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Analysis of Simian Hemorragic Fever Virus Proteins and the Host Cell Responses of Disease Resistant and Susceptible Primates

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ABSTRACT

African monkey species are natural hosts of simian hemorrhagic fever virus (SHFV) and develop persistent, asymptomatic infections. SHFV was previously shown to also cause a rapid onset fatal hemorrhagic fever disease in macaques. Infection of macaques with a new isolate of SHFV from persistently infected baboon sera, that showed high nucleotide identity with the lab strain LVR, resulted in viremia, pro-inflammatory cytokine and tissue factor production, and symptoms of coagulation defects. Primary macrophages and myeloid dendritic cell cultures from disease-susceptible macaques efficiently replicated SHFV and produced pro-inflammatory cytokines, including IL-6 and TNF-α, as well as tissue factor. Cells from disease resistant baboons produced low virus yields and the immunomodulatory cytokine IL-10. IL-10 treatment of macaque cells decreased IL-6 levels but had no effect on TNF-α levels, tissue factor or virus production suggesting that IL-10 plays a role in modulating immunopathology in disease-resistant baboons but not in regulating the efficiency of virus replication.
SHFV is a member of the family Arteriviridae. The SHFV genome encodes 8 minor structural proteins. Other arteriviruses encode 4 minor structural proteins. Amino acid sequence comparisons suggest that the four additional SHFV minor structural proteins resulted from gene duplication. A full-length infectious clone of SHFV was constructed and produced virus with replication kinetics comparable to the parental virus. Mutant infectious clones, each with the start codon of one of the minor structural proteins substituted, were analyzed. All eight SHFV proteins were required for infectious virus production.

The SHFV nonstructural polyprotein is processed into the mature replicase proteins by several viral proteases including papain-like cysteine proteases (PLPs). Only one or two PLP domains are present in other arteriviruses but SHFV has three PLP domains. Analysis of in vitro proteolytic processing of C- and N-terminally tagged polyproteins indicated that the PLP in each of the three SHFV nsp1 proteins is active. However, the nsp1α protease is more similar to a cysteine protease than a PLP. Analysis of the subcellular localization of the three SHFV nsp1 proteins indicated they have divergent functions.

INDEX WORDS: Simian hemorrhagic fever virus (SHFV), Arterivirus replication, Pro-inflammatory cytokines, Interleukin-10 (IL-10), Infectious clone, Minor structural proteins
ANALYSIS OF SIMIAN HEMORRHAGIC FEVER VIRUS PROTEINS AND THE HOST
CELL RESPONSES OF DISEASE RESISTANT AND SUSCEPTIBLE PRIMATES

by

HEATHER ANNE VATTER

A Dissertation Submitted in Partial Fulfillment of the Requirements for the Degree of
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May 2013
DEDICATION

This dissertation is dedicated to my loving family. To my amazing son Aiden, everything I do is always for you and I hope you learn to dance down whatever path you chose to take in life. To my wonderful husband, Travis, you stood by my side throughout this long journey. You pushed me when I wanted to quit, hugged me when I wanted to cry and celebrated with each success. I hope our journey together continues to be filled with love and support. To my parents, Joyce and Kent Hardcastle, thank you for the love and support you gave me from the moment we met. You have taught me not only how to dance but also to understand the humor in life. I am the person I am today only because each of you loved me in your own unique way. Thank you for that love and support.

“When you get the choice to sit it out or dance, I hope you dance”

Leanne Womack, I Hope You Dance

After 408 tries, two Detroit scientists finally got the formula for their cleaner right

Truth Behind Formula 409
I would like to thank my principle advisor, Dr. Margo A. Brinton. I have valued and your advice and support throughout my time in this lab. You have pushed me to a better scientist than I first thought possible. I would like to thank my committee member, Dr. Richard Dix and Dr. Teryl Frey, your insight and suggestions over the years have been invaluable. Finally, I would like to thank all of the members of the Brinton lab, both past and present. I could not have completed this program without your friendship and support through the years. May the bonds we made here last through our lives not matter where we travel after.
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LIST OF ABBREVIATIONS

Simian hemorrhagic fever virus (SHFV)
Porcine reproductive and respiratory syndrome virus (PRRSV)
Equine arteritis virus (EAV)
Lactate dehydrogenase-elevating virus (LDV)
Subgenomic message RNA (sg mRNA)
Open reading frame (ORF)
Glycoprotein (GP)
Membrane (M)
Nucleocapsid (N)
Nuclear localization sequence (NLS)
Nucleolus localization sequence (NoLS)
Nuclear export signal (NES)
Double membrane vesicle (DMV)
Replication/transcription complex (RTC)
Papain-like protease (PLP)
Cysteine protease (CP)
3C-like serine protease (3CLSP)
Serine protease (SP)
RNA-dependent RNA polymerase (RdRp)
Nidovirus endoribonuclease (NednoU)
Transcription regulatory sequence (TRS)
Viral hemorrhagic fever (VHF)
Disseminated intravascular coagulopathy (DIC)
Ribosomal frame shift (RFS)
Endoplasmic reticulum (ER)
Macrophage (MΦ)
Dendritic cell (90)
Myeloid dendritic cell (mDC)
Porcine aveolar macrophage (PAM)
Peripheral blood monocyte (PBMC)
Real-time reverse transcription polymerase chain reaction (qRT-PCR)
Interferon (IFN)
Interleukin (IL)
Tumor necrosis factor alpha (TNF-α)
Ribosomal RNA (rRNA)
Double stranded RNA (dsRNA)
Suppressor of cytokine signaling (SOCS)
Multiplicity of infection (MOI)
SHFV infectious clone (SHFVic)
Immunoprecipitation (IP)
TNF-related apoptosis-inducing ligand (TRAIL)
Severe acute respiratory syndrome coronavirus (SARS-CoV)
Nuclear factor κB (NF-κB)
1 INTRODUCTION

1.1 Arteriviruses

The Family Arteriviridae currently includes four members, simian hemorrhagic fever virus (SHFV), porcine reproductive and respiratory syndrome virus (PRRSV), equine arteritis virus (EAV) and lactate dehydrogenase-elevating virus (LDV) (103). Arteriviruses belong to the Order Nidovirales that also includes the Families Coronaviridae and Roniviridae. In Latin “nido” means nested and this nomenclature refers to the 3’ nested set of subgenomic messenger RNAs (sg mRNAs) produced by all Nidoviruses. The Nidovirales families were grouped based on similarities in sg mRNA production, genome organization, replication strategy and several conserved enzymatic domains in the replicase genes (7, 89). Despite these similarities, members of the different virus families differ in virion morphology, protein composition and genome length with the arteriviruses containing the smallest genomes.

Arteriviruses have a limited host range. SHFV only infects some non-human primate monkey species, EAV only infects horses, PRRSV only infects pigs and LDV only infects mice. EAV and PRRSV infected animals develop either an asymptomatic, persistent infection or acute disease characterized by respiratory distress, fever and artery necrosis (60, 83). Both EAV and PRRSV infections can also cause reproductive defects including spontaneous late term abortion, stillborn offspring or weak foals and piglets (103). Interestingly, both EAV and PRRSV have been shown to persist in semen and these viruses can be sexually transmitted in addition to being spread via aerosols (6). LDV causes lifelong, asymptomatic infections in mice that are characterized by increased serum levels of lactate dehydrogenase. This feature is used as the endpoint for virus titration because there is no plaque assay for this virus (20). Some strains of LDV are able to induce a fatal age-related poliomyelitis in specific inbred murine strains (78). SHFV infection of several macaque species causes a rapid onset fatal hemorrhagic fever disease (115). In contrast, African monkey species develop either an asymptomatic infection or a non-fatal acute disease after SHFV infection (51).
1.2 Genome Organization and Expression

The arterivirus genome is a 12.7 to 15.7 kb positive-sense, single-stranded RNA with a type I 5´ cap and a 3´ polyA tail (103). The 5´ most two-thirds of the genome encode open reading frames (ORFs), ORF1a and ORF1ab, which are translated by host ribosomes into two polyproteins, pp1a and pp1ab, that are predicted to be subsequently processed by viral proteases into fourteen non-structural replicase proteins (142). The overlapping 3´ structural protein ORFs are expressed from a set of nested sg mRNAs. With the exception of SHFV, all currently identified arterivirus genomes contain seven structural ORFs that are translated into eight structural proteins; E, glycoprotein 2 (GP2), GP3, GP4, GP5, GP5a, a non-glycosylated membrane protein (M) and a nucleocapsid protein (N) (Fig. 1.1) (103). In addition to these ORFs, the SHFV genome also encodes four more ORFs which are thought to have arisen by duplication of the ORFs located immediately downstream of ORF1ab. These ORFs subsequently diverged through the accumulation of mutations. SHFV is therefore predicted to encode eight minor structural proteins (GP2a, GP2b, GP3, GP4, GP5, GP6, and the E1 and E2 translated from alternative AUGs in ORF2a and ORF4, respectively), and three major structural proteins (GP7, M and N) (Fig. 1.1) (46).

Arteriviruses, with the exception of SHFV, produce a set of six 3´ and 5´ co-terminal sg mRNAs), designated sg mRNA 2 through sg mRNA 7, through discontinuous transcription (103). These sg mRNAs are translated to produce the three major and four minor structural proteins.

1.3 Virion

The arterivirus virion is a spherical particle of approximately 45-55 nm in diameter that contains a host-derived lipid bilayer envelope (103). The nucleocapsid protein, N, forms an inner shell of 25-35 nm in diameter that contains the RNA genome. The outer surface of arterivirus particles are relatively smooth with only a few small protrusions that are thought to be heterodimers formed by disulfide-linked GP5 and M proteins (Fig. 1.2) (48). The three minor structural proteins also form heterotrimeric complexes of GP2, GP3 and GP4 as minor components on the virion surface (22, 36, 103).
1.4 Viral Proteins

1.4.1 Major structural proteins

The N protein, encoded by ORF9 for SHFV, has a predicted molecular mass of 15 kDa which is similar to that of the N proteins encoded by other arteriviruses (103). Based on the PRRSV data, N is expected to form non-covalently bound dimers through C-terminal domain interactions that multimerize and form an isometric capsid shell. The genomic RNA is thought to be encapsidated through interactions with the N-terminal half of the N protein (23). The N proteins of EAV and PRRSV have been shown to shuttle between the nucleolus and cytoplasm of infected cells due to a bipartite pat7 nuclear localization signal (NLS), a nucleolar localization signal (NoLS) and a nuclear export signal (NES) located in the N-terminal region of the protein (94, 121). A study of PRRSV NLS mutants in pigs indicated that blocking nuclear localization of the N protein resulted not only in decreased viral replication and viral persistence but also in increased production of neutralizing antibodies (91).

The 19 kDa M and 26 kDa GP5 (GP7 in SHFV) proteins form a disulfide-linked transmembrane heterodimer on the surface of arterivirus particles (Fig. 1.2) (103). The highly conserved M protein is predicted to have three transmembrane domains and a short ectodomain that results in low immunogenicity as shown for the PRRSV M protein despite its lack of glycosylation (36). The less conserved GP5 (GP7 of SHFV) is also predicted to have four transmembrane domains and a short ectodomain with several N-linked glycosylation sites. Studies on the role of the major structural protein heterodimer complex during PRRSV infection of porcine macrophages has demonstrated that the M protein binds to heparin sulfate on the cell surface during initial adherence and sialic acid on the GP5 (GP7) ectodomain is then responsible for a more specific interaction with sialoadhesin on the macrophage surface (29, 128). In addition to its role during attachment, the major heterodimeric complex is also thought to regulate virion structure by inducing membrane curvature during virion budding from the ER membrane of host cells (36).
1.4.2 Minor structural proteins

The minor glycoproteins, GP2, GP3, GP4, form covalently and non-covalently linked heterotrimeric complexes that create small periodic 10-15 nm protrusions on the surface of PRRSV virions (Fig. 1.2) (36, 134). Each of these proteins is an integral membrane protein that is highly glycosylated prior to incorporation into progeny virions (132). Previous data suggested that GP3 was not a structural protein for all PRRSV strains due to detection of this protein in culture fluid of cells infected with North American PRRSV strains but not European strains (36). However, recent data indicated that GP3 is indeed present in the virions of both European and North American PRRSV strains suggesting that all arterivirus virions contain heterotrimeric complexes (22). A previous study with an EAV infectious clone showed that the minor glycoproteins are required for the production of infectious progeny virus but are not critical for formation of non-infectious viral particles (133). The minor protein heterotrimeric complex was shown to regulate cell tropism due to the interaction of the GP2 and GP4 proteins with the CD163 receptor during viral entry (21, 118).

A fourth non-glycosylated minor structural protein, E, is encoded by either ORF2a (for PRRSV strains) or ORF2b (for EAV and LDV) and oligomerizes to form an ion channel in the virion membrane during virus entry (Fig. 1.2) (67, 135). The myristoylation motif (MGXXXS/T) of arterivirus E proteins was shown to be not required for virus entry, as predicted, but required for virus particle formation (50, 117).

Recently, a fifth minor structural protein was identified that is encoded from an ATG present in the +2 reading frame of ORF5 (ORF7 of SHFV) (42). It has been suggested that this protein has a role in viral replication because mutation of this ATG in an EAV infectious clone resulted in decreased viral titers and smaller sized plaques (42). However, the functions of this protein have not yet been determined.

The SHFV-LVR genome encodes 12 structural proteins including two E proteins produced from an ORF 3’ to the ORF2a AUG and from an ORF 5’ to ORF4 (67, 105). Alignment of the SHFV and PRRSV ORF sequences indicated that SHFV contained four additional 3’ ORFs and that the proteins produced by the SHFV ORFs 2a, 2b and 3 had the highest degree of homology to those produced by the
PRRSV ORFs 2, 3 and 4, respectively (46). However, the more 3’ E protein contains a myristoylation motif and based on this putative myristoylation motif, the E protein encoded by ORF4 would be more homologous with the E proteins of EAV and PRRSV (117).

1.4.3 Nonstructural Proteins

The two nonstructural polyproteins, pp1a and pp1ab, encoded by all known arterivirus genomes are proteolytically processed by viral proteases into eleven to fourteen mature nonstructural proteins (nsp). The number of nsp1 proteins produced by the different arterivirus genomes varies. EAV produces only 1 (nsp1α), PRRSV and LDV produce two (nsp1α and nsp1β) and SHFV produces three (nsp1α, nsp1β and nsp1γ) (142). Some of these nsps assemble on double membrane vesicles (DMVs) to form the replication/transcription complex (RTC) that transcribes either full-length or sg RNA minus strand RNA from the genome. The sg RNAs are generated by a discontinuous transcription mechanism (103). The structure and function(s) of several, but not all, arterivirus nsps have been investigated using EAV and PRRSV infectious clones and proteins (41, 109, 142).

The nsp1 proteins have papain-like protease (PLP) activity and cleave at a Gly/Gly dyad. The N-terminus of the N-terminal nsp1 protein contains a zinc finger motif that is required for sg RNA transcription but not for genome replication (122). The processing and functions of the three SHFV nsp1 proteins were analyzed as a part of the current study and are discussed in detail in Chapter 5.

The nsp2 protein contains a cysteine protease (CP) (107), that cleaves at a Gly/Gly dyad located at the nsp2/nsp3 junction in PRRSV strains (53). Nsp2 has recently been shown to function as a deubiquitinating enzyme and this activity may explain how nsp2 is able to suppress IFNβ expression and signaling (41). Analysis of hydrophobicity plots of the PRRSV nsps suggested that nsp2, nsp3 and nsp5 have extensive membrane spanning domains and in vitro expression of nsp2 and nsp3 alone was sufficient to produce DMVs suggesting that these two viral proteins are responsible for forming the perinuclear DMVs and the remaining RTC proteins anchor onto either nsp2 or nsp3 (36, 41).
The main protease is a 3C-like serine protease (3CLSP) that is located in nsp4. The catalytic triad of this protease is His-Asp-Ser and it cleaves at Glu residues immediately followed by either a Gly, Ser or Ala residue that are located at the junctions of all downstream nsps (41). Interestingly, despite its critical role in processing the polyprotein, nsp4 is not required in viral RTCs for efficient replicase activity (131). Currently, nsp5, nsp6, nsp7, nsp8 and nsp12 have no known functions in viral replication or modulation of host responses besides their association with viral RTCs either as mature monomers or as intermediates formed during processing of the polyprotein (41, 131).

The viral RNA dependent RNA polymerase (RdRp) is located at the C-terminus of nsp9 and is responsible for de novo (primer-independent) initiation of viral full-length and sg RNA transcription (7). The RdRp of all nidoviruses has a unique catalytic core of Ser/Asp/Asp compared to the Gly/Asp/Asp catalytic core of all other viral RdRps indicating a unique functional evolutionary relationship between arteriviruses and coronaviruses (31). Additionally, all nidoviruses encode a functional helicase that is located in nsp10 in arteriviruses (101). Nsp10 also contains a zinc binding domain and an ATPase domain. In vitro studies of nsp10 function indicate that the zinc binding domain is required for binding to the viral RNA and the ATPase domain is required for the energy dependent 5´-to-3´ unwinding of the viral RNA by the helicase (41, 101). Nsp11 contains an endoribonuclease (NendoU) that has only been observed in nidoviruses and was shown to cleave 3´ of unpaired pyrimidines (126). While nsp11 is not required for viral replication, mutation of this enzyme resulted in significantly decreased viral replication and low levels of sg mRNA (41). These data along with the observation that NendoU is able to efficiently cleave host ssRNA suggesting that the role of this protein may be to modulate the host innate immune response by cleaving cellular RNA substrates (41).

1.5 Arterivirus Replication Cycle

All known arteriviruses are able to productively infect macrophages and dendritic cells. With the exception of LDV, they can also infect MA104 cells and the MA104-derived Marc145 cells (Fig. 1.4) (103). The ectodomain of M within the GP5 (GP7 in SHFV) heterodimers of arterivirions binds to hepa-
rin sulfate on the cell surface (25, 29). Then a second, more specific interaction between α2-3 or α2-6-linked sialic acids on GP5 (GP7 in SHFV) and the N-terminal immunoglobulin domain of sialoadhesin occurs. The minor structural protein complex then interacts with the CD163 receptor leading to virus internalization by clathrin-dependent endocytosis (14, 25-28, 86). Fusion of the viral membrane with the endosomal membrane is expected to occur only after acidification of the endosome and formation of an ion-channel by E protein oligomers (67, 82). After release of the capsid into the cytoplasm and uncoating, the 5’ two-thirds of the genome is translated into two polyproteins, pp1a or pp1ab. The pp1ab is produced when a -1 ribosomal frameshift occurs at a ‘slippery’ heptanucleotide sequence during approximately 15 to 20% of the translation events (31). Both pp1a and pp1ab are autocatalytically processed by the viral proteases (7, 126). Viral nsps induce DMV formation via the autophagy pathway and assemble on these DMVs to form the viral RTCs that synthesis viral RNAs (Fig. 1.3) (102, 103, 113, 131). Translation of the predominantly monocistronic sg mRNAs produces the major and minor structural proteins (23). Virion assembly begins with localization of the N protein with the replicase complexes where it oligomerizes to form a capsid shell around a nascent strand of genomic RNA. Capsids then acquire envelopes containing major protein heterodimers, minor protein heterotrimers and E protein oligomers by budding through cytoplasmic membranes. Mature virions are then transported to the plasma membrane in vesicles. The vesicle membrane fuses with the plasma membrane releasing progeny virions into the extracellular space (103).

1.6 Discontinuous Transcription

Each of the arterivirus sg mRNAs has the same a 5´ leader sequence which contains a transcription regulating sequence (TRS) located at the top of a hairpin loop. The currently accepted model of synthesis of subgenomic minus strand RNA by discontinuous transcription is shown in Fig. 1.4. According to this model, the viral replication complex transcribes the positive-sense genomic RNA into a minus-sense RNA. Transcription can either proceed to the end of the template or stop at a TRS located at the 5´ end of one of the 3´ structural ORFs. The RdRp and the nascent minus strand are then translocated to the 5´ end
of the template where the minus sense TRS of the transcript hybridizes with the complementary 5’ leader TRS and the RdRp extends the nascent strand by copying sequence from the 5’ end of the genome template. The nascent minus-sense sg RNA is then transcribed into positive sense sg mRNA by viral RTCs and the sg mRNAs are subsequently translated into viral structural proteins by host ribosomes (89).

The majority of the polycistronic sg mRNAs are functionally monocistronic because only the 5’ most ORF is translated. Exceptions are sg mRNA2 of PRRSV, EAV and LDV that produces both GP2 as well as the E protein due to ribosomal choice of an alternative AUG located either upstream or downstream of the AUG for GP2 (36). Recently, ORF5 of EAV (ORF7 in SHFV) was shown to be polycistronic. It encodes the major structural protein GP5 and also a shorter C-terminal GP5a protein that was shown to be required for efficient viral replication (42).

Despite the relatively high conservation of the sequences of each 3’ TRS, evidence suggests that different 3’ minus-sense anti-body TRSs have different affinities for the leader positive-sense TRS. Also, the frequency of RdRp stopping at different 3’ TRSs varies (7, 89). These differences regulate the abundance of the various sg mRNAs. The sg mRNA encoding the nucleocapsid was shown to have the highest affinity for the leader TRS and is the most efficiently transcribed minus sg RNA and most abundant sg mRNA (89). The regulated use of discontinuous transcription by all nidoviruses not only allows the addition of a 5’ translation enhancer sequence (leader sequence) to each sg mRNA but also increases the chance of alternative copy-choice recombination that can lead to the acquisition of new genes or the duplication of viral genes (89).

1.7 Viral Hemorrhagic Fever Disease

Viral hemorrhagic fever (VHF) disease in humans is caused by several single-stranded RNA viruses, including the filoviruses Ebola and Marburg viruses (10). While VHF in humans can result in a wide range of symptoms, disease is typically characterized by a rapid onset of fever, headache and malaise with some level of hemorrhagic manifestation such as rash, epitaxis and petechia (44, 79). Laboratory findings indicate that human VHF can also be associated with disseminated intravascular coagulopathy.
DIC, lymphocytopenia, and proteinuria (44). Hemorrhagic fever viruses have been shown to primarily infect macrophages and dendritic cells resulting in production of high viral titers and pro-inflammatory cytokines as well suppression of the adaptive immune response (10). The host inflammatory response is thought to be responsible for the observed pathogenesis and the induction of fatal shock in humans since the lesions observed are not severe enough to induce terminal shock (10, 44, 77). Similarly, the host responses to hemorrhagic fever virus in macaques include production of pro-inflammatory cytokines that result in bystander apoptosis as well as increased TF expression which leads to increased vascular permeability and coagulation defects (10, 11, 43, 96). The observation that some humans without a clinical history of hemorrhagic disease were found to be positive for Ebola specific antibodies suggested that human responses to this virus can differ and result in either a symptomatic or asymptomatic infection (70, 71). Early and appropriate immune responses leading to activation of adaptive immunity were shown to be associated with a favorable disease outcome.

SHFV was first isolated in 1964 as the causative agent of outbreaks of a fatal hemorrhagic fever in captive macaque colonies (115). Symptoms of SHFV infection in macaques included rapid onset fever, facial edema, anorexia, dehydration, proteinuria, petechia, epitaxis, retrobulbar hemorrhages and death occurring by three weeks after infection (74, 87). Several macaque monkey species, including rhesus (Macaca mulatta), cynomolgus (Macaca fascicularis) and stump-tailed (Macaca arctoides) macaques, were shown to be susceptible to SHFV infection (87). In contrast, African monkey species, including patas (Erythrocebus patas), African green monkeys (Cercopithecus aethiops) and baboons (Papio anubis and Papio cynocephalus), develop either an asymptomatic, persistent infection or a mild, non-fatal, acute disease after SHFV infection (51). Host factors, as well as viral factors, may be involved in determining the outcome of an SHFV infection. However, the mechanisms that contribute to determining whether an SHFV infection will be asymptomatic or symptomatic are not well understood.
1.8 Goals of this Dissertation

1.8.1 Specific Aim 1: To compare the responses of cells from disease-resistant and disease-susceptible monkeys to SHFV infection.

African monkeys, such as patas monkeys, baboons and African green monkeys are the natural hosts of SHFV. Previous outbreaks of SHFV in macaques were caused by accidental transmission of the virus from a persistently infected patas monkey to susceptible macaques through the use of multi-dose syringes (74, 87). For other types of viral hemorrhagic fever viruses as well as other arteriviruses, the timing and spectrum of cytokine response elicited in infected macrophages and dendritic cells has been linked to disease severity (11, 77, 83). Host responses to SHFV in disease-resistant and susceptible primates had not been investigated. Studies were performed to compare viral replication and the host responses in disease-resistant and susceptible monkeys to SHFV infection to gain insights about the mechanisms contributing to the differential disease outcomes in animals.

