PRESENCE OF EBOLA STRUCTURAL PROTEINS IN EXOSOMES CAN CAUSE IMMUNE SYSTEM DYSREGULATION

by

Allison Heath

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of
Master of Science
Molecular Biology

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by

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Bachelor of Science
Florida State University, 2013

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Fall Semester 2015
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DEDICATION

This is dedicated to my loving husband Kenny, my mostly good dog Oscar Wilde, and the Florida State Seminoles, GO NOLES!!!!

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ABSTRACT

PRESENCE OF EBOLA STRUCTURAL PROTEINS IN EXOSOMES CAN CAUSE IMMUNE SYSTEM DYSREGULATION

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George Mason University, 2015

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The Ebola virus (EBOV) is a member of the *Filoviridae* family and is the virus responsible for the most recent world health emergency. The first outbreaks of the disease occurred in 1976 in Sudan and in the Democratic Republic of Congo. The most recent outbreak has included the countries of Guinea, Sierra Leone, Liberia, Nigeria, Mali and Senegal.

Within days of infection with the virus, patients begin to experience fever, chills, and myalgia. As the infection progresses, symptoms advance to nausea, vomiting, diarrhea, chest pain, cough, bloody feces, vomiting blood, distention and/or hemorrhage of sclerotic arterioles in the eyes, gastrointestinal bleeding from the mouth and rectum and hemorrhage from the nose and mouth may also be observed. Spread of the virus occurs through breaks in the skin, contact with mucosal surfaces, or after direct contact with persons infected or recently deceased from the virus. It is crucial that we gain a

greater understanding of the mechanisms of the pathenogenesis of this virus; both to prevent future outbreaks of the disease and to better understand other similar RNA viruses.

In this project, we have focused on biology of three proteins critical for Ebola pathogenesis. Ebola GP, NP and VP40 are proteins associated with egress and entry into the cells. Our data suggests that exposure of VP40 to immune cells can result in cell death, and that this protein may be transmitted via exosomes. Our study aids in deciphering the role of VP40 in immune cell death and suggests that some FDA approved drugs that inhibit the cell death may reverse at least some pathogenic processes instigated by the VP40 associated exosomes. While the effects of exosomes on the course of viral infection and host response have yet to be fully explored, we have provided evidence that these exosomes may play a role in the spread of Ebola infection to immune privileged sites and a possibility of the establishment of latent Ebola infection.

INTRODUCTION

Ebola Virus

Ebola virus (EBOV) is a negative sense single strand RNA virus that is membrane-enveloped and filamentous. EBOV is composed of seven genes that encode eight proteins including: the nucleoprotein (NP), viral proteins VP24-VP30-VP35-VP40, (L) polymerase and glycoprotein (GP). These proteins may be contained in any of three viral compartments: the nucleocapsid, the matrix space, and the envelope[1].

The EBOV GP protein is the sole protein expressed on the surface of the virus; it is essential in the attachment of EBOV to host cells[2,3]. The envelope protein GP is thought to be responsible for the hemorrhagic symptoms of EBOV infection due to its role in the destruction of endothelial cells[4]. Immunochemistry assays using monoclonal antibodies have uncovered the presence of GP antigen in the plasma membranes of infected cells in Nonhuman-primates (NHP) and guinea pigs. A notable exception were macrophages and blood monocytes that rarely have detectable levels of GP[5]. The mechanism of entry of EBOV into host cells is mainly via macropinocytosis and then trafficking via endosomal vesicles.[6] The factors that direct the movement of endosomal virus vesicles are not fully understood[6].

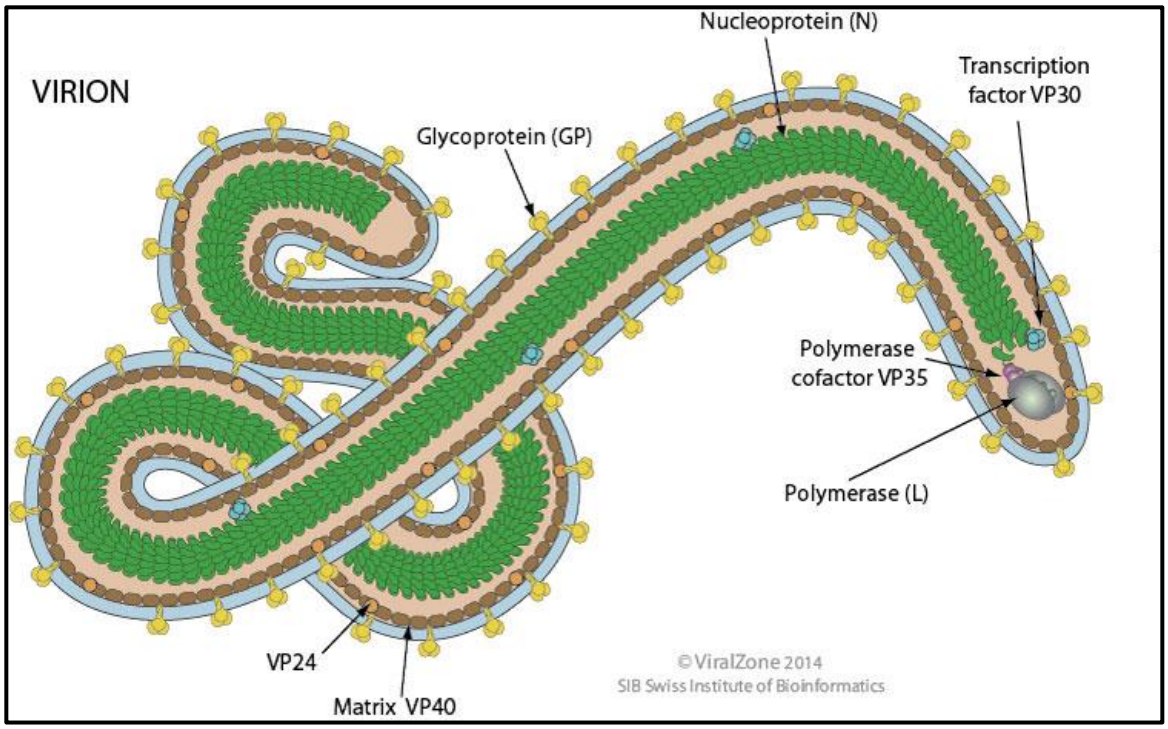

Figure 1: Structure of Ebola Virus[7].

VP40 is the EBOV matrix protein. It is responsible for forming a coating with the inner leaflet of the lipid bilayer[8] where it then binds to lipids and controls the development of new viral particles[9]. VP40 plays a critical role in viral assembly. It has been shown that Vp30, Vp35 and VP40 act as RNA silencing suppressors, further supporting the idea that EBOV proteins actively resist degradation by RNAi in order to ensure successful viral replication[8].

Evasion of Host Immune Defenses

The principle target cells of EBOV infection are macrophages and dendritic cells[3]. These infected immune cells are unable to induce the required "cytokine storm" that would be otherwise produced in course of a healthy immune response[3]. The body has many sophisticated methods of defense against viral pathogens. Cellular RNA interference (RNAi) delivers a natural response against viral infection[10]. RNAi was not initially accepted as a means of immune response in mammals. However, the identification of suppressor proteins of RNAi in animal viruses and the experimental determination that engineered RNAi has the capacity to impede viral infection in mammalian cells have provided supportive evidence for the function of RNAi as an inherent antiviral mechanism[10]. EBOV, like other viruses, has evolved a mechanism that can suppress this method of antiviral defense. VP35, VP30 and VP40 of EBOV independently act as suppressors of RNAi silencing (SRSs)[10].

A product of the RNAi pathway, microRNA (miRNA) are short non-coding RNA sequences that can bind to and therefore restrict transcription or translation of target mRNA homologs[11]. miRNAs are believed to be generated by the DICER endonuclease[12]. miRNA associates with the RNA induced silencing complex (RISC) that assists miRNA-mediated target recognition which either contributes to the degradation or silencing of the target message[11]. It is thought that some EBOV proteins may act at suppressors of this RNA silencing. In particular, EBOV proteins VP30, 35 and 40 may serve as suppressors of RNA silencing (SRSs), while VP30 may inhibit the activity of Dicer[10].

Figure 2: Physician thought to have recovered from Ebola infection presented with ocular EBOV infection resulting in the permanent change in iris color from blue to green[13].

