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Camouflage and Misdirection: The Full-On Assault of Ebola Virus Disease

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Abstract

Ebolaviruses cause a severe hemorrhagic fever syndrome that is rapidly fatal to humans and non-human primates. Ebola protein interactions with host cellular proteins disrupt Type I and Type II interferon responses, RNAi anti-viral responses, antigen presentation, T-cell mediated antibody responses, humoral antibodies and cell mediated immunity. This multifaceted approach to evasion and suppression of innate and adaptive immune responses in their target hosts leads to the severe immune dysregulation and “cytokine storm” that is characteristic of fatal ebolavirus infection. Here we highlight some of the processes by which Ebola interacts with its mammalian hosts to evade anti-viral defenses.

Introduction

The *Filoviridae* family consists of three genera: Marburgvirus, Ebolavirus and the newly identified Cuevavirus. Within the Ebolavirus genus, there are five species including Zaire ebolavirus, Sudan ebolavirus, Bundibugyo ebolavirus, Tai Forest ebolavirus and Reston ebolavirus. Filoviruses were first identified as the causative agent of a hemorrhagic fever syndrome in Marburg, Germany in 1967. Nine years later, the first two ebolaviruses were described in the Democratic Republic of Congo (formerly Zaire) and Sudan. Since then, over 30 Ebola virus disease outbreaks have infected thousands with a mean case fatality rate of around 65% in humans (Hartman et al., 2010). The recent Ebola virus disease outbreak in West Africa began in March of 2014 and has thus far caused >7500 confirmed and probable cases with a case fatality rate of about 50% (for latest information see: <http://www.who.int/csr/disease/ebola/en/>).

Virus Life Cycle

Ebola particles are enveloped, filamentous, and contain a monopartite negative-sense RNA genome. While Ebola initially targets macrophages and dendritic cells it is able to infect almost all cell types with the exception of lymphocytes (Wool-Lewis and Bates, 1998).

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Virus particles have been proposed to attach to host cells through multiple plasma membrane surface-expressed proteins (e.g., C-type lectins, DC-SIGN, integrins, TIM-1, Axl) (Alvarez et al., 2002; Baribaud et al., 2002; Kondratowicz et al., 2011; Lin et al., 2003; Schornberg et al., 2009; Shimojima et al., 2006; Simmons et al., 2003; Takada et al., 2000). Once attached to the plasma membrane, the viral envelope glycoprotein induces particle uptake via macropinocytosis. The induction of macropinocytosis appears to be dependent on the action of cell surface proteins, including TIM-1 and Axl (Aleksandrowicz et al., 2011; Brindley et al., 2011; Hunt et al., 2011; Moller-Tank et al., 2013; Mulherkar et al., 2011; Nanbo et al., 2010; Quinn et al., 2009; Saeed et al., 2010; Shimojima et al., 2007, 2006; Wen et al., 2013). After uptake into macropinosomes, particles travel to low pH compartments of late endosomes and lysosomes where the viral envelope glycoprotein (GP) is proteolytically cleaved by endosomal cysteine proteases (i.e., cathepsin B and L). This cleavage removes a heavily glycosylated region from GP (Chandran et al., 2005; Dube et al., 2009; Hood et al., 2010; Misasi et al., 2012; Schornberg et al., 2006) and exposes a domain in GP that binds specifically to the endosomal/lysosomal resident filovirus receptor Niemann-Pick C1 protein (NPC1) (Carette et al., 2011; Côté et al., 2011). While current evidence suggests that NPC1 binding may be sufficient to trigger fusion of the viral and cellular membranes (Miller et al., 2012), it is as yet unclear whether additional host proteins or intracellular conditions are necessary (e.g., reducing conditions, altered pH, additional protease cleavage) (Brecher et al., 2011; Chandran et al., 2005).

Once the viral and internal cell membranes fuse, the virus particle uncoats and its anti-genome is transcribed into mRNA using nucleocapsid-associated viral proteins. The virus genome consists of seven viral genes, VP24, the nucleoprotein (NP), VP30, VP35, the matrix protein (VP40), the RNA-dependent RNA polymerase (L) and the glycoprotein gene, which are transcribed into mRNA resulting in the production of at least ten proteins. Transcription of the genome is mediated via a complex of VP30, VP35 and the viral polymerase L bound to an NP-coated genome (Bharat et al., 2012; Hartlieb et al., 2003, 2007; Modrof et al., 2003; Mühlberger et al., 1999; Sanchez and Kiley, 1987; Sanchez et al., 1993). VP30 phosphorylation leads to its dissociation from the VP35/L complex and is the signal to switch from transcription to replication (Biedenkopf et al., 2013; Martinez et al., 2011a). Following this switch, virus genomes are replicated and coated by NP, VP24, VP30 and VP35 (Mühlberger et al., 1999). During assembly, L associates with the ribonucleoprotein complex via an interaction with VP35. The ribonucleoproteins then associate with the matrix protein VP40 and viral particles are extruded through the plasma membrane within lipid raft microdomain regions (Stahelin, 2014).

