MOLECULAR BASIS OF EXOSOMAL FUNCTION DURING RIFT VALLEY FEVER VIRUS INFECTION

by

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DEDICATION

I dedicate this to my wonderful parents who supported me as I completed this critical chapter of my life.

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I would like to thank my mentors, Dr. Hakami and Dr. Kashanchi, and the many members of the Hakami and Kashanchi labs for teaching me experimental techniques and guiding me over the past three years. I would like to thank my family and friends for supporting me during this challenging and yet rewarding process.

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LIST OF ABBREVIATIONS

Dulbecco's Modified Eagle Medium ..... DMEM
Eagle's Minimum Essential Medium ..... EMEM
Fetal Bovine Serum ..... FBS
Multiplicity of Infection ..... MOI
Polymerase Chain Reaction ..... PCR
Phosphate Buffered Saline ..... PBS
Plaque Forming Unit ..... PFU
Post Infection ..... P.I.
Tris Buffered Saline ..... TBS
Quantitative Reverse Transcriptase Polymerase Chain Reaction ..... qRT-PCR
Rift Valley Fever Virus ..... RVFV

ABSTRACT

MOLECULAR BASIS OF EXOSOMAL FUNCTION DURING RIFT VALLEY FEVER VIRUS INFECTION

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Exosomes are small "bioactive" extracellular vesicles that play a central role in intercellular communication and have garnered tremendous interest given the recent discovery of their critical role in a variety of diseases, including infectious diseases. Among these are immunomodulation, alteration of dissemination/infectivity during infection, and modulation of pathogenesis. However, their mechanisms of action remain largely unknown and their role during infections with biodefense agents remains unexplored. We have examined the role of host exosomes during infection with the Rift Valley Fever Virus (RVFV), a Category A priority Pathogen that carries the potential for both devastating public health and agricultural impacts. To assess the role of exosomes, clones of Vero cells that show resistance to RVFV infection and are unable to release functional virions were first generated. Exosomes from these clones contained exosomal markers such as CD63 and were able to activate the TLR3 pathway in recipient reporter cells. Interestingly, these

exosomes contained viral RNA (signal for L, M, and S segments). Furthermore, exosomes derived from some of the resistant clones contained viral proteins such as N. Finally, treatment of immune recipient cells (T cells and monocytic cells) with some of the exosome preparations showed a drastic rate of apoptosis through PARP cleavage and caspase-3 activation. We have already published these resistant clone findings (Ahsan et al., 2016). To further investigate these findings, we also analyzed the role of exosomes derived from cells susceptible to RVFV infection, and as part of this effort developed a highly effective purification scheme that allowed complete separation of the exosomes from the released virions. Similar to the resistant clones, we find the presence of RVFV RNA and N protein in purified exosomes from cells susceptible to RVFV. Additionally, the exosomes from infected origin are capable of inducing cell death in recipient monocytic and lymphocytic cells. Collectively, our data suggest that exosomes from RVFV-infected cells alter the dynamics of the neighboring cells and may contribute to disease pathology.

CHAPTER ONE: INTRODUCTION

Rift Valley Fever Virus

Background and Significance

Geography

Rift Valley Fever (RVF) is an arthropod-borne zoonotic disease responsible for epidemics in Africa and the Arabian Peninsula. The causative agent, Rift Valley Fever Virus (RVFV; *Bunyaviriade: Phlebovirus*), primarily utilizes a mosquito vector to infect both humans and ruminants. Initially identified in the Rift Valley region of Kenya in 1930, RVFV has been isolated from over 30 species of mosquitos with the geographical range spanning the southern coast of the Mediterranean Basin to Egypt and Saudi Arabia, parts of western Africa, sub-Saharan Africa including Madagascar, and southern Africa (Nanyingi et al., 2015).

Disease Cycle and Economic Impact

RVFV outbreaks predominantly occur after heavy rainfalls and flooding events. Infected *Aedes* mosquito eggs resist desiccation and larvae emerge once the eggs have come into contact with water. Post flooding, the hatched eggs develop into infectious adult mosquitoes where they act as vectors and allow the spread of RVFV to livestock (Himeidan et al., 2014). Infection may spread through vertical transmission between

animals as well. RVFV has been shown to infect a wide range of animal hosts including cattle, goats, sheep, buffaloes, and camels. The incubation period varies in length (few hours to few days) and depends on multiple factors: virus strain, dose and route of inoculation, age, and species of the animal. Clinical signs appear and last up to five days (Pepin et al., 2010). The resulting consequences are high rates of abortion and mortalities amongst newborns. A major consequence of RVF outbreaks include heavy economic costs due to loss of livestock. For instance, the most recent major outbreak in Kenya (2006/2007) resulted in a ban on livestock trade and mandatory quarantine yielded a loss greater than 9.3 million USD (Muga et al., 2015).

Human Pathogenesis

The virus is generally transmitted to humans through bites from infected mosquitoes or through contact with blood, excrements, or consumption of meat or raw milk of domestic animals (Seufi and Galal, 2010). The main site of RVFV induced lesions is observed in the liver and a constant rise of liver enzymes and a decrease in total leukocyte counts are associated with severe RVF disease. In the course of severe infections, the virus can be found in nearly all tissues and cell types which may be due to an undiscovered, ubiquitous cellular receptor (Pepin et al., 2010). Symptoms may vary from a mild, flu-like syndrome to potentially fatal ocular, encephalitic, or hemorrhagic syndromes, with fatality rates that may reach as high as 50% (Kahlon et al., 2010).

Threat/Bioterrorism

RVFV has been considered to have a high colonization capacity due to its capability of surviving a wide range of environments. This raises many concerns in regards to humans and animals since it has the potential of being intentionally introduced into western regions as a biological weapon or natural exotic pathogen (Rolin et al., 2013). The potential threat that RVFV poses has led the National Institute of Allergy and Infectious Diseases (NIAID) to classify RVFV as a category A pathogen, a high consequence pathogen by the World Organization for Animal Health, and the third most dangerous animal threat (following avian influenza and foot-and-mouth disease) by the United States Department of Agriculture Animal and Plant Health Inspection Service (Rolin et al., 2013).

Molecular Biology

RVFV is an enveloped virus approximately 80-120 nm in diameter and containing a ribonucleocapsid. Its lipid bilayer envelope consists of heterodimers of the Gn and Gc glycoproteins (Ikegami, 2012). The 12 Kb viral genome comprises three single stranded RNA segments: L, M, and S segments. The L and M segments are of negative polarity and the S segment is of ambisense polarity (Boshra et al., 2011).

RVFV Gene Products

The S segment encodes the nucleocapsid (N) protein, which encapsulates the viral RNA to form the ribonucleoprotein (RNP) complex. In addition to providing structural stability to the RNA genome, the N protein also plays a critical role in the life cycle of RVFV (Lopez et al., 1995). The N protein interacts with the membrane glycoproteins and RdRp, allowing easy access to the RNP during RNA synthesis. Therefore, N is necessary for the RNA genome transcription and replication.

Additionally, the S segment encodes the nonstructural protein NSs, which serves as a critical virulence factor for RVFV. NSs is known to form filaments in the nuclei of infected cells and possesses the ability to tamper with the anti-viral response of the host cell through modulation of innate immunity pathways (Yadani et al., 1999; Walter and Barr, 2011). Studies have proven NSs to inhibit the synthesis of interferon beta mRNA, most likely by forming a multiprotein complex with Sin3A-associated protein (SAP30) on the IFN-β promotor (Le May et al., 2004). Alternatively, activation of IFN regulatory factor 3, NF-κB, and activator protein 1 is observed (Billecocq et al., 2004). NSs also possesses the capability to suppress the host cell's mRNA transcription by sequestering the p44 subunit of a transcription factor for RNA polymerase I and II (TFIIH) (Le May et al., 2004). NSs also evades the host's innate immune response by inducing degradation of PKR, which allows the translation of cellular and viral mRNA (Habjan et al., 2009). Overall, the role of NSs is to prevent an antiviral response by halting the transcription of host mRNA and prevents shut-off of translation in order to maintain viral protein synthesis (Ikegami and Makino, 2011).

The M segment encodes a polyprotein precursor which is later cleaved into Gn, Gc, NSm, NSm-Gn, and a 78 kDa protein (Ikegami, 2012). The Gn/Gc viral envelope proteins are also responsible for facilitating virion particle formation and aiding in the attachment of RVF virions to target cells. Similar to Gn and Gc, the NSm protein may play a role in the virus assembly. It is also thought the protein is important for pathogenesis and may aid in the regulation of apoptosis (Won et al., 2007).

The L segment encodes the viral RNA dependent RNA polymerase (RdRp), which is responsible for synthesizing viral mRNA (Muller et al., 1994). The L protein is packaged into virions and synthesizes initial transcripts in the RVFV infected cells (Piper et al., 2011).

RVFV Life Cycle

RVFV virions enter the cell in a pH dependent manner after binding to a cell receptor. It has been proposed that DC SIGN acts as a mediated receptor in regards to entry in macrophages and dendritic cells (Lozach et al., 2011). Upon entry, the viral polymerase and encapsidated genome are released into the cytoplasm where the RdRp initiates transcription to synthesize viral mRNA. All three RNA segments act as templates for both mRNA transcription and RNA replication (Ikegami and Makino, 2011). Transcription may begin within 40 minutes of infection and viral genomic replication begins approximately 1-2 hours following infection (Ikegami et al., 2005). Each template strand has complimentary non-translated regions (NTRs) at the 5’ and 3’ terminal sequences, allowing the formation of pan handle-like structures. (Walter and Barr, 2011).

These structures serve as promotors for RNA transcription and the synthesis of mRNA occurs through a cap-snatching mechanism where capped host mRNAs are cleaved and the capped 5' end serves as a primer for viral mRNA synthesis (Lopez et al., 1995). The cleaved polypeptides for Gn and Gc form a heterodimer and remain in the Golgi in order to associate with the three different viral RNA segments to mediate virus assembly, maturation, and budding from the host cell (Won et al., 2007). Interestingly, the NSs and NSm proteins are not packaged into the exiting virions and are considered dispensable for viral replication (Ikegami and Makino, 2011).

Exosomes

Exosomes are cell derived membrane bound vesicles secreted into the neighboring extracellular environment. Exosomes were originally considered to be a vehicle of cellular waste removal from the cytosol; however, they are now being recognized to aid in cell-cell communication, play a major role in immunomodulation, and enhance infectivity during viral infections (Fleming et al., 2014; Luga et al., 2012; Colino and Snapper, 2007; Izquierdo-Useros et al., 2010). These extracellular vesicles are characterized as having a diameter ranging from 50-150 nm and a density of 1.23 – 1.16 g mL^{-1} (Théry et al., 2006). They are thought to originate from late endosomes and contain specific mRNAs, microRNAs, proteins, and lipids but the exact content of the exosomes vary depending on the cellular origin; however, some of the more common exosomal markers include CD63, Alix, and actin (Harding et al., 1984; Izumi et al.; Jaworski et al., 2014a; Ailawadi et al., 2015; Logozzi et al., 2009).

