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Characterization of Innate Immune Mechanisms Against RVFV and a Potential Target for Antivirals

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Characterization of Innate Immune Mechanisms Against RVFV and a Potential Target for Antivirals

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Dedication

For my husband, you are my inspiration and my strength.

For my biggest fans, Mom and Dad, you never stopped believing in me.

And for the beagles, who always stayed close while I wrote late into the night and stayed in on the weekends.

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CHARACTERIZATION OF INNATE IMMUNE MECHANISMS AGAINST RVFV AND A POTENTIAL TARGET FOR ANTIVIRALS

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Rift Valley fever virus (RVFV) is a mosquito-borne zoonotic pathogen, which affects both humans and livestock. RVFV belongs to the *Bunyaviridae* family, genus *Phlebovirus* and consists of a tripartite genome (L-, M- and S- segments). NSs, encoded in the S- segment promotes degradation of PKR and general transcription shutoff including IFN-β mRNA synthesis. Currently, no effective measures have been developed to prevent or treat RVFV disease and very little is understood about what takes place during the initial stages of RVFV infection. The objective of this series of studies was to characterize RVFV infection in human dendritic cells (DC) and to develop a dominant-negative method to attenuate RVFV NSs protein as a treatment for RVFV. Human DC displayed low permissiveness to RVFV but initiated high levels of pro-inflammatory mediator secretion in response to the virus. DCs infected with rMP12-C13type virus, encoding a non-functional NSs protein, were able to initiate increased levels of maturation marker presentation of CD40, CD86 and CD83 and were also able to induce higher levels of proinflammatory molecules while rMP-12 and rMP12-delNSm/78 viruses (expressing functional NSs protein but lacking 78 kDa protein and NSm) did not. We found that NSm does not play a major role in DC activation during RVFV infection. We then attempted to develop a dominant-negative strategy to inhibit RVFV NSs. NSs forms filamentous inclusions within the nucleus and self-associates at the C-terminus of the NSs protein. We characterized 11 different MP-12 mutants, each encoding a 17-25 amino acid truncation in NSs. Unexpectedly, each of the mutants lacked NSs functions to inhibit innate immune responses due to the truncations. Although the NSs proteins were still able to accumulate within the cells, they did not interact with MP-12 NSs when co-expressed in cells. In cells infected with MP-12 and treated with *in vitro* synthesized RNA encoding truncated nonfunctional NSs, MP-12 NSs was still able to inhibit IFN-β and was able to degrade PKR protein, indicating that co-expression of non-functional NSs does not exhibit dominant-negative phenotypes.

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293	human embryonic kidney 293 cells
T-705	6-fluoro-3-hydroxy-2-pyrazinecarboxamide
2x MEM	2x Modified Eagle Medium
DAPI	4',6-diamindino-2-phenylindole
A549	adenocarcinomic human alveolar cells
BSA	bovine serum albumin
C-terminus	carboxyl-terminus
CAT	chloramphenicol acetyltransferase
C13	clone 13
cpm	counts per minute
CBP	Creb binding protein
°C	degrees Celcius
DC	dendritic cells
DNA	Deoxynucleic acid
dsRNA	double stranded RNA
DMEM	Dulbecco's minimum essential medium
eIF2α	eukariotic initiation factor 2 alpha
FCS	fetal calf serum
Flag	Flag protein tag
FITC	fluorescein isothiocyanate
Gc	glycoprotein c
Gn	glycoprotein n
GTP	guanosine-5' triphosphate
hpi	hours post infection
IFA	indirect immunofluorescence assay
IMP	inosine 5'-monophosphate
IMPDH	inosine monophosphate dehydrogenase
IFN α	interferon alpha
IFN β	interferon beta
IFN γ	interferon gamma
IRF3	interferon regulatory factor 3
IL-6	interleukin 6
IL-8	interleukin 8
i.m.	intramuscular injection
IND	investigational new drug
VeroE6	African green monkey kidney cells
kDa	kilodalton
kg	kilogram

MFI	mean fluorescence intensity
mRNA	messenger RNA
µg/ml	micrograms per milliliter
mg	milligram
MEM-α	minimal essential medium - alpha
m.w.	molecular weight
MCP-1	monocyte chemotactic protein-1
MP-12	A live-attenuated RVFV strain
moi	multiplicity of infection
NSm	non-structural protein M
NSs	non-structural protein S
ORF	open reading frame
PBMC	peripheral blood monocytic cells
PBS	phosphate buffered saline
PFU	plaque forming unit
Poly (ICLC)	carboxymethyl cellulose, polyinosinic-polycytidylic acid, poly-L-lysine dsRNA
PIC	preinitiation complex
PKR	dsRNA-dependent protein kinase
PTV	Punta Toro virus
rads	radiation unit
RNA	Ribonucleic acid
RVF	Rift Valley fever
RVFV	Rift Valley fever virus
RPMI-1640	Roswell Park Memorial Institute - 1640
SAP30	Sin3A-associated protein 30
SF	Strep-Tactin and Flag tandem tag
s.c.	subcutaneous injection
TSI-GSD-200	The Salk Institute - Government Services Division-200 (a formalin-inactivated RVFV vaccine)
TCID50	tissue culture infectious dose 50%
tRNA	tranfer RNA
TFIIH	transcription factor II H
TPB	tryptose phosphate broth
U/kg	units per kilogram
VSV	vesicular stomatitus virus
wt	wild type

CHAPTER 1: INTRODUCTION

1.1 HISTORY OF RIFT VALLEY FEVER

Rift Valley fever (RVF) was first recognized in an outbreak affecting sheep in a farm located in Kenya in 1930. It was first identified when a population of pregnant ewes had aborted with a large number of newborn lambs found dead (1). In human cases, Rift Valley fever virus (RVFV) causes acute biphasic febrile illness while some patients also develop encephalitis, blindness or lethal hemorrhagic fever (1-4). It has been suggested that RVFV first appeared on the African continent in the late 1800's, and it was endemic to sub-Saharan Africa until the 1970's. Then, RVFV spread across geographical barriers into new regions of Africa into the Arabian Peninsula (5, 6). Since the 1930 outbreak, over 30 new outbreaks have been recorded in Africa; e.g. East Africa (Kenya, Tanzania, or Somalia) in 1960, 1978-79, 1997-98, and 2006-7 Southern Africa (South Africa, Namibia, Zimbabwe, Zambia or Mozambique) in 1950-51, 1952-53, 1955-59, 1969-71, 1973-74, 1978, 1981, 1985, 1996 and 2007-8, Western Africa (Mauritania, or Senegal) 1987, 1993, and 1994-5, other parts of Africa (Sudan in 1973, 76, and 2006, Egypt in 1977-78, 1993 and 1997, Madagascar in 1990-1991 and 2008, Comoro in 2007-8), and the Arabian Peninsula (Saudi Arabia and Yemen) in 2000 (7-12) **(Figure 1.1)**.

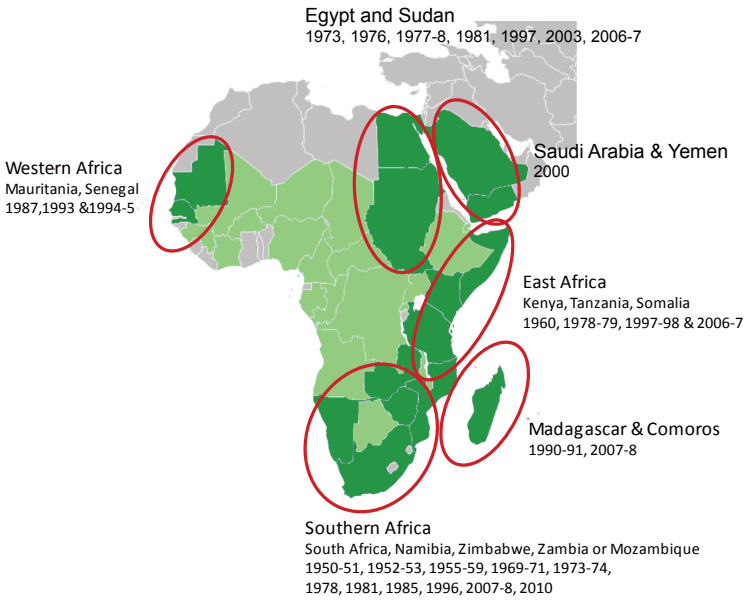


FIGURE 1.1: GEOGRAPHICAL DISTRIBUTION OF RVF
RVFV is endemic to sub-Saharan Africa. Outbreaks of RVF are indicated in coutnries marked Dark Green. Those countries with evidence of RVFV without major RVFV outbreak are marked in Light Green.

After its appearance in the 1930 Kenyan outbreak, RVFV remained in areas of the eastern region of Africa later appearing in new locations of the continent. In 1951, RVFV made its first appearance in South Africa within the regions of Western Free State and South-Western Transvaal (13). Sheep were mainly affected with an estimated death of over 100,000 adult sheep and abortion of an estimated 500,000 pregnant ewes (2, 14). Adult sheep had an estimated mortality rate of 60% while cattle showing 20% in abortion rate (13). Farmhands, ranchers and laborers later showed signs of illness after coming into contact with afflicted animals. Infection was confirmed in only 50 individuals with more expected to have been affected (15). The origins of this outbreak remain unknown, but it has become endemic in South Africa and has periodically reemerged since then (2).

In 1977-78, RVFV made its first geographical jump across the sub-Saharan into Egypt causing a large outbreak affecting an estimated 200,000 individuals and resulting in 594 deaths (14). In addition to the unexpected health effects on the human population, estimated $115 million US dollars was lost due to loss of livestock during outbreak (2, 16, 17). It has been suggested that initiation of the 1977-78 outbreak in Egypt was caused by transport of infected camels and livestock into Egypt but no definitive cause has been identified (2).

The largest outbreak in Eastern Africa occurred in Kenya in 1997-98 after a period of unusual heavy rainfall. A reported 27,500 human infections occurred, making this outbreak the largest in Sub-Saharan Africa with a recorded 170 humans deaths (18). It is unknown if human infection was primarily caused by mosquito transmission or contact with infected animal tissues, but most humans contracting the virus were involved in animal care, dissection and slaughter suggesting aerosolization as one of sources of infection for human RVFV infection (19).

In 2000, a RVFV outbreak appeared outside of the African continent for the first time in Saudi Arabia and Yemen (20-23). The source of the outbreak remains unknown, but genomic analysis has suggested a link between the outbreak in Saudi Arabia / Yemen and that in Eastern Africa in 1997-8 (20, 24). Possibly, introduction had occurred by import of infected cattle from endemic areas years prior to the outbreak and had remained unnoticed until favorable conditions ignited an outbreak (24). An estimated 516 clinical cases were identified in affected areas of Saudi Arabia with a reported 17% mortality rate in hospitalized cases (21). Most cases identified were found to have originated in the

flood plains at the foothills of the Sarawat mountains and into Jeddah further south nearing the border of Yemen (22). In neighboring Yemen, numbers of individuals suspected to have the disease climbed to 1087 cases with 11% mortality rate. These cases were found mainly in the coastal plain near the village of Wadi Mawr and a common characteristic of those infected was exposure to infected livestock, body fluids or tissue (23).

Spread of RVFV from original endemic area to new area has been suggested to be an effect of either transport of infected ruminants into new regions or possible wind displacement of infected vectors (mosquitos) (2, 8, 14, 24). Due to difficulty of enforcement of animal trade regulations and movement of the public in and out of endemic areas in addition to periodic flooding of endemic areas, outbreaks remain a constant threat. Spread beyond current endemic borders is expected since mosquito species known as RVFV vectors are found in various locations outside of the African continent, threatening the possibility of establishment of RVFV among mosquito populations in regions of Europe, Australia and even in the U.S. (2, 8, 25). Currently there are no commercially available vaccines or treatments to the public for RVF so spread into non-endemic countries has become a concern due to the effects related to panic in the populations and economic loss in agricultural industry.

1.2 RVFV TRANSMISSION

RVFV is transmitted through mosquito vector. Outbreaks occur during heavy flooding in areas with low-lying flats or dambos. RVF outbreaks are related to an increase in mosquito numbers after heavy rainfall or even in result of dam construction

causing unnatural flooding in endemic areas (24, 26). During the wet season, mosquitos are able to flourish and establish themselves on the populations of herbivores drawn to the flooded areas for water intake. As the infected mosquitos feed, the virus is spread to new hosts.

RVFV was first isolated from mosquitos in uninhabited forests of Uganda in 1944 (27). Since then, RVFV was also found in over 30 species of mosquitos, most well known: *Anopheles, Culex, Mansonia, Aedes, or Eretmapoites* mosquitos, which have been shown to carry RVFV in the wild and to transmit the virus into new hosts within the laboratory (24, 28). Vectors of RVFV can be categorized into two different types of vectors, the first – reservoir or maintenance vector, and the second – the amplifying vector. *Aedes* mosquitos, *A. mcintoshi, A. vexans,* and *A. dentatus* are known for their role as a reservoir vectors for RVFV. *Aedes* mosquitos spread the virus to their eggs transovarily. Females lay infected eggs on the moist soil and vegetation surrounding flooded areas which survive periods of desiccation and cooler climates for periods of years until the next heavy rainfall (29). To hatch, these eggs must go through a period of desiccation before they are able to continue their life cycle ensuring maintenance of RVFV until the next flood event (14). Newly hatched mosquitos feed on local ruminant populations until the second part of the transmission cycle takes place. After 2 weeks the amplifying vectors, *Culex* mosquitos, begin to hatch from egg rafts present at the surface of the floodwaters and begin to feed. *Culex* contract RVFV from already infected ruminants having high levels of viremia (30, 31) and aggressively bites humans or other animals. RVFV amplification occurs within the ruminant population and as mosquitos take blood meals from these animals, become infected and later introduce the virus to

human hosts during subsequent feedings **(Figure 1.2)**. As flooded areas dry up and the temperatures drop, mosquito populations dwindle and decrease until the next sequence of heavy rains (2, 3, 8, 14).

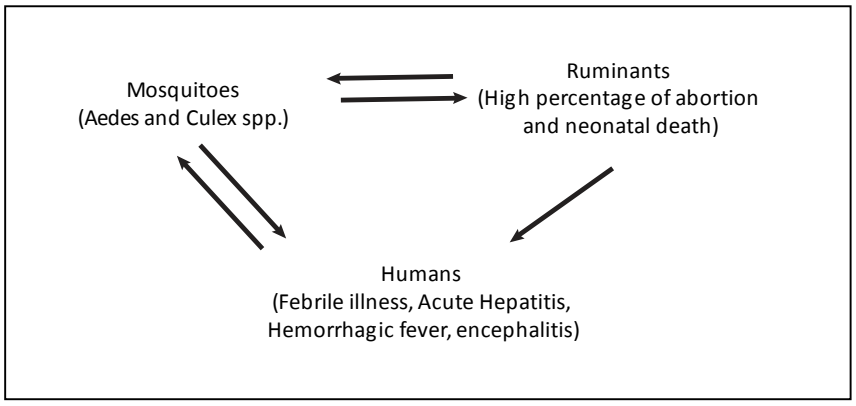


FIGURE 1.2: RVFV TRANSMISSION CYCLE
RVFV is transmitted through mosquito bite. Initially, transovarily infected *Aedes spp.* mosquitos infect ruminant populations drawn to flooded areas for water. Weeks later, amplifying vectors *(Culex spp.)* contract the virus when taking a blood meal from an infected ruminant. As the amplifying vector moves into new regions looking for new food sources, virus is then transmitted to new hosts (ruminants and humans) as it feeds. Humans are also able to contract the virus through contact with tissues or body fluid of infected animals.

In addition to vector transmission, humans become infected following direct contact with body fluids, tissues or aborted tissues of infected animals (veterinarians, veterinary staff, ranchers, farmers, etc.) Individuals working in slaughterhouses are also exposed to the virus by aerosolization of infected body fluids during animal slaughter (24, 32, 33).

1.3 RVFV PATHOGENESIS

RVFV is contracted by humans through either mosquito bite, contact with infected tissues or through respiratory route via aerosol inhalation. Human infection of RVFV mainly presents as a self-limiting febrile illness. After an incubation period of 4-6 days patients experience malaise, fever, headache, nausea and muscle pains (4, 31, 34-36). RVF presents with a biphasic febrile illness phase which usually resolves after two weeks (4). A small percentage of those afflicted (1%) go on to develop neurological or hemorrhagic complications of disease (4, 31, 35, 37). Symptoms of neurological illness present usually 1-2 weeks into the illness with fever, stiffness of the neck, confusion and convulsions (4, 31, 38, 39). In addition to the febrile illness, patients go on to develop macular rashes, ecchymosis on the limbs and eyelids as well as bleeding from the gums and mucosal surfaces. In addition, acute hepatitis will occur creating jaundice (34, 36, 40). Death can occur in hemorrhagic fever patients between 3-6 days after appearance of symptoms, and patients develop macular rashes, ecchymosis on the limbs and eyelids as well as bleeding from the gums and mucosal surfaces (34, 36). In some RVF patients, an increase in levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and lactate dehydrogenase (LDH) occurs while platelets and hemoglobin are decreased (4). In addition, 1 to 10% of patients develop retinitis and chorioretinal scarring, which may result in blindness.