Sneaking in with the Trash: Apoptotic mimicry

Ebolavirus particles can be up to a micron in length, making it difficult for the viruses to enter via classic clathrin or caveolin mediated endocytosis pathways. Recent evidence using pseudotyped viruses, virus-like particles and infectious Ebola particles suggests that after attachment to the plasma membrane, ebolavirus induces macropinocytosis in a process that is enhanced by the presence of TIM-1 and Axl (Hunt et al., 2011; Moller-Tank et al., 2013; Mulherkar et al., 2011; Nanbo et al., 2010; Quinn et al., 2009; Saeed et al., 2010; Shimojima et al., 2006). Interestingly, an increased amount of phosphatidylserine (PS) may be present

on the surface of Ebola virus-like particles (Jemielity et al., 2013; Moller-Tank et al., 2013). PS is a lipid primarily present on the inner leaflet of plasma membranes (Zachowski, 1993). Upon cell death via apoptosis, PS is exposed to the outer leaflets of plasma membranes and apoptotic bodies. This alerts nearby cells, including phagocytic cells, to begin “eating” the debris via macropinocytosis in a process that is mediated by TIM-1 and Axl, and does not induce an inflammatory response (Biermann et al., 2013; Morizono and Chen, 2014; Zagórska et al., 2014). This suggests the possibility that, similar to other large viruses such as vaccinia virus (Mercer and Helenius, 2008), Ebola may induce macropinocytic uptake by appearing to be an apoptotic body to phagocytic cells. This “apoptotic mimicry” is anti-inflammatory and induces rapid uptake of a large virus into cells, thus avoiding humoral and cell surface immunity factors.

Snipping the Alarm-wire: Preventing the interferon alarm

Evasion of cellular innate immune responses is of particular importance to viruses and many have evolved mechanisms to circumvent innate immunity. Ebola inhibits both Type I and Type II interferon responses in target cells, especially macrophages, monocytes and dendritic cells. The ultimate result is a defect in dendritic cell maturation and diminished T-cell activation and proliferation along with apoptosis leading to lymphopenia, a key characteristic of Ebola virus disease. Studies in animal models and in tissue culture suggest that both pathogenesis and interferon antagonism are linked to VP35 and VP24 (Cilloniz et al., 2011; Ebihara et al., 2006; Hartman et al., 2008a, 2008b; Mateo et al., 2011; Prins et al., 2010; Reid et al., 2007).

VP35

VP35 is a viral polymerase cofactor that functions in RNA synthesis and has been proposed to link L to NP. In addition to these roles, VP35 plays a prominent role in Ebola’s inhibition of alpha and beta interferon induction through multiple mechanisms.

RIG-I and MDA-5 are innate pattern recognition receptors that detect foreign cytosolic RNA. RIG-I recognizes 5'-triphosphates of blunt-ended RNA and MDA-5 senses long double stranded RNA (dsRNA). Both signal via the downstream adapter IPS-1 (a.k.a. MAVS, VISA, Cardif) resulting in NF- κ B, IRF-3 and IRF-7 activation and subsequent expression of Type-I interferon and pro-inflammatory cytokines. Activation of IRF-3/7 is the result of a signal cascade through which they are phosphorylated by TANK-binding kinase 1 (TBK-1) and I κ B kinase- ϵ (IKK ϵ) (Chiang et al., 2014). Early experiments determined that VP35 disrupted the RIG-I pathway by preventing IRF-3 phosphorylation (Basler et al., 2000, 2003). Later, VP35 was shown to interact with the N-terminal kinase domain of IKK ϵ in preventing IRF-3 phosphorylation and acting as a decoy substrate for IKK ϵ /TBK-1 kinases. Furthermore, binding of VP35 to IKK ϵ prevents interactions with other proteins, including IRF-7 and IPS-1 (Prins et al., 2009). The net result of these interactions is inhibition of the induction of genes with interferon response promoters.

In addition to these downstream events in the RIG-I pathway, VP35 interacts with dsRNA to prevent RIG-I and MDA-5 responses (Cárdenas et al., 2006). Structural and biochemical studies revealed that VP35 contains a C-terminal interferon inhibitory domain (IID) with

two clusters of basic amino acids. One cluster centers on residue R312 and participates in binding to dsRNA. Further analysis revealed that VP35 binds to blunt ended dsRNA in a manner very similar to that seen with RIG-I (Cárdenas et al., 2006; Leung et al., 2009, 2010a). Structural studies of VP35 dsRNA binding are consistent with the finding that VP35 prevents both RIG-I and MDA-5 responses. Observations from RNA bound and unbound structures revealed that VP35 is able to bind both the phosphate backbone of dsRNA and end-capped RNA in VP35 dimers. Mutations of the basic patch centering on R312 abrogate dsRNA binding and structural analysis suggests that R312 mutations disrupt VP35 dimerization (Kimberlin et al., 2010). Experiments using recombinant viruses incorporating mutant VP35_{R312A} showed attenuation of virulence and impairment of both virus growth and interferon antagonism, suggesting that IID binding to dsRNA and VP35 dimerization play a key roles in the virus life cycle and pathogenesis (Hartman et al., 2008a, 2008b; Kimberlin et al., 2010; Prins et al., 2010).