Exosome Biogenesis

The biogenesis of multivesicular endosomes (MVEs) involves the inward budding of an endosome which pinches off into a vesicle (which is now considered an exosome) within the endosomal lumen (Raposo and Stoorvogel, 2013). The endosomal sorting complex responsible for transport (ESCRT) are the multiprotein machineries that may be responsible for the formation of MVEs. The ESCRT 0, I, and II complexes recognize and sequester membrane proteins at the endosomal membrane and the ESCRT III complex allows the budding and pinching off of the endosomal membrane into intraluminal vesicles (Raiborg and Stenmark, 2009; Hurley, 2011). Proteins observed in exosomes suggest them as being present in the cytosol and membranes of endocytic compartments or the plasma membrane. The mechanism responsible for packaging and sorting select proteins into the internal vesicles of late endosomes is not fully understood (Théry et al., 2002). The fusion of the multivesicular endosomes with the plasma membrane at the cell surface allows the secretion of exosomes into the extracellular environment. It is thought that specific protein machinery (i.e. NSF, SNAP, and SNARE complexes) are involved in the fusion of both membranes but this is not fully understood (Pelham, 2001).

Exosomes and Viruses

The role of exosomes from virally infected cells has been explored over the recent years. For instance, virally infected cells secrete exosomes with major changes in protein composition as compared to exosomes from uninfected cells (Meckes et al., 2013). This is demonstrated in exosomes purified from B cells infected with Kaposi sarcoma

herpesvirus (KSHV), Epstein-Barr Virus (EBV), or cells that are dually infected. Mass spectrometry results indicated that 871 proteins were identified and approximately 360 of these proteins were unique to exosomes isolated from virally infected B cells. Previous research has suggested that viral latent membrane protein 1 (LMP1) upregulates the protein content in exosomes from EBV infected cells, which can affect the interferon and NFκB signaling pathways, lipid raft organization, and trafficking of proteins. On the other hand, the effects of exosomes released from KSHV infected cells differed in terms of protein translation, metabolism, and cell migration. In addition, exosomes released from virally infected cells have been shown to have varying compositions of RNA (Canitano et al., 2013). For example, exosomes released from EBV infected lymphoblastoid and nasopharyngeal cells contain EBV encoded latent phase mRNAs.

Current RVFV Vaccines

There are currently no licensed vaccines to protect against RVFV; however, three licensed veterinary vaccines are in use to protect the ruminant population: inactivated virus vaccine, live attenuated virus vaccine, and the clone-13 attenuated virus vaccine (Mansfield et al., 2015). The use of the inactivated vaccine (Entebbe vaccine) requires multiple doses. Although data from a 12 year trial proved the longevity and long term immunity of the vaccine, it was considered an impractical approach in resource-limited agricultural settings in rural Africa (Randall et al., 1962). The live attenuated vaccine (Smithburn) was widely used and deemed as successful in Kenya and South Africa. However, problems were reported of the vaccine causing abortions in some ruminant

species (Botros et al., 2006). The possibility of the vaccine reverting to virulence due to reassortment with the wild type virus could lead to an increase in the diversity of RVFV strains in the surrounding environment (Ikegami, 2012). The Clone 13 attenuated vaccine (contains a significant deletion of the NSs virulence gene) has proven to be avirulent and safe to use in mice and ruminants but intranasal inoculation led to neurological disease in mice. This suggests that the vaccine virus still retains some of its virulence, which decreases its desirability for human and ruminant use (Dodd et al., 2014; Mansfield et al., 2015). The live attenuated MP-12 vaccine (contains mutations in all three genomic segments) has been given a conditional licensure in the United States due to its slight teratogenic effects in ruminants. Further studies are needed to deem this as a competent human vaccine (Mansfield et al., 2015). All of the available licensed options have the potential of co-infecting the host and demonstrating reassortment between wild type and vaccine viruses.

The absence of a competent vaccine highlights the need for a new, safe, cost effective, and efficient vaccine for both animal and human use. Exosomes contain the potential to be used as vectors for vaccinations. Exosomes possess the capability to be used as a delivery system for nucleic acids and proteins with high efficacy and low levels of toxicity and immunogenicity (Andaloussi et al., 2013; Batrakova and Kim, 2016). The data presented here suggest that exosomes from RVFV infected cells may alter the dynamics of the neighboring cells and can potentially contribute to disease pathology. Therefore, it is intriguing to speculate that exosomes released from RVFV infected cells may have the potential to serve as a novel vaccine candidate against RVFV, especially

since they are naturally produced in the course of infection and therefore are not anticipated to pose the type of safety concerns that can be associated with synthetic platforms.

CHAPTER TWO: MATERIALS AND METHODS

Cell Culture and Reagents

Vero (African green monkey kidney) cells were maintained in Dulbecco's modified minimum essential medium (DMEM) supplemented with 10% heat inactivated Fetal Bovine Serum (FBS), 1% L-glutamine, and 1% penicillin/streptomycin. Jurkat (human T cells) and U937 (monocytic) cells were grown in RPMI-1640 (Roswell Park Memorial Institute) supplemented with 10% FBS, 1 % L-glutamine, and 1% penicillin/streptomycin. All cell lines were maintained at 37°C in 5% CO_2. Exosome-depleted media was appropriately supplemented as aforementioned except FBS was ultra-centrifuged at 40,000rpm (Beckman Type 70Ti rotor) for 3h in a ratio of 1:1 (FBS:media).

Western Blot Analysis

Thirty µl of cell lysate in lysis buffer were heated at 70°C for 10 minutes before separation on NuPAGE 4-12% Bis-Tris gels (Novex). The proteins were transferred with either the dry transfer method using iBlot gel transfer nitrocellulose stacks (Novex), or the wet transfer method. An Immobilon PVDF membrane (Millipore) was cut according to gel size and soaked in 100% methanol for five minutes before being placed on top of the protein gel. Both items were placed between 3M paper and sponges and placed within a wet transfer apparatus. The transfer buffer consisted of 10% Tris Glycine Transfer Buffer (Quality Biological) and 20% methanol in diH_2O and allowed to transfer at 250 mA for 2 hours at room temperature or overnight at 80 mA at 4°C. Membranes were blocked in 5% milk in TBS + 0.1% tween-20 (TBS-T) for 2 hours at room temperature,

and then incubated overnight at 4°C with appropriate antibody: α-CD63 (ab8219, 1:500), α-TSG101 (BD612696, 1:1000), α-N protein (generous gift or Dr. C. Schmaljohn-USAMRIID, 1:500), α-V5 (Serotec MCA1360, 1:1000), α-Flag (Sigma-Aldrich F1804, 1:1000), and α-beta actin (ab49900 1:5000). The membrane was washed five times with TBS-T for five minutes each and incubated with the appropriate secondary HRP-coupled antibody at 1:1000 ratio in 5% milk in TBS-T for 1 hour. The membrane was washed 5 times for 5 minutes each before protein bands were visualized using Super Signal West Femto Maximum Sensitivity Substrate kit (ThermoScientific) in a ChemiDoc XRS System (Bio-Rad).

Exosome Purification

Vero cells were expanded into 2 T-225 flasks and either infected or mock-infected at MOI 1 for 1 hour at 37°C, 5% CO_2. Inoculum was removed and cells washed once with PBS prior to the addition of one hundred ml of exosome free DMEM. Supernatants were collected 36 hours post infection (unless otherwise noted) and passed through a 0.22 µm filter to remove any residual apoptotic bodies and dead cells. The filtrate was ultracentrifuged at 10,000g for 30 minutes at 4^0C. The supernatants were transferred to a clean ultracentrifuge tube and were ultracentrifuged at 40,000rpm (Beckman Type 70Ti rotor) for 2 hours at 4^0C. Supernatants were removed and exosome pellets were resuspended in PBS without calcium and magnesium and ultracentrifuged again at 40,000 rpm for 2 hours at 4°C. Exosome pellets were resuspended in 200 µl of sterile PBS without calcium and magnesium. These semi-purified exosomes were stored at -80°C with Halt Protease Inhibitor Cocktail 100x (ThermoFisher Scientific) for subsequent

purification or analysis. The protein concentrations of exosome preparations were determined by BCA protein assay kit (Pierce).

Sucrose Gradient Purification

A 2M sucrose stock was used to prepare four sucrose fractions with densities ranging from 1.0465 g/ml to 1.25 g/ml. Two milliliters of each fraction was pipetted into a Beckman centrifuge tube (Item no. 331372) beginning with the densest fraction. Semi-purified exosome samples were layered on top and ultracentrifuged at 100,000 x g for three hours. Each fraction (taking into account the exosome sample) was removed and desired fractions containing exosomes were resuspened in 20 mls of PBS and ultracentrifuged again at 100,000 x g for 2 hours to remove the sucrose. The final purified exosome pellets were resuspened in PBS with Halt Protease Inhibitor Cocktail 100x (ThermoFisher Scientific) and stored at -80°C.

Plaque Assay

Vero cells were plated in a 6 well plate at 1 x 10^6 cells per well to reach 90%-100% confluency in 24 hours. RVFV supernatant was serially diluted 10 fold in DMEM from 10^{-1} to 10^{-7} dilutions. 400 µL of diluted inoculums were used to infect each well in duplicates. Vero cells were infected for 1 hour at 37°C in 5% CO_2. The infection was followed by a 3ml overlay of a 1:1 mixture of 0.6% agarose in diH_2O and 2X EMEM supplemented with 5% FBS, 2% penicillin/streptomycin, 1% L-Glutamine, 1% Sodium Pyruvate, and 1% nonessential amino acids. The overlay was allowed to solidify at room temperature before incubation at 37°C, 5% CO_2. After 72 hours, 10% formaldehyde in diH_2O was used to fix cells for 1 hour at room temperature. The agarose plugs were

gently removed and the cells were stained using a 1% crystal violet, 20% methanol solution in diH_2O for 30 minutes at room temperature. Average number of plaques were counted for each treatment (wells with plaques greater than 300 were not counted), and the following equation used to deduce viral titer: pfu/ml = average number of plaques x dilution factor x 2.5.

RNA Isolation and Quantitative RT-PCR

Total RNA was isolated from 100 µg of sucrose gradient purified exosome samples (unless otherwise noted) using the trizol-chloroform method. Approximately 400 ng of isolated RNA was utilized for cDNA synthesis with GoScript Reverse Transcriptase System (Promega) using Random Primers. Primer pairs for each of the three segments are as follows: for L segment: RVFV L Polymerase Forward (5'-GGT GGC ATG TTC AAT CCT TT-3'; Tm = 54°C) and RVFV L Polymerase Reverse (5'-GCA TTC TGG GAA GTT CTG GA-3'; Tm = 54°C); for M segment: Gn Forward (5'-AAA GGA ACA ATG GAC TCT GGT CA-3', Tm =58°C) and Gn Reverse (5'-CAC TTC TTA CTA CCA TGT CCT CCA AT-3'; Tm = 58°C); for S segment: RVFV NSs Forward (5'-TCT GAA AGA AGC CAT ATC CT-3'; Tm = 54°C) and RVFV NSs Reverse (5'-CTC GCT ATC ATC CTG TGT AA-3'; Tm = 54°C) and RVFV N Forward (5'-CAT GGT GGA TCC TTC TCT AC-3'; Tm = 54°C) and RVFV N Reverse (5'-CTA TTC ACT GCT GCA TTC AT-3'; Tm = 54°C).). The cycles conditions used were: 1 cycle at 95°C for 2.5 min, 39 cycles at 95°C for 15 sec and 58°C (for L and Gn primer sets) or 54°C (for N and NSs primer sets) for 40 sec. The relative RNA count of the samples were based on the cycle threshold (Ct) value relative to the standard curve.