1.4 NSS PROTEIN

RVFV is a negative-stranded RNA virus consisting of three segments, Small (S-), Medium (M-) and Large (L-). The L- segment encodes the RNA-dependant RNA polymerase. The S-segment encodes the N (nucleoprotein) and non-structural protein S

(NSs), while the M-segment encodes the glycoproteins Gn, Gc, NSm and 78kDa protein. NSs is the major virulence factor for RVFV and is dispensable for RVFV replication (41, 42). NSs plays a role in inhibition of the host antiviral responses as follows: (1) NSs inhibits the activation of interferon (IFN)-β promoter (43), (2) NSs induces general transcription suppression (44, 45), and (3) NSs promotes degradation of dsRNA-dependent protein kinase (PKR) (46).

The function of IFN-β inhibition is related to specific binding of NSs to the Sin3A-associated protein 30 (SAP30) (43). NSs attaches to SAP30, which binds to YY1 at IFN-β promoter **(Figure 1.3)**. Forming a co-repressor complex with several co-repressor factors (Sin3A, NCoR, and HDA3), NSs inhibits binding of the CREB binding protein (CBP), which limits the activation of IFN-β promoter (43). Interferon regulatory factor 3 (IRF3), a transcription factor important in the initiation of IFN-β transcription, is still able to be transported into the nucleus and bound to the IFN-β promoter yet is unable to induce activation of IFN-β promoter (43). This suggests that interaction of the co-repressor complex with NSs affects IFN-β promoter activation. The SAP30-binding domain was identified at amino acid (aa.) 210-230 of NSs, and recombinant RVFV lacking the SAP30-binding domain failed to inhibit IFN-β promoter activation (43).

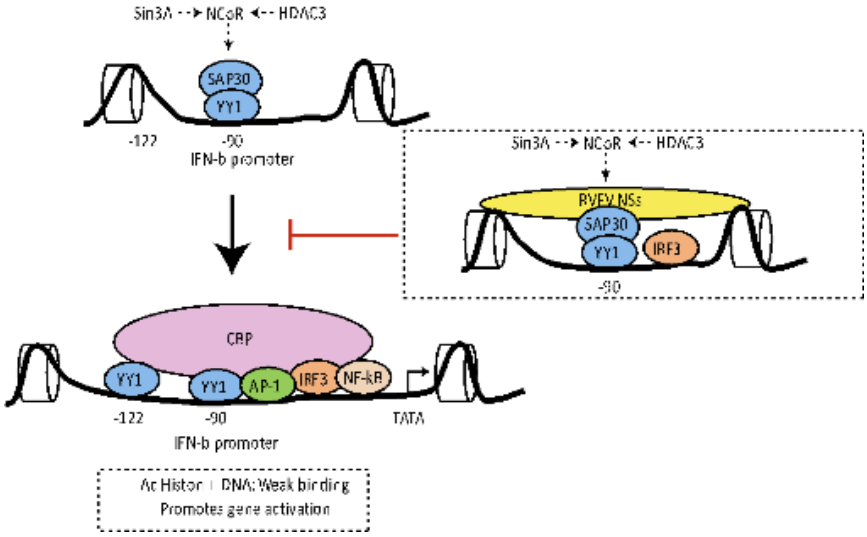


FIGURE 1.3: NSS INTERACTION WITH SAP30 CO-REPRESSOR COMPLEX
NSs protein binds to the IFN-β repressor protein YY1 forming a complex with SAP30 and the SAP30 associated proteins (Sin3A, NCoR and HDAC3), inhibiting Creb binding protein attachment and activation of IFN-β transcription (43). (Image courtesy of Tetsuro Ikegami, UTMB)

NSs has also been shown to affect general transcription through degradation or sequestration of subunits of the transcription factor IIH (TFIIH). TFIIH is an important subunit of the transcription pre-initiation complex (PIC) and is vital to the activation of polymerase II transcription activation (44, 45, 47). TFIIH is a basal transcription factor consisting of 10 subunits creating two bound subcomplexes: the ring-like core (XPB, p62, p52, p44, p34, p8 and XPD) and the cdk activating kinase (CAK) (cdk7, cyclin H and MAT1). Any absence of these subunits affects assembly of TFIIH and its function leading to inhibition of PIC activation and transcription activity **(Figure 1.4)**. NSs protein binds to TFIIH p44 subunits, sequestering it from TFIIH assembly (44). This function is only effective to newly synthesized TFIIH, while already assembled TFIIH cannot be suppressed by this mechanism. In addition, NSs binds to TFIIH p62, and

promotes post-translational degradation of p62, both at the nucleus and cytoplasm, resulting in p62 becoming undetectable early in infection (45). TFIIH plays a vital role as a transcription factor for both RNA polymerase I and II, thus suppression of TFIIH by RVFV NSs will induce a suppression of the host general transcription (47, 48),.

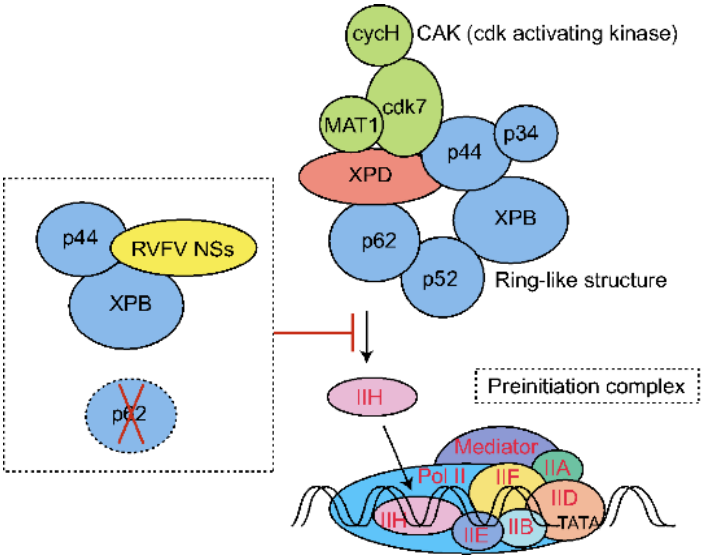


FIGURE 1.4: RVFV NSS AFFECTS FUNCTION OF TFIIH THROUGH SEQUESTRATION OR DEGRADATION OF SUBUNITS
NSs negatively affects assembly of the TFIIH transcription factor by degrading p62 subunits and sequestering p44 subunits inhibiting association of TFIIH with the preinitiation complex (PIC), affecting activation of transcription within the cell (44, 45). (Image courtesy of Tetsuro Ikegami, UTMB)

NSs also promotes degradation of PKR, which regulates host translational initiation; i.e., delivery of Met tRNA into initiation codon of mRNA through the phosphorylation of eIF2α **(Figure 1.5)**. Due to the lack of PKR, both host and viral translation remain active and uninhibited. Previous studies have shown cells infected with authentic MP-12 did not induce phosphorylation of eIF2α (46, 49).

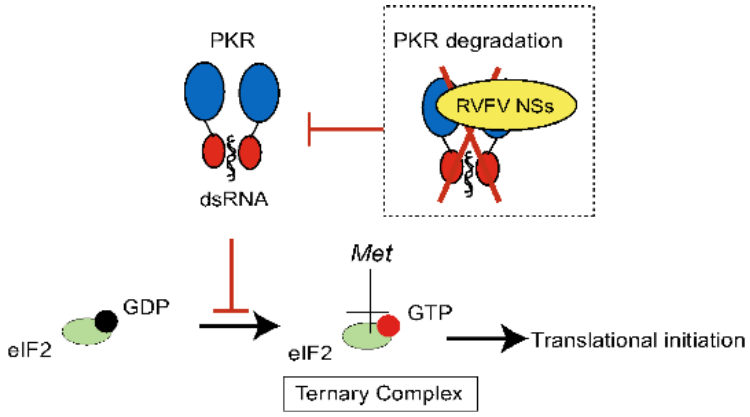


FIGURE 1.5: RVFV NSS PROMOTES DEGRADATION OF PKR RESULTING IN UNINHIBITED VIRAL TRANSLATION
RVFV NSs protein promotes degradation of PKR, preventing phosphorylation of eIF2α protein and allowing for active viral and host cell translation (49). (Image courtesy of Tetsuro Ikegami, UTMB).

Characteristically, RVFV NSs forms filaments exclusively within the nucleus of infected cells (50). With use of recombinant Semliki Forest viruses expressing RVFV NSs protein encoding a deletion or point mutation, Yadani et. al. studied NSs domains required for formation of these filaments as well as translocation of NSs into the nucleus. With a yeast-two hybrid system, it was discovered that the C-terminus 17 aa. is required for NSs oligomerization (51). The deletions and point mutations within the 17 aa. C-terminus of NS did not affect translocation activity into the nucleus (51). It was concluded that the self-association domain within the C-terminus of NSs is required for filament formation in nucleus, while the requirement for NSs translocation into the nucleus remains unknown.

1.5 VACCINE CANDIDATES

Currently, only one formalin-inactivated Entebbe strain of RVFV is available as an investigational new drug (IND) for humans, but is not of use for the general public. This vaccine, TSI-GSD (The Salk Institute – Government Services Division)-200, has been found safe and induces antibody titers of 1:40 or more against RVFV (52, 53). TSI-GSD 200 requires multiple booster immunizations to induce 1:40 or more of neutralizing antibodies against RVFV, while the production is limited due to the use of high containment facility, and thus the vaccine manufacturing cost is expensive and the shelf-life is also short (54). Although live-attenuated vaccines induce stronger immune responses than that of TSI-GSD 200, they retain the possibility of reversion to virulence. The Smithburn neurotropic strain was originated from Entebbe strain isolated from mosquito samples collected in Ugandan forest (27, 52, 55). Viscerotropic Entebbe strain was passaged intracranially in mice and eventually became neurotropic. The resulting Smithburn strain has been used as the first licensed veterinary vaccine for RVF starting in the 1950's whereas the use has been limited to endemic countries; e.g., South Africa, Kenya and Egypt. Smithburn vaccine retains significant residual virulence in pregnant animals, and causes abortion and fetal malformations in pregnant animals (56, 57).

During an Egyptian outbreak during 1977/78, ZH548 and ZH501 strains were isolated from febrile and lethal human cases of RVF, respectively. MP-12 strain has been generated by 12 serial passages of ZH548 strain in the presence of a chemical mutagen, 5-fluorouracil (58). Originally, it was reported that all three segments of MP-12 genome were attenuated, while recent studies suggest attenuation is attributed to the mutations in

the M- and L- segments, with the S-segment still encoding virulence phenotype (52, 59, 60). Despite a single amino acid substitution in the NSs gene, MP-12 NSs remains fully functional (59).

74HB59 strain was isolated from a non-fatal human case in a 1974 outbreak in the Central African Republic, and was passaged 5 times in suckling mice and twice in Vero cells. Clone 13 strain (C13) was plaque-purified from from viral sample derived from Vero cells infected with 74HB59 strain. Attenuated from a large in-frame deletion (69%) in the NSs gene, C13 lacks a functional NSs gene (24, 52, 61, 62). C13 vaccine is able to induce type-I IFN in infected cells while unable to replicate as efficiently as RVFV encoding an intact NSs in type-I-IFN competent cells (62). Studies in animal models have shown that C13 vaccine possesses high immunogenicity, and is able to induce long-term immunity in sheep without teratogenic effects or abortions in pregnant sheep making it a promising candidate for veterinary vaccines (61, 63).

Recently, reverse genetics was used to create highly immunogenic vaccine candidates with deletion of RVFV virulence genes, which have less potential of reversion. Bird et. al. developed a recombinant RVFV lacking NSs and NSm, which induces protection against wild-type RVFV. RVFV lacking NSs and NSm does not cause viremia, while effectively initiating immune response against infection in sheep, without any teratogenic or abortive effects in pregnant ewes (64).

Recombinant virus vectors encoding protective epitopes of RVFV have also been tested with successful results: e.g., lumpy skin virus (65), alphavirus (66), or Newcastle disease (67) expressing RVFV glycoproteins. Virus-like particles (VLPs) have also been

generated with use of recombinant baculoviruses expressing the nucleocapsid and glycoproteins (Gn and Gc) of RVFV. VLPs are safer to make with potentially lower levels of side effects in those vaccinated (68). DNA vaccines are ideal candidates that are easy to manufacture, and DNA vaccine expressing RVFV Gc, Gn and N protein of RVFV (69-71) have shown promise, while one has found potential use of nucleoprotein as the immune target against RVFV (72). Despite their potential, DNA vaccines for RVF are still in early stages of research and are not ideal for immunization upon RVFV outbreak due to their requirement of multiple vaccinations for production of neutralizing antibodies (69, 70, 72).

1.6 Antiviral Therapeutics for RVF Treatment

Several antivirals have been studied for their effects in animal models (hamsters, mice and monkey). A pyrimidine analog, ribavirin, has been shown to limit RVF in hamster (60 mg/kg two times daily s.c. at day 0 and then treated with 20 mg/kg two times daily on days 1-10), Rhesus monkey (50 mg/kg i.m. 2 hours after infection then 10mg/kg i.m. every 8 hours for 9 days) and mouse (75 mg/kg s.c. at 8 hours post infection, then 25 mg/kg s.c. two times a day for 10 days) models (73, 74). Ribavirin is a broad-spectrum drug, which affects replication of both RNA and DNA viruses. It has become a recommended drug for most hemorrhagic fevers, including RVFV, despite the lack of thorough testing (75-78). Antiviral mechanisms of ribavirin are as follows; (1) inhibition of inosine 5'-monophosphate (IMP) dehydrogenase, which decreases levels of guanosine-5' triphosphate (GTP) within treated cells thus limiting pools of the nucleotide for replication of new virons (79), (2) interference with mRNA translation during treatment

possibly due to an incorporation into the mRNA caps (80) (3) inhibition of the viral RNA polymerase complex (81) and ability to induce accumulations of mutations within the viral genome (as seen in poliovirus, Hantaan virus and foot and mouth disease virus) (82). Studies with Punta Toro virus (PTV) (a *Phlebovirus*) and RVFV have shown that ribavirin successfully suppressed viremia *in vivo* and *in vitro* (73, 83, 84) demonstrating the potential of using ribavirin as RVF treatment. In fact, the Working Group for Civilian Biodefense recommends the use of ribavirin during periods of mass outbreaks and disease in areas with dense populations (1, 78). Ribavirin alone is effective for antiviral therapy; combination of this therapeutic with others would merge different modes of treatment for novel approaches for RVF therapy. Ribavirin was combined with Poly (ICLC), poly-L-lysine and carboxymethyl cellulose - a potent inducer of interferon, in treatment of RVFV in mice and proved successful when compared to results obtained in studies conducted on ribavirin or Poly (ICLC) alone (1-4, 85). Treatment of mice with a combination of ribavirin and poly (ICLC) resulted in 92% protection when compared to 50% with either ribavirin or Poly (ICLC) alone at 24 hours post infection.

IFN is capable of limiting RVFV viremia and clinical manifestations as either a preventative treatment or post-infection treatment using dosages of $5x10^3$ U/kg or $5x10^5$ U/kg (for 24 hour intervals for 5 days beginning at 24 hours prior to inoculation or 6 hours after inoculation and every 24 hours for 5 days after inoculation) in rhesus monkey model (5, 6, 86). Later when the pathology of the IFN responses were monitored in an infected non-human primate model, a direct correlation between IFN response and severity of RVFV was found, demonstrating that initiation of IFN response within 24

hours after inoculation had lessened disease severity, with the subject more likely to survive infection (87).

Although ribavirin is an effective antiviral for RVFV, there are concerns regarding its safety as it has been shown to cause anemia and is associated with teratogenic effects, suggesting the need for alternative therapies for RVFV or other diseases. T-705 (6-fluoro-3-hydroxy-2-pyrazinecarboxamide) is a compound that inhibits the RNA-dependent RNA polymerase of RNA viruses. After infection with PTV, T-705 was able to protect mice after challenge with a dosage of 30 mg/kg initiated 24 hours post infection and given twice daily. When side effects are compared to that of ribavirin, mice had a lower levels of weight loss suggesting a lack of toxicity possibly due to T-705 not affecting inosine monophosphate dehydrogenase (IMPDH) levels (83, 88).

1.7 DENDRITIC CELLS

Dendritic cells (DC) are important elements of the innate immune system. DC facilitate regulation of the interplay between innate and adaptive immune responses with use of important effector functions: phagocytosis, antigen presentation and cytokine production, (89, 90). Immature DCs are found throughout the body; e.g., blood, skin, gastrointestinal tract and respiratory tract. DCs are one of the first cells to interact with pathogens, making them one of the first and ideal targets for viral infection (91, 92). Pathogen-related antigens or apoptotic cells activate DCs, which induces the maturation. Upon stimulation, DCs produce cytokines to attract other immune cells to the site of infection (89, 92). As DCs are activated, they then express surface marker CD40 and

CD86 co-stimulatory molecules to initiate signaling for the maturation of DC. CD206 expression and phagocytosis decrease as the cells mature. DC will then travel to the lymph nodes and present antigen to naïve $CD4^+$ T cells to initiate humoral and further cellular immune responses (92-94). Understanding of how DCs interact with RVFV and what responses are initiated after contact with them is vital for better understanding of how RVFV infects and how live-attenuated vaccines are able to initiate immune responses efficiently.