Interestingly, comparisons of VP35 IID from the pathogenic Zaire ebolavirus and Reston ebolavirus, thus far only pathogenic in monkeys, revealed a slight decrease in interferon antagonism and dsRNA binding by Reston. However, these decreases did not appear to contribute significantly to the differences in virulence between the Zaire and Reston viruses (Leung et al., 2010b). Furthermore, comparison of the structures of the Zaire and Reston VP35 did not reveal substantial differences between the dsRNA recognition mechanisms (Kimberlin et al., 2010). Together, these data suggest additional viral factors likely play a role in the differential host responses between these two viruses.

Recently, VP35 was found to interact with the PKR Activator (PACT) (Fabozzi et al., 2011). In addition to having activity in RNA silencing and PKR activation, PACT also binds to and activates RIG-I. Subsequent work showed that VP35 binding to PACT prevents PACT binding to RIG-I and inhibits RIG-I activation (Luthra et al., 2013). VP35 binding to PACT is mediated via the same central basic patch in IID that abrogates dsRNA binding described above, suggesting a possible role for dsRNA in this interaction. Surprisingly, PACT interaction with VP35 inhibits the binding of VP35 with L, causing a decreased efficiency of viral RNA synthesis and genome replication, a phenotype of “mutual antagonism”(Luthra et al., 2013). Taken together with previous data, these experiments point to the critical importance of VP35 antagonism of the RIG-I pathway during ebolavirus infection.

Additional VP35 interactions with cellular proteins have been explored using a yeast 2-hybrid system. These studies found that VP35 interacts with IRF-7, Ubc9 and PIAS1 (Chang et al., 2009). Ubc-9 and PIAS1 are key components of the small ubiquitin-related modifier (SUMO) system of post-translational modification, which regulates a variety of cellular pathways and proteins. During SUMOylation, SUMO proteins are activated by SUMO-specific proteases and transferred to a SUMO conjugating E2 enzyme (e.g., Ubc9). Next, an E3 ligase, such as PIAS1, is used to transfer the SUMO domain to a lysine on the target protein (Wimmer et al., 2012). Studies with Ebola VP35 found that it was able to block CpG-induced interferon induction involving the IRF3/7 pathway. Subsequent investigation revealed that PIAS1 is able to SUMOylate IRF7. VP35 expression enhanced SUMOylation of IRF7, leading to suppression of its activity and a decrease in interferon promoter activity.

Similar findings were noted with IRF3 following expression of VP35 (Chang et al., 2009). Thus, VP35-induced SUMOylation of IRF-3 and IRF-7 leads to a further reduction in interferon α/β gene transcription.

Arenaviruses (e.g., Lassa, LCMV) have taken a similar multipronged approach to preventing type I interferon responses. Lassa hemorrhagic fever has many similar characteristics to Ebola virus disease, including the absence of interferon production and lymphoid depletion. On the molecular level, arenaviruses have been shown to suppress interferon production by targeting both upstream (i.e., RIG-I/MDA-5) and downstream signaling events (i.e., IKK ϵ interactions, IRF-3 phosphorylation) (Koma et al., 2013). In this way each virus disrupts multiple access points in the pathways that lead to increased interferon production. Given the similarity in the clinical syndromes and the common approach to interferon antagonism, further investigations may provide insights into the underlying pathogenic mechanisms of hemorrhagic fever syndromes.

VP24

When innate immunity is intact, the host response to virus infection causes secretion of interferon in order to generate antiviral responses in neighboring cells, signal hematopoietic cell responses and increase antigen presentation in APC's. Secreted interferon binds to Type I and II interferon receptors, inducing signaling via adapter proteins and results in the phosphorylation and subsequent dimerization of signal transducer and activator of transcription (STAT) proteins (e.g., STAT-1, STAT-2). Next, dimerized phosphorylated STATs are transported to the nucleus where they bind to interferon response elements and induce gene expression (Ivashkiv and Donlin, 2014; Plataniias, 2005). Given the importance of these pathways for inducing anti-viral gene expression in response to interferon, they are commonly targeted by viruses. For example, Dengue virus blocks STAT-1 phosphorylation and acts to degrade STAT-2 via proteasomal degradation pathways (Green et al., 2014). Early experiments with Ebola found that the virus not only blocked the production of interferon, but also inhibited cellular responses that normally result from both interferon α/β (Type I) and interferon γ (Type II) signaling. This signaling block was associated with the expression of the Ebola VP24 protein, which was later shown to prevent the nuclear accumulation of dimerized phosphorylated STAT-1 (Reid et al., 2006), which participates in both Type I (i.e., STAT-1/STAT-2 phosphorylated-dimer) and Type II (STAT-1/STAT-1 phosphorylated- dimer) signal propagation cascades (Ivashkiv and Donlin, 2014; Plataniias, 2005).

