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Immune barriers of Ebola virus infection

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Abstract

Since its initial emergence in 1976 in northern Democratic Republic of Congo (DRC), Ebola virus (EBOV) has been a global health concern due to its virulence in humans, the mystery surrounding the identity of its host reservoir and the unpredictable nature of Ebola virus disease (EVD) outbreaks. Early after the first clinical descriptions of a disease resembling a 'septic-shock-like syndrome', with coagulation abnormalities and multi-system organ failure, researchers began to evaluate the role of the host immune response in EVD pathophysiology. In this review, we summarize how data gathered during the last 40 years in the laboratory as well as in the field have provided insight into EBOV immunity. From molecular mechanisms involved in EBOV recognition in infected cells, to antigen processing and adaptive immune responses, we discuss current knowledge on the main immune barriers of infection as well as outstanding research questions.

Introduction

Ebola virus (EBOV, species *Zaire ebolavirus*) is a non-segmented negative-sense RNA virus belonging to the *Filoviridae* family within the order *Mononegavirales*. The EBOV genome harbors seven genes that encode at least eight proteins [1]. EBOV has been a mystery for laymen and scientists alike, since its high virulence and origin have been difficult to explain. Initial reports described a case-fatality ratio (CFR) of up to 90% in humans and virtually 100% in experimentally-infected non-human primates (NHP), and laboratories around the world have devoted efforts during the last forty years to defining the molecular mechanisms responsible for EBOV disease (EVD) pathophysiology.

Early studies during and after the Yambuku outbreak reported evidence of necrosis in liver biopsies as well as disseminated intravascular coagulation (DIC) in patients who died from EVD [2,3]. Extensive necrosis in lymphoid and non-lymphoid tissues as well as DIC was

shortly after observed in NHP infected with EBOV via parenteral inoculation [4,5]. As the NHP evolved as the ‘gold-standard’ filovirus infection model, many of the initial features of EBOV immunology were described in this model, including the coincident detection of virus-specific antibodies and viral clearance [6], EBOV-associated neutrophilia and lymphopenia, and the first notion that immune-based mechanisms could be responsible for endothelial dysfunction [7]. Later on, the NHP model also served to identify macrophages and dendritic-like cells as putative primary targets for EBOV infection [8–10]. With the confirmation of EBOV-induced lymphopenia and lymphocyte apoptosis in humans [11] and the identification of viral protein (VP) 35 and VP24 as type I interferon (IFN-I) antagonists (reviewed in [12]), the prevailing model for many years has been that EBOV infection is associated with strong immune suppression. However, the research community recognized that this model did not reconcile well with the overwhelming inflammation associated with fatal ebolavirus infection [13–15]. Here we discuss how our understanding of EBOV-induced immune responses has evolved throughout the years. In particular we will focus on cell-intrinsic, innate and adaptive EBOV immunity and will identify still-unresolved research questions.

Innate barriers to EBOV infection

All animal species, including humans, are equipped with natural barriers to infection, which include cellular sensors, innate cytokines and innate immune cells among others. Here we briefly summarize innate immune barriers to EBOV infection in humans and animal models of infection.

Cellular checkpoints — attachment factors and EBOV receptor

Permissiveness to EBOV infection is rather rare in the animal kingdom. Only a few species are susceptible to infection, unfortunately this includes humans. Why humans are so vulnerable to EVD in contrast to, for example, rodents is not well understood. However, intrinsic barriers that prevent or inhibit EBOV infection at different steps of the viral replication cycle have been identified. Attachment of EBOV particles to the cell surface is mediated by binding of the highly glycosylated EBOV glycoprotein (GP) to two distinct groups of carbohydrate-binding cellular surface proteins, C-type lectin receptors and glycosaminoglycans (reviewed in [16]). In addition to carbohydrate binding proteins, EBOV particles interact with surface proteins that directly or indirectly bind to phosphatidylserine, including TIM and TAM receptors. Thus, attachment to the target cell is a promiscuous process, that is only partially blocked by knocking down individual attachment factors [16]. After internalization by macropinocytosis [17], EBOV engages its receptor in the endosome, the cholesterol transporter Niemann-Pick C1 (NPC1) protein [18,19]. In contrast to the various surface attachment factors that are involved in EBOV entry, NPC1 is indispensable for EBOV infection. Thus, cells derived from patients with homozygous mutations in NPC1 and NPC1 knockout mice are resistant to EBOV infection [18,20]. Interestingly, sequence polymorphism of the *npc1* locus in different animal species affects NPC1-GP interaction, thus preventing EBOV entry in a species-specific manner as shown for the NPC1 proteins of Russell’s vipers, King cobras and African straw-colored fruit bats [21,22].