Samples containing sequences of the segments of RVFV genomic cDNA were diluted and used as quantitative standards.

Cell Viability Assay

Approximately 15,000 Jurkat or U937 cells (unless otherwise noted) were seeded in approximately 100µl of exosomes free RPMI in a 96 well plate. Cells were treated with varying concentrations of exosome samples and allowed to incubate for five days at 37°C in 5% CO_2. Both plate and Cell Titer Glo reagent (Promega) were brought to room temperature for 30 minutes. Equivalent volumes of reagent was added to wells and manually shaken for 2 minutes followed by a 10 minute incubation at room temperature. Fluorescence values from media alone served as background and used to normalize sample values. Percent Viability was calculated based on value of untreated cells. Assays were conducted in technical duplicates.

CHAPTER THREE: RESULTS

Generation of RVFV Resistant Clones

The plan for generation of RVFV resistant clones was devised by Dr. Kashanchi, and I provided assistance to Dr. Kashanchi in carrying out the experimental protocols for the generation of these clones. Exosomes are known entities that are involved in communication between cells resulting in spread of information including cytokines and miRNA, some of which aid in infection of adjacent cell (Fleming et al., 2014; Kalamvoki et al., 2014). We were interested in characterizing exosome-enriched preparations from RVFV resistant clones that were incapable of releasing functional virions and asked whether they could influence the neighboring cells. We decided to generate resistant clones since RVFV infection causes cell death in a majority of infected cell types *in vitro* within 24-48 hours. Therefore, we devised a scheme to generate reliable clones that contained viral RNA and/or proteins that could potentially be secreted from the cells as components of extracellular vesicles, particularly exosomes. Our rationale comes from our previous work where HIV-1 or HTLV-1 infected resistant clones (i.e. latent cells) produced exosomes that contained both viral RNA and/or proteins (Narayanan et al., 2013; Jaworski et al., 2014a). Specifically, we infected Vero cells with RVFV at multiplicity of infection (MOI) of 3 and allowed the cells to incubate for 10-14 days at 37°C days and checked for presence of small surviving resistant clones. These cultures contained individual patches of resistant cells despite having high virus titer in the media. Following a two-week incubation, plates were washed and cells were removed using a micropipette with a solution of trypsin and diluted in a microtiter plate to achieve a 1-10

cell/well cultures (Figure 1A). We created 90 resistant clones using this dilution cloning procedure. These cells were next expanded and supernatants (5 day incubation at 37°C) were added to TLR3 indicator cells. We and others have previously shown that molecules within the exosomes activate various cascades of signal transduction as evident by increase in pro-inflammatory cytokines mediated by TLRs (O'Neill and Quah, 2008; Bhatnagar and Schorey, 2007). We focused on seven of 90 clones that were amongst the top 30 activators of the TLR3 reporter cell line and survived 50 subsequent passages to increase their clonal purity (Supplementary Figure 1). To further assure that the passaged clones were truly resistant to infection, they were re-infected with RVFV (MOI: 3). After 48-72 hours, none of these clones demonstrated signs of cell death following secondary viral infection. However, all of the clones were sensitive to secondary infection by another cytoplasmic RNA virus, the Venezuelan Equine Encephalitis Virus (data not shown). We finally performed a set of confirmatory assays including RNA PCR to validate presence of RVFV genome in these clones (Supplementary Figure 2). Collectively, these data imply that RVFV infection can allow generation of cells that are resistant to RVFV infection.

We next asked whether we could isolate resistant clones again, this time using another RVFV strain that has been reconstructed to contain epitope-tags in the viral genome. The clone epi-RVFV contains V5-tagged L, and C-terminal Flag-tagged NSs. The virus was synthesized by reverse genetics and was replicated in Vero cells to obtain high titers (generous gift of Drs. Terasaki and Makino, UTMB). Again, similar to experimental design mentioned above, Vero cells were infected with wild type epi-RVFV

and resistant clones were isolated and screened using TLR3 indicator cells. Results in Figure 1B indicate that resistant clones were able to survive viral infection and subsequently few of the clones were isolated for passages and dilution cloning. Four clones were chosen for the characterization mainly due to ease of isolation and maintenance of clones in cell culture for an extended period of time.

Effect of Resistant Clone Supernatants on Vero Cells

These experiments were mainly performed by Dr. Kashanchi and I provided assistance by performing the qRT-PCR analysis. We asked whether the activation of the TLR3 cells (Figure 1B) was due to the presence of potential mutant viruses contaminating our resistant clone preparations. We designed the following experiment to address this issue: unfiltered supernatants from both clones H6 and #14 were added to fresh Vero cells and the cells were washed 24 hrs later. Fresh media was then added and samples (both supernatants and cells) were collected at the end of weeks 1, 2, and 3. Then, independent supernatants from week 0 (starting material) and weeks1, 2, and 3 were treated with nanoparticles NT080, NT082 (to concentrate exosomes) and NT086 (to concentrate potential virus) prior to qRT-PCR for presence of Rift genome (Figure 2A). We have previously shown that dilute viral or exosomal samples can be concentrated and enriched using specific nanoparticles (Jaworski et al., 2014b). Data in Figure 2B shows that Rift RNA was present in the resistant clone supernatants (starting material; S0) but was almost completely absent in supernatants from treated Vero cells in weeks 1-3 (S1-3). We also looked at the intracellular RNA level from these treated cells and found that the Rift

RNA was only present in week 1 samples and completely absent in weeks 2 and 3 samples (Figure 2C). Cells at weeks 1-3 were actively growing as evident by duplication and presence of GAPDH. We next asked whether exosome from clone #14, that were involved in the induction of apoptosis in immune recipient cells (Figure 5), had the capacity to replicate its genomic RNA in these cells. Our rationale for these experiments is based on data demonstrating that exosomes from HCV infected cells contain viral genomic RNA that can enter into uninfected cells and replicate independent of its specific receptor (Masciopinto et al., 2004; Bukong et al., 2014). This would be in comparison to an active viral replication, where viral RNA replicates to ~10^6 copies in 24-48 hours after infection. Therefore, we treated both Jurkat and U937 cells with clone #14 or control exosomes from uninfected Vero and isolated total RNA at 2 and 4 days followed by qRT-PCR for presence of viral genomic RNA. Results in Figure 2D indicate that the Rift RNAs did not replicate in either cell type, further implying that components of replication machinery may be missing in these exosome-enriched samples, hence resulting in an eventual loss of genomic RNA in the recipient cells. Collectively, these data further imply that the resistant clones do not contain mutant viruses that could potentially replicate with slower kinetics in susceptible cells.

Presence of Viral Genome in Exosome-Enriched Preparations

I performed the qRT-PCR experiments for this analysis. We next isolated exosomes from the 2nd set of clones using differential centrifugation and compared their RNA content with exosomes from the 1st generation un-tagged clones. Results in Figure 3 indicate that

indeed the exosome-enriched preparations contained viral RNA in both the 1st and 2nd set of clones. Two clones, H6 from the 1st set and #14 from the 2nd set, showed the highest level of genomic RNA (all 4) in the exosome-enriched preps. Collectively, these results imply that Rift RNA are present not only in intracellular compartment of the resistant clones but also in exosomes.

Presence of Viral Proteins in Exosome-Enriched Preparations

These experiments were performed by myself and Alex Barclay. Presence of Rift viral RNA promoted us to ask whether viral proteins were also present in these exosome preparations. We performed western blots against the critical viral protein N. Antibody against N protein was readily available as a generous gift of Dr. Connie Schmaljohn, USAMRIID. Results in Figure 4 indicate that clones E1, E5, H6, and #14 contained varying amounts of N protein in the exosome-enriched materials. The presence of N protein was independent of N RNA, as apparent in clones #1, 21 and 36. Other exosomal markers including CD63 and actin were expectedly present in all tested clones.

Effect of Exosome-Enriched Materials on Recipient Cells

I assisted Dr. Kashanchi with these by conducting the cell viability assays. We and others have previously shown that exosomes from latently HIV-1 or HTLV-1 infected cells are capable of controlling survival of recipient uninfected cells (Narayanan et al., 2013; Jaworski et al., 2014a; Lenassi et al., 2010; Aqil et al., 2014). The effects were largely due to either viral proteins or RNA present in their exosomes. Here, we asked whether the exosome-enriched preparations obtained from the Rift resistant clones also were able to control cell survival in recipient cells.

We designed an experiment on indicator cells using concentrated exosomes. Approximately 50 -100,000 cells were plated in a 96 well plate and exosomes enriched through differential ultracentrifugation from 1st/2nd set of resistant clones and Vero control cells were added to monocytes and lymphocytes and allowed to incubate at 37°C for five days. Viability was subsequently assayed using CellTiter-Glo. No additional culture supernatant or media was added during the experiment.

Results in panel A indicate that clones H6 and #14 were effectively inhibiting cell viability in both cell types following 5 day incubation. Interestingly, clones H6 and #14 contain high levels of all 4 RNAs from RVFV. Other clones including #1, 21, and 36 had lower levels of all RNAs with minimal cell death. Clone E5 was missing Gn altogether with no apparent cell death. Collectively, these results suggest that the death of recipient cells may be related to high level of viral RNA in exosomes.

Effect of RVFV on Immune Cells

One of the fundamental questions regarding our work on exosomes is whether exosomes released from infected cells could play a critical role in the pathology of the disease in comparison to the viral release. This is an important question since viral infection usually results in release of high titer viruses over a period of time, which may overwhelm and mask any potential exosomal effect on the recipient cells. Along these lines, data in Figures 5A indicate that exosomes can regulate the recipient immune cells and cause apoptosis over a period of time. However, it was not clear to us whether RVFV could also cause apoptosis of immune cells *in vitro*. To answer this, we performed an experiment where T-cells (Jurkat) and monocytes (U937) were treated at MOI 1.0 and

incubated with the virus for 5 days. In our initial set of experiments, we monitored these cells everyday by microscopy and trypan blue staining (data not shown). Subsequent quantitative experiments were performed in microtiter plate and assayed for viability using Cell Titer- Glo. Interestingly, we observed complete resistance of the immune cells to apoptosis by RVFV over an extended period of time (Figure 5B). As expected, positive control Vero cells died following infection. Collectively, these results imply that immune cells may be resistant to RVFV-induced cell death, whereas exosomes could potentially target immune cells for destruction. This dual action of viral infection of specific target cells and apoptosis of immune cells from exosomes could contribute to the overall pathology of RVFV infection.

Effect of Exosomes on the Recipient Cell Apoptosis Machinery

These experiments were performed by Robert Barclay. We next asked whether classical markers of apoptosis were present in the recipient cell extracts treated with exosome-enriched populations. We performed western blots for markers of PARP, Caspase 3, as well as PKR degradation, a known marker of NSs target in cells (Habjan et al., 2009). We treated both T-cells (Figure 6A) and monocytes (Figure 6B) for 5 days and subsequently obtained whole cell extracts for western blot analysis. We used Rift infection (MOI=0.1) and uninfected Vero exosomes as positive and negative controls respectively. Our rationale for these experiments was based on the fact that Cell Titer Glo assay alone (data in Figure 5) does not differentiate between various stages of cell viability. Data in panel A indicate that PARP and Caspase 3 cleavage was mostly seen in recipient T-cells treated with exosome clone #14. This was also somewhat consistent in

monocytes treated with clone #14 exosomes (panel B). Interestingly, clone H6 treated cells didn't show appreciable levels of PARP or Caspase 3 cleavage in either of the cell types, indicating that the mechanism of action for these two clones may be very different in treated recipient cells. Finally, for clone #14 lane, degradation of PKR was much more pronounced in monocytes as compared to T-cells, implying that the NSs activity may be higher in these cells. Collectively, these results imply that exosome treated recipient cells may undergo apoptosis.