1.8.2 Specific Aim 2: To functionally analyze SHFV-specific structural and non-structural proteins.

One method of investigating the roles of individual proteins encoded by an RNA virus is to construct a full-length cDNA clone of the viral genome and use it to substitute or delete nucleotides to modify the expression of a particular viral protein and analyzing the effects on an infection initiated by transfection of an in vitro transcribed mutant infectious genome RNA. Previous studies of arterivirus replication and pathogenesis have focused on EAV and PRRSV due both to the agricultural economic impact of these viruses and the availability of infectious clones for several EAV and PRRSV strains (39, 40, 81, 85, 125, 136). Infectious clones had not been made for LDV or SHFV. A strategy similar to that used to construct several coronavirus infectious clones (137-139) was used to construct the first full-length infectious cDNA clone of SHFV. This clone was used to analyze the functional relevance of the duplicated SHFV 3′
ORFs. In addition, experiments were performed to analyze the functions of the duplicated SHFV nsp1 proteins.
Figure 1.0.1 Arterivirus genomic gene organization. The ORFs of the SHFV and EAV genomes are shown as boxes. ORF1a and ORF1ab are translated from the genomic RNA. Pp1ab is translated from the genome only after a -1 ribosomal frame shift (RFS) occurs. The structural protein ORFs are translated from a nested set of 3′ and 5′ coterminial sg mRNAs. EAV, LDV and PRRSV genomes encode three major (38) and four minor (purple) structural protein ORFs. The SHFV genome encodes four additional minor structural protein ORFs (blue).
Figure 1.0.2 Virion structure of arteriviruses. (A). The predicted membrane topology of the two major structural proteins, M and GP5, and four minor structural proteins, GP2, GP3, GP4 and E, of the type I PRRSV. Rectangles indicate transmembrane domains, dotted rectangles indicate signal peptides. Hexagons indicate predicted glycosylation sites on the ectodomain of each protein. The disulfide linkage between M and GP5 that forms the heterodimer are indicated. This figure was reproduced from Dokland 2010. (B) Virion of a prototypic arterivirus (EAV) showing GP5 and M heterodimers and GP2, GP3 and GP4 heterotrimers and the associated E ion-channel on the outer surface of the lipid bilayer membrane. The nucleocapsid forms a shell enclosing the viral RNA genome. Adapted from Gorbalenya et al (2006).
**Figure 1.0.3 Arterivirus Replication Cycle.** The virus attaches to cell surface receptors and enters the host cell by endocytosis in a clathrin dependent manner. After uncoating, the viral RNA is translated to produce pp1a and pp1ab which are proteolytically processed into the nonstructural proteins that form viral replication complexes on virus-induced DMVs. Replicase complexes synthesize full-length and sg minus-strand RNA, positive-strand genomic RNA and sg mRNAs. The sg mRNAs are translated to produce envelope associated structural proteins that assemble on the endoplasmic reticulum (ER) and nucleocapsid that encapsidates the genomic RNA. Viral capsids are enveloped by budding through the ER membrane proteins and trafficking through the Golgi complex before release from the host cell. Figure reproduced from Snijder and Spaan (2006).
Figure 1.0.4 Model for the generation of Nidovirus sg RNAs by discontinuous transcription. Replication refers to synthesis of the positive-sense genome from the anti-genome and transcription refers to the synthesis of minus-sense sg RNAs from the positive-sense genome. The RdRp can copy a full-length minus strand or stop at one of the 3’ TRS. The anti-TRS (anti-body TRS) located at the 3’ end of each nascent minus-sense sg RNA transcript then pairs with the complementary leader TRS at the 5’ end of the positive-sense genomic RNA. The nascent minus-sense sg RNA is then extended from the leader sequence. The minus-sense sg RNAs are subsequently transcribed into sg mRNAs that are translated by host ribosomes. Figure modified from Pasternak, et al (2006).
ISOLATION AND CHARACTERIZATION OF A NEW SIMIAN HEMORRHAGIC FEVER VIRUS ISOLATE FROM PRESISTENTLY INFECTED BABOONS

2.1 Introduction

SHFV was isolated in 1964 and shown to be the causative agent of fatal hemorrhagic fever outbreaks in macaque colonies in the United States, Russia and Europe characterized by mortality approaching 100% by 3 weeks after infection (52, 115). Initial symptoms in macaques include fever and facial edema followed by depression, anorexia and dehydration and then by signs of coagulation defects such as skin petechia, epitaxis, melena, retrobulbar hemorrhages and subcutaneous hematomas (2, 87). Necropsy revealed splenomegaly due to follicular hemorrhage and necrosis of the liver, adrenal glands and lymphatic tissue (2). The symptoms of SHFV infections in macaques closely resemble those of other hemorrhagic fever viruses, such as Ebola viruses, Marburg and Lassa virus (10, 76). The pathology of viral hemorrhagic fevers has been associated with the host innate immune responses to viral infection because infected macrophages (MΦs) and dendritic cells (DCs) release pro-inflammatory cytokines that induce tissue factor and subsequent disseminated intravascular coagulopathy (10, 43, 72). In previous primate facility SHFV outbreaks, the virus was transmitted to disease-susceptible macaques by accidental exposure to blood from African monkeys, such as patas monkeys (Erythrocebus patas), that had asymptomatic, persistent infections (74). It was previously estimated that 1-10% of wild-caught African monkeys, including patas, baboons (Papio papio) and African green monkeys (Cercopithecus aethiops), are persistently infected with SHFV (74).

SHFV is a member of the Family Arteriviridae, which also includes EAV, PRRSV and LDV. Arteriviruses have highly restricted host ranges and cell tropism. Only MΦs and DCs are infected in horses and donkeys by EAV, in pigs by PRRSV, in mice by LDV and in African and Asian monkeys by SHFV (103). Infections with both EAV and PRRSV cause disease characterized by fever, anorexia, tissue necrosis, inflammation of the respiratory tract and reproductive failure such as spontaneous abortions or delivery of weak offspring (104). However, LDV typically causes lifelong, asymptomatic, persistent infections
that are identified by increased serum levels of lactate dehydrogenase (13, 104). Due to the significant agricultural impact of EAV and PRRSV induced diseases, the majority of arterivirus research has focused on these two viruses.

Peripheral blood monocytes (PBMCs) were needed for studies comparing responses to SHFV infection in disease resistant and disease susceptible primate MΦ and DC cultures and an SHFV-specific RT-PCR assay was developed to identify possible persistently infected baboons. To validate this assay, a survey of archived serum samples from wild-caught baboons was performed and indicated that 10% were SHFV positive. Random sampling of baboons currently in the primate colony suggested that approximately 20% of the captive-born baboons were SHFV positive. Virus was isolated from the sera of several persistently infected baboons. Sequences obtained for two of the isolates differed by less than 0.2% from each other and from the LVR consensus sequence. Infection of disease-susceptible Japanese macaques with one of the isolates resulted in coagulation defects, production of tissue factor and pro-inflammatory cytokines, and high levels of virus replication indicating that the new SHFV isolates induce persistent asymptomatic infections characterized by a low level viremia in disease-resistant baboons and fatal hemorrhagic disease in disease-susceptible macaques.

2.2 Materials and Methods

2.2.1 Cells

MA104 cells, obtained from O. Nainin, Centers for Disease Control and Prevention, were grown in minimum essential medium (MEM) supplemented with 10% FBS, 1% L-glutamine and 1% gentamicin at 37°C in 5% CO₂. PBMCs were isolated from whole blood collected in acid citrate dextrose (ACD) BD Vacutainer® blood collection tubes (BD Bioscience) from rhesus macaques (Yerkes Regional Primate Research Center, Atlanta, GA) by Ficoll® 400 (Mediatech Inc., Manassas VA) density gradient centrifugation according to standard protocols. Monocytes were seeded at 10⁶ cells/well in 24-well plates and allowed to adhere for 2 h before gentle washing with Hanks Buffered Saline Solution (HBSS, Gibco). Immature mDCs were cultured from adherent cells by incubation with Roswell Park Memorial Institute
(RPMI) 1640 culture media (Gibco) supplemented with 10% autologous serum or 10% heat-inactivated fetal bovine serum (FBS), 50 U/ml of penicillin, 50 µg/ml of streptomycin, human recombinant granulocyte-MΦ colony stimulating factor (R&D Systems; 1000 U/ml) and recombinant human interleukin 4 (R&D Systems; 500 U/ml) for 11 days at 37°C in 5% CO₂. MΦs were cultured from adherent cells by incubation with RPMI-1640 culture media supplemented with 10% autologous serum or 10% heat-inactivated FBS, 50 U/ml of penicillin, 50 µg/ml of streptomycin, human recombinant MΦ colony stimulating factor (R&D Systems; 5000 U/ml) for 11 days at 37°C in 5% CO₂. Two-thirds of the culture media was replaced with fresh growth media every three days to replenish growth factors. Cell identity was confirmed and cells were counted using a FACSCanto flow cytometer and analyzed using FACSDiva software (BD Bioscience) after cells were stained with fluorescently-labeled antibodies directed against surface markers for HLA-DR, DC-SIGN, CD11c and CD83 for mDCs or CD91 and CD163 for MΦs (BD Bioscience). Greater than 90% of cells in mDC cultures were HLA-DR⁺, DC-SIGN⁺, CD11c⁺, and CD83⁻ as analyzed by flow cytometry. Greater than 95% of cells in MΦ cultures were CD91⁺ and CD163⁺ as analyzed by flow cytometry.

2.2.2 Virus

An aliquot of SHFV, strain LVR 42-0/M6941, (American Type Culture Collection) was sequentially plaque-purified three times and then amplified once on MA104 cell monolayers. Stock pools of SHFV-LVR were prepared by infecting confluent MA-104 monolayers at an MOI of 0.2. Culture media was harvested at 32 h after infection. Clarified virus pools containing titers of ~10⁷ PFU/ml was aliquoted and stored at -80°C.

Baboon-derived SHFV isolates were amplified by a single passage on primary MΦ and mDCs cultures. Cells were incubated with 200 µl of baboon serum at 37°C in 5% CO₂ for 1 h. After virus adsorption, cells were washed three times with HBSS before incubation with cell-specific culture fluid at 37°C in 5% CO₂.
SHFV infectivity was quantified by plaque assay on confluent monolayers of MA104 cells in six-well plates. After adsorption for 1 h at room temperature, the virus inoculum was removed and the wells were overlaid with 1% SeaKem ME agarose (Bio-Whittaker Molecular Applications) mixed 1:1 with 2x MEM containing 5% FCS, and incubated at 37°C for 72 h. After removal of the agarose, cells were stained with 0.05% crystal violet in 70% ethanol.

2.2.3 SHFV one-step RT-PCR assay

Whole blood samples from baboons were collected in ACD tubes at Texas Biomedical Research Institute and shipped at room temperature overnight to Georgia State University. ACD tubes were centrifuged at 2,000 rpm for 5 min, plasma was removed and stored at -80°C. Whole blood was also collected from baboons in clot tubes (BD) and serum was collected from these tubes and stored at -80°C. Archived serum samples collected from whole blood were aliquoted and stored in Tygon tubing at -80°C at Texas Biomedical Research Institute and shipped on dry ice overnight to Georgia State University. RNA was isolated from 200 µl serum collected from clot tubes (BD Bioscience) by TRI Reagent LS (Molecular Research Center, Inc.) according to manufacturer’s protocol. Isolated RNA was quantified by spectrophotometry and 100 ng was used as template for RT-PCR. Pairs of primers from either the nucleocapsid (forward primer, 5’-ggcaaaccaaaaccaataaaggg-3’ and reverse primer, 5’-ataatcagtctgctgctgggg-3’) and the helicase (forward primer, 5’-ctctgtataacattttgtgctgggg-3’ and reverse primer, 5’-ttgctgagcctgataggcagg-3’) regions of the genome were designed based on the SHFV-LVR Genbank sequence (accession number AF180391.1). RNA was amplified using the SuperScript® III One-Step RT-PCR system with Platinum® Taq High Fidelity (Invitrogen). The cycling parameters were RT at 48°C for 30 min, denaturation at 94°C for 2 min, 37 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec and elongation at 68°C for 30 sec. The resulting DNA was electrophoresed on a 1% agarose gel containing ethidium bromide in TBE buffer (90 mM Tris, 90 mM boric acid and 2 mM EDTA, pH 8.0) and visualized with a LAS3000 Luminescent Image Analyzer (GE Healthcare).
2.2.4 454 Sequencing of viral RNA

2.2.5 Quantification of intracellular cell and viral mRNA by real-time RT-PCR (qRT-PCR)

A primer-probe set targeting the nucleocapsid region of SHFV was designed from the SHFV-LVR Genbank consensus sequence. The primer sequences were as follows: forward primer 5'-tcceacctcageacacatca-3'; TaqMan probe 5'-6FAMaacagctgctaggtgMGBNFQ-3'; reverse primer 5'-ccgctccggtgtctagt-3'. Total cellular RNA from SHFV-infected PBMCs was isolated with TRI Reagent (Molecular Research Center, Inc.) according to manufacturer’s protocol. Total cellular RNA (100 ng) was used for one-step qRT-PCR to quantify IFNβ mRNA, viral RNA or 18S rRNA in a single-plex format with the TaqMan one-step RT-PCR master mix reagent kit (Applied Biosystems) using gene specific 20x primer mixes and TaqMan MGB probes (IFNβ Rh03648734_s1 FAM dye labeled or eukaryotic 18S rRNA VIC/TAMRA dye labeled; Applied Biosystems). The cycling parameters were RT at 48°C for 30 min, AmpliTaq activation at 95°C for 10 min, denaturation at 95°C for 15s and annealing/extension at 60°C for 1 min (repeated 40 times). RNA was quantified with an Applied Biosystems 7500 sequence detection system. Triplicate Ct values were analyzed by the comparative Ct (ΔΔCt) method (Applied Biosystems) for IFNβ. The amount of target \(2^{-\Delta\Delta Ct}\) was determined by normalization to the endogenous control (18S rRNA) in each sample and relative to a calibrator (day 0). Intracellular genomic SHFV RNA was quantified using a standard curve generated with 10-fold serial dilutions of a known concentration of SHFV genomic RNA in vitro transcribed with an SP6 mMMessage mMMachine kit (Ambion) from a SHFV nucleocapsid cDNA template. After in vitro transcription, the DNA template was digested with Turbo DNase at 37°C for 5 min. The RNA was purified with lithium chloride, precipitated with ethanol, washed with 70% ethanol, resuspended in RNase-free water, and quantified by UV spectrophotometry.

2.2.6 Western blotting

PBMCs were lysed in RIPA buffer (1x phosphate-buffered saline, 1% Nonidet P-40, 0.5% sodium deoxycholate and 0.1% SDS) containing Halt protease inhibitor cocktail (Thermo Scientific). Following separation by SDS-PAGE, cell proteins were electrophoretically transferred to a nitrocellulose mem-
brane. Membranes were blocked with 1x TBS containing 5% non-fat dry milk and 0.1% Tween 20 before incubation with a 1:1000 dilution of sheep anti-human tissue factor antibody (Cedarlane Labs) in the presence of blocking buffer. Actin was used as a loading control and was detected with a 1:20,000 dilution of antibody C-11 (Santa Cruz Biotechnology). Blots were washed with 1x TBS containing 0.1% Tween-20 and incubated with a 1:2,000 dilution of secondary antibody (horseradish peroxidase-conjugated anti-sheep or anti-mouse; Santa Cruz). Washed blots were then processed for chemiluminescence using a Super-Signal West Pico detection kit (Pierce Scientific) according to manufacturer’s protocol.

2.2.7 Quantification of secreted cytokines

Pro-inflammatory cytokines in macaque plasma were quantified using a Luminex® microsphere based ELISA as previously described (57). Briefly, polyclonal antibodies (Table 1) were conjugated to polystyrene xMAP microspheres (Luminex ® Corp.) using the xMAP® antibody coupling kit (Luminex ® Corp.). One hundred microspheres for each cytokine were then incubated with 25 µl of plasma at 4°C for 16 h. Microspheres incubated with 25 µl of serially diluted human recombinant cytokines were used to construct a standard curve (from 400 pg/ml to 0.128 pg/ml). Microspheres were washed three times in PBS containing 1% BSA and then incubated with biotinylated antibody for each analyte at 25°C for 1 h. Microspheres were washed three times in 1% BSA, PBS and then incubated with 5 µg/ml streptavidin-R-phycoerythrin (Sigma-Aldrich) at 25°C for 30 min. Microspheres were washed three times in 1% BSA, PBS and resuspended in Luminex xMAP systems fluid and analyzed on a Luminex 100 analyzer (Qiagen).

2.2.8 Experimental infection and evaluation of macaques

Experimental animal infections and sample collections were performed at Oregon National Primate Research Center, Beaverton, OR by members of Dr. S. Wong’s laboratory. All aspects of the experimental animal infection studies were performed according to the institutional guidelines for animal care and use at the Oregon National Primate Research Center, Beaverton, OR. Four adult (9-12 years old)
male Old World Japanese macaque (*Macaca fuscata*) monkeys were inoculated intravenously with 100 PFU and evaluated by physical examination on a daily basis by a clinical veterinarian. A clinical score sheet was designed similarly as described (59) with five clinical categories to assess the disease course and involved overall clinical appearance, respiratory abnormalities, activity and species specific behavior, responsiveness, and core body temperature. Each category possessed a rating of zero to ten, with 0 as normal and 10 being severe.

Animals were humanely euthanized when their clinical assessment scores achieved a total of 10. Complete necropsies were performed and tissue samples were collected from all organs and fixed in neutral-buffered formalin, and prepared for histopathological analysis.

### 2.2.9 Flow cytometry analysis of PBMCs

Prior to experimental SHFV inoculation blood, bronchoalveolar lavage (BAL) and lymph node biopsy were collected to serve as pre-infection control samples. Following inoculation blood samples were collected daily to obtain plasma and/or PBMCs for analysis and BAL was performed on days 5 or 6 post-infection for isolation of tissue specific cells for immunological analysis. PBMCs and BAL cells were stained for extracellular markers CD8b (Beckman Coulter, Brea, CA) and CD4 (eBioscience, San Diego, CA) to define CD4⁺ and CD8⁺ T cell subsets, as well as CD20⁺ (Biolegend, San Diego, CA), CD27 (eBioscience), and IgD (Southern Biotech, Birmingham, AL) to define naïve, marginal zone (MZ)-like, and memory B cell subsets, as described previously (80). Cells were subsequently fixed and permeabilized per manufacturer’s protocol (BioLegend) and then stained for Ki67 (BD Pharmingen, San Jose, CA), a nuclear antigen involved in DNA replication. Samples were acquired on an LSRII instrument (BD), and data were analyzed using FlowJo software (TreeStar, Ashland, OR). Complete blood counts were obtained to determine peripheral lymphocyte numbers for each RM, and used to convert the percentages of Ki67⁺ CD4⁺ and CD8⁺ T cells into cell numbers/µl blood. Total lymphocyte counts in the BAL cannot be accurately determined, so percentages of Ki67⁺ CD4⁺ and CD8⁺ T cells were used. Baseline levels of Ki67⁺ cells were measured at two time points prior to RRV infection, averaged, and set to
1. Subsequent time points were then calculated and expressed as a fold change in Ki67+ population compared to baseline in each animal.

2.3 Results

2.3.1 Survey of wild-caught and current baboons at Texas Biomedical Research Institute.

A previous study predicted that between 1 and 10% of individuals in populations of several African monkey species are asymptomatic, persistent carriers of SHFV (74). Because PBMCs from SHFV-negative baboons were required for a study comparing host responses to SHFV infection in disease-resistant and disease-susceptible monkeys, RT-PCR assays were developed to detect SHFV-positive animals. Two assays, one using primers from the SHFV nucleocapsid gene region and the other using primers from the SHFV helicase gene region, were first tested using RNA isolated from a stock pool of plaque purified SHFV-LVR. A single 300 bp PCR product was detected with the nucleocapsid primers (Fig. 2.1A, lane 1). The expected 700 bp PCR product was detected with the helicase primers and a second unexpected 350 bp product was also observed (Fig. 2.1B, lane 1). No PCR products were present in the RNA-free water lanes (Fig. 2.1A and B, lane 2). To test the ability of the RT-PCR assays to detect SHFV RNA in monkey sera, archived serum samples from wild-caught baboons previously introduced into the colony were obtained. RNA isolated from the sera of 120 wild-caught baboons was amplified using each primer set. Thirteen baboons tested positive in both the nucleocapsid and helicase assays (Fig. 2.1A and B, lane 3). Only the 700 bp band was observed in the helicase assay with all of the positive baboon sera. It is likely this band was generated from a defective RNA with a deletion in the helicase region present only in the LVR stock. No PCR bands of the expected sizes were detected with RNA from the remaining 107 baboon serum samples with either the nucleocapsid or helicase assay (Fig. 2.1A and B, lane 4). These data indicate that ~11% of the wild-caught baboons entering this colony between 1981 and 1997 were positive for SHFV.

Whole blood samples periodically collected from baboons currently living at the primate center were used to differentiate MΦs and mDCs for studies of the cellular host responses to SHFV infection.
The animals sampled were typically in the primate center clinic either for their yearly check-up or because they had lacerations due to fighting. Prior to cell isolation, plasma was also collected from each animal in ACD tubes and tested for the presence of SHFV using the RT-PCR assays. SHFV positive samples were not used to prepare cell cultures. Of the 79 randomly sampled baboon blood samples tested, 16 (20.3%) were positive for both nucleocapsid and helicase SHFV RNA. Interestingly, a single shipment of blood collected from 8 males all tested positive. Colony records indicated that these 8 animals were housed in the same corral and at least two of these males had lacerations from fighting. These data indicate that SHFV continues to be transmitted from SHFV-infected animals to other colony animals born in captivity. However, since the sampling was not performed in a truly random fashion, the percent of positive animals may be lower across the entire colony.

Data from a previous study suggested that SHFV-infected, disease-resistant patas monkeys could be viremic for several years (51). To study the length of time baboons could remain SHFV-positive, serial archived serum samples were tested for four of the animals that were previously found to be SHFV positive using a single sample. Each of the three available samples from B11661 and each of the four from B11662 were positive for SHFV RNA (Table 1). Despite similarities in the identification numbers, these two wild-caught animals were procured from different sources and are not likely to be related. However, these two animals were both housed in Egypt at the same facility from 1992 to 1994 before being moved in 1994 to the US primate facility. Serial archived serum samples for two captive-bred animals were also tested. All three of the samples for baboon 19927 and all three of the samples for baboon 25360 were positive for SHFV RNA (Table 1). The data indicate that SHFV can persist in baboons for more than 10 years.

2.3.2 **Analysis of two new SHFV isolates from persistently infected baboon sera**

Initial attempts were made to amplify SHFV from the sera of persistently infected baboons by infection of MA104 cells. No cytopathic effect was observed by 120 h after infection (data not shown). To quantify the SHFV RNA present in the serum samples, RNA isolated from B11661 and B11662 serum
was serially diluted and analyzed using the nucleocapsid RT-PCR assay for nucleocapsid. Each serum sample contained ~10^2 genome copies/ml (data not shown). Sera from B11661 and B11662 were then analyzed directly by 454 sequencing. Serum was first filtered to remove bacteria and then virus particles in serum were concentrated by centrifugation. The pellet was treated with RNase before extraction of the viral RNA to eliminate cellular RNA. Viral RNA was then extracted and subjected to 454 sequencing as described in Materials and Methods. SHFV sequence fragments representing a low percentage of genome coverage were obtained. Sequences for other known primate viruses and bacteria were also detected. The high abundance of some of the other infectious agents efficiently competed for the random 454 primer sets. To increase the amount of SHFV RNA available for sequencing, virus in persistently infected baboon sera was amplified by a single passage on macaque MΦs. Serum (200 µl) from baboon B11661, B11662, B19927 or B25360 was used to infect MΦ cultures derived from four different rhesus macaques and culture fluid collected at 24 h intervals was analyzed for infectivity by plaque assay on MA104 cells. By 120 h after infection, culture fluid contained 50 to 7.5x10^3 PFU/ml of virus. Both the nucleocapsid and helicase RT-PCR assays detected the presence of SHFV RNA in these culture fluids (data not shown).

The genome sequences of the rhesus MΦ amplified, baboon SHFV isolates were next analyzed by 454 sequencing. Complete sequences were obtained from both isolates with 94.8 to 99.7% coverage. The 15.7 kb genome of the B11661 isolate varied at 29 nts while the genome of the B11662 isolate varied at 25 nts from the SHFV-LVR Genbank consensus sequence. Nineteen of the single nt variations observed in the B11661-derived virus were also present in the B11662-derived virus (Table 2). Interestingly, 454 sequencing of the stock SHFV-LVR identified the same 19 nt mutations as well as an additional T5453C mutation (Table 2). These data suggest that the Genbank consensus sequence, which was generated by “shot gun” cloning contained mistakes at 19 positions. The very high sequence conservation observed between the isolates form two long-term persistently infected baboons and between these isolates and SHFV-LVR which was isolated in the 1970s from a patas monkey was unexpected.
2.3.3 Infection of Japanese macaques with a new SHFV isolate

To determine whether the new SHFV isolates were able to induce hemorrhagic fever in disease-susceptible macaques, two Japanese macaques were infected intravenously with 100 PFU of the B11661 isolate. Animals were observed and scored daily 0 (normal) to 10 (worst) based on clinical appearance, respiratory abnormalities, activity/behavior, responsiveness and core body temperature. Animals were euthanized when they reached a body score of 10. Plasma and PBMCs were harvested from collected blood samples.

Within 9 days after infection, one macaque (JM-23333) showed clinical signs of pneumonia and sepsis most likely due to SHFV-induced lymphocytopenia and was humanely euthanized. The second macaque (JM-24054) did not have clear signs of sepsis but was treated with an antibiotic to prevent bacterial infection. JM-24054 survived for a total of 13 days before hemorrhagic fever disease symptoms were observed and the animal was humanely euthanized.

Two additional macaques (JM-22015 and JM-23328) were prophylactically treated with antibiotic to prevent bacterial sepsis and then infected with SHFV isolate. Both of these animals displayed clinical symptoms of hemorrhagic fever such as fever, epistaxis, petechia and edema by 8 days after infection and were humanely euthanized on day 9 due to clinical scores of 10 or greater (Table 3). Due to the observation that neither animal in the second experiment developed sepsis, plasma and PBMC samples from these two animals were used to analyze the host responses to SHFV infection. High levels of infectious virus was detected in the plasma by plaque assay on MA104 cells by 2 days after infection with a peak virus titer in JM-22015 of 2x10⁶ PFU/ml at 4 days after infection and in JM-2338 of 2x10⁶ PFU/ml at 6 days after infection (Fig. 2.2A). Although previous data suggested that not all SHFV isolates lytically infect MA104 cells (51), clear plaques were observed by 72 h after infection (data not shown). Intracellular viral RNA levels in PBMCs were analyzed by qRT-PCR. A peak viral RNA level of 48,775 pg/µl was observed for JM22015 at 4 days after infection while JM-23328 had peak a viral RNA level of 28,025 pg/µl at 6 days after infection (Fig. 2.2B).
IFNβ mRNA levels in PBMCs from the SHFV-infected Japanese macaques were also measured by qRT-PCR at different times after infection. IFNβ mRNA expression levels increased after infection in both animals but, peak IFNβ mRNA levels were not observed until 8 days after infection which did not correlate with peak viremia and suggested the possibility of virus induced suppression of type I IFN induction (Fig. 2.2C). Plasma levels of IFNα protein were quantified by ELISA. Peak IFNα protein levels of 246 pg/ml was detected in JM-23328 and of 716 pg/ml in JM-22015 at 2 days after infection and the levels rapidly declined thereafter (Fig. 2.2D).

To determine whether pro-inflammatory cytokines were produced in response to SHFV infection, a multiplexed ELISA was performed as described in Materials and Methods. There was no detectable IL-1β, IL-10 or IL-12/23(p40) in the plasma of either JM-23328 or JM-22015 (data not shown). However, in both animals, an increase in IL-6, MIP-1α, MIP-1β and TNF-α levels after infection was observed with time after infection. These data support the hypothesis that pro-inflammatory cytokine production is a major contributor to SHFV-induced pathology.

Disseminated intravascular coagulopathy (DIC) is one of the hallmarks of viral hemorrhagic fever and is characterized by coagulation defects initiated by increased expression of tissue factor on monocytic cell surfaces induced by pro-inflammatory cytokine production (10). Attempts to measure coagulation using commercially available human-specific kits were inconclusive likely due to the small volume of the blood samples. Lysates of PBMCs collected at different times after infection were analyzed by Western blotting using an anti-human tissue factor antibody. By day 8 to 9 after infection, both JM-23328 and JM-22015 PBMCs contained detectable levels of tissue factor (Fig. 2.2I). The detection of tissue factor in PBMCs at the time when clinical symptoms of coagulation defects were observed in both animals strongly suggests that both animals were developing disseminated intravascular coagulopathy.