Viruses can affect the immunological functions of DC. by either enhancing or weakening effector functions of DC, to initiate different types of immunopathogenesis and/or immune evasion. Persistent viruses, such as human and murine CMV, are sequestered within DC while impairing cellular function (91). Measles and influenza virus are able to induce cytopathic effects of DC (95). Additionally, DC can act as "Trojan horses" while concealing infectious viruses, to later act as a reservoir for further dissemination within the host (89, 90).

Previous studies have shown that human monocyte-derived macrophages (MDM) are permissive to RVFV infection. RVFV encoding functional NSs (wt ZH501 strain) was able to replicate more efficiently in MDM than NSs deficient viruses. This may be due to the ability of wt RVFV ZH501 to limit type-I IFN production and proinflammatory response in these cells thus allowing for uninhibited viral replication (96). Another study suggests that infection of ZH501 in mouse model causes a decrease of populations of lymphocytes, monocytes and platelets during infection (97). These results suggest the ability of RVFV to modulate immune responses. Other studies have suggested the possibility of RVFV to infect DC via DC-specific intercellular adhesion

molecule 3-grabbing nonintegrin (DC-SIGN) as a receptor for RVFV infection and its ability to infect dermal DC (98). In vivo, recombinant virus lacking functional NSs was found to accumulate in mouse DC in the local draining lymph nodes while MP-12 infected mice did not show this characteristic. It is possible that MP-12 has the ability to escape host immune responses at the draining lymph nodes and infected DCs travel directly to the liver or spleen without accumulation at draining lymph node (99). These studies suggest DCs have an important role to determine the pathogenesis of RVF during the initial stages of RVFV infection *in vivo*. Since DC play a role in establishing cellular and humoral immune responses during infection, it is important to know if RVFV can interfere with DC functions which would identify new targets for antivirals and treatments.

1.7 SPECIFIC AIMS

Rift Valley fever virus is a zoonotic mosquito-borne virus endemic to sub-Saharan Africa and has spread into Egypt, Madagascar, and the Arabian Peninsula. RVFV causes high levels of abortion in infected ruminants and is capable of causing disease in humans resulting in hemorrhagic fever, blindness, acute hepatitis or encephalitis. Spread of RVFV outside endemic areas is of great public health concern due to its effects on public health as well as economy related to animal industry. Currently, there is no effective therapeutic treatment for RVF, and RVFV is classified as a Category A Priority Pathogen by NIAID and HHS/USDA overlap select agent. RVFV is a negative-stranded RNA virus consisting of three segments, Small (S-), Medium (M-), and Large (L-) segment. The S- segment encodes N and NSs proteins in an ambi-sense manner while the M- segment encodes Gn, Gc, NSm and 78kDa protein. The L- segment

encodes RNA-dependent RNA polymerase. The NSs protein is a major virulence factor for RVFV known for its ability to 1) suppress host general transcription, 2) inhibit activation of IFN-β promoter and 3) promote degradation of PKR. NSm protein is a potential virulence factor, which may delay apoptosis of infected cells while another study suggested that RVFV lacking NSm lessens the occurrence of acute hepatitis, but increases the occurrence of neurological disease in a rat model. My long-term goal is to identify novel targets for future applications in preventative and therapeutic measures for human infections with highly virulent pathogens such as RVFV. DCs are cells responsible for primary recognition of pathogens and are key in connecting the innate and adaptive immune responses. It is unknown how RVFV NSs protein affects these cells and how it may influence the outcome of infection. I hypothesize that NSs is responsible for inhibiting innate immune responses in human DCs, and the attenuation of NSs inhibits the replication of RVFV. First, I will characterize the host response of DC against RVFV lacking functional NSs and then I will characterize the functions of truncated NSs proteins and the effect of coexpression with functional RVFV NSs to attenuate NSs functions in infected cells. This study will lead to develop new strategies to combat RVFV infection.

AIM 1: IMPACT OF NSS ON DC RESPONSES TO RVFV INFECTION

DCs are one of the initial innate immune cells to come into contact with invading pathogens. DCs are found throughout the host's body and are located in areas exposed to the environment (e.g. skin, gastric system, respiratory system) and constantly sample the environment in search of pathogens or apoptotic cells. DCs located in the dermis are one of the first cells to come into contact with a virus transmitted via mosquito bite and to react against infection to initiate cellular and adaptive immune responses. I hypothesize

NSs protein shuts off host transcription in DC, impacts phenotypic and functional maturation and the ability to uptake antigen and production of inflammatory mediators. I will characterize the maturation of DC, cytokine production and DC ability to stimulate T-lymphocytes in response to RVFV infection.

AIM 2: ATTENUATION OF RVFV NSS FUNCTIONS BY TARGETING NSS SELF-ASSEMBLY PROCESS

RVFV NSs self-assembles, forms filamentous structures within the nucleus and exhibits multiple biological functions. NSs inhibits host general transcription including IFN-β mRNA synthesis, and promotes degradation of TFIIH p62 and PKR proteins. I hypothesize that interference of the NSs self-association process by co-expression of truncated NSs proteins causes an attenuation of NSs function, including the inhibition of IFN-β promoter and degradation of PKR, limiting viral replication in type-I IFN competent cells. Using eleven rMP-12 viruses encoding 17-25 aa. deletions within the NSs gene, I will describe the characterization of the interference of MP-12 NSs assembly and the resulting attenuation of NSs functions.

Chapter 2: Impact of NSs on DC Responses to RVFV Infection

2.1 ABSTRACT

To determine the effect of the NSs protein on DC function and maturation, we characterized the response of human DCs to infections with rMP-12, rMP12-C13type or rMP12-delNSm/78 viruses. To understand host responses after infection in overall populations which include both infected and uninfected cell populations, we used 48 hours post infection (hpi) samples using low multiplicity of infection (moi) 0.01. Using FACS analysis, we found that known DC maturation surface markers (CD40, CD83 and CD86) were increased in cells infected with rMP12-C13type compared to rMP-12 or rMP12-delNSm/78 infected DC. Phagocytosis of DC was decreased more efficiently in those infected with rMP12-C13type than those infected with rMP-12 or rMP12-delNSm/78. DCs infected with rMP12-C13type efficiently secreted type-I IFNs, IFN-γ, IL-6, MCP-1 and IL-8, while those infected with rMP-12 or rMP12-delNSm/78 did not show any increases. Importantly, DCs infected with rMP12-C13type effectively stimulated proliferation of naïve $CD4^+$ T cells. Collectively, this study suggests that DCs infected with rMP12-C13type undergo maturation and induce cytokines/chemokines. Thus, RVFV NSs inhibits host innate immune responses, and the subsequent maturation of DC, which might be important for efficient viral replication and pathogenesis.

2.2 INTRODUCTION

DCs are primary innate immune cells responsible for recognition of antigens and initiation of immune responses against pathogens playing an important role in connecting innate and adaptive immune responses against pathogens (89, 90, 93, 100). Residing in

areas of the body exposed to the environment (e.g. gastrointestinal tract, respiratory system, and skin), DC continuously sample the environment for traces of pathogen or apoptotic cells (89). Upon attachment to surface receptors (e.g. DC-SIGN or CD206) (98, 101) or through endocytosis, pathogens or apoptotic cells are recognized by DC which activate cellular maturation initiating innate and adaptive immune responses (89, 93). DC play a key role for initiation of innate and adaptive immune responses through activation of naïve $CD4^+$ and $CD8^+$ T cells (89, 90, 93, 100). Taking advantage of the roles DCs play in initiating immune response, viruses are capable of enhancing or diminishing DC effector functions to modulate the host response resulting in immunopathogenesis and/or immune evasion (89, 92, 100). Also, DC are capable of harboring viruses to serve as a reservoir to further distribute virus throughout the host (92, 102-104).

Two types of DCs are involved in innate and adaptive immune responses. Myeloid DCs originate in the bone marrow from progenitor cells. Once the cells are formed, they move around and localize all over the body in various tissues (the blood, the dermis, and in the mucosal surface). This ability provides DC with the unique advantage to become one of the first cells to come into contact with the pathogens (90, 93). Plasmacytoid DCs are commonly found in the lymph node and are well known to express higher levels of IFN-α in response to infection than myeloid DCs and are important in maturation induction of other DCs to induce adaptive immune responses (93, 105-107) allowing for the host to mount a response against disease and prevent replication of the pathogen. Understanding of what takes place at the site of infection and how DCs affect events in the preliminary stages of infection will allow for better interpretation of how

infection is initiated and how innate immune responses are activated. This might eventually lead to identification of targets for effective treatments and vaccines against RVFV.

RVFV is able to modulate host immune responses. A recent study has shown that human monocyte derived macrophages are susceptible to RVFV infection and NSs protein is able to limit type-I IFN production and pro-inflammatory response (96). In a mouse model, RVFV ZH501 strain induced lymphocytopenia with a decrease of lymphocytes, monocytes and platelets which later recovered in numbers before the hosts had expired (97). Despite these studies, there is still much to learn of how immune cells are involved in the role of initial host immune response against RVFV.

Study of the early immune response of monocyte-derived myeloid DC against RVFV will provide for a better understanding of the effect of DC on the initial steps of RVFV infection, and will provide new targets for therapeutics.

A major virulence factor of RVFV, NSs acts as an antagonist of IFN-β. This ability to suppress IFN-β modulates monocyte-derived macrophage responses by limiting their response to infection (96, 108). With this in mind, it is possible that NSs plays a valuable role modulating innate immune responses of infected DCs as well. We hypothesize that RVFV NSs can downregulate host gene expression in primary monocyte-derived human DC, limiting the ability of DC to respond to infection, resulting in a diminished innate and adaptive immune response against infection. We were also interested in characterizing the role NSm plays during RVFV infection of DC. Although not required for viral replication, NSm has shown to play a role in limiting viral induced apoptosis within infected cells but its overall function remains unknown (109, 110).

Studies have also demonstrated a decrease in acute hepatitis but an increase in neurotropic disease in rats infected with recombinant virus possessing a deletion of NSm protein suggesting a possible role in the pathogenesis (111). The overall objective of this study is to characterize RVFV infection of human DC in the presence or absence of NSs proteins and to evaluate whether these proteins can affect maturation and innate immune responses of DC. In this series of experiments we used recombinant MP-12 (rMP-12) and rMP12-C13type (encoding non-functional NSs protein) and rMP12-delNSm/78 virus (encoding functional NSs protein but lacking functional NSm and 78kDa protein).

2.3 MATERIALS AND METHODS

VIRUS

In this study, rMP-12, rMP12-C13type and rMP12-delNSm/78 viruses were used. rMP12-C13type virus is a recombinant MP-12 encoding a in-frame truncation of NSs gene at aa.16-198 similar to that of authentic Clone 13 strain (41). Another recombinant virus, rMP12-delNSm/78 virus lacks a functional NSm protein and 78kDa protein but still possesses a functional NSs protein (a kind gift from Dr. Makino at UTMB). The original stocks of these viruses were subjected to 2-3 additional passages in VeroE6 cells and then centrifuged to generate a cell-free working viral stock with a titer expressed as 50% tissue culture infectious dose ($TCID_{50}$)/ml sample and was then stored at -80°C. In addition, aliquots of infectious stock were irradiated ($2x10^6$ rads) from a cobalt 60 source according to a pre-determined kill curve, and were then used as an inactivated rMP-12, rMP12-C13type or rMP12-delNSm/78 as controls for these studies. All experiments

involving infectious virus were conducted at the University of Texas Medical Branch (Galveston, TX) in a biosafety level 2 laboratory.

VIRUS TITRATIONS

Cell culture supernatants were collected at different time points post-infection prior to measurement of infectious viral titers with use of $TCID_{50}$ assay on permissive Vero E6 cell monolayers. VeroE6 cells were grown in 96-well plates with a serial 10-fold dilution of samples. The titer of individual samples in this chapter are expressed as $TCID_{50}$ per milliliter of samples as described previously (112).

DENDRITIC CELLS

Primary human DCs were prepared from highly enriched peripheral blood monocytic cells (PBMC) from healthy donors using centrifugation on a Histopaque 1077 gradient. $CD14^+$ monocytes were purified from PBMC by negative selection magnetic column separation system (EasySep® Human monocyte $CD14^+$ Enrichment Kit, StemCell Technologies). DCs were generated from highly enriched $CD14^+$ monocytes. Harvested monocytes were cultured in complete RPMI 1640/10%FCS (R-10) medium with an added mix of GM-CSF (1000 U/ml) and IL-4 (500 U/ml) for differentiation of the monocytes into DCs. Cytokines were replenished every 3-4 days with cells harvested after 7 days of culture for morphological and phenotypic characterization. GM-CSF/IL-4 stimulated monocytic cells resulted in the appearance of nonadherent or loosely adherent cells, and the majority of them expressed $CD14^{low/}CD1^+CD40^{+/low}CD86^{+/low}$ HLA-$DR^{+/low}CD83^-$ cells, typical of immature DC.

VIRAL INFECTIONS

DC were either mock-infected or infected with rMP-12, rMP12-C13type, or in some cases rMP12-delNSm/78 virus with an moi of 0.1 (unless indicated) or with γ-inactivated virus at 37°C for 1 hour. Cells were cultured after washing to remove unbound virions. DC were then cultured at 37°C in RPMI 1640 medium supplemented with 10% heat-inactivated FCS medium. The cell-free supernatants and cells were harvested at indicated time points during infection for viral titration, cytokine profiling and phenotyping. At 0 and 48 hpi, cells were harvested and counted using a hemocytometer and 1% trypan blue to quantify number of viable cells during infection to compare DC survival for each virus type.

PHENOTYPING

DCs were mock-infected or infected with rMP-12, rMP12-C13type or rMP12-delNSm/78, harvested, pelleted and resuspended in 100µl of staining solution (PBS with 2% FCS and 0.2% sodium azide) containing FITC conjugated mouse antibodies directed against human CD40, CD86, CD83, CD206, or HLA-DR molecules. For a non-specific antibody-binding control, cells were stained with FITC- labeled control antibodies. All antibodies were purchased from Caltag Laboratories. Cells were washed and then fixed in 500µl of 2% paraformaldehyde in PBS overnight. Data were acquired on a FACScan and analyzed by CellQuest software (BD Biosciences). In addition, mean fluorescence intensity (MFI) values of each molecule type was used to monitor the changes of surface molecule expression.

ASSESSING PHAGOCYTOSIS BY FLOW CYTOMETRY

After harvest at 48 hpi, infected DC were pelleted and resuspended in 500µl of R-10 medium in 1.5ml Eppendorf tubes containing FITC-dextran (m.w.=40,000, 40 µg/ml; Sigma Aldrich) prior to incubation at 37°C (or 4°C for negative control) for 1 hour. Cells were washed three times and then fixed with 2% paraformaldehyde,. Quantities of intracellular fluorescent probe were measured with use of FACScan flow cytometry (BD Biosciences).

CYTOKINE AND IFN PROFILING

Supernatant collected at each time period post infection were irradiated ($2x10^6$ rads) from a cobalt-60 source (located at UTMB), according to a predetermined kill curve to eliminate viable virus. Cytokine levels in samples were quantified using a BioPlex Human 27-Plex kit (BioRad) following the manufacturer's protocols and provided wash station and resulting software (Biorad, Hurcules, CA) (112). All results were then analyzed in Excel and Graphpad Pro (Prism) for statistical significance. Levels and activity of type I IFN (or α/β IFN) in culture supernatant were measured by using a VeroE6/vesicular stomatitis virus (VSV) based plaque-reduction assay as performed previously (112, 113) (114). Each culture supernatant sample was serially diluted 1:3 and incubated on VeroE6 cells 18 hours before infection with 40 plaque-forming units (pfu) per well of VSV. After a one-hour incubation with virus, wells were overlaid with methylcellulose and VSV plaques were allowed to grow for 24-48 hours before staining with crystal violet. Titers of type-I IFN were calculated from the highest dilution, which gave 50% plaque reduction of VSV compared to control column, which was not treated

with interferon. For a standard control we used a recombinant IFN-β that was diluted 1:3 (The PBL Biomedical Laboratories, Piscataway, NJ).

INDIRECT IMMUNOFLUORESCENCE ASSAY (IFA)

For IFA, human DCs were cultured in collagen coated 96 well plates. Cells were infected with a moi of 3 of rMP-12 virus. Cells were fixed 16-24 hours with 4% paraformaldehyde and prepared for IFA. Cells are permeabilized with 0.2% of Triton-100/PBS, and then labeled using anti-RVFV Gn protein antibodies (monoclonal antibody provided by U.S. Army Medical Research Institutes of Infectious Disease) in 0.5% BSA-PBS and incubated 1 hour at 37°C. Cells were washed with PBS prior to addition of DAPI (Molecular Probes). Fluorescent images were captured with conventional UV microscopy using Olympus IX71.