Phosphorylated STAT-1 dimer transport to the nucleus is mediated via interactions with members of the nucleoprotein interactor 1 family (i.e., karyopherin- α 1, - α 5 or - α 6). Karyopherin- α binds to nuclear localization signals (NLS) on cargo destined for the nucleus. Downstream interactions with karyopherin- β and other proteins allow the cargo to cross the nuclear membrane. Unlike most nuclear proteins, dimerized phosphorylated STAT-1 interacts with karyopherin- α through a unique non-canonical NLS, allowing it preferential access to the nucleus. Biochemical and structural studies have shown that VP24 binds to karyopherin- α in the non-canonical NLS binding domain. This results in a block in phosphorylated STAT-1 dimer transport to the nucleus. Furthermore, VP24 binding to

karyopherin- α does not appear to block access to the canonical NLS binding site (Mateo et al., 2010; Reid et al., 2007; Xu et al., 2014). Therefore, the virus is able to block both Type I and Type II interferon signaling without disrupting routine trafficking to the nucleus of the infected cell.

Unphosphorylated STAT-1 is able to enter the nucleus via a karyopherin- α independent mechanism, where it is able to induce and prolong the expression of interferon-induced immune regulatory genes (Cheon and Stark, 2009; Meyer and Vinkemeier, 2004). Biochemical and structural studies using a STAT-1 C-terminal truncation mutant demonstrated that VP24 binds efficiently to STAT-1 lacking the tyrosine target for phosphorylation (Zhang et al., 2012). This suggests an additional mechanism unrelated to phosphorylated STAT-1 dimer nuclear transport by which VP24 is able to inhibit the induction of the interferon response (i.e., by blocking the action of both forms of STAT-1).

In addition to the Jak-STAT pathway, Type I interferon receptors signal via the p38 MAP kinase pathway. In this pathway a signal cascade of MAP kinases results in the phosphorylation of p38- α (Ivashkiv and Donlin, 2014; Plataniias, 2005). One study found that in HEK-293T cells, VP24 was able to block the interferon- β induced phosphorylation of p38- α . However, this finding was cell type-specific and the blocking mechanism has yet to be demonstrated or generalized to cells that are primary targets of infection (e.g., macrophages, dendritic cells) (Halfmann et al., 2011).

Like VP35, VP24 has been linked to Ebola pathogenesis. In particular, experiments using mouse- and guinea pig-adapted variants of Ebola demonstrated that VP24 in conjunction with other proteins such as NP are critical to virus lethality (Cilloniz et al., 2011; Ebihara et al., 2006; Mateo et al., 2011; Reid et al., 2007). Structural analysis and comparison of Sudan and Reston VP24 identified two conserved structural pockets that contain residues implicated in the pathogenesis of VP24. Solvent exchange studies showed that this region may be near a proposed STAT-1 binding region on VP24, suggesting that it may play a role in STAT-1 binding and pathogenesis (Zhang et al., 2012). Taken together with previous studies on karyopherin- α , VP24 plays a role in pathogenesis through the disruption of both Type I and Type II interferon signaling. Thus, Ebola not only diminishes the interferon alarm but inhibits the alarm response after it has been heard. This coordinated approach between VP35 and VP24 leads to a highly effective antagonism of the innate immune responses mediated by interferons.

Silence Will Fall: Disrupting the RNAi response

Infection of cells by RNA viruses activates an RNA-specific inhibition (RNAi) pathway in host cells that silences viral gene expression by cleaving viral RNA into small interfering RNAs (siRNA) that bind and disrupt complementary RNA transcripts. Therefore, this host antiviral response is often a target for viral proteins. For example, HIV-1 Tat is thought to suppress RNAi responses by blocking Dicer activity, a key component of the RNA silencing complex. Ebola VP35 suppresses cellular RNAi silencing and can complement HIV-1 Tat⁻ mutants. This suppression was lost when mutations were made in the dsRNA binding domains of VP35, suggesting that VP35 may bind to siRNA or the dsRNA precursors of

siRNA (Haasnoot et al., 2007). While subsequent studies found that the C-terminal domain of VP35 bound to siRNA and not to dsRNA, the RNAi silencing suppression activity of VP35 did not correlate with binding to siRNA. It was hypothesized that suppression via RNA binding-independent mechanisms could instead occur through binding to the RISC complex or sequestration of RISC complex proteins prior to their incorporation into the complex (Zhu et al., 2012). This theory is supported by prior experiments that found VP35 interacts with transactivation response RNA-binding protein (TRBP) and PACT (Fabozzi et al., 2011), both components of the RISC RNAi complex and thus proposed to mediate the VP35-dependent RNA silencing suppressor activity. Interestingly, another Ebola protein, VP30, also binds to components of the RISC complex and acts as a suppressor of RNAi silencing (Fabozzi et al., 2011). Similar to Ebola's tactics for interferon antagonism, the virus uses two different viral proteins to disrupt the mammalian RNAi innate immune response.

