses, Langerhans cells and/or dermal myeloid dendritic cells (mDC’s) appear to be an early target of viral replication following transmission from the mosquito vector (21, 36). mDC’s are professional antigen presenting cells that rapidly respond to viruses in the absence of viral replication and are capable of producing type I interferons (IFN-αβ) to high levels (7, 10). Understanding how these viruses target to and successfully establish infection in DC’s is an important and rapidly developing area of research.

Several reports indicate that viral N-linked glycans are major mediators of DC tropism, where the presence of high mannose N-linked glycans on mosquito-cell-derived Sindbis virus and West Nile virus (WNV) enhances infection of mDC’s due to interactions with the mannose binding C-type lectin receptor (CLR) DC-SIGN (5, 16). These results suggest that CLR’s act as attachment receptors to enhance infection of DC’s and macrophages, though it is likely that other molecules act as entry receptors. In addition to enhancing infection, high mannose N-linked glycans on mosquito-cell-derived virions are linked to poor IFN-αβ induction in mDC’s (29) and plasmacytoid DC (pDC) cultures (31). Furthermore, mosquito-cell-derived WNV has been shown to actively inhibit poly I:C induced IFN-αβ responses in macrophages (1). Virus inhibition of the innate immune response is not limited to arthropod borne viruses, since high mannose N-
linked glycans on the gp120 molecule of HIV suppress mDC responses following DC-SIGN binding (30).

While a growing body of evidence suggests that high mannose N-linked glycans negatively regulate antiviral responses, other studies indicate that viral glycoproteins either assembled as virus-like particles (VLP’s) or glycoproteins fixed on the surface of infected cells can stimulate type I IFN through interactions with lectins (15). Charley et al. demonstrated that complex, but not high mannose, N-linked glycans on the transmissible gastroenteritis virus (TGEV) M protein were responsible for IFN induction in porcine peripheral blood mononuclear cells (4). TGEV mutants lacking N-linked glycan sites were poor inducers of IFN-α (19). Additional studies demonstrated that both the highly ordered structure of an assembled virus-like-particle (2) and the glycan linkage (N-linked but not O-linked glycans) were critical for robust IFN-αβ production (6). Similar results obtained with influenza virus demonstrated a requirement for interactions between sialic acid on the virus particle and mouse spleen cells for IFN-αβ production (23). Finally, Ebola VLP stimulation of human mDC’s requires the heavily glycosylated mucin domain (22). Taken together, these data suggest that interactions between lectin receptors and viral N-linked glycans are capable of both suppressing and activating antiviral responses in IFN producing cells.

Previous work from our laboratory demonstrated that mosquito-cell-derived alphaviruses were poor inducers of IFN-αβ, while mammalian-cell-derived alphaviruses induced robust IFN-αβ responses in mDC’s (29). This effect was at least partly regulated by the glycosylation of the virion, where high mannose glycans were poor inducers of IFN-αβ, while virus with complex sugars induced robust IFN-αβ responses. In this study
Ross River virus (RRV) was used to assess whether IFN-αβ induction differences represented active suppression, or if complex sugars were required for efficient IFN-αβ induction in mDC’s. A panel of RRV N-linked glycan deficient viruses was developed and compared to wild type RRV for infectivity and IFN-αβ induction following mDC infection. These studies showed that the N-linked glycans on the E2 glycoprotein of mammalian-cell-derived RRV were required for robust IFN-αβ induction in mDC cultures.

MATERIALS AND METHODS

Recombinant virus design. Ross River virus (T48 strain) was kindly provided by Richard Kuhn, Purdue University. The pRR64 plasmid, which encodes the entire cDNA genome of RRV (17) was used to generate recombinant viruses containing either single or multiple N-linked glycosylation site mutations on the E2 and E1 glycoprotein. Asparagine to glutamine mutations at each glycosylation site were introduced by standard site directed mutagenesis at amino acids 200 and 262 in the E2 glycoprotein and amino acid 141 in the E1 glycoprotein. All mutant clones were sequenced and mutated DNA fragments were subcloned into the backbone of both RRV and RRV expressing GFP between the unique ApaI site (located in NSP4, nucleotide 6,746) and the unique XmaI site (located in E1, nucleotide 10,693).

Generation of virus stocks, plaque assays. Plasmid pRR64 was used to generate recombinant virus stocks as described previously (29). Briefly, pRR64 was linearized by Sac I digestion, full length 5’ capped, poly-A tail RNA transcripts were generated by in vitro transcription (Ambion) and RNA was electroporated into baby hamster kidney-21 cells (BHK-21, ATCC CCL-10). Twenty-four hours post electroporation, mammalian-
cell-derived (mam-RRV) virus containing supernatants were harvested and clarified via centrifugation at 3000 rpm. Clarified supernatants were either aliquoted, or concentrated through a 20% (w/vol) sucrose cushion at 72,000 x g and virus was resuspended in phosphate-buffered saline (PBS) supplemented with 1% Fetal Bovine Serum (FBS), calcium and magnesium. To generate mosquito-cell-derived virus (mos-RRV), mam-RRV was passaged at a multiplicity of infection (MOI) of 0.1 in C6/36 Aedes Albopictus cells (ATCC CRL-1660). After a 1 hour attachment period, cells were washed twice to remove unattached virus. At 18-24 hours post infection, supernatant was harvested and concentrated in the same manner as described above for mam-RRV. Both mam- and mos-RRV were titered by a standard plaque assay on BHK-21 cells. Briefly, BHK-21 cells were seeded in 60 mm dishes and infected with serial 10-fold dilutions of virus stocks for one hour. Cell monolayers were then overlayed with 0.4% immunodiffusion agarose (MP Biomedicals) in media for 40-44 hours, and then stained with neutral red (Sigma). Plaques were counted to determine plaque forming units (PFU)/ml for each virus stock.

BHK-21 cells were grown in minimal essential alpha medium (Gibco) supplemented with 10% FBS, 10% Tryptose phosphate, L-glutamine, penicillin and streptomycin. C6/36 cells were grown in minimal essential medium containing Earl’s salts, FBS, penicillin, streptomycin, and non-essential amino acids.

**UV Inactivation.** Both mock and virus samples were subjected to 254 nm shortwave UV light (Mineralight lamp UVG-54) at a distance of 5 cm from the source. Virus inactivation was confirmed by both a loss of GFP expression from a second 26S
subgenomic promoter following mDC’s infection as well as a loss in plaque formation on BHK-21 cells.

**Bone Marrow Dendritic cell (mDC) cultures and infection conditions.** Bone marrow derived dendritic cells were prepared as described previously (28). Briefly, the tibia and femur of a 129 Sv/Ev mouse were removed and bone marrow was isolated. Red blood cells were lysed, and remaining cells were differentiated in granulocyte macrophage colony stimulating factor (GM-CSF) and interleukin-4 IL-4 (Peprotech) for 7 days in ultra-low-cluster 6-well plates (Costar). Bone marrow derived dendritic cells were cultured in RPMI-1640 supplemented with 10% Fetal Bovine Serum, L-glutamine, Penicillin and Streptomycin, and 2-Mercaptoethanol. Immature DC’s were confirmed as described previously (28). Surface expression of CD11c, CD11b, MHC II, and the absence of B220 were all quantified by FACS. At 7 days post isolation, cells were either frozen or used for infectivity studies. Infections were initiated in 0.2 ml total volume for two hours, and then 0.5 ml media was added to each well of a 24 well plate. At 12 hpi, cells were harvested and GFP expression was quantified by flow cytometry.

**Type I Interferon (IFNαβ) Bioassay.** IFN-αβ levels in cell culture supernatants were measured by interferon bioassay as described previously (29, 35). L929 mouse fibroblasts (ATCC CCL-1) were seeded in 96-well plates and grown in the same media as BHK-21 cells. Samples were acidified to a pH of 2.0 for 24 hours and then neutralized to pH 7.4. Additional virus inactivation by UV light (as described above) was performed prior to titration by two-fold serial dilutions down the plate. Twenty four hours later, encephalomyocarditis virus (EMCV) was added to each well at an MOI of 5. At 18-24 hours post infection, 3-[4,5-Dimethylthiazol-2-yl]-2,5-Diphenyltetrazolium bromide
(MTT) (Sigma), was used to determine viability in each well. The MTT product produced by viable cells was dissolved in isopropanol/0.4% HCl and quantified by absorbance readings on a microplate reader at 570 nm. Each plate contained an IFN-β standard (Chemicon or R&D systems) which was used to determine the international units (IU/ml) of IFN-αβ in the unknown samples.

**Single step, multi-step growth curves.** BHK-21 cells were seeded in 24-well plates and infected with wild type RRV and each mutant virus for 1 hour at a MOI of 5 or 0.01. Virus inocula were removed and cells were washed to remove unbound virus. At 1, 3, 6, 9, 12, 24, and 30 hpi 100 µl aliquots were removed and virus titers were determined by plaque assay as described above but were performed in 6-well plates.

**Real Time PCR.** For RRV genome analysis Sybr green primers described previously (29), or a TaqMan primer-probe set (designed with the primer express software) specific for the Nsp 3 region of RRV was used. The primer and probe sequences were (Forward: 5’-CCGTGGCGGGTGTTATCAAT-3’ Reverse: 5’-AACACTCCCGTCGACAACAGA-3’ Probe: 5’-ATTAAGGTGTAGCCATCC-3’). Purified virion RNA was isolated with the 96-well MagMax viral RNA extraction kit (Ambion). Virion RNA was reverse transcribed using Mouse Murine Leukemia Virus Reverse Transcriptase (Invitrogen), and a dilution of cDNA was used for quantitative Real Time PCR (Applied Biosystems). A DNA standard curve of the RRV genome was generated to ensure optimal primer/probe efficiency and to assign relative genome numbers to directly compare to each sample. The data obtained from the real time PCR assay was normalized to the number of PFU from the same virus stock to generate a relative genome number to PFU ratio.
RNA specific infectivity assays. RNA from each RRV glycoprotein mutant virus was transcribed from the corresponding mutant pRR64 plasmid. The DNA template digested with DNase, RNA precipitated with LiCl and quantified by absorbance at 260 nm. Serial dilutions of RNA were transfected into BHK-21 cells in 60 mm dishes using Lipofectamine 2000 (Invitrogen) for 15 minutes and cells were overlayed with alpha minimal essential medium and 0.4% immunodiffusion agarose. Forty hours post transfection, dishes were stained with neutral red, plaques were enumerated, and the PFU/µg RNA was determined.