Case Studies of Novel Routes of EBOV Transmission or Recurrence

While EBOV has not been yet been acknowledged as a disease exhibiting latency, the following cases suggest that the virus is capable of staying dormant in the body for longer than previously believed. In 2014, a physician contracted EBOV after treating an infected patient. He was treated with an experimental siRNA antiviral agent, convalescent donor plasma and supportive care; after completion the treatment, blood and urine samples of patient were tested negative for EBOV by quantitative real time polymerase chain reaction (RT-PCR). Ten weeks after discharge, the patient began to suffer from new symptoms including ophthalmic discomfort, i.e., "bilateral ocular

burning, foreign-body sensation, and photophobia". Aspirate of the aqueous humor tested positive for EBOV RNA on quantitative RT-PCR, and EBOV was isolated by means of a viral culture of the specimen. There was no EBOV detected in a conjunctival swap or in a peripheral blood test either by viral culture or by RT-PCR. Hence, it is imperative that investigation be done into the mechanisms by which EBOV remained in the ocular fluid. It is likely that this is not an exclusive occurrence, and there may be pockets of virus remaining latent in other immune privileged sites such as the central nervous system, gonads and articular cartilage[14].

Further consequence of EBOV persisting in immunologically privileged sites for far longer than previously established is the continued possibility of sexual transmission of the virus long after a patient has recovered from the disease. In fact, EBOV has been observed and isolated from seminal fluid up to 82 days after onset of Ebola symptoms, while the detection of viral EBOV RNA has been possible for as long as 101 days after the appearance of disease symptoms[15]. In 2015, a Monrovian woman was confirmed by RT-PCR testing of her blood to have contracted EBOV. The woman had no known contact with persons with symptoms consistent with Ebola, nor had she traveled to any are where the virus was endemic[15]. She had, however engaged in sexual intercourse with a man who had contracted and subsequently been treated for Ebola. The man had been discharged from treatment when viral RNA was no longer detectable in his blood vie RT-PCR. The man's blood was also negative for Ebola specific antibodies IgG and IgM. The couple had engaged in sexual intercourse on 3-5 occasions. It was later discovered that EBOV was detectible in the man's semen by RT-PCR 199 days after the onset of his

initial EBOV symptoms[15]. Previous CDC and WHO guidelines had recommended that Ebola patients abstain or use condoms for three months after recovering from Ebola, however in light of recent evidence of extended transmission risk, these organizations are now recommending that these precautions be taken indefinitely.

Ebola recurrence was also reported in a few occasions. A Scottish nurse contracted Ebola while administering care in Sierra Leone in December 2014. She underwent medical treatment and appeared to have a complete recovery from the infection. In October 2015, the nurse was readmitted to medical care after complaining of feeling "unwell". Medical staff at NHS Greater Glasgow and Clyde confirmed that the nurse was suffering from a recurrent episode related to a prior Ebola infection[16,17].

The occurrence of an ocular EBOV infection in a patient who showed no viral RNA in RT-PCR examination of his blood and urine[14], transmission of EBOV via sexual intercourse by a man 199 days after RT-PCR examination of his blood showed no detectable viral RNA, and a recurrent episode after a patient was thought to have been cleared of the virus suggests the presence of viral reservoirs within the body. It is possible that a latent Ebola infection may conceal itself in immune privileged sites.

Exosomes

Exosomes are small membrane bound vesicles that originate from the late endosomes.[18] Exosomes transport biological macromolecules to targets adjacent to their point of origin and to distant locations in the body[19]. Human DNA and RNA viruses are known to modify host proteins and integrate their own viral proteins and other cargo into

secreted exosomes[19]. RNA viruses gain an advantage in the commandeering host exosomes to enhance viral spread[19]. In the case of HIV-1 infections, infected monocyte-derived macrophages (MDMs) increase the number of exosomes and micro vesicles secreted from the cell[19]. If there is, indeed, latency in Ebola infection, it is possible that the virus uses exosomes to transport viral genomic material to other cells (neighboring or peripheral).

Study Aims

Here we tested the hypothesis that Ebola proteins might be capable of promoting apoptosis of immune cells. We also inquired if Ebola proteins could be contained in exosomes and whether these exosomes are capable in transmitting viral proteins in a manner that could result in destruction of immune cells. Finally, we asked whether it were possible to eliminate the danger of infection by Ebola while maintaining its protein integrity, thus, allowing scientific research Biosafety-2 or 3 rather than Biosafety-4 containments. Our findings are discussed below.

MATERIALS AND METHODS

Cell culture and reagents

CEM, Jurkat and U937 cells were grown and cultured to mid log phase of growth in RPMI-1640 media containing 10% heat-inactivated fetal bovine serum (FBS), 1% L-glutamine, and 1% streptomycin/penicillin (Quality Biological, Gaithersburg, MD). All cells were incubated at 37°C in the presence of 5% CO_2.

Transfections and luciferase assay

293T cells were transfected with plasmids using the Attractene reagent (Qiagen, Chatsworth, CA, USA) according to the manufacturer's instructions. The next day, cells were treated with DMSO or the indicated compound at 1.0 μM. Forty-eight hours post drug treatment, firefly luciferase activity was measured with the BrightGlo Luciferase Assay (Promega, Madison, WI, USA) and luminescence was read from a 96-well plate on an EG&G Berthold luminometer (Berthold Technologies, Oak Ridge, TN, USA).

Cell viability assay

Fifty thousand cells were plated per well in a 96-well plate and treated the next day with 1.0 or 10 μM compound or DMSO. Forty-eight hours later, CellTiter-Glo (Promega,

Madison, WI, USA) was used to measure viability following the manufacturer's recommendations. CellTiter-Glo is a luminescent assay used to measure cell viability by ATP level. The reagent was added to the wells (1:1 reagent: media) and incubated at room temperature for 10 minutes protected from light. The luminescence was detected using the GloMax-Multi Detection System (Promega).

Protein extracts and immunoblotting

Cells were collected, washed once with PBS and pelleted. Cells were lysed in a buffer containing Tris–HCl pH 7.5, 120 mM NaCl, 5 mM EDTA, 0.5% NP-40, 50 mM NaF, 0.2 mM Na_3VO_4, 1 mM DTT and one tablet of complete protease inhibitor cocktail per 50 ml. Lysis was performed on ice, incubated on ice for 30 minutes and spun at 4°C for 5 minutes at 14,000 rpm. The protein concentration for each preparation was determined with a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). Cell extracts were resolved by SDS PAGE on a 4-20% tris–glycine gel (Invitrogen, Carlsbad, CA, USA). Proteins were transferred to polyvinylidenedifluoride microporous membranes using the iBlot dry blotting system as described by the manufacturer (Invitrogen). Membranes were blocked with Dulbecco's phosphate-buffered saline (PBS) 0.1% Tween-20 + 3% BSA. Primary antibody against the specified protein was incubated with the membrane in blocking solution overnight at 4°C. Membranes were washed twice with PBS + 0.1% Tween-20 and incubated with HRP-conjugated secondary antibody for 1 hour in blocking solution. Presence of secondary antibody was detected by SuperSignal

West Dura Extended Duration Substrate (Pierce, Rockford, IL, USA). Luminescence was visualized on a Kodak 1D image station (Carestream Health, Rochester, NY, USA).

Nanotrap particles binding conditions

Each target analyte was captured by nanotrap particles as described below. A 30% slurry (30 µl) of each type of nanotrap particles was incubated with target molecule spiked in 100 µl of appropriate binding buffer for 30 min at room temperature. After 2–3 washes (each 5 min) nanotrap particles containing captured molecules were assayed using immunoblotting[20].

RESULTS

Treatment of immune cells with VLP and Ebola proteins causes inhibition of immune cells.

In this part of the study, we investigated whether Ebola virus proteins as such, either as free purified proteins or protein-containing VLPs, are able to produce apoptotic effects in recipient cells. The rationale for this was derived from the findings that Ebola virus is capable of creating massive cell death as well as cytokine storm in animals[18,19]. To evaluate the hypothesis that one or more of EBOV proteins may serve as direct pro-apoptotic agents, we incubated increasing concentration of these proteins with three types of immune cells, including two types of T-cells (early and late developmentally deregulated cells being represented by CEM and Jurkat cell cultures, respectively) and one monocyte cell culture for 5 days followed by assaying their viability using cell titer Glo kits. Figure 3A depicts results of this experiment. Minimal amounts of cell death were observed in lymphocytes, while in monocytes, an increase in apoptosis was observed when VLPs containing all three proteins were used as treatment agent. On the other hand, free VP40 was able to decrease viability of lymphocytes, as evident on Figure 3B. The testing aimed at finding out the range of apoptosis-promoting concentrations of free VP40, and VP40 containing VPLs are currently under way in Dr. Kashanchi lab. However, our preliminary data are indicative that Ebola proteins, especially VP40, are capable of direct destruction of immune cells.