MIXED LYMPHOCYTE CULTURE

Primary human DCs were prepared from highly enriched PBMC as described above. $CD4^+$ cells were purified by negative selection using StemSep Human CD4+ T cell Enrichment Kit and magnetic column (StemCell Technologies). DCs were infected with an moi of 1 of either rMP-12, rMP12-C13type, rMP12-delNSm/78 or γ-inactivated virus, or left untreated for 2 days in 200μl of RPMI media with 10%FCS in 96-well plates. Treated DCs were then harvested and co-cultured in triplicate with a fixed number of allogenic CD4+ T cells at various ratios (1/10, 1/20, 1/40, 1/80, 1/160, or 1/320) and then incubated for a total of 6 days. Cultures were pulsed with 1 μCi/well of [^{3}H] thymidine (New England Nuclear) for the last 12-16 hours in culture. Mock-infected cells were used as a negative control. The incorporation of the [^{3}H] thymidine was measured with use of liquid scintillation counting with Top Count NXT

(PerkinElmer) microplate scintillation and luminescence counter and expressed as counts per minute (cpm).

STATISTICAL ANALYSIS

Unpaired Student's t-test was completed using Graphpad Prism 5.03 program (Graphpad Software Inc.) for the comparison of two groups.

2.4 RESULTS

A. RVFV INFECTION OF HUMAN DC RESULTS IN LOW PERMISSIVE INFECTION

In this study, responses of primary human DCs to rMP-12, rMP12-C13type and rMP12-delNSm/78 were characterized to determine if the NSs and NSm proteins modulate antiviral responses of DCs. We used viruses expressing functional NSs (rMP-12, rMP12-delNSm/78), nonfunctional NSs (rMP12-C13type), and virus lacking 78kD/NSm protein (rMP12-delNSm/78). We analyzed samples harvested at 24 or 48 hpi with a moi of 0.1 to monitor overall host innate immune responses including both infected and uninfected populations. The rationale of using 24 and 48 hpi time points was that cells infected with RVFV undergo apoptosis at later time points of infection such as 72 or 96 hpi (110). Initially, a high moi of 3 was used to confirm the susceptibility of human DCs to rMP-12 virus. Cells were fixed at 48 hpi and indirect immunofluorescence assay (IFA) was performed to detect Gn proteins. Although cells were infected with a high moi, human DCs poorly expressed Gn proteins (~10%) at 48 hpi **(Figure 2.1)**. Thus, it was possible that virus does not actively replicate at 48 hpi. To test viral replication kinestics at initial stages of infection, human DCs were infected with

rMP-12, rMP12-C13type or rMP12-delNSm/78 with a moi of 0.1. Supernatants were collected over a course of 48 hours and the $TCID_{50}$ was determined. Titers of rMP-12, rMP12-C13type or rMP12-delNSm/78 were not increased during the observation period confirming that little to no infection occurred in DCs **(Figure 2.2)**.

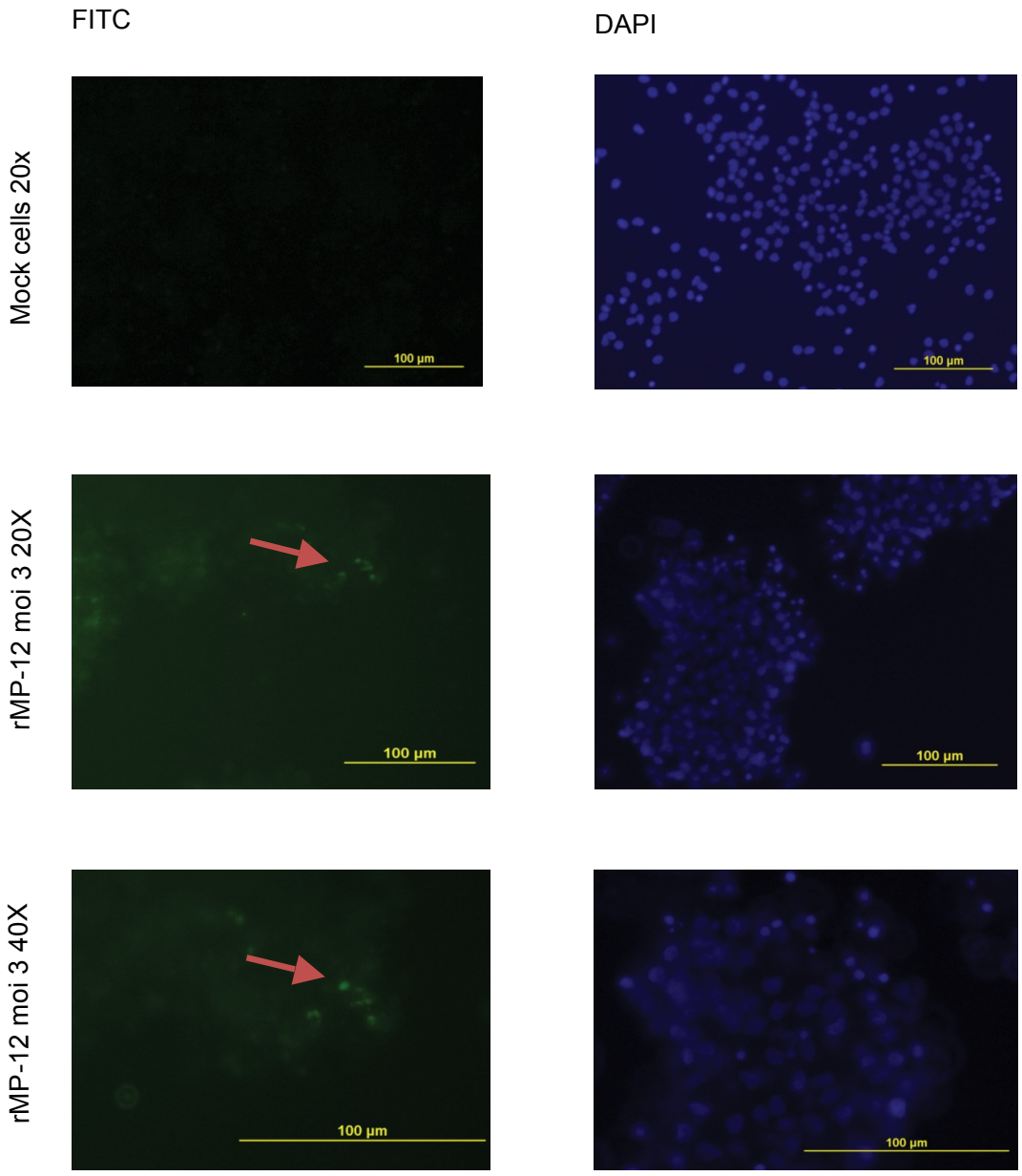


FIGURE 2.1: HUMAN DCS ARE PERMISSIVE TO RMP-12 INFECTION
Human DCs were infected with rMP-12 virus at an moi of 3. Cells were collected after 48 hpi then stained with mouse ascites monoclonal antibodies directed against RVFV surface glycoprotein Gn (hyper-immune ascites fluid, Precision Antibody) in 0.5% BSA-PBS for detection of viral inclusions. DAPI staining was performed to assess cellular status as control. Positive stained cells are marked with an arrow.

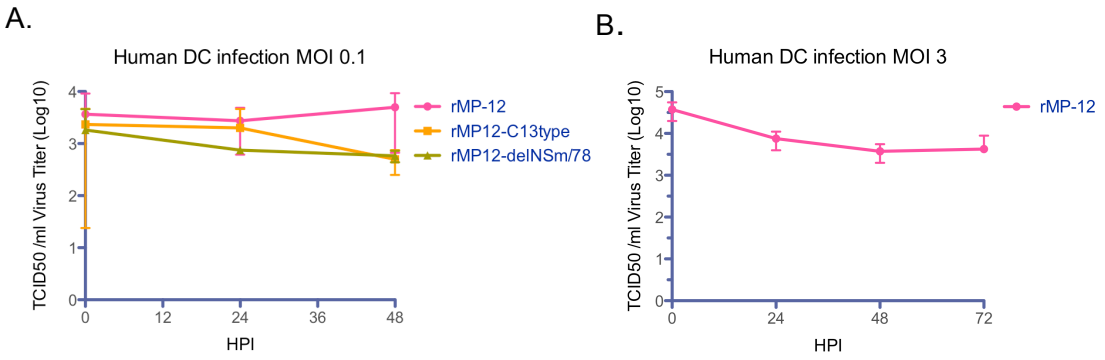


FIGURE 2.2 : HUMAN DENDRITIC CELLS DO NOT SUPPORT EFFICIENT RVFV REPLICATION

Human DCs were infected with rMP-12, C13type or delNSm/78 virus with a moi of 0.1 (A) or moi of 3 (B). Supernatant was sampled at the time points indicated (hpi) to determine yields of infectious virus by the standard infectivity assay in VeroE6 cells. Titers are expressed as Log_{10} TCID50 virus per ml. Results of 3 independent experiments are displayed. Error bars represent standard deviation.

Cell viability was then tested to see if DC viability was affected by RVFV infection. Human DCs were either mock-infected or infected with rMP-12, rMP12-C13type or rMP12-delNSm/78 with a moi of 0.1. At 0 and 48 hours, infected cells were harvested and stained with trypan blue for visualization of viable cells under microscope with use of a hemocytometer. The viability of cells infected with the mentioned viruses was not decreased when compared to mock control **(Figure 2.3)**.

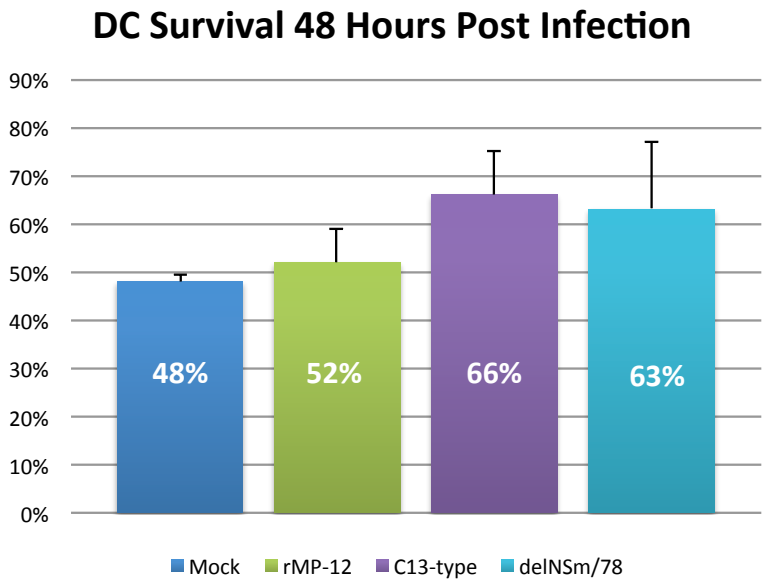


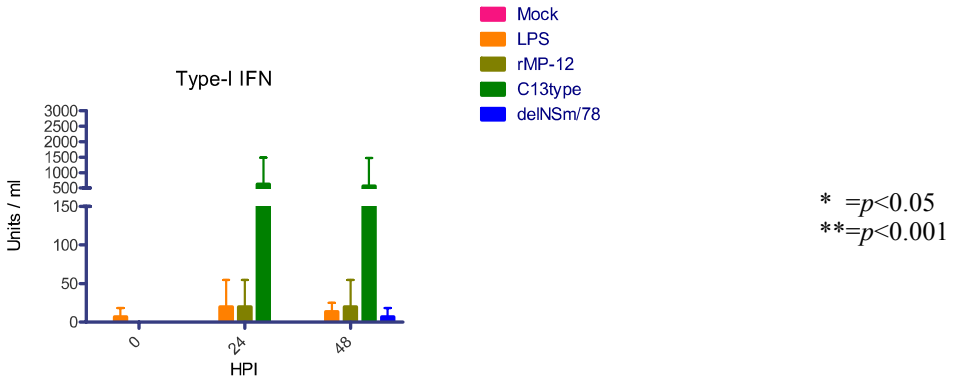
FIGURE 2.3: CELL VIABILITY OF CELLS INFECTED WITH RECOMBINANT MP-12
Viability of DC mock-infected or infected with moi 0.1 of rMP-12, rMP12-C13type or rMP12-delNSm/78 was assessed using trypan blue exclusion technique. Percentages were calculated in DC numbers present in culture at 0 and 48 hpi. Results are the outcome of 3 independent experiments and error bars are representative of standard deviation.

B. IMMUNOLOGICALLY IMPORTANT CYTOKINES AND CHEMOKINES ARE SECRETED FROM INFECTED HUMAN DENDRITIC CELLS AFTER RVFV INFECTION.

To test secretion of type-I IFN or cytokines from human DCs infected with rMP-12, rMP12-C13type or rMP12-delNSm/78, DCs were infected with a moi of 0.1. Culture supernatants were collected at 0, 24, or 48 hours and were then γ-irradiated to inactivate viable virus. BioPlex (BioRad) and type-I IFN bioassay were performed for measurement of pro-inflammatory response against infection. Rationale to use human Bioplex 27-Plex cytokine assay is that we tried to understand the release of biologically active cell mediators. As an alternative method, we can analyze transcription profile of infected cells. However, RVFV NSs modifies host proteins at post-translational level (i.e., PKR and TFIIH p62), and host mRNA levels do not necessarily reflect the amounts

of host proteins released from infected cells. Despite the low progeny virus production in RVFV infected cells, DCs infected with rMP12-C13type virus secreted high levels of type-I IFNs, IFN-γ, MCP-1, IL-6 and IL-8, while those infected with rMP-12 or rMP12-delNSm/78 did not increase those cytokines at high level **(Figure 2.4 A and B)**. Although we could observe the cytokine changes among infected cells, the differences were not statistically significant (Student's t-test) due to a large variation in reaction to RVFV among human DCs. The rest of the cytokine panel did not show any increase or change in expression after infection. These results suggest lack of a functional NSs protein, but not NSm, increased type-I IFN and host cytokine productions.

A.



B.

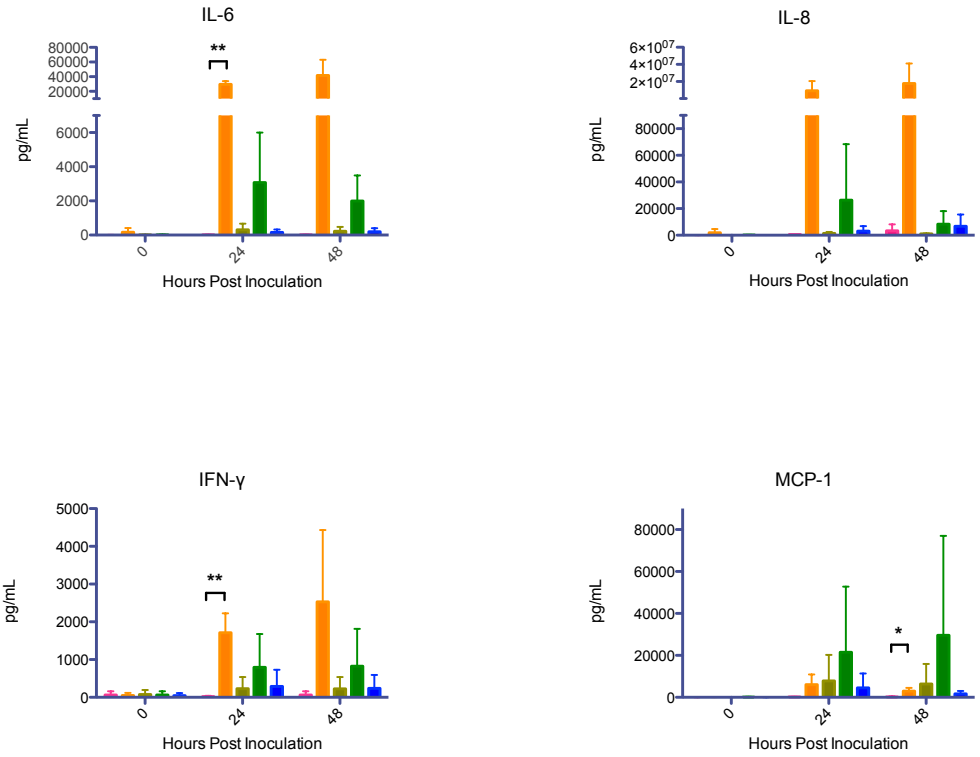


FIGURE 2.4: RVFV NSS INHIBITS TYPE-I IFN AND PROINFLAMMATORY CYTOKINE PRODUCTION IN HUMAN DC

(A) Supernatants harvested at 0, 24, and 48 hpi were irradiated and used for type-I IFN bioassay. Results are presented as titers of IFN units per ml of culture supernatant and are shown as means +/- standard deviations for three separate infection bioassay results. Statistical differences were analyzed by Student's t-test. (B) Supernatant was harvested

from 24-hour increments during infection and irradiated with $2x10^6$ rads from a Cobalt-60 source before assaying on BioPlex human 27-plex kit. Statistics were analyzed with Student's unpaired t-test.