Endoglycosidase Digestions and Western Blot. Equal PFU from each purified virus produced in either BHK-21 or C6/36 cells (ranging from $2 \times 10^5$-$1 \times 10^6$ PFU) were either mock digested or digested with PNGase or EndoH (New England Biolabs) overnight according to the manufactures protocol. An equal volume of each digested virion preparation was analyzed on a 10% SDS-PAGE gel followed by western blot analysis using an anti-RRV mouse hyperimmune ascites fluid (ATCC VR-1246AF). Protein bands were visualized by ECL Plus immunofluorescence (Amersham) and developed with film (Amersham Hyperfilm ECL).

Statistics. All relevant groups were compared using a one-way analysis of variance (ANOVA) or unpaired students T-test where indicated using GraphPad Instat software, version 3.06.

RESULTS

Mosquito-cell-derived Ross River virus (mos-RRV) does not actively suppress the IFN-αβ response in mDC’s induced by mammalian-cell-derived RRV (mam-RRV).
Studies from other groups demonstrated that N-linked glycans from C6/36-derived WNV could suppress IFN-αβ induction in response to dsRNA stimulation of macrophages (1), and high mannose residues on HIV gp120 have immunosuppressive effects on DC’s (30). Our studies have demonstrated that mos-RRV more efficiently infected murine mDC’s, but was a poor inducer of IFN-αβ when compared to mam-RRV. This effect was at least partly mediated by the presence of high mannose N-linked glycans on mos-RRV (29). However, our previous studies did not evaluate whether the poor IFN-αβ induction by mos-RRV was the net result of active inhibition of IFN-αβ induction by mos-RRV or a failure of mos-RRV to induce IFN-αβ at all. Therefore, to determine whether mos-RRV could inhibit IFN-αβ induction by mam-RRV, we performed a series of co-infection studies using both replication competent as well as UV inactivated viruses.

Consistent with previous studies (29), mam-RRV induced IFN-αβ from mDC’s to higher levels than mos-RRV even when a 5-fold excess of mos-RRV was used (Figure 3.1a, 444.0 International Units(IU)/ml mam-RRV MOI=10 versus 96.3 IU/ml mos-RRV MOI=50). If mos-RRV were actively suppressing IFN-αβ induction in mDC’s, we hypothesized that mos-RRV co-infected with mam-RRV would result in a loss of high level IFN-αβ induction. However, co-infection with 5-fold excess mos-RRV had no effect on mam-RRV mediated IFN-αβ induction (Figure 3.1a, 419.6 IU/ml). These studies were confirmed with UV inactivated RRV. We have previously shown that UV inactivated mam-RRV induces IFN-αβ, while UV mos-RRV fails to induce, thereby allowing us to evaluate effects of mosquito-cell-derived virus independently of productive viral replication. As shown in Figure 1B, UV mos-RRV at a 5-fold excess
was still a poor inducer if IFN- αβ compared to UV mam-RRV (16.6 IU/ml mos-RRV MOI=50 versus 197.6 IU/ml mam-RRV MOI=10). Co-infection of UV mam-RRV at an MOI of 10 with UV mos-RRV at an MOI of 50 had no effect on IFN- αβ induction (186.4 IU/ml). Though these results do not absolutely rule out active inhibition of IFN- αβ induction by mos-RRV, they do strongly suggest that mos-RRV is unable to induce a general block on IFN-αβ induction in mDC’s.

**Generation and Characterization of Ross River virus Glycan Mutants.**

The co-infection studies suggested that mos-RRV did not inhibit mam-RRV IFN-αβ induction in murine mDC’s. To further evaluate the role of viral N-linked glycans in this process, we generated a panel of viruses lacking one or more of the 3 viral envelope N-linked glycosylation sites. Amino acid substitutions from asparagine (N) to glutamine (Q) were introduced at each glycosylation site in the T48 infectious clone of RRV (E2 amino acids 200 and 262 and E1 amino acid 141) (17, 39) by site directed mutagenesis and changes were confirmed by sequence analysis. A diagram of each mutant virus generated is shown in Figure 3.2.

To test if each glycan mutant virus was viable, recombinant viral RNA was in vitro transcribed and transfected into BHK-21 cells. The RNA specific infectivity for each virus was determined by agarose overlay of transfected BHK-21 cells and plaques from each virus were counted. Each mutant virus produced plaques with similar efficiency to wild type RRV and had specific infectivity values ranging between the wild type value of 6.40x10³ PFU/ µg and 1.50x10³ PFU/µg of RNA (data not shown).

To confirm that the introduced mutations into the RRV genome ablated each glycosylation site, western blot analysis using anti-RRV antisera against the RRV
envelope glycoproteins was performed on purified virions. Previous studies with Dengue virus demonstrated that removal of an N-linked glycan resulted in a 2 kD mobility shift by SDS-PAGE analysis (32). Figure 3.3a depicts migration differences of the E1 and E2 glycoproteins between wild type RRV and each glycan mutant. Ablation of each glycosylation site resulted in a corresponding mobility shift indicative of the loss of each N-linked glycan. The same pattern of the E2 and E1 glycoproteins was also observed with each mos-RRV glycan mutant produced in C6/36 cells (data not shown). PNGase treatment of virions, which removes all N-linked oligosaccharides indicated that the differences in mobility were due to a loss of one or more oligosaccharides (Figure 3.3b).

To determine whether loss of one or more N-linked glycan sites adversely affected viral specific infectivity, we calculated the relative number of RRV genomes per PFU (as determined on BHK-21 cells) by quantitative PCR and established a relative genome: PFU ratio for each virus. The left two columns in Table 3.1 display the ratio of genomes per PFU of wild type and each mutant mam-RRV normalized to wild type mam-RRV (therefore, the value for wild type RRV is set to 1.000). All mam-RRV values were within two-fold of each other. We also evaluated the genome: PFU ratio of each RRV glycan mutant when derived from mosquito cells. All mos-RRV mutants produced virus particles with similar genome to PFU ratios to wild type mos-RRV (Table 3.1, right two columns). Within both cell lines, no mutant virus had a genome/PFU ratio that differed by more than 2-fold. In addition, the ratio of genomes per PFU of wild type mos-RRV to wild type mam-RRV was 0.502, which is consistent with previous studies (29).
To further evaluate if the loss of N-linked glycans impaired RRV replication, we performed single and multi-step growth curves (Figure 3.4). Removal of one or both E2 N-linked glycans had little to no effect on viral replication in single or multi-step growth curves (Figure 3.4a and c). All RRV glycan mutants missing the E1 141 sugar were viable but did exhibit a reduction in viral titers of approximately 1 to 1.5 logs compared to wild type RRV in both single and multi-step growth curves at late times post infection (Figure 3.4b and d). In addition to a slight reduction in endpoint titer, RRV missing all three N-linked glycan sites displayed a small plaque phenotype on BHK-21 cells (Figure 3.4e).

Identification of the predominant oligosaccharide at each glycosylation site of mam- and mos-RRV

A major difference between virus particles produced from mammalian cells versus virus particles produced from insect cells is that insect-cell-derived viruses have only terminal mannose sugars attached to their viral glycoproteins, while mammalian-cell-derived viruses can have complex, mannose, or a mixture of both (hybrid) oligosaccharides (13, 14). We utilized the RRV glycan mutant panel to determine the predominant oligosaccharide at each glycosylation site of mam- and mos-RRV. Individual mutants of mam- and mos-RRV were digested with either PNGase F or EndoH. PNGase F nonspecifically cleaves all complex, mannose, and hybrid N-linked glycans. EndoH cleaves high mannose and hybrid sugars, but does not cleave pauci mannose or complex sugars (33). Following digestion with each endoglycosidase, the glycans on the E2 and E1 glycoproteins were analyzed by western blot using anti-RRV antisera (Figure 3.5 and summarized in Table 3.2). PNGase and EndoH digestion of the
mam-RRV mutant E2 N262Q (one E2 sugar at amino acid 200) demonstrated the E2 amino acid 200 sugar is PNGase and EndoH sensitive (Figure 3.5a, lanes 7-9). This indicated that the predominant oligosaccharide at amino acid 200 is either a hybrid (both complex and high mannose) or high mannose. mam-RRV also has two PNGase sensitive, EndoH resistant sugars (E2 amino acid 262 and E1 amino acid 141) indicating complex sugars at both of these sites (Figure 5A lanes 4-6 and 10-12).

Since arthropods do not produce complex glycans (3), the mos-RRV envelope has either high mannose or pauci-mannose (man-3) oligosaccharides. PNGase and EndoH digestion of mos-RRV glycan mutants revealed that both E2 sugars are EndoH sensitive, indicative of high mannose (man-4 to 9) sugars at these sites (Figure 3.5b panel second and third panel from the left). mos-RRV also had one PNGase sensitive, EndoH resistant sugar (E1 amino acid 141) indicative of a pauci-mannose (man-3) (Figure 3.5b far right panel). Based on a greater molecular weight difference between undigested and the PNGase digested E1 glycoprotein of mam- and mos-RRV, it is likely that the 141 sugar is complex in mammalian cells and pauci-mannose in insect cells (compare far right panels of 3.4a and 3.4b). Table 2 summarizes the results in figure 3.5 listing the EndoH sensitivity for each N-linked glycan and the predominant oligosaccharide at each site for both mam- and mos-RRV.

