

# The Role of Exosomal VP40 in Ebola Virus Disease

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Ebola virus (EBOV) can cause a devastating hemorrhagic disease, leading to death in a short period of time. After infection, the resulting EBOV disease results in high levels of circulating cytokines, endothelial dysfunction, coagulopathy, and bystander lymphocyte apoptosis in humans and nonhuman primates. The VP40 matrix protein of EBOV is essential for viral assembly and budding from the host cell. Recent data have shown that VP40 exists in the extracellular environment, including in exosomes, and exosomal VP40 can impact the viability of recipient immune cells, including myeloid and T cells, through the regulation of the RNAi and endosomal sorting complexes required for transport pathways. In this study, we discuss the latest findings of the impact of exosomal VP40 on immune cells *in vitro* and its potential implications for pathogenesis *in vivo*.

**Keywords:** Ebola, VP40, exosomes, ESCRT, RNAi, apoptosis

## Introduction

**E**BOLA VIRUS (EBOV) IS a single-stranded, enveloped, negative sense RNA virus of the *Filoviridae* family (Foster, 1999). EBOV infection can cause EBOV disease (EVD) in humans and nonhuman primates, resulting in an acute febrile illness (Singh *et al.*, 2015). Cases of EVD can progress to hemorrhagic fever characterized by cytokine storm, coagulopathy, leaky vessels, bystander lymphocyte apoptosis, and high rates of mortality in those affected (Messaoudi and Basler, 2015; Messaoudi *et al.*, 2015; Rougeron *et al.*, 2015).

Recently, increasing numbers of studies of the survivors from Sierra Leone and Liberia have identified populations of seropositive individuals that either presented with or reported no symptoms of EVD (Leroy *et al.*, 2000; Dean *et al.*, 2016; Richardson *et al.*, 2016). This could represent an important means of invisible transmission during outbreaks and has significant repercussions for the epidemiology and spread of the virus. In addition, those that recover from EVD do not always clear the virus completely.

Recent studies of survivors from Sierra Leone have shown that as many as one in four infected males can contain viral RNA in their semen for up to 6–9 months after disease onset (Christie *et al.*, 2015; Deen *et al.*, 2015; MacIntyre and Chughtai, 2016). Viral persistence has been documented in other compartments as well, including the eyes, brain, breast milk, and vaginal secretions (Rodriguez *et al.*, 1999; Varkey *et al.*, 2015; Billioux *et al.*, 2016; Chancellor *et al.*, 2016).

The survival of EBOV in these localized tissues can result in delayed or transient sequelae, including ocular and neurological symptoms that endure long after the virus is undetectable (World Health Organization, 2016).

Viruses have evolved many strategies to evade host immune surveillance and enhance persistence in host tissues. One mechanism of recent focus has been the viral utilization of the exosomal pathway within infected host cells. Exosomes are small, membrane-bound microvesicles produced by host cells and originate from the late endosomal pathway. They can act as intercellular messengers through the delivery of proteins and nucleic acids from the parent cell to target recipient cells, and thereby can affect change in the recipient cell (Théry *et al.*, 2002; Février and Raposo, 2004; Akers *et al.*, 2013). In infected cells, viral proteins, mRNAs, and miRNAs can be packaged into exosomes to impact recipient neighboring cells (Fleming *et al.*, 2014).

It has become clear in recent years that exosomes can play a significant role in the pathogenesis and progression of disease in viral infections, such as HIV-1, HTLV-1, and Rift Valley Fever virus (Lenassi *et al.*, 2010; Narayanan *et al.*, 2013; Jaworski *et al.*, 2014a; Schwab *et al.*, 2015; Ahsan *et al.*, 2016; Sampey *et al.*, 2016). In the case of Ebola, the viral matrix protein VP40 has recently been shown to be packaged into exosomes, which in turn can decrease the viability of recipient immune cells (Pleet *et al.*, 2016). In this study, we review the recent findings regarding exosomal VP40 and the potential role of these exosomes in EVD pathogenesis.