C. NSs Protein Affects DC Phagocytosis

Changes in phagocytic activity are a hallmark function of matured DCs. It has been described that immature DCs are more active in phagocytosis than mature DC (115-117). To determine if levels of phagocytosis changed in response to RVFV infection, FITC labeled Dextran (FITC-Dextran) uptake of DC was evaluated after infection with rMP-12, rMP12-C13type or rMP12-delNSm/78. The intensity of FITC-Dextran uptake by those infected DCs was then analyzed by Flow-Cytometry. DCs infected with rMP-12 and rMP12-delNSm/78 showed slightly decreased phagocytosis when compared to mock, while those infected with rMP12-C13type had largely reduced percentages of FITC-positive cells (26%) **(Table 2.1)**. These results suggest that rMP12-C13type-infected DCs mature in higher numbers when compared to those infected with rMP-12 or rMP12-delNSm/78.

TABLE 2.1 Intracellular FITC-Dextran

Primary DC

Infection Type	% Positive	MFI
Mock	78	3,981
rMP-12	53	2,667
delNSm/78	64	3,035
C13type	26	2,816

TABLE 2.1: LEVELS OF PHAGOCYTOSIS IN DC DECREASE IN CELLS INFECTED WITH RMP12-C13TYPE VIRUS.
Cells were mock-infected or infected with moi 0.1 of rMP-12, rMP12-C13type or rMP12-delNSm/78 then harvested 48 hpi and incubated with FITC labeled dextran sulfate. Cells were stained and uptake of FITC Dextran by DC was measured using FACScan Flow Cytometer. MFI; Mean Fluorescence Intensity. Data is representative of 3 independent experiments.

D. EFFECT OF NSS EXPRESSION ON SURFACE EXPRESSION OF DC MATURATION MARKERS AND CO-STIMULATORY MOLECULES.

The maturation phenotype of human DCs infected with rMP-12, rMP12-C13type or rMP12-delNSm/78 was further tested by measuring expression of surface co-stimulatory molecules and DC maturation markers including CD40, CD86, CD83, HLA-DR and CD206 by flow-cytometry. The increased expression of several costimulatory molecules (CD40, CD83, CD86 and HLA-DR) serves as a marker of DC maturation (94, 118-122). CD206 facilitates endocytosis of dextran-sulfate and therefore may be responsible for the observed phenotypic alteration in phagocytosis activity (101). Previously we observed varied levels of FITC-Dextran uptake in rMP-12, rMP12-C13type and rMP12-delNSm/78 infected cells. CD206 mannose receptor surface expression is considered constitutive on immature DC, although expression is modulated during activation through the presence of pro-inflammatory cytokines or toll-like

receptors stimulation (101). Thus, if phagocytosis is reduced due to DC activation then presentation of CD206 should also become reduced. Human DCs were mock-infected or infected with rMP-12, rMP12-C13type or rMP12-delNSm/78 at an moi of 0.1, then collected at 48 hpi for antibody staining for flow-cytometry analysis. Compared to mock-infected cells, rMP-12, rMP12-C13type or rMP12-delNSm/78 infection resulted in increased surface expression of CD40 and CD86 **(Table 2.2)**, while rMP12-C13type also increased CD83. The highest levels of CD40 (99%), CD83 (31.5%) and CD86 (88.9%) were found in DCs infected with rMP12-C13type. On the other hand, DCs infected with rMP12-C13type had shown the lowest levels of CD206 expression (15.4%) when compared to DCs infected with rMP-12 or rMP-12-delNSm/78 (25.8% and 31.2%). Together, these results suggest that an increased number of DCs infected with rMP12-C13type undergo maturation and the reduced levels of CD206 is consistent with the phagocytosis phenotype as shown in **Table 2.1**.

TABLE 2.2 Surface Expression of DC Maturation Markers

Infection Type	Human DC (% Positive/MFI)				
	CD40	CD86	CD83	HLA-DR	CD206
Mock	37.7/679	5.6/527	4.97/390	99.5/4099	42.7/472
rMP-12	97.3/1157	56.1/592	3.98/416	99.4/4011	25.8/450
delNSm/78	92.0/806	21.4/575	4.6/889	99.7/3676	31.2/438
C13type	99.0/2143	88.9/800	31.5/404	99.4/4200	15.4/458
LPS	96.1/2269	92.9/1234	49.9/612	99.6/5054	30.9/634

TABLE 2.2: MATURATION MARKERS UPREGULATE FASTER IN CELLS INFECTED WITH RVFV LACKING FUNCTIONAL NSS PROTEIN THAN IN RVFV POSSESSING FUNCTIONAL NSS PROTEIN.
Human DC were stained with FITC labeled mouse antibody directed at CD40, CD83, CD86, CD206 and HLA-DR (Caltag Laboratories) and analyzed by flow cytometry. Results are representative of 3 independent experiments.

E. PRIMING OF $CD4^+$ T CELLS BY HUMAN DCS INFECTED WITH RMP-12, RMP12-C13TYPE OR RMP12-DELNSM/78.

Next, DC ability to activate naïve T cells in response to infection was monitored. Mixed lymphocyte cultures were used to measure $CD4^+$ T cell proliferation in the presence of DCs infected with rMP-12, rMP12-C13type or rMP12-delNSm/78. In agreement with the maturation phenotypes, active proliferation of $CD4^+$ T cells was observed in the presence of DCs infected with rMP12-C13type, while no detectable proliferation occurred in the presence of DCs infected with rMP-12 or rMP12-delNSm/78 **(Figure 2.5)**. These results suggest that NSs inhibits the maturation of human DCs and the subsequent priming of $CD4^+$ T cells.

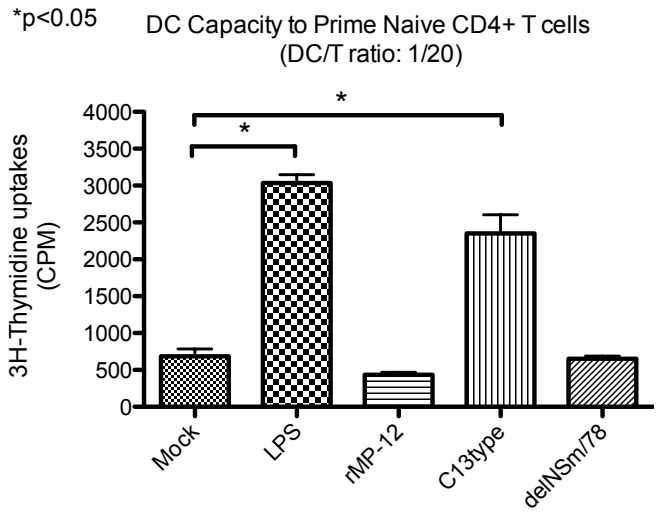


FIGURE 2.5: CD4+ T CELL ACTIVATION AND PROLIFERATION BY DC INFECTED WITH RECOMBINANT RVFV.
DCs were mock-infected or infected with LPS (positive control), rMP12-, rMP12-C13type, or rMP12-delNSm/78 viruses. At 48 hpi, DCs were then cultured with a ratio of 1:20 of $CD4^+$ naïve T cells. After 6 days, levels of [^{3}H] thymidine were measured determining levels of T cell proliferation in response to activated DC. Statistics were measured with use of Student's t-test.

2.5 DISCUSSION

The ability of RVFV to dysregulate or to limit DC maturation during infection is not only of great importance for the initial establishment of infection, but also for subsequent establishment of long term immunity. The importance of NSs as a virulence factor for RVFV has been well documented in epithelial and parenchymal cells, but the impact of this phenomenon on the cellular functions of DC has not been previously detailed. This series of experiments has characterized the response of monocyte-derived human DCs in response to RVFV infection. Unexpectedly, we found human DCs prepared in vitro do not support active replication of MP-12 and the variants. Past studies suggest that MP-12 primarily infect and replicate in mouse DCs (99) while DC-SIGN was identified as a receptor for RVFV (98). Our results suggest that RVFV does not

replicate very efficiently in human DCs. However, further confirmation will be required to conclude this, because the infectivity of RVFV is different between *in vivo* and cultured cells. The present study indicates that NSs protein of RVFV restricts maturation and activation of human DC. Maturation and co-stimulatory cell surface markers (CD40, CD83, and CD86) were all up-regulated in response to RVFV infection, while higher levels of expression were detected in human DCs infected with rMP12-C13type, which lacks a functional NSs gene. As DCs mature, they migrate to local lymph nodes to activate naïve $CD4^+$ T cells to initiate adaptive and cellular immune responses. With this in mind, recent studies suggest that mice inoculated with rMP12-C13type at the footpad accumulated viral proteins in DCs at the popliteal lymph node, while those inoculated with MP-12 do not accumulate viral proteins at the draining lymph nodes (123). Considering that MP-12 NSs inhibits the maturation of DCs, NSs might support the evasion of RVFV- infected DCs from the host immune system. On the other hand, MP-12 lacking functional NSs (C13type) might be an immunogenic vaccine candidate since it can effectively induce human DC maturation and prime $CD4^+$ T cells. The virus lacking NSm protein did not present any effect on DC function or maturation suggesting it has no significant role in these sequences of events. Our results also suggest that MP-12 lacking 78kDa/NSm inhibits the expression of CD86 (Table 2.2). It suggests that 78 kDa/NSm expression might promote the maturation of human DCs in the experiment. However, the detailed mechanism of this phenomenon is not clear, because we consistently used low moi infection to see total effect induced by small number of infected DCs.

The maturation of DCs infected with rMP12-C13type could be triggered by the induction of type-I IFNs and cytokine/chemokines. Cytokines and the chemokines secreted from DCs infected with RVFV may possibly contribute to macrophage and T cell activation for initiation of cellular innate immune response; e.g. IL-6, IL-8, IFN-γ,

type-I IFN or MCP-1 **(Figure 2.4 and 2.5)**. It is surprising that human DCs, although supporting only low viral replication, make substantial quantities of cytokine/chemokine secretion and induce DC maturation events. We expect a similar event may occur at the local lymph node upon RVFV infection. The consequence of the inhibition of cytokine/chemokine secretion by NSs and the resulting pathogenesis should be studied in the future for better understanding of the role NSs protein during initial infection stages. Such information will be useful to uncover potential new targets for RVF treatments and also provide further insight on the effects of this virulence protein on primary innate immune cells.

NSs protein is well known as the major virulence factor allowing for RVFV to cause systemic infection and disease *in vivo*, but it is only one of the defining factors required for pathogenesis of RVFV in immune-competent hosts. For example, MP-12 is attenuated by mutations at M- and L- segments, while the S- segment and the NSs gene encode virulence phenotype (59). Thus, it is very likely that the virulence of RVFV is determined by multiple factors. A lack of NSs abolishes viral ability to counteract host innate immune responses and promotion of an efficient stimulation of T cells. These results suggest a prerequisite role of NSs to allow virus replication before being sensed by the host immune system. An ability to replicate in a limited time frame might be controlled by other viral proteins such as M- or L- segment, which may be worth studying in the future.

Chapter 3: Co-expression Analysis of Nonfunctional NSs in Cells Infected with RVFV

3.1 ABSTRACT

Major virulence factor NSs of RVFV suppresses host general transcription, inhibits IFN-β promoter activation, and promotes degradation of PKR. In this study, we determined if co-expression of non-functional NSs mutants could attenuate the functions of RVFV NSs. Each mutant encodes a 17-25 aa. truncation at aa. 6-30, 31-55, 56-80, 81-105, 106-130, 131-155, 156-180, 181-205, 206-230, 231-248 or 249-265 of NSs. Recombinant (r) MP-12 encoding these truncations were unable to inhibit IFN-β mRNA synthesis or degrade PKR *in vitro*. When co-expressed with functional MP-12 NSs, truncated mutants did not exhibit a dominant-negative effect to suppress IFN-β mRNA or to degrade PKR while NSs lacking aa. 240-265 could marginally attenuate PKR degradation by MP-12 NSs. Truncated NSs did not interact with MP-12 NSs regardless of C-terminus self-association domain. This study suggests that NSs functions are highly susceptible to conformational change, and RVFV NSs can exert biological functions even in the presence of non-functional truncated NSs, regardless of the C-terminus self-association domain.

3.2 INTRODUCTION

To develop an effective antiviral strategy, we hypothesized that co-expression of non-functional NSs in RVFV-infected cells lead to the attenuation of viral replication because of dominant-negative inhibition of NSs functions. It is known that NSs self-associates at the C-terminus, 17 amino acids (aa. 249-265), and forms filamentous

structures within the nucleus of infected cells (50, 51). Thus, non-functional NSs encoding the C-terminus self-association domain may be incorporated into NSs oligomers and interfere with NSs functions. To generate non-functional NSs, we used a series of NSs mutants each encoding a short truncation located at a different position within the protein **(Figure 3.1)**. A previous study suggests that absence of SAP30 domain at the aa. 210-230 abolishes IFN-β suppression function (43), implying that deletions within NSs will disrupt some NSs functions but allow for it to retain its ability to self-associate into NSs filaments. The objective of this study was to determine if co-expression of non-functional NSs would abolish or attenuate the function(s) of MP-12 NSs in infected cells with the use of a dominant-negative effect. This study will provide new insight on a possible method to attenuate RVFV NSs in infected cells for use in creating new treatments for RVF.

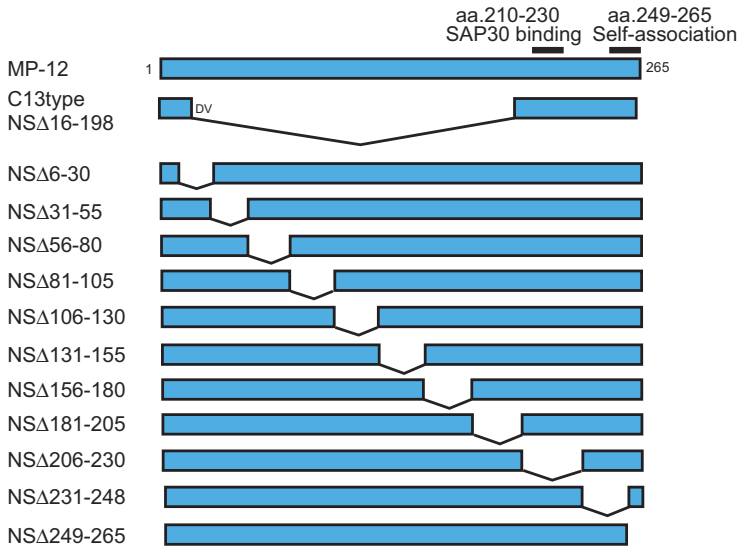


FIGURE 3.1: SCHEMATICS OF NSS ENCODING A SERIAL DELETION
11 different rMP-12 viruses were created, each possessing a 17-25aa. serial deletion within the NSs gene. For this study, MP-12 and rMP12-C13type viruses were used as functional and nonfunctional NSs controls. MP-12 encodes both the SAP30 (aa. 210-230) (43) and the self-association domain (aa.249-265) (51) which are absent in NSsΔ206-230 and NSsΔ249-265 respectively. rMP12-C13type possesses a 69% deletion within the NSs gene (aa. 16-198) rendering the protein nonfunctional.

3.3 MATERIALS AND METHODS

MEDIA AND CELLS

Vero E6 cells and wild type (wt) A549 cell cultures cultivated and maintained in Dulbucco's Modified Medium (DMEM) (Gibco) containing 10% inactivated fetal calf serum (FCS) and 100μg/ml penicillin and 100 μg/ml streptomycin were added to the culture media. BHK/T7-9 cells stably expressing T7 RNA polymerase (124) were maintained in MEM-α containing 10% FCS and 600 μg/ml of hygromycin B.

PLASMIDS

The plasmid encoding anti-viral sense of MP-12 S-segment at the downstream of the T7 promoter, pProT7-S(+), was described previously (41). Serial deletion of 75bp (25

aa.) was introduced into the NSs open reading frame (ORF) of pProT7-S(+)-NSsΔ6-30, NSsΔ31-55, NSsΔ56-80, NSsΔ81-105, NSsΔ106-130, NSsΔ131-155, NSsΔ156-180, NSsΔ181-205, NSsΔ206-230 or NSsΔ231-248, referred as NSsΔ1-11 respectively. For C-terminus mutant, the PCR fragment encoding NSs ORF with C-terminus 51bp (17 aa) deletion was amplified and clones between *Hpal* and *Spel* of pProT7-S(+) (41) designated at NSΔ249-265. The alanine substitutions of NSs-E253-255A/D257-259A, NSs-E253-255A or NSsD257-259A were made onto pProT7-S(+) plasmid by site-directed mutagenesis with PFU Ultra (Agilent Technologies), and designated as pProT7-NSs-E253-255A/D257-259A, NSs E253-255A or NSs D257-259A respectively. NSs ORF of those NSs mutants were amplified by PCR with Phusion High Fidelity DNA polymerase (New England Biolab), and cloned into pcDNA3.1mycHisA (Invitrogen) between *Kpn*I and *Xho*I, and designated as pcDNA2.1mycHisA - NSΔ6-30, NSΔ31-55, NSΔ56-80, NSΔ81-105, NSΔ106-130, NSΔ131-155, NSΔ156-180, NSΔ181-205, NSΔ206-230, NSΔ231-248, NSΔ249-265, NSs-E253-255A/D257-259A, NSs-E253-255A or NSsD257-259A.