**Removal of N-linked oligosaccharides on the E2 glycoprotein of mos-RRV reduces its infection efficiency of murine mDC’s, but does not affect IFN-αβ production.**

Co-infection studies presented in Figure 3.1 indicated that mos-RRV did not actively inhibit IFN-αβ induction by mam-RRV in mDC’s. To assess this further, we
grew the panel of N-linked glycan mutants in mosquito cells and evaluated these viruses for their ability to induce IFN-αβ. If high mannose N-linked glycans on the mosquito-cell-derived virus were actively inhibiting IFN-αβ, we hypothesized that viruses lacking one or more of these glycosylation sites would restore IFN-αβ induction. As shown in figure 3.6a, wild type mos-RRV infected more mDC’s than wild type mam-RRV (21% versus 3% infected). Infection percentages were consistently reduced with mos-RRV lacking all three N-linked glycans (12% infected), but removal of all three N-linked glycans did not reduce infection efficiency of the mosquito-cell-derived virus to that seen with wild type mam-RRV. This suggested that glycan independent effects may also contribute to the enhanced infection efficiency of murine mDC’s by mos-RRV, though this requires further evaluation. Most importantly, none of the mosquito-cell-derived viruses lacking one, two, or all three N-linked glycans exhibited high IFN-αβ induction (25-50 IU/ml) in mDC’s compared to mam-RRV (320 IU/ml, Figure 3.6b), further suggesting that the presence of high mannose N-linked glycans on mos-RRV does not inhibit IFN-αβ induction in mDC’s.

**N-linked oligosaccharides on the E2 glycoprotein of mam-RRV are required for robust IFN-αβ induction in mDC’s.**

An alternative hypothesis to high mannose N-linked glycans suppressing IFN-αβ is the possibility that complex N-linked glycans on mam-RRV promote IFN-αβ induction in mDC’s. Studies with other viruses have demonstrated a role of N-linked glycans on the virus particle in induction of IFN-αβ in cultured cells (4, 23, 37). Therefore, each glycan mutant virus was grown in BHK-21 cells and evaluated for its ability to infect mDC’s and induce IFN-αβ. Wild type mam-RRV and each envelope glycan mutant infected...
mDC’s to similar levels (5-7% infected Figure 3.6c and data not shown), however, while wild type virus induced IFN-αβ to high levels (1000-1100 IU/ml), viruses lacking both E2 glycans showed a reduction in IFN-αβ (220-240 IU/ml), while removal of the E1 glycan had no effect on IFN-αβ induction (1300-1700 IU/ml) (Figure 6D).

To ensure that the diminished IFN-αβ induction was not simply due to reduced infection efficiency within the culture, we infected mDC’s with wild type or mos-RRV E2 N200, 262Q at a higher MOI of 50 and measured IFN-αβ induction. As shown in figures 3.6e and f, even when a large percentage of mDC’s within the culture were infected by both viruses, the E2 glycan deficient virus still showed reduced IFN-αβ induction compared to the wild type virus. These results suggest that the failure of mos-RRV to elicit strong IFN-αβ responses in mDC’s is at least partly due to a lack of complex N-linked glycans on mos-RRV rather than active inhibition of IFN-αβ induction by mos-RRV.

DISCUSSION

Understanding how alphaviruses cause disease is essential for designing successful strategies for therapeutics and vaccines. One important step in the disease process is the transmission of the virus from the mosquito vector to the vertebrate host, which often involves virus-dendritic cell interactions (20, 21, 36). Therefore we have focused on viral interactions with mDC’s and have demonstrated previously that mos-RRV more efficiently infected yet induced less IFN-αβ in these cells when compared to mam-RRV. Studies presented here further dissected the role of N-linked glycans in these early virus-host interactions and demonstrated that complex glycans on the E2 envelope protein of mam-RRV are required for robust IFN-αβ induction (Figure 3.6d).
The previously observed lack of high level IFN-αβ induction by mosquito-cell-derived alphaviruses suggested that they are either actively suppressing IFN-αβ induction or are not as potent inducers of IFN-αβ as the mammalian-cell-derived virus. Arjona et al. recently demonstrated that C6/36-derived WNV can actively suppress murine macrophage activation following stimulation with dsRNA, and that the suppression was dependent on high mannose N-linked glycans (1). In our system mos-RRV was unable to actively suppress the IFN-αβ induced by mam-RRV infection of mDC’s (Figure 1). Furthermore, a mos-RRV mutant lacking N-linked glycans was still a poor inducer of IFN-αβ, indicating that high mannose sugars on mos-RRV did not suppress the ability of the DC to respond to viral infection. Instead our results suggest that the presence of complex N-linked sugars on the E2 glycoprotein of mam-RRV leads to enhanced IFN-αβ induction and that mos-RRV is a poor inducer, at least in part, due to its lack of complex N-linked glycans. Furthermore, our results demonstrate that the complex sugar on the E1 glycoprotein of mam-RRV does not play a role in IFN-αβ induction, since mam-RRV E1 N141Q induces equivalent levels of IFN-αβ compared to wild type mam-RRV (Figure 6D). However, all mutants lacking E1 glycosylation displayed a minor but similar growth defect in BHK-21 cells (Figure 3.4) suggesting that E1 141 is required for optimal virus particle production but not for IFN-αβ induction from mDC’s. Based on the alphavirus structure (25), E1 potentially lies below the E2 glycoprotein in the surface of the virion. Therefore E1 may not contact the cellular receptor/sensing molecule responsible for inducing IFN-αβ, though this requires further investigation.

Our data are consistent with several studies which have demonstrated virion glycosylation can promote IFN-αβ induction (2, 6, 19, 23, 38) and that complex but not
high mannose N-linked glycans are responsible for IFN-αβ induction (4). However, it is certainly possible that mosquito-cell-derived alphaviruses actively suppress IFN-αβ induction, but that we were unable to detect this effect, or that different results might be observed in other cell types, such as macrophages or human DC’s. For example C6/36 derived WNV actively suppressed receptor-interacting protein 1 (RIP-1) signaling in murine macrophages (1). Additional reports demonstrated that IFN-αβ produced in pDC’s by C7/10-cell derived WNV was drastically reduced compared to vero-cell-derived WNV, but C7/10-cell derived WNV was not able to actively inhibit IFN-αβ induced by Sendai virus (31).

Our results strongly suggest that viral complex/hybrid N-linked glycans promote IFN-αβ responses in mDC’s, which implicates a couple of potential mechanisms underlying this process. Lectin receptors on the surface of mDC’s may differentially direct the mosquito and mammalian-cell-derived viruses to different compartments within the mDC. For example, the mammalian-cell-derived virus may come into contact with host sensor molecules, such as TLR’s or RIG-I with greater efficiency than the mosquito-cell-derived virus. Alternatively, rather than redirecting the viruses, lectin receptors on mDC’s may be triggered by the glycans of mam-RRV, but not mos-RRV, to enhance IFN-αβ induction through an undefined co-stimulatory molecule to upregulate the IFN-αβ response. Finally, IFN-αβ induction may be independent of lectin receptors. For example, a pattern recognition molecule, such as a TLR, could also recognize the mam-RRV, but not mos-RRV envelope. Complex glycans arranged in the compact and highly ordered structure of a virus particle can be recognized by the host cell as foreign (40), and TLR 4 can recognize viral glycoproteins and trigger signaling pathways leading
to IFN-αβ production (9, 11, 18, 26). It is therefore possible that envelope glycosylation patterns on mam-RRV, but not mos-RRV trigger a CLR, TLR 4, or another cellular protein to induce IFN-αβ following interaction with mDC’s.

In addition to allowing the dissection of viral glycosylation in regulating IFN-αβ induction by mosquito- and mammalian-cell-derived viruses, the generation of this panel of RRV mutants provided a useful set of tools for identifying the predominate type of glycosylation at each N-linked site. A similar panel of glycan mutants in Sindbis virus demonstrated that ablation of 2 or more N-linked glycosylation sites resulted in severe growth restriction (25). In these studies, RRV glycan mutants were viable and displayed some growth defects but were not as severely impaired for particle production as Sindbis.

As expected, EndoH digestions verified that the mos-RRV envelope contains both high mannose and pauci-mannose oligosaccharides. In contrast, mam-RRV has two complex sugars as the predominant form at the E1 141 and E2 262 glycosylation sites, while the E2 200 glycosylation site contains hybrid (a site with both mannose and complex sugars) or high mannose sugars (Figure 5). The presence of an EndoH sensitive sugar on the E2 protein of mam-RRV is likely due to E1 or the immature precursor of E2 (PE2) masking the E2 200 site to prevent complete carbohydrate processing during glycoprotein assembly in the endoplasmic reticulum and Golgi (27, 34). Incomplete glycan processing also occurs with Sindbis glycoproteins (14) and it would be interesting to determine whether is has any impact on IFN-αβ induction.

In summary, we have found that N-linked glycans on the E2 glycoprotein of mam-RRV are required for robust IFN-αβ responses against Ross River virus. This suggests that mosquito-cell-derived alphaviruses may avoid induction of IFN-αβ simply
due to the lack of complex N-linked oligosaccharides on the virion, rather than an active inhibition of IFN-αβ production. Additional studies are underway to determine whether this effect is common to multiple alphaviruses and to define the mechanism underlying the role of complex N-linked glycans in promoting virus induced IFN-αβ responses.