Lymphocyte populations in collected PBMCs were quantified by flow cytometry (Fig. 2.3). In both JM-22015 and JM-23328, the total number of CD4+, CD8+, CD14+ and CD20+ cells decreased between 2 and 6 days after infection. The number of these cells increased between 8 and 9 days after infection compared to uninfected levels suggesting an early transient lymphocytopenia after SHFV infection.
2.4 Discussion

European PRRSV strains are classified as Type I and North American strains as Type II. Genomes of the two types of PRRSV share only 55-70% sequence identity and differ in cell tropism. Although there is a single serotype of EAV, field isolates showed significant sequence variation (5, 54). Two sequenced strains of LDV, LDV-P and LDV-C, share approximately 80% sequence identity (88). Recently, multiple variants of SHFV that showed significant sequence variation from each other as well as from the SHFV-LVR Genbank consensus sequence were reported in colobus monkeys (65, 66). The high sequence conservation observed between two recent SHFV isolates from long term persistently infected baboons and between these isolates and the SHFV-LVR isolated in the 1970s from a patas monkey was inconsistent with previous studies with other arteriviruses that identified multiple strains of each virus and the existence of quasispecies within the same host. A possible reason for the low diversity observed in SHFV from persistently infected baboons is the very low level of viremia in these animals. It was estimated that there was approximately 100 copies of viral RNA per ml which is consistent with the low genome coverage obtained after direct 454 sequencing of baboon sera. Although the baboon isolates could not be amplified by a single passage in MA104 cells, the virus that was amplified in macaque MΦs was able to efficiently replicate in MA104 cells and produced plaques. The SHFV-LVR isolate required seven serial blind passages on MA104 cells before CPE at 72 h after infection was observed (115). Additional data indicated that SHFV replicates to low levels (10^3 and 10^2 PFU/ml in MΦ and mDC cultures, respectively) in cells isolated from baboons compared to higher replication levels (10^6 PFU/ml in both MΦ and mDC cultures) in cells isolated from disease-susceptible rhesus macaques (Vatter and Brinton, unpublished data). In contrast to the low levels of viremia observed in persistently infected baboons, persistent LDV titers are approximately 10^4 ID_{50}/ml (95). The viremic titers of SHFV in species of colobus monkeys appear to be high since complete genome SHFV coverage was obtained by 454 sequencing di-
rectly from the sera of several infected monkeys (65, 66). It is not known whether the colobus monkeys were persistently or acutely infected with SHFV.

Previous studies of macaques experimentally infected with SHFV indicated that disease was characterized by rapid onset fever, edema, depression, proteinuria and manifestations of hemorrhagic defects (88). In addition, these early experiments noted lymphocytopenia in infected animals but did not mention observing sepsis. A recent study, of SHFV-LVR infection of rhesus macaques infected with high doses of SHFV produced similar clinical symptoms including edema, proteinuria and hemorrhagic defects, such as petechia, hematuria and epitaxis, in both survivors and non-survivors (59). This study observed lymphocytopenia as well as sepsis in both survivor and non-survivors. Clinical data presented in the present study indicate that Japanese macaques infected with the new baboon isolate of SHFV display clinical symptoms similar to those observed in rhesus macaques infected with SHFV-LVR. Interestingly, the recent study on SHFV-LVR infection of rhesus macaques used high virus doses and showed only 40 to 70% mortality but peak viremic viral titers of $10^5$ PFU/ml in both survivors and non-survivors (59), while the earlier studies showed 100% mortality of rhesus macaques infected with very low levels of SHFV-LVR (88). The new SHFV isolate produced 100% morbidity and produced higher viral titers with an inoculum of 100 PFU (59).

Hemorrhagic fever disease in macaques caused by Ebola virus is characterized by fever, malaise and coagulation defects that result from release of cytokines, including IL-6 and TNF-α, from infected cells (10). This production of cytokines has been suggested to cause lymphocyte apoptosis and induce tissue factor expression that results in coagulation defects (10, 43, 96). In agreement with these findings, Japanese macaques infected with the baboon SHFV isolate produced several pro-inflammatory cytokines, including IL-6 and TNF-α, and showed increased tissue factor levels in PBMCs. Increased tissue factor production has been correlated with the development of clinical symptoms indicative of coagulation defects. The recent study on experimental infection of rhesus macaques with SHFV-LVR reported higher levels of pro-inflammatory cytokines, specifically IL-6 and IL-18, in non-survivors compared to survivors but tissue factor expression was not analyzed (59). However, the authors did observe hematological evi-
idence indicating coagulopathy in both survivors and non-survivors. These data suggest that the disease induced by the new SHFV isolate in Japanese macaques closely resembles the disease induced by SHFV-LVR infection of rhesus macaques.
**Figure 2.1 Detection of SHFV RNA by RT-PCR in baboon sera.** RNA (100 ng) isolated from baboon serum was analyzed by one-step RT-PCR sing primers to a region of the SHFV (A) nucleocapsid or (B) helicase genes. RNA extracted from an aliquot of a stock pool of SHFV-LVR containing $10^7$ PFU/ml of virus was used as a positive control and nuclease-free water was used as a negative control. PCR products were separated on 1% agarose gels. Lane 1, SHFV-LVR RNA; lane 2, water; lane 3, SHFV-positive baboon serum; lane 4, SHFV-negative baboon serum. The results shown for SHFV positive and negative baboon serum are representative of results from 28 positive and 171 negative baboon sera.
Table 1 RT-PCR analysis of serial serum samples from SHFV-positive baboons.

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Table 2. Conserved majority nt in the three SHFV isolates.

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Figure 2.2 Analysis of viral replication and cytokine production in SHFV infected Japanese macaques. (A) Plasma samples collected at the indicated times after infection were titrated for infectivity by plaque assay. Each data point is the average of duplicate titrations. JM-23328 (■) or JM-22015 (●). Total cell RNA was extracted from PBMCs at the indicated times after infection. (B) Plus-sense viral RNA was quantified by real-time RT-PCR with a reference viral genomic RNA of known concentration. (C) Relative quantification of IFNβ mRNA was detected by real-time RT-PCR. RNA levels are expressed in RQ units as the relative change in expression compared to the level of IFNβ mRNA in mock-infected cells. JM-23328 (gray) or JM-22015 (black). The amounts of each RNA measured were normalized by comparison to 18S rRNA levels. Values shown are the averages of assays done in triplicate. Error bars represent standard error. (D – H) Serum levels of pro-inflammatory cytokines were quantified by multiplexed ELISA. Data points represent averages of each time point measured in triplicate. Error bars represent standard deviation. (I) Tissue factor expression in PBMCs was analyzed by Western blotting using anti-tissue factor antibody. Actin was used as a loading control.
Figure 2.3 Quantification of lymphocyte populations after SHFV infection. PBMCs collected after SHFV infection and stained with the indicated cell surface marker. Flow cytometry was used to quantify the number of surface marker positive cells. JM-22015 (●), JM-23328 (■).
3 DIFFERENTIAL RESPONSES TO SHFV INFECTION IN MACROPHAGES AND MYELOID DENDRITIC CELLS ISOLATED FROM DISEASE-RESISTANT AND DISEASE-SUSCEPTIBLE PRIMATES

3.1 Introduction

SHFV was isolated in 1964 as the causative agent of fatal hemorrhagic fever outbreaks in macaque colonies in the United States, Russia and Europe that were characterized by mortality approaching 100% by 3 weeks (52, 115). Initial symptoms in macaque are fever and facial edema followed by depression, anorexia and dehydration and finally signs of coagulation defects, such as skin petechiae, epitaxis, melena, retrobulbar hemorrhages and subcutaneous hematomas (2, 87). Necropsy revealed hemorrhage in the spleen and necrosis of the liver, adrenal glands and lymphatic tissue (2). The symptoms of SHFV infection closely resemble those caused by other hemorrhagic fever viruses, such as Ebola, Marburg and Lassa viruses in macaques, particularly the coagulation defects and hemorrhage (10, 76). The pathology of viral hemorrhagic fevers is mediated by the host innate immune response to a viral infection. Infected MΦs and DCs release pro-inflammatory cytokines that induce tissue factor and subsequent disseminated intravascular coagulopathy (10, 43, 72). Infected individuals show a marked impairment of the adaptive immune response due to impaired DC function and lymphocyte apoptosis (10).

In previous macaque colony SHFV outbreaks, the virus was transmitted to disease-susceptible macaques by accidental exposure to the blood of an African monkey that was persistently infected but asymptomatic (74). It was previously estimated that 1-10% of wild-caught African monkeys, including patas (Erythrocebus patas), baboons (Papio papio) and African green monkeys (Cercopithecus aethiops) are persistently infected with SHFV (74).

SHFV is a member of the family Arteriviridae that also includes EAV, PRRSV, and LDV. Arteriviruses have a polycistronic, single stranded RNA genome of positive polarity that is 12.7 to 15.7 kb in length and contains a 5' type I cap and 3' poly(A) tail (104). Arteriviruses have highly restricted host and cell ranges. Only MΦs and DCs are infected in horses and donkeys (EAV), in pigs (PRRSV), in mice...
(LDV) or in monkeys (SHFV) (103). Both EAV and PRRSV infections can cause disease characterized by fever, anorexia, tissue necrosis, inflammation of the respiratory tract and spontaneous abortions or delivery of weak offspring (104). LDV typically causes asymptomatic, lifelong, persistent infections (104). Due to the significant agricultural impact of EAV- and PRRSV-induced disease, the majority of the research done on arterivirus infections has focused on these two viruses.

The efficiency of SHFV replication and virus-induced cytokine production in cells isolated from disease-resistant baboons and disease-susceptible macaques were compared. Virus replication and virus-induced cell death was more efficient in both mDCs and MΦs isolated from macaques compared to those from baboons. Tissue factor and pro-inflammatory cytokines were produced in response to SHFV infection by cells isolated from macaques but not by cells isolated from baboons. Interestingly, compared to macaque cells, baboon cells produced higher basal IL-10 levels and IL-10 protein levels increased after SHFV infection. Treatment of macaque mDCs or MΦs with physiological levels of IL-10 resulted in decreased production of IL-6, IL-1β and MIP-1α but did not decreased tissue factor expression, viral replication or virus-induced cell death. These data suggest that IL-10 may contribute to the suppression of pro-inflammatory cytokine production in response to SHFV infection in disease-resistant African monkeys.

3.2 Materials and Methods

3.2.1 Cells

PBMCs were isolated from whole blood obtained from baboons (Southwest National Primate Research Center, San Antonio TX) or rhesus macaques (Yerkes Regional Primate Research Center, Atlanta, GA) by Ficoll® 400 (Mediatech Inc., Manassas VA) density gradient centrifugation according to standard protocols. Monocytes were seeded at 10^6 cells/well in a 24-well plate or at 10^6 cells/well on an 8-chamber slide and allowed to adhere for 2 h before gentle washing with Hanks Buffered Saline Solution (HBSS, Gibco). Immature mDCs were cultured from adherent cells by incubation with Roswell Park Memorial Institute (RPMI) 1640 culture media (Gibco) supplemented with 10% autologous serum or 10% heat-inactivated fetal bovine serum (FBS), 50 U/ml of penicillin, 50 ug/ml of streptomycin, human recombi-
nant granulocyte-macrophage colony stimulating factor (R&D Systems; 1000 U/ml) and recombinant human interleukin 4 (500 U/ml) for 11 days at 37°C in 5% CO2. MΦs were cultured from adherent cells by incubation with RPMI-1640 culture media supplemented with 10% autologous serum or 10% heat-inactivated FBS, 50 U/ml of penicillin, 50 ug/ml of streptomycin, human recombinant macrophage colony stimulating factor (R&D Systems; 5000 U/ml) for 11 days at 37°C in 5% CO2. Two-thirds of the culture media was replaced with fresh growth media every three days to replenish growth factors. Cell identity was confirmed and cells were counted using a FACSCanto flow cytometer and analyzed using FACSDiva software (BD Bioscience) after cells were stained with fluorescently-labeled antibodies directed against surface markers for HLA-DR, DC-SIGN, CD11c and CD83 for mDCs or CD91 or CD163 for MΦs (BD Bioscience). Greater than 90% of cells in mDC cultures were HLA-DR+, DC-SIGN+, CD11c+, and CD83- as analyzed by flow cytometry. Greater than 95% of cells in MΦs cultures were CD91+ and CD163+ as analyzed by flow cytometry.

MA104 cells were obtained from O. Nainin, Centers for Disease Control and Prevention and grown in minimum essential medium (MEM, Gibco) supplemented with 10% FBS, 1% L-glutamine and 1% gentamicin at 37°C in 5% CO2.

3.2.2 Virus

SHFV, strain LVR 42-0/M6941, was obtained from American Type Culture Collection and sequentially plaque-purified three times and then amplified once on MA104 cell monolayers. Stock pools of SHFV-LVR were prepared by infecting confluent MA-104 monolayers at an MOI of 0.2 and harvesting culture fluid at 32 h after infection. Clarified virus pools contained titers of ~10^7 PFU/ml and were aliquoted and stored at -80°C. Primary cell cultures were infected at an MOI of 1 or 10. Virus was diluted in cell specific media. After adsorption for 1 h at 37°C in 5% CO2, virus inoculum was removed and cells were washed three times with HBSS before incubating with cell specific media at 37°C in 5% CO2.

Plaque assays were performed on confluent monolayers of MA104 cells in six-well plates. After adsorption for 1 h at room temperature, the virus inoculum was removed and the wells were overlaid with
1% SeaKem ME agarose (Bio-Whittaker Molecular Applications) mixed 1:1 with 2x MEM containing 5% FCS and incubated at 37°C for 72 h. After removal of the agarose, the cells were stained with 0.05% crystal violet in ethanol. Each virus dilution was assayed in duplicate.

3.2.3 **Preparation of UV-inactivated virus**

An aliquot of SHFV was inactivated by exposure to UV light (4.75 J/cm²) for 10 min at a distance of 10 cm. The complete loss of detectable infectivity after UV exposure was confirmed by plaque assay.

3.2.4 **Quantification of secreted cytokines**

Culture fluid was collected from mock infected and SHFV infected cultures at various times after infection. Three to 27 independent experiments were performed and each experiment was assayed a single time. Pro-inflammatory cytokines in culture fluid were quantified using a Luminex® microsphere based ELISA as previously described (57). Briefly, polyclonal antibodies were conjugated to polystyrene xMAP microspheres (Luminex® Corp.) using the xMAP® antibody coupling kit (Luminex® Corp.). A total of 500 coupled microspheres per cytokine were incubated at 4°C for 16 h with 25 μl of culture fluid or phosphate buffered saline (PBS), pH 7.4 buffer (Sigma) containing 1% bovine serum albumin (BSA) and a non-human primate cytokine standard (IFNα from Mabtech, IL-10 from BioLegend, remaining cytokines from Millipore Corp., Billerica, MA) diluted to concentrations from 400 pg/ml to 0.128 pg/ml. Microspheres were washed three times in PBS + 1% BSA, pH 7.4 buffer and then incubated with a biotinylated antibody for each cytokine (Table 4) at 25°C for 1 h. Microspheres were then washed three times in PBS + 1% BSA, pH 7.4 buffer and incubated with 5 μg/ml streptavidin-R-phycoerythrin (Sigma) at 25°C for 30 min. Microspheres were then washed three times in PBS + 1% BSA, pH 7.4 buffer, resuspended in Luminex xMAP systems fluid and analyzed on a Luminex 100 analyzer (Qiagen). At least 100 events were measured for each cytokine. Alternatively, pro-inflammatory cytokines in culture fluids were screened using the 14-plex non-human primate cytokine Milliplex panel (Millipore) according to the manufacturer’s protocol.
3.2.5 Quantitative real-time RT-PCR (qRT-PCR)

Total cellular RNA from SHFV-infected or mock-infected primary cells was isolated using an RNeasy Mini kit (Qiagen) according to the manufacturer’s protocol. IFNβ mRNAs were detected using specific primer mixes and TaqMan MGB probes (IFNβ Rh03648734_s1; IL10 Rh02621709_m1 FAM dye labeled; Applied Biosystems) and an Applied Biosystems 7500 sequence detection system. One-step RT-PCR was performed for each target gene and for the endogenous control (eukaryotic 18S rRNA VIC/TAMRA dye labeled; Applied Biosystems) in a single-plex format with 100 ng of cellular RNA and the TaqMan one-step RT-PCR master mix reagent kit (Applied Biosystems). The cycling parameters were 48°C for 30 min, 95°C for 10 min, and 40 cycles of 95°C for 15 sec and 60°C for 1 min. Six independent experiments were performed and each experiment was measured in triplicate. The triplicate Ct values were analyzed with Microsoft Excel using the comparative Ct (ΔΔCt) method of the SDS Applied Biosystems software which also applied statistical analysis to the data (TINV test in Microsoft Excel). The values were normalized to those for 18S rRNA and presented as the relative fold change compared to the uninfected 24 h calibrator sample in relative quantification units (RQUs). Error bars represent the standard error of the mean (32) and indicate the calculated minimum (RQmin) and maximum (RQmax) of the mRNA expression levels based on an RQmin/max of the 95% confidence level.

SHFV genomic RNA was quantified by real-time RT-PCR. A primer-probe set targeting the nsp9 (helicase) region of the SHFV genome was designed from the sequence of SHFV-LVR strain (GenBank accession number AF180391.1). The primer sequences used were as follows: forward primer 5'-cgtacacccgcgtctct-3'; TaqMan probe 5'-6FAMttgacgttctacaagggMGBNFQ-3'; reverse primer 5'-cggcaagtggcatccta-3'. The reaction mixture contained 200 ng of cellular RNA, the primer pair (1 µM) and the probe (0.2 µM) in a total volume of 20 µl. Intracellular genomic SHFV RNA was quantified by using a standard curve generated with serial dilutions of a known concentration of SHFV RNA. SHFV RNA was in vitro transcribed with a SP6 mMessage mMachine kit (Ambion) from a SHFV nsp9 cDNA template, the DNA template was digested with Turbo DNase at 37°C for 5 min and the product RNA was
purified with lithium chloride, precipitated with ethanol, washed with 70% ethanol, resuspended in RNase-free water, and quantified by UV spectrophotometry.

3.2.6 Confocal microscopy

Cultures of mDCs grown in 8-chamber slides were mock-infected or infected with SHFV-LVR at an MOI of 10 for 1 h at room temperature. At various times after infection, cells were fixed with 4% paraformaldehyde in PBS for 5 min, permeabilized with 0.01% Triton-X in PBS for 5 min and blocked in 5% horse serum overnight at 4°C. Cells were incubated with murine anti-dsRNA, washed with PBS, and then incubated with Alexa Fluor 594 rabbit anti-mouse IgG (Invitrogen) and Hoechst 33342 nuclear stain (0.5 µg/ml; Invitrogen). Cells were washed with PBS before applying a coverglass with ProLong® Gold anti-fade reagent (Invitrogen). Cells were visualized with a 40x oil-immersion objective on a LSM700 laser confocal microscope (Zeiss) and images were analyzed with the Zeiss LSM version 4.2 software.

3.2.7 Western blotting

Cell lysates were collected in RIPA buffer (1x PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate and 0.1% SDS) containing Halt protease inhibitor cocktail (Thermo Scientific). Following separation by SDS-PAGE, cell proteins were electrophoretically transferred to a nitrocellulose membrane. Membranes were blocked with 1x Tris boric acid saline buffer (TBS) containing either 5% bovine serum albumin or 5% non-fat dry milk and 0.1% Tween 20 before incubation with either sheep anti-human tissue factor antibody (Cedarlane Labs), mouse anti-SOCS3 (L210) antibody (Cell Signaling Technology) or mouse anti-IκBa (L35A5) antibody (Cell Signaling Technology) in the presence of blocking buffer. Actin was used as a loading control and was detected with C-11 antibody (Santa Cruz Biotechnology). Blots were washed with 1x TBS and incubated with anti-sheep or anti-mouse horseradish peroxidase-conjugated antibody (Santa Cruz). After washing, blots were processed for chemiluminescence using a Super-Signal West Pico detection kit (Pierce Scientific) according to the manufacturer’s protocol.
3.3 Results

3.3.1 Analysis of viral replication kinetics in primary cell cultures

Although it was previously reported that SHFV peak yields from cultured peritoneal MΦs isolated from disease-susceptible macaques were 10-fold higher than those from disease-resistant patas, viral replication in mDCs had not been previously compared (51). Both MΦs and mDCs were cultured from PBMCs isolated from baboon and rhesus macaque whole blood samples as described in Materials and Methods. Comparable numbers of cells were generated from each type of monkey (data not shown). Cells were infected with SHFV at an MOI of 1 and viral yields in culture fluid were determined by plaque assay in MA104 cells. Macaque-derived MΦ cultures produced a peak titer of $8 \times 10^4$ PFU/ml by 24 h after infection while baboon-derived MΦ cultures produced a peak titer of $7 \times 10^2$ PFU/ml at 12 h after infection (Fig. 1A). qRT-PCR was next done to assess the levels of intracellular viral RNA replication. Macaque MΦ cultures produced higher levels of intracellular viral RNA at all times analyzed (Fig. 3.1B). Macaque mDCs produced a peak titer of $9 \times 10^4$ PFU/ml by 24 h after infection while baboon mDCs produced a peak titer of $2 \times 10^2$ PFU/ml by 8 h after infection (Fig. 3.1D). Macaque mDC cultures produced higher intracellular viral RNA levels at all times analyzed compared to baboon mDC cultures (Fig. 3.1E). The data indicate that SHFV replicates more efficiently in both types of macaque cell cultures than in the baboon cell cultures.

Both EAV and PRRSV infections of African green monkey kidney cell lines induce apoptosis and necrosis that result in the death of cultured cells (68, 111). To determine whether SHFV infection of disease-resistant and susceptible cells caused a decrease in cell viability, an MTT assay was performed. Macaque and baboon MΦ and mDC cultures were infected with SHFV at a MOI of 1. Macaque MΦ cultures showed a 25% decrease in cell viability by 12 h after infection with a progressive decline to an ~20% viability by 48 h compared to mock-infected cells. Baboon MΦ cultures showed a 13% decrease in cell viability only at 24 h after infection (Fig. 3.1C). Infected macaque mDC cultures showed a decline in
cell viability similar to that observed in macaque MΦ cultures. Baboon mDC cultures showed a 25% decrease in cell viability at 12, 24 and 48 h after infection compared to mock-infected cells (Fig. 3.1F).

3.3.2 Analysis of the percentage of cells infected

The differences in viral replication observed between macaque- and baboon-derived cells could be due to differential viral replication efficiencies in cells from the two types of monkeys or to differences in the number of cells infected. Cell cultures were infected with SHFV at an MOI of 10 and the number of infected cells as well as the relative intensity of viral RNA was assessed by immunofluorescence. Antidouble stranded RNA antibody (α-dsRNA) was used to detect intracellular replicating viral RNA. The majority of macaque MΦs were infected while only about 5% of baboon MΦs were infected (Fig. 3.2A and 3.2B). Infected macaque and baboon MΦ cultures had comparable perinuclear α-dsRNA staining intensities. The data suggest that SHFV replicates efficiently in only a subset of baboon MΦs while the majority of macaque MΦs are permissive to SHFV infection.

The percentages of infected baboon and macaque mDCs was similar at each time point analyzed (Fig. 3.2D). However, the α-dsRNA staining intensity was higher in macaque mDCs at all times analyzed compared to the baboon mDCs (Fig. 3.2C). The data suggest that SHFV replicates less efficiently in cultured baboon mDCs compared to macaque mDCs but that similar percentages of cells in each culture are permissive to SHFV infection.

3.3.3 Analysis of SHFV-induced type I interferon (IFN) production by primary primate cell cultures

Previous data indicated that several PRRSV proteins were able to suppress type I IFN production and signaling (15, 41, 110). To analyze the kinetics of SHFV-induced type I IFN production by disease-resistant and disease-susceptible cell cultures, primary MΦ and mDC cultures were mock infected or infected with SHFV at an MOI of 1 and IFNβ mRNA levels were quantified by qRT-PCR at different times after infection. IFNβ expression in macaque MΦs increased 3-fold by 12 h and 11.6 fold by 24 h after infection compared to mock infected cell levels assayed at 24 h. In baboon MΦs, IFNβ expression increased 1.7 fold by 8 h and 3.7 fold by 24 h after infection compared to the levels in mock infected cells.
at 24 h (Fig. 3.3A). IFNβ expression in SHFV-infected macaque mDCs progressively increased between 8 and 24 h after infection while IFNβ expression in baboon mDCs did not increase through 48 h compared to mock infected levels (Fig. 3.3B).

IFNα protein levels in culture fluid from mock and SHFV infected cultures were quantified by ELISA. IFNα levels in macaque MΦ culture fluids increased 96-fold compared to those from time-matched, autologous mock-infected cultures at 24 h after infection but the levels were not significantly above those from control cells at 36 h after infection (Fig. 3.3C). In baboon MΦ culture fluids, IFNα levels increased 2.4 and 4.8-fold at 24 and 48 h after infection, respectively (Fig. 3.3C). IFNα levels increased in macaque mDC cultures at 36 and 48 h after infection. However, no increase in IFNα levels were observed in baboon mDC culture fluids at any time analyzed (Fig. 3.3D). These data indicate that both macaque mDCs and MΦs show increased IFNβ gene expression and IFNα protein expression in response to SHFV infection while only baboon MΦs showed a detectable type I IFN response.

3.3.4 Analysis of pro-inflammatory cytokine production by SHFV-infected primary cell cultures

Infection of primates with either Ebola or Lassa viruses induces the production of several pro-inflammatory cytokines including TNF-α, IL-6, IL-8, IL-1β, MIP-1α and MIP-1β (55, 77). Also, infection of equine MΦs with EAV induced mRNA upregulation for a similar set of cytokines (83). To compare pro-inflammatory cytokine production in cells from disease-resistant and disease-susceptible monkeys in response to SHFV infection, baboon and macaque MΦ cultures were infected with SHFV at an MOI of 1 or mock infected and cytokines in culture fluids were quantified by ELISA. No significant change in the levels of IFNγ, IL-2, IL-5, IL-8, IL-13, MCP-1, MIP-1α and MIP-1β in culture fluids from SHFV-infected macaque or baboon MΦ cultures compared to those from time-matched, autologous, mock-infected cultures was observed (data not shown). However, the levels of IL-1β, IL-6 and IL-12/23(p40) increased significantly in culture fluids from SHFV-infected macaque MΦs compared to those from mock infected cultures (Fig. 3.4). TNF-α levels also increased significantly in SHFV infected macaque MΦ culture fluids but the increase was less than 2-fold compared to mock levels and may not have been biologi-
cally relevant. The levels of IL-1β, TNF-α and IL-12/23(p40) in baboon MΦ cultures did not increase by 48 h after infection and only a minimal increase (<2-fold) in IL-6 at 36 h after infection was observed which may not be biologically significant. Infected macaque MΦ cultures did not produce significantly increased levels of RANTES after infection while at 36 and 48 h baboon MΦ cultures produced increased RANTES. To determine whether viral replication was required for pro-inflammatory cytokine production by MΦ cultures, cells were infected with UV-inactivated virus and cytokines present in culture fluid were quantified. No increase in the levels of the cytokines tested was detected in culture fluids from baboon MΦ cultures incubated with UV-inactivated virus. A 3.7-fold increase in IL-6 levels at 12h and a 6.8-fold increase in RANTES at 24 h were observed in culture fluid from macaque MΦs treated with UV-inactivated virus. These data suggest that viral replication is increases the production of pro-inflammatory cytokines by macaque MΦs.