Purification of Exosomes from Cells Susceptible to RVFV

I fully conducted all the experiments for the RVFV-susceptible cells. The role of exosomes from RVFV-infected Vero cells that have not been selected for resistance to infection were also analyzed. Similar to the exosomes released from resistant clones, the specific goals were to obtain exosomes derived from susceptible RVFV-infected cells and characterize them to identify if any viral RNA/proteins were present, in addition to determining their functional properties. Successfully doing so would yield completely novel information about the process of RVFV infection through analysis of intercellular communication mechanisms that previously had not been studied, opening a new window of understanding into the RVFV pathogenesis.

Two T225 flasks of Vero cells were infected/mock-infected with double-tagged RVFV for 1 hour and supernatants were collected approximately 36 hours post infection and passed through a 0.22 µm filter unit. Due to their similar size, ultracentrifugation of exosomes also brings down RVF virions and therefore further purification is required to separate out the exosome population. To address this critical issue, we began testing

different sucrose density distributions based on the known densities of exosomes and Bunyaviridae, 1.16-1.23 g/ml and 1.16-1.18 g/ml, respectively (Théry et al., 2006; Schmaljohn et al., 1983; El Mekki et al., 1981). Through these studies, we were ultimately able to optimize the density distribution and identify a suitable density gradient profile for our purification purposes. The unpurified exosome pellet was placed on two successive sucrose density gradients and the five fractions were separated based on varying densities and labeled as 1, 2, 3, 4, and 5.

In order to ensure proper isolation of exosomes, it was necessary to perform Western blot analysis of the double sucrose gradient purified exosome samples by probing for well-established exosomal markers (i.e., CD63 and TSG101). A similar analysis was previously conducted with exosomes isolated from resistant clones using antibodies against CD63, Actin, and Alix. The profile for the exosomes (Figure 7A) is similar for both exosomes originating from uninfected cells (Ex_U) and from RVFV-infected cells (Ex_I). All exosomes preparation demonstrated the same profile in that fractions 2, 3, and 4 would contain exosomes. For the remaining experiments, these three fractions containing highly purified exosomes were combined and used a single preparation. The western blot probing for TSG101 was conducted on three independent preparations (Figure 7A). Next, it was necessary to quantify the number of RVF virions that may be present in the Ex_I preps to verify that the sucrose gradients were indeed purifying the exosomes away from the virus. To address this issue, plaque assays were conducted on three independent preps both after ultracentrifugation (before sucrose gradient purification) and also after each of the two sucrose gradient purifications (Figure

7B). The results for all the three independent preparations show that after purifying the Ex_I population with the second sucrose gradient approximately 99.99% of the virus population is removed (Figure 7B). This indicates that conducting two successive sucrose gradients post ultracentrifugation using our optimized densities allows quantitative separation of the CD63+, TSG101+ exosomes from the remaining RVFV virions.

Sucrose Gradient Purified Exosomes Contain Viral RNA

We determined whether the RVFV genome (whole or fragments) was packaged in exosomes of infection origin similar to the exosomes released from resistant clones. RNA was isolated from purified exosomes of uninfected and infected origin and analyzed by qRT-PCR using primer pairs for the three viral genome segments (L, M, and S). Similar to Figure 3, the Ex_I population contained signal for all segments of viral RNA at varying levels for two independent preps (Figure 8). The negligible amount of virus present in the Ex_I preps was taken into account by also including a condition in which an identical amount of virions was tested as control. The Ct signal for this control condition was similar to that of the Ex_U population (negative control), demonstrating that as expected the amount of virions present in the purified EX_I preps is indeed negligible and does not contribute a signal. The data for the Ex_I population reflects the viral RNA present in just the Ex_I populations.

Sucrose Gradient Purified Exosomes Contain Viral Proteins

In addition to testing for viral RNA, it is imperative to establish whether the exosomes released from cells susceptible to RVFV infection contain viral proteins. Preliminary data from the resistant clone studies (Figure 4) suggested that exosomes may contain, or are

associated with, various RVFV proteins such as N. As previously conducted, isolated exosomes were subjected to Western blot analysis using antibodies against the N protein, C terminal FLAG-tagged NSs, and V5-tagged L polymerase. Figure 9 shows that two independent preparations of Ex_I did not contain the L or NSs proteins but both did contain N (similar to the exosomes released from resistant clones). These results suggest selective packaging of RVFV proteins into exosomes and indicate that the presence of exosome-associated viral proteins is independent of the presence of viral RNA segment expressing them, as shown in Figure 8.

Effect of Purified Exosomes on Viability of Recipient Cells

As seen in Figure 5A, the addition of exosomes derived from the resistant clones induced cell death in recipient cell lines (T cells and monocytes). We tested whether this is also observed with the Ex_I preps. Similar experiments were performed by adding various amounts of purified exosomes of infected and uninfected origin to monocytes and lymphocytes. The cells were incubated for five days without renewing the media and assayed for cell viability using the CellTiter-glo assay (measuring the levels of ATP in viable cells). As the concentration of Ex_I increased from 2ug to 10ug the viability of both cell lines decreased as well, to as low at 10% cell viability. On the other hand, the control Ex_U population did not have any appreciable effect on the viability (Figure 10). These results are similar to what was observed with exosomes isolated from certain resistant clones (Figure 5A). As seen in Figure 5B, when RVFV was added to the immune cells there was no significant effect on viability. Therefore, the decrease in viability observed

is due to the exosomal population and not the minute amount of virions present in the sample.

CHAPTER FOUR: FIGURES

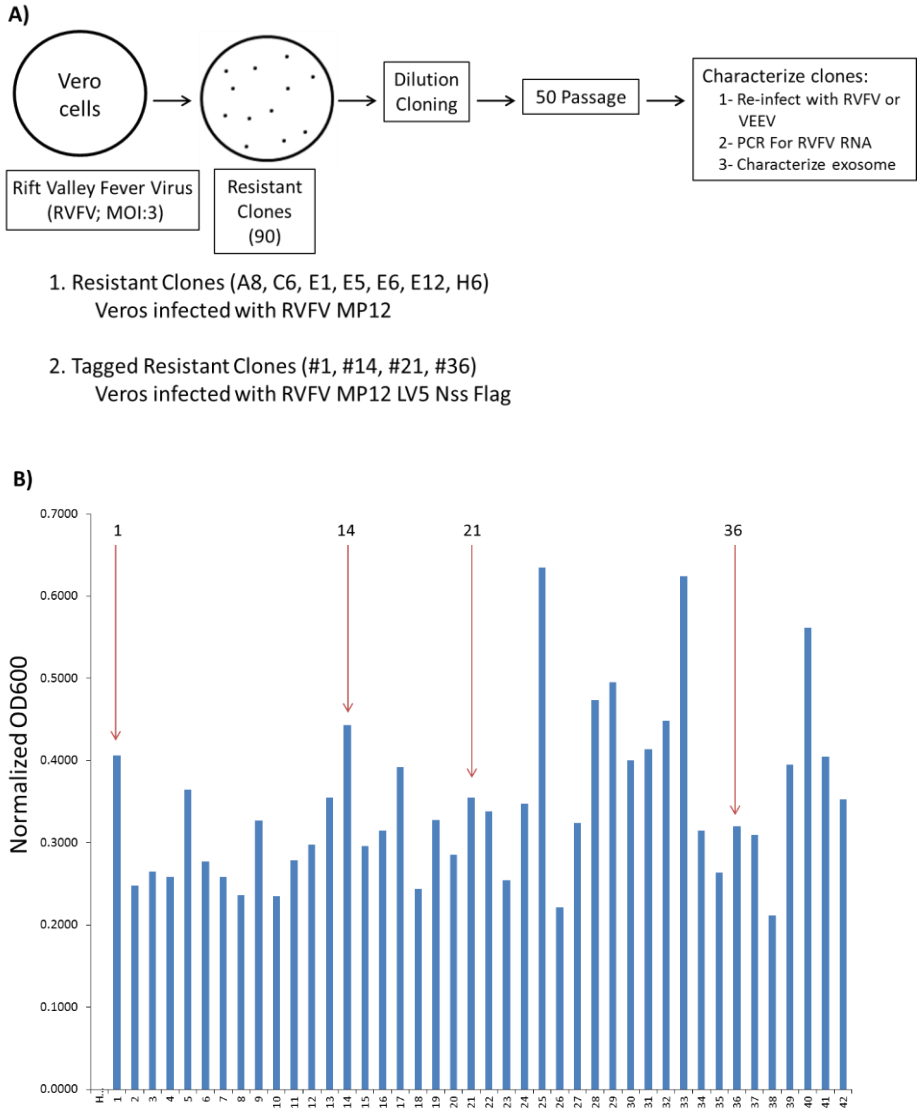
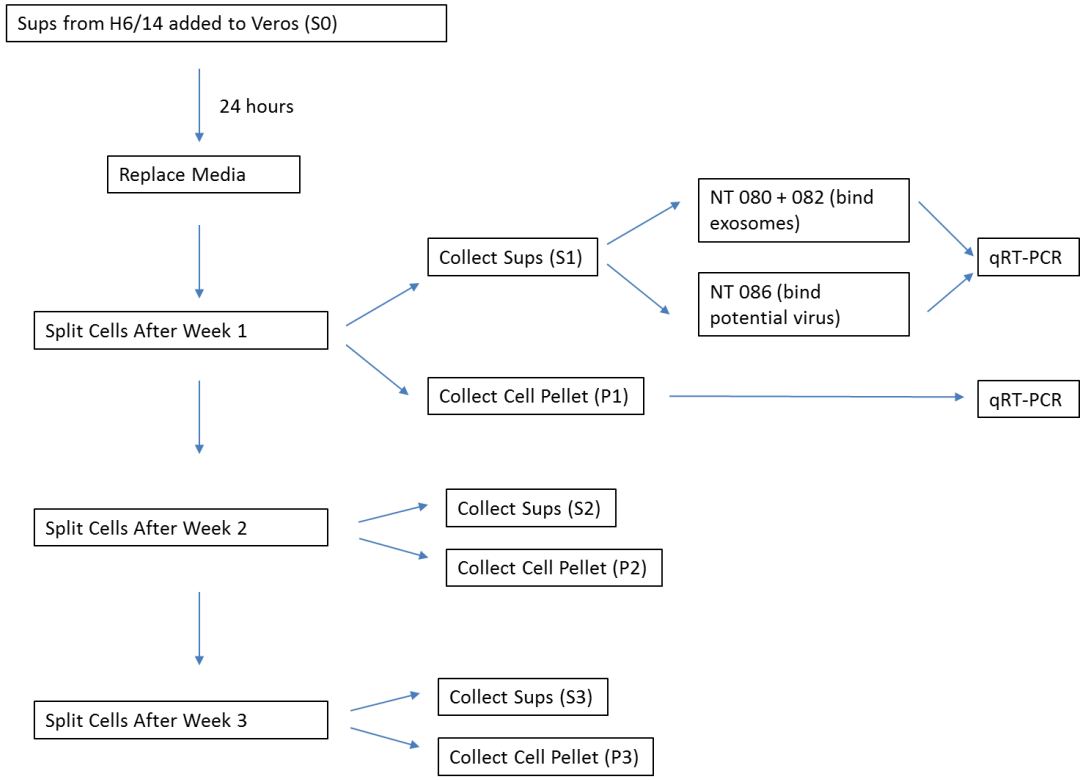
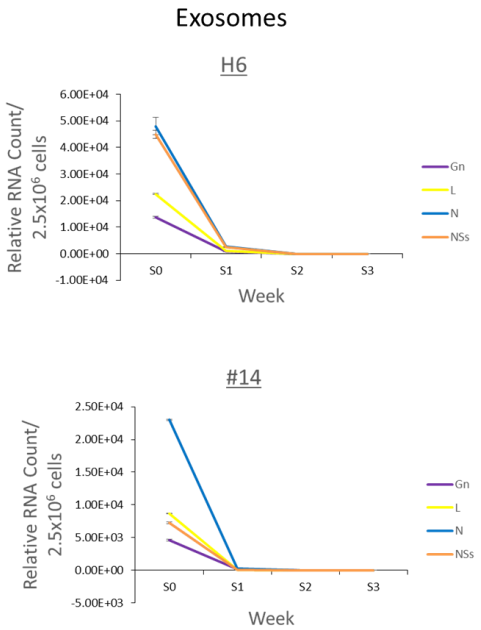