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### *Role of VP40 in RNAi dysregulation and bystander lymphocyte apoptosis*

During pathogenesis of EVD, the loss of T cell populations by bystander lymphocyte apoptosis has been well documented, despite the inability of EBOV to directly infect these cells (Geisbert *et al.*, 2000; Bradfute *et al.*, 2007; Wauquier *et al.*, 2010). The molecular mechanisms of this bystander apoptosis are unknown, but a number of hypotheses including induction of cell death by dysfunctional cell-cell interactions, exposure to chemical mediators produced by infected cells (including TNF- $\alpha$ , NO species, and IL-1 $\beta$ ), and interactions with viral proteins such as GP or sGP have been suggested (Geisbert *et al.*, 2000; Bradfute *et al.*, 2010).

Recently, we have found that 293T cells transfected with VP40-encoding plasmids generate exosomes containing VP40 protein. Furthermore, when these VP40-containing exosomes were placed on recipient immune cells (T cells and monocytes), cell death was readily observed (Pleet *et al.*, 2016). This is similar to previous observations that exosomes from cells infected with HIV-1, HTLV-1, and Rift Valley Fever virus have been shown to negatively impact the viability of naive recipient immune cells (Lenassi *et al.*, 2010; Jaworski *et al.*, 2014a; Ahsan *et al.*, 2016; Sampey *et al.*, 2016). As exosomes from EBOV-infected cells contain VP40 and can cause apoptosis in recipient T cells *in vivo*, this could represent a novel mechanism of inducing apoptosis in uninfected lymphocytes.

Interestingly, it was observed that components of the miRNA machinery, including Dicer, Drosha, and Ago proteins, were downregulated in both donor 293T cells transfected with VP40 plasmids and naive recipient T cells (Pleet *et al.*, 2016). Dicer, Drosha, and Ago are integral RNAi pathway proteins involved in the production of miRNAs and siRNAs for the silencing or degradation of target mRNA transcripts (Novina and Sharp, 2004). Previous studies have linked the downregulation of these components to the induction of apoptosis (Su *et al.*, 2009; Han *et al.*, 2013; Bian *et al.*, 2014; Lombard *et al.*, 2015). Combined, these studies suggest that EBOV VP40-laden exosomes from infected cells may be able to induce bystander lymphocyte apoptosis in uninfected, recipient immune cells, potentially through the modulation of RNAi components.

### *Exosomal VP40 and the endosomal sorting complexes required for transport pathway*

The Endosomal Sorting Complexes Required for Transport (ESCRT) pathway consists of four complexes (ESCRT-0, -I, -II, and -III) that are largely responsible for the recognition and selective packaging of cargo into nascent exosomes (Henne *et al.*, 2011). Previously, it has been shown that EBOV VP40 can recruit TSG101 and Alix proteins of the ESCRT pathway to aid in viral budding (Licata *et al.*, 2003; Panchal *et al.*, 2003; Timmins *et al.*, 2003; Silvestri *et al.*, 2007; Han *et al.*, 2015). This is not unique to Ebola, as other viruses, including HIV-1, have likewise been shown to use ESCRT proteins such as TSG101 for this purpose (Garrus *et al.*, 2001; Martin-Serrano *et al.*, 2001). In addition, 293T cells transfected with VP40 increase in intracellular levels of TSG101 (ESCRT I), and EAP20 and EAP45 (ESCRT II) proteins (Pleet *et al.*, 2016). Increases in ESCRT com-

ponents may suggest an increase in exosomal biogenesis in the presence of VP40.

Along these lines, both intracellular and concentrated exosomal preparation levels of exosomal marker CD63 were also found to be increased in cells transfected with VP40 plasmid (Pleet *et al.*, 2016). The upregulation of various host molecules derived from exosomes (i.e., CD81, CD63, and CD9) by several viruses and incorporation into the viral membrane for various purposes have been previously described (Dongen *et al.*, 2016). For example, CD63 upregulation during HIV-1 infection has been suggested to mediate CD63 integration into HIV-1 membranes to aid in both viral fusion and replication (Li *et al.*, 2011, 2014; Narayanan *et al.*, 2013; Fu *et al.*, 2015; Sampey *et al.*, 2016). It is possible that EBOV VP40 may play a role in the upregulation of CD63 for a similar purpose.