To determine whether baboon and macaque mDCs also produce pro-inflammatory cytokines in response to SHFV infection, mDC were mock infected or infected with SHFV at an MOI of 1 and cytokines present in culture fluid were quantified by ELISA. Culture fluids from neither macaques nor baboons showed significant changes in the levels of IFNγ, IL-2, IL-5, IL-8, IL-12/23(p40), IL-13, MCP-1, MIP-1β or RANTES after SHFV infection compared to those from time-matched, autologous, mock-infected cultures (data not shown). Macaque mDC culture fluids contained significantly increased levels of IL-6, MIP-1α and TNF-α compared to those from time-matched, autologous, mock-infected cultures (Fig. 3.5). Macaque mDC cultures also produced significantly increased levels of IL-1β by 24 h after infection but the 1.5- and 1.6-fold increases observed may not be biologically significant. A small (1.5-fold) increase in MIP-1α levels at 36 h after infection was detected in baboon mDC culture fluids. The levels of IL-6, MIP-1α and IL-1β did not increase after incubation of mDC cultures from either macaques or baboons with UV-inactivated virus. TNF-α levels increased 4.5-fold at 24 h after UV-inactivated virus incubation with macaque mDC cultures. These data indicate that viral replication is required for production of IL-6 and MIP-1α but not TNF-α production by macaque mDCs.
A previous study showed that the production of pro-inflammatory cytokines, specifically IL-6 and MCP-1, by macaques in response to Ebola virus infections was associated with increased tissue factor expression, which led to coagulation defects and mortality (43). To determine whether the production of pro-inflammatory cytokines by SHFV-infected primate cells induced tissue factor production, SHFV infected and mock infected baboon and macaque MΦ and mDC lysates were analyzed for tissue factor by Western blotting. Mock-infected macaque MΦ and mDC cultures had no detectable tissue factor. Increased levels of tissue factor were detected by 12 h after infection in macaque MΦs (Fig. 3.6A) and mDCs (Fig. 3.6C) compared to mock infected lysates collected at 24 h after infection. Tissue factor was not detected in macaque MΦ or mDC cultures at 24 or 48 h after infection. This is most likely due to loss of cells. The MTT assay showed that there was a ~50% decrease in macaque cell viability by 24 h after infection (Fig. 3.1C and 3.1F). Mock-infected baboon MΦ (Fig. 3.6B) and mDC (Fig. 3.6D) cultures expressed relatively high levels of tissue factor and the levels decreased after infection. These data indicate that SHFV infection of macaque cells induces tissue factor expression. Interestingly, while uninfected baboon cells express tissue factor, the levels of tissue factor protein decreased after infection.

3.3.5 Analysis of IL-10 signaling in SHFV infected primary cell cultures

Although EAV infection of equine MΦs induces expression of pro-inflammatory cytokine genes, PRRSV infection of porcine mDCs and MΦs does not and the PRRSV infection was reported to also induce transcription of the immune-regulatory cytokine, interleukin 10 (IL-10) (75, 110, 114). To determine whether IL-10 plays a role in modulating the pro-inflammatory cytokine response to SHFV infection in baboon cells, the expression of *IL10* mRNA was first examined by qRT-PCR. *IL10* mRNA levels increased by 3.7-fold by 24 h after infection in baboon MΦs while in macaque MΦs, *IL10* mRNA levels increased by 2.2-fold by 12 h (Fig. 3.7A). *IL10* mRNA expression also increased in baboon mDCs by 2.5 to 9.5-fold and 12 and 48 h, respectively, but no increase in *IL10* mRNA expression after SHFV infection was observed in macaque mDCs (Fig. 3.7B).
To determine whether the increased *IL10* mRNA expression observed in response to SHFV infection resulted in increased levels of secreted IL-10 protein, IL-10 protein in culture fluids was quantified by ELISA. IL-10 protein levels in macaque mock infected MΦ culture fluids ranged from <0.2 to 1.1 pg/ml at different times after infection with the highest level observed at 24 h (Fig. 3.7C). Culture fluids from SHFV infected macaque MΦ cultures contained IL-10 protein levels between <0.2 and 2.1 pg/ml with the peak observed at 24 h after infection. However, the levels in SHFV infected culture fluids were not significantly different from those in time-matched mock infected culture fluids. IL-10 protein levels in culture fluids from mock infected baboon MΦs ranged from 0.5 to 2.1 pg/ml with the highest level observed at 4 h while the levels in SHFV infected baboon MΦ culture fluids ranged from 0.3 to 3.4 pg/ml with a peak observed at 24 h after infection. However, the level of IL-10 was only significantly increased compared to time-matched mock infection levels at 36 h.

Detectable IL-10 protein levels of >0.2 pg/ml were only observed at 4 h in culture fluid from mock infected macaque mDCs and only very low levels of IL-10 protein (<0.2 pg/ml) were detected in macaque mDC culture fluids after SHFV infection (Fig. 3.7D). IL-10 levels in mock infected baboon mDC culture fluids ranged from 1.0 to 2.9 pg/ml with the peak at 24 h while IL-10 protein levels in SHFV infected baboon mDC culture fluids ranged between 0.7 and 1.5 pg/ml with the highest level observed at 48 h after infection. The levels of IL-10 in mock-infected baboon mDC culture fluids were significantly higher than those in SHFV-infected baboon mDC culture fluids between 12 and 48 h after infection. These results suggest that although SHFV infection increases IL-10 mRNA expression in baboon mDCs, IL-10 protein levels in the culture fluids of these cells decreased compared to those in mock infected culture fluids.

The higher levels of IL-10 observed in baboon mock infected mDC culture fluids indicated that these cultures produced higher levels of IL-10 prior to infection. As an alternative means of assessing the basal levels of IL-10 produced by the various types of cell cultures, cells were cultured as described in Materials and Methods with 2/3 of the media changed every three days with culture media. After 11 days of culturing, culture fluid was collected and IL-10 protein levels were quantified by ELISA. Macaque MΦ
culture fluids had basal IL-10 levels of 0.5 pg/ml ± 0.2 pg/ml (n = 6) while baboon MΦ culture fluids contained 1.7 pg/ml ± 0.2 pg/ml (n = 6). Similarly, macaque mDC culture fluids contained IL-10 levels <0.2 pg/ml (n = 8) while baboon mDC culture fluids contained 5.4 pg/ml ± 3 pg/ml (n = 9) (data not shown).

IL-10 is a regulatory cytokine that can suppress pro-inflammatory cytokine production by inducing expression of suppressor of cytokine signaling 3 (SOCS3) (84). To determine whether the high IL-10 levels produced before and during SHFV infection by baboon mDC and MΦ cultures were sufficient to induce SOCS3 expression, cell lysates from macaque and baboon cell cultures were mock infected or infected with SHFV at an MOI of 1 and cell lysates collected at various times after infection were analyzed by Western blotting using an anti-SOCS3 antibody. Both baboon MΦ and mDCs showed increased SOCS3 expression after SHFV infection while MΦ and mDCs isolated from macaques did not (Fig. 3.7E). Interestingly, even though baboon MΦ and mDC mock-infected culture fluids contained IL-10, no SOCS3 expression was observed in the lysates of these cells.

IL-10 binds to a heterodimeric surface receptor composed of IL-10R1 and IL-10R2. The IL-10R2 subunit is constitutively expressed at high levels on monocytic cell surfaces while the IL-10R1 subunit is expressed at low levels (<100 units/cell surface) until its expression is up-regulated by various stimuli (84). To determine whether the lack of SOCS3 expression in mock infected baboon cells was due to decreased IL-10R1 expression in mock infected cells compared to SHFV infected cells, primary cell cultures were mock infected or infected with SHFV at an MOI of 1 and proteins in cell lysates were analyzed by Western blotting using anti-IL-10R1 antibody. IL-10R1 was detected in mock infected baboon mDCs and macaque MΦs and mDCs but not in baboon MΦs (Fig. 3.7F). The levels of IL-10R1 decreased in both macaque MΦs and mDCs after SHFV infection but increased in baboon MΦs and mDCs after SHFV infection at 24 and 48 h, suggesting that SHFV infection induced IL-10R1 expression only in baboon cells.
3.3.6 Analysis of IL-10 modulation of host responses to SHFV infection in disease-susceptible cells

Based on the finding that disease-resistant baboon mDCs produce high levels of IL-10 both before and after SHFV infection compared to disease-susceptible macaque mDCs and produce significantly lower amounts of pro-inflammatory cytokines in response to infection, we hypothesized that IL-10 played a role in suppressing pro-inflammatory cytokine production in response to SHFV infection in baboon cultures. Expression of the IL-10R1 receptor by both mock-infected macaque MΦ and mDCs suggested that all of these cells should be able to induce SOCS3 expression if treated with IL-10. To determine whether IL-10 could suppress SHFV-induced pro-inflammatory cytokine expression, macaque MΦ and mDC cultures were treated with 5 pg/ml of rhIL-10 starting immediately after the 1 h virus adsorption period or for 1 h prior to viral adsorption and then for 48 h after virus adsorption. Pro-inflammatory cytokines were quantified by ELISA in both mock infected and SHFV infected (MOI of 1) culture fluids with and without rhIL-10 treatment. Macaque MΦ culture fluids contained significantly decreased IL-6 levels at 36 and 48 h after SHFV infection when treated with rhIL-10 under either condition compared to untreated infected culture fluids (Fig. 3.8A). However, there was no significant change in IL-1β, TNF-α, IL-12/23(p40) or RANTES levels with rhIL-10 treatment (Fig. 3.8B, 3.8C, 3.8D, 3.8E). Macaque mDC cultures treated with rhIL-10 only after or both before and after SHFV infection also produced decreased IL-6 levels at 12, 24 and 48 h after SHFV infection compared to untreated SHFV infected cells (Fig. 3.9A). MIP-1α levels from macaque mDC cultures were also decreased at 12, 24 and 36 h after infection (Fig. 3.9D). However, IL-1β and TNF-α levels did not significantly decrease with rhIL-10 treatment of macaque mDC cultures (Fig. 3.9B and 3.9C).

Based on the observation that rhIL-10 treatment resulted in reduced IL-6 levels in both macaque mDCs and MΦ culture fluids and that previous studies have shown that reduced pro-inflammatory cytokine production correlated with reduced tissue factor levels as well as increased survival after Ebola virus infection of macaques, it was hypothesized that IL-10 treatment would decrease tissue factor production in SHFV infected macaque cells (10). Macaque MΦs and mDCs tissue factor expression was analyzed by Western blotting in cell lysates from mock or SHFV infected (MOI of 1) cells that had been incubated
with or without rhIL-10. Tissue factor levels were still observed to increase at 12 h after infection in IL-10 treated MΦs (Fig. 3.8F) and mDCs (Fig. 3.9E). However, the levels were slightly decreased in the IL-10 treated cells.

To determine whether IL-10 treatment affected viral replication, viral yields from SHFV infected (MOI of 1) macaque MΦ and mDC cultures that were incubated with or without rhIL-10 were assessed by plaque assay. IL-10 treatment did not decrease virus yields from either MΦs or mDCs (Fig. 3.8G and 3.9F, respectively). To determine whether IL-10 treatment reduced SHFV-induced cell death, the viability of SHFV infected (MOI of 1) macaque cells that were incubated with or without rhIL-10 was analyzed by MTT assay. Neither SHFV-infected macaque MΦs nor mDCs showed a significant increase in viability with IL-10 treatment (Fig. 3.8H and Fig. 3.9G, respectively). Treatment of macaque MΦs and mDCs with higher concentrations, 10 pg/ml or 20 pg/ml, of rhIL-10 also did not decrease viral replication or cell viability (data not shown). The data indicate that IL-10 used reduce the production of some pro-inflammatory cytokines, including IL-6, in response to SHFV infection in macaque MΦ and mDC cultures but did not suppress the levels of other pro-inflammatory cytokines, such as TNF-α, and was able to only partially inhibit tissue factor expression. IL-10 had not effect on viral replication or improve cell viability.
3.4 Discussion

Macaque MΦs produce high yields of SHFV and 93% of these cells were shown to be permissive for SHFV replication while only a subset of baboon MΦs (<10% of the total MΦ population) were productively infected. It is likely that this subpopulation of baboon MΦs act as the reservoir for virus replication that is required to maintain a chronic low level viremia within persistently infected animals. Interestingly, other arteriviruses, such as PRRSV and LDV have been shown to productively infect only 5 to 10% of cultured MΦs suggesting SHFV infection of baboon MΦs is typical while the permissiveness of the majority of macaque MΦs is atypical for arterivirus infections (37, 63, 93). Despite similar percentages of infected cells in the baboon and macaque mDC cultures, extracellular viral yields were very low in baboon mDC cultures suggesting there may be a block in a later stage of the virus replication cycle in these cells. The observation that baboon mDCs had much lower dsRNA staining intensity compared to macaque mDCs supports the hypothesis that the efficiency of intracellular virus replication but not virus attachment and entry is less efficient in baboon mDCs.

In contrast to the fatal disease induced in macaques, SHFV infection of several African monkey species results in a persistent, asymptomatic disease (52, 74). The mechanisms regulating this differential host response were not previously studied. In response to infection with various types of hemorrhagic fever viruses, macaques were previously reported to produce pro-inflammatory cytokines that resulted in bystander apoptosis as well as increased TF expression that in turn lead to increased vascular permeability and coagulation defects (10, 11, 43, 96). The similar clinical manifestations of SHFV infections in macaques to those of other hemorrhagic fever virus infections suggested that similar host responses resulted in the development of disease (1, 51, 59, 74, 87). Data from the present study showed that SHFV infection of macaque MΦ and mDC cultures induced the production of pro-inflammatory cytokines including IL-6. Infection of baboon MΦ and mDC cultures did not induce pro-inflammatory cytokine production and the data suggested that the high basal production of the immunoregulatory cytokine IL-10 by the baboon cells suppressed virus induction of pro-inflammatory cytokines. It was previously suggested that IL-
10 modulates pro-inflammatory cytokine production by PRRSV infection in porcine mDCs (35, 75, 114). In contrast, pro-inflammatory cytokine production was correlated with EAV virulence and IL-10 was not induced by EAV infected equine cells (6, 83). In agreement with these data, baboon MΦs and mDCs showed high basal levels of IL-10 as well as up-regulation of IL10 mRNA levels after SHFV infection and did not produce pro-inflammatory cytokines in response to SHFV infection. The basal IL-10 levels observed in baboon cell cultures were comparable to those previously reported in human monocyte cultures (17). Basal IL-10 production in human cells was shown to be due to a constitutive level of IL10 mRNA expression and post-transcriptional regulation of protein production by destabilizing motifs in the 3´ untranslated region of the mRNA that lead to rapid mRNA degradation in unstimulated cells (92, 99, 123). The observation that SHFV infection of baboon mDCs decreased IL-10 protein levels despite elevated IL-10 mRNA levels suggests that SHFV infection may increase IL10 mRNA degradation.

Despite the high basal levels of IL-10 in baboon MΦ and mDC culture fluids, SOCS3 expression was only observed in these cells after SHFV infection. Previous data suggested that IL-10 suppression of cytokine production via SOCS3 expression is accomplished by signaling through the heterodimeric receptor complex of IL-10R1 and IL-10R2 (84). While IL-10R2 is constitutively expressed at high levels on monocytic cell surfaces, IL-10R1 expression must be induced (17). The observation that IL-10R1 levels in baboon mDCs increased after SHFV infection suggests that infection causes these cells become permissive to IL-10 signaling and this leads to upregulation of SOCS3 expression which blocked pro-inflammatory cytokine production. However, while SOCS3 signaling increased in baboon MΦ cultures after SHFV infection, IL-10R1 levels remained constant indicating that SOCS3 expression is regulated by alternative mechanisms. Previous data demonstrated that SOCS3 is transcriptionally up-regulated by IL-6-dependent activation of STAT1/STAT3 in murine pituitary cells to generate negative feedback regulation of IL-6 (3). It is possible that SHFV-infected baboon MΦs utilize STAT1/STAT3-dependent signaling to regulate IL-6 and SOCS3 in an IL-10 independent manner.

Several studies have shown that IL-10 treatment of human and murine MΦ and DC cultures block pro-inflammatory cytokine production including IL-6, IL-1β and TNF-α in response to viral infection or a
sustained inflammatory response (84). Although IL-10 treatment of macaque mDCs and MΦs decreased IL-6 and MIP-1α production in response to SHFV infection, a significant decrease in TNF-α, IL-1β or IL-12/23(p40) production was not observed. It is likely that SHFV infection of macaque cells induces pro-inflammatory cytokine production by the activation of multiple pathways there are not all blocked by rhIL-10 treatment.

While rhIL-10 treatment of macaque cells decreased the levels of some SHFV-induced pro-inflammatory cytokines, it had no effect on either virus yields or SHFV-induced cell death. Several previous studies showed that both EAV and PRRSV infections induce apoptosis via TNF receptor signaling (68, 111). Based on the finding that TNF-α is produced by macaque MΦs and mDCs after SHFV infection with and without rhIL-10 treatment, it is likely that TNF-α also plays a role in apoptosis induction after SHFV infection.

Overall, the results of this study strongly suggest that disease in SHFV-infected macaques is mediated by the production of pro-inflammatory cytokine production by mDCs and MΦs and that IL-10 produced by cells of disease-resistant baboons is partially responsible for down regulating this inflammatory response.
Table 4. Antibody Clones Used in the Luminex ELISA Assays

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Capture antibody</th>
<th>Biotinylated Detection antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α</td>
<td>MAb1(^1)</td>
<td>MAb11(^1)</td>
</tr>
<tr>
<td>IFN-α</td>
<td>MT1-3-5(^2)</td>
<td>MT2/4/6(^2)</td>
</tr>
<tr>
<td>IL-1β</td>
<td>JK1B-1(^1)</td>
<td>JK1B-2(^1)</td>
</tr>
<tr>
<td>IL-6</td>
<td>MQ2-13A5(^1)</td>
<td>MQ2-39C3(^1)</td>
</tr>
<tr>
<td>IL-10</td>
<td>JES3-9D7(^1)</td>
<td>JES3-12G8(^1)</td>
</tr>
<tr>
<td>IL-12/23(p40)</td>
<td>MT86/221(^2)</td>
<td>MT618(^2)</td>
</tr>
<tr>
<td>MIP-1α</td>
<td>AF-270-NA(^3)</td>
<td>BAF270(^3)</td>
</tr>
<tr>
<td>MIP-1β</td>
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<tr>
<td>RANTES</td>
<td>AF-278-NA (^3)</td>
<td>BAF-278(^3)</td>
</tr>
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\(^1\) Antibody purchased from BioLegend, San Diego, CA
\(^2\) Antibody purchased from Mabtech, Inc., Mariemont, OH
\(^3\) Antibody purchased from R&D Systems, Inc. Minneapolis, MN
Figure 3.1 Kinetics of SHFV replication in primary cells isolated from disease-resistant and disease-susceptible primates. MΦ (A, B, C) and mDCs (D, E, F) isolated from baboon (black) or macaque (gray) PBMCs were mock infected or infected with SHFV-LVR at an MOI of 1.0. (A, D) Culture fluid was collected at the indicated times after infection and viral yields were quantified by plaque assay on MA104 cells. Each data point is the average of duplicate titrations done on samples collected from cells isolated from 14 baboons or 28 macaques. (B, E) Total cellular RNA was isolated from cell lysates prepared at the indicated times after infection and the copies of viral helicase region RNA were quantified by qRT-PCR as described in Materials and Methods. Each experiment was performed in triplicate and representative data from one of the three independent experiments are shown. The mRNA level of the helicase gene was normalized to the level of the 18S rRNA in the same sample and compared to data from a standard curve. The error bars represent standard error of means (32) (n=6). (C, F) Cell viability was determined by MTT assay. Values shown are averages from six independent experiments. Each sample was assayed in triplicate. Error bars indicate standard error of means (32). *p≤0.05, **p≤0.01, ***p≤0.005.
Figure 3.2 Number of primary cells infected by SHFV. (A) MΦs and (C) mDCs cultured from baboons or macaques were mock infected or infected with SHFV at an MOI of 10. Cells were fixed at indicated times after infection, permeabilized, stained with anti-dsRNA antibody and visualized by confocal microscopy. Images shown are representative of three independent experiments. The same microscope settings were used to obtain all of the images. Percentage of infected (B) MΦs and (D) mDCs. The total number of cells, as well as the number of infected cells, were counted in four confocal image fields for each time point from cells isolated from three macaques and three baboons. Error bars indicate SEM. *p≤0.05, ***p≤0.005.
Figure 3.3 Induction of type I interferon in SHFV-infected primary cell cultures. Cultured MΦs and mDCs were mock infected or infected with SHFV-LVR at an MOI of 1. Total cell RNA isolated from (A) MΦs or (B) mDCs and used to assay IFNβ mRNA by real-time qRT-PCR. Each experiment was performed in triplicate and the average values shown are the average of seven independent experiments. The mRNA level of the IFNβ gene was normalized to the level of 18S rRNA in the same sample and is shown as the fold change over the amount of mRNA in the mock at 25 h samples expressed as relative quantification units (RQUs). The error bars represent the calculated SEM (n=7) and are based on an RQ_{min/max} of the 95% confidence level. Culture fluid from (C) MΦs or (D) mDCs was analyzed for IFNα protein by multiplexed ELISA. The data shown are average fold change levels in SHFV-infected culture fluids from cells from 4 baboons (black) and 4 macaques (gray) compared to the levels in autologous time-matched mock infected culture fluid. Error bars represent SEM. *p≤0.05, **p≤0.01 and ***p≤0.005, ns : not significant.
Figure 3.4 Pro-inflammatory cytokine production by MΦs isolated from disease-resistant and disease-susceptible primates in response to SHFV infection. Culture fluid from MΦs mock infected, infected with SHFV (MOI of 1.0) or infected with UV-inactivated SHFV virus was analyzed for pro-inflammatory cytokines by multiplexed ELISA. Values represent average fold change in SHFV-infected culture fluid compared to autologous time-matched mock infected culture fluid. Culture fluid from 20 baboons infected with SHFV (black), 21 macaques infected with SHFV (gray), 3 baboons infected with UV-inactivated SHFV (white) or 3 macaques infected with UV-inactivated SHFV (stippled) were each measured a single time. Error bars represent SEM from. *p ≤ 0.05, **p ≤ 0.01 and ***p ≤ 0.005, ns: not significant.
Figure 3.5 Pro-inflammatory cytokine production by mDCs isolated from disease-resistant and disease-susceptible primates in response to SHFV infection. Culture fluid from mDCs mock infected, infected with SHFV (MOI of 1.0) or infected with UV-inactivated SHFV virus was analyzed for pro-inflammatory cytokines by multiplexed ELISA. Values represent average fold change in SHFV-infected culture fluid compared to autologous time-matched mock infected culture fluid. Culture fluid from 25 baboons infected with SHFV (black), 22 macaques infected with SHFV (gray), 4 baboons infected with UV-inactivated SHFV (white) or 4 macaques infected with UV-inactivated SHFV (stippled) were each measured a single time. Error bars represent SEM from. *p ≤ 0.05, **p ≤ 0.01 and ***p ≤ 0.005, ns : not significant.
Figure 3.6 Tissue factor expression from cultured cells. Cultured MΦs (top panels) and mDCs (bottom panels) isolated from macaques (A and C) or baboons (B and D) were mock infected or infected with SHFV-LVR (MOI of 1.0). Cells were lysed at the indicated times after infection and proteins were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes. Tissue factor (24) expression was detected by western blotting. Actin was used as a loading control. The blots shown are representative of three independent experiments.
Figure 3.7 Interleukin 10 signaling in primary cells following SHFV infection. Total cell RNA was isolated from cultured (A) MΦs or (B) mDCs at different times after mock infection or infection with SHFV (MOI of 1.0) and IL10 mRNA levels were analyzed by real-time RT-PCR. Values were normalized to the levels of 18S rRNA in the same sample. Average values shown were obtained from cells isolated from 6 macaques (gray) and 7 baboons (black), each measured in triplicate. Culture fluids collected from the (C) MΦs or (D) mDCs were used to quantify IL-10 protein by ELISA. Values shown are averages obtained from 8 macaques (mock - stippled, infected - gray) and 10 baboons (mock - white, infected - black). Error bars represent SEM. *p≤0.05. Proteins in cell lysates from MΦs and mDCs were separated by 10% SDS-PAGE and analyzed for (E) SOCS3 or (F) IL-10RA by Western blotting. Actin was used as a loading control. Blots shown are representative of three independent experiments.
Figure 3.8 Effects of IL-10 treatment on SHFV-induced pro-inflammatory cytokine production and tissue factor expression from macaque MΦs. (A-E) Macaque MΦ cultures were treated with rhIL-10 starting 1 h before infection as well as after infection (white bars), treated with rhIL-10 only after infection (gray) or left untreated (black). Pro-inflammatory cytokines present in culture fluids collected at the indicated times after infection were analyzed by multiplexed ELISA. Average fold change in cytokine levels in SHFV-infected culture fluids above the levels in autologous time and treatment-matched mock infected culture fluid are shown. Values shown are averages from cultures from 6 macaques. Error bars represent SEM. *p ≤ 0.05, **p ≤ 0.01 and ***p ≤ 0.005, ns : not significant. (F) Proteins in cell lysates from untreated or treated MΦs were separated by 10% SDS-PAGE and analyzed for tissue factor by Western blotting. Actin was used as a loading control. Blots shown are representative of three independent experiments. (G) Extracellular viral yields were quantified by plaque assay on MA104 cells. Macaque MΦ cultures were treated with rhIL-10 starting 1 h before infection as well as after infection (●), treated with rhIL-10 only after infection (▲) or left untreated (■). Average values of duplicate titrations from 6 independent experiments are shown. Error bars represent SEM. (H) Viability of infected cells with and without IL-10 treatment was determined by MTT assay. Average values of six independent experiments each measured in triplicate. Error bars represent SEM.
Figure 3.9 Effects of IL-10 treatment on SHFV-induced pro-inflammatory cytokine production and tissue factor expression from macaque mDCs. (A-E) Macaque mDC cultures were treated with rhIL-10 starting 1 h before infection as well as after infection (white bars), treated with rhIL-10 only after infection (gray) or left untreated (black). Pro-inflammatory cytokines present in culture fluids collected at the indicated times after infection were analyzed by multiplexed ELISA. Average fold change in cytokine levels in SHFV-infected culture fluids above the levels in autologous time and treatment-matched mock infected culture fluid are shown. Values shown are averages from cultures from 6 macaques. Error bars represent SEM. *p ≤ 0.05, **p ≤ 0.01 and ***p ≤ 0.005, ns : not significant. (F) Proteins in cell lysates from untreated or treated MΦs were separated by 10% SDS-PAGE and analyzed for tissue factor by Western blotting. Actin was used as a loading control. Blots shown are representative of three independent experiments. (G) Extracellular viral yields were quantified by plaque assay on MA104 cells. The mDC cultures were treated with rhIL-10 starting 1 h before infection as well as after infection (●), treated with rhIL-10 only after infection (▲) or left untreated (■). Average values of duplicate titrations from 6 independent experiments are shown. Error bars represent SEM. (H) Viability of infected cells with and without IL-10 treatment was determined by MTT assay. Average values of six independent experiments each measured in triplicate. Error bars represent SEM.
4 ANALYSIS OF DUPLICATED OPEN READING FRAMES DURING SHFV REPLICATION USING A FULL-LENGTH cDNA INFECTIOUS CLONE

4.1 Introduction

SHFV was identified as the causative agent of several fatal hemorrhagic fever virus outbreaks in captive rhesus macaque colonies in the United States, Russia and Europe (115). Experimental infection of several different macaque species with SHFV produced clinical symptoms including fever, anorexia, adipsia, cyanosis, skin petechial and nose bleeds and ultimately lead to death around 7 to 13 days after infection (2, 74, 87). The symptoms induced by SHFV infection in rhesus macaques closely resemble those induced by other types of hemorrhagic fever viruses, such as Ebola Zaire and Marburg viruses (10). African monkeys are the natural hosts of SHFV and SHFV infection of baboons, patas, vervets, and African green monkeys resulted in either a mild acute or persistent, asymptomatic infection (51). The initiation of the previous macaque colony SHFV outbreaks is thought to have been due to inadvertent mechanical transfer of SHFV from a persistently infected African monkey to a macaque followed by virus transmission between macaques (87). Humans exposed to SHFV-infected macaques did not develop disease symptoms or seroconvert (19, 87).