Plundering the Factories: Coopting host cellular functions

Protein Translation

Host cells thwart viral infection by decreasing cellular protein synthesis in an effort to prevent or slow viral replication. This is accomplished through the double stranded RNA-dependent Protein Kinase, PKR. Upon binding of dsRNA and activation, PKR phosphorylates the alpha subunit of the eukaryotic translation initiation factor-2 (eIF-2 α). Ebola VP35 expression has been shown to block PKR activity and enhance expression of proteins after cells were treated with Type I interferons. VP35 blocks PKR by impairing phosphorylation of both PKR and the eIF-2 α . Current data suggests this effect is mediated by the VP35 IID domain; however, further work to elucidate the molecular mechanism is required (Feng et al., 2007; Schümann et al., 2009).

RNA stability and replication

The heterogeneous nuclear protein complex C1/C2 proteins (hnRNP C1/C2) are typically found in the nucleus where they bind poly-U regions (>4) in mRNA and assist splicing prior to mRNA export to the cytoplasm. They have also been shown to be able to participate in cap-independent, IRES-dependent translation in the cytoplasm during mitosis (Shabman et al., 2011). Several viruses, including Dengue and poliovirus, have been found to co-opt this function to enhance viral protein synthesis and replication (Brunner et al., 2005, 2010; Noisakran et al., 2008; Pettit Kneller et al., 2009). Ebola VP24 expression causes the relocalization of hnRNP C1/C2 from the nucleus to the cytoplasm. Interestingly, hnRNP C1/C2 also binds to karyopherin- α via the same non-canonical NLS sequence STAT-1 and VP24 use, suggesting a possible mechanism for redistribution. It was hypothesized that since the Ebola genomic and mRNA sequences contain many poly-U tracts, hnRNP C1/C2 may interact with Ebola RNA to stabilize viral mRNA and enhance genome replication (Shabman et al., 2011). Thus, through PKR inhibition and hnRNP C1/C2 relocalization, ebolaviruses coopt cellular machinery to optimize translation of gene products and potentially prolong the half-life of viral messenger and genomic RNA.

Escape from Alcatraz: The tetherin release program

Tetherin is a Type I interferon inducible cellular factor that has been shown to prevent enveloped virus budding from plasma membranes. Tetherin contains two membrane-anchored domains and is thought to mediate inhibition of budding by having one anchor linked to the cellular and viral membranes (Neil et al., 2008). Tetherin was first described to antagonize retrovirus and HIV-1 particle budding. These studies found that particle budding was rescued by the expression of HIV-1 Vpu (Neil et al., 2008). When Ebola virus-like particles (VLP) were made with VP40 in the presence of overexpressed tetherin, a similar decrease in VLP release was noted and simultaneous expression of HIV-1 Vpu rescued particle release. VLPs were then expressed with or without GP, and GP was found to antagonize tetherin activity and co-immunoprecipitate tetherin (Kaletsy et al., 2009). Recent experiments suggest that residues within the transmembrane domain of GP contribute to GP-dependent tetherin antagonism (Gnirß et al., 2014; Kühl et al., 2011). Mechanisms proposed for GP-tetherin antagonism include interference with tetherin integrity, steric hindrance by GP that interferes with the formation of the tetherin connection between the viral and cellular membranes, and GP-dependent exclusion of tetherin from the region of the plasma membrane from which Ebola virus particles bud (Kaletsy et al., 2009). In HIV-1, Vpu mediates cell surface downregulation, relocalization and degradation of tetherin (Lopez et al., 2012). However, Ebola GP does not appear to downregulate or alter tetherin localization (Lopez et al., 2010, 2012), suggesting a novel anti-tetherin mechanism.

The Host Strikes Back: Blocking the entrances and exits

The trade-off between efficient viral replication and pathogenicity is a delicate balance that when mismatched can lead to host demise. Therefore, viruses have evolved to suppress immune responses in a selective and regulated manner that facilitates controlled virus replication and progeny transmission. In this section we highlight two interferon inducible genes that to date are not known to be directly countered during Ebola infection.