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Figure 3.1. C6/36 cell-derived Ross River virus (mos-RRV) co-innoculated with
BHK-21 cell-derived RRV (mam-RRV) does not suppress IFN-αβ induction by
mam-RRV in murine myeloid dendritic cells (mDC’s). A. Wild type mam-RRV and
wild type mos-RRV were used to infect mDC’s either alone or in combination at the
indicated multiplicity of infection (MOI). At 12 hours post infection (hpi), supernatants
were harvested and IFN-αβ levels were measured by IFN-αβ bioassay. Type I IFN levels
of mDC’s incubated with mam-RRV (MOI 10) and mam-RRV (MOI 10) plus mos-RRV (MOI 50) were statistically significant (P <0.05) from mos-RRV (MOI 50) as determined by one-way ANOVA. mam-RRV was not significant from mam-RRV coinfectected with mos-RRV. B. UV irradiated mam- and mos-RRV were used at the indicated MOI either alone or in combination to infect mDC’s. Supernatants were harvested as in (A) at 12 hpi to measure IFN-αβ levels. Each bar represents the mean and standard error of the mean of triplicate samples. Type I IFN levels of DC’s incubated with mam-RRV (MOI 10) and mam-RRV (MOI 10) plus mos-RRV (MOI 50) were statistically significant (P <0.05) from mos-RRV (MOI 10, 20, and 50) as determined by one-way ANOVA. This experiment is representative of at least three independent experiments.
Figure 3.2. Diagram of the panel of Ross River virus (RRV) N-linked glycosylation mutants. A diagram of the RRV structural genes, with the locations of the E2 and E1 N-linked glycosylation sites indicated by an asterisk. Each combination of RRV glycan mutants was generated by PCR site directed mutagenesis. Each segment was sequenced and subcloned back into both the pRR64 and the pRR64-GFP plasmid backbones.
Figure 3.3. Ross River virus (RRV) mutants missing either a single or a combination of N-linked glycosylation sites on the E2 and E1 glycoproteins are viable.

A. Proteins from purified RRV virions were separated through a 10% SDS-PAGE gel and western blot analysis of the E2 and E1 proteins was performed with anti-RRV polyclonal antisera. Mutation of each glycosylation site resulted in an approximate 2 kD size reduction of either E1 or E2. The mobility shift of E1 and E2 for each glycan mutant virus corresponds with the predicted shift in molecular weight. B. RRV purified glycan mutants digested with PNGase migrate approximately at the same rate, indicative that migration differences in (A) are due to loss of N-linked glycans. Data is representative of at least three independent experiments.
Table 3.1. Relative number of genomes per plaque forming unit (PFU)\(^b\) of mam- and mos-RRV normalized to WT mam- and mos-RRV

<table>
<thead>
<tr>
<th>Virus</th>
<th>Relative Genome number: PFU ratio normalized to WT mam-RRV</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Mam-RRV</td>
</tr>
<tr>
<td></td>
<td>AVG(^b)</td>
</tr>
<tr>
<td>WT</td>
<td>1.000</td>
</tr>
<tr>
<td>N200Q</td>
<td>0.643</td>
</tr>
<tr>
<td>N262Q</td>
<td>1.232</td>
</tr>
<tr>
<td>N200,262Q</td>
<td>0.645</td>
</tr>
<tr>
<td>N141Q</td>
<td>1.690</td>
</tr>
<tr>
<td>N200,141Q</td>
<td>0.628</td>
</tr>
<tr>
<td>N262,141Q</td>
<td>0.996</td>
</tr>
<tr>
<td>N200,262,141Q</td>
<td>0.946</td>
</tr>
</tbody>
</table>

\(^a\) PFU for each virus was determined on BHK-21 cells.
\(^b\) Ratios were determined by calculating the number of genomes (Taq-man Real Time PCR) for each plaque forming unit on BHK-21 cells.
\(^c\) Each mam-RRV value was normalized to the WT mam-RRV Genome: PFU ratio. Each mos-RRV value was normalized to the WT mos-RRV Genome: PFU ratio (e.g. average WT mam-RRV value = 1).
\(^d\) Each sample represents the mean and standard error of the mean of triplicate RNA extractions from each virus preparation.
\(^e\) The ratio of relative number of genomes of WT mos-RRV to WT mam-RRV in this experiment was 0.502, consistent with previous results (29).
Figure 3.4. Multi-step and single step growth curves of wild type Ross River virus (RRV) and RRV glycan mutants on BHK-21 cells. BHK-21 cells were infected with wild type RRV and each glycan mutant at a multiplicity of infection (MOI) of 0.01 (multistep, A and B) or a MOI of 5 (single step, C and D) for one hour. Output titers at
1, 3, 6, 9, 12, 24 and 30 hours post infection are plotted. Each data point represents the mean and standard error of the mean of three independent samples. E. Crystal violet plaque assay staining of wild type and glycoprotein mutant RRV from a separate multistep growth curve at 9 hours post infection demonstrate differences in plaque morphology of each glycan mutant compared to wild type RRV. All data is representative of at least three independent experiments.
Figure 3.5. Identification of the predominant oligosaccharide at each glycosylation site of Ross River virus (RRV) produced in BHK-21 and C6/36 cells. From left to right, wild type RRV (panel 1), RRV E2 N200Q (panel 2), RRV E2 N262Q (panel 3), and RRV E2 N200,262Q (panel 4) were either undigested (U), or digested with either PNGase (P) or EndoH (E) overnight and then analyzed by SDS-PAGE followed by western blot with anti-RRV polyclonal antisera. **A.** Analysis of RRV digestions grown in BHK-21 cells (mam-RRV). **B.** Analysis of RRV digestions grown in C6/36 cells (mos-RRV). The letters R and S on the lower left side of the E2 and E1 glycoproteins in the EndoH (E) lane indicate if the oligosaccharide on that glycoprotein is EndoH resistant or sensitive when compared to the corresponding glycoprotein in the uncut (U) lane. Data is representative of at least three independent experiments.
Table 3.2. Identification of predominant oligosaccharide at each N-linked glycosylation site in BHK-21 and C6/36 cells.

<table>
<thead>
<tr>
<th>Glycosylation Site</th>
<th>Endo H Sensitivity</th>
<th>Predominant Oligosaccharide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mam-RRV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E2 200</td>
<td>Sensitive</td>
<td>High/Mannose/Hybrid</td>
</tr>
<tr>
<td>E2 262</td>
<td>Resistant</td>
<td>Complex</td>
</tr>
<tr>
<td>E1 141</td>
<td>Resistant</td>
<td>Complex</td>
</tr>
<tr>
<td>Mos-RRV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E2 200</td>
<td>Sensitive</td>
<td>High Mannose</td>
</tr>
<tr>
<td>E2 262</td>
<td>Sensitive</td>
<td>High Mannose</td>
</tr>
<tr>
<td>E1 141</td>
<td>Resistant</td>
<td>Pauci-Mannose</td>
</tr>
</tbody>
</table>
Figure 3.6. The robust of IFN-αβ induction by BHK-21 cell-derived Ross River virus (mam-RRV) on DC’s correlates with the presence of the E2 N-linked glycans on the virus particle.

A. Infection percentages of mDC’s with wild type mos-RRV, mos RRV E2 N200,262Q E1 N141Q and wild type mam-RRV at a multiplicity of infection (MOI) of 10. Infection was quantified by FACS analysis counting GFP positive cells at 12 hours post infection
(hpi). Each bar is significantly different from each other as determined by one-way ANOVA. **B.** The same samples as in (A), with the addition of each RRV glycan mutant grown in C6/36 cells. Murine mDC’s were infected at a MOI of 10 and at 12 hours post infection supernatants were harvested for an IFN-αβ bioassay. Each bar represents the mean and standard error of the mean of triplicate samples. The level of type I IFN produced by mam-RRV is significantly different from each mos-RRV mutant virus as determined by one-way ANOVA. **C.** Infection percentages of mDC’s following infection with wild type mam-RRV, mam-RRV E2N200, 262Q E1 N141Q, and wild type mos-RRV at an MOI of 20. Each mam-RRV bar represents the mean and standard error of the mean of triplicate samples, while mos-RRV represents the mean of duplicate samples. Infectivity differences comparing mos-RRV to mam-RRV and mam-RRV E2 N200, 262Q E1 N141Q are statistically significant as determined by one-way ANOVA. **D.** The same samples as in C, with the addition of each individual glycan mutant virus produced in BHK-21 cells. Each N-linked glycan mutant of mam-RRV was used to infect mDC cultures at an MOI of 20. At 12 hours post infection, supernatants were for an IFN-αβ bioassay. Asterisks indicate two groups are statistically significant by unpaired students t-test (* P<0.01). NS indicates two groups are not significant from each other. **E.** Wild type mam-RRV and mam-RRV E2N200Q were used to infect mDC’s at an MOI of 50 for 12 hours and supernatants were measured by IFN-αβ bioassay (F). Each bar in A-D represents the mean and standard error of the mean of triplicate samples, except for mos-RRV in figures 6C and D which are representative of duplicate samples. Each bar in E and F represents a single sample. All data is representative of at least three independent experiments.
CHAPTER FOUR

EVALUATING BOTH THE TYPE AND PRESENCE OF N-LINKED GLYCOSYLATION ON ROSS RIVER VIRUS FOR THEIR ROLE IN SEVERE DISEASE

Reed S Shabman\textsuperscript{1,2,3}, Thomas E Morrison\textsuperscript{1,2,3}, and Mark T Heise\textsuperscript{1,2,3}

\textsuperscript{1} The Department of Genetics, \textsuperscript{2}The Department of Microbiology and Immunology, \textsuperscript{3}The Carolina Vaccine Institute, The University of North Carolina-Chapel Hill, Chapel Hill, NC 27599
ABSTRACT

Alphavirus disease in humans is a significant problem world-wide and currently very few vaccines and treatments exist. However, the details of alphavirus disease are not well understood. Previous in vitro work with Ross River virus (RRV) demonstrated that mosquito-cell-derived RRV (mos-RRV) was a poor inducer of type I IFN (IFN-αβ) in myeloid dendritic cells, while mammalian-cell-derived virus (mam-RRV) was a potent IFN-αβ inducer, and this was at least partly mediated by viral N-linked glycosylation. Therefore, studies were performed to further evaluate the role of viral N-linked glycans in the in vivo pathogenesis of RRV-induced disease. Though mos-RRV and mam-RRV did not differ in their ability to induce downstream disease in infected mice, mos-RRV did exhibit higher levels of replication in the draining lymph node at early times post infection, suggesting that mos-RRV might have an early replication advantage in the draining lymph node. Additional analysis of viral mutants lacking one or more viral N-linked glycosylation sites demonstrated that viruses lacking N-linked glycans were attenuated for virus-induced disease, suggesting that viral glycosylation plays a major role in the in vivo pathogenesis of RRV.
INTRODUCTION

New and old world alphaviruses are responsible for significant morbidity worldwide. New world alphaviruses are generally associated with encephalitis while old world alphaviruses are associated with arthritis/arthralgia (3, 18). Currently the old world alphaviruses Chikungunya and Ross River virus (RRV) impact millions of people in Asia, Australia and the south pacific, but vaccines and therapeutics to treat these infections are not well developed (2, 5). One key aspect for designing successful treatments for old world alphaviruses is to gain a better understanding of the molecular basis of how these viruses cause disease.