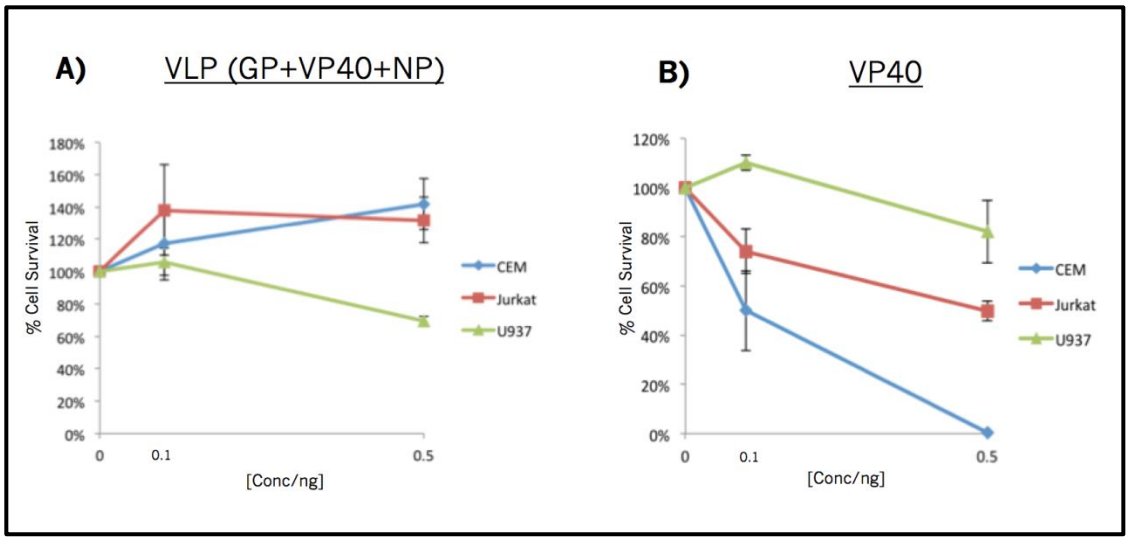

Figure 3: The results of the treatment of various immune cells with VLP-bound or free Ebola proteins.

Treatment of immune cells with cell-free media conditioned by 293T cells transfected with various EBOV proteins can lead to immune cells dysregulation.

We then asked if cells transfected with VP40, GP or NP producing plasmids would express these proteins and if so, would that expression cause cell death *in vitro*. In particular, we were interested in finding out whether exosome-enriched preparation from EBOV GP resistant clones incapable of releasing functional virions could influence neighboring cells. The rationale for this study comes from the findings that Ebola infected cells are capable of the production of EBOV proteins[5]. We performed a transfection of 293T cells with VP40, GP and NP producing plasmids (Fig 4), then the cells were kept under selection by the antibiotics described in Fig 5A. After 5 days of selection, cell-free media were recovered from each transfection and used to treat either CEM, or Jurkat or U937 cells. The cells were incubated for 5 days, followed by CellTiter

Glo assays to measure cell viability. Fig 5B depicts the result of this experiment. In lymphocytes (CEM), a minimal amounts of cell death were observed after exposing them to unfiltered cell-free media conditioned by control cells, or cells transfected with expression constructs with VP40, GP, NP, VP40 + GP, GP+NP and VP40+ GP+NP inserts. A moderate amount of cell death were observed after exposing CEM lymphocytes to unfiltered cell-free media conditioned by cells transfected with constructs expressing a combination of VP40 and NP proteins. Fig 5C indicates that there is a significant cell death in CEM lymphocyte cultures exposed to unfiltered cell-free media conditioned by cells transfected with constructs expressing VP40, GP, VP40 + GP, VP40 + NP, GP + NP, or VP40 + GP + NP inserts. Similar treatment of the Jurkat lymphocytes resulted in minimal death when Jurkats were treated with the unfiltered cell-free media conditioned by cells transfected by expression construct with NP insert. Fig 5D demonstrates that U937 cells show substantial amount of apoptosis when exposed to unfiltered cell-free media conditioned by cells transfected with constructs expressing VP40, GP, or VP40 + GP + NP combination of EBOV proteins. When U937 cells were exposed to unfiltered cell-free media conditioned by cells transfected with constructs expressing VP40+GP and VP40 + NP combinations of EBOV proteins, a moderate amount of cell death was observed. Collectively, the data presented here suggest that Ebola proteins produced by 293T cells transfected by respective expression constructs are capable of human lymphocyte killing.

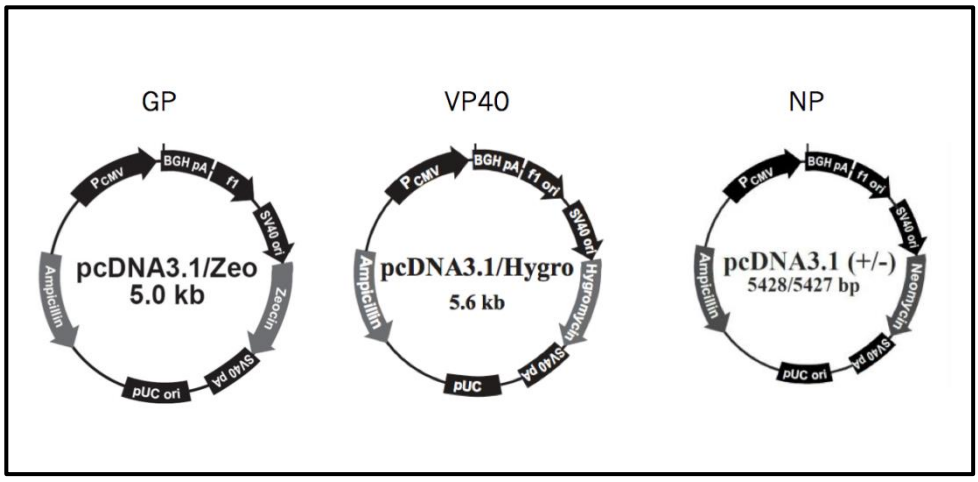

Figure 4: Expression constructs producing GP, VP40, or NP proteins of Ebola virus

Table 1: Drugs Used for Antibiotic Selection of Transfections

Plasmid	Antibiotic(s)
VP40	Hygromycin
GP	Zeocin
NP	Genticin
VP40 + GP	Hygromycin + Zeocin
VP40 + NP	Hygromycin + Genticin
GP + NP	Zeocin + Genticin
VP40 + GP + NP	Hygromycin + Zeocin + Genticin

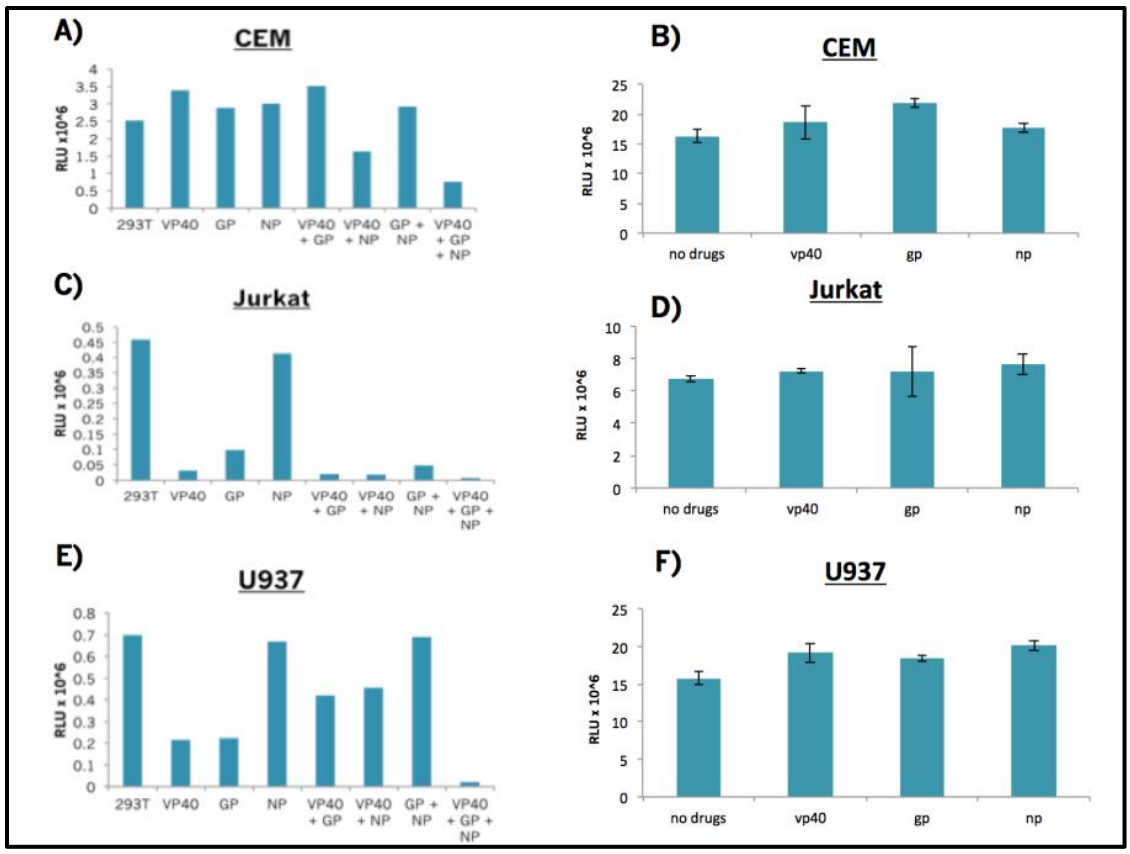

Figure 5: Treatment of Immune Cells with Cell-free media from 293T cells transfected with plasmids expressing various EBOV proteins or treated with antibiotics alone.