Interferon-induced transmembrane proteins (IFITMs) are broadly active interferon responsive restriction factors that were first described as inhibitors of influenza A and have been shown to be active against many enveloped viruses including Ebola and its cousin Marburg virus (Huang et al., 2011). IFITMs are transmembrane proteins that are found in multiple membrane surfaces along cellular uptake pathways and have been proposed to act by blocking virus entry (Huang et al., 2011; Perreira et al., 2003). Several anti-viral mechanisms have been proposed including cholesterol accumulation, inhibition of back fusion of virus containing vesicles in multivesicular bodies, changes to membrane fluid dynamics, or alterations in membrane curvature (i.e., restricting the capacity for fusion between the virus and cellular membranes)(Amini-Bavil-Olyae et al., 2013; Perreira et al., 2013). Recent experiments with IFITM-3 and influenza A virus suggest that IFITM-3 acts by blocking an as yet undefined step between virus-cell membrane hemi-fusion and fusion pore formation (Desai et al., 2014). Thus, IFITM-3 allows the virus and cellular membrane lipids to mix but does not allow the formation of a complete fusion pore. This prevents the ribonucleoprotein core of the virus from gaining access to the cytoplasm and blocks infection. Future experiments will need to be performed to determine if this mechanism is

applicable to Ebola and how some enveloped viruses (i.e., arenaviruses) are able to avoid restriction by IFITMs.

Another interferon-stimulated gene that blocks Ebola infection is ISG-15, a broadly active antiviral gene product that, when conjugated to a target protein, causes alterations to target protein activity, cellular location and stability. It has been shown to affect many viruses including influenza A/B, hepatitis B, HIV-1, herpes simplex type-1, LCMV and Ebola (Harty et al., 2009). In the case of Ebola, the anti-viral activity of ISG-15 was linked to a disruption in VP40-mediated budding using virus-like particle (VLP) assays. Ebola budding is in part mediated by Nedd4 ubiquitination of VP40. Two groups found that ISG-15 interacts with Nedd4 and prevents the ubiquitination of VP40, leading to inefficient particle budding (Liu and Harty, 2010). Thus, together with IFITMs and tetherin, these observations suggest that interferon stimulated genes have potent antiviral activities targeted against ebolavirus during both entry and egress.

The resistance of freshly isolated monocytes to Ebola infection illustrates one example where these factors may be playing a role during entry. Experiments with Ebola GP-pseudotyped VLPs found that particles were able to bind but not enter freshly isolated monocytes. However, once the monocytes underwent differentiation, the previously bound virus was able to infect the monocytes, a result recapitulated using replicating Ebola viruses (Martinez et al., 2013). Detailed analysis found that, as the monocytes matured, IFITMs were downregulated while both cysteine protease cathepsin B and NPC1 expression were upregulated. These findings demonstrate how IFITM restriction factors might play a role in the relative resistance to infection of interferon-activated cells, and also confirm the importance of NPC1 for Ebola infection.

Balancing the scales of the immune response

A paradox in Ebola virus disease is that both survival and mortality are linked to the generation of strong immune signaling responses in the host. Survivors and asymptomatic patients have increased numbers of T cells and an early cytotoxic T-cell mediated response (Baize et al., 1999; Sanchez et al., 2004; Wauquier et al., 2010). Rapid uptake of Ebola by macrophages and dendritic cells results not only in translocation to lymphoid and peripheral tissues, but may explain the deficit in non-survivors of inflammatory cytokine secretion needed early after exposure for the control of infection. Experiments using *in vitro* cultured monocyte-derived macrophages found that Ebola infection inhibited the secretion of TNF- α , IL-6, and IL-10, consistent with impairments observed in association with fatal disease outcome (Mahanty et al., 2003). Furthermore, dendritic cells exposed to either live or inactivated virus failed to upregulate molecules needed for T-cell co-stimulation, resulting in an inhibition of CD4 T-cell proliferation. Studies using blood samples obtained from infected human subjects found that IL-6 and TNF- α responses were higher in survivors than non-survivors at early time points. At later time points, IL-6 remained higher in survivors, but the difference from non-survivors was less dramatic due to increases in the latter group. In contrast to IL-6, TNF- α in non-survivors was much higher than survivors late after disease onset. It is interesting to note that IL-10 may play a critical role in modulating these responses. While IL-10 was mildly elevated in survivors, likely as a feedback mechanism to

control the inflammatory response, the increase was short lived, as would be expected once cytokine levels return to normal. However, IL-10 was 6–10 fold higher in fatal cases and remained elevated until death. In addition, monocyte/macrophage activation as measured by neopterin levels was 2–10 fold higher and consistently elevated in fatal cases (Baize et al., 2002; Leroy et al., 2000, 2001), suggesting unregulated immune activation. While more recent studies showed different patterns for specific cytokines (Wauquier et al., 2010), there is a general trend toward survivors having a short-lived, balanced pro- and anti-inflammatory response and non-survivors having a prolonged inflammatory response that leads to “cytokine storm”.