Our understanding of old world alphavirus-induced disease has improved through the development of mouse models that mimic human disease. Previous work illustrates alphaviruses initially target to dendritic cells (DC’s) within the skin and draining lymph node (DLN) near the site of inoculation (10). DC targeting is enhanced by interactions with C-type lectin receptors (CLR’s) and high mannose glycans on the virus particle, which are present in greater abundance on mosquito-cell-derived virions (6, 16). Replication in the DLN then seeds a serum viremia and replication in tissues specific to the particular alphavirus.

In the mouse model, RRV preferentially replicates in the ankle and quadriceps muscle, which are the most severely damaged tissues. Virus titers quickly approach $1 \times 10^9$ PFU/gram by 24 hours post infection (hpi) (12, 21). Peak virus replication in the skeletal muscle subsides by 7 days post infection, before the most severe clinical signs are observed in RRV-infected animals. Around day 7 post infection, activated macrophages and natural killer (NK) cells infiltrate to the hind limb tissue. Inflammatory
infiltrates control viral replication, but have also been shown to contribute to disease since ablation of macrophages and macrophage factors reduce disease severity (8, 9). Furthermore, mice deficient in the complement pathway display less severe disease signs which are potentially attributed to changes in the activation status of inflammatory macrophages (11 and Tem Morrison personal communication).

Established mouse models have also highlighted the critical role for an intact type I interferon (IFN-αβ) pathway to control alphavirus replication. Removal of the IFN-αβ receptor changes the disease outcome from a self-limiting nonlethal disease to a lethal infection within days post inoculation (13, 20). These data combined with in vitro studies (14, 15) implicate that IFN-αβ signaling is the most important factor to control alphavirus replication which is why alphavirus modulation of IFN-αβ is of interest in our studies.

We previously demonstrated that mosquito-cell-derived Alphaviruses induce less IFN-αβ on mDC’s than mammalian-cell-derived Alphaviruses (15). Additionally, the presence of E2 N-linked glycans on mammalian-cell-derived Ross River virus (mam-RRV) but not mosquito-cell-derived RRV (mos-RRV) was required for robust levels of measured IFN-αβ from mDC’s (16). Therefore, we tested 1) if mice infected with mam- or mos-RRV differed in disease severity and 2) if the presence of N-linked glycans contributed to RRV-induced disease. Initial experiments indicated that mammalian- and mosquito-derived viruses did not differ in their ability to cause downstream disease, but that the mosquito-cell-derived virus may have an early replication advantage in the draining lymph node. Further studies using a panel of N-linked glycan deficient viruses in mice indicated that one or more viral N-linked glycosylation sites is required for full RRV virulence in the mouse model and potential mechanisms for this are discussed.
MATERIALS AND METHODS

Generation of virus stocks. Plasmid pRR64 was used to generate recombinant virus stocks as described previously (15). Briefly, pRR64 was linearized by Sac I digestion and full length 5’ capped, poly-A tail RNA transcripts were generated by in vitro transcription (Ambion) and RNA was electroporated into baby hamster kidney-21 cells (BHK-21, ATCC CCL-10). Twenty-four hours post electroporation, virus containing supernatants were harvested and clarified via centrifugation at 3000 rpm. Clarified supernatants were stored at -80°C. BHK-21 cells were grown in minimal essential alpha medium (Gibco) supplemented with 10% FBS, 10% Tryptose phosphate, L-glutamine, penicillin and streptomycin. To generate mosquito-cell-derived virus (mos-RRV), mam-RRV was passaged at a multiplicity of infection (MOI) of 0.1 in C6/36 Aedes Albopictus cells (ATCC CRL-1660). After a 1 hour attachment period, cells were washed twice to remove unattached virus. At 18-24 hours post infection, supernatant was harvested and concentrated in the same manner as described above for mam-RRV.

Recombinant virus design. Ross River virus (T48 strain), provided by Richard Kuhn (Purdue University), was used as a wild type backbone for all mutant virus stocks (7). Recombinant viruses containing either single or multiple N-linked glycosylation site mutations (Asparagine to Glutamine) on the E2 and E1 glycoprotein were introduced by standard site directed mutagenesis at amino acids 200 and 262 in the E2 glycoprotein and amino acid 141 in the E1 glycoprotein. All mutant clones were sequenced and sub cloned into the backbone of RRV at the unique ApaI site (located in NSP4, nucleotide 6,746) and the unique XmaI site (located in E1, nucleotide 10,693).
**Plaque assays.** Virus stocks were titered by a standard plaque assay on BHK-21 cells. Briefly, BHK-21 cells were seeded in 60 mm dishes and infected with serial 10-fold dilutions of virus stocks for one hour. Cell monolayers were then overlayed with 0.4% immunodiffusion agarose (MP Biomedicals) in media for 40-44 hours, and then stained with neutral red (Sigma). Plaques were counted to determine plaque forming units (PFU)/ml for each virus stock.

**Mouse infections, disease scoring.** C57Bl/6J, 129 Sv/ev and 129 type I interferon receptor deficient mice were obtained from Jackson Labs (Bar Harbor, Maine). Animal breeding and housing was located at UNC-CH and in compliance with all guidelines. All RRV infected mice were housed in a biosafety level 3 laboratory.

Virus stocks were diluted to standard dose for animal injections as described previously (11, 12). Stocks were diluted to a final concentration of $1 \times 10^5$ PFU/ml (1000 PFU/10µl) in phosphate buffered saline (PBS) supplemented with 1% Donor Calf Serum. Alternatively, virus inoculums was serially diluted to achieve input doses ranging from 1000 PFU/10 µl to 0.01 PFU/10 µl. C57Bl/6, 129 Sv/ev and 129 type I interferon receptor deficient (IFN-αβ RKO) mice were anestitized and injected with 10 µl of the diluted virus stock (1000 PFU per injection). Clinical disease signs were determined by altered gait and a loss in hind limb gripping ability. Clinical score was assessed with the following scale: 0, no outward signs of disease; 1, ruffled fur slight weight loss; 2, mild hind-limb weakness; 3, mild hind limb weakness; 4, moderate hind limb weakness and slight dragging of hind limbs; 5, severe hind limb dragging and loss of all hind limb function; 6, moribound; 7, death.
To determine virus titers in the draining lymph node and skeletal muscle, mice were euthanized and perfused with PBS to remove residual blood from tissues. Tissues were harvested, homogenized in PBS 1% DCS Ca++/Mg++, and stored at -80C. Viral titers were determined from each tissue by plaque assay on BHK-21 cells as described above.

**IFN-αβ Bioassay.** IFN-αβ levels in cell culture supernatants were measured by interferon bioassay as described previously (15, 20). L929 mouse fibroblasts (ATCC CCL-1) were seeded in 96-well plates and grown in the same media as BHK-21 cells. Samples were acidified to a pH of 2.0 for 24 hours and then neutralized to pH 7.4. Additional virus inactivation by UV light was performed prior to titration by two-fold serial dilutions down the plate. Twenty four hours later, encephalomyocarditis virus (EMCV) was added to each well at an MOI of 5. At 18-24 hours post infection, 3-[4,5-Dimethylthiazol-2yl]-2,5-Diphenyltetrazolium bromide (MTT) (Sigma), was used to determine viability in each well. The MTT product produced by viable cells was dissolved in isopropanol/0.4% HCl and quantified by absorbance readings on a microplate reader at 570 nm. Each plate contained an IFN-β standard (Chemicon or R&D systems) which was used to determine the international units (IU/ml) of IFN-αβ in the unknown samples.

**RESULTS**

**Mammalian or mosquito-cell-derived Ross River virus cause equivalent levels of disease in infected mice.**

Previous studies demonstrated mammalian-cell-derived alphaviruses were more potent inducers of IFN-αβ than mosquito-cell-derived alphaviruses and we hypothesized
that mosquito-cell-derived viruses may replicate more efficiently at early times post infection, thereby leading to more severe virus-induced disease. However, mice infected with mam- and mos-RRV displayed similar weight loss and clinical scores following infection (Figures 4.1a and b). In the RRV model, disease does not occur until relatively late in the infection process (Day 4-5 post infection). Therefore, it was possible that the mosquito and mammalian derived viruses differed in their ability to establish infection in the draining lymph node, but disease induction was too crude a readout to sensitively differentiate between early differences between these virus preparations. To assess differences in viral replication and IFN-αβ, mice were infected with mam- and mos-RRV and at 6, 12, and 24 hpi the DLN and serum were harvested. At 6 hpi, DLN titers from mice infected with mos-RRV were approximately 1 log higher than mice infected with mam-RRV (Figure 4.1c). DLN titers from mos-RRV infected mice were slightly elevated at 12 hpi, and there was no difference in virus replication in the DLN by 24 hpi (Figure 4.1c). However, this experiment represents only two animals per group and larger numbers of animals are required to identify any statistically significant differences. Serum levels from each of these mice were also evaluated for systemic IFN-αβ. While there were no detectable levels of IFN-αβ measured by bioassay at 6 and 12 hpi, IFN-αβ was detected at 24 hpi in both groups of mice but the levels did not differ (Figure 4.1d). These data indicated that mos-RRV may establish replication in the DLN more efficiently than mam-RRV at early times post infection but downstream disease was unaffected, at least at the dose of virus used in these studies.