Treatment of immune cells with filtered and unfiltered cell-free media from 293T cells transfected results in inhibition of T-cells and monocytes.

We then asked if Ebola proteins in transfected cells might be packaged in exosomes that would be capable of cell destruction. We reasoned that because exosomes are known to assist in the spread of some viruses to adjacent or peripheral cells it may be

possible that EBOV is spread to new cells via a similar mechanism 16–19. Because exosomes are known to be smaller than 0.22 μM, we reasoned that passing transfection cell-free media through a 0.22 μm filter would remove larger VLPs and apoptotic bodies, letting exosomes pass through the filter. We isolated cell-free media from the transfections and then divided it into filtered (with 0.22μm filter) and unfiltered groups. We then treated CEM, Jurkat and U937 with the filtered and unfiltered cell-free media. The cells were incubated for 5 days, then a CellTiter Glo was performed to measure cell viability. Results in Fig 6A showed minimal cell death in CEM lymphocytes treated with either filtered or unfiltered cell-free media from control 293T cells, and GP and NP transfections. There was significant cell death following treatment with either filtered or unfiltered VP40 transfection cell-free media. As a control, recipient cells were treated with supernatant from 293T cells that were not transfected, however were placed under antibiotic selection similar to cells in Figure 6A. Results in Figure 6B reveal no inhibition due to antibiotic selection. Results in Fig 6C showed minimal cell death of Jurkat lymphocytes following treatment with either filtered or unfiltered control cell-free media and VP40, GP, and NP transfection cell-free media. As a control, recipient cells were treated with supernatant from 293T cells that were not transfected, however were placed under antibiotic selection similar to cells in Figure 6C. Results depicted in Figure 6D show that there is no inhibition due to antibiotic selection. Results in Fig 6E showed significant cell death in U937 monocytes following treatment with either filtered or unfiltered VP40, GP and NP transfection cell-free media. As a control, recipient cells were treated with supernatant from 293T cells that were not transfected, however were

placed under antibiotic selection similar to cells in Figure 6E. Results in Figure 6F reveal no inhibition due to antibiotic selection. Collectively, these data obtained in these experiments suggest that U937 cells are particularly susceptible to destruction by Ebola VP40, GP and NP proteins.

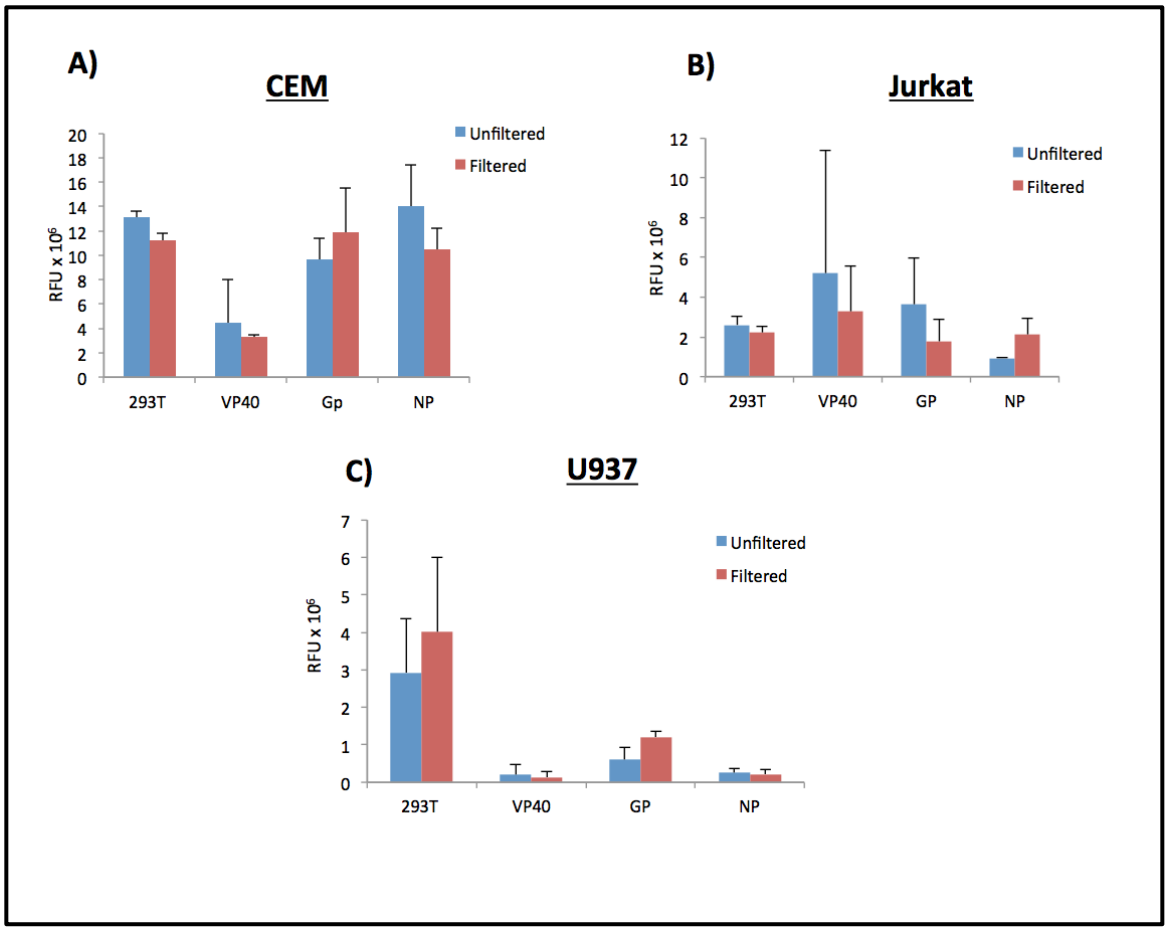

Figure 6: Treatment of Immune cells with filtered and unfiltered cell-free media conditioned by cells transfected with plasmids expressing various EBOV proteins. isolated cell-free media from the transfections and then divided it into filtered (with 0.22µm filter) and unfiltered groups. We then treated CEM, Jurkat and U937 with the filtered and unfiltered cell-free media. The cells were incubated for 5 days, then a CellTiter Glo was performed to measure cell viability.