Figure 1: Generation of Rift infected resistant clones. A) Vero cells were infected with RVFV at MOI 3. Approximately 1% of cells survived the infection and isolated using trypsin diluted with PBS. Clones were plated and passaged 50 times before characterization. **B)** The HEK-293T based reporter cell line, HEK-Blue hTLR3 (InvivoGen), was used to detect activation of TLR3 by supernatant of RVFV resistant Vero cells. After 18 hours of incubation at 37^0C in 5% CO2, the absorbance (600 nm) of each control and test condition in the 96-well plate was measured using the GloMax Multi Detection System (Promega). Readings from all positive controls (sample 2, 10ng/mL Poly I/C) and experimental samples were normalized using the mean reading from three sterile water treated negative controls. Four clones (as indicated) were selected for follow up experiments. Error bars on the first two samples (negative and positive controls) indicated ± 1 SD of biological triplicates.

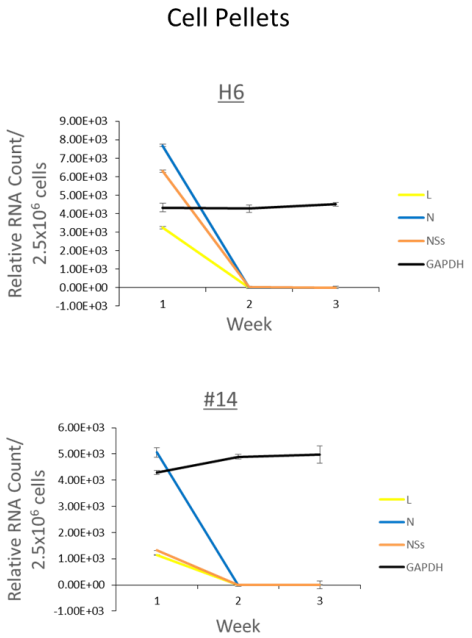
A)


Sups from H6/14 added to Veros (S0)
24 hours
Replace Media
Split Cells After Week 1
Collect Sups (S1)
NT 080 + 082 (bind exosomes)
NT 086 (bind potential virus)
qRT-PCR
Collect Cell Pellet (P1)
qRT-PCR
Split Cells After Week 2
Collect Sups (S2)
Collect Cell Pellet (P2)
Split Cells After Week 3
Collect Sups (S3)
Collect Cell Pellet (P3)

B)


Exosomes
H6
Relative RNA Count/ 2.5x10^6 cells
6.00E+04
5.00E+04
4.00E+04
3.00E+04
2.00E+04
1.00E+04
0.00E+00
-1.00E+04
S0
S1
S2
S3
Week
Gn
L
N
NSs
#14
Relative RNA Count/ 2.5x10^6 cells
2.50E+04
2.00E+04
1.50E+04
1.00E+04
5.00E+03
0.00E+00
-5.00E+03
S0
S1
S2
S3
Week
Gn
L
N
NSs

C)


Cell Pellets
H6
Relative RNA Count/ 2.5x10^6 cells
9.00E+03
8.00E+03
7.00E+03
6.00E+03
5.00E+03
4.00E+03
3.00E+03
2.00E+03
1.00E+03
0.00E+00
-1.00E+03
1
2
3
Week
L
N
NSs
GAPDH
#14
Relative RNA Count/ 2.5x10^6 cells
6.00E+03
5.00E+03
4.00E+03
3.00E+03
2.00E+03
1.00E+03
0.00E+00
-1.00E+03
1
2
3
Week
L
N
NSs
GAPDH

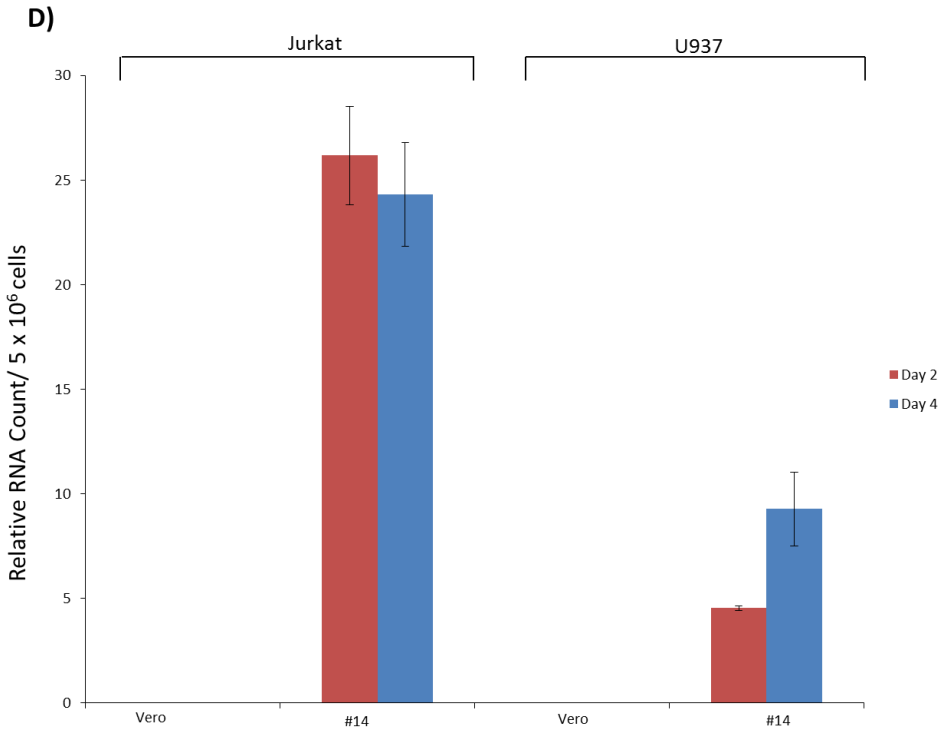


Figure 2: Effect of Resistant Clone Supernatants on Vero Cells. A) Diagram depicting experimental outline in panels **(B)** and **(C)**. **B)** Supernatants (1 ml) from clones H6 and #14 were added to Vero cells (2.5 X 10^6/ml in complete media in a 24 well plate; ~35% confluency) overnight, washed 24 hrs later and supplemented with complete media. Each week, supernatants (1 ml) were collected and concentrated using mixture of nanoparticles NT080 + NT082 (to concentrate exosomes; 50 ul of 30% slurry) and NT086 (to concentrate potential virus; 50 ul of 30% slurry) overnight at 4˚C. Samples were pelleted the next day, washed, and total RNA was isolated for qRT/PCR. "S0" denotes the starting material and "S1-3" was supernatants from treated Vero cells in weeks 1-3. **C)** Similar to panel (**B**), except that the total RNA was isolated from the cell pellets prior to qRT/PCR for Rift RNAs. GAPDH served as internal control for RNA expression. **D)** Total RNA isolated from Jurkat and U937 cells were treated with exosome-containing preparations from Vero or resistant clone #14 after 2 and 4 days. qRT-PCR analysis were conducted with primers specific for NSs.

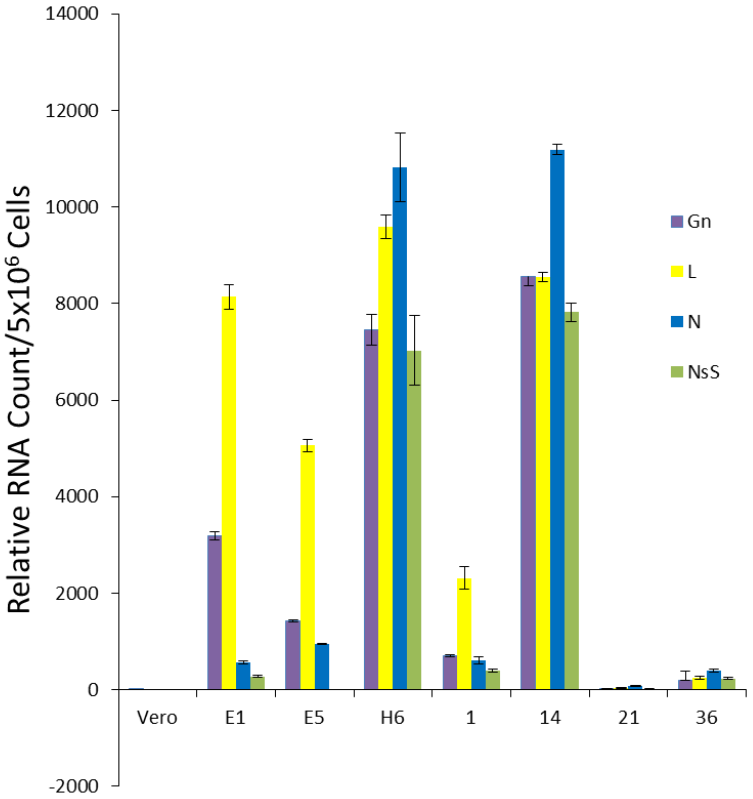


Figure 3: Presence of RVFV Genomic RNA in Exosomes. Exosome-enriched preparations were isolated from first and second sets of clones via differential ultracentrifugation method. Total RNA was extract to test for the presence of RVFV genome.

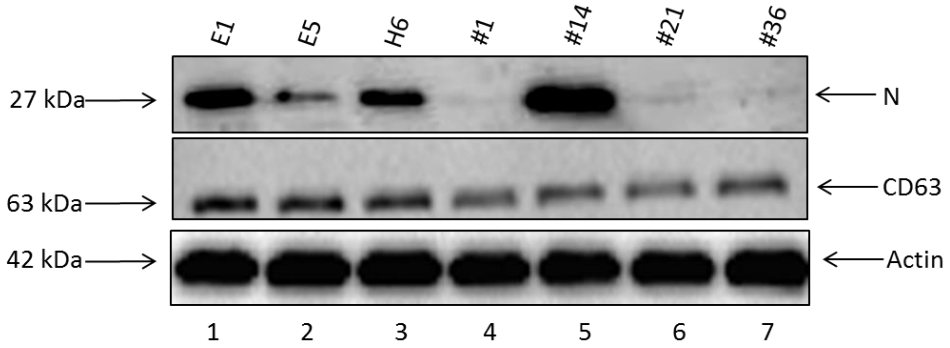


Figure 4: Presence of Viral and Exosomal Proteins in Exosomes. Enriched exosomes were isolated through differential centrifugation and were analyzed by Western Blotting with antibodies to N protein (generous gift from Dr. Connie Schmaljohn; 1:500), CD63 (ab8219; 1:500), and Actin (ab49900; 1:5000).