RECOVERY OF RECOMBINANT MP-12

Recovery of recombinant MP-12 encoding NSs truncation was completed with use of a plasmid combination of pProT7-M(+), pProT7-L(+), pT7-IRES-vN, and pT7-IRES-vL and either of pProT7-S(+)-NSΔ6-30, NSΔ31-55, NSΔ56-80, NSΔ81-105, NSΔ106-130, NSΔ131-155, NSΔ156-180, NSΔ181-205, NSΔ206-230, NSΔ231-248, NSΔ249-265, NSs-E253-255A/D257-259A, NSs-E253-255A or NSsΔ257-259A. BHK/T7-9 cells were transfected with those plasmids as described in previous study (41).

NORTHERN BLOT ANALYSIS

Total cellular RNA was extracted from infected or mock-infected wt A549 using TRIzol® reagent. RNA samples were denatured and then separated using a 1.2% denaturing agarose-formaldehyde gels and which were then transferred onto positively charged nylon membranes (Roche Applied Sciences). Northern blot analysis was completed as described previously with strand specific RNA probes to detect anti-sense S-segment/N mRNA, human IFN-β or human ISG56 mRNA (125, 126).

WESTERN BLOT ANALYSIS

Mock-infected or infected cells (293 or VeroE6) were lysed using a 2xSDS sample buffer and boiled at 100°C for 10 minutes. Samples were then exposed to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The gel was then electro-blotted onto polyvinylidene difluoride membranes (immobilon; Millipore) and blocked for one hour using 0.5% bovine serum albumin with 1% Tween 20, 75mM NaCl, 10mM Tris-HCL; pH 7.6 at room temperature. Afterwards, membranes were then treated with primary antibodies; anti-RVFV mouse polyclonal antibody – a kind gift of R.B. Tesh at University of Texas Medical Branch, anti-Flag tag M2 monoclonal antibody (Sigma), anti-actin goat polyclonal antibody (I-19; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-PKR mouse monoclonal antibody (cat 610764; BD biosciences, BS transduction laboratories), anti-NSs rabbit polyclonal antibody (a kind gift from Dr. Makino at UTMB) and incubated overnight at 4°C. Membranes were then washed and incubated with secondary antibody for one hour at room temperature. The membranes were then developed with an enhanced chemiluminescence kit (Amersham Biosciences) according to the provided manufacturers instructions.

ANALYSIS OF VIRAL REPLICATION

A549 cells were infected with rMP12-NSs-Flag at a moi of 0.01, and then mock-transfected or immediately transfected with *in vitro* synthesized RNA encoding chloramphenicol acetyltransferase (CAT), NSΔ249-265 or NSs-E253-255A. At 72 hpi, culture supernatants were collected and plaque assay was performed.

PLAQUE ASSAY

Plaque assays were conducted using VeroE6 cells and after adsorbing samples at 37°C for 1 hour, inocula were removed and cells were overlaid with overlay medium which is a 1:1 mixture of 2x Modified Eagle Medium (MEM) containing 10% FBS, 10% tryptose phosphate broth (TPB), 100μg/ml penicillin and streptomycin, and 1.2% agar. Plates were incubated at 37°C for three days and then a second layer of the overlay medium containing 0.0013% Neutral Red were laid in each well and allowed to incubate an additional 16 hours. Virus titers (pfu/ml) were calculated based on plaque counts.

IN VITRO RNA SYNTHESIS

The pcDNA3.1mycHisA plasmids encoding CAT (49), NSΔ6-30, NSΔ31-55, NSΔ56-80, NSΔ81-105, NSΔ106-130, NSΔ131-155, NSΔ156-180, NSΔ181-205, NSΔ206-230, NSΔ231-248, NSΔ249-265, NSs-E253-255A/D257-259A, NSs-E253-255A or NSsD257-259A were linearized and transcribed *in vitro* using the mMESSAGE mMACHINE T7 Ultra kit (Ambion) following manufacturer's protocol. Linearized CAT DNA possesses a myc-His tag at the 3' end.

TRANSFECTION

Transfection of *in vitro* synthesized RNA was performed using TransIT-mRNA Transfection Kit (Mirus) according to manufacturer's protocol. RNA was transfected into cultured 293 cells or A549 cells by mixing linear RNA encoding intact NSs or each deletion mutant of NSs with the provided kit reagents (mRNA boost reagent and TransIT-mRNA Reagent). After a 3 minute incubation at room temperature the mixtures were then dropped into the indicated cell cultures containing DMEM medium with 10% FBS.

VIRUS INFECTION PROCEDURE

Both type I IFN competent (wt A549) and incompetent (VeroE6 or 293) cells were used for infections. After infection, cell lysate was harvested for either Western blot or Northern blot assays. For Northern blot assays, wt A549 cells were grown in 6 cm plates in DMEM + 10% FBS and 100 μg/ml of penicillin and 100 μg/ml of streptomycin. Media was removed and a high moi (3) of authentic MP-12, rMP12-C13type, rMP12-NSs-Flag, or NSs truncation mutants was added to each well and allowed to incubate at 37°C for one hour. Virus inocula were replaced with 5ml of DMEM 10% FBS and incubated for 7 hours. Total cellular RNA was extracted using TRIzol®. For Western blot, 293 or VeroE6 cells were grown in 12-well plates and then infected the same as above, but cell lysate was harvested at 16 hpi using 2x SDS sample buffer. Samples were boiled for 10 minutes and stored at -20°C until use.

NUCLEAR AND CYTOPLASMIC FRACTIONATION

VeroE6 cells were mock-infected or infected with MP-12 or rMP-12 virus encoding NSs serial deletions: NSΔ6-30, NSΔ31-55, NSΔ56-80, NSΔ81-105, NSΔ106-

130, NSΔ131-155, NSΔ156-180, NSΔ181-205, NSΔ206-230, NSΔ231-248 in 6-well plates. After 16 hours, cells were harvested and lysed with lysis buffer (PBS containing 1% TritonX-100) and allowed to incubate 5 min on ice. Samples were spun down at 10,000xg at 4°C for 10 minutes and supernatant (cytoplasmic fraction) was harvested and mixed with 1:1 ratio of 2xSDS sample buffer. Cell pellets (nuclear fraction) were washed once with cold PBS, and suspended into lysate buffer. Both cytoplasmic and nuclear lysates were mixed with 2xSDS sample buffer, and boiled for 10 minutes before subjected to SDS-PAGE.

CO-PRECIPITATION ASSAY

293 cells were infected with rMP12-NSs-SF, in which NSs is tagged with SF-tag (a tandem Strep-Tactin and Flag-tag) (45) (moi 3) and then transfected with *in vitro* synthesized capped RNA (encoding MP-12 NSs, truncated NSs or CAT). Proteins were labeled with [^{35}S] methionine/cysteine (MP Biomedicals) between 6 and 10 hpi, and cell lysates were prepared. Samples were mixed with Strep-Tactin beads (Qiagen) and SF-tagged NSs proteins were extracted from supernatant. NSs-SF and co-precipitated proteins were analyzed by SDS-PAGE and subsequent autoradiography.

STATISTICAL ANALYSIS

Unpaired Student's t-test was completed using Graphpad Prism 5.03 program (Graphpad Software Inc.) for the comparison of two groups.

3.4 RESULTS

A. GENERATION OF RECOMBINANT MP-12 ENCODING NSS WITH TRUNCATION

To determine if co-expression of truncated NSs expression would affect functional NSs protein in infected cells, 11 recombinant MP-12 viruses encoding NSs with 17 or 25 aa. deletions **(Figure 3.1)** were recovered using reverse genetics. The plaques of the NSΔ6-30 and NSΔ56-80 viruses were clearer and less turbid than other mutants showing turbid plaques **(Figure 3.2)**, suggesting that each truncated NSs has a unique effect on cytopathology.

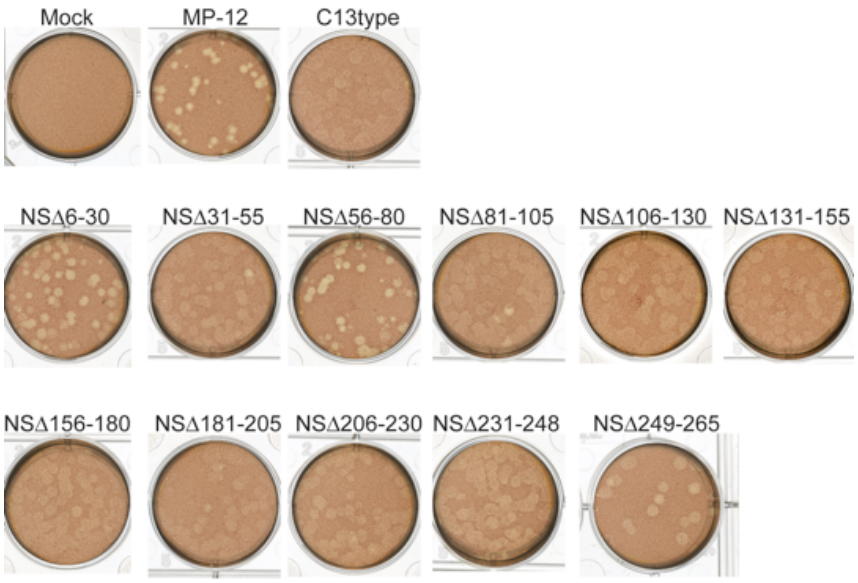


FIGURE 3.2: PLAQUE PHENOTYPES OF RECOMBINANT MP-12 ENCODING TRUNCATED NSS
Plaque assay was performed as explained in materials and methods section. Picture shows plaques at 78 hpi.

Next, the effects of NSs truncations on major NSs functions were evaluated. Vero E6 cells were either mock-infected or infected with MP-12, rMP12-C13type or an NSs

truncation mutants with an moi of 3. At 16 hpi, whole cell lysates were collected 16 hpi. Accumulation of PKR was analyzed by Western blot **(Figure 3.3 A)**. All of the truncated NSs had accumulated abundantly in infected cells except for NSΔ249-265 **(Figure 3.3 A)**. The C-terminus is recognized as a highly soluble and immunogenic region (123, 125), so it could be due to the lack of antibody sensitivity to detect the linear epitopes of NSs lacking the C-terminus. On the other hand, the polyclonal antibody used for detection might contain antibodies reacting to conformational epitopes on NSs protein. IFA was performed to detect the conformational epitope of NSΔ249-265. 293 cells were cultured and then transfected with *in vitro* synthesized RNA encoding NSΔ249-265. After incubation, cells were stained with anti-RVFV mouse polyclonal antibody. NSΔ249-265 could be detected abundantly within the cytoplasm and nucleus **(Figure 3.4)**. Therefore, all of the truncated NSs could be accumulated in the cells.

As expected, authentic MP-12 virus (functional NSs protein) promoted the degradation of PKR whereas cells infected with rMP12-C13type or any of those NSs truncation mutants did not decrease PKR levels **(Figure 3.3 A)**. The function to inhibit IFN-β mRNA synthesis was also tested. A549 cells were mock-infected or infected using an moi of 3 of MP-12, rMP12-C13type or NSs truncation mutants. At 7hpi, total RNA was extracted. Northern blot was then used for detection of mRNA for IFN-β or ISG56 and RVFV S RNA and N mRNA. IFN-β or ISG56 mRNA was not induced in cells infected with MP-12, whereas those infected with rMP12-C13type induced those mRNA abundantly **(Figure 3.3 B)**. NSs truncation mutant viruses induced IFN-β mRNA and ISG56 mRNA abundantly in infected cells **(Figure 3.3 B)**. Therefore, truncation of NSs abolished NSs ability to degrade PKR and suppress IFN-β mRNA synthesis.

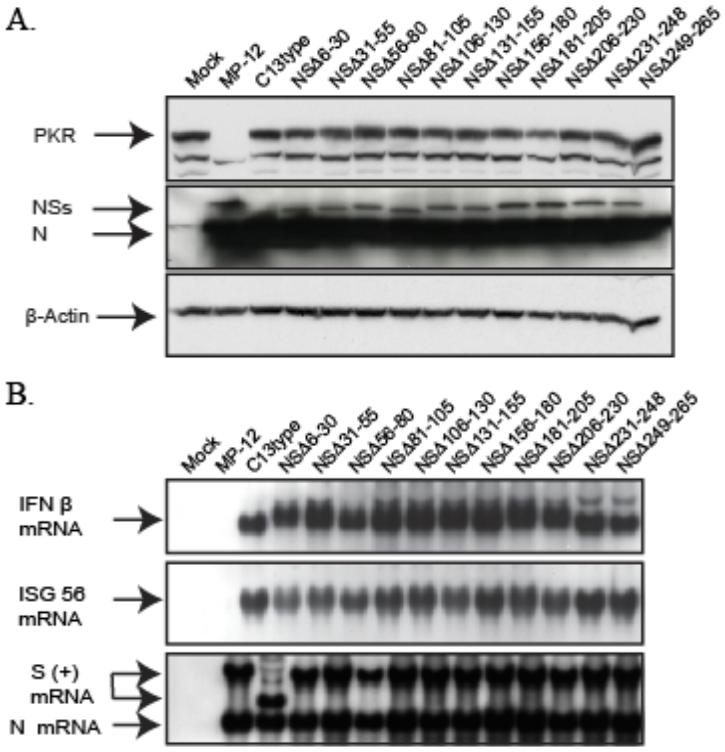


FIGURE 3.3: CHARACTERIZATION OF NSS FUNCTIONS ENCODING A PARTIAL IN-FRAME TRUNCATION

(A) Vero E6 cells were mock-infected or infected with the indicated viruses with an moi 3. Cell lysate was harvested 16hpi and then treated with antibodies specific to PKR (anti-PKR), RVFV NSs (anti-RVFV) or β-actin (anti-Actin) for Western blot. (B) For Northern blot analysis, wt A549 cells were infected or mock-infected with the viruses indicated using an moi of 3. Cellular RNA was collected 7 hpi and Northern blot was conducted to measure abundance of IFN-β, ISG56, or RVFV anti-viral sense S RNA/ N mRNA using a specific probe as mentioned in the materials and methods section of this chapter.

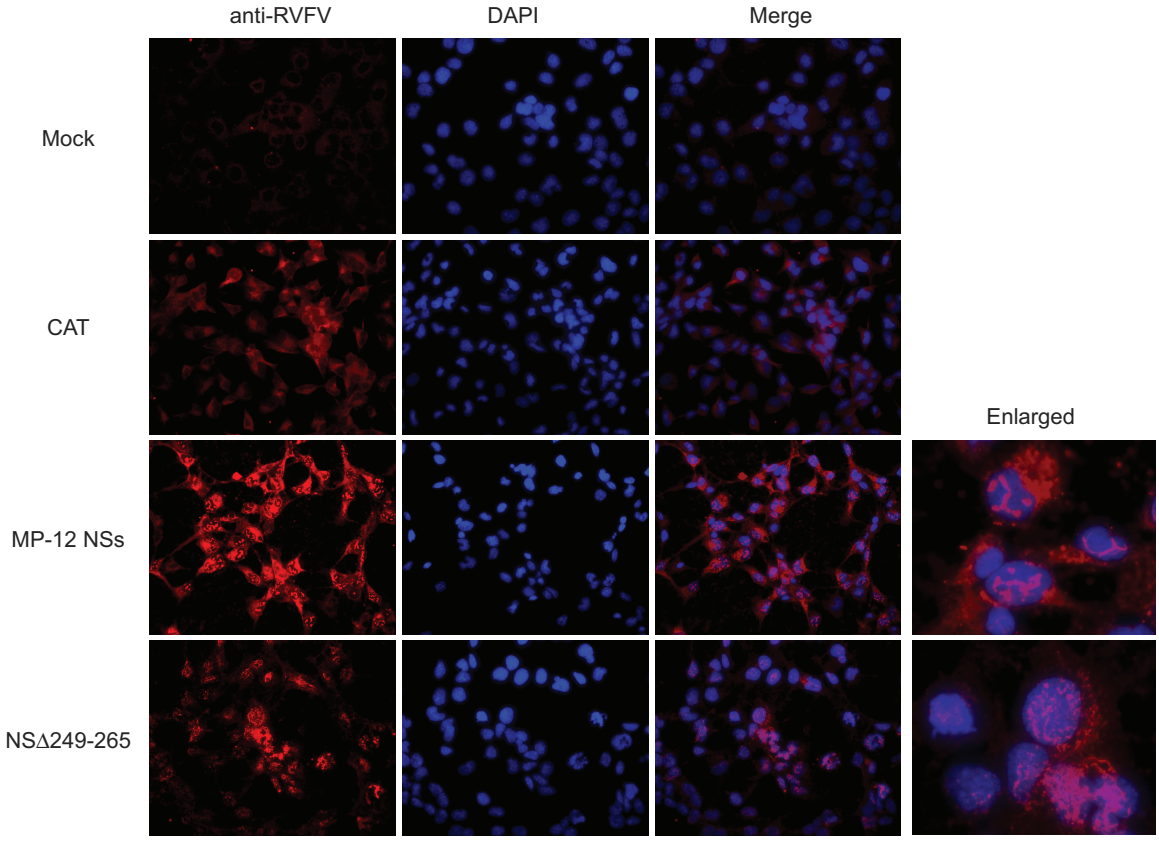


FIGURE 3.4: MP-12 AND NSSΔ249-265 NSS PROTEIN EXPRESSION
Using *in* vitro synthesized RNA encoding CAT (negative control), NSs of MP-12 or NSΔ249-265, 293 cells were mock-transfected or transfected. After 16 hours, cells were fixed and stained with anti-RVFV antibody and Alexa-Fluor 594 (goat anti-mouse IgG) before viewing under fluorescent microscope.

