

Ebola Virus Disease in Humans: Pathophysiology and Immunity

César Muñoz-Fontela and Anita K. McElroy

Abstract Viruses of the *Ebolavirus* genus cause sporadic epidemics of severe and systemic febrile disease that are fueled by human-to-human transmission. Despite the notoriety of ebolaviruses, particularly Ebola virus (EBOV), as prominent viral hemorrhagic fever agents, and the international concern regarding Ebola virus disease (EVD) outbreaks, very little is known about the pathophysiology of EVD in humans and, in particular, about the human immune correlates of survival and immune memory. This lack of basic knowledge about physiological characteristics of EVD is probably attributable to the dearth of clinical and laboratory data gathered from past outbreaks. The unprecedented magnitude of the EVD epidemic that occurred in West Africa from 2013 to 2016 has allowed, for the first time, evaluation of clinical, epidemiological, and immunological parameters in a significant number of patients using state-of-the-art laboratory equipment. This review will summarize the data from the literature regarding human pathophysiological and immunologic responses to filoviral infection.

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C. Muñoz-Fontela (✉)

Laboratory of Emerging Viruses, Heinrich Pette Institute, Leibniz Institute for Experimental Virology, Martinstraße 52, 20251 Hamburg, Germany
e-mail: cesar.munoz-fontela@hpi.uni-hamburg.de

A.K. McElroy (✉)

Infectious Diseases, Department of Pediatrics, Emory University School of Medicine, 2015 Uppergate Drive NE, Atlanta, GA 30322, USA
e-mail: akmcclr@emory.edu

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1 Introduction

Ebola virus (EBOV) is the prototypic member of the *Ebolavirus* genus in the *Filoviridae* family of negative-sense, single-stranded RNA viruses. Discovered in 1976 during the first documented outbreak of Ebola virus disease (EVD) in the town of Yambuku in northern Zaire (today Democratic Republic of the Congo), EBOV has since caused sporadic human disease outbreaks of varying magnitude in Equatorial African countries (Sanchez et al. 2007a). In March 2014, an EBOV variant later named EBOV Makona was first detected in Guinea. This variant was responsible for a 3-year-long epidemic that affected tens of thousands of people in several West African countries, collapsing the healthcare systems of three of them. EBOV Makona rampaged through both rural and urban areas, and underscored previously poorly characterized features of EVD, like sexual transmission and virus persistence after recovery (Bausch et al. 2007; Rowe et al. 1999; Chughtai et al. 2016; Deen et al. 2015; Fischer et al. 2016; Rodriguez et al. 1999; Varkey et al. 2015; Uyeki et al. 2016a).

The scientific and clinical knowledge of human EVD before its appearance in West Africa was very limited. The scarcity of human cases and their occurrence in rural areas of Equatorial Africa limited research, as did confinement of filovirus research to biosafety level 4 containment laboratories. In addition, basic studies on EVD pathophysiology have been hampered by the lack of susceptible small animal models with competent immunity. For example, laboratory mice, a commonly used disease model, are completely resistant to nonadapted EBOV.

Before 2014, EVD was described as an acute hemorrhagic fever, thus earning its former name Ebola hemorrhagic fever (EHF); case fatality rates of up to 90% had been reported. The disease was characterized by lymphopenia, disseminated intravascular coagulation (DIC), immunosuppression, and a systemic inflammatory response resembling septic shock (Feldmann and Geisbert 2011). While many of these observations have been strengthened by findings from the West African EVD outbreak, some of the previous hypotheses have been revised. Perhaps one of the most surprising findings has been the low overall number of human cases presenting with bleeding (Schieffelin et al. 2014), as well as the lack of correlation between bleeding and disease severity (Schieffelin et al. 2014; McElroy et al. 2014a, b). These findings triggered the change in disease nomenclature from Ebola hemorrhagic fever to Ebola virus disease. Moreover, the finding that EVD correlates with robust immune activation rather than immunosuppression (Ruibal et al.