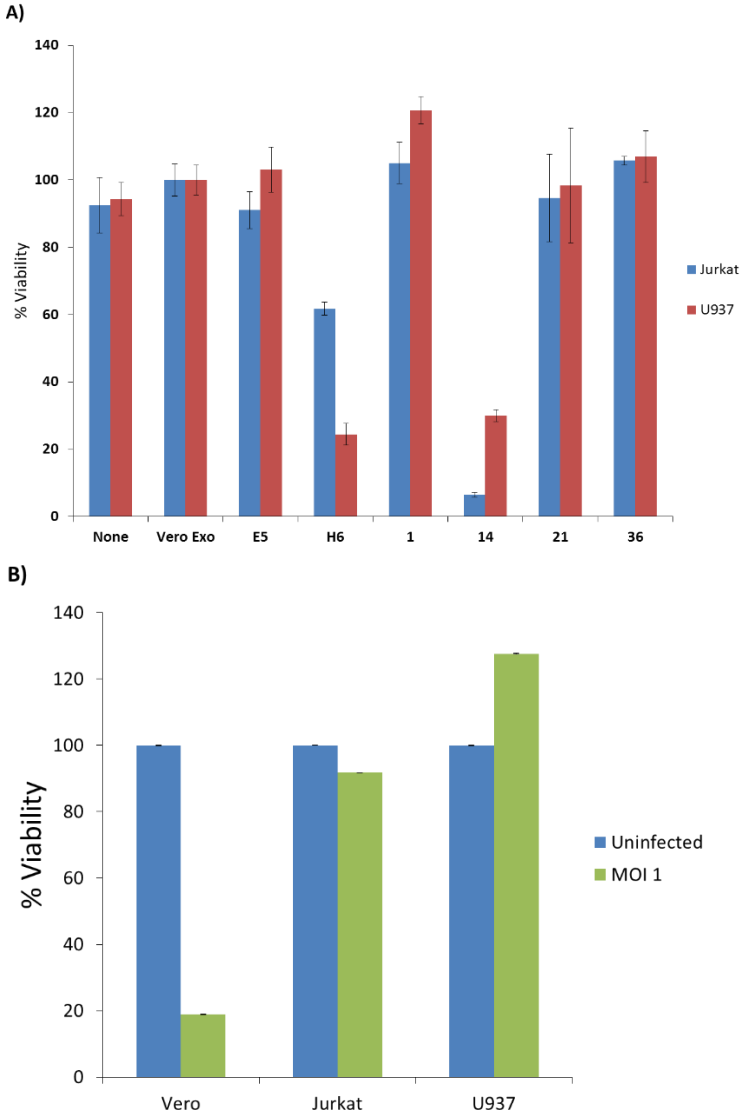


Figure 5: Effect of Exosomes on Recipient Cells. A) Exosomes were isolated through differential ultracentrifugation and 2µg was added to 50,000-100,000 cells in a 96 well plate. Cells were allowed to incubate at 37°C for 5 days and viability was subsequently assayed using CellTiter-Glo assay. Values for exosome-free DMEM (control) were used to subtract background and samples treated with exosomes from uninfected Vero cells were set to 100% and used to normalize the experimental values. The assay was conducted using biological triplicates and error bars indicate ± 1 SD. **B)** Immune cells including T-cells (Jurkat) and monocytes (U937) were grown to log phase of growth in complete media (1.5 x 10^6 cells/ml). RVFV (epitope-tagged MP12) at MOI of 1.0 was used for infection of immune cells and Vero cells as a positive control. Cultures were incubated for 5 days as 37°C (without removing the virus) and cell viability were assayed using CellTiter-Glo. Results in panel B are from 2 independent experiments. Similar results were also observed with the wild type un-tagged MP12 (data not shown).

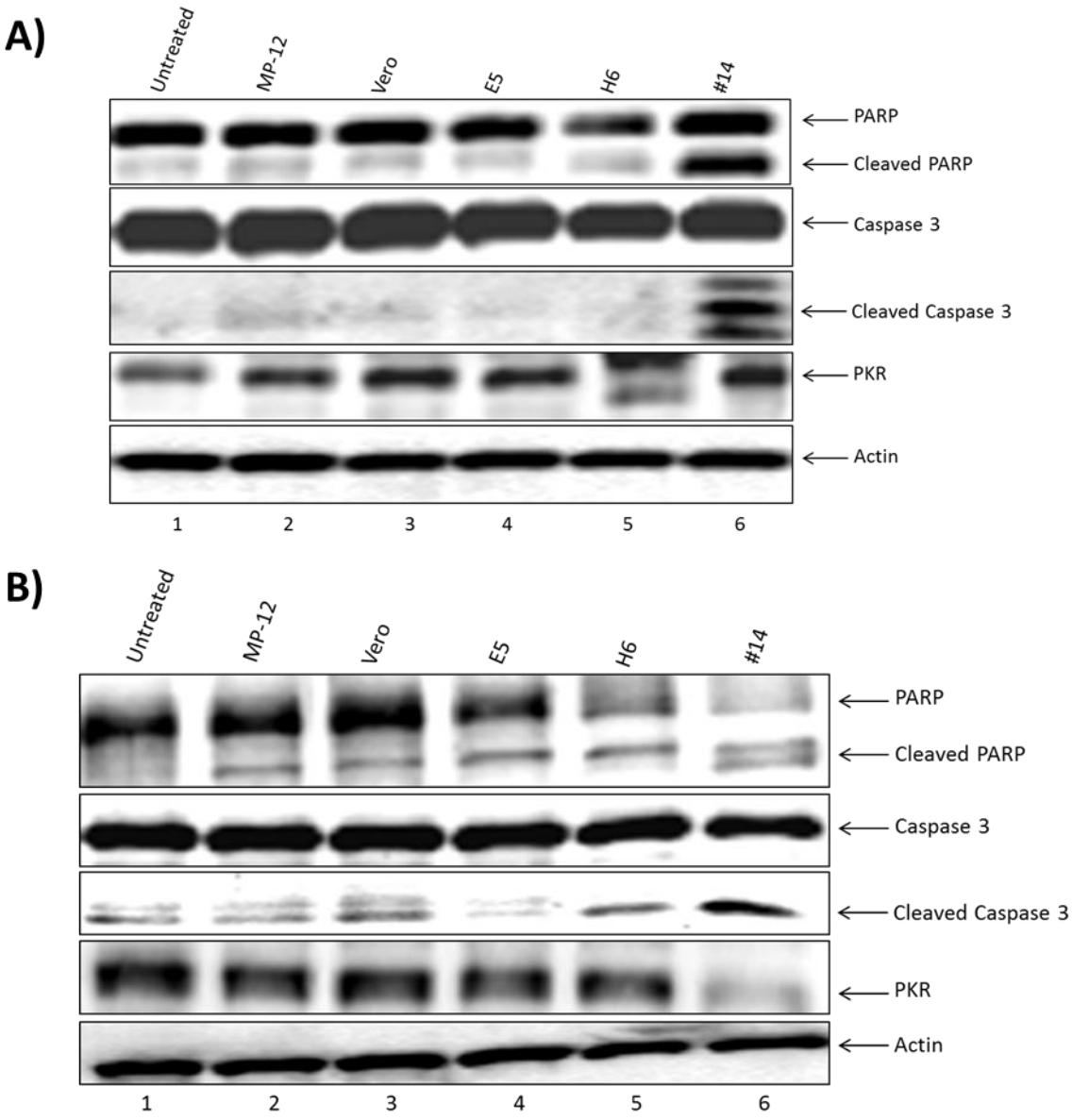


Figure 6: Effect of exosomes on the recipient cell apoptosis machinery. A) Jurkat cells treated with exosome-enriched preparations from resistant clones were analyzed by Western blotting with antibodies to markers of apoptosis, which included Caspase 3 (sc-7148 1:200), PARP (sc-7150 1:200), and PKR (sc-707 1:200), or Actin (ab49900 1:5000). **B)** U937 cells were treated similar to panel A and extracts were used for western blot analysis probing for the same apoptosis markers.

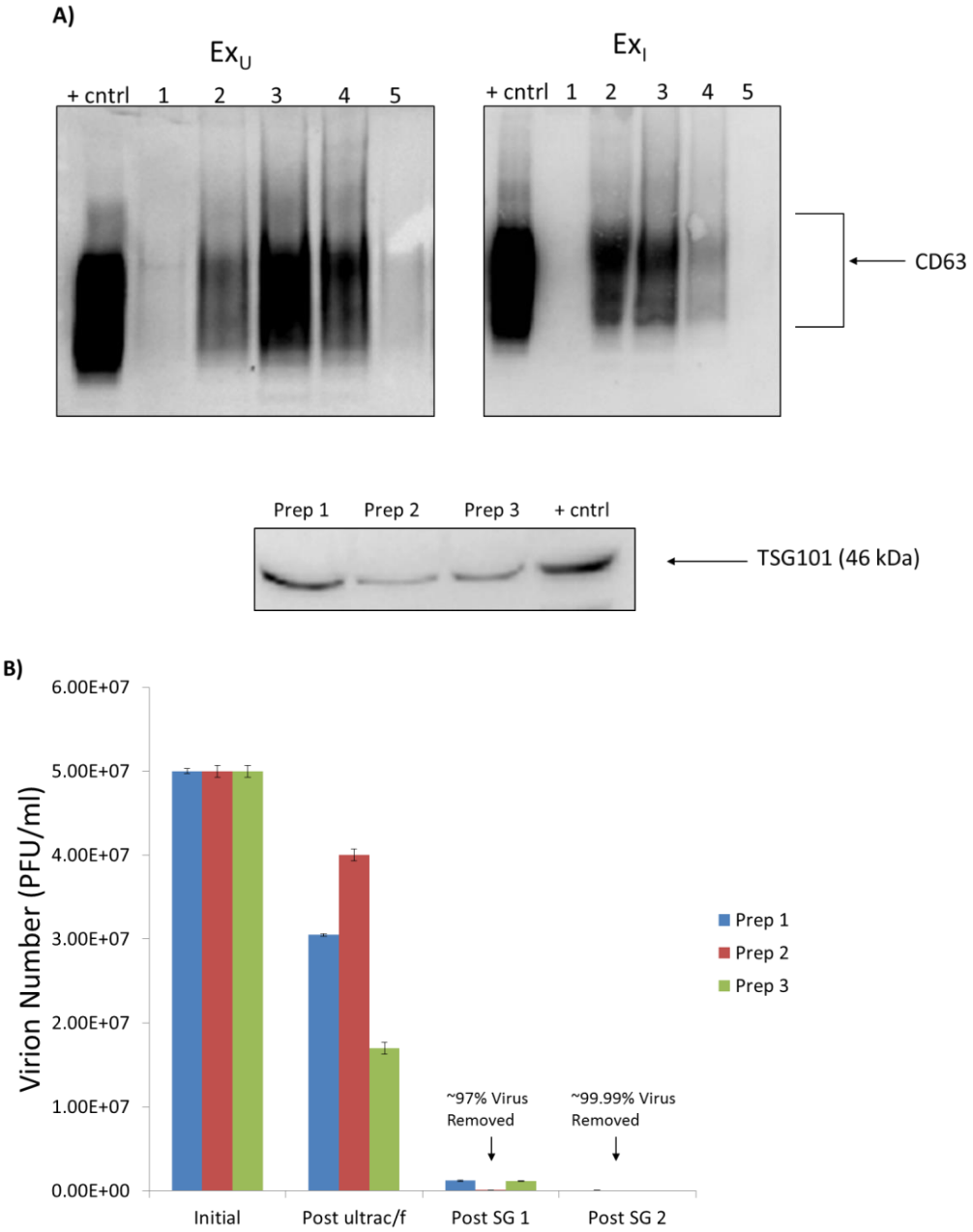


Figure 7: Purification of Cells Susceptible to RVFV. A) Exosomes were spun on two successive sucrose gradients and fractions 1-5 were separated. Protein content was analyzed by western blot for exosomal marker CD63 (ab8219, 1:500). Ex_U and Ex_I denote exosomes harvested from uninfected and infected origin. Fractions 2, 3, and 4 were combined and denoted as a prep. Ex_I preps were also analyzed by western blot for exosomal marker TSG101 (BD612696, 1:1000). **B)** Plaque assays were conducted for three independent Ex_I preps after the initial ultracentrifugation spin and two sucrose gradient (SG) purifications.