Role of antigen presenting cells — immunity versus virus dissemination

EBOV has a broad cell tropism including endothelial and epithelial cells, hepatocytes, fibroblasts and some types of antigen-presenting cells (APCs) such as macrophages and dendritic cells (DCs) (reviewed in [23]). The preference of EBOV to infect APCs and, in particular, DCs has important implications for the host immune response. DCs provide a bridge between innate and adaptive immunity as they are necessary to process pathogen material into defined epitopes and present them to cognate naïve T cells [24]. Considering that most of the human population is immunologically naïve to EBOV [25–28], proper APC function during EVD is essential to initiate virus-specific responses.

In an early study that evaluated the susceptibility of NHP to aerosolized EBOV, alveolar macrophages, an important subset of respiratory APCs, were shown to contain EBOV antigen [8]. Later on, using a combination of immunohistochemistry and electron microscopy techniques, another type of APCs, dendritic cells (DCs) (or rather, ‘DC-like’ cells), were shown to contain EBOV nucleocapsids, which strongly suggested that these cells supported EBOV replication *in vivo* [9,10]. Preferential infection of macrophages was also observed in other filovirus infections, including a patient fatally infected with Marburg virus (MARV) [29], a chimpanzee fatally infected with Tai Forest virus (TAFV) [30] and NHPs infected with Reston virus (RESTV) [31] (Figure 1).

One major limitation in interpreting the results of these initial studies is that it is difficult to morphologically distinguish macrophages from DCs, in particular inflammatory DCs derived from monocytes, without the aid of flow cytometry-based immunophenotyping. Moreover, due to the phagocytic capacity of APCs, it is also challenging to discriminate between infection and antigen-capturing (i.e. engulfment of infected-cell debris). One strategy to discriminate between infection and antigen capturing is co-labeling with antibodies against internal proteins and surface glycoproteins (as readout of active virus shedding) [32]. In NHP and guinea pigs infected with EBOV, VP40, the viral matrix protein, was readily stained in tissue macrophages while only some subpopulations showed evidence of surface GP [33]. Following this strategy, it was recently observed that intranasal inoculation of EBOV allowed detection of surface GP in murine alveolar macrophages and DCs but not in immature monocytes and neutrophils. Further characterization of the infected DC subsets showed that DC-SIGN-expressing inflammatory and resident DCs were infected with EBOV in the lung as well as lung-draining lymph nodes. Conversely, DC subsets expressing langerin did not stain for GP suggesting that these cells may be spared from infection [34•]. These results raise two important questions: Do infected DC-SIGN⁺ DCs participate in EBOV dissemination? And, since langerin⁺ DCs are highly specialized in the activation of CD8 T cells, what role do these cells play in bridging innate and adaptive immune responses to EBOV? [35,36] Interestingly, langerin⁺ DCs might be resistant to fusion of enveloped viruses including HIV and influenza virus [37,38]. These findings suggest a division of labor by which, while DC-SIGN⁺ DCs would be directly infected with EBOV, langerin⁺ DCs would specialize in capturing debris from infected cells to recirculate antigens for presentation via MHC class I; the so-called cross-presentation pathway. Whether this is the case during EBOV infection is an important research question.