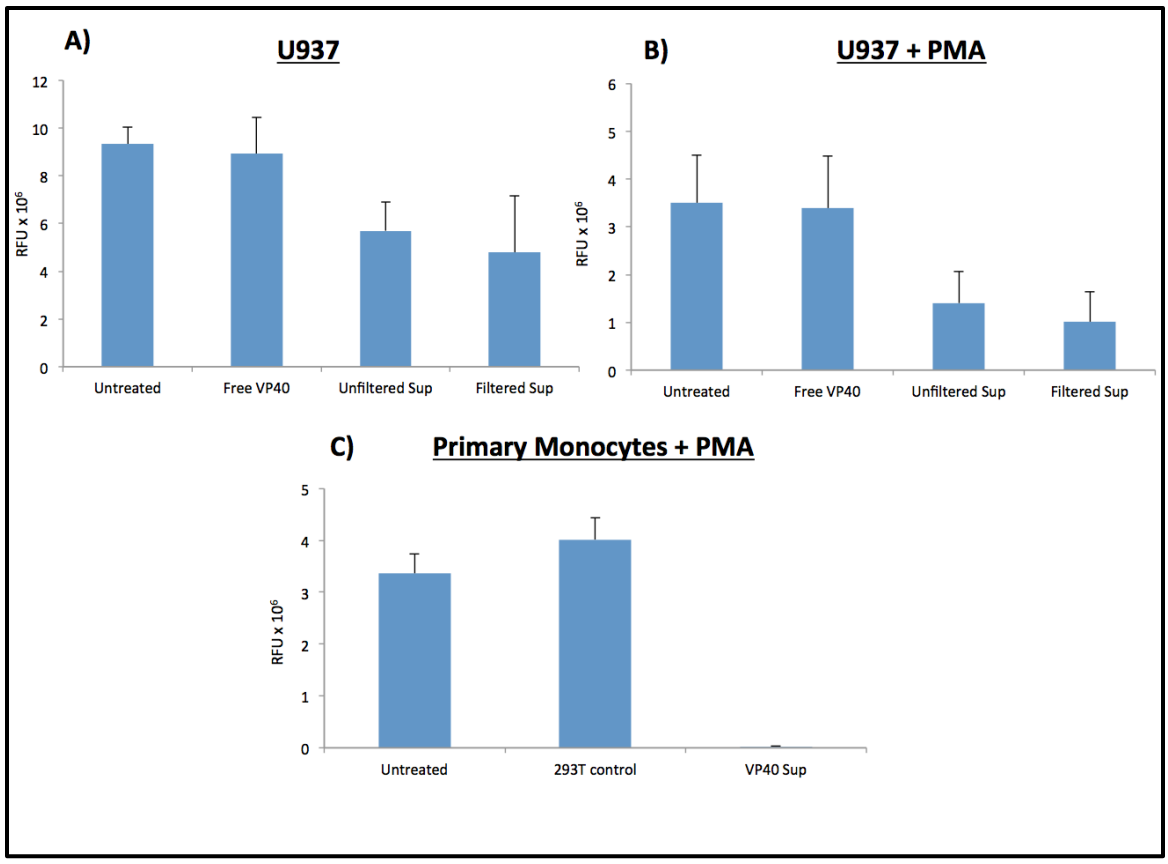

Figure 7: Treatment of differentiated and undifferentiated monocytes with VP40

Treatment of differentiated and undifferentiated monocytes with VP40 reveals strong inhibitory effect of VP40 on macrophages.

We then asked if VP40 poses greater toxicity in relation to macrophages than it does in relation to other immune cells. The rationale for this study is that the macrophages are the natural targets of Ebola; that is the reason why EBOV is unable to trigger a forceful immune response[3]. We differentiated U937 monocytes into macrophages using phorbol 12-myristate 13-acetate (PMA) for five days. We then treated both the differentiated U937 cells and undifferentiated U937 cells with free VP40, unfiltered VP40 transfection

cell-free media and filtered transfection cell-free media. The cells were incubated for 5 days, followed by a CellTiter Glo assay to measure cell viability. Results in Fig 7A show that while free VP40 is minimally toxic to U937 cells, cell-free media from VP40 transfected 293T cells causes significant cell destruction. Results in Fig 7B show that free VP40 is minimally toxic to PMA activated U937 cells, and while either filtered or unfiltered VP40 transfection cell-free media demonstrate significant cell killing. Interestingly, the treatment of cell with the cell-free media that was passed through a 0.22μm filter resulted in the greatest amount of cell death. The data suggests that free VP40 exhibits little toxicity to both inactivated and activated U937 cells. The data also reveals that U937 cells that have been differentiated into macrophages are more susceptible to VP40 produced by transfected 293T cells, and that cell killing is greater in the cells treated with the filtered transfection cell-free media. The results of this experiment are consistent with the suggestion that VP40 could be contained in exosomes and potentially released as free VP40. Results in Fig 7C show that cell-free media from VP40 transfected 293T cells is capable of causing the death of primary cells.

Ebola proteins can be trapped using specially designed nanoparticles under reducing conditions.

We asked if it was possible to capture Ebola proteins in a manner that would bypass the neccessity of working in Biosafety Level-4 conditions. The inherent risk associated with virulence of the virus is a major limitation in the field of Ebola research. In order to assure the safety of researchers, work with the virus must be performed in a Biosafety

level-4 laboratory. Limiting research to this high safety level is both costly and labor intensive. To remedy this problem, we asked whether nanoparticles could be used to trap denatured Ebola proteins. Our rationale was that altering the structure of the viral proteins would eliminate the risk of infection to researchers and allow experiments to be performed at low Biosafety safety levels.

In order to determine which particles would capture the greatest amount of protein, we attempted to trap VLP bound GP, free GP protein and free VP40 with nine different nanoparticles[20]. We allowed the nanoparticles to bind proteins when the samples were rotated overnight at 4°C. We then performed immunoblotting to assess the quality of the binding. Once the nanoparticles that captured the greatest amount of proteins were determined, we then performed the binding both in the presence and absence of Laemmli buffer to determine whether or not the proteins would be detectable in these conditions**.** Western blotting experiments (data not shown) demonstrated that, in absence of Laemmli buffer, VLP bound GP, free GP and free VP40 could be trapped by nine different nanoparticles. VLP bound GP was optimally trapped by nanoparticle preparations 213 and 224. Free GP was strongly bound by nanoparticles 210, 213, 217, 219, 223, 224, and 229. Free VP40 was most strongly captured by nanoparticles 224 and 229. We determined that the most consistently strong binders between all three samples were nanoparticles 213 and 224.

Western Blotting experiments (data not shown) also revealed that both free GP and VLP bound GP could be trapped by nanoparticle preparations 212, 213 and 224 in presence of Laemmli buffer. The strongest binder of both free GP and VLP bound GP

was nanoparticle preparation 213. Results in Figure 8C show bands that correspond to free E. coli derived VP40 trapped to nanoparticle preparations 210, 212, 213, 217, 219, 222, 223, 224, and 229. This was done both in the presence and absence of Laemmli buffer. In the absence of Laemmli buffer, nanoparticles 224 and 229 were the most adept at capturing the free proteins. In the presence of Laemmli buffer, protein was captured by nanoparticles 213, 217, 219, 222, 223, and 224, with the greatest protein capture by nanoparticles 213 and 224. Collectively, these data suggest that capturing of free GP, free VP40 and VLP bound GP with nanoparticles is possible under the conditions of detergent or reducing buffer treatment.

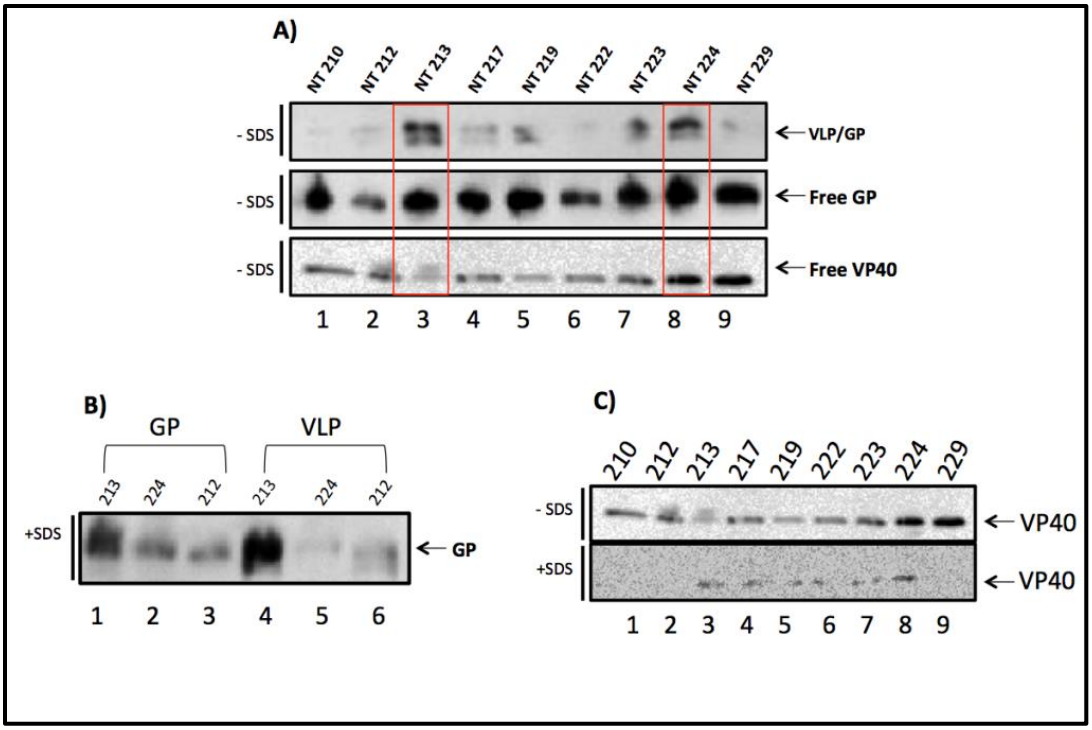

Figure 8: Binding of EBOV Proteins under reducing conditions. Specially designed nanoparticles were used to bind VLP or free Ebola proteins and immunoprecipitated to determine strongest binding(Panel A). We then repeated this binding in the presence of SDS buffer to determine which nanoparticles would continue to trap proteins in reducing conditions (Panels B & C).