To test the cellular localization of truncated NSs within the cell, 293 cells were mock-infected or infected with MP-12 or rMP-12 virus encoding deletions within the NSs gene; NSΔ6-30, NSΔ31-55, NSΔ56-80, NSΔ81-105, NSΔ106-130, NSΔ131-155, NSΔ156-180, NSΔ181-205, NSΔ206-230 or NSΔ231-248. We did not use NSΔ249-265 since RVFV antibody does not detect this NSs protein by Western blot. As expected, MP-12 NSs (control) was distributed in both the nuclear and cytoplasmic fractions and N protein had accumulated dominantly within the cytoplasm. Samples infected with NSΔ6-

30, NSΔ31-55, and NSΔ56-80 had abundantly accumulated NSs protein in the nucleus while other mutants (NSΔ81-105, NSΔ106-130, NSΔ131-155, NSΔ156-180, NSΔ181-205 and NSΔ 231-248) had lower levels of NSs abundance within the nucleus **(Figure 3.5)**.

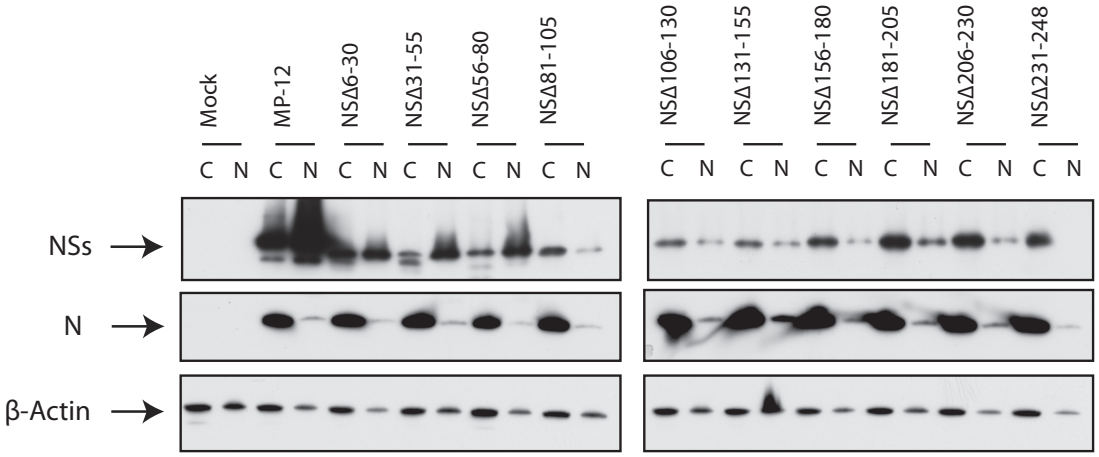


FIGURE 3.5: NUCLEAR LOCALIZATION OF TRUNCATED NSS
VeroE6 cells were mock-infected or infected with the specified virus. Nuclear (N) and cytoplasmic (C) fractions were removed and collected as described in materials and methods. NSs (anti-NSs antibody), N (anti-RVFV antibody) and β-actin (anti-actin antibody) abundance were then detected by Western blot.

B. EFFECTS OF CO-EXPRESSION OF TRUNCATED NSS WITH MP-12

To verify inhibition of MP-12 NSs function in cells co-expressing non-functional truncated NSs in infected cells, VeroE6 cells were co-infected with an moi of 3 of MP-12 and either rMP12-C13type or a truncated NSs mutants. At 16hpi, cells were collected and PKR and RVFV protein abundance was measured within infected cells using Western blot. PKR was not degraded by co-infection with rMP12-C13type or any of NSs truncation mutants **(Figure 3.6)**. In cells co-infected with NSΔ6-30 or NSΔ56-80, two

distinct bands were observed, one of which was MP-12 NSs and the other faster migrating band was the truncated NSs **(Figure 3.6)**. However, other truncation mutants did not allow an accumulation of MP-12 NSs in the presence of truncated NSs. The results indicate that the expression of MP-12 NSs gene was inhibited by presence of NSs mutant replication.

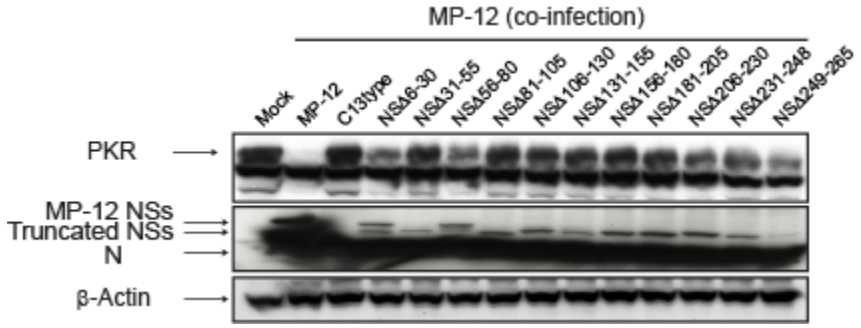


FIGURE 3.6: CO-INFECTION OF MP-12 WITH TRUNCATED NSS MUTANTS
VeroE6 cells were mock-infected or infected with MP-12 and one of the indicated viruses using an moi of 3. After 16 hours, cell lysate was harvested and Western blot was performed to measure abundance of PKR (anti-PKR antibody), NSs and N (anti-NSs antibody) and β-actin (anti-actin antibody).

The experiment was repeated using *in vitro* synthesized RNA encoding the truncated mutants in place of live MP-12 mutants. 293 cells were either infected with rMP12-NSs-Flag (encoding NSs tagged with Flag-tag at the C-terminus) at an moi of 3 or mock infected, and then were transfected with *in vitro* synthesized RNA encoding either CAT or truncated NSs, or mock-transfected. Similar to previous experiments, cells were harvested at 16 hpi and then PKR and Flag protein abundance was measured. All cells infected with rMP12-NSs-Flag could accumulate NSs-Flag **(Figure 3.7 A)**. Thus, no apparent interference of MP-12 NSs expression was observed at this time. Interestingly, PKR was still degraded by NSs derived from rMP12-NSs-Flag in the presence of NSΔ6-30, NSΔ31-55, NSΔ56-80, NSΔ81-105, NSΔ106-130, NSΔ131-155, NSΔ156-180,

NSΔ181-205, NSΔ206-230 or NSΔ231-248, while the abundance of PKR was slightly increased in the presence of NSΔ249-265 (lacking C-terminus) **(Figure 3.7 A)**.

The levels of IFN-β mRNA were measured to determine the effects of MP-12 NSs and truncation NSs mutant co-expression. None of the truncated NSs attenuated rMP12-NSs-Flag ability to suppress of IFN-β mRNA synthesis **(Figure 3.7 B)**. ISG56 mRNA expression was also tested. ISG56 is expressed under interferon-stimulated response element (ISRE) enhancer, which can be induced by type-I IFN, while it can be also induced by IRF3 activation independent of type-I IFNs (127). It was noted that cells co-expressing NSΔ249-265 slightly increased the abundance of ISG56 mRNA **(Figure 3.7 B)** whereas the other truncated mutants did not. Therefore, all truncated NSs retaining the C-terminus self-association domain; NSΔ6-30, NSΔ31-55, NSΔ56-80, NSΔ81-105, NSΔ106-130, NSΔ131-155, NSΔ156-180, NSΔ181-205, NSΔ206-230 and NSΔ231-248 did not inhibit PKR degradation, IFN-β or ISG56 gene suppression, while a truncated NSs specifically lacking the C-terminus domain, NSΔ249-265, slightly inhibited PKR degradation and ISG56 gene suppression, but not IFN-β gene suppression.

We then measured interaction of truncated NSs with MP-12 NSs with co-precipitation assay using rMP12-NSs-SF (recombinant MP-12 with a tandem Strep-tag and Flag tag) virus. Using an moi of 3, 293 cells were infected with rMP12-NSs-Flag virus. Immediately after infection, cells were mock-transfected or immediately transfected with *in vitro* synthesized RNA encoding MP-12 NSs (NSv), CAT, or truncation mutants; NSΔ6-30, NSΔ31-55, NSΔ56-80, NSΔ81-105, NSΔ106-130, NSΔ131-155, NSΔ156-180 and NSΔ181-205. Proteins were labeled with [^{35}S] methionine/cysteine between 6 and 10 hpi and subsequently precipitated with magnetic beads covalently bound to Strep-Tactin. If truncated NSs can bind to NSs-SF, those NSs could be co-precipitated with NSs-SF. However, none of the truncated NSs could be co-

precipitated with NSs-SF **(Figure 3.8)**. Input data had shown less amounts of truncated NSs than MP-12 NSs. In particular, NSΔ206-230 and NSΔ231-248 poorly accumulated in cells. We repeated the experiments using freshly prepared RNA, but the same result was obtained. Thus it is possible that those NSs expressions may have interfered with co-expression of rMP12-NSs-Flag **(Figure 3.8 B)**. Overall, none of the truncated mutant NSs had been found to interact with the NSs of rMP12-NSs-SF virus. The co-precipitation and fractionation results show that NSs truncation could result in mislocalization and reduction of NSs expression, which might lead to poor interaction of those NSs with MP-12 NSs in cells.

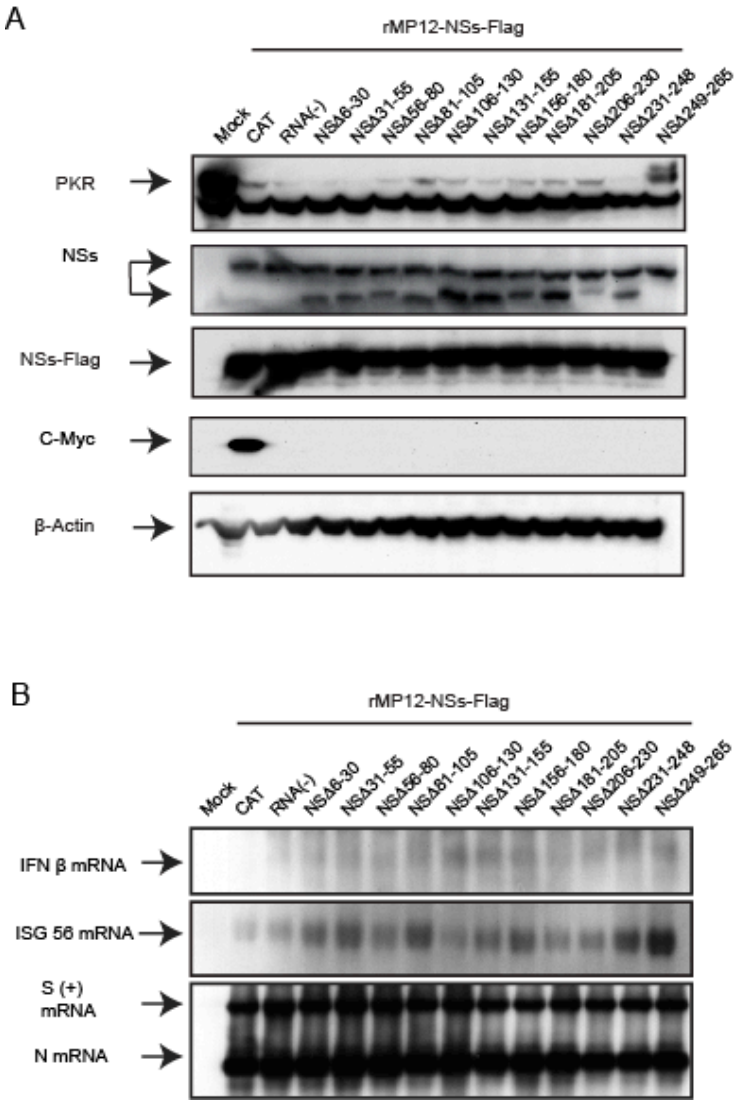


FIGURE 3.7: CO-EXPRESSION OF TRUNCATED NSS IN CELLS INFECTED WITH RVFV
(A) 293 cells were either mock-infected or infected with rMP12-NSs-Flag virus (moi of 3). Cells were then directly mock-transfected or transfected with *in vitro* transcribed RNA encoding CAT (transcription control) or NSs of indicated truncation. Cells were collected after 16 hpi and Western blot was performed to measure abundance of PKR (anti-PKR antibody), NSs-Flag (anti-Flag antibody), β-actin (anti-actin antibody), c-myc (anti-c-myc antibody) and RVFV-NSs (anti-RVFV antibody). (B) For Northern blot, A549 cells were mock-infected or infected with rMP12-NSs-Flag (moi of 3) and then directly mock-transfected or transfected with *in vitro* synthesized RNA encoding CAT or the indicated truncated NSs mutant. Cellular RNA was collected and abundance of IFN-β, ISG-56 and RVFV antiviral sense S RNA/N mRNA were detected using a specific probe as mentioned in materials and methods of this chapter.

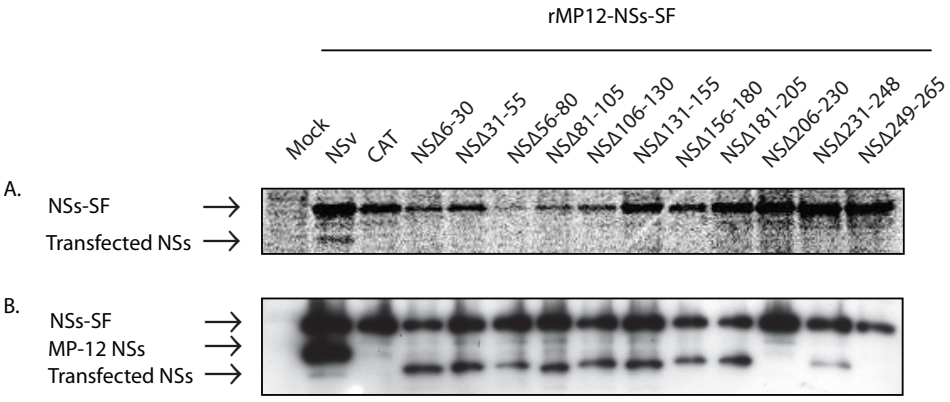


FIGURE 3.8: CO-PRECIPITATION OF TRUNCATED NSS AND MP-12 NSS.
(A) 293 cells were infected with rMP12-NSs-SF (recombinant rMP-12 tagged with StrepTactin/Flag tandem tag) (moi of 3) and were then mock-transfected or transfected with *in vitro* synthesized RNA encoding MP-12 NS (NSv), CAT or indicated truncated mutant. At 6 hpi, newly synthesized proteins were labeled with [^{35}S] methionine/cysteine. After 4 hours, SF-tagged proteins were then precipitated using Strep-Tactin magnetic beads. Samples were then run on a SDS-PAGE gel and bands were visualized with use of autoradiography. (B) Input control samples were collected prior to co-precipitation assay and abundance of NSs (anti-NSs antibody) was measured by Western blot.

C. EFFECTS OF CO-EXPRESSION OF NON-FUNCTIONAL NSS POSSESSING POINT MUTATIONS AT THE C-TERMINUS WITH FUNCTIONAL MP-12 NSS

Since NSΔ249-265 was able to slightly affect PKR degradation and ISG56 gene expression, we hypothesized that truncated NSs marginally compromises NSs function by an almost intact NSs gene except for the C-terminus domain. We intended to generate non-functional NSs, which retains most of the NSs sequence intact. Since sequences of acidic residues are a unique feature of the NSs C-terminus; i.e., aa. 253-355 (EEE) and aa. 257-259 (DDD), the region was replaced with alanine which resulting in NSs-D257-259A, NSs-E253-255A and NSs-E253-255A/D257-259A (mutations at both regions) **(Figure 3.9 A)**. Interestingly, substitution of alanine at aa. 257-259 destabilized the NSs proteins and accumulation of NSs was dramatically decreased **(Figure 3.9 B)**. The accumulation of NSs-D257-259A and NSs-E253-255A/D257-259A could not be detected by IFA, suggesting that the poor detection of NSs is due to the poor expression or

stability of NSs mutants. We confirmed that NSs-D257-249A and NSs-E253-255A/D257-259A do not promote PKR degradation and do not inhibit IFN-β mRNA synthesis, but this may be due to poor accumulation of NSs **(Figure 3.9 B&C)**. Unlike the others, NSs-E253-255A could accumulate in cells abundantly, form clear filamentous inclusions in the nucleus (data not shown) and was still able to degrade PKR and to suppress IFN-β mRNA synthesis. To see inhibitory effects of NSs-D257-259A and NSs-E253-255A/D257-259A, 293 cells were mock-infected or infected with rMP12-NSs-Flag (moi of 3) and then immediately transfected with *in vitro* synthesized RNA encoding CAT, rMP-12 NSs, NSs-D257-259A, NSs-E253-255A or NSs-E253-255A/D257-259A. As expected, NSs-D257-259A and NSs-E253-255A/D257-259A did not inhibit PKR degradation or IFN-β gene suppression while NSΔ249-265 slightly increased PKR abundance in rMP-12 NSs-Flag infected cells **(Figure 3.10 A&B)**.