SHFV is a member of the family Arteriviridae that also includes EAV, PRRSV and LDV (103). The Arteriviridae, Coronaviridae and Roniviridae families have been classified together in the Order Nidovirales based on similar genome organization and gene expression strategies (104). The SHFV genome is a 5’ capped and 3’ polyadenylated, positive-sense, single-stranded RNA of approximately 15.7 kb that encodes 14 ORFs (ORF1a, ORF1ab, and ORF2a, E1, 2b, 3, E2, 4, 5, 6, 7, 7a, M and N). The 5’ two-thirds of the genome is translated into the ORF1a and ORF1ab polyproteins (Fig. 1A). The 14 non-structural proteins that are proteolytically cleaved from these polyproteins are required for genome and 3’ sg RNA transcription and replication (7, 103). The 3’ coterminal, nested, sg mRNAs encode the structural proteins.
The 3’ most gene, ORF 9, encodes the abundant 15 kDa nucleocapsid (N) protein that forms disulfide-linked homodimers that interact to form the capsid shell (23, 103). The N protein interacts with genome RNA through its basic N-terminal domain facilitating packaging of the viral RNA into progeny virions (23). Two additional major structural proteins produced by all arteriviruses are the 19 kDa non-glycosylated M protein and the 26 kDa major glycoprotein (GP7 in SHFV and GP5 in other arteriviruses) that interact to form disulfide-linked heterodimers. The M protein is the most conserved arterivirus structural protein and is essential for progeny virion assembly (29, 103). The M-GP5 heterodimers function in virion attachment by interacting with heparin sulfate as well as with the N-terminal domain of sialoadhesin on the surface of target cells (28, 29). However, it is not known whether this heterodimer also interacts with the activated monocyte-specific CD163 receptor that is required for virion entry/uncoating (130). Recently, a novel protein, GP5a (GP7a in SHFV) that is initiated at an alternative start site in ORF5 (ORF7 in SHFV) was shown to be required for the replication of both EAV and PRRSV (42, 58). The role of this newly identified protein has not yet been studied in either LDV or SHFV.

The functions of the arterivirus minor structural proteins, E, GP2, GP3 and GP4, have not been fully characterized and these proteins are not as well conserved as the major structural proteins. The E protein is translated from the bicistronic sg mRNA 2 in LDV, EAV and PRRSV, but the position of the E start site differs between these viruses. The PRRSV E protein is encoded by a downstream AUG (ORF2b) and the GP2 protein is translated from the 5’ AUG (ORF2a). However, the E proteins of LDV and EAV are initiated from the 5’ most AUG (ORF2a) while GP2 is translated from a downstream AUG (ORF2b). Regardless of the positioning of the E ORF compared to GP2 protein, the E proteins produced by each of these viruses is a minor virion component that functions as an ion-channel required for virion uncoating (117, 132, 135). All of the arterivirus structural proteins are predicted to have one or more transmembrane domains (36). GP2, GP3 and GP4 were reported to form heterotrimers located on the outer surfaces of EAV and European PRRSV strain virions. For LDV and North American PRRSV strains, GP2 and GP4 form heterodimers and GP3 was reported to be a soluble protein (6, 103, 118, 132). However, a subsequent study indicated that GP3 is a virion component of a highly pathogenic North American
PRRSV isolate (NVSL 97-7895) (22). The minor structural protein complexes are postulated to be involved in receptor binding and virion uncoating for each virus (67, 135).

SHFV is unique among the other arterviruses in having nine 3’ ORFs instead of six as do other members of the arterivirus family (Fig. 4.1A) (102, 140). Based on a greater degree of amino acid sequence homology between SHFV ORF2a, 2b and 3 and PRRSV ORF2, 3 and 4, respectively, it was suggested that these are the SHFV minor structural protein ORFs and ORF5, 6 of SHFV are a duplicated set. It was also suggested that this gene duplication might be a laboratory artifact unique to the LVR strain (46). However, these additional SHFV ORFs were recently reported to be present in SHFV isolates from naturally infected colobus monkeys (65, 66) and persistently infected baboons (Vatter and Brinton, unpublished data). A second overlapping ORF is predicted both in the SHFV ORF2b and ORF4 suggesting that a minor glycoprotein and an E protein could be translated from each of these bicistronic sg mRNAs. The predicted E protein AUG in ORF2b is downstream of the GP2b AUG as is the case in the PRRSV genome, while the E protein AUG in ORF4 is upstream of the GP4 AUG similar to the EAV and LDV gene order. The E proteins of EAV and PRRSV contain myristoylation motifs and inhibition of myristoylation during EAV replication significantly decreased virus yields suggesting that myristoylation of the N-terminal glycine of E is essential for efficient viral replication (117). The SHFV ORF4 E protein has a myristoylation motif but the ORF2b E protein does not.

A full-length cDNA infectious clone of SHFV-LVR was constructed. Virus produced after transfection of cells with viral RNA in vitro-transcribed from the infectious clone (SHFVic) had similar replication kinetics to virus produced after transfection of cells with viral RNA isolated from the parental SHFV-LVR stock. Northern blot analysis of sg mRNAs produced in cells infected with SHFVic virus indicated that eleven sg mRNAs were produced. A set of mutant infectious clones, each lacking the expression of one of the duplicated 3’ ORFs, was made and analyzed for replication after RNA transfection. Only four of the mutants produced progeny virus but this virus was not infectious. The data indicate that the duplicated SHFV 3’ proteins are not functionally redundant.
4.2 Materials and Methods

4.2.1 Cells and virus

The MA-104 cell line, a gift from O. Nanin, Centers for Disease Control and Prevention (Atlanta, GA), was cultured in minimal essential medium (MEM) supplemented with 10% heat-inactivated fetal bovine serum and 10 µg/ml gentamicin.

SHFV, strain LVR 42-0/<6941 (American Type Culture Collection) was sequentially plaque-purified three times and amplified once on MA104 cells. A stock pool of SHFV-LVR was prepared by infecting confluent MA104 monolayers at an MOI of 0.2. Culture media harvested at 32 h after infection was clarified, aliquoted and stored at -80°C. The titer of this stock was approximately 10^7 PFU/ml.

4.2.2 RNA extraction and generation of genomic fragments

Viral RNA was extracted from the parental virus pool with TRI Reagent (Molecular Research Center, Cincinnati, OH) and 5 overlapping cDNA fragments were synthesized using SuperScript II reverse transcriptase (Invitrogen) and the primers listed in Table 5. The fragment junctions were selected based on the location of PflMI recognition sequences (CCANNNN/NTGG). A unique SpeI cut site (for cloning into the pACYC184 vector) and the SP6 promoter were added to the 5’ end of fragment I. A 47 nt poly(A) tail, a unique Pvul site (for linearization) and a NotI site (for cloning into the pACYC184 vector) were added to the 3’ end of fragment V. Each cDNA was amplified by PCR. Cycling conditions were 48°C for 30 min, 94°C for 2 min, then 37 cycles of 94°C for 30 s, 55°C for 30 s and 65-72°C for 1 to 5 min followed by a final cycle at 72°C for 7 min for TA cloning. PCR fragments were gel purified and cloned into the pCR-XL-TOPO vector and maintained in TOP10 or INV110 E.coli cells (Invitrogen) grown in LB media containing kanamycin (50 mg/ml). Multiple clones of each fragment were generated and sequenced. Mutagenesis of fragment clones was performed using the QuikChange Lightning Site-Directed Mutagenesis kit (Agilent) according to manufacturer’s protocol.
4.2.3 Assembly of full-length cDNA clones

Plasmid DNA of each fragment was digested with PflMI alone or in combination with SpeI (fragment I), NotI (fragment V) or RsrII (fragment III and V) before gel purification. The pACYC184 vector was cleaved with XbaI and EagI to generate compatible cohesive ends with SpeI and NotI, respectively, and then gel purified. All of the fragments and the vector were simultaneously ligated using the T4 Rapid Ligation kit (Thermo Scientific) and used to transform XL10-Gold KanR Ultracompetent cells (Stratagene). The cells were grown overnight at 37°C in LB media containing chloramphenicol. Full-length clones were sequenced to insure that no mutations had occurred during cloning.

4.2.4 In vitro transcription and transfection

Full-length cDNA clones were linearized with PvuI, purified on Minelute columns (Qiagen) and used as templates for in vitro transcription of capped mRNA with the mMessage mMACHINE kit (Ambion) according to the manufacturer’s protocol and incubated at 37°C for 8 h before DNase treatment and isopropanol precipitation. The final pellet was resuspended in nuclease-free dH2O. A sample of each RNA was denatured with a formamide-based RNA sample buffer (Ambion) and analyzed for size and quality on a RNase-free denaturing gel.

MA104 cells were seeded in a six-well plate (70,000 cells/ml) and grown overnight to <50% confluence. Cells were washed once with serum-free Opti-MEM (Gibco). The in vitro transcribed RNA was mixed with 10 µl DMIRE-C (1,2-dimyristoylpropyl-3-dimethyl-hydroxy ethyl ammonium bromide and cholesterol) (Invitrogen) in serum-free Opti-MEM (Gibco) and added to the cells. DMIRE-C without RNA was used as a negative control and DMIRE-C with RNA isolated from the SHFV parental pool was used as a positive control. The transfection media was removed after 4 h and 2 ml of fresh media were added to each well. Culture fluid (500 µl) harvested at 5 days was used to infect a fresh monolayer. Three serial passages of each sample were performed.
4.2.5 **Plaque Assay**

Plaque assays were performed on confluent monolayers of MA104 cells in six-well plates. Briefly, culture fluid was centrifuged at 1000 rpm for 5 min at 4°C, serially diluted in growth media and 100 µl of diluted sample was added to each well. After adsorption for 1 h at room temperature, inoculum was removed and wells were overlaid with 1% SeaKem ME agarose (Bio-Whittaker Molecular Applications), mixed 1:1 with 2x MEM containing 5% FCS, and incubated at 37°C for 72 h. After removal of agarose, cells were stained with 0.05% crystal violet in ethanol. Plaque titrations were performed in duplicate for each sample.

4.2.6 **Western blot**

Culture media was removed from infected monolayers of MA104 cells in 6 well plates and RIPA buffer (1x phosphate-buffered saline, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) containing Halt protease inhibitor cocktail (Thermo Scientific) was added to lyse cells. Proteins in the lysate were separated by SDS-PAGE, and then transferred to a nitrocellulose membrane by electrophoresis. Membranes were blocked with 1x Tris-buffered saline (TBS), pH 8.0, containing 5% non-fat dry milk and 0.1% Tween 20 before incubation with a polyclonal primary antibody specific for SHFV capsid (NETH-YVFAEPGDLC), nsp1β (FAQKVITAFPEGVLC) or actin (C-11; Santa Cruz Biotechnology, Santa Cruz, CA) in the presence of blocking buffer. Blots were washed with 1x TBS and incubated with secondary antibody (horseradish peroxidase-conjugated anti-rabbit or anti-mouse; Santa Cruz) and were then processed for chemilluminescence using a Super-Signal West Pico detection kit (Pierce, Rockford, IL) according to manufacturer’s protocol.

4.2.7 **Digoxigenin (DIG) -labeled RNA probes for sg mRNAs**

Primers were designed based on the locations of the transcription regulatory sequences (TRS) for each of the eight SHFV sgRNAs previously predicted (46) and used to amplify templates by RT-PCR for making negative sense sg mRNA probes. Primers were also designed to amplify a template for a 5'-leader probe (Table 6). Each reverse primer contained a T7 promoter. The PCR products were validated by se-
sequencing and used as templates for T7 polymerase *in-vitro* transcription of the RNA probes. The probes were labeled by incorporation of DIG using a DIG Northern Starter Kit (Roche) according to the manufacturer’s protocol. The concentration of each labeled probe was determined by a dot-blot assay using different dilutions of a DIG-labeled human actin RNA probe of known concentration as a control (Roche). Briefly, 1 µl of each dilution was spotted onto an Amersham Hybond-N+ membrane (GE Healthcare) and then UV-crosslinked. The membrane was blocked with DIG blocking solution (Roche), incubated with anti-DIG antibody (1:10,000 dilution, Roche) and then with CDP-Star (Roche). The signal was detected with an LAS4000 mini Luminescent Image Analyzer (GE Healthcare). Spot intensities were determined using Multi Gauge V2.3 software.

### 4.2.8 Northern Blot hybridization

MA-104 cell monolayers in 6 well plates were either mock infected or infected with SHFV infectious clone virus at a MOI of 1. At different times after infection, total cell RNA was extracted with TRI reagent (Molecular Research Center, Inc.). NorthernMax formaldehyde loading dye (Ambion) was added to 1 µg of RNA and the RNA was denatured at 80°C for 10 min, and then separated on a 1% formaldehyde agarose gel for 2.5 h at 100V. RNA Millennium Markers-Formamide (Ambion) were run on one lane of the gel. The capillary transfer method was used to transfer the RNA overnight onto an Amersham Hybond-N+ membrane (GE Healthcare) and the RNA was then UV-crosslinked to the membrane. The lane containing the RNA standards was cut from the membrane and stained with methylene blue buffer. The rest of the membrane was pre-hybridized with DIG Easy Hyb buffer (Roche) at 68°C for 30 min and then hybridized with 100 ng/mL of a DIG-labeled, denatured RNA probe at 68°C overnight. The hybridized membrane was washed first with low stringency buffer (2 x SSC + 0.1% SDS) at room temperature, then with high stringency buffer (0.1 x SSC + 0.1% SDS) at 68°C followed by blocking with DIG blocking solution (Roche). To detect the RNA bands, membranes were incubated with anti-DIG antibody
(1:10,000 dilution, Roche), developed with CDP-Star (Roche) and imaged with an LAS4000 mini Lumin-nescent Image Analyzer (GE Healthcare).

### 4.2.9 Quantitative real-time RT-PCR

SHFV genome RNA in culture fluid was isolated with TRI Reagent. Primer/probe sequences targeting the helicase (nsp9) region were forward primer 5'-cgtacaccegccgtctgct-3'; TaqMan probe 5'-6FAMttgacgttctcacaaggMGBNFQ-3'; reverse primer 5'-cggcaagtggcatccaa-3'. Reaction mixtures contained 500 ng of RNA, the primer pair (1 µM) and probe (0.2 µM) in a total of 10µl. Total extracellular SHFV genomic RNA was quantified using a standard curve generated with serial dilutions of a known concentration of SHFV genomic RNA that had been in vitro transcribed with MAXIscript SP6 transcription kit (Ambion) from a cDNA clone of nsp9. Transcribed RNA was purified by DNase digestion, phenol-chloroform extraction and ethanol precipitation before quantification by UV spectrophotometry to measure the number of RNA molecules present in the sample. One-step RT-PCR was performed and analyzed as previously described (100).

### 4.3 Results

#### 4.3.1 Construction of a full-length cDNA infectious clone of SHFV-LVR

An SHFV infectious clone was required to analyze the functional requirement of the duplicated SHFV minor structural proteins. A full-length cDNA clone of the SHFV, strain LVR, genome was constructed using a previously described strategy (40, 85, 137, 139) (Fig. 4.1). Five overlapping cDNA fragments covering the entire genome were amplified as described in Materials and Methods. Multiple nt mutations were present in each fragment compared to the Genbank consensus sequence and each mutation was changed to the Genbank consensus sequence by site-directed mutagenesis in the fragment clones. The corrected fragment clones were cut with PflMI and simultaneously ligated in the correct orientation into the large capacity pACYC184 vector (Fig. 4.2A). Full-length infectious clone plasmids were maintained in XL10 Gold ultracompetent cells and sequenced. Plasmids with the correct full-length sequence
were linearized by restriction enzyme cleavage and \textit{in vitro} transcribed with the mMessage mMMachine SP6 kit followed by DNase digestion and lithium chloride precipitation as described in Materials and Methods. The resulting RNA was analyzed on an RNase-free denaturing agarose gel (Fig. 4.2B). Six RNA bands were detected with sizes of 15, 13, 12, 7, 4 and 3 kb. The 15 kb band was expected to be the full-length viral RNA while the shorter products were the result of premature termination or aberrant transcription.

MA104 cells transfected with \textit{in vitro} transcribed RNA were observed for 120 h for cytopathic effect (CPE). Transfected cell lysates were collected after 120 h and analyzed by Western blotting for both capsid and nsp1\(^\beta\). Transfected cells did not show obvious CPE and viral proteins were not detected in cell lysates. The harvested culture fluid was serially passaged four times in an attempt to recover virus but neither CPE nor intracellular viral protein was not detected.

Since the Genbank consensus sequence of SHFV was obtained by shot gun cloning, it was possible that it contained some mistakes. RNA extracted from the parental SHFV pool was subjected to 454 sequencing. In addition, the original uncorrected fragment clones were re-amplified and sequenced. The 454 sequencing and fragment sequencing revealed the same 18 nt changes compared to the consensus sequence (Table 7). Genomic fragments were constructed with these 18 nt changes and these fragments were used to construct a new full-length clone (SHFVic). MA104 cells transfected with 100 or 500 ng of \textit{in vitro} transcribed RNA from this infectious clone showed CPE by 72 h and Western blot analysis detected nsp1\(^\beta\) in cell lysates (Fig. 4.3A). To confirm the production of infectious virus after SHFVic RNA transfection, 100 \(\mu\)l of undiluted culture fluid from transfected cells was passaged onto fresh MA104 cells. These cells showed CPE by 24 h after infection and Western blot analysis confirmed the presence of intracellular nsp1\(^\beta\) protein by 72 h.

To determine whether the rescued virus had replication kinetics comparable to the parental LVR virus stock, MA104 cells were infected with either the first passage infectious clone virus or with the parent virus at an MOI of 1 and viral titers were quantified by plaque assay on MA104 cells (Fig. 4.3B). The parental virus and the infectious clone virus showed similar replication kinetics and both produced \(10^6\)
PFU/ml by 72 h after infectious. Western blot analysis detected similar levels of nsp1β and capsid proteins by 24 h after infection in both parental virus and infectious clone virus infected cell lysates (Fig. 4.3C). These data indicate that the replication kinetics of virus derived from the infectious clone are comparable to those of the parental LVR virus.

4.3.2 Analysis of sg mRNA production during SHFV infection

Arteriviruses were previously reported to produce six sg mRNAs in infected MA104 cells (103, 127). The completion of the sequence of the 3′ structural ORF region indicated that the SHFV genome contained additional 3′ ORFs and analysis of the sg mRNA 5′ leader-body junction by RT-PCR amplification of intracellular RNA indicated that nine SHFV sg mRNA were produced (47, 140). To determine the expression kinetics of the SHFVic virus sg mRNAs in MA104 cells, cellular RNA isolated at different times after infection was analyzed by Northern blotting using probes directed against the 5′ leader or 3′ terminal region of the genome (Fig. 4.4A and 4.4B). A total 12 instead of the expected 9 bands were detected by 8 h after infection with sizes of 15.7, 5.0, 4.3, 4.0, 3.5, 2.8, 2.6, 2.0, 1.6, 1.5, 1.2 and 0.7 kb. With the exception of the 4.3, 1.6 and 1.5 kb bands, the detected sg mRNAs corresponded to the previously described 9 sg mRNAs produced during an SHFV-LVR infection (46). The intensities of the sg mRNA bands differed with sg mRNA 2, 4, 7, 8 and 9 being the most abundant at all times after infection. Previous Northern blot analyzes of SHFV sg mRNAs detected these 5 strong bands and reported the sg mRNA 5 and 6 bands together as the single additional sg mRNA band (47, 140). Interestingly, the relative abundance patterns of the sg mRNAs of the two sets of duplicated SHFV 3′ ORFs were similar with sg mRNAs 2 and 4 abundantly expressed and sg mRNA 3, 4, 5 and 6 expressed at lower levels. To further analyze the three additional sg mRNAs detected, additional Northern blot analyses of total cellular RNA isolated at 12 h after infection were done with probes directed against the known 9 sg mRNAs. The results confirmed the identification of the sg mRNAs 2 through 9 bands (Fig. 4.4C). The additional 4.3 kb band was detected with the probes for sg mRNA 2 through sg mRNA 9 indicating that this sg mRNA includes sequences located downstream of the sg mRNA 2 TRS and upstream of the predicted sg mRNA 3
TRS which suggested that this novel sg mRNA may be sg mRNA2b. The possibility of a 2b sg mRNA was predicted by the previous TRS sequence analysis but evidence of the sg mRNA was not previously obtained by RT-PCR amplification (46). The 1.5 and 1.6 kb bands were detected with probes targeting sg mRNA 7 through sg mRNA9 indicating that these RNAs include sequence located downstream of the predicted sg mRNA 7 TRS and upstream of the sg mRNA 9 TRS. These two sg mRNAs contain two possible start codons located at nts 14559 and 14632. Translation initiating from the first AUG would produce a 23 aa peptide from an alternative frame from ORF7. Translation from the second AUG, which is in frame with ORF7, would produce a 69 aa C-terminal GP7 peptide.

4.3.3 Analysis of the functional relevance of the individual minor structural proteins

The SHFV genome is predicted to encode 8 minor structural proteins while only 4 minor structural proteins are encoded by EAV and PRRSV genomes (46). Northern blot analysis indicated that sg mRNAs were produced for each of the minor structural proteins in SHFV-infected cells. To inhibit protein expression, the translational start site for each of the 8 minor structural proteins was mutated individually to an Ala residue without altering the coding of overlapping ORFs in the appropriate fragment plasmid. An additional replication defective mutant was constructed by mutating the RNA-dependent RNA polymerase catalytic Ser-Asn-Asn (SDD) residues to Ser-Ala-Ala (SAA). Full-length mutant clones were then assembled using the same methods used to construct the wildtype SHFVic. Three full-length clones for each mutant were made and separately transfected into MA104 cells. Transfected cells were observed for CPE for 120 h. Only a single ΔGP3 clone showed obvious CPE at 72 h after transfection comparable to that observed after transfection with the wild type SHFVic RNA suggesting a spontaneous reversion of this clone had occurred. Cells transfected with RNA from all of the other minor structural protein mutant clones tested did not show CPE by 120 h after transfection similar to cells transfected with RNA from the SAA mutant. Culture fluid from transfected cells was passaged onto naïve MA104 cells and cells were observed daily for CPE. Again, the same ΔGP3 clone induced CPE by 120 h after passage while all of the other mutants as well as the other two ΔGP3 clones did not induce obvious CPE. Five se-
rial passages of culture fluid for each of the mutants also did not result in the detection of CPE on MA104 cells.

To determine whether the single ΔGP3 clone had reverted, RNA was isolated from both transfected culture fluid and 1st passage culture fluid and a 500 nt region surrounding the AUG mutation was sequenced. The original transfected RNA was also sequenced to determine whether a spontaneous mutation had occurred during *in vitro* transcription. The expected Met-Ala mutation was present in the *in vitro* transcribed RNA as well as in RNA isolated from both the transfection plate culture fluid and passage culture fluid. A single c11773t substitution was observed only in the passage culture fluid RNA. This nt change did not create a new AUG but did change an Ala to a Val in the first E protein (E1) ORF but was silent in the overlapping GP2b protein ORF. The data suggest that the phenotype change observed was due to a second site mutation in another part of the genome.

To determine whether viral proteins were produced after transfection with RNA from the mutated clones, transfected cell lysates were collected at 120 h after transfection and analyzed by Western blotting for nsp1β protein. Nsp1β was not detected in cells transfected with any of the mutant RNAs (Fig. 4.5A). Recent data suggested that all four of the minor structural proteins of EAV and PRRSV are functionally required during viral attachment/entry to host cells (118). To determine whether a low level of non-infectious progeny virus was produced from the mutant RNA transfected cells but was not amplified because it was unable to infect cells, RNA was isolated from culture fluid at various times after transfection and helicase RNA was quantified by qRT-PCR. As expected, transfection of SHFVic RNA resulted in a large increase in extracellular viral RNA by 96 h after transfection that increased by 120 h while the SAA mutant did not produce an increase in extracellular RNA above background levels. The GP2a and GP2b mutant RNA transfections also did not produce significant increases in extracellular viral RNA by 120 h after transfection indicating that progeny virions were not released (Fig. 4.5B and 4.5C). The GP3, GP4 and GP5 mutants all produced sustained levels of extracellular viral RNA that was first detected at 24 h after transfection indicating that progeny virions were produced (Fig. 4.5D, 4.5E and 4.5F). The GP6 mutant clone also produced extracellular viral RNA by 24 h after transfection but the levels decreased by 48
suggesting the possibility that the virions produced were unstable (Fig. 4.5G). However, the amount of extracellular virus produced by the GP3, GP4, GP5 and GP6 mutant RNAs was very low compared to the amount produced by the wildtype RNA.