Together these observations suggest that subjects able to overcome innate immune response blocking by VP24, VP30 and VP35 are more likely to establish an early, balanced and beneficial secretion of pro-inflammatory/anti-inflammatory cytokines. In contrast, when early host anti-viral innate responses are blocked, uncontrolled virus replication and lysis of hematopoietic cells leads to late, unbalanced cytokine release, overall dysregulation of immune responses and the development of advanced Ebola virus disease.

Camouflage & Misdirection: Avoiding adaptive immune responses

A fatal irreversible consequence of hematopoietic cell destruction by Ebola is reduced antigen presentation. This outcome is consistent with the observation of poor IgG responses observed in fatal infection, while high levels of IgG are associated with survival (Baize et al., 1999; Ksiazek et al., 1999). Compounding inhibition of IgG production, Ebola has evolved multiple properties that could circumvent antibody effectiveness. Large filamentous virions containing high density, stable glycoprotein present a potential obstacle for efficient inhibition by antibodies, and virus filament folding may create “pockets” of glycoprotein inaccessible to antibody binding. Furthermore, heavy glycosylation in the mucin-like domain of GP may limit access to critical epitopes required for efficient neutralization (Martinez et al., 2011b). Antibody access is restricted further due to rapid virion uptake via macropinocytosis and intracellular receptor binding. Additionally, ebolavirus hides its critical receptor binding domain beneath a glycan cap, with exposure and receptor binding occurring only after cathepsin mediated removal of the cap. This protection of a critical functional domain is analogous to HIV where CD4 engagement of gp120 exposes the co-receptor binding site (Harrison, 2008). Furthermore, the use of an intracellular receptor is a novel immune evasion strategy that may be important for other viruses (e.g., Lassa fever virus (Jae et al., 2014)).

Besides membrane anchored GP, the glycoprotein gene of Ebola encodes sGP, a 364 a.a. protein which is identical to GP in its 205 N-terminal residues but is secreted by infected cells and is not present in virions. The default transcript of the glycoprotein gene is surprisingly not the virion-associated GP trimer, but instead dimeric GP, a process that is governed by polymerase stuttering at an RNA editing site. This has led to speculation that sGP functions to modulate or misdirect host immune responses (Kindzelskii et al., 2000; Yang et al., 1998). The best evidence for this hypothesis comes from a recent paper showing in mice that sGP promotes immune evasion by serving as an antibody decoy for GP or by presenting alternative non-neutralizing antibody epitopes for the humoral immune response

(Mohan et al., 2012). Remarkably, evidence suggests that about 80% of glycoprotein gene expression is sGP. This may be due, in part, to selective pressures seeking to balance toxicity due to cytopathic effects of GP with the requirement for GP on virus particles, and avoidance of host immune responses directed against GP (Yang et al., 2000, Volchkova et al., 2011). Taken together, these data suggest that Ebola expression of sGP and GP is a tightly regulated process in which immune shielding and virus particle production are balanced via RNA editing.

In addition to immune evasion achieved through virion and GP structural characteristics, GP exhibits direct immunosuppressive properties (Chepurnov et al., 1999). sGP interacts with neutrophils and disrupts the linkage between Fc gamma RIIIB and CR3 (Kindzelskii et al., 2000). Furthermore, the Ebola transmembrane glycoprotein, GP2, bears structural similarity to retroviral glycoproteins possessing an immunosuppressive peptide motif (Volchkov et al., 1992) that was found to inhibit lymphocyte activation and proliferation (Yaddanapudi et al., 2006). Of particular interest was the observation that the analogous immunosuppressive peptide from Reston virus was inhibitory only in macaque, but not human, peripheral blood cells, implicating this motif in Ebola pathogenicity.

Studies measuring *in vivo* antigen-specific T-cell responses are limited by the difficulty in obtaining and preserving viable lymphocyte samples from Ebola-infected subjects and survivors. Nonetheless, data suggests that there is a dramatic decrease in the absolute numbers of T cells due to bystander apoptosis. This impairs both direct cell-mediated killing of virus-infected cells and the T-cell dependent antiviral antibody responses (Sanchez et al., 2004). Indirect measures of T-cell function based on serum cytokine levels and RNA expression in isolated lymphocytes suggest, as with immunoglobulin levels, an association between intact cell-mediated immunity and survival (Baize et al., 1999; Ksiazek et al., 1999; Sanchez et al., 2004; Wauquier et al., 2010). In macaques, CD8⁺ T cells are essential for vaccine-induced immune protection (Stanley et al., 2014; Sullivan et al., 2011). Antibodies clearly play a beneficial role in containing virus but are not required for protection against some Ebola species (Hensley et al., 2010; Stanley et al., 2014; Sullivan et al., 2011), but participate in effective virus clearance and likely require the presence of intact host cell-mediated responses (Wong et al., 2012). One speculative interpretation of both human and macaque data is that immunoglobulin and innate immune responses participate in containing early viral loads, but that cell-mediated immunity is needed for efficient virus clearance. The interplay and necessity of broad immune response mechanisms is also suggested by Ebola evolution of strategies to counteract each of these host antiviral defenses. Taken together these data suggest a complex interplay between pro- and anti-inflammatory factors resulting in either a balanced immune response and host survival or dysregulation and death.