Macrophages might also play an important role in the induction of the strong inflammatory response observed in EBOV infection. Although innate responses are suppressed in many cell types when infected with EBOV (see below), macrophages are remarkable exceptions. In these cells, EBOV infection elicits a proinflammatory response including strong upregulation of IFN-I signaling pathways [39,40,41•,42,43]. Activation of monocyte-derived macrophages occurs at a very early stage of infection and is mediated by the viral surface protein GP through toll-like receptor 4 (TLR4) [44–46]. Activation of TLR4, a classic sensor for bacterial infections, is common to several viral proteins such as Dengue NS1, HBsAg and RSV F [47–49], but its consequences on antiviral immune responses are not well understood. GP-mediated activation of TLR4 could skew downstream T-cell responses away from the typical Th1 response seen during most viral infections [50•,51••] toward a Th2 phenotype [52], which could influence patient outcome; alternatively the GP-TLR4 interaction might also play a role in the induction of T-cell death during EBOV infection [53]. Of note, T cells are not infected by EBOV [54], but EBOV particles seem to be able to bind to T cells and trigger the activation of an inflammatory response, including TLR4 and Tim-1 signaling [53,55].

Approaches to suppress the inflammatory response in EBOV infection show promising results [56]. This includes the use of TLR4 antagonists which have been shown to dampen the proinflammatory response during EBOV infection in primary human macrophages and in infected mice, opening up novel supportive treatment options for EVD [57,58].

Interferon-replication barriers and viral counter attack

The interferon system (IFN) is a major innate barrier for virus infection as well as a chief driver of APC function [59,60]. As many other viruses, EBOV is sensitive to interferon (IFN). Pre-treatment of cells *in vitro* with IFN-I or type II IFN (IFN-II) significantly reduces EBOV replication [61–63]. Although IFN-I treatment of EBOV-infected nonhuman primates did not protect the animals from lethal disease [64] it led to prolonged survival in one study [65]. Protection was achieved when IFN was administered in combination with monoclonal antibodies [66]. Mice treated with IFN γ survived an otherwise lethal EBOV infection, supporting the *in vitro* data [62•]. Among the identified antiviral interferon-stimulated genes (ISGs) that block EBOV infection are IFN-induced transmembrane (IFITM) proteins and Zinc finger antiviral protein (ZAP) (for review see [67]).

Not surprisingly, EBOV has evolved strategies to counteract the IFN response. Out of seven structural proteins, at least three, GP, VP35 and VP24, are involved in dampening the innate immune response with VP35 and VP24 being the central players [67]. VP35, a cofactor of the viral polymerase, blocks RIG I-mediated antiviral responses including IFN-I induction, while VP24, a protein involved in nucleocapsid formation, interferes with IFN-I and IFN-II signaling (reviewed in [12]). Work with recombinant EBOV expressing mutant VP35 and/or VP24 shed some light on the role of these proteins as virulence factors. *In vitro*, both proteins interfere with the maturation of infected monocyte-derived dendritic cells (DC), potentially suppressing T cell responses [68,69••]. Infection with recombinant EBOV containing mutations in VP35's IFN-inhibitory domain not only led to increased activation of antiviral pathways and reduced viral replication in cell culture but also to complete

attenuation in a mouse model of EBOV infection [70–73]. This shows that VP35-mediated dampening of the innate immune response is critical for pathogenesis. Interestingly, adaptation of EBOV to guinea pigs or mice results in adaptive mutations in VP24, while VP35 is not affected [74,75]. However, since wild-type and rodent-adapted VP24 proteins block IFN signaling in both human and rodent cells equally well [76,77], it is questionable if the ability to block innate immune responses is a determinate of EBOV permissiveness.