Titration of mam- and mos-RRV to optimize replication studies in the DLN
We hypothesized that an input dose of 1000 PFU may mask early replication differences between mam- and mos-RRV. Therefore studies were designed to determine an optimal dose of mam- and mos-RRV for measuring replication in the DLN at early times post infection. Based on our in vitro studies, we predicted that mos-RRV would initially induce less IFN-αβ and therefore replicate more efficiently than mam-RRV. Furthermore, if a mos-RRV replication advantage was due to differences in IFN induction, this advantage would be abolished in IFN-αβ receptor deficient (RKO) mice. IFN-αβ RKO mice are extremely susceptible to alphavirus infection and succumb within days of inoculation (20). Therefore, 129 Sv/ev wild type and 129 IFN-αβ RKO mice were infected at doses ranging from 1000 to 0.1 PFU and viral titers in the draining lymph node assessed. Though previous work suggested that mos-RRV had a replication advantage at 6 hours post infection, we reasoned that this effect might be extended at lower doses, and therefore initially assessed replication at the 12 hour time point. As expected, all DLN titers were elevated in IFN-αβ RKO mice when compared to wild type mice at each dose (Figure 4.2). Slight differences were observed between mos and mam RRV at the 1 PFU dose, but the group sizes were too small to determine whether this difference was real (Figure 4.2). Therefore, additional studies are planned to evaluate this effect more closely using larger groups of mice and looking at earlier times (6 hours) post infection.

**Viral N-linked glycosylation sites are required for full RRV-virulence in vivo.**

To further assess the role of viral N-linked glycans in RRV-induced disease, we next tested the requirement for the presence of N-linked glycans to cause disease using a previously constructed panel of RRV envelope glycan mutants (16). A diagram of each
glycan mutant is depicted in Figure 4.3. As previously described, each mutant was viable, had similar genome to PFU ratios and similar replication (in BHK-21 cells) to wild type RRV (16). Using this panel, we infected 24-day old C57Bl/6 mice with 1000 PFU, monitored body weight change and evaluated for hind limb dysfunction by a previously established scoring system (11, 12). Mice infected with each glycan mutant had less severe body weight changes and a lower clinical score than mice infected with wild type RRV (Figure 4.4). However the degree of attenuation varied with each mutant virus. Mice infected with RRV lacking either one or both E2 glycans had a modest reduction in starting body weight clinical signs compared to wild type RRV infected mice (Figure 4.4a and b). Mice infected with RRV glycan mutants missing the E1 glycan had a greater reduction in both starting body weight and clinical signs compared to wild type RRV (Figure 4.4c and d). Strikingly, mice infected with RRV missing all three N-linked glycans displayed no disease signs and gained weight at a similar rate to a mock infected mouse (Figure 4.4d).

Since each mutant virus was not severely impaired for replication in vitro (16), we also tested if each RRV mutant virus was as lethal as wild type RRV in IFN-αβ RKO mice. With the exception of the RRV glycan mutant missing all three N-linked glycans (AST 5.50 days), each virus was as lethal as wild type RRV following infection of IFN-αβ RKO mice (AST ranging from 2.43 to 2.88 days, figure 4.4e). This suggested that each virus replicates to levels similar to wild type RRV in the absence of an intact IFN-αβ system, but more detailed studies are needed to verify this observation.

**Skeletal muscle titers are reduced in a subset of the RRV glycan deficient viruses compared to wild type RRV.**
Since removal of N-linked glycans on mam-RRV ameliorated RRV-induced disease (Figure 4.4), we tested if this difference was attributed to defects associated with viral replication. For these studies, mice were infected with wild type RRV and a subset of the glycan mutants including RRV E2 N200,262Q, RRV E1 N141Q and RRV E2 200,262Q E1 N141Q. This group represented viruses that result in severe, intermediate, and no disease in the C57Bl/6 mouse model (Figure 4.5a). At 24 and 72 hpi virus titers in the quadriceps muscle were measured since previous studies demonstrated that RRV preferentially targets to and replicates to high titers in the quadriceps muscle (12).

Mice infected with wild type mam-RRV replicated to high titers (6 to 6.5 log10 PFU/gram) in the quadriceps muscle at 24 hpi (Figure 4.5c). Mice infected with RRV E2 N200,262Q had no reduction in virus titers at 24 hpi, however viral titers were reduced at 72 hpi by approximately 1 log. Mice infected with RRV E1 N141Q or RRV E2 N200,262Q E1 N141Q had a reduction in skeletal muscle titers of 1 to 2 logs at 24 hpi. At 72 hpi, the E1 N141Q infected mice had a 1.5 log reduction in viral titers in the skeletal muscle compared to wild type mam-RRV, while the triple mutant virus was not tested at this time point (Figure 4.5c). From these preliminary data, it appears that attenuated disease associated with RRV E2 glycan mutants could be either dependent or independent of viral replication. However, the attenuation of the E1 glycan mutants are at least partially due to defects in viral replication.

**DISCUSSION**

Alphavirus infections are a serious health concern, yet very few therapeutics and vaccines exist. Therefore, understanding the mechanism of alphavirus induced disease is extremely important to both developing and improving existing treatments. These studies
focus on the old world alphavirus RRV, and evaluate the role of viral N-linked glycans in virus-induced disease. These studies are extensions of two previous in vitro studies that demonstrated mos-RRV infected mDC’s more efficiently, yet induced less IFN-αβ than mam-RRV (15) and robust IFN-αβ levels from myeloid DC cultures infected with mam-RRV required the presence both E2 N-linked glycans (16). The results of these studies suggested that there may be differences in replication at early times post infection between mam- and mos-RRV in vivo, and that the presence of N-linked glycans are required for severe disease.

Studies in other systems show that mosquito delivery of West Nile virus (WNV) replicated to higher levels than vero-cell-derived virus inoculated by needle injection (19). Additionally, our data in combination with two independent studies with WNV indicate that mosquito-cell-derived viruses are able to avoid host antiviral responses in DC’s and macrophages (1, 15, 17). These results implicate a role for both the cell source of the virus and salivary gland extracts to have immunosuppressive effects to enhance replication following mosquito delivery.

The fact that mam- and mos-RRV infected mice developed similar disease is not particularly surprising. A virion derived from a mosquito (or a mosquito cell in our system) only retains the properties of that host cell for a single round of replication. If a dose of both viruses that establishes infection is used, then downstream disease would not differ. Attempts were made to titrate each virus to determine if mos-RRV replicates more efficiently in the DLN than mam-RRV. However, unlike the initial difference observed in figure 1C, we measured no major differences in replication between mam- and mos-RRV in the DLN at 12 hpi (Figure 4.2). It was interesting to note that at an
input dose of 1 PFU, there were slight replication differences between mos- and mam-RRV. Future experiments using earlier time points and larger numbers of animals are planned.

There are several potential explanations for the discrepancies in these results. First, there may be no difference between mam- and mos-RRV replication in the DLN. Alternatively, we have not examined the correct time point in these studies, or a more sensitive measurement of replication (e.g. viral RNA) may be needed in future studies. Therefore, future experiments with mam- and mos-RRV are required to test if mos-RRV can more efficiently establish infection and ultimately cause disease at lower doses than mam-RRV.

Our second line of studies revealed the requirement for RRV envelope glycosylation to cause severe disease. RRV-induced disease in 24-day old mice was dramatically different between wild type RRV and the glycan mutant missing all envelope glycosylation (Figure 4.4c and 4.4d). The panel of glycan mutants identified that both the E2 and E1 N-linked glycans were required for the most severe disease. Interestingly each RRV glycan mutant was as lethal as wild type RRV in IFN-αβ RKO mice with the exception of RRV missing all three N-linked glycans. These results imply that while reduction in viral replication may contribute to differences in disease outcome, N-linked glycosylation of the virus particle may play a role in promoting viral virulence.

Viral N-linked glycans have several potential roles in RRV induced disease. Specifically, these glycans can interact with C-type lectin receptors that promote infection of important target cells and also modulate IFN-αβ responses. With this in mind, we are designing future studies to compare infection efficiencies, the phenotype of
the cells infected, and the innate immune response in the draining lymph node between wild type and N-linked glycan mutant viruses. Furthermore, viral glycans could activate the host complement cascade which may contribute to the development of disease. There are previous reports that demonstrate alphavirus envelope glycans directly activate the host complement system (4). This is of particular interest in the RRV mouse model since complement contributes to severe RRV-induced disease. Thus, the glycans on RRV could activate the complement pathway which in turn would activate and/or recruit inflammatory macrophages to the hind limbs. This sequence of events could trigger the well-characterized hind limb dysfunction in RRV infected mice and is a focus of future studies.