Table 2: Nanoparticles Used in EBOV Binding experiments

NT #	Affinity Bait	Core	Shell	Shell Type
NT210	Reactive Blue 4	Yes	No	
NT212	Reactive Red 120	Yes	No	
NT213	Acrylic Acid	Yes	Yes	pNIPAm
NT217	Reactive Blue 4	Yes	Yes	VSA
NT219	Cibacron Blue F3G-A	Yes	Yes	VSA
NT222	Cibacron F3G-A Core	Yes	No	
NT223	Acrylic Red	Yes	Yes	Acrylic acid "arms"
NT224	Acrylic Acid (saponified methacrylate)	Yes	No	
NT229	Reactive Yellow	Yes	Yes	VAS

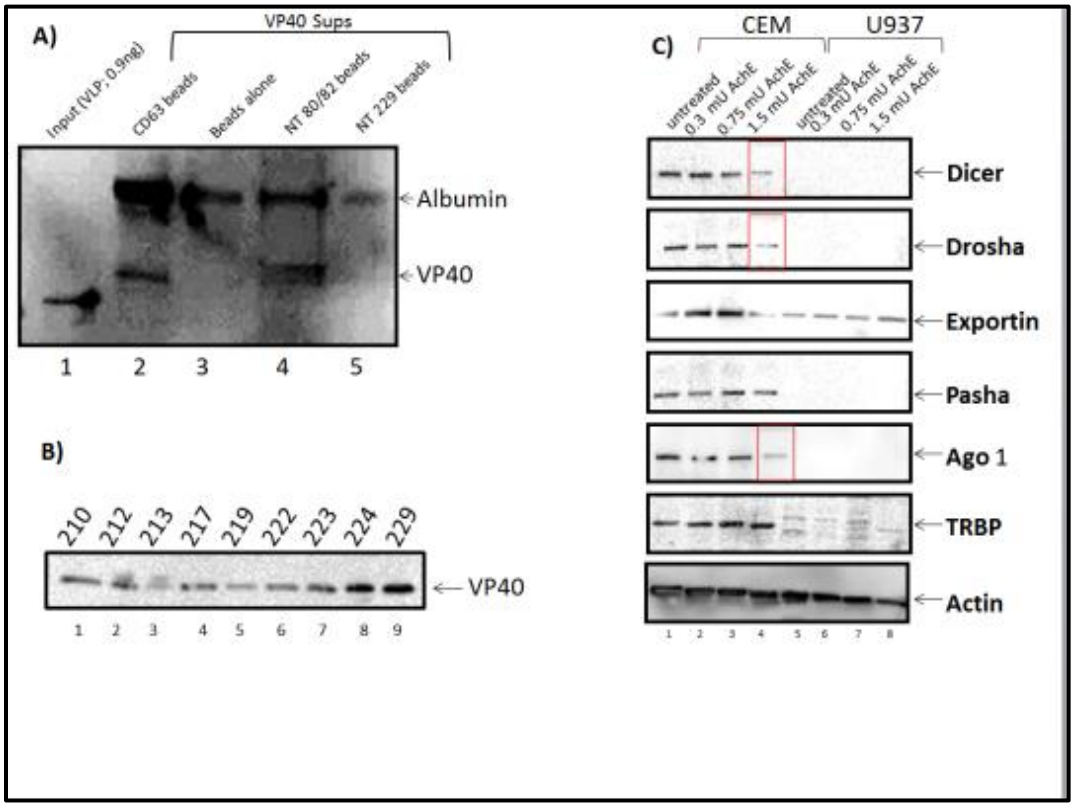

Figure 9: Binding of VP40 293T transfected cell-free media and recipient cell miRNA machinery inhibition. Transfection supernatant was bound using CD63 beads, beads alone, NT 80/82, or NT229. Binding was then performed at 4C rotating for 72 hours and immunoprecipitated for VP40 (Panel A). Nanoparticles were chosen based on previous experiments revealing strongest binders of Ebola proteins (Panel B). CEM and U937 cells were then treated with transfection supernatant and immunoprecipitated for miRNA machinery components (Panel C)

VP40 may be bound to exosomes in transfection supernatant

We asked whether VP40 found in transfection cell-free media was in a form of free protein or it was exosome bound. We reasoned that because inhibitory effects were

observed in cells that were treated with cell-free media that had been passed through a 0.22 μm filter, it might be possible that VP40 was contained within exosomes rather than existing as free protein. We isolated VP40 transfection cell-free media and treated it with either CD63 beads, beads alone, Nanoparticles 80/82, or Nanoparticles 229.

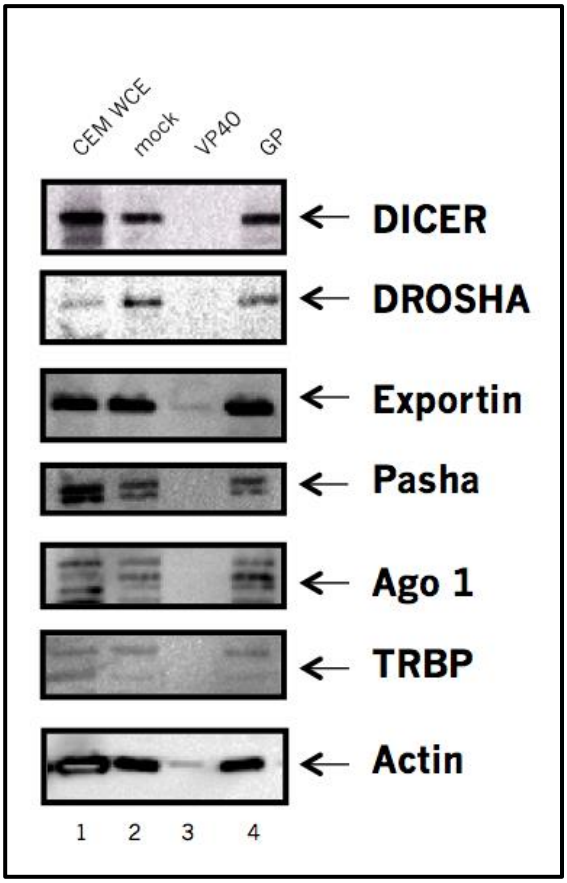

Figure 10: Inhibition of miRNA machinery in donor cells

We then incubated the samples at 4°C, rotating for 72 hours. Samples were then immunoprecipitated with antibodies specific to VP40. Results in Fig 8A revealed the presence of VP40 in samples bound with CD63 beads and Nanoparticles 80/82, which have been shown in previous studies to bind to exosomes. VP40 was absent from samples treated with beads alone, and samples treated with Nanoparticle 229, which had previously been shown to be a strong binder of free VP40 (Figure 8B). Collectively, these data suggest that VP40 may be associated with exosomes rather than as free protein.

Presence of VP40 may result in inhibition of miRNA machinery in donor and recipient cells.

It has been previously shown that some viruses are able to interfere with miRNA machinery as a means of evading host immune response[10–12]. We asked whether VP40 might exert a similar effect on elements of miRNA machinery components.

In order to examine the involvement of host cells' miRNA machinery, we transfected 293T cells with plasmids producing either VP40 or GP. Cells were kept under antibiotic selection for 16 days and cell pellets were harvested and immunoprecipitated with antibodies specific to Dicer, Drosha, Exportin, Pasha, Ago-1, TRBP and actin. We isolated the cell-free media from VP40 transfected cells and passed the cell-free media through a 0.22 μm filter in order to remove VLPs and apoptotic bodies. We then treated either CEM or Jurkat cells with 100, 250 or 500 μl of transfection cell-free media.

Recipient cells were then incubated at 37°C for 5 days, then cell pellets were harvested and immunoprecipitated with antibodies specific to miRNA machinery components. Results in Fig 8C show an absence of all miRNA machinery components in cells transfected with VP40, and a reduction in actin. Cells transfected with GP show no reduction in miRNA machinery components and no reduction in actin. Results depicted in Fig 9 reveal a decrease in the expression of Dicer, Drosha and Ago 1 in CEM cells treated with 500 µl of VP40 transfection cell-free media. Collectively, these data suggest that VP40 may be capable of interfering with miRNA machinery in host and recipient cells.