Given the inhibitory effects of IFNs on EBOV replication and the requirement of the virus to block IFN responses for optimal replication, it came as a surprise that IFN signaling pathways were more strongly upregulated in patients, who succumbed to EBOV disease, than in those who survived the infection [78•,79,80]. This might be partially explained by the activation of immune-relevant cells through TLR4 or other physiological events that cannot be reproduced by *in vitro* studies. Alternatively, since patients generally arrive for care well into the course of their illness, the elevated IFN seen in fatal cases may be a direct reflection of uncontrolled viral loads stimulating an exaggerated IFN response.

Adaptive immunity against Ebola virus

Acute infection — mechanisms of virus clearance

Early work on EVD in humans demonstrated decreased numbers of total and HLA-DR+ CD8 T cells as well as increased levels of Fas, Fas ligand and associated lymphocyte apoptosis in patients with fatal outcomes [11,81,82]. These data, along with the finding of persistently elevated viral loads in patients who succumb to disease [83], suggested a role for T cells in control of viral replication and clearance. In fact, despite T-cell lymphopenia, CD8 T cells proliferated, expressed activation markers and adoptive transfer of CD8 T cells from moribund mice could protect 100% of naive mice from EBOV challenge [84]. Studies also indicated that lymphocyte apoptosis in itself was likely not a determinate of outcome since Bcl-2 KO mice still died despite a significant decrease in lymphocyte apoptosis [85]. Finally, T-cell depletion in infected mice and NHP resulted in loss of protection and increased virus replication and dissemination [10,34•,86].

There is substantial T-cell activation during acute infection in humans, however, the frequency of CD8 T cells expressing inhibitory markers are elevated in patients who succumb to disease, suggesting that T cells play a functional role in EVD outcome [87••]. Nevertheless, while a high magnitude of T cell activation has been demonstrated in acute EVD, the specificity of this response namely, virus-specific versus bystander T-cell activation, has yet to be reported.

Individuals who succumb to disease often do so with either very low or no EBOV-specific antibodies [11], and viral loads are often already declining before detection of EBOV-specific IgG [83,88], suggesting that during natural infection, the humoral response may play a minimal role in recovery. While EBOV-specific IgM is present in most individuals by 10 days post symptom onset, it is not until almost three weeks after symptom onset that most patients are positive for EBOV-specific IgG [89]. There is limited data on the development of neutralizing antibodies during acute infection and survivors do not have significant neutralizing titers until well into convalescence [90]. Finally, in mouse models of EVD, B

cells are completely dispensable for survival from infection [86]. Despite the lack of evidence for a role of the native virus-specific IgG response in survival during natural infection, there is a large amount of data suggesting that specific combinations of passively transferred antibodies, even when provided during acute infection, can improve clinical outcomes [91].

Convalescence and beyond — waning immunity and hidden antigens

Following recovery from acute EVD, both virus-specific T-cell responses as well as virus-specific humoral responses can last at least up to 10 years [92]. However, the magnitude of these responses wanes over time with only 29% having neutralizing antibodies at the 10-year time point [93]. The T-cell responses of this same patient population demonstrated long lived EBOV-specific effector CD4 T cells, but only in those survivors that also had EBOV neutralizing activity [94]. The authors were not able to detect significant CD8 T cell responses in these long-term survivors. Whether this is a technical challenge or an indication of a lack of persistence of CD8 T cell memory remains to be seen. In repatriated EVD survivors, virus-specific CD8 T cells are still detectable, albeit at low frequencies at least out to 18 months post-discharge (Rama Akondy, personal communication).

Significant clinical sequelae following EVD have been noted since the first outbreaks [89,95–97]. The prolonged sequelae of disease could be secondary to immune phenomena rather than virally mediated [98], but virus persistence has been observed, specifically in sites that are classically thought of as immune privileged, including the brain, eye and testicle (semen) [78,99,100]. It seems reasonable to conclude that the virus is able to persist in these locations and evade immune surveillance because these sites are, by definition, able to tolerate foreign antigens [101]. However, it is clear that there can be a break in this tolerance of foreign viral antigen since inflammation was noted in both the eye and the brain in two patients with clinical recurrence of EVD in these locations [99,100]. Furthermore in the NHP model, investigators were able to demonstrate that local mononuclear phagocytic cells in these locations are the sites of virus persistence [102]. When and how virus reactivation and immune recognition occurs in these sites remains a mystery.