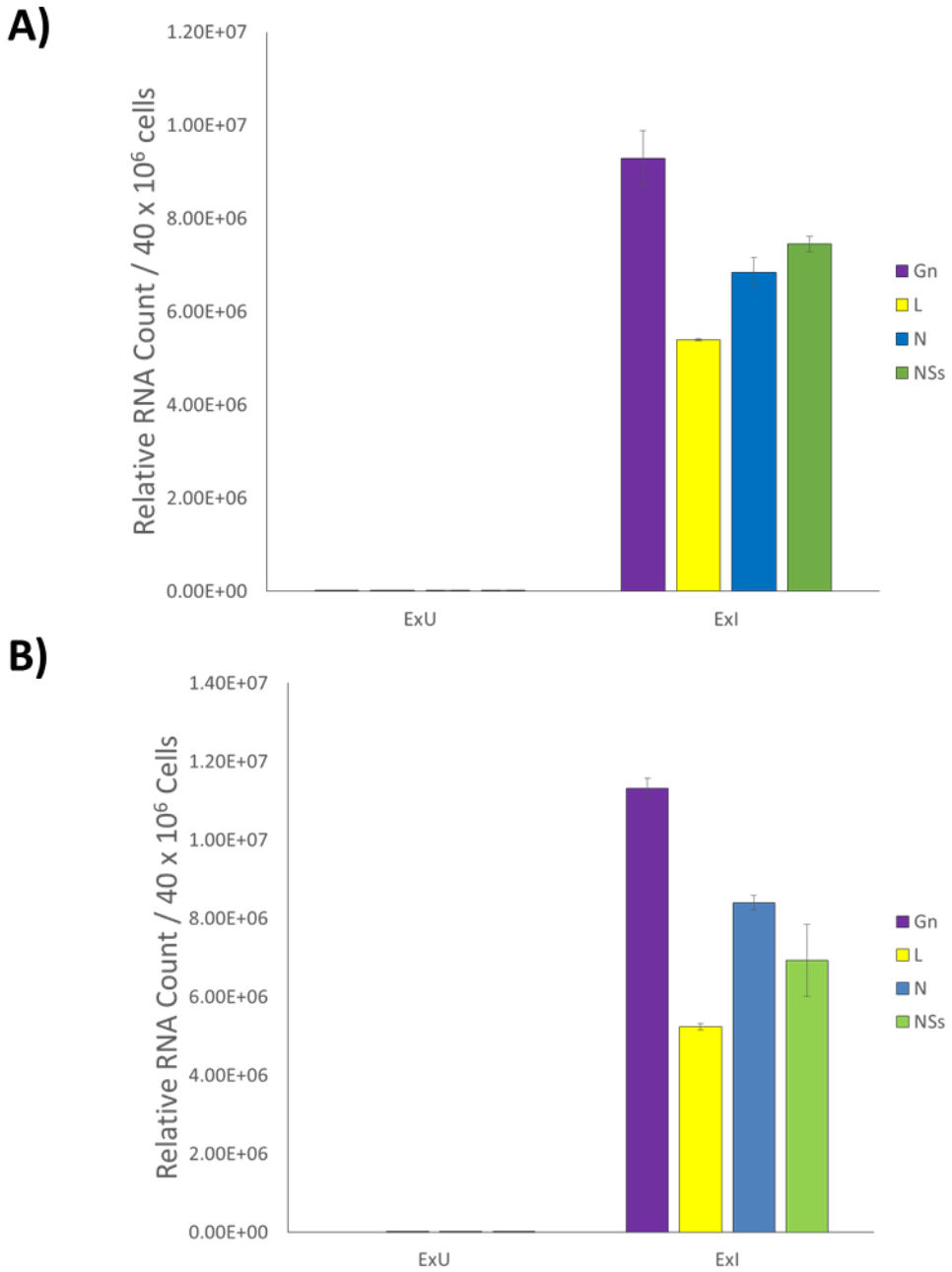


Figure 8: Sucrose Gradient Purified Exosomes Contain Viral RNA. A) Total RNA was extracted from Ex_U and Ex_I samples using the trizol-chloroform method. Approximately 400ng/µl of RNA was utilized for cDNA synthesis using the GoScript Reverse Transcriptase System. qRT-PCR was performed and the absolute quantification of the samples was determined based on the cycle threshold (Ct) value relative to the standard curve. **B)** Similar to (A), qRT-PCR was performed on a second set of biological replicate.

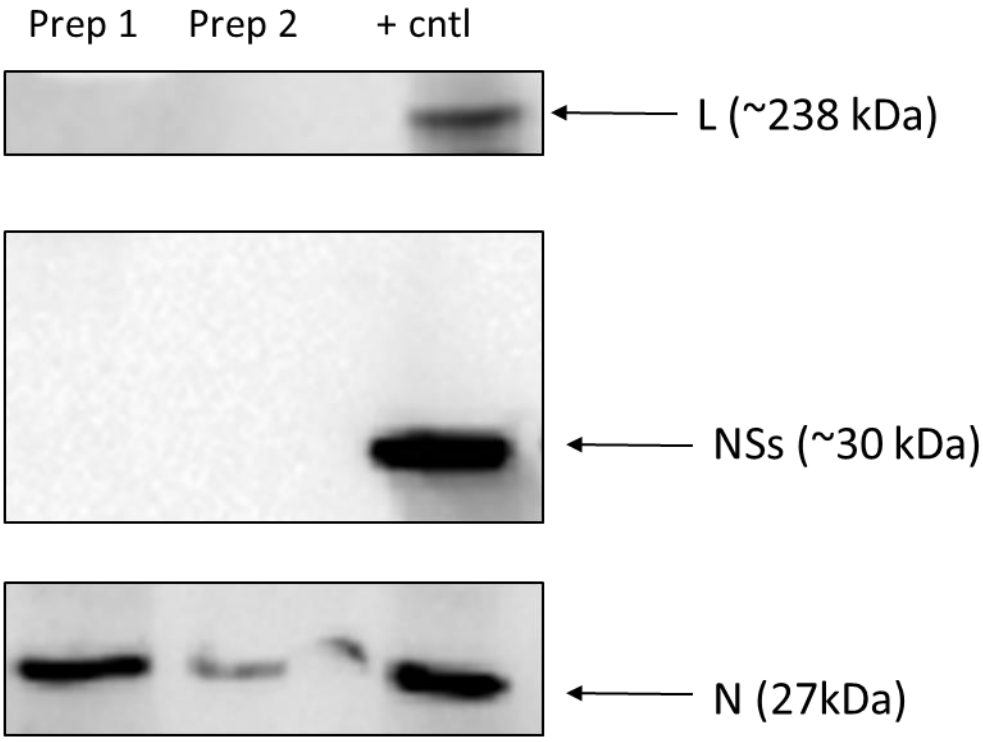


Figure 9: Sucrose Gradient Purified Exosomes Contain the Viral Protein N. Ex_I samples were analyzed by western blot with antibodies against V5 (Serotec MCA1360, 1:1000), Flag (F1804, 1:1000), and N protein (generous gift or Dr. C. Schmaljohn-USAMRIID, 1:500). Corresponding positive controls were whole cell lysates of RVFV infected Vero cells.