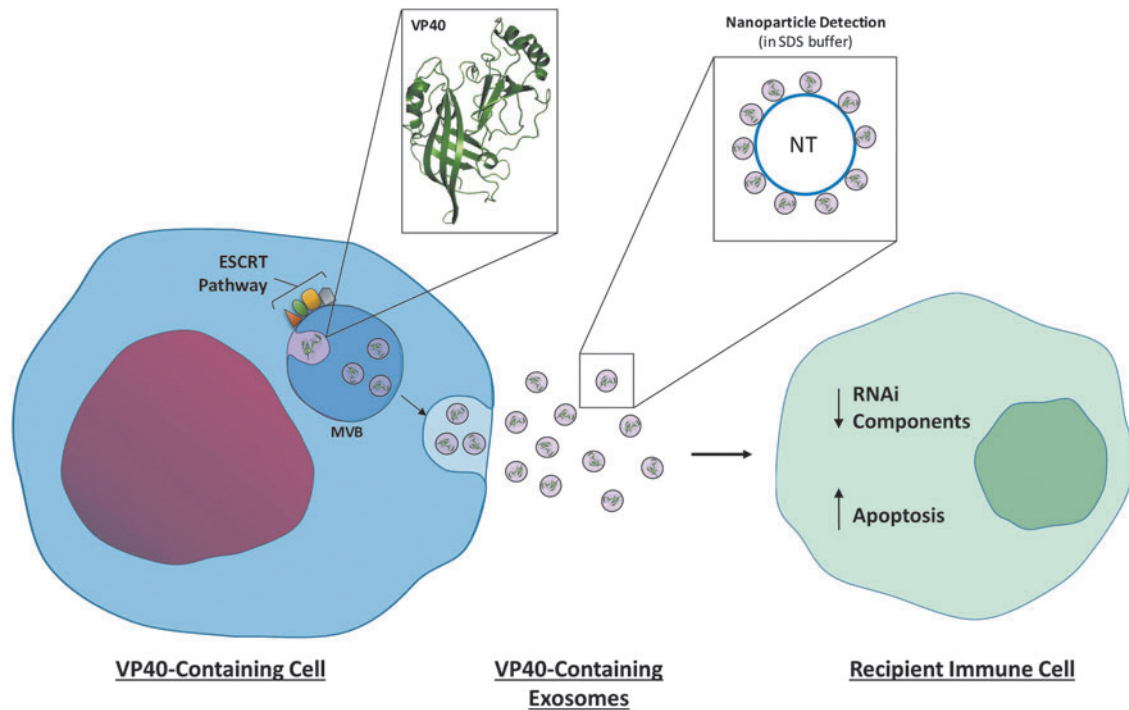
Together, these observations could indicate that the presence of Ebola VP40 may upregulate the biogenesis of exosomes through manipulation of exosomal tetraspanins and the ESCRT pathway by an unknown mechanism. Increases in exosomes containing VP40 may then induce bystander apoptosis in immune cell populations and contribute to unregulated viral replication in infected hosts.

### *Potential therapeutic and diagnostic implications*

Loss of T cell populations has been strongly associated with poor prognosis and fatality in EVD patients (Ryabchikova *et al.*, 1999; Martines *et al.*, 2015; Agrati *et al.*, 2016). As previously described, exosomes containing VP40 from infected cells may contribute to this phenotype and potential mortality. Previous data have shown that treatment with Food and Drug Administration (FDA)-approved drugs such as oxytetracycline may be capable of downregulating the biogenesis and/or release of exosomes, as evidenced by a decrease in exosomal markers (i.e., CD63) in the extracellular milieu posttreatment (Pleet *et al.*, 2016). Recipient cells incubated with supernatants (containing exosomes) from VP40-transfected cells that were treated with oxytetracycline demonstrated a recovery in cell viability in a dose-dependent manner.