Sequence homology between GP2a and GP4 and between GP3 and GP6 of SHFV was previously reported and it was suggested that these proteins might be functionally redundant (46). However, the data shown in Figure 4.5 indicates that GP2a and GP4 are not functionally redundant since different effects on virus production were observed with the loss of each protein. The data also indicated that GP2b and GP5 do not have redundant functions. The observation that non-infectious virus was present in culture fluid after transfection with the GP3 or GP6 individual mutant RNA suggests that these two proteins may have some functional redundancy. Infectious clones were next made that had the AUGs of both the GP2a and GP4 or GP2b and GP5 or GP3 and GP6 ORFs mutated. No CPE was observed by 120 h after transfection of the GP2a/GP4 or GP2b/GP5 double mutants or during 4 serial passages and no detectable intracellular viral protein was observed after transfection or passage (Fig. 4.6A). Also, no extracellular viral RNA was detected after transfection with these double mutants (Fig. 4.6B and 4.6C). A mutant with the AUGs of both ORF3 and ORF6 mutated produced no CPE by 120 h after transfection or after 4 serial passages (data not shown). Also, no intracellular viral protein was detected 120 h after transfection or after passage (Fig. 4.6A). Interesting, no extracellular viral RNA was detected after transfection suggesting the possibility that these two proteins may have functional redundancy (Fig. 4.6D).

Previous work indicated that the E protein translated from the sg mRNA2s of PRRSV and EAV is essential for virus entry but not for virion production (117). To determine whether either of the SHFV E proteins is required for extracellular virus production, infectious clones with the AUG of one of the E ORFs mutated were made and in vitro transcribed RNA was transfected into MA104 cells. CPE was not observed in the transfection plates or during 5 serial passages with either E mutant. There was no detectable intracellular viral protein produced by 120 h after transfection or after passage indicating that both E1 and E2 are required for virus replication (Fig. 4.7A). Quantification of viral RNA in transfected culture fluid indicated that the E1 mutant produced a low level of extracellular viral RNA at 24 h after transfection.
tion, but this level was comparable to the level of extracellular viral RNA observed at 24 h after transfection with SAA RNA (Fig. 4.7B). The E2 mutant did not produce detectable extracellular viral RNA by 120 h after transfection (Fig. 4.7C).

4.4 Discussion

Multiple infectious clones for EAV and PRRSV strains have been constructed (34, 85) and used to investigate viral replication as well as the induction of host immune responses. The SHFVic constructed as part of this study is the first full-length cytopathic cDNA infectious clone of SHFV. The SHFV clone also represents the longest known full-length RNA genome that has been stably maintained within a single plasmid because the infectious cDNA clones of coronaviruses are maintained as separate fragment clones due to instability of maintaining full-length clones in a single plasmid (137, 139). This clone represents a valuable tool for further studies of virus replication in the MA104 cell line as well as virus replication and induction of host responses in primary cell cultures from disease-resistant and susceptible primates.

Previous reports have suggested that SHFV produces a total of 8 sg mRNAs that would result in the production of 3 major structural proteins and 6 minor structural proteins due to the sg mRNA2 encoding for two proteins (46, 47, 140). Northern blot analysis indicated that a total of 12 sg mRNAs were produced between 8 and 32 h after infection and while the lengths of 9 of the sg mRNAs closely match those predicted for the 9 previously identified sg mRNAs, three additional sg mRNAs with molecular weights of 4.3, 1.6 and 1.5 kb were also identified. The long RNA probes used in the present study detected the 4.3 kb sg mRNA2b, but the level of this RNA was low compared to other sg mRNAs. These data indicate that SHFV GP2b is not translated from a bicistronic sg mRNA2 but is translated from an independent sg mRNA that would have the previously predicted TRS at nts 11337 to 11345.

The current Northern analysis also revealed the presence of two novel sg mRNA of ~1.5 kb and 1.6 kb in length that both would have a TRS located 3’ of the sg mRNA 7 TRS and 5’ of the sg mRNA 8
TRS. Two potential TRS sequences are located at nts 14251 to 14260 (AACACTCACT) and at nts 14294 to 14302 (ACTCTGACT). Translation initiation at an AUG located at 14559 in both of these sg mRNAs would result in a 23 aa protein. Translation initiation from a second AUG located at 14632 in both sg mRNAs would produce a 69 aa C-terminal protein of GP7. Further work is required to determine whether either of these proteins are produced during an SHFV infection or required during viral replication.

Analysis of the effect of inhibiting the expression of each of the minor structural proteins using the SHFVic indicated that all six of the minor glycoproteins and both of the E proteins are required for production of infectious virions. While none of the mutant constructs were able to produce infectious virions, low levels of extracellular virions were produced when GP3, GP4, GP5 or GP6 was not expressed but these virions were non-infectious. The data also suggested that virions lacking GP6 were not stable.

Although the data indicate that each of the SHFV minor proteins are functionally important for the production of infectious virions, it is not yet know whether all of them are structural components of virions. EAV and European PRRSV virions contain heterotrimers of GP2, GP3 and GP4 while US PRRSV and LDV virions contain heterodimers of GP2 and GP4 with GP3 released as a soluble protein but still critical for heterodimer formation (6, 103, 118, 132). However, another study obtained data indicating that GP3 is a structural component in virions produced by the vFL12 infectious clone that is based on a US strain of PRRSV (22). When either SHFV GP2a or GP2b was not expressed, extracellular virions were not detected suggesting the likelihood that these two proteins form a heterodimer similar to the GP2 and GP3 proteins of other arteriviruses that is required for virion assembly and egress. This was not the case for the SHFV homologs of these two proteins, GP4 and GP5 because neither of these proteins was absolutely required for extracellular virus production. However, GP4 and GP5 do appear to be required for virion infectivity.

Interestingly, while knockout of either GP3 or GP6 resulted in the release of non-infectious progeny virus the GP3/GP6 double knockout mutant did not produce detectable levels of extracellular virus, suggesting that GP3 and GP6 may have a redundant function. It is possible that this redundant function may be related to efficient formation of the GP2a/GP2b heterodimer. A previous study reported that the
GP3 of European PRRSV strains is required for formation of the GP2/GP4 heterodimer (23). The data obtained provide some additional support for the hypothesis that the 5′ set of minor protein ORFs has retained the original functions while the homologous 3′ ORFs have functionally diverged but are still required for efficient virion production.

Previous studies have indicated that the E protein of other arteriviruses forms an ion-channel that functions during entry (67, 135). The SHFV genome is predicted to encode for two E proteins in the 5′ (E1) and 3′ (E2) sets of minor protein ORFs. When E1 was mutated, only a minimal amount of extracellular viral RNA was detected while when E2 was mutated, no extracellular viral RNA was observed. These data indicate that the two SHFV E proteins have divergent functions but both are required for production of infectious progeny virions. Interestingly, a recent study indicated that the myristoylation motif present on the E protein of EAV is not required for virion entry but is critical for virus replication (117). Data presented in the current study supports this hypothesis because the E2 protein mutant, which contains the myristoylation motif, did not produce extracellular virus RNA while the mutant lacking non-myristoylated E1 protein was still able to produce a very low level of extracellular viral RNA. It is likely that the E1 protein is required for formation of stable virion particles due to the observation that the extracellular RNA observed at 24 h after transfection quickly decreased, likely as a result of unstable virion degradation.
Table 5. Primers used to generate overlapping fragments from SHFV-LVR RNA.

<table>
<thead>
<tr>
<th>Primer ID</th>
<th>Sequence (5’ to 3’)\textsuperscript{a,b}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fragment I</td>
<td>SP6-1 act agt ATT TAG GTG ACA CTA TAG ATT AAA ATA AAA GTG TGA AGC</td>
</tr>
<tr>
<td>Fragment I</td>
<td>232R GTT GGT GGG TTA CCA GTC TCA GTT CC</td>
</tr>
<tr>
<td>Fragment II</td>
<td>2307 TAC GTT GTG CGC CTT GAC TCT GAC</td>
</tr>
<tr>
<td>Fragment II</td>
<td>7991 GCT TGC CAG ACA GAA ATT TGA GAC TG</td>
</tr>
<tr>
<td>Fragment III</td>
<td>7856 TGG TCT CTC CTC AGG TGA TC</td>
</tr>
<tr>
<td>Fragment III</td>
<td>12306 AGT CAT GTT GCC TGT AAT TGT CTC</td>
</tr>
<tr>
<td>Fragment IV</td>
<td>12213 TAT GTC TAT CGT CCA CCA CTT GTC</td>
</tr>
<tr>
<td>Fragment IV</td>
<td>12960 AGG TAT TTA GAA AGT CCA GTC ACG</td>
</tr>
<tr>
<td>Fragment V</td>
<td>12662 TCT GCT GGT TGG TAA AAT GCT CTC ACT CAC</td>
</tr>
<tr>
<td>Fragment V</td>
<td>15717 GCG GCC GCC GAT CGT (T)\textsubscript{42} cga tcc gcg gcc gcA A</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Recognition sites for restriction enzyme SpeI, PvuI and NotI are indicated by lowercase.

\textsuperscript{b} SP6 promoter consensus sequence is indicated by underlined nts.
Table 6. Primers used to generate probes to detect SHFV sg mRNAs.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5’ to 3’)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Target</th>
<th>Probe length (nt)&lt;sup&gt;b&lt;/sup&gt;</th>
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</thead>
<tbody>
<tr>
<td>sgRNA2-F</td>
<td>TTGTATCTGCCTACAAACAATTTGGGTCGCGCG</td>
<td>sg mRNA2</td>
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<tr>
<td>sgRNA2-R</td>
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<td>sgRNA3-F</td>
<td>CCGCAACTACACCTCGGCTCTAACAATCAAGCG</td>
<td>sg mRNA3</td>
<td>520nt (11902-12421)</td>
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<tr>
<td>sgRNA3-R</td>
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</tr>
<tr>
<td>sgRNA4-F</td>
<td>CCATCATTTTGCATGCTTGGCGATCTTTAGAG</td>
<td>sg mRNA4</td>
<td>529nt (12509-13037)</td>
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<tr>
<td>sgRNA4-R</td>
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</tr>
<tr>
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<tr>
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</tr>
<tr>
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<td>sg mRNA8</td>
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<tr>
<td>5'-leader-F</td>
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<td>5'-leader</td>
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<td>cggtagttaaac gcacactatagggtTCTGCAATCCCAAGGCCAC</td>
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</tbody>
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<sup>a</sup>T7 promoter sequence is shown in lowercase.

<sup>b</sup>Nt numbering according to the SHFV infectious clone genome RNA.
Figure 4.1 Construction of a full-length SHFV-LVR cDNA infectious clone. (A) SHFV genome organization. The SHFV ORFs are shown as boxes. The 3’ ORFs include the minor structural ORFs (gray) and the three major structural ORFs (white). (B) Single step strategy for SHFVic assembly. Overlapping fragments were generated by RT-PCR and cloned into pCR-XL-TOPO. Fragment clones were cleaved with PfoI alone (fragments II, III or IV) or in combination with SpeI (fragment I) or with NotI (fragment V) before ligation into the large capacity vector pACYC184 cut with XbaI and EagI to pair with the SpeI sticky end in fragment I and the NotI sticky end in fragment V. RFS - ribosomal frameshift sequence. M - non-glycosylated membrane protein. N - nucleocapsid protein.
Figure 4.2 Assembly and transcription of the SHFV infectious clone. (A) The five viral genome fragment clones in pCR-XL-TOPO were cut with PfIMI alone (fragment 2 and 4) or in combination with RsrII (fragment 3), SpeI (fragment 1) or NotI and RsrII (fragment 5). * indicates viral genome fragments. The pACYC184 vector was cut with XbaI and EagI (V). (B) In vitro transcribed RNA was treated with DNase and precipitated with lithium chloride before analysis on an RNase-free denaturing agarose gel. Arrow indicates full-length RNA generated from the infectious clone cDNA. Arrowheads indicate shorter RNA products.
Table 7. Nt differences between Genbank consensus and 454 consensus of parental virus stock.

<table>
<thead>
<tr>
<th>Position</th>
<th>Nt in parent</th>
<th>Nt in cDNA clone</th>
<th>Amino Acid Change</th>
<th>Gene</th>
</tr>
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<td>511</td>
<td>C</td>
<td>T</td>
<td>P101L</td>
<td>nsp1α</td>
</tr>
<tr>
<td>849</td>
<td>G</td>
<td>T</td>
<td>V214F</td>
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<td>2503</td>
<td>G</td>
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<td>G765A</td>
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<tr>
<td>3726</td>
<td>C</td>
<td>-</td>
<td>10 aa</td>
<td>nsp2</td>
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<td>14033</td>
<td>C</td>
<td>T</td>
<td>P11S</td>
<td>GP7</td>
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Figure 4.3 Growth kinetics of RNA from SHFV parental virus and the infectious clone. MA104 cells were transfected with 0, 100 or 500 ng RNA transcribed from the SHFV infectious clone. Culture fluid from transfected cells was collected at 72 h and passaged onto naïve MA104 cells for 72 h. (A) Cell lysates from transfection or passage 1 cells were analyzed for viral nsp1β protein by Western blot. (B) Virus yields from MA104 cells infected with parental virus or SHFVic virus harvested from transfected cells at an MOI of 1 were quantified by plaque assay on MA104 cells. Parental virus (■); SHFVic virus (●). Data points indicate averages of samples from three independent experiments each measured in duplicate. Error bars represent standard deviation of mean (124). (C) Cell lysates were analyzed for nsp1β and capsid by Western blot. Blots shown are representative of three independent experiments. Actin was used as a loading control.
Figure 4.4. Northern blot analysis of sg mRNAs produced in SHFVic virus infected MA104 cells. Total cellular RNA was isolated from MA104 cells that were mock infected or infected with SHFVic at an MOI of 1 for 24 h. Total cellular RNA (1 µg) was separated by electrophoresis on a 1% denaturing agarose gel before transfer to an Hybond-N+ membrane and hybridized with DIG-labeled RNA probes specific for the (A) 5’ leader sequence, (B) sg mRNA9 or (C) sg mRNA2 through sg mRNA8. Full-length or specific sg mRNAs were identified based on size. M - mock infected, 1,2,3 – biological replicates.
Figure 4.5 Analysis of the functional relevance of GP2a, GP2b, GP3, GP4, GP5 and GP6. MA104 cells were transfected with RNA generated from the parental infectious clone (IC), the replication defective mutant clone (SAA) or the minor structural protein mutant clones for 120 h. Culture fluid was collected at 120 h and passaged onto naïve MA104 cells for 120 h. (A) Western blotting of cell lysates collected 120 h after transfection or passage with antibodies against nsp1β. Blots shown are representative of three independent experiments. β-actin was used as a loading control. (B to G) Analysis of extracellular viral RNA. RNA was extracted from culture fluid at various times after transfection with wildtype RNA (gray), replicase mutant (white) or (B) GP2a, (C) GP2b, (D) GP3, (E) GP4, (F) GP5, or (G) GP6 mutant (black) RNA. Extracellular viral RNA was quantified from 100 ng of isolated RNA by qRT-PCR. Values shown are representative of two independent experiments and each sample was assayed in triplicate. Error bars represent SD. The full range of the wildtype viral RNA production is only shown in panel B.
Figure 4.6 Analysis of the effect of mutation of the AUGs of pairs of duplicated minor structural proteins. MA104 cells were transfected with RNA generated from the parental infectious clone (IC), the replication defective mutant clone (SAA) or the minor structural protein mutant clones for 120 h. Culture fluid was collected at 120 h and passaged onto naïve MA104 cells for 120 h. (A) Western blotting of cell lysates collected 120 h after transfection or passage with antibodies against nsp1β. Blots shown are representative of three independent experiments. β-actin was used as a loading control. (B to D) Analysis of extracellular viral RNA. RNA was extracted from culture fluid at various times after transfection with wildtype RNA (gray), replicase mutant (white) or (B) GP2a/4, (C) GP2b/5 or (D) GP3/6 mutant (black) RNA. Extracellular viral RNA was quantified from 100 ng of isolated RNA by qRT-PCR. Values shown are representative of two independent experiments and each sample was assayed in triplicate. Error bars represent SD. The full range of the wildtype viral RNA production is only shown in panel B.
Figure 4.7 Analysis of the functional relevance of the SHFV E proteins. MA104 cells were transfected with RNA generated from the parental infectious clone (IC), the replication defective mutant clone (SAA) or the minor structural protein mutant clones for 120 h. Culture fluid was collected at 120 h and passaged onto naïve MA104 cells for 120 h. (A) Western blotting of cell lysates collected 120 h after transfection or passage with antibodies against nsp1β. Blots shown are representative of three independent experiments. β-actin was used as a loading control. (B and C) Analysis of extracellular viral RNA. RNA was extracted from culture fluid at various times after transfection with wildtype RNA (gray), replicase mutant (white) or (B) E1 or (C) E2 mutant (black) RNA. Extracellular viral RNA was quantified from 100 ng of isolated RNA by qRT-PCR. Values shown are representative of two independent experiments and each sample was assayed in triplicate. Error bars represent SD. The full range of the wildtype viral RNA production is only shown in panel B.
5 FUNCTIONAL ANALYSES OF THE THREE SIMIAN HEMORRHAGIC FEVER VIRUS PAPAIN-LIKE PROTEASES

5.1 Introduction

SHFV is a member of the Family Arteriviridae. Other members include the prototype EAV, LDV and PRRSV. Based on genomic organization and replication strategy similarities, this virus family has been classified in the Order Nidovirales with the Families Coronaviridae and Roniviridae (33, 104). Arterivirus genomes are polycistronic, positive polarity, single-stranded RNA with a 5´ type I cap and a 3´ poly (A) tract (97, 98). The SHFV genome, which is approximately 15.7 kb in length and contains a 5´ non-coding region (NCR) of 209 nt and a 3´ NCR of 76 nt, is the longest known arterivirus genome. The structural proteins are encoded at the 3´ end of the genome and expressed from a nested set of 5´ and 3´ co-terminal sg mRNAs (46, 47). Two nonstructural polyproteins (1a and 1ab) are encoded by ORF1a and 1ab located at the 5´ end of the genome. Translation of polyprotein 1a terminates at the first in-frame UAA. Polyprotein 1ab is produced when a -1 ribosomal frameshift occurs just before the 1a stop codon (30, 103, 104).

All arterivirus polyproteins contain cysteine protease (CP), serine protease (SP) and papain-like cysteine protease (PLP) domains (129). Each of the different arterivirus genomes encodes one CP and one SP, but the number of PLP varies (142). Arterivirus PLPs contain the catalytic Cys-His dyad present in all PLPs encoded by other positive-sense RNA viral genomes, including coronaviruses. However, the linker region between the cysteine and histidine residues in arterivirus PLPs is half the length of that in coronavirus PLPs making the arterivirus PLPs the smallest currently known (49, 142). The coronavirus PLPs cleave at one or two sites upstream of their catalytic domains while the arterivirus PLPs studied to date cleave at a single site downstream of their catalytic domains (8, 103, 142).

ORF1a of EAV encodes an N-terminal PLP (PLPα) that is inactive due to a Cys to Lys substitution in the catalytic site, but a second active PLP (PLPβ) located downstream of PLPα cleaves at a Gly-Gly dipeptide located 31 aa downstream of the PLPβ catalytic His residue (106, 142). Due to the inactive
PLPα, a fusion protein (PLPα + PLPβ) of approximately 30 kDa and named nsp1 is produced after translation of the EAV ORF1a (106). Despite the lack of a known nuclear localization signal, this fusion protein can localize to the nuclei of infected cells but is also known to be essential for transcription of viral RNA as well as efficient virion assembly in the cytoplasm (119, 121). The ORF1a regions of the LDV and PRRSV genomes encode active PLPα and PLPβ enzymes that cleave at an unknown site or at an identified Tyr-Gly dipeptide, respectively (30, 142). Although a previous report suggested that both PRRSV nsp1 proteins can localize to the nuclei of infected cells (15), another study reported that nsp1α shuttles between the cytoplasm and nucleus while nsp1β is only localized to the cytoplasmic compartment (109). The zinc-finger of EAV nsp1 and PRRSV nsp1α was shown to be required for sg mRNA production while the PRRSV nsp1β protein was reported to be involved in viral genome replication (15, 64, 122).

The SHFV genome encodes three PLP domains (PLPα, PLPβ and PLPγ) at the 5' end of ORF1a that have not previously been functionally analyzed (142). Sequence alignment and homology modeling of the three SHFV nsp1 sequences on a PRRSV nsp1β structure (41) predicted that each of these proteins could fold into a structure similar to that of PRRSV nsp1β with a cysteine and a histidine at comparable positions to the catalytic residues of PRRSV nsp1β and canonical cleavage site dyads were predicted downstream of each PLP. The activity of each SHFV PLP was assessed following mutation of its predicted catalytic Cys residue. In addition, the cleavage dyads at each nsp1 protein junction were functionally analyzed. Finally, processing of the polyprotein and subcellular localization of each nsp1 protein was analyzed in vivo.

5.2 Materials and Methods

5.2.1 Sequence and bioinformatics analyses

Sequence analysis was performed using the ORF1A protein sequence that was conceptually translated from a modified SHFV genome (accession no. AF180391). The SHFV PLPα, PLPβ and PLPγ boundaries were estimated using putative cleavage sites and active site domains for the known PLPs of
LDV and PRRSV. A conserved motif was identified and used as an anchor for determining the upstream portion of the putative PLPα sequence (Fig. 5.1). All sequences were manipulated in the Geneious software suite version 5.6.3 (BoiMatters, LTD; www.geneious.com) and multiple alignments of the PLP amino acid sequences were performed using ClustalX in Geneious, with similarity determined using the PAM250 substitution matrix. Once the putative boundaries were predicted, homology models were generated using the crystal structure of the PRRSV PLP protein (pdb code: 3IFU) as the template (ref: pubmed 19706710). Homology models were generated using Modeler (J. Mol. Biol. 234, 779-815, 1993; http://salilab.org/modeller/) as implemented in the Max Planck Institute’s Bioinformatics Toolkit (http://toolkit.tuebingen.mpg.de/), and the structures were rendered and compared using MacPymol (Delano Scientific, LLC; http://www.pymol.org).

5.2.2 Cells and virus

MA-104 cell line obtained from the Centers for Disease Control and Prevention (Atlanta, GA) was cultured in a 5% CO₂ atmosphere in minimal essential medium supplemented with 10% fetal bovine serum and 10 µg/ml gentamicin. An aliquot of strain LVR 42-0/M6941 passage 2 was obtained from the American Type Culture Collection, sequentially plaque-purified three times and then amplified once on MA104 cell monolayers. Stock pools of SHFV-LVR were prepared by infecting confluent MA-104 monolayers at an MOI of 0.2. Culture media was harvested at 32 h after infection. Clarified virus pools containing titers of ~10⁷ PFU/ml were stored at -80°C.

5.2.3 Construction of SHFV nsp1 clones

A region consisting of the 5´ 1933 nts of the SHFV genome, which included the 5´ NCR (209 nts) and the 5´ 1724 nts of ORF1a, was amplified by RT-PCR from purified SHFV-LVR genomic RNA using the EcoRI and TaqI tailed forward primer (5’-AGgaattetegaGATTAAAATAAAAGTGTGAAG-3’, restriction sites are indicated by lower case) and the XbaI tailed reverse primer (5’-GCtctagaACCGGCAGTACAGCATGGGT-3’). This region of ORF1a contains the three PLP domains and the putative cleavage sites for nsp1α, β and γ as well as the first 201 nts of nsp2. To generate con-
structs with an in-frame N-terminal FLAG tag and a C-terminal c-Myc tag, the coding region (nts 210 through 1933) was subcloned into the pFLAG/myc expression vector (Sigma) to generate pFlagSHFVmyc. To construct plasmid DNA for use as template in coupled in vitro transcription/translation reactions, the FLAG-SHFV-c-Myc region of the pFlagSHFVmyc clone was subcloned into the pTNT expression vector (Promega) to generate pTNT-FWm-wt. Mutagenesis of the pTNT-FSm-wt construct was performed using a QuickChange site-directed mutagenesis kit (Stratagene) according to the manufacturer’s protocol. The primers used to change the putative PLP catalytic Cys residues to Ala or to mutate the -2 and -1 residues of each predicted cleavage site with either Val or Ala are listed in Table 1. All wild type and mutant sequences generated were confirmed by sequencing.

5.2.4 **In vitro, coupled transcription/translation reactions**

Wild type and mutated pTNT-FSm cDNA constructs were the templates for in vitro, coupled transcription/translation reactions done using the TNT® Coupled Wheat Germ Extract System (Promega) according to manufacturer’s protocol. Briefly, 1 µg of the plasmid DNA was mixed with 25 µl of wheat germ extract, 2 µl of TNT® reaction buffer, 1 µl of amino acid mixture without Cys, 1 µl of TNT® SP6 RNA polymerase and 20 µCi of 35S Cys (Cys L-[35S] 1mCi/mmol, Perkin Elmer). Reactions were incubated at 30°C for 2 h prior to use for immunoprecipitation.

5.2.5 **Immunoprecipitation**

In vitro translation reactions were divided into four equal aliquots. One aliquot was stored at -20°C until use as the lysate control during gel electrophoresis. One aliquot was incubated with murine anti-IgG antibody and another with anti-c-Myc monoclonal antibody (Sigma) for 1 h with rotation at 4°C, after which Protein G agarose (Roche) was added and the samples were rotated at 4°C overnight. The other aliquot was incubated with anti-FLAG® M2 affinity agarose beads (Sigma) with rotation at 4°C overnight. Beads were washed 3 times in lysis buffer (1% Triton X-100, 0.1% SDS, 150 mM NaCl, and 50 mM Tris HCl pH 7.4) containing HALT protease and phosphatase inhibitor cocktail (Pierce Scientific), and then centrifuged. After addition of 30 µl of 2x sample loading buffer (20% SDS, 25% glycerol,
0.5 M Tris-HCl pH 6.8, 0.5% bromophenol blue and 5% β-mercaptoethanol), the samples were boiled for 5 min to denature the protein complexes attached to the agarose beads. The proteins were then separated by SDS-PAGE on a 13% polyacrylamide gel. Gels were fixed (10% acetic acid and 30% methanol), incubated first in AutoFluor (National Diagnostics), and then in anti-cracking buffer (7% acetic acid, 7% methanol and 1% glycerol), dried and autoradiographed.