Summary

Fatal Ebola infection is marked by a catastrophic failure of innate and adaptive immunity that is mediated by virus-encoded proteins as well as properties associated with virus structure. At the heart of Ebola-induced immune dysregulation is a multipronged attack on host antiviral immunity. Early and coordinated disruption of host innate responses by VP24, VP30 and VP35 leads to elevated levels of virus replication, a cascade of inappropriately

timed cytokine release and death of both antigen-presenting and responding immune cells. This results in a poorly activated adaptive immune response that is further compromised by the induction of lymphocyte apoptosis and antibody decoy mechanisms. In incidental hosts, this multifaceted approach to subversion of the immune system results in high mortality that would be expected to limit virus persistence in the absence of a distinct reservoir species.

Since first being identified almost 40 years ago, the genome of Ebola has shown remarkable stability, an unusual feature for RNA viruses with an error-prone polymerase. This suggests Ebola is highly adapted in its reservoir host. One intriguing hypothesis is that additional host restriction factors limit pathogenicity in a natural reservoir species. These factors would play a role in the balance between immunity and sustained viral replication, allowing the virus to propagate and persist over time in the reservoir species. Outside this reservoir, sustained large outbreaks such as the one in West Africa may lead to ebolavirus adaptations being observed longitudinally, resulting in altered pathogenicity as the virus adapts to humans. Overall, the multifaceted approach of Ebola to selectively regulate immune responses and its variable pathogenicity in different host species makes this virus both scientifically interesting and a challenging foe.

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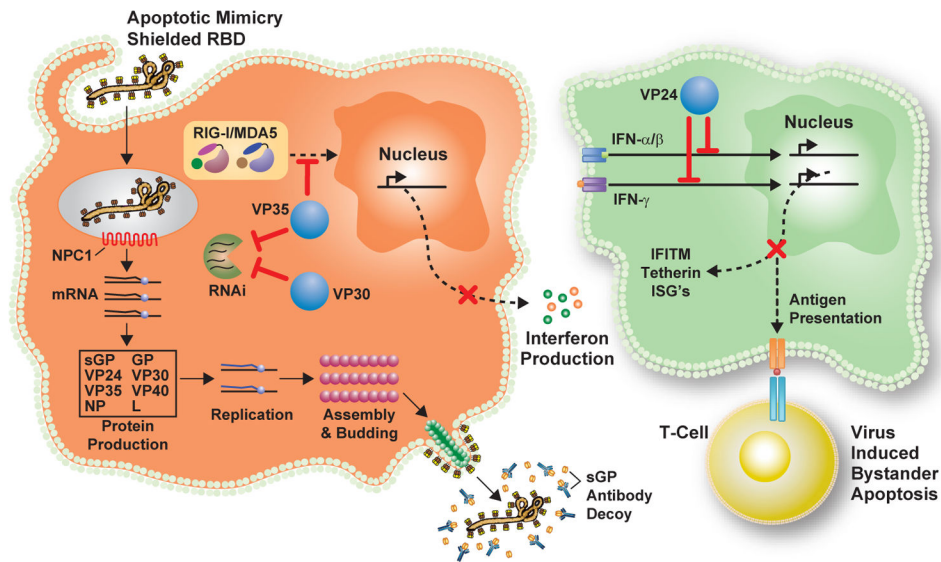


Figure 1.

Ebolavirus life cycle: (Left) Virus particles attach to cell surface, inducing macropinocytosis and virus uptake, possibly using apoptotic mimicry to suppress inflammatory responses. The particle is brought to a low pH compartment where it is cleaved by cysteine proteases to reveal its NPC1 receptor binding domain (RBD). Following fusion and uncoating, the viral genome is transcribed into mRNA and viral proteins produced. Eventually, a signal to begin genome replication occurs, followed by particle assembly and budding.

Immune avoidance mechanisms: Expression and secretion of sGP serves as an antibody decoy for antibodies generated against GP. Viral proteins VP35, VP30 and VP24 are expressed and mediate innate immune avoidance in all cell types. (Left) VP35 interferes with RIG-I/MDA-5 signaling and induction of interferon. Additionally, VP35 and VP30 block the RNAi response against viral gene expression. (Right) VP24 acts to inhibit Type I and II interferon (IFN) signaling. This prevents interferon-induced gene expression and in antigen presenting cells blocks enhancement of antigen presentation to T cells.