Furthermore, treatment with oxytetracycline resulted in a decrease in CHMP6 (ESCRT-III) and VP40 protein levels within VP40-transfected cells (Pleet *et al.*, 2016). This could indicate a possible mechanism for oxytetracycline inhibition of exosome production and a potential therapeutic option by repurposing of an FDA-approved drug. Although eukaryotic cells are not the normal target for antibiotics, other tetracycline-class drugs such as minocycline have demonstrated efficacy with other infections (Si *et al.*, 2004; Zink *et al.*, 2005; Szeto *et al.*, 2010; Dutta and Basu, 2011). A true validation of the use of oxytetracycline would require animal pre-dosing to potentially target the microbiome before observation of potential inhibition of exosome regulation in eukaryotic host cells *in vivo*.

Diagnostic methods in the field prove challenging for EBOV. Often, detection of virus by polymerase chain reaction or ELISA is the method of choice; however, samples should be shipped to a proper BSL-3 or -4 biocontainment facility for diagnostics to safely take place (Reusken *et al.*, 2015; Kaushik *et al.*, 2016). Several techniques geared toward better diagnostics are being developed, but few are capable of being simply and safely carried out at the point-of-care. Some potential methods for POC diagnosis,

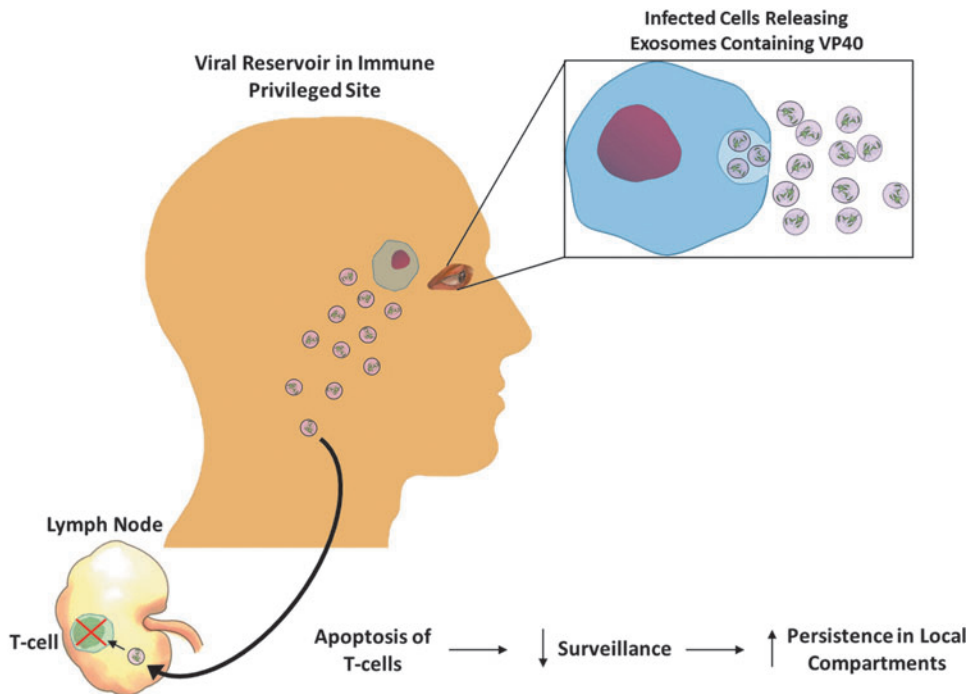


**FIG. 1.** Impact of exosomes containing Ebola VP40 on recipient immune cells. Ebola VP40 protein becomes integrated into exosomes with the modulation of ESCRT pathway components. Upon release, these exosomes can be received by immune cells (i.e., T cells). RNAi components, including Drosha, Dicer, and Ago, in both exosome donor and recipient cells can become downregulated. Recipient immune cells can then take part in programmed cell death. Nanoparticles may be used for the concentration of exosomes to detect viral proteins (and potentially RNAs) from samples, including human material using SDS buffer. ESCRT, endosomal sorting complexes required for transport; SDS, sodium dodecyl sulfate.

including an electrochemical immune-sensing approach, are highlighted by Kaushik *et al.* (2016), but another new method involves the use of nanoparticles.