5.2.6 Western blotting

Confluent MA104 monolayer cells were lysed by addition of RIPA buffer (1x phosphate-buffered saline, 1% Nonidet P-40, 0.5% sodium deoxycholate and 0.1% SDS) containing Halt protease inhibitor cocktail (Thermo Scientific). Following separation by SDS-PAGE, cell proteins were electrophoretically transferred to a nitrocellulose membrane. Membranes were blocked with 1x TBS containing 5% non-fat dry milk and 0.1% Tween 20 before incubation in the presence of blocking buffer with a monospecific polyclonal antibody made for this study by Abgent to detect one of the SHFV nsp1 proteins. The peptides used to make these antibodies were: nsp1α (NH2-GDLTRPEETPLPGGC-CONH2), nsp1β (a:NH2-FAQKVIT-AFP-EGVLC-CONH2 or b:NH2-DESVPDCQIIARF-CONH2), nsp1γ (NH2-FPPLSRK-SEAQRAIL-CONH2). Actin was used as a loading control and was detected with antibody C-11 (Santa Cruz Biotechnology). Blots were washed with 1x TBS (0.01 M Tris and 0.15 M NaCl, pH 8) and then incubated with secondary antibody, either anti-rabbit-horseradish peroxidase (Santa Cruz Biotechnology) or anti-mouse-horseradish-peroxidase (Santa Cruz Biotechnology). Washed blots were then processed for chemiluminescence using a Super-Signal West Pico detection kit (Pierce Scientific) according to manufacturer’s protocol.

5.2.7 Confocal microscopy

MA-104 cells grown on coverslips were mock-infected or infected with SHFV-LVR at an MOI of 10. At various times after infection, cells were fixed with 4% paraformaldehyde in PBS, permeabilized with 0.01% Triton-X in PBS and blocked in 5% horse serum overnight at 4°C. Cells were incubated with two primary antibodies; a rabbit anti-SHFV nsp1 (Abgent) and a murine anti-dsRNA antibody (English &
Scientific Consulting, Szirak, Hungary) at RT for 1 h, washed with PBS and then incubated with Alexa Fluor 488 goat anti-rabbit IgG, Alexa Fluor 594 rabbit anti-mouse IgG (Invitrogen) and Hoechst 33342 nuclear stain (Invitrogen) at RT for 1 h. After washing, the coverslips were mounted on microscope slides with ProLong® Gold anti-fade reagent (Invitrogen). Cells were visualized with a 40x oil-immersion objective on a LSM700 laser scanning confocal microscope (Zeiss) and images were analyzed with Zeiss LSM version 4.2 software.

5.3 Results

5.3.1 Analysis of SHFV nsp1 sequences by alignment and homology modeling

The catalytic residues previously identified for all studied coronavirus and arterivirus PLPs are a cysteine followed by a histidine. In all coronavirus PLPs the catalytic Cys is followed by an aromatic residue (Trp or Tyr) (49), while in all known active arterivirus PLPs a Trp is adjacent to the catalytic Cys (30). Alignment of the amino acid sequences of the three SHFV PLP catalytic domains revealed a single Cys-Trp dyad upstream of a histidine in each: Cys115/Trp116/His130 in PLP_α, Cys246/Trp247/His309 in PLP_β and Cys378/Trp379/His443 in PLP_γ (Fig. 5.1A). However, the predicted PLP_α Cys-Trp dyad was located only 14 aa upstream from the catalytic His residue while the PLP_β and PLP_γ dyads were located 62 and 64 aa residues from their respective histidines. A conserved GV[Q/T]G motif was identified approximately halfway between the catalytic Cys and His residues in the putative PLP_β and PLP_γ. The position of this motif in PLP_α (aa position 93 to 96) was used as an anchor to identify Cys63 in PLP_α as the more likely catalytic Cys for PLP_α. Cys63 aligned with the predicted catalytic cysteines in PLP_β and PLP_γ and was located 67 aa residues from the PLP_α catalytic histidine (Fig. 5.1A). However, Cys63 is followed by alanine instead of by an aromatic residue and a catalytic Cys/Ala dyad had not previously been identified in any known PLP. A recently resolved crystal structure of the PRRSV PLP_α region (41) was next used to generate a homology model of each of the SHFV PLP domains (Fig. 5.1B). In the modeled structure of SHFV PLP_α, Cys63 was located near the catalytic His130 in a position that was spatially
similar to those predicted for the putative catalytic Cys of both SHFV PLPβ and PLPγ (Fig. 5.1B) as well as for PRRSV PLPα (data not shown). These data supported the hypothesis that the SHFV PLPα catalytic residues are Cys63 and His130.

Both coronavirus and arterivirus PLPs cleave at Tyr or Gly residues that are adjacent to a Gly residue (142). Analysis of the SHFV nsp1 region sequence revealed seven Gly/Gly dyads and seven Tyr/Gly dyads. Sequence alignment and structure homology modeling of the three SHFV PLP regions suggested that PLPβ and PLPγ each cleave at a downstream Gly/Gly dyad and that PLPα cleaves at a unique Gly/Thr dyad (Fig. 5.2A). However, two Gly/Gly dyads (positions 150/151 and 164/165) detected 5 and 19 aa upstream from the predicted PLPα cleavage site are also possible PLPα cleavage sites. Also, three adjacent Gly residues were present at the predicted PLPγ cleavage site. The molecular masses of the peptides that would be produced by cleavage at the various sites were predicted (Fig. 5.2B).

A cDNA construct that expressed a polyprotein consisting of the N-terminal region of SHFV ORF1a (nts 210 through 1933) with an N-terminal Flag tag and a C-terminal c-Myc tag was made as described in Materials and Methods. The polyprotein encoded by this construct included the complete nsp1α, nsp1β and nsp1γ sequences and 93 aa of nsp2. Wild type and mutant plasmid DNAs were used as templates in coupled in vitro transcription/translation reactions done in the presence of 35S-methionine. The products of PLP processing of polyproteins produced were immunoprecipitated (IP) using either an anti-Flag or an anti-c-Myc antibody and separated by SDS-PAGE. The masses of the precipitated proteins were compared to the those of the predicted protein products (Fig. 5.2B). The wild type construct produced 9 bands with molecular masses of approximately ~63, ~54, ~39, ~30, ~28, ~27, ~23, ~18, and ~15 kDa. The 63, 54, 39, 30, 28, 27, 23, and 18 kDa bands were pulled down by anti-FLAG antibody, while the 63, 39, 30, and 23 kDa bands were pulled down by the anti-c-Myc antibody (Fig. 5.3A). The 63 kDa band pulled down by both antibodies is the full-length polyprotein, while the 54 kDa band pulled down by only anti-FLAG antibody contains nsp1α, nsp1β and nsp1γ but not nsp2 sequence. The FLAG-tagged 39 kDa band most likely contains both nsp1α and nsp1β, while the FLAG-tagged 18 kDa band most likely contains only nsp1α. These data indicate that the nsp1α protein is efficiently cleaved from the polypro-
tein. The c-Myc-tagged 23 kDa band most likely contains nsp1γ and nsp2. The 15 kDa band is most likely nsp1γ because neither the anti-FLAG nor anti-c-Myc antibody pulled down this peptide (Fig. 5.2B). These data indicate that nsp1γ is also efficiently cleaved from the polyprotein and nsp2.

The predicted molecular mass for nsp1β is 22.1 kDa. Because this is an internal peptide it would not be tagged. The nsp1γ-nsp2 band was predicted to be 24.5 kDa. Comparison of the intensity of the ~23 kDa band detected in the lysate and c-Myc pulldown lanes indicated that the band intensity was greater in the lysate than in the c-Myc IP lane suggesting that the lysate band contained both nsp1β and the nsp1γ-nsp2 precursor. In an attempt to verify that the ~23 kDa band contained nsp1β, two different antibodies specific for nsp1β were used for IP of the lysate. Unfortunately, neither of these pulldowns produced detectable bands (data not shown).

A 30 kDa band produced by the wild type construct was present in the lysate as well as in the FLAG and the c-Myc IP lanes, but not in the IgG IP lane, suggesting that this band contained both nsp1α and nsp2 peptides. The 30 kDa bands in each of the IP lanes had decreased intensity compared to same band in the lysate lane. It is likely that the 30 kDa lysate band contained two precursor peptides, one consisting of nsp1α plus part of nsp1β and a second that consisted of nsp2 and nsp1γ plus part of nsp1β. These bands were thus designated as nsp1α+tβ and tβ+nsp1γ+nsp2, respectively. The detection of these bands suggested that the catalytic region of nsp1β contains a functional cleavage site but no Gly/Gly or Tyr/Gly dyads were present in this region. However, a Gly271/Val272 dyad was present within the PLPβ catalytic domain.

Three additional unexpected bands of 23, 27 and 28 kDa were present in both the lysate and the FLAG IP lane. Based on the presence of the FLAG tag and the molecular masses of these bands, these peptides were likely produced by cleavage at non-canonical sites located downstream of the predicted nsp1α cleavage sites but before the predicted cleavage site Gly271/Val272 in the nsp1β catalytic region. However, the intensity of each of these protein bands was low suggesting that cleavage at these sites was not efficient. These bands were designated nsp1α+ttβ due to the further truncation of nsp1β. The presence of a 39 kDa band in the c-Myc IP lane supports cleavage of at least one of these non-canonical sites be-
cause a peptide with the C-terminal region of ttβ attached to nsp1γ and nsp2 would have an approximate molecular mass of 37 kDa. These bands were thus designated as ttβ+nsp1γ+nsp2. The intensity of each of these protein bands was also low suggesting that cleavage at these sites was not efficient.

5.3.2 Functional analysis of the predicted catalytic Cys residues

To determine whether the predicted nsp1α catalytic Cys residue was functional, Cys63 was substituted with alanine and the resulting construct was transcribed and translated in vitro. The products were either analyzed directly or after IP with anti-FLAG or anti-c-Myc antibody (Fig. 5.3B). The 18 kDa band observed with the wild type construct was absent from both the lysate and FLAG IP lanes indicating that Cys63 is required for cleavage of nsp1α from the polyprotein. In addition, while a 30 kDa band was present in the lysate and c-Myc IP lanes, a 30 kDa band was not observed in the FLAG IP lane indicating that a peptide containing nsp1α and the N-terminal region of nsp1β was not produced. Interestingly, the 27 kDa and 28 kDa bands were present in both the lysate and FLAG lanes and the band intensities were increased compared to the wild type construct suggesting that cleavage at two of the non-canonical sites located after the Gly171/Thr172 dyad but before the predicted nsp1β catalytic region was increased when the nsp1α catalytic residue was mutated. The 23 kDa band, however, was not present in the FLAG IP lane suggesting that PLPα is required for cleavage of the C-terminal most cleavage site between the Gly171/Thr172 dyad and the predicted nsp1β catalytic region.

When the predicted nsp1β catalytic site Cys246 was substituted with alanine, seven peptide bands were detected in the lysate with approximate molecular masses of 63, 54, 39, 30, 23, 18 and 15 kDa (Fig. 5.3C). The c-Myc IP lane contained the 63 and 23 kDa bands, while the FLAG IP lane contained the 63, 54, 39, 30, and 18 kDa bands. The observed increased intensity of the full-length polyprotein (63 kDa) and the polyprotein precursor without nsp2 (54 kDa) indicate that further processing of these large precursors was decreased when the nsp1β catalytic Cys was mutated. However, the detection of an 18 kDa band in the lysate and FLAG IP lanes and also a 15 kDa band only in the lysate lane indicated that the nsp1α and nsp1γ proteins were cleaved from the polyprotein. The observation of a 23 kDa band in the
lysate and c-Myc lanes indicated that nsp1β was also cleaved from nsp1γ. The 27 and 28 kDa bands observed in the lysate and FLAG IP lanes with the wild type construct were not detected with the Cys246 mutant construct indicating that cleavage between the C-terminus of nsp1α and the nsp1β catalytic region did not occur. However, the 30 kDa band was detected in the lysate and FLAG construct indicating that cleavage within the catalytic region of nsp1β had occurred. The observation that the nsp1β/nsp1γ junction was cleaved even though the PLPβ catalytic Cys was mutated suggested that one of the other PLPs could cleave at the nspβ/nsp1γ junction.

Analysis of the peptides produced from a construct with the predicted nsp1γ catalytic Cys378 substituted with an alanine revealed seven bands in the lysate with approximate molecular masses of 63, 39, 30, 28, 27, 23, and 18 kDa (Fig. 5.3D). The c-Myc lane contained the 63, 39 and 30 kDa bands while the FLAG IP lane contained the 63, 39, 30, 28, 27, 23, and 18 kDa bands. These data indicated that nsp1α and nsp1β were produced but nsp1γ was not. The nsp1α plus nsp1β precursors (39 kDa band in lysate and FLAG lanes) and precursors containing nsp1α plus various lengths of the nsp1β N-terminal region (30, 28, and 27 kDa bands in the lysate and FLAG lanes) were also detected indicating that cleavage before and within the nsp1β catalytic region still occurred when the nsp1γ catalytic Cys was substituted. Additionally, the detection of various truncated forms of nsp1β plus nsp1γ plus nsp2 (39 and 30 kDa bands in the lysate and c-Myc lanes) indicate that nsp1γ was not cleaved from nsp2. Taken together, these data indicate that the predicted PLPγ Cys378 is required for cleavage of nsp1γ from nsp2.

Substitution of Cys246 in PLPβ did not completely block cleavage of nsp1β from nsp1γ while mutation of Cys63 or Cys378 prevented cleavage of nsp1α and nsp1γ, respectively. The cleavage of nsp1β when Cys246 was mutated suggested that either PLPα or PLPγ could cleave at the nsp1β/nsp1γ junction.

To obtain a clearer picture of the sites each PLP could cleave, double mutant constructs were next made which produced polyproteins with only one active PLP. A mutant with both PLPα Cys63 and PLPβ Cys246 substituted with alanines produced peptides in the lysate lane with molecular masses of 63, 54, 39, 30, 28, 27, 23, and 15 kDa. The 63, 54, 39, 30, 28, and 27 kDa bands were present in the FLAG IP
lane while the 63 and 23 kDa bands were present in the c-Myc IP lane (Fig. 5.4B). The 54 and 39 kDa bands were present in the c-Myc IP and IgG IP lanes at comparable intensities indicating that these proteins were not specifically binding to the α-c-Myc antibody. Detection of the 39 kDa band in the FLAG IP lane and the 23 kDa band in the c-Myc IP lane indicated that the nsp1β/nsp1γ and the nsp1γ/nsp2 junctions were cleaved when only PLPγ was active. Interestingly, the 30, 28, and 27 kDa bands were also in the FLAG IP lanes indicating that PLPγ can also cleave at multiple sites within nsp1β. The absence of an 18 kDa band in either the lysate or FLAG IP lanes and the observed increase in the intensity of the 39 kDa band in the lysate and FLAG IP lanes indicated that the nsp1α/nsp1β junction was not cleaved by PLPγ.

A double mutant constructed with both PLPβ Cys246 and PLPγ Cys378 substituted with alanines produced a very strong intensity 63 kDa band that was present in the lysate, c-Myc IP and FLAG IP lanes and 30 and 18 kDa bands that were in the lysate and FLAG IP lanes but not in the c-Myc IP lane (Fig. 5.4C). Multiple weak bands in the lysate, FLAG IP and c-Myc IP lanes with masses that did not correlate with those of any of the peptides predicted to be produced during polyprotein processing were detected. The observation of the 30 kDa band in both the lysate and FLAG IP lanes indicated that PLPα is also able to cleave within the catalytic region of nsp1β. However, when only PLPα is active, the efficiency of its cleavage at the nsp1α/nsp1β junction was decreased compared to the wild-type polyprotein.

A double mutant with both PLPα Cys63 and PLPγ Cys378 substituted with alanines produced bands of 63, 39, 30, 28 and 27 kDa in the lysate and FLAG IP lanes and of 63, 39 and 30 kDa in the c-Myc IP lane. The detection of the 30, 28 and 27 kDa bands indicated that PLPβ can cleave not only at the nsp1β/nsp1γ junction but also within the PLPβ catalytic region and between the nsp1α/nsp1β junction and the PLPβ catalytic region. However, because self-cleavage of a protease within its own catalytic region seemed unlikely, additional bioinformatics analyses were done to determine the possible existence of an additional protease with a catalytic core between Cys115/Trp116 and His181 or His185. However, neither sequence alignment nor homology modeling supported the existence of an additional protease between aa 100 and 203.
5.3.3 Functional analysis of the cleavage sites utilized by the SHFV PLPs

Arterivirus PLPs have previously been reported to cleave at Gly/Gly or Tyr/Gly dyads (30, 106, 142). Of the fourteen possible Gly/Gly or Tyr/Gly dyads present in the SHFV nsp1 region, seven (Tyr40/Gly41, Tyr72/Gly73, Gly108/Gly109, Tyr110/Gly111, Tyr404/Gly405, Tyr418/Gly419, Tyr427/Gly428) were ruled out because proteins with the predicted molecular masses for the products of cleavage at these sites were not present in the wild type lysate lane (Fig. 5.3A). Two additional cleavage sites (Tyr149/Gly150 and Tyr479/Gly480) could not be ruled out based on these criteria. However, the sequence alignment predicted alternative cleavage dyads close to these locations and these were tested first (Fig. 5.2A). Previous studies had shown that substitution of the +1 and +2 amino acids of PLP cleavage sites prevented recognition and cleavage (8, 129). The +1 and +2 amino acids of each of six putative sites (Gly150/Gly151, Gly164/Gly165, Gly171/Thr172, Gly271/Val272, Gly350/Gly351, Gly484/Gly485, and Gly485/Gly486) were substituted with alanines or valines. Mutation of the Gly150/Gly151 site, located at the predicted nsp1α/nsp1β junction, resulted in loss of the 18 kDa nsp1α band in both the lysate and FLAG IP lanes (Fig. 5.5B). Mutation of the second predicted nsp1α/nsp1β junction dyad, Gly164/Gly165, resulted in a significant decrease of the 18 kDa band in the lysate and FLAG IP lanes (Fig. 5.5C). These data indicate that both Gly150/Gly151 and Gly164/Gly165 are required for efficient cleavage of nsp1α from nsp1β by PLPα. In contrast, when Gly171/Thr172, the cleavage site predicted by the bioinformatics analyses was substituted, the 18 kDa band was detected in the lysate and FLAG IP lanes but the level of the 63 and 54 kDa precursor proteins increased compared to the wild-type polyprotein (Fig. 5.5D). The data indicate that this site is not recognized by PLPα but that a mutation at this position decreases overall cleavage efficiency, possible due to an effect on folding.

Gly350/Gly351 was predicted to be the cleavage site between nsp1β and nsp1γ (Fig. 5.2). A construct with this site substituted produced seven bands in the lysate lane of 63, 54, 30, 28, 27, 23 and 18 kDa, bands of 63, 54 and 23 kDa in the c-Myc IP lane and bands of 63, 54, 30, 28 and 27 kDa in the FLAG IP lane (Fig. 5.5F). The intensity of the 63 kDa band in the lysate, c-Myc IP and FLAG IP lanes, as well as the intensity of the 54 kDa band in the lysate and FLAG IP lanes was increased compared to
those produced by the wild type construct while the intensity of all the other bands was decreased. These results indicated that substitution of the Gly350/Gly351 site efficiently reduced but did not block cleavage of nsp1β from nsp1γ and also decreased cleavage at the nsp1α/nsp1β and nsp1γ/nsp2 junctions.

Three adjacent glycine residues (Gly484/Gly485/Gly486) are present at the predicted cleavage site at the C-terminus of nsp1γ (Fig. 5.1A). To determine whether the Gly484/Gly485 dyad is cleaved, the +2 and +1 aa of this putative site were substituted. Nine bands (63, 54, 39, 30, 28, 27, 24, 23 and 18 kDa) were detected in the lysate lane. All of these bands with the exception of the 23 kDa band were also present in the FLAG IP lane while the 63, 30 and 23 kDa bands were present in the c-Myc IP lane (Fig. 5.5G). The increase in the 23 kDa band and the lack of a 15 kDa band in the lysate lane indicated that the nsp1γ protein was not efficiently cleaved at the nsp1γ/nsp2 junction. The detection of a 24 kDa band in the FLAG IP lane indicated that cleavage between the nsp1α/nsp1β junction and the catalytic region of PLPβ increased when the Gly484/Gly485 site was mutated. To determine whether the Gly485/Gly486 dyad could also function as a cleavage site, the +2 and +1 aa residues of this site were substituted with alanines. This mutant construct produced a band pattern similar to that of the Gly484/Gly485 mutant construct including increased detection of the 24 kDa band in the FLAG IP lane (Fig. 5.5H). These data indicate that efficient cleavage at the nsp1γ/nsp2 junction requires all three glycine residues (Gly484/Gly485/Gly486).

Several constructs produced the 30 kDa nsp1α+tβ and tβ+nsp1γ+nsp2 bands indicating cleavage occurred within the catalytic region of PLPβ. However, sequence analysis did not reveal a Gly/Gly or Tyr/Gly dyad present in this region (Fig. 5.1A). Both the PLP of murine hepatitis virus and the human coronavirus PL1pro have been reported to recognize Gly/Val, Gly/Asn or Ala/Gly dyads (142). Analysis of the nsp1β sequence indicated the presence of a Gly271/Val272 dyad within the predicted nsp1β catalytic region (Fig. 5.1A). A construct with the +2 and +1 aa residues of this site substituted with valines produced seven proteins (63, 54, 39, 28, 27, 23, and 18 kDa) in the lysate lane. The FLAG IP lane contained bands of 63, 54, 39, 28, 27, and 18 kDa while the c-Myc IP lane contained bands of 63, 54, and 23 kDa.
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(Fig. 5.5E). The absence of the 30 kDa band in all three lanes confirmed that the N- and C-terminal 30 kDa bands were produced by cleavage at the Gly271/Val272 site.

The wild type construct also produced two unexpected bands at 28 and 27 kDa that were only precipitated by FLAG antibody indicating that these two proteins contained nsp1α and part of nsp1β and so were labeled as α+ttβ. Based on their sizes, these two proteins are likely to result from cleavage occurring at sites located after the nsp1α cleavage site (Gly164/G165) but before the nsp1β catalytic region cleavage site (Gly271/Val272). The N-terminal sequence of nsp1β was searched for alternative cleavage dyads and three potential cleavage sites Ala186-Gly187, Gly187-Val188 and Gly213-Val214 were found. Each of these sites was substituted with alanines at the +1 and +2 positions. Both the 27 kDa and 28 kDa bands were produced by each of these mutant clones indicating that none of these sites were used (data not shown).

5.3.4 Analysis of nsp1 processing and localization in vivo

To analyze the kinetics of nsp1α, nsp1β and nsp1γ production in infected cells, MA104 cells were infected with SHFV-LVR at an MOI of 1 and cell lysates were collected at various times after infection and analyzed by Western blotting using anti-nsp1α, N- and C-terminal specific anti-nsp1β or anti-nsp1γ antibodies. Only mature proteins with slightly different molecular masses were detected for each nsp1 protein suggesting that nsp1 proteins are post-translationally modified in infected host cells (Fig. 5.6A, 5.6B and 5.6C). The anti-nsp1α antibody detected only a single band at 22 kDa, both the N and C-terminal anti-nsp1β antibodies detected only a single band at 22 kDa and the anti-nsp1γ antibody detected only a single 18 kDa band. No precursor proteins were observed indicating rapid processing of the nsp1 proteins from the polyprotein in infected cells. Also, no truncated forms of nsp1β or longer forms of nsp1α were detected in cell lysates at any time during the course of the infection.

It was previously reported that the nsp1 fusion protein of EAV and the nsp1α of PRRSV localize to the nucleus of infected cells even though neither of these proteins contains a predicted nuclear localization signal (15, 64, 121). Analysis of the sequences of the individual SHFV nsp1 proteins also did not
reveal any nuclear localization sequences. To examine the localization of the three SHFV nsp1 proteins in infected cells, MA104 cells were infected with SHFV at an MOI of 10 and analyzed by confocal microscopy at 12 and 24 h after infection with one of the SHFV nsp1 specific antibodies and an anti-dsRNA antibody used to detect viral RNA replication intermediates. The nsp1α protein was found in the cytoplasm of infected cells and showed strong colocalization with dsRNA but was also detected in the nucleus of infected cells by 12 h after infection (Fig. 5.6D). Although background nsp1β antibody staining was detected in the nucleoli in both infected and uninfected cells, the nsp1β protein was only detected in the cytoplasm of infected cells and colocalized with dsRNA in the perinuclear region (Fig. 5.6E). The nsp1γ protein was detected in the cytoplasm of infected cells by 12 and 24 h after infection but only weakly colocalized with dsRNA and was also detected in the nuclei of infected cells (Fig. 5.6F).

5.4 Discussion

The N-terminal region of the arterivirus polyproteins 1a and 1ab contains three different types of protease domains, the main 3CLSP, a single accessory CP and at least one accessory PLP which together are responsible for processing the polyprotein into 12 to 14 mature nonstructural proteins (142). The PLP domains are required for auto-cleavage of the N-terminal nsp1 proteins. Although the genomes of the arteriviruses, EAV, LDV and PRRSV each encode two PLP domains the EAV PLPα is inactive (106). The SHFV genome was predicted to encode three PLP domains (PLPα, PLPβ and PLPγ). The data obtained in the current study showed that all three of the predicted SHFV PLPs are active and result in the production of the nsp1α, nsp1β and nsp1γ proteins in both in vitro translation reactions and in infected cells. Functionally active PLP domains encoded by all previously studied arteriviruses and coronaviruses are composed of a catalytic Cys adjacent to an aromatic Trp or Tyr and a distal His residue (142). The catalytic Cys residues of SHFV PLPβ and PLPγ were determined to be Cys246 and Cys378, respectively. Both of these cysteine residues are adjacent to an aromatic Trp residue and are 62 and 65 aa, respectively, upstream of the predicted catalytic His residue. However, the catalytic Cys of PLPα was shown to be Cys63
which is adjacent to a non-aromatic Ala residue that is 67 aa upstream of the predicted catalytic His residue. The SHFV PLPα catalytic region is therefore more similar to the CP domain located in arterivirus nsp2 proteins. The CP catalytic core consists of a Cys residue adjacent to a Gly and is located 63 to 69 aa upstream of a catalytic His residue (53, 107). The activity of known arterivirus CPs was shown to be dependent on additional Cys residues located throughout the protease that can form a zinc finger structure required for protease activity (53, 142). There are several Cys residues located in nsp1α at positions 115, 173 and 195 that could function to form a zinc finger scaffold. Although additional work is required to confirm that SHFV PLPα is a CP and not a PLP, amino acid alignment and mutagenesis data indicate that Cys63 is the catalytic residue of the nsp1α encoded protease that cleaves at the nsp1α/nsp1β junction.