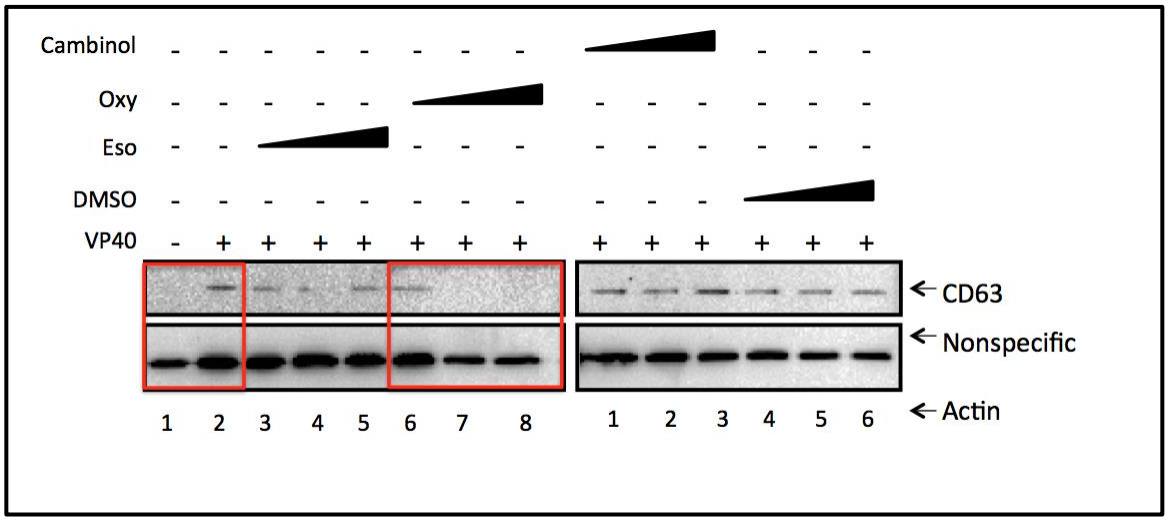

Figure 11: Treatment of VP40 transfected 293T cells with FDA Approved Drugs. VP40 transfected 293T cells were treated with FDA approved drugs and immunoprecipitated for CD63 and actin.

Table 3: Drugs Used for the Suppression of Exosomal Release

Drug	Dosage		
Cambinol	0.1 μM	1.0 μM	10 μM
Oxytetracycline	0.1 μM	1.0 μM	10 μM
Esomeprazole	0.1 μM	1.0 μM	10 μM
DMSO	0.1 μM	1.0 μM	10 μM

Treatment of VP40 transfected 293T cells with FDA approved drugs may result in a decrease in exosomal release.

We then asked whether treatment of FDA approved drugs previously shown to reduce exosomes release (unpublished data) may reduce exosomes release in VP40 transfected 293T cells. 293T cells were either un-transfected or transfected with VP40. Transfected cells were then either placed under antibiotic selection alone or antibiotic selection and treatment with 0.1, 1.0 or 10 μM Oxytetracyline, Esomeprazole, Cambinol or DMSO (control) (Fig 10). Cells were incubated for 3 days at 37°C, immunoprecipitated with antibodies specific to CD63 and actin. Results in Fig 8A show an increase in CD63 and actin in cells transfected with VP40, and a decrease in CD63 and actin in transfected cells treated with 1.0 and 10.0 μM Oxytetracycline. These data suggest an increase in exosomes release in cells transfected with VP40 and a subsequent decrease in exosomes release in VP40 transfected cells with treatment of 1.0 and 10 μM Oxytetracycline.

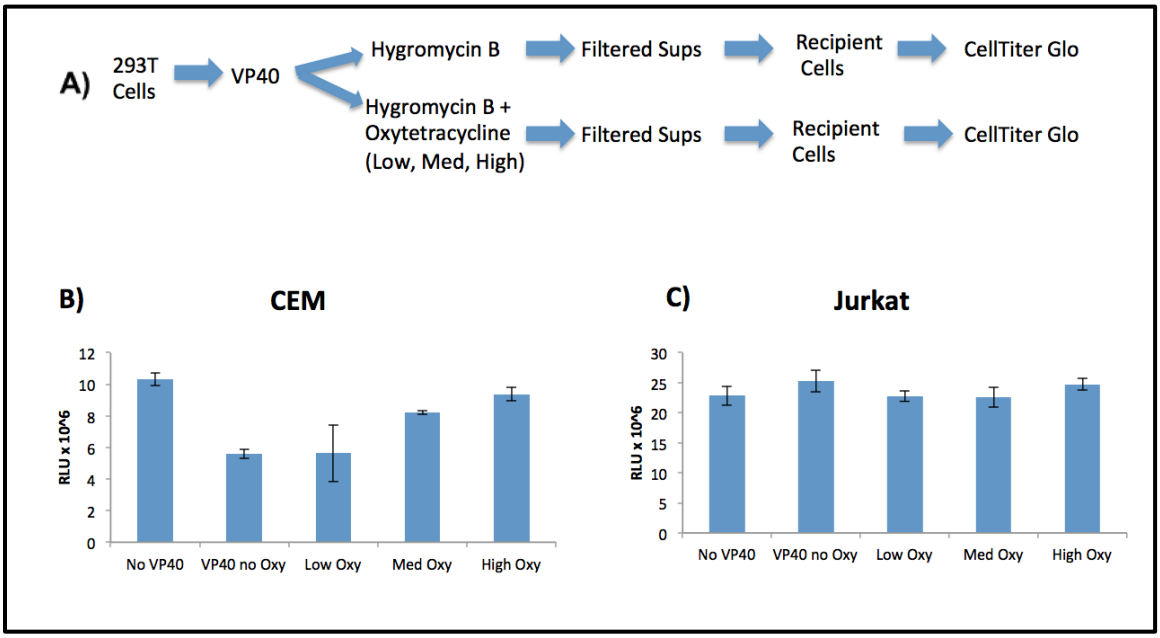

Figure 12: Treatment of recipient T-cells with VP40 transfected 293T cells under treatment with Oxytetracycline

Treatment of recipient T-cells with VP40 transfected 293T cells under Oxytetracycline treatment may compensate for VP40 inhibitory effects.

We then asked whether treatment of donor cells with Oxytetracycline would reduce inhibitory effects of exosomes with EBOV proteins on recipient T-cells. We reasoned that if treatment with Oxytetracycline might reduce exosomes release in donor cells, it might also reduce inhibitory effects of donor cells on recipient immune cells. We transfected 293T cells with VP40 and then placed those cells under antibiotic selection. Cells were then either untreated or treated with 0.1, 1.0, or 10 μM Oxytetracycline and

incubated at 37°C for 3 days. Cell-free media samples were then harvested and passed through a 0.2 μm filter. After filtering, cell-free media samples were then used to treat either CEM or Jurkat cells (Figure 12A) . Recipient T-cells were then incubated at 37°C for 5 days and assayed via CellTiter Glo (Figure 12B). Results shown in Fig 12B reveal a decrease in metabolism of CEM cells treated with VP40 transfection cell-free media alone. Recipient cell-free media were harvested and passed through a 0.2 μm filter and bound to Nanoparticles 80/82, rotating at 4°C for 72 hours. Cell-free media samples were then centrifuged to isolate Nanoparticle pellet, which was then immunoprecipitated with antibodies specific to CD63 and actin. CEM cells treated with 0.1, 1.0, and 10 μM Oxytetracycline showed a corresponding increase in cell metabolism relative to the concentration of Oxytetracycline. Results in Fig 11C show no significant change in cell metabolism in Jurkat cells treated with untransfected cell-free media, VP40 transfected cell-free media with no Oxytetracycline treatment, and VP40 transfected cell-free media treated with Oxytetracycline. These data suggest that treatment of host cells with Oxytetracycline may reduce inhibitory effects on recipient early developmentally deregulated (CEM) cells, but not on recipient late developmentally deregulated (Jurkat) cells.

DISCUSSION

Exosomes are known to be involved in communication between cells; release of exosomes may result in spread of a variety of biologically active molecules including cytokines and miRNA, some of which aid in infection of adjacent cell[18 21]. Exosomes are also known to play a significant role in the spread of retroviruses, since viral hijacking of exosomes has now been observed[19]. The presence of Ebola virus within immune privileged sites such as the ocular fluid compartment while it is no longer detectable in RT-PCR examination of blood samples and the continued transmission of Ebola in seminal fluid long after blood and urine RT-PCR found no trace of viral genomic material are highly suggestive of intercellular transmission of Ebola virus. This route of transmission is consistent with exosome mediated transfer of viral proteins and nucleic acids[14,15,18]. Previous experiment performed in Dr. Kaschanci lab established that infected cells with latent virus are capable of influencing cell viability and gene expression in recipient cells[22]. Here, we attempted to test whether Ebola proteins could be spread between cells through exosomes and whether these proteins could functionally control cell growth in recipient cells.