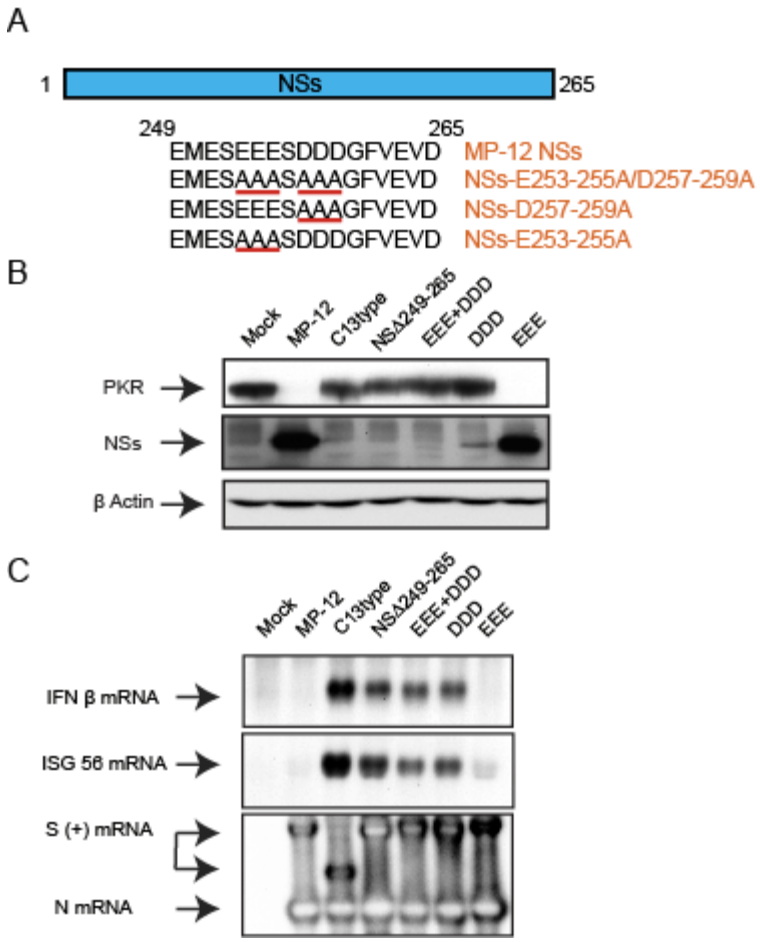


FIGURE 3.9 CHARACTERIZATION OF NSS ENCODING POINT MUTATIONS WITHIN THE C-TERMINUS

(A) Layout of the three NSs mutations NSs-E253-255A/D257-259A, NSs-D257-259A and NSs-E253-255A. Acidic residues were substituted using Alanine at aa.253-255 (EEE), aa.257-259 (DDD). Underlines mark the substituted amino acids. (B) VeroE6 cells were either mock-infected or infected with MP-12, rMP12-C13type or indicated NSs mutants with an moi of 3. Cell lysate was collected 16 hpi and Western blot was performed to measure abundance of PKR (anti-PKR antibody), NSs (anti-RVFV antibody) or β-actin (anti-actin antibody). (C) For Northern blot, A549 cells were mock-infected or infected with the indicated viruses (moi of 3). Cell lysate was collected at 7 hpi to measure abundance of IFN-β and ISG56 mRNA and RVFV antiviral-sense S RNA/N mRNA using the specific probes defined in materials and methods of this chapter.

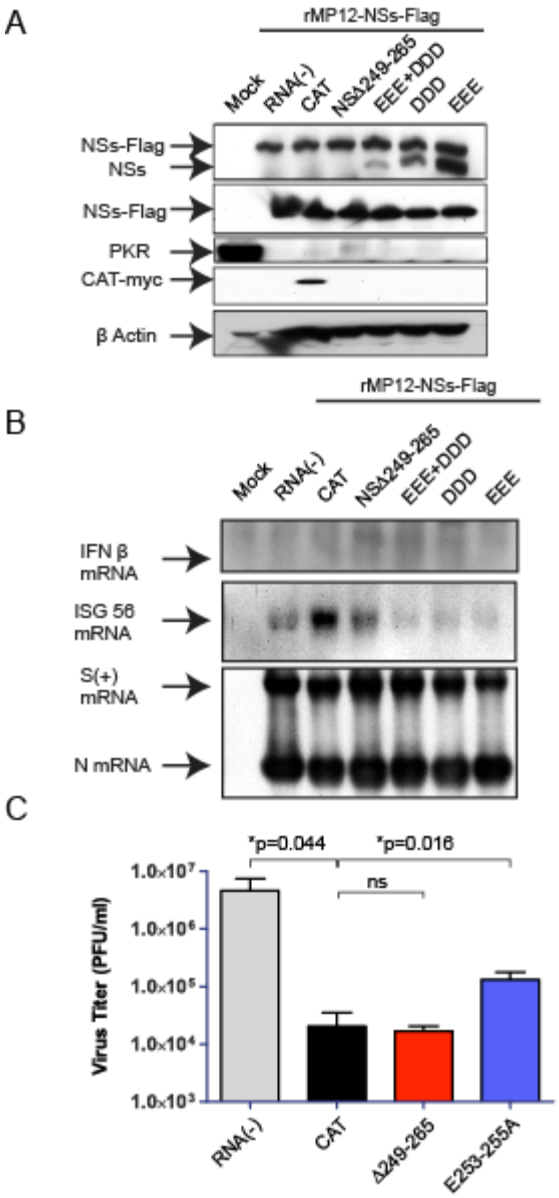


FIGURE 3.10: CO-EXPRESSION OF RMP12-NSS-FLAG WITH NSS POINT MUTATION MUTANTS

(A) Using rMP12-NSs-Flag with an moi of 3, 293 cells were mock-infected or infected then immediately mock-transfected or transfected with in *vitro* transcribed RNA encoding CAT or NSs of the indicated truncation mutant. At 16hpi, cell lysate was collected and Western blot was performed to measure abundance of PKR (anti-PKR antibody), NSs (anti-RVFV antibody) or β-actin (anti-actin antibody). (B) For Northern blot, A549 cells were mock-infected or infected with rMP12-NSs-Flag using a moi of 3 and then either mock-transfected or transfected with *in vitro* synthesized RNA encoding CAT or the indicated truncated NSs mutant. Cellular RNA were collected and abundance of IFN-β, mRNA, ISG56 mRNA, and RVFV antiviral-sense S RNA/N mRNA was

measured using specific probes as described in materials and methods of this chapter. (C) VeroE6 cells were infected with MP-12 virus using a moi of 0.01 and then mock-transfected or transfected with *in vitro* synthesized RNA encoding either CAT or the indicated NSs mutant. After 72 hours, cleared culture supernatant was harvested and virus titer was quantitated with plaque assay. P values for unpaired Student's t-test are shown (*:$p<0.05$, ns: not significant).

With the effect of NSΔ249-265 on IFN-β and PKR, it is important to understand if replication of MP-12 is inhibited by co-expression of this NSs mutant. A549 cells were infected with MP-12 virus (moi of 0.01) and then mock-transfected or transfected with *in vitro* synthesized RNA encoding CAT (transcription control), NSΔ249-265 or NSs-E253-255A. After 72 hours, samples were collected and plaque assay was used to quantify titers. Cells transfected with CAT RNA had significantly lowered MP-12 virus titer when compared to the mock-transfected control **(Figure 3.10 C)**. Cells transfected with NSΔ249-265 RNA did not further decrease viral replication but expression of NSs-E253-255A had significantly increased viral titer of MP-12 **(Figure 3.10 C)**. These results suggest that NSΔ249-265 does not inhibit MP-12 replication.

3.5 DISCUSSION

Dominant-negative effects of non-functional viral proteins have been observed in a number of different virus infections (128-132) and have shown promise as an anti-viral strategy. This study focuses on the ability to use a truncated non-functional RVFV NSs to induce an increase of IFN-β or PKR. RVFV NSs protein self-associates and forms filamentous inclusion bodies within the nucleus of infected cells (50, 51). We hypothesized that co-expression of truncated non-functional NSs with functional RVFV NSs will attenuate NSs functions with a dominant-negative effect through incorporation of the nonfunctional NSs into the functional NSs oligomers. Unexpectedly, co-

expression of nonfunctional NSs deficient in C-terminus self-association domain (NSΔ249-265) showed marginal inhibition of PKR degradation and increased levels of ISG56 protein in co-expressed cells. NSΔ249-265 was shown to be present in both the nucleus and cytoplasm of the infected cells. The results suggest that this truncated mutant could induce a competition with functional NSs for host factors, which are required for PKR degradation, resulting in moderate inhibition of PKR degradation.

Truncated mutants (NSΔ6-30, NSΔ31-55, NSΔ56-80, NSΔ81-105, NSΔ106-130, NSΔ131-155, NSΔ156-180, NSΔ181-205, NSΔ206-230, NSΔ231-248, and NSΔ249-265) in this study all demonstrated inability to suppress IFN-β mRNA synthesis and PKR degradation. Co-precipitation assay also showed that other than NSΔ6-30, NSΔ31-55 and NSΔ56-80, the truncated mutants accumulated mainly within the cytoplasm and did not localize in the nucleus. Collectively, these results suggest that deletions within the NSs protein may affect protein conformation resulting in mislocalization of protein within the cell and reducing interactions between functional NSs and truncated NSs, which lead to a lack of interference of truncated NSs with functional NSs in infected cells.

NSΔ249-265 (lacking the C-terminus) allows for marginal increases of PKR in the presence of RVFV NSs, inducing possible competition for any host factors that may be required for PKR degradation allowing for accumulation. The inhibitory mechanism of PKR degradation by NSΔ249-265 is unknown. We had hypothesized the intact polypeptide of aa. 1-248 can compete with authentic NSs for host factors, which may be required for initiation of PKR degradation. However, we could not reproduce similar inhibition with use of two other non-functional NSs (NSs-D257-259A or NSs-E253-255A/D257-259A) possessing a fully intact NSs gene, although those NSs share the same sequences except for the C-terminus 17 amino acids. Unexpectedly, poor accumulation

of NSs-D257-259A or NSs-E253-255A/D257-259A was observed in the experiment. Thus, it is likely that the C-terminus aa.257-259 contributes to the stability of NSs. We also found that over-expression of NSΔ249-265 did not inhibit MP-12 replication, suggesting that its dominant-negative effect could be very weak.

Despite truncated NSs protein expression being unable to inhibit MP-12 NSs function, co-infection of MP-12 and either rMP12-C13type or NSs truncation mutants had clearly restrained PKR degradation and accumulation of MP-12 NSs was clearly suppressed in cells co-infected with NSΔ31-55, NSΔ81-105, NSΔ106-130, NSΔ131-155, NSΔ156-180, NSΔ181-205, NSΔ 206-230, NSΔ231-248 and NSΔ249-265. Although the mechanism is unknown, it is possible that co-existence of two different S-segments may induce competition of RNA translation or transcription within the cell.

In conclusion, functions of NSs are highly susceptible to amino acid truncation, which abolishes NSs function of degrading PKR, inhibition of IFN-β or association with other NSs. Co-expression of these non-functional NSs with MP-12 NSs did not interrupt the functions of MP-12 NSs. Thus, future strategy to design antivirals should target the conformational structure of NSs.

Chapter 4: Future Directions

This study laid a foundation for future studies involving RVFV NSs protein. The two aims presented in this paper characterized the effect of RVFV NSs protein on DC maturation and activation and then looked into the effects of short truncations throughout the NSs gene on NSs function. Better understanding of host innate immune response against RVFV and NSs protein can provide new targets for use of antivirals and therapeutics for RVF.

DCs have a key role in the induction of innate and adaptive immune responses, important for vaccines to induce humoral immunity (103, 133). For aim 1, we demonstrated that although human DCs *in vitro* have low permissiveness to RVFV infection, these cells are capable of mounting a strong proinflammatory cytokine and chemokine response against infection. Cytokines stimulate cellular response against infection resulting in DC maturation and activation of CD4+, CD8+ T cells and B cells which induce cellular response and long-term immunity against the virus (92, 93, 134, 135). We found that human DCs infected with virus encoding a functional NSs (rMP-12) had resulted in decreased levels of DC maturation and activation when compared to those infected with virus lacking functional NSs (rMP12-C13type). Despite the low permissiveness to infection, DCs are capable of mounting a pro-inflammatory response which suggests that they have a role in the initial stages of RVFV infection. We suggest that NSs protein is important for RVFV to inhibit DC maturation, which will lead to attenuation of the subsequent cellular and humoral response induction against infection. Future studies should focus on the activity of DC *in vivo*, to better understand the role of NSs protein in evading immune response. Characterization of the effects of RVFV on

DC response is required to better understand host innate immune responses against RVFV to lead to better understanding of current vaccines and possible treatments for disease.

The second aim of this study focuses on partial in-frame truncations which affect NSs ability to localize within the nucleus of the cell and had abolished its ability to inhibit IFN-β expression and to degrade PKR. These results suggest that conformation of NSs is important for its function and localization within the cell. In addition, we found that co-expression of these truncated mutants with functional NSs do not have any effect of NSs function indicating that NSs dominant-negative activity does not inhibit NSs function. Eleven NSs mutants possessing short deletions had inhibited ability to suppress IFN-β or to promote PKR degradation. Interestingly, only Δ6-30, Δ31-55, Δ56-80 and Δ249-265 mutants were able to localize within the nucleus of the cell suggesting nuclear localization was also affected by the partial truncation of NSs. These four mutants encode a SAP30 binding domain. In a previous study, a mutant encoding a deletion of the SAP30 binding domain (aa. 210-230) was unable to fully interact with SAP30 co-repressor complex thus inhibiting the ability for NSs to suppress IFN-β (43). It was also indicated that despite this deletion, NSs lacking aa.210-230 was able to form stable NSs proteins within the nucleus suggesting that the protein was able to localize within the nucleus (43). Our study had a similar mutant lacking the SAP30 binding domain, NSsΔ206-230, but unlike the previous study, it had mainly localized within the cytoplasm of the infected cell. This suggests that in addition to binding to SAP30, NSs requires another structural requirement to inhibit IFN-β transcription. Overall, our results indicate that co-expression of truncated NSs protein with functional NSs results in no

significant attenuation of NSs ability to suppress IFN-β or degrade PKR. Future studies should be directed to the conformation of NSs as a target for potential therapeutics for RVF. The findings of this study suggest that modifying conformation could attenuate NSs affecting the virus' ability to replicate.

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RESEARCH ACTIVITIES:

Rift Valley fever virus is a devastating mosquito-borne virus native to parts of Africa and the Arabian Peninsula. RVFV causes high-rates of abortion in infected ruminants and is capable of causing disease in humans resulting in hemorrhagic fever, blindness or encephalitis. Currently there is no available vaccine and few methods of treatment for infection increasing chances of death among infected patients during an outbreak. Due to these threats and the lack of treatments or vaccines, the CDC, USDA and the NIAID have classified RVFV as a Category A select agent. My research focuses on innate immune response against RVFV and analyzing attenuated NSs protein (a well-known RVFV virulence factor) for use as an antiviral to inhibit unattenuated NSs function when co-cultured with virulent

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PUBLICATIONS:

1. **Head, J.A**., Kalveram, B. and Ikegami, T. (2012) Functional Analysis of Rift Valley Fever Virus NSs Encoding a Partial Truncation. ***PLoS ONE*** 7(9): e45730. doi:10.1371/journal.pone.0045730.

2. Kalveram B, Lihoradova O, Indran SV, Lokugamage N, **Head JA**, Ikegami T. **2012**. Rift Valley fever virus NSs inhibits host transcription independently of the degradation of dsRNA-dependent protein kinase PKR. ***Virology***. *In press*.

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4. **Head, J.A**., Hill, T.E., Garron, T., Tseng, M.A., Khahanchi, B., Chopra, A., Morrill, J., Ikegami, T., Peters, C.J., and Tseng. C.T. "Expression of Rift Valley Fever Virus (RVFV) NSs, But Not NSm, Profoundly Modulates Intrinsic Functions of Human Dendritic Cells Triggered by RVFV". (Manuscript in preparation).

ABSTRACTS

Head, J.A., T.E. Hill, S. Fukushi, M.A. Tseng, T. Ikegami, C.J. Peters and C.T. Tseng. "Mechanisms of Host Innate Immune Response to Rift Valley Fever Virus : Impact of NSs and NSm on the effector Functions of Dendritic Cells". Annual IHII/McLaughlin Colloquium (2011). University of Texas Medical Branch. Galveston, TX.

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