ACKNOWLEDGEMENTS

We would like to thank Dr. Robert Johnston and Dr. Nancy Davis allowing us to perform the in vivo studies with in the VEE biosafety level 3 laboratory. We would like to thank Bianca Trollinger for assistance with cell culture. The work was supported by research grant number 5R21AI064645-02 awarded to M.T.H. R.S.S. was supported by the UNC Virology Training Grant and the UNC dissertation completion fellowship.
REFERENCES


Figure 4.1. Evaluation of 24-day old C57Bl/6 mice infected with either mammalian- or mosquito-cell-derived Ross River virus (mam- and mos-RRV). Individual mice were mock infected or infected with 1000 PFU of either, mam- or mos-RRV. A. Change in percent starting body weight. Each line represents a single mouse. B. Clinical score of representative mock infected, mos- and mam-RRV infected mice. C. Replication kinetics in the draining lymphnode and serum (D) at 6, 12, and 24 hours post infection. At each indicated time post infection, mice were euthanized and the DLN from the injected leg was harvested in addition to serum from the superior vena cava. Viral titers from the DLN were determined by plaque assay on BHK-21 cells, while serum interferon-αβ levels determined by bioassay. Each line in C and bar in D represents an individual mouse.
Figure 4.2. Titers in the draining lymphnodes (DLNs) of mice infected with mammalian or mosquito-cell-derived Ross River virus (mam- and mos-RRV).

Either mam- or mos-RRV was used over a range of doses from 1000 to 0.1 to infect 24-day old 129 Sv/ev (A) or 129 type I interferon receptor deficient mice (B). At 12 hours post infection, mice were sacrificed and the DLN from the injection leg was harvested and homogenized. Plaque assays for virus titers from each DLN was determined on BHK-21 cells. Each bar represents an individual mouse.
Figure 4.3. Diagram of the structural region of wild type and individual N-linked glycan mutants of Ross River virus. The 3' structural region of RRV with asterisks indicate the locations of N-linked glycosylation sites. An X indicates the ablation of that glycan by site directed mutagenesis.
Figure 4.4. Morbidity and Mortality scoring following infection of 24-day old C57Bl/6 and 129 interferon receptor deficient mice with wild type and RRV N-linked glycan mutants. A-D. Weight loss and clinical scores of wild type and RRV infected C57Bl/6 mice. A. Percent change in starting body weight in mice infected with wild type RRV and each E2 glycoprotein mutant. B. Clinical scoring of mice infected with wild type RRV and each E2 glycan mutant. C. Percent change in starting body weight in mice infected with wild type and each E1 glycoprotein mutant. D. Clinical scoring of mice infected with wild type RRV and each E1 glycan mutant. Each bar in A-D represents the mean and standard error of the mean of four mice. E. Average survival times of 129 type I interferon receptor deficient mice infected with wild type RRV and each N-linked glycan mutant. Each line represents the average survival time of at least 3 mice.
Figure 4.5. RRV skeletal muscle titers following infection with wild type and RRV N-linked glycan mutants. A and B. The same data as in Figure 5, displaying percent change in starting body weight and clinical score of mice infected with RRV wild type, RRV E2 N200,262Q, RRV E1 N141Q, and RRV E2 N200,262Q E1 N141Q. C. Skeletal muscle titers from mice infected with each of the viruses shown in A and B. At 24 and 72 hours post infection, mice were euthanized, perfused with PBS and quadriceps muscle from the injected leg was harvested. Tissue was homogenized and viral titers were determined on BHK-21 cells. Each bar represents the mean and standard error of the mean of between 3 and 5 mice.
CHAPTER FIVE

DISCUSSION AND FUTURE DIRECTIONS
The transition of a virus from mosquitoes to the vertebrate host represents a crucial stage in determining whether arthropod borne viruses successfully establish infection. Of particular importance is the initial interaction of the virus with myeloid dendritic cells (mDCs), which are the initial site of replication for many arboviruses. Interactions at this initial site (e.g. infection efficiency) and immune responses (e.g. type IFN-αβ induction from the infected cell) can determine whether or not the virus will establish infection and ultimately cause disease in the animal. Many arboviruses target mDCs during dissemination from the vector to the host, however, they are also a central player in orchestrating antiviral activities to clear the virus from an infected animal. Therefore, this presents an interesting phenomenon since many mosquito borne viruses target to mDCs, cells that play a central role in eradicating the infection.

There are several possibilities as to why viruses target to mDCs in the skin and draining lymph node (DLN). First, this could be a strategy used by the virus to establish a systemic infection. Infecting resident mDCs in the skin will result in DC activation and migration into the DLN and the virus could utilize this to gain access into the DLN. The virus could then spill into the blood resulting in a systemic infection. Furthermore, viruses are well documented in their ability to antagonize IFN-αβ and other antiviral responses (21). By infecting mDCs, the virus has the ability to suppress an antiviral response early in infection and/or kill DC’s which produce IFN-αβ to high levels. Alternatively, DC targeting could be a strategy used by the host to capture the virus in an antigen presenting cell so that viral antigens can be presented to the immune system to expedite its removal (26). Either way, virus-DC interactions represent a key step early in
infection that can ultimately determine disease outcome. Therefore, we established a model to mimic the vector to host transition and specifically test if mosquito-cell-derived virions differed from mammalian-cell-derived virions in their ability to interact with mDCs.

The experiments in this dissertation identified that Ross River virus generated in a mosquito cell line (designated mos-RRV) more efficiently infected mDCs and induced less IFN-αβ than RRV grown in a mammalian cell line (designated mam-RRV). We observed similar results with VEEV and BFV indicating that mosquito-cell-derived alphaviruses can initially evade or suppress an early IFN-αβ in mDCs to potentially provide the virus with an advantage to establish a successful infection. Differential IFN-αβ responses were specific to the virus particle and not due to non specific contaminating factors. Furthermore, the particle to PFU ratios of each virus stock are roughly equivalent and do not solely account for differences in IFN-αβ production.

Since a major difference between mammalian and mosquito derived virus is the presence of complex glycosylation (mammalian) versus high mannose carbohydrates (mosquito) on the virus, studies were performed to determine if differential glycosylation contributed to differences in IFN-αβ production. Our studies indicated that mam- and mos-RRV are differently glycosylated and that RRV generated in mutant Chinese hamster ovary (CHO) cells that produce high mannose glycans induced significantly less IFN-αβ than RRV generated in wild type CHO cells which have complex glycans. This data suggests that the presence of high mannose glycosylation on mosquito derived arboviruses may allow the virus to avoid induction of antiviral responses during the initial transition from the mosquito to the vertebrate host.
After our initial observations, another report provided evidence that mosquito-cell-derived West Nile virus (mos-WNV) was able to suppress dsRNA stimulation of murine macrophages (1). These data, in addition to our observations suggested two potential models to explain why mosquito-cell-derived viruses were poor inducers of IFN-αβ. The mosquito derived viruses could either actively suppress an IFN-αβ response from an initially infected cell or simply fail to induce IFN-αβ. Support for this second hypothesis was recently demonstrated with WNV by Silva et al. (25), where WNV derived from the C7/10 insect cell line was a poor inducer of IFN-αβ in pDC’s when compared to vero-cell-derived WNV. Additionally C7/10-cell-derived WNV did not actively suppress IFN-αβ induction by Sendai virus in pDC’s.

To determine if poor IFN-αβ induction in mos-RRV infected mDC cultures was attributed to either active suppression or a failure to induce IFN-αβ, we performed co-infection studies using mam- and mos-RRV. These results indicated that mos-RRV was unable to suppress IFN-αβ induction by mam-RRV in mDC cultures. Additionally, a panel of RRV N-linked glycan mutants demonstrated that complex glycans on the E2 glycoprotein of mam-RRV, but not mos-RRV, were essential for robust IFN-αβ responses on mDCs. Therefore, the failure of mos-RRV to induce IFN-αβ is most likely due to a lack of complex carbohydrates on the virion rather than active suppression of the DC antiviral response.

However, several additional experiments are required to definitively prove mos-RRV is unable to actively suppress IFN-αβ production from mDCs following infection. First, mos-RRV may suppress DC activation following stimulation with a synthetic stimulus such as PolyI:C or LPS. mos-RRV may also actively suppress IFN-αβ
production following infection of a different cell type, such as murine macrophages or human DCs. mos-RRV infection of mDCs PRIOR to infection with mam-RRV may inhibit mam-RRV IFN-αβ induction, however, these studies are complicated due to previous work demonstrating homologous exclusion of alphavirus infection in a cell previously infected with an alphavirus. Finally, mDC co-infection studies using mam- and mos-RRV have not fully evaluated if mam-RRV reduces the number of infected mos-RRV cells in a mDC culture. Preliminary experiments examining infection qualitatively by fluorescence microscopy indicated that infection percentages of mos-RRV versus mos-RRV co-infected with mam-RRV were not dramatically different. Therefore, confirming these results by flow cytometry is a focus of future studies. Even if these studies did indicate that mos-RRV is capable of actively suppressing a DC antiviral response, it does not take away from the observation that complex glycans on mam-RRV are required for robust IFN-αβ responses by DCs.

This work is not the first report that complex glycans on virus particles can mediate IFN-αβ responses, however, to our knowledge it is the first report with an arbovirus. In agreement with the WNV data discussed above, high mannose glycans on mosquito-cell-derived viruses may represent an early immune evasion mechanism. However, it is likely that interactions between DC’s and macrophages with other mosquito borne viruses could differ from the observations with RRV and WNV. For example, studies with the related Flaviviridae members WNV and DV indicate that WNV, but not DV envelope glycoproteins suppress dsRNA stimulation of macrophages (1). Furthermore studies by Kong et al. indicate that resting macrophages fail to produce measurable levels IFN-β or IL-1β following WNV infection, while Chen et al.
demonstrated high levels of IL-1β shortly after infection of macrophages with DV (3, 11). There are potential discrepancies between the two experimental systems described above, but it suggests that mechanisms to suppress innate antiviral responses are common among many but not all mosquito borne viruses.