To test whether or not free Ebola proteins could cause cell death of immune cells, we designed the following set of experiments. Using recombinant VLPs and free VP40,

we were able to demonstrate that VLPs are capable of causing cell death in monocytes and that free VP40 is able to decrease viability of lymphocytes, specifically of CEM and Jurkat cells. These data, collectively, helped to establish that Ebola proteins, especially VP40, are capable of direct destruction of immune cells.

We then attempted to test whether the cells transfected with recombinant constructs expressing Ebola proteins could secrete Ebola proteins that may be able to control the fate of recipient cells. We determined that the treatment of CEM lymphocytes with VP40 + NP transfection cell-free media conditioned by cells expressing Ebola proteins results in moderate cell death. We also demonstrated that exposure of Jurkat lymphocytes exposed to unfiltered cell-free media from VP40, GP, VP40 + GP, VP40 + NP, GP + NP, and VP40 + GP + NP expressing cells resulted in pronounced cell death. Finally, when U937 monocytes were treated with transfection cell-free media, media conditioned by VP40, GP, or VP40 + GP + NP expressing cells, we observed remarkable cell death, while same type of cells exposed to unfiltered cell-free media from VP40+GP or VP40 + NP expressing cells, moderate cell death was observed.. We were also able to establish that inhibitory effects exerted on immune cells were not a result of antibiotic selection of donor cells. Collectively these data suggest that Ebola proteins produced by transfected cells are capable of lymphocyte killing. These findings are consistent with the suggestion that secreted Ebola proteins can be transmitted between cells and are capable of direct induction of apoptosis in recipient cells.

We then asked whether secreted Ebola proteins could be transmitted to other cells by exosomes. To uncover the presence of exosomes in media conditioned by cells expressing Ebola virus proteins, the cell-free media was passed through a 0.22 μm filter in order to remove VLPs and apoptotic bodies. We then treated immune cells with either filtered or unfiltered cell-free media. CEM lymphocytes treated with either filtered or unfiltered cell-free media conditioned by control cells displayed minimal amounts of cell death, while cells treated with cell-free media conditioned by GP or NP expressing cells displayed significant cell death following treatment. In Jurkat lymphocytes following treatment with either filtered or unfiltered cell-free media conditioned by control cells and cell-free media conditioned by cells expressing Ebola proteins VP40, GP, or NP. Additionally, significant amounts of cell death were detected in U937 monocyte cultures following treatment with either filtered or unfiltered cell-free media conditioned by cells expressing Ebola proteins VP40, GP or NP. Together, these data indicate that U937 cells are particularly susceptible to destruction by Ebola proteins VP40, GP and NP, and that Ebola protein are being carried to these cells via an exosome mediated transport.

It has been previously established that one of the natural targets of Ebola infection is the macrophage. Moreover, macrophage targeting by Ebola explains why this virus is unable to trigger a robust immune response[3]. We asked whether U937 differentiated into macrophages would be more susceptible to the direct toxic effects of Ebola proteins. We found out that while free VP40 is minimally toxic to undifferentiated U937 cells, cell-free media conditioned by cells expressing EBOV protein VP causes significant cell death.

When the U937 cells were differentiated, however, free VP40 is minimally toxic to PMA activated U937 cells, and while either filtered or unfiltered cell-free media conditioned by VP40 expressing cells demonstrate significant cell killing, the cell-free media that were passed through a 0.22 μm filter resulted in the greatest amount of cell death. Importantly, it is clear that free VP40 has minimal toxicity to both differentiated and undifferentiated U937 cells, whileVP40 transmitted via treatment with cell-free media, including filtered cell-free media. These observations confirm that exosome transported Ebola proteins, in general, and the VP40 contained in these exosomes, in particular, are functionally intact.

Due to the highly virulent nature of the Ebola virus, its studies may only be performed in a Biosafety level-4 laboratory. In order to broaden the research opportunities in this filed and lower the cost of Ebola research, it is necessary to develop means of examining the virus without risk of infection to the researcher. This is an imperative considering the fact that a great deal of initial contact with the virus by the scientific community occurs at hospitals, which may or may not have Biosafety level-4 containment facilities. By subjecting the virus-like particles to reducing conditions, we were able to denature and thereby deactivate the virus. We have used SDS buffer and nanoparticles to verify the conservation of the desired viral proteins. First, we determined the most efficient nanoparticles for Ebola viral capture under non-reducing conditions. We determined that the strongest binders of VLP and free GP and VP40 samples are the nanoparticle preparations 213 and 224. We then attempted VLP and free GP capture in reducing conditions using nanoparticles 213, 224, and 212. The strongest binder of both

free GP and VLP bound GP was the nanoparticle preparation 213. Finally we performed nanoparticle binding of free VP40 in both reducing and non-reducing conditions. In the absence of SDS buffer, nanoparticle preparations 224 and 229 were the most adept at capturing the free proteins. In the presence of SDS buffer, protein was captured by nanoparticle preparations 213, 217, 219, 222, 223, and 224, with the greatest protein capture by nanoparticle preparationss 213 and 224. These data demonstrate the utility of denaturing Ebola proteins in reducing the risk of infection while allowing expanded access to viral research at a Biosafety level-2 or level-3 facility.

We questioned whether VP40 may be located in exosomes rather than being secreted by host cells as free protein. We reasoned that because inhibitory effects were observed in cells that were treated with cell-free media that had filtered to remove VLPs and apoptotic bodies, it might be possible that VP40 was contained within exosomes. Results in Figure 9A are consistent with the presence of exosomes-associated VP40, and consistent with the absence of free VP40.

It has been previously shown that EBOV proteins VP30 and VP35 are capable of functioning as suppressors of RNA silencing; thus, providing for a virus a mean to evade host immune response[10]. We then asked if VP40 might also be capable of suppressing activity of miRNA machinery. CEM cells treated with the greatest concentration of exosomes suffered an inhibition of Dicer, Drosha and Ago 1. We then asked if EBOV VP40 and GP would exhibit a suppression effect on 293T, host cell miRNA machinery. 293T cells transfected with VP40 appeared to have an inhibition of all miRNA machinery components, however, a decreased level of actin expression suggests a lack of cellular

material may be the cause of the apparent lack of miRNA machinery activity. 293T cells transfected with GP protein revealed no loss of miRNA machinery activity.

Several FDA approved drugs have been found to lower exosomal release in virus infected cells (unpublished data). In particular, Cambinol, Oxytetracycline and Esomeprazole have all been found to have an inhibitory effect on HIV, HTLV, or RIFT infected cells (Data not shown). We asked whether these drugs might have the same effect on EBOV infected cells. Results depicted in Figure 11 show that VP40 transfected cells increase production of CD63 and actin, while VP40 transfected cells treated with Oxytetracycline show a decrease in CD63 and actin that is directly correlated to the concentration of Oxytetracycline used to treat the cells. We then asked whether treatment of host cells with Oxytetracycline might reduce EBOV host cell inhibitory effect on recipient T cells. Results in Figure 12B reveal that CEM is inhibited when treated with VP40 transfection cell-free media, but treatment with Oxytetracycline appears to resolve the inhibitory effect exerted by VP40 transfection cell-free media. Results in Figure 12C reveal no significant inhibitory effect on Jurkat cells, which is consistent with previous data presented in Figure 6B in which treatment of Jurkat cells with unfiltered and filtered VP40 transfection cell-free media presented no inhibitory effect.

In conclusion, our data suggests that free VP40, but not VLPs are inhibitory to T-cells, and supernatants conditioned by cells expressing Ebola proteins (containing either free protein, VLPs, apoptotic bodies, or exosomes) display inhibitory effects on both T-cells and monocytes. Filtering the supernatants, and therefore potentially removing VLPs and apoptotic bodies, does not result in decrease of inhibitory effects of cell-free media

on recipient immune cells. We have also shown that VP40 is contained in exosomes and Ebola proteins may be transmitted between cells via exosome mediated transport and that exosomes from Ebola infected cells may contain viral proteins. We have also established that Ebola proteins are capable of cell killing. We have also presented evidence that VP40 may exhibit an inhibitory effect on miRNA machinery in both recipient T-cells and host cells. In addition, we have established that treatment with FDA approved drugs, previously shown to lower exosomal release (unpublished data) may also have the effect of reducing exosomal release in host cells and may reduce the inhibitory effects of VP40 on recipient T cells. While the effects of exosomes on the course of viral infection and host response have yet to be fully explored, we have provided evidence that these exosomes may play a role in the spread of Ebola infection to immune privileged sites and a possibility of the establishment of latent Ebola infection.

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BIOGRAPHY

Allison Heath received her Bachelor of Science from Florida State University, Tallahassee Fl, in 2013. She hates the Gators and the Caines; she bleeds garnet and gold! GO NOLES!!!!!