Active or passive vaccination against EBOV — the need for correlates of immunity

Immunity that contributes to survival during natural infection may not be the same as immunity that can protect following active or passive vaccination. In passive vaccination, only the humoral component is at work and this can be protective when given pre-exposure and can rescue from death when given well into the disease [91]. These antibodies appear to work not only by direct neutralization but also by engaging effector functions such as ADCC [103].

A wealth of literature exists on EBOV vaccines in animal models [104], so we will focus on correlates of immunity in vaccines that have been trialed in humans and NHP's. Using a rAd5 EBOVGP-based vaccine, it was clearly demonstrated that protection from challenge was mediated by CD8 T cells in NHPs [105]. In contrast, using an rVSV EBOVGP-based vaccine, investigators suggested that antibodies were the correlate of protection since CD4

depletion prior to vaccination but not at time of challenge led to loss of vaccine-mediated protection [106]. Therefore the mechanistic correlate of protection can be different depending on the vaccine platform chosen to deliver the antigen.

Multiple reports of human clinical trials focus on two promising vaccine platforms, rVSV or rAdenovirus (either rAd5 or rChAd3) to deliver the viral GP, and all show induction of GP-specific humoral immunity using a variety of different assays [107–118]. The only human EBOV vaccine study to date that evaluated efficacy used the rVSV vectored GP [119]. Unfortunately, while some of the rAdenovirus vectored vaccine studies examined T cell responses, there are very little data about T cell responses in any of the rVSV studies, and all studies utilized different types of assays for quantitation of immune responses, making comparisons inappropriate. A recently published study evaluated both ChAd3-EBO-Z and rVSVG-ZEOBV-GP side-by-side as compared to placebo, and all vaccinated individuals developed robust GP-specific antibody responses [117]. However, despite all of these trials in humans, no immunologic correlate of protection (either mechanistic or non-mechanistic) has been defined; a clear gap that needs to be addressed.

Summary and perspective

Many studies during the last forty years have provided insight into the filovirus host-range, the mechanisms of infection and virus antagonism of the host response, and the role of both arms of the adaptive immune response in virus clearance. There are however many outstanding open questions. For example, it will be important in the near future to reconcile the *in vitro* findings regarding EBOV-mediated type I IFN antagonism with the observation that fatal EVD is associated with higher production of IFN. To address this question, it will be necessary to establish *in vivo* platforms that permit a dissection of the interaction between EBOV proteins and the IFN system early during infection. Along the same lines, it is of great interest to determine the mechanisms by which EBOV disseminates from the initial points of infection (presumably skin and mucosae) to the body. Studies in animal models will be helpful to determine the molecular mechanisms responsible for the observed dysregulation of T-cell homeostasis in humans as well as to compare the relative contribution of antibody neutralization and Fc-mediated functions to virus clearance. One important challenge ahead is to convert this knowledge into antiviral strategies and highly needed post-exposure medical countermeasures.

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Current Opinion in Virology

Figure 1.

Barriers to EBOV infection. **(1)** Neutralizing Abs and Ab-mediated functions; **(2)** Attachment factors such as C-type lectin receptors allow EBOV binding to the membrane of target cells. **(3)** EBOV enters through macropinocytosis and binds its cellular receptor, the cholesterol transporter Niemann-Pick C1 (NPC1) in the endosome; **(4)** In infected cells, viral proteins VP35 and VP24 play an important role targeting the type I IFN response both at the production and signaling levels; **(5)** EBOV infects discrete DC subsets (inflammatory DCs derived from monocytes) which also capture cell debris from other infected cells; **(6)** DCs provide a bridge between innate and adaptive immunity by priming of EBOV-specific CD8 T cells; **(7)** CD4 T cell mediated help is necessary for activation of the humoral immune response and anti-EBOV antibody production.