Nanotrap (NT) particles are small hydrogel particles 700–800 nm in diameter with a bait core surrounded by a sieving

shell. Pores in the shell allow for selective passage of particles attracted to the baits, and result in the concentration and protection from degradation of target molecules (Jaworski *et al.*, 2014b). NTs have previously been used to reliably concentrate and capture exosomes from supernatants for



**FIG. 2.** Potential role of exosomes containing Ebola VP40 in viral persistence. Infected cells in immune privileged sites (i.e., ocular fluid) could release exosomes containing viral proteins such as VP40. These exosomes may then travel to distant areas such as lymph nodes, where they may induce apoptosis in immune and T cell populations. Increased cell death in surveilling immune cells could allow for increased viral replication and persistence in local compartments.

downstream analyses (Jaworski *et al.*, 2014a; Ahsan *et al.*, 2016; Sampey *et al.*, 2016). Along these lines, specific NTs have recently been explored as a method to detect Ebola viral proteins GP and VP40 from virus-like particles (VLPs) spiked into human samples with inactivating sodium dodecyl sulfate (SDS) buffer. Promisingly, NT219 particles (Cibacron Blue F3G-A affinity bait) have been successful in detecting VP40 protein from VLPs in human saliva and urine when using SDS buffer (Pleet *et al.*, 2016).

This method of inactivating human samples with SDS buffer and capturing viral proteins (and potentially RNA molecules) with NTs could pose a safer alternative to other common methods, and represents a useful method to concentrate diagnostic targets and increase sensitivity as well as specificity for downstream assays. Another benefit of the use of these particles may be the potential to bypass a BSL-4 facility altogether; samples could be inactivated first with the SDS buffer and then subsequently utilized for diagnosis in BSL-1 or BSL-2 conditions with NTs. In summary, the development and exploration of novel therapeutics and diagnostics for EVD should be considered and encouraged.

## Conclusion

The overall role of exosomes containing VP40 in EVD pathogenesis may be quite complex (Fig. 1). Since exosomal membranes contain lipid rafts (Gassart *et al.*, 2003; Janas *et al.*, 2015), it is perhaps not surprising that VP40 can be integrated into exosomes, as VP40 normally oligomerizes in lipid rafts under the plasma membrane prior to viral budding (Bavari *et al.*, 2002; Panchal *et al.*, 2003). It is also therefore possible that *in vivo* exosomes from infected cells may package additional viral proteins such as GP and/or NP. It could be speculated that the combined effect of these viral proteins on recipient immune cells could be intensified, resulting in even more dramatic damage *in vivo*.

During pathogenesis, EBOV can pass through the blood–brain barrier (Sagui *et al.*, 2015; Billioux *et al.*, 2016) and enter the brain. Cases of clinical latency or persistence of EBOV in ocular fluid or the brain may be due to migration of virally infected cells into these areas during this time. Once recovery takes place and the blood–brain barrier heals, infected cells may become trapped. When infected cells are no longer subjected to constant surveillance, they could be allowed to freely release exosomes containing Ebola proteins. These exosomes may originate from cells that are resistant to the cytopathic effects of EBOV, or it may be from cells with latent or integrated virus, which have been seen in other hosts (Belyi *et al.*, 2010; Taylor *et al.*, 2010, 2011). Then, these exosomes could migrate to distant compartments, such as lymph nodes, where immune cells could be inhibited or destroyed, thus allowing for unregulated replication or persistence of the hidden virus (Fig. 2).

The potential impact of exosomes containing viral proteins in EVD could be substantial. Further *in vivo* research on this subject is needed to determine the true extent of the significance of exosomes containing Ebola VP40 during pathogenesis.

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## Disclosure Statement

No competing financial interests exist.

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