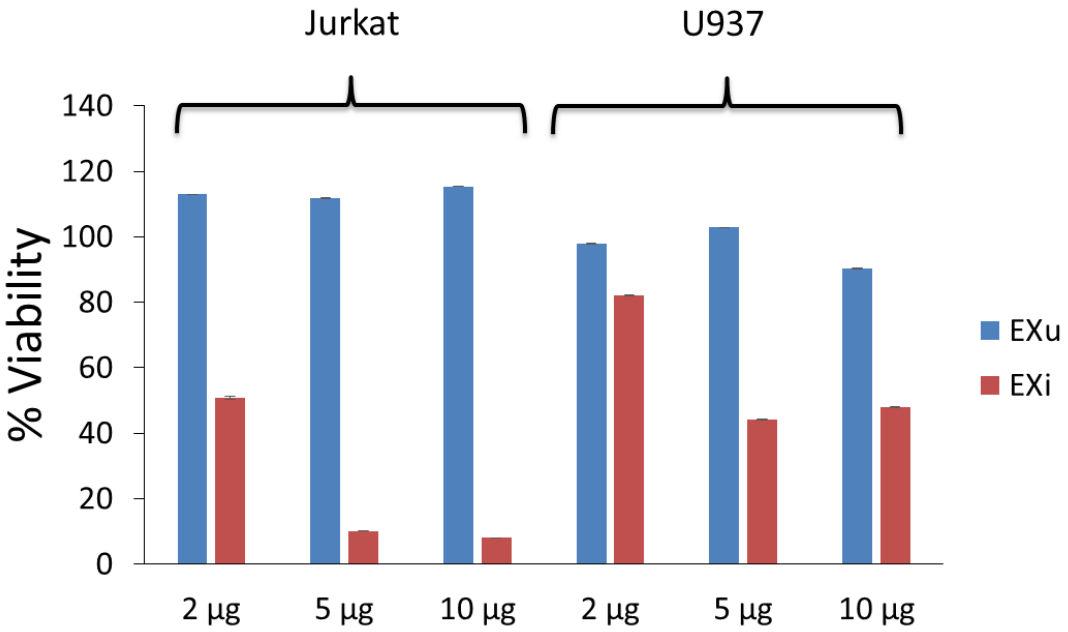
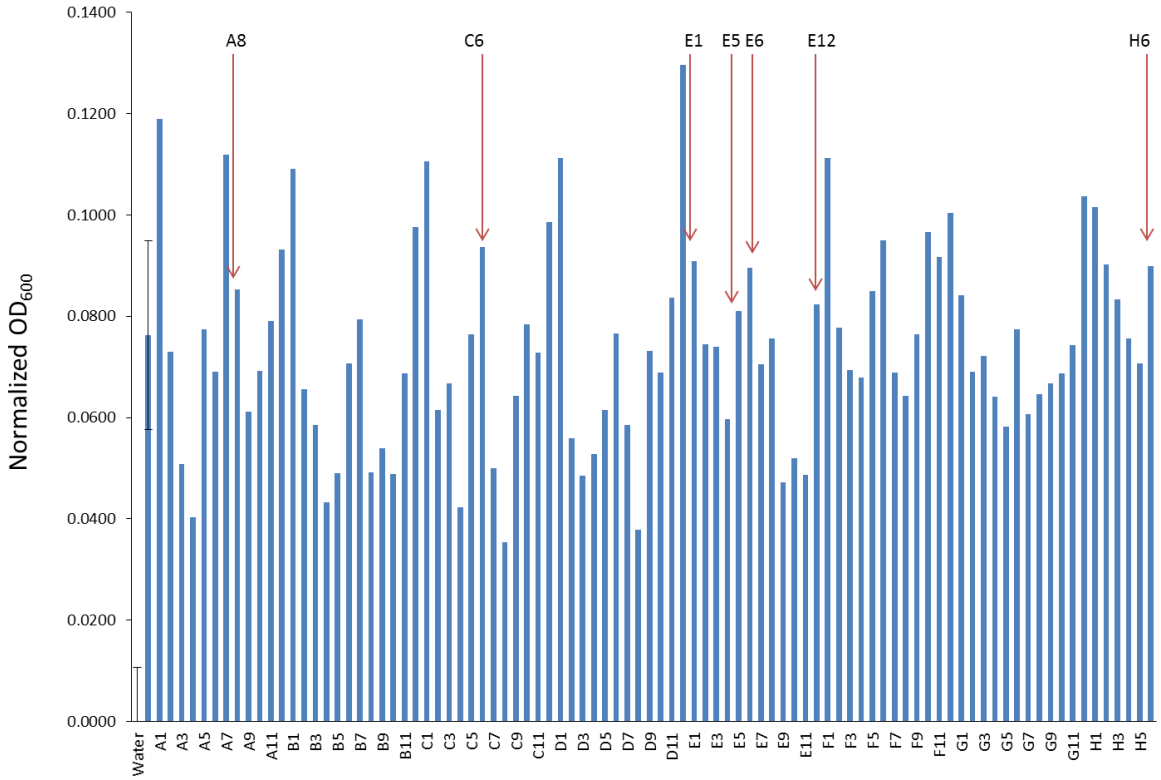
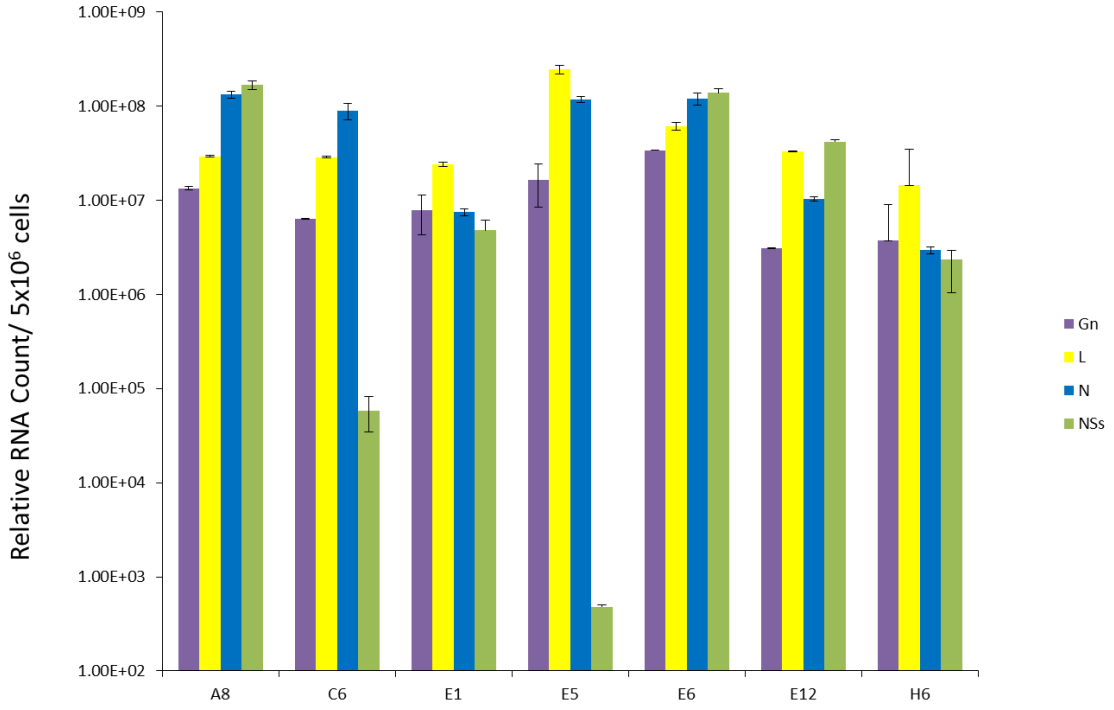


Figure 10: Effect of Purified Exosomes on Viability of Recipient Cells. Approximately 15,000 Jurkat or U937 cells were seeded and treated with Ex_U or Ex_I and allowed to incubate for 5 days at 37°C. Viability of cells was subsequently assayed using Cell Titer-Glo. Values from exosome-free RPMI were used to subtract background and untreated cells were set to 100% and used to normalize experimental values.

CHAPTER FIVE: SUPPLEMENTARY FIGURES



Supplementary Figure 1: Generation of Rift Infected Resistant Clones. The HEK-293T based reporter cell line, HEK-Blue hTLR3 (InvivoGen), was used to detect activation of TLR3 by supernatant of RVFV resistant Vero cells. After 18 hours of incubation at 37^{0}C in 5% CO2, the absorbance (600 nm) of each control and test condition in the 96-well plate was measured using the GloMax Multi Detection System (Promega). Readings from all positive controls (sample 2, 10ng/mL Poly I/C) and experimental samples were normalized using the mean reading from three sterile water treated negative controls. Seven clones (as indicated) were selected for follow up experiments. Error bars on the first two samples (negative and positive controls) indicated ± 1 SD of biological triplicates.



Supplementary Figure 2: Presence of RVFV genome in resistant clones. Total RNA was extracted from seven RVFV resistant Vero cells using the trizol-chloroform method. For each sample, approximately 400ng/µl of RNA was used for cDNA synthesis Reverse Transcriptase System using Random Primers. The absolute quantitation of the samples was determined based on the cycle threshold (Ct) value relative to the standard curve.

CHAPTER SIX: DISCUSSION

In recent years, exosomes have emerged as essential and critical components of intercellular communication during viral infection related to disease states, including cancers and spreading of viral infections. Previous work has shown that exosomes from virally infected latent cells have the ability to control gene expression and cell viability in recipient cells (Narayanan et al., 2013; Jaworski et al., 2014a). Here, we attempted to test whether Rift infected cells could also secrete exosomes that may be able to control the fate of the recipient cells. We utilized a scheme to generate reliable clones that survived RVFV infection and that their exosomes contained potential viral RNA and/or proteins. Using *in vitro* infection of Vero cells, we were able to generate multiple resistant clones that could be passaged up to at least 50 times and still retained both viral mRNA and proteins in the exosome-enriched preparations. We performed these experiments in two independent sets of infections: with wild type MP12 and with an epitope-tagged MP12. Using TLR3 as an initial read out assay, we obtained clones that produced exosome-containing secretions in 5 day cultures that contained varying levels of genomic RNA and viral proteins. These clones could be grown to large scale, which simplifies purification schemes for downstream functional assays. Interestingly, these clones were all resistant to Rift infection, but not to other viruses including VEEV, indicating that the mechanism of resistance may be unique and not general for most incoming RNA viruses. These data also indicate that the Rift replication/transcription machinery must still be somewhat intact in these resistant clones, as both RNA and proteins were present after continuous

passage and replication. It is therefore tempting to speculate that the viral assembly in these cells may be blocked, and/or that there is a competition between viral or exosomal release as both entities presumably utilize the same exit machinery.

Previous work with HCV (Masciopinto et al., 2004) demonstrated the presence of viral RNA in exosomes released from infected cells. These exosomes spread viral infection to the neighboring cells, indicating that the genomic RNA was capable of replicating in cells. We asked whether the same findings may be true for the RVFV resistant clones. Consistent with our previous work from HIV-1 and HTLV-1, we were unable to observe Rift replication in the recipient cells, indicating that the full replication machinery is not transferred in these exosome-containing secretions. Therefore, HCV RNA transfer in exosomes may have a different packaging set of signals compared to other nuclear or cytoplasmic viral RNAs, which may cause it to transfer complete replication machinery through exosomes. We also performed a time course study where supernatants from resistant clones were added back to uninfected Vero cells. These cells were actively replicating for the duration of the study (3 weeks) and did not produce wild type viral progeny. Importantly, supernatants from these newly treated cells did not show presence of viral RNA or virus in the supernatants. We did however observe cell associated viral RNA (10 fold less) in samples from week one, which may be due to presence of the input exosomal RNA or a very slow growing mutant virus population. Either way, the effect on host Vero cells were not deemed significant compared to the results from immune Jurkat and U937 cells which showed apoptosis when treated for few days.

Using HIV-1 as our model system for presence of viral RNA in exosomes (Narayanan et al., 2013), we have recently observed binding and activation of number of TLRs to these RNAs when using the exosomes from latently-infected cells (Sampey et al., 2015). Here, we used a functional TLR3 assay as a generic readout for TLR and NF-κB activation. We consistently have observed activation of TLR3 in these assays when using exosomes obtained from resistant clones. This further suggests that the exosomal cargo may have functional properties in the recipient cells, including activation of signal transduction for genes involved in cell fate.

Exosomes from our individual resistant clones, and exosomes from cells susceptible to RVFV infection, also demonstrate a role in apoptosis of recipient cells that may depend on the expression of viral RNA and proteins. For instance, clone H6 shows appreciable amounts of all RNAs and some N protein whereas clone #14 contained viral RNAs as well as increased N protein levels. It is important to note that treatment with exosomes from clone #14 exhibits all the classical signs of Rift infection in recipient cells, including presence of apoptosis markers and PKR cleavage. Although we cannot rule out the presence of other viral proteins in clone #14, presence of all viral RNAs and N protein may be responsible for apoptosis in both T cells as well as in monocytes.

Our data using resistant clones clearly suggested that if exosomes from cells susceptible to RVFV infection (i.e., regular infection) were isolated they would also contain viral RNA and proteins. Therefore, we devised a purification scheme that allowed successful

isolation of exosomes during regular infection that are free of RVF virion contamination. Indeed, these exosomes also contain viral RNA and proteins. Similar to the exosomes from the resistant clones, the exosomes from infected cells had the capability of decreasing cell viability in recipient cells. We speculate that Rift infection *in vivo* may also allow formation of exosomes that persist for an extended period of time. The question of how these exosomes affect the overall pathology in animals or humans remains to be clarified; however, we have recent data demonstrating that the EX_I can cause a decrease in the replication of the virus (unpublished), suggesting that the EX_I is a means by which the host controls viral spread. Future experiments using animal models and inhibitors that allow blocking of exosome formation from infected cells may uncover their true potential during *in vivo* pathogenesis.

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