There are several possibilities why complex glycans on the RRV envelope induce IFN-αβ responses. First, lectin receptors on the surface of mDCs may be triggered by N-linked glycans on mammalian, but not mosquito-cell-derived viruses to elicit an IFN-αβ response. Identifying the cellular sensor that detects the glycosylated mam-RRV glycoprotein is a focus of future studies. Our current hypothesis is that a C-type lectin (CLR) interacts with viral glycoproteins to induce IFN on mDCs, but whether or not mam- and mos-RRV interact with a single CLR or multiple CLR’s remains unknown. Studies utilizing CLR deficient mice to generate mDCs are currently underway. We hypothesize that the absence of one or more CLR on DC’s will alter either infection percentages or IFN-αβ responses. If this was the case, it would provide insight into which receptor(s) is/are involved in infection IFN-αβ induction differences.

Alternatively, a toll like receptor (TLR) could recognize the mam-RRV, but not mos-RRV envelope. Complex glycans arranged in the compact and highly ordered structure of a virus particle can be recognized by the host cell as foreign (27). Interestingly, TLR-4 can recognize viral glycoproteins and trigger signaling pathways leading to IFN-αβ production (7, 13, 22). It is therefore possible that envelope glycosylation patterns on mammalian but not mosquito-cell-derived alphaviruses trigger a CLR, TLR-4, or another cellular protein to induce IFN-αβ following interaction with mDCs. However, recently generated data with TLR-4 deficient DC’s suggests TLR-4
does not fully account for differences in IFN-αβ induction since differences in IFN-αβ levels between wild type and TLR-4 deficient DC’s were identical at low input doses of virus, while a modest IFN-αβ reduction (2-fold) in TLR-4 deficient DC’s was observed at higher input doses of virus. TLR-4 deficient DC’s were more permissive to virus infection and these modest differences in infection and IFN-αβ responses warrant further investigation.

mam-RRV glycosylation, which is mostly complex is required for robust IFN-αβ induction, while the mannose glycans on mos-RRV do not induce IFN-αβ. Thus, it is interesting that RRV has a mannose/hybrid sugar on its E2 glycoprotein even when produced in mammalian cells. This occurs with other arboviruses, including Dengue and Sindbis virus (9, 18). Perhaps this is an evolutionary adaptation by these viruses to minimize IFN-αβ production during transmission from the mosquito to the vertebrate host. However, it is possible that the E2 200 site does contain complex or hybrid sugars that are not detectable by our methods. Each mature RRV particle contains 720 potential glycosylation sites, and it is conceivable that minor carbohydrate populations may present. This would be similar to a previous study with WNV, where particles contained high mannose sugars that were not visible by western blot (4) demonstrating minor species of oligosaccharides at each glycan site are possible.

Therefore, a future direction of this project is to more carefully evaluate the specific type of complex carbohydrate present on mam-RRV that is critical for IFN-αβ induction. There are established methods to digest carbohydrates on intact virions in order to remove different types of sugars (2, 4, 5). An older study showed that digestion of mannose sugars alone (using EndoH and EndoF) did not hinder the ability of
Coronavirus particles to induce IFN-αβ in porcine peripheral blood mononuclear cells (PBMC’s). However, removal of both mannose and complex sugars (PNGase digestions) resulted in a virion that poorly induced IFN (2). Furthermore, several enzymes specific for different types of oligosaccharides can be used in these studies. One of these is neuraminidase which cleaves terminal sialic acid (SA) residues on glycoproteins. SA on the surface of a virion is a likely candidate to induce IFN-αβ, since the presence of SA on influenza promotes IFN-αβ production (17).

In addition to mos-RRV inducing low levels of IFN-αβ in mDC cultures, we also have consistently observed that mos-RRV infects more mDCs than mam-RRV. While we do not know the exact mechanism for these infectivity differences, we can speculate as to why this happens. First, we have demonstrated that high mannose glycans on mos-RRV are important for infection, since N-linked glycan mutant viruses missing both N-linked glycans infect mDC cultures less efficiently. While mam-RRV does contain some EndoH sensitive sugars, it is not clear whether or not they are high mannose or hybrid sugars. Furthermore, mam-RRV also contains complex sugars on the E2 site 262 site on the virus particle which may prevent virus-lectin interaction required for efficient infection. Another interesting observation in these studies was that mos-RRV missing all N-linked glycans still infected more mDCs than a non glycosylated mam-RRV particle. Similar to other systems (10, 23), structural differences between mam- and mos-RRV such as viral RNA and lipid content could contribute to differential IFN-αβ induction, leading to differences in infection efficiency.

Second, enhanced infection by mos-RRV in mDCs could be linked to poor IFN-αβ induction. This would allow the virus to productively infect a larger proportion of
cells in the culture. We have demonstrated that an intact IFN-αβ signaling pathway contributes to infectivity differences since mDCs lacking a functional IFN-αβ receptor produce little IFN upon viral infection and show reduced infection differences between mam- and mos-RRV. Therefore, the presence of 2 high mannose glycan sites on mos-RRV AND the fact that mam-RRV induces more IFN-αβ both contribute to infection differences between these two virus preparations.

In addition enhanced infection efficiency by mos-RRV, previous studies with RRV indicate that interactions between RRV and sub neutralizing levels of RRV antibody result in antibody-dependent enhancement (ADE) of infection (16). Infection by ADE occurs following the formation of virus-antibody complexes that bind to Fc-γ receptors (Fc-γRs) on monocytes, macrophages, and DCs. Following ligation, these complexes are engulfed via endocytosis. Upon entry, the virus is able to productively replicate since the antibodies bound to the incoming virus do not completely neutralize the particle. Previous work demonstrated that RRV infection via ADE resulted in active suppression of LPS induced NFκB and interferon stimulated gene (ISG) activation when compared to RRV infection in the absence of ADE (16). Therefore in addition to differences in cell source, ADE may be another mechanism utilized by RRV to both enhance infection efficiency and dampen the innate immune response. Taken together, our observations with mammalian and mosquito-cell-derived RRV combined with immune suppression by RRV infection via ADE represent two innate immune evasion mechanisms by the RRV in DCs and macrophages.

The majority of the studies in this dissertation were performed in vitro, but another obvious question was whether or not differential N-linked glycosylation of
mammalian and mosquito-cell-derived RRV have any consequences in vivo. Furthermore, we wanted to evaluate whether the absence of N-linked glycosylation on the virus particle contributed to RRV-induced disease. To address these issues we utilized the previously established RRV mouse model. Initial experiments indicated that animals infected with a standard dose (1000 PFU) of either mam- or mos-RRV both developed similar disease scores, but mos-RRV infected mice had slightly higher DLN titers at 6 hours post infection (hpi). In a more detailed follow up study we were unable to detect differences in viral replication in the DLN at early times post infection. Future experiments examining earlier time points as well as more sensitive assays to measure viral replication may be required to reconcile these different results.

We did observe differences in disease in mice infected with either wild type RRV or a panel of RRV mutants that lack N-linked glycans. Specifically, the loss of N-linked glycosylation correlated with the loss in disease severity. A virus missing both N-linked glycans displayed no measurable hind limb dysfunction or weight loss. Both viral replication and an aberrant immune response contribute to RRV disease pathology (14, 15, 20). Therefore we are currently trying to determine if a reduction in viral replication in target tissues, a reduction in immune pathology, or both contribute to the attenuated phenotype observed in mice infected with RRV N-linked glycan mutants. Comparing replication in the quadriceps muscle of infected animals with a subset of glycan mutants indicated that the RRV E1 glycan mutants are likely attenuated as a result of viral replication defects. It is possible that the attenuation of the RRV E2 N-linked glycan mutants, which display similar replication kinetics to wild type RRV, results from a lack of host immune pathology. Therefore, we are designing experiments to determine if wild
type and N-linked glycan mutant viruses infect different cell types in the draining lymph node and if they induce different innate immune responses. RRV E2 N-linked glycan mutants could also fail to activate the complement pathway, which has been shown to be directly activated by alphaviruses (8) and contribute to RRV-induced disease (19).

Another interesting set of studies will be to produce mosquito and mammalian-cell-derived alphavirus replicon particles (VRP). VRP are well characterized single hit vectors that have identical physical features to a wild type virus but are unable undergo multiple rounds of replication (6). Producing VRP from both mosquito and mammalian cells would allow further dissection of the initially infected cell without subsequent spread. Furthermore, VRP can be used in combination with a recently developed “ribonomics” technology. Konopka et al demonstrated that VRP expressing an epitope tagged pA binding protein can measure transcriptional changes in both infected and uninfected cells within the same culture (12). Producing mam- and mos-VRP expressing a tagged pA binding protein could be used in vivo to measure differences between infected and uninfected cells both cell culture and in the draining lymph node of mice. It is possible that IFN-αβ differences between mam- and mos-VRP infected cultures or animals would show more dramatic differences between the two groups. Additionally, our inconclusive in vivo studies between mam- and mos-RRV may be resolved by using mam- and mos-VRP. This technique is more sensitive and may reveal subtle differences that we were not able to detect with our assays.

Altogether, the observations presented in this report provide new insight into the early interactions between mosquito borne viruses and the innate immune system of the vertebrate host. At first glance, it may appear that evasion of an early IFN-αβ response
by a mosquito-cell-derived virion would have only a small impact on downstream disease. However, the properties of mosquito-cell-derived virions may be instrumental in determining whether or not the virus will establish an infection. If the input virus is confronted with a strong antiviral IFN-αβ response, viral replication would be limited and the infection will resolve quickly. This is a likely the scenario during a lot of viral infections, since not every individual exposed to virus by a mosquito bite develops disease. It would be beneficial for the virus to have a “head start” against the host immune system and suppress responses at very early times, even before the virus begins de novo protein synthesis. This could be achieved by the mosquito-derived virus which lacks complex sugars on the envelope glycoproteins which may act in concert with the well documented immunosuppressive properties of mosquito saliva (24). Both of these non-genetic factors would be extremely useful for a mosquito borne virus since their small genomes limit their ability encode a large number of accessory IFN antagonists. Hopefully the data in this dissertation will provide the basis for additional study regarding how mosquito borne viruses successfully establish infection and ultimately cause disease.
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