The cleavage sites for PLPα in both the PRRSV and LDV polyproteins have yet to be identified. However, the PLPβs encoded by these two viruses have been shown to cleave at Tyr/Gly dyads while the single active PLP in the EAV polyprotein cleaves at a Gly/Gly dyad (30, 45, 106). Mutagenic analysis of predicted cleavage sites within the nsp1 region of the SHFV genome indicated that each of the three nsp1 proteases cleaves at a Gly/Gly dyad downstream of the predicted catalytic His residue (Fig. 5.7). Data indicate that the SHFV PLPβ and PLPγ require a single Gly/Gly dyad for efficient cleavage while efficient cleavage of the nsp1α/nsp1β junction by PLPα requires two Gly/Gly dyads suggesting that folding of the polyprotein around the nsp1α/nsp1β junction is critical for protease cleavage. Alternatively, it is possible that mutation of either the predicted Gly150/151 or Gly164/Gly165 cleavage sites resulted in a misfolded polyprotein that blocked recognition of the nsp1α/nsp1β junction by PLPα. In addition to cleaving the nsp1α/nsp1β junction, PLPα was also shown to be able to cleave at the Gly271/Val272 dyad within the catalytic region of PLPβ but not at non-canonical sites within the N-terminal region of nsp1β. Cleavage at this distal site further supports the hypothesis that PLPα is a CP as previous in vitro data showed that the CP encoded by EAV nsp2 cleaves at a dyad approximately 500 aa residues downstream from the catalytically active Cys while PLPs typically cleave at more proximal cleavage dyads (107).

The EAV PLP, the PRRSV PLPβ and the LDV PLPβ are only able to cis cleave at a single dyad located C-terminal to their respective catalytic His residues in in vitro cleavage assays (30, 106). Alt-
hough each SHFV nsp1 protease efficiently cleaved at a Gly/Gly dyad located C-terminal to their respective catalytic His residues both \textit{in vitro} and \textit{in vivo}, they can each cleave at additional sites \textit{in vitro} (Fig. 5.7). SHFV PLP\(\gamma\) is also able to back cleave at multiple sites including at the nsp1\(\beta\)/nsp1\(\gamma\) junction and within the PLP\(\beta\) catalytic region. This activity suggests that PLP\(\gamma\) is able to \textit{trans} cleave at sites N-terminal to the catalytic Cys residue after cleavage occurs at the canonical dyad located C-terminal to the catalytic His residue. Previous work showed that PLPs encoded by the coronaviruses, murine hepatitis virus strain A59 and human coronavirus 229E, are also able to \textit{trans} back cleave at a cleavage dyad located N-terminal to their respective catalytic Cys residues in \textit{in vitro} cleavage assays (56, 116). The ability to \textit{trans} cleave was correlated with the identity of the aa residues adjacent to the cleavage dyad. After the Sindbis CLSP cleaves at a C-terminal cleavage dyad, an aromatic Trp residue located immediately N-terminal to the cleavage site remains in the catalytic fold after the cleaved peptide has exited the protease which prevents subsequent \textit{trans} cleavage (16). The three arterivirus PLPs that do not exhibit \textit{trans} cleavage in \textit{in vitro} cleavage assays cut at dyads adjacent to Trp or Tyr residues suggesting that their inability to \textit{trans} cleave may be due to the presence of a bulky aromatic residue blocking the substrate site (142). The two coronavirus PLPs that have \textit{trans} cleavage activity cut at dyads adjacent to non-aromatic Lys or Val residues which would not block the catalytic site and allow \textit{trans} activity. Also, PRRSV nsp2 CP has been shown to possess \textit{trans} cleavage activity at a Gly/Gly dyad but substitution of the adjacent non-aromatic aa residues with prolines decreased \textit{in vitro} cleavage at this site suggesting that the ability of this CP to \textit{trans} cleavage is also dependent on the properties of the residues adjacent to the cleavage dyad (53).

The cleavage dyad Gly271/Val272 in the catalytic region of PLP\(\beta\) was recognized and cut during polyprotein processing to produce N-terminal and C-terminal peptides of \(~30\) kDa that were pulled down by the FLAG and c-Myc antibodies, respectively. Mutagenic analysis of the individual PLP catalytic Cys residues suggested that PLP\(\alpha\) is partially responsible for cleavage at this dyad because when Cys63 of this PLP was mutated, the 30 kDa band intensity decreased compared to wild type and was not detected in the FLAG IP lane indicating that the nsp1\(\alpha\) and truncated nsp1\(\beta\) peptide was not present. However, when
Cys63 was mutated in combination with either Cys246 or Cys378, the 30 kDa band was still detected indicating that both PLP\(\beta\) and PLP\(\gamma\) can cleave at Gly271/Val272. However, the observation that the intensity of the 30 kDa band did not differ when either PLP\(\beta\) or PLP\(\gamma\) were individually inactivated by mutation indicates that PLP\(\alpha\) is the primary protease that cleaves Gly271/Val272.

Cleavage at the nsp1\(\gamma\)/nsp2 junction was reported to be required for release of nsp2 and the nsp2 CP leads to release of nsp4 which contains the main 3CLSP that cleaves at all the remaining nonstructural protein sites in the polyprotein (41, 108). Cleavage at Gly271/Val272 may represent an alternative site for releasing PLP\(\gamma\) in the event that the canonical Gly350/Gly351 cleavage site is blocked.

Production of the 27 and 28 kDa bands detected in both the lysate and FLAP IP lanes during processing of the wild type polyprotein indicates that viable by non-canonical cleavage sites are located in the region between the nsp1\(\alpha\)/nsp1\(\beta\) junction and the PLP\(\beta\) catalytic region. The data suggest that PLP\(\beta\) is able to cleave at these non-canonical sites because these bands were not present in assays done with the PLP\(\beta\) catalytic Cys mutant polypeptide but were present when both the PLP\(\alpha\) and PLP\(\gamma\) catalytic Cys residues were mutated. PLP\(\gamma\) may also be able to cleave at these non-canonical site since the intensity of the 27 and 28 bands decreased in assays with the PLP\(\gamma\) catalytic Cys mutant construct compared to wild type construct. Also, both bands were present but at a decreased level compared to the wild type when the catalytic Cys of both PLP\(\alpha\) and PLP\(\beta\) were mutated.

The N-terminus of nsp1 in all known arteriviruses contains a zinc finger motif that is required for sg mRNA transcription and viral RNA replication (119, 122). The presence of two alternative cleavage sites and the ability of multiple proteases to back cleave at these sites allowing for release of the zinc-finger containing nsp1\(\alpha\) proteins from the remainder of the SHFV polyprotein may provide a secondary mechanism for release of the zinc finger protein in the absence of the canonical cleavage by PLP\(\alpha\).

Despite the production of multiple precursor proteins and alternative cleavage products during \textit{in vitro} processing of the polyprotein, only the mature nsp1\(\alpha\), nsp1\(\beta\) and nsp1\(\gamma\) proteins were observed in cell extracts at both early and late times after infection. In contrast, previous work demonstrated that the EAV nsp1 protein was rapidly released from the polyprotein during \textit{in vitro} processing suggesting that
cleavage of this single nsp1 protein was cotranslational (106). The detection of precursor proteins as well as alternative peptides in the SHFV *in vitro* reactions but not in infected cells suggests that either interaction of the polyprotein with viral or host proteins or of post-translational modification of the polyprotein occurring only in infected cells regulate nsp1 cleavage. Each SHFV nsp1 protein is predicted to contain multiple post-translational modification motifs including myristoylation, phosphorylation and glycosylation sites (data not shown). Modification at one or more of these sites could affect the folding of the SHFV polyprotein and the lack of these modifications/interactions *in vitro* may result in exposure of cleavage dyads not accessible in the *in vivo* folded polyprotein. However, the possibility of proteosomal degradation of uncleaved precursor peptides and alternatively cleaved peptides during *in vivo* infections cannot be ruled out.

The nsp1 fusion protein of EAV and at least one of the PRRSV nsp1 proteins have been shown to shuttle between the nucleus and cytoplasm of infected cells (109, 121). The single nsp1 protein of EAV suppresses anti-viral host responses by inhibiting NF-κB activation, but is also required for sg mRNA transcription and viral RNA replication (119, 120). The nsp1α protein of PRRSV, while not required for viral genome replication, is essential for sg mRNA transcription as well as suppression of IFN production by inhibition of NF-κB activation (62, 64, 109). The PRRSV nsp1β protein suppresses IFNβ expression by blocking STAT1 nuclear translocation but is also required for viral RNA replication (15, 64). Analysis of the cellular localization of SHFV nsp1 proteins indicated that both nsp1α and nsp1γ localize to both the cytoplasm and nucleus of infected cells while nsp1β is only in the cytoplasm. Additionally, while nsp1α and nsp1β strongly associates with viral replication complexes, nsp1γ only weakly colocalizes with viral replication complexes. It is possible that the three nsp1 proteins of SHFV have divergent roles in viral replication and evasion of the host anti-viral responses. Additional work is underway to analyze the functions of each of the SHFV nsp1 proteins in viral RNA replication, sg mRNA transcription and modulation of host anti-viral responses.
Figure 5.1 Amino acid analysis of the three SHFV PLPs. (A) Amino acid alignment of the three SHFV PLP catalytic regions with the predicted C-terminal cleavage sites located between the nsp1α/nsp1β, nsp1β/nsp1γ and nsp1γ/nsp2 junctions. Asterisks indicate predicted catalytic residues in each protease. (B) Protein folding of the three SHFV nsp1 proteins based on resolved crystal structure of PRRSV nsp1α protein. The predicted catalytic Cys and His residues for each protein are indicated in yellow.
Figure 5.2 SHFV nsp1 construct cleavage map. The first 1722 nucleotides of SHFV ORF1a/b were cloned into pTnT vector with an N-terminal FLAG tag and a C-terminal c-myc tag. (A) The N-terminal region of the polyprotein 1a/b. Black boxes indicate relative positions of the predicted PLP motifs based on the folds shown in Figure 1B. Predicted catalytic residues for PLPα, PLPβ and PLPγ are indicated with arrows above and possible cleavage sites are indicated by dotted lines. (B) Predicted lengths of possible fragments generated after cleavage of the polyprotein at the various predicted cleavage sites.
<table>
<thead>
<tr>
<th>Construct</th>
<th>Mutation(s)</th>
<th>Primer Sequence (5’ - 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>mα</td>
<td>Cys63 → Ala</td>
<td>GGGCGCTATGctGCTCTTGAATGATA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TATCATCTCAGAGTGATGAGCAGCC</td>
</tr>
<tr>
<td>mβ</td>
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<tr>
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<td></td>
<td>GAAACAACCTCAGGGCAGCCGGCCATGCCCTCAAG</td>
</tr>
<tr>
<td>mγ</td>
<td>Cys378 → Ala</td>
<td>CCTCACTGCGGGITTGCGTGTCGACGATTTCC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GAAATAGCTGCAAACGAGAGCaATGTGG</td>
</tr>
<tr>
<td>G150/G151</td>
<td>Tyr149 → Val / Gly150 → Val</td>
<td>CACATTGGCTACgtGCGGACATGAGC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GGTTGATGCGGCGCATGAGGCAATTCGG</td>
</tr>
<tr>
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<td></td>
<td></td>
<td>GATAAAAAGCTCAACCCATGCGTATGG</td>
</tr>
<tr>
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<td></td>
<td></td>
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</tr>
<tr>
<td>G265/Y266</td>
<td>Phe264 → Val / Gly265 → Val</td>
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<td></td>
<td></td>
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<td>G271/V272</td>
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<td></td>
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<td>GCCAAATTTATTTTCGGACGCCATGACCAATGGTTAACCC</td>
</tr>
<tr>
<td>G350/G351</td>
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<td>CGTTTCATGTTAGACGTGTCGCTCAAGGAAAGATACATTG</td>
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<tr>
<td></td>
<td></td>
<td>GCCAAATTTATTTTCGGACGCCATGACCAATGGTTAACCC</td>
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<tr>
<td>G484/G485</td>
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<tr>
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<td></td>
<td>CGATTGCGCCGCGCgtGcGcGAAGGTCACGGCCATCG</td>
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<tr>
<td>G485/G486</td>
<td>Gly484 → Ala / Gly485 → Ala</td>
<td>CGTTACCGTCGCGCGGcGcGcGAAGGTCACGGCCATCG</td>
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<tr>
<td></td>
<td></td>
<td>CGATTGCGCCGCGCgtGcGcGAAGGTCACGGCCATCG</td>
</tr>
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</table>

Lowercase indicates mutated nucleotides in each primer.
Figure 5.3 Analysis of cleavage products produced by polyprotein containing mutations of single PLPs. Wild type (A), PLPα (B), PLPβ (C) or PLPγ (D) single cysteine mutant clones were \textit{in vitro} transcribed and translated using wheat germ extract lysates in a coupled TnT reaction (Promega). Protein products were labeled by incorporations of $[^{35}\text{S}]$ cysteine, immunoprecipitated with murine IgG, anti-c-myc or anti-FLAG antibody before separation by 13% SDS-PAGE. Molecular weight markers are shown on the right and the possible nsp1 proteins are shown on the left. tβ indicates protein produced by cleavage at the predicted G265/Y266 cleavage site; ttβ indicates protein produced by cleavage at unknown sites between the nsp1α/nsp1β junction and before predicted cleavage sites within nsp1β. A decreased exposure was used for FLAG IP lanes in A and C.
Figure 5.4 Analysis of cleavage products produced by polyprotein containing mutations of two PLPs. Wild type (A), PLPα/β (B), PLPβ/γ (C) or PLPα/γ (D) double cysteine mutant clones were in vitro transcribed and translated using wheat germ extract lysates in a coupled TnT reaction (Promega). Protein products were labeled by incorporations of [35S] cysteine, immunoprecipitated with murine IgG, anti-c-myc or anti-FLAG antibody before separation by 13% SDS-PAGE. Molecular weight markers are shown on the right and the possible nsp1 proteins are shown on the left. tβ indicates protein produced by cleavage at the predicted G265/Y266 cleavage site; ttβ indicates protein produced by cleavage at unknown sites between the nsp1α/nsp1β junction and before predicted cleavage sites within nsp1β. A decreased exposure was used for FLAG IP lanes in A and B.
Figure 5.5 Analysis of cleavage products produced by polyprotein containing mutations of predicted PLP cleavage sites. Wild type (A), nsplα/np1β junction cleavage site (B-D), PLPβ catalytic region cleavage site (E), nsplβ/np1γ junction cleavage site (F) or nsplγ/np2 junction cleavage site (G-H) mutant clones were in vitro transcribed and translated using wheat germ extract lysates in a coupled TnT reaction (Promega). Protein products were labeled by incorporations of [35S] cysteine, immunoprecipitated with murine IgG, anti-c-myc or anti-FLAG antibody before separation by 13% SDS-PAGE. Molecular weight markers are shown on the right and the possible nspl proteins are shown on the left. tβ indicates protein produced by cleavage at the predicted G265/Y266 cleavage site; ttβ indicates protein produced by cleavage at unknown sites between the nsplα/np1β junction and before predicted cleavage sites within np1β. A decreased exposure was used for FLAG IP lanes in A and B.
Figure 5.6. *In vivo* processing and subcellular localization of the nsp1 proteins. Western blot analysis of MA104 cells infected with SHFV LVR strain at an MOI of 1. Lysates were collected in radioimmuno-precipitation assay (RIPA) buffer at the indicated times after infection and analyzed using antibodies to SHFV viral proteins and actin. Arrows indicate viral protein specific bands. (D-F) MA104 cells were infected with SHFV LVR strain at an MOI of 10, fixed at indicated times after infection and analyzed by confocal microscopy. Anti-dsRNA antibody (red) was used to detect SHFV-infected cells, anti-SHFV antibodies (38) were used to detect viral protein localization and Hoeschst dye (blue) to detect nuclei.
Figure 5.7. Model for polyprotein cleavage by the SHFV nsp1 proteases. The three SHFV nsp1 proteases are indicated by red, blue and green boxes and the recognized cleavage dyads are indicated by dashed lines. The protease present in the nsp1α region is predicted to be a cysteine protease (CP) and the proteases present in both nsp1β and nsp1γ regions are predicted to be PLPs. Red, blue and green arrows indicate cleavage dyads that are cleaved in vitro by the proteases present in nsp1α, nsp1β and nsp1γ, respectively. ZF indicates the predicted zinc finger domain present in the N-terminal region of nsp1α.
6 CONCLUSIONS

6.1 Consequences of SHFV-induced Pro-inflammatory Cytokine Production

Previous studies suggested that fatal viral hemorrhagic fever disease is induced by an uncontrolled systematic inflammatory response leading to suppression of the adaptive immune response due to lymphocyte apoptosis and coagulation defects exemplified by development of DIC (12, 72). Emerging evidence suggests that a non-fatal hemorrhagic fever virus infection is characterized by a controlled inflammatory response and activation of adaptive immunity. However, mechanisms contributing to the induction of a regulated response have not been elucidated and may depend to some degree on co-evolution between host and virus (10, 12). The current study confirmed that disease-susceptible macaques and macaque MΦs and mDCs produce high levels of several pro-inflammatory cytokines after SHFV infection while disease-resistant baboons develop low titer, persistent infections and their MΦs and mDCs do not produce pro-inflammatory cytokines. Baboon cells were shown to produce high levels of the anti-inflammatory cytokine IL-10. However, IL-10 signaling through IL-10R to induce SOCS3 production was observed only after SHFV infection. Neither IL-10R1 nor SOCS3 levels increased in macaque cell cultures after SHFV infection. To our knowledge, this is the first study to show not only higher basal levels of IL-10 protein production by disease-resistant baboons compared to disease-susceptible macaques but also virus-dependent induction of SOCS3 by IL-10 receptor activation. Additionally, this study demonstrated the regulation of several, but not all, SHFV-induced pro-inflammatory cytokines by IL-10 signaling.

Data presented in the current study demonstrated that TNF-α was produced in response to SHFV infection by macaque MΦs and mDCs but not by baboon cells. TNF-α was produced after incubation of cells with either live or UV-inactivated virus, indicating TNF-α production by macaque cells was not dependent upon virus replication. Additionally, neither TNF-α levels nor cell viability after SHFV infection of macaque cells was altered by IL-10 treatment. A previous study reported that another arterivirus,
PRRSV, induces caspase-mediated apoptosis of porcine MΦs (18). More recent data indicated that the induction of apoptosis by both EAV and PRRSV is partially dependent on extrinsic signaling, such as TNF-related apoptosis inducing ligand (TRAIL) mediated signaling, that results in caspase 8 cleavage (69, 111). Cultured human monocytes and mDCs infected with live and inactivated Ebola virus produced TNF-α that induced TRAIL-mediated apoptosis (55). TNF-α production in Ebola virus infected primates was reported to induce lymphocyte apoptosis and increased vascular permeability. Interestingly, the retrovirus, simian immunodeficiency virus (SIV), also induces TRAIL expression in MΦs and mDCs isolated from disease-susceptible primates, such as rhesus macaques, but not from ones isolated from disease-resistant primates such as African green monkeys, sooty mangabeys and chimpanzees (61) suggesting that apoptosis of monocytes induced by SIV from disease-susceptible primates is also dependent on TRAIL signaling. The activation of TRAIL-mediated apoptosis should be investigated in SHFV infected macaque MΦs and mDCs to determine whether TNF-α production in macaque cells is responsible for the induction of apoptosis after SHFV infection.

Non-fatal Ebola virus infections in humans have been associated with early, transient plasma levels of pro-inflammatory cytokines while fatal infections were characterized by delayed and less controlled production of similar cytokines (4). In contrast, asymptomatic Ebola virus infections in humans are associated with an early, transient production of pro-inflammatory cytokines that coincides with the production of IL-10 as well as rapid activation of the adaptive immune response leading to development of virus neutralizing IgG and IgM (70, 71). Several studies have indicated that IL-10 enhances IgG and IgM production from cultured B-cells by promoting B-cell survival and proliferation (84). It is possible that the high IL-10 levels observed in baboon MΦ and mDC cultures could increase B-cell proliferation and production of anti-SHFV specific IgG and IgM in animals. Interestingly, pigs infected with PRRSV that produce IL-10 rapidly produce IgM antibodies specific for the major viral structural proteins of GP5, M and N by 7 to 21 days after infection and virus-specific IgG by 21 days after infection that can persist for up to one year after infection (73). This extended duration of neutralizing antibody production was significantly longer than the estimated 6 weeks of viremia and 2-3 months of virus persistence (103, 114). EAV
and LDV, which can establish lifelong persistence in horses and mice, respectively, induce neutralizing antibodies typically targeting the GP5 N-terminal ectodomain (6). While the present study demonstrated that SHFV can persist in baboons for more than 10 years, production of neutralizing antibodies directed against the SHFV GP7, or any other viral proteins, has not yet been analyzed. Analysis of SHFV neutralizing antibodies in persistently infected baboons may provide further understanding of the differential immune response to SHFV infection in disease-resistant and disease-susceptible monkeys. Several hemorrhagic fever viruses are able to productively infect human DCs but induce only a limited set of pro-inflammatory cytokines in these cells compared to MΦs (9). Interestingly, hemorrhagic fever virus infection of human DCs was shown to inhibit expression of several cell surface receptors required for activation of T and B-cells, including CD86, HLA-DR, CD11c and CD83 while surface expression of the CCR5 cytokine/chemokine receptor was maintained suggesting that DC maturation is suppressed and these cells are more sensitive to cytokine signaling (9). It remains to be determined whether expression of these surface receptors are altered by SHFV infection in mDCs isolated from macaques and/or baboons. It is possible that the ability of macaque mDCs to activate T and B-cells is suppressed after SHFV infection while infected baboon mDCs are still able to induce T and B-cell mediated immunity. The observation of CCR5 down-regulation and increased surface expression of co-stimulatory markers, such as CD83, CD86 and/or HLA-DR on SHFV-infected baboon mDCs would suggest that mDC maturation is not inhibited by infection.

Previous studies of LDV and PRRSV infections in cultured murine or porcine MΦs, respectively, indicated that less than 10% of the cells are infected by 24 h suggesting that only a subpopulation of these cells are permissive to infection (37, 63, 93). This limited host cell tropism was suggested to be due either to differential expression of cell surface receptors required for viral attachment and entry or to specific host proteases required for a productive viral infection. Data from the present study showed that 10% of MΦs from disease-resistant baboons but the majority of MΦs from disease-susceptible macaques were permissive to SHFV infection. These data indicate that SHFV infection of baboon MΦs is similar to what is observed for infections by other arteriviruses of MΦs obtained from their natural hosts while the
highly efficient infection of macaque MΦs is the exception. Arterivirus attachment to host cells is dependent on interaction with heparin sulfate as well as sialoadhesin followed by CD163 binding which is needed for virus entry (14, 26). It has not yet been determined whether MΦ populations isolated from baboons and macaques differ in the levels of the three critical cell surface proteins. Cellular proteases, such as aspartic protease cathepsin E and an unidentified trypsin-like serine protease, are required for PRRSV uncoating in MΦs (82). Whether macaque MΦs are more permissive to SHFV infection due to differences in host proteases present in macaque and baboon MΦs could also be analyzed.

6.2 Modulation of Host Responses by Viral Proteins

Multiple infectious clones for EAV and PRRSV strains have been constructed (34, 85) and used to investigate viral replication as well as the induction of host immune responses. The SHFVic contracted as part of this study is the first full-length cytopathic cDNA infectious clone of SHFV. The SHFV clone also represents the longest known full-length RNA genome that has been stably maintained within a single plasmid because the infectious cDNA clones of coronaviruses are maintained as separate fragment clones due to instability of maintaining full-length clones in a single plasmid (137, 139). This clone represents a valuable tool for further studies of virus replication in the MA104 cell line as well as virus replication and induction of host responses in primary cell cultures from disease-resistant and susceptible primates.

Attempts to construct an infectious clone based on the Genbank consensus sequence did not produce infectious progeny virus suggesting the Genbank consensus, which was derived from shot-gun cloning and sequencing using radioactive chain termination sequencing contained errors that prevented the formation of infectious virus after transfection of full length in vitro transcribed RNA (102). Re-sequencing of the entire genome directly from RNA isolated from a virus pool, as well as sequencing of multiple cDNA clones by 454 sequencing, identified multiple changes from the Genbank consensus, including an insertion-deletion that changed a stretch of ten amino acids. All 18 nt changes were incorporated into the cDNA clone and transfection of RNA transcribed from this clone produced virus compara-
ble to RNA isolated directly from the parental SHFV LVR pool. However, all 18 nts may not be required for production of infectious virus and additional work is needed to determine which of the substitutions is critical.

Previous studies indicated that the nsp1 proteins of EAV and PRRSV inhibit cell anti-viral responses such as NF-κB and IFNβ as well as have functions in viral genome replication and sg mRNA transcription (62, 64, 109, 119, 120). Also, both nsp1α and nsp1β of PRRSV were reported to be able to transcriptionally suppress TNF-α expression (112). Data from the present study indicate that SHFV produces three nsp1 proteins in infected MA104 cells. The nsp1α and nsp1γ proteins were observed in both the cytoplasm and nucleus of infected cells, while nsp1β was only present in the cytoplasm. It has not yet been determined which, if any, of the SHFV nsp1 proteins may affect the differential IFNβ expression observed in the primary cell cultures from disease-resistant and disease-susceptible monkeys. The functions of each nsp1 protein can now be investigated in the context of a virus infection using mutated infectious clones to transfect primary cell cultures.

Previous studies also showed that the nucleocapsid proteins of both PRRSV and EAV localize to the nucleolus of infected cells due to a bipartite pat7 NLS and an adjacent NoLS (91, 94, 121). Analysis of pigs infected with a mutant PRRSV that had the NLS of the nucleocapsid protein substituted indicated that nuclear localization of the nucleocapsid protein is required for efficient viral replication as well as for modulation of host responses such as suppression of antibody production (91). Interestingly, the nucleocapsid protein of the coronavirus, severe acute respiratory syndrome (SARS-CoV) also localizes to the nucleus of infected cells by a similar bipartite NLS where it binds to the NF-κB element in the il6 gene promoter leading to induction of IL-6 (141). IL-6 production during SARS-CoV infection has been associated with the cytokine storm and damaging inflammatory response observed in SARS patients. Data from the present study showed that IL-6 production increased in macaque-derived cells after SHFV infection but not in baboon-derived, infected cells suggesting differential NF-κB activation in cells isolated from the two types of primates. In data not shown, the SHFV nucleocapsid was found to localize to the nucleolus of MA104 cells by 12 h after infection. It remains to be determined whether nucleocapsid sub-
cellular localization is similar in MΦs and mDCs isolated from baboons and macaques. Also, additional preliminary data (data not shown) suggest that NF-κB is activated in macaque cells but not in baboon cells. Mutant SHFVic-derived viruses could be used to analyze nucleocapsid activation of NF-κB in primary cell cultures.
REFERENCES