2016; McElroy et al. 2015a), and the ability of the virus to persist in several body fluids long after recovery (Varkey et al. 2015; Uyeki et al. 2016a; Sow et al. 2016; Green et al. 2016; Deen et al. 2015) have changed our current view of EVD and have prompted new directions in research and new public health policies. Here we will aim to integrate these novel findings within the current human EVD model, and will discuss future research directions.

Several ebolaviruses cause EVD, and while differences may exist between the diseases caused by the individual viruses, this review will focus on EVD as a disease caused by all known viruses in the *Ebolavirus* genus (ebolaviruses) that are pathogenic for humans. The reader will note that most of the available data come from infections caused by EBOV rather than the other pathogenic viruses in this genus: Sudan virus (SUDV), Bundibugyo virus (BDBV), and Taï Forest virus (TAFV). The related marburgviruses, Marburg virus (MARV) and Ravn virus (RAVV), will be mentioned where appropriate data are available, but unfortunately, information on Marburg virus disease (MVD), which is caused by both of them, is still lacking.

2 Portals of Ebolavirus Entry

Epidemiological data collected over the last 40 years indicate that human infection with EBOV occurs mainly through close contact with infected body fluids. This probably occurs during both spillover events (e.g., contact with infected blood during butchering of bushmeat) and human-to-human transmission. There is no evidence that direct contact with bats causes EBOV spillover into humans (Mari Saez et al. 2015; Leroy et al. 2009), but infection with MARV and RAVV via direct or indirect contact with Egyptian rousettes (fruit bats of the species *Rousettus aegyptiacus*) (Amman et al. 2012; Schuh et al. 2017), has been documented. Human visits to caves or mines in which these bats roost have been directly associated with the development of MVD (Bausch et al. 2003; Centers for Disease and Prevention 2009; Adjemian et al. 2011), strongly indicating that mucosal or skin contact with bat droppings is sufficient to initiate MARV infection in humans.

With the exception of the first EVD outbreaks in Zaire, which were linked to substantial percutaneous needle transmission (Ebola haemorrhagic fever in Zaire 1976/1978), most of the data since the early 1990s suggest that exposing skin and mucosae to EBOV while conducting activities like body washing during traditional funerals or caring for sick relatives in the household is sufficient for human-to-human transmission of EBOV (Bausch et al. 2007; Dowell et al. 1999; Francesconi et al. 2003). Early data collected from a laboratory exposure to SUDV even suggest that skin abrasions may not be necessary to allow ebolavirus entry through the skin (Emond et al. 1977). These findings raise questions regarding how ebolavirus infection takes place in skin and mucosae, and which cells are involved in the primary amplification of the virus.

Antigen-presenting cells are a putative initial target of EBOV infection and previous research in animal models of disease has indicated that dendritic cells (DCs) and macrophages are early and preferred targets of EBOV and support virus replication (Geisbert et al. 2003a). Both DCs and macrophages can also be productively infected by EBOV *in vitro* (Gupta et al. 2001, 2007; Mahanty et al. 2003; Bosio et al. 2003), and EBOV prevents activation of *in vitro*-derived DCs, mainly through the action of VP24 and VP35 (Yen et al. 2014; Jin et al. 2010; Ilinykh et al. 2015).

To further complicate things, a great deal of research over the last decade has been devoted to defining the ontogeny and specific function of DC subsets in mice and humans. The emerging picture is that several cell subsets exist with overlapping and nonoverlapping functions, and these subsets can be roughly classified into classical, plasmacytoid, and inflammatory DCs in humans (see (Haniffa et al. 2013) for an excellent review). Whether EBOV can equally infect different DC subsets is not known, but some of the existing evidence suggest that it cannot (Leung et al. 2011). For example, a number of cellular receptors have been involved in the attachment of EBOV virions to target cells. These receptors include several C-type lectins present on the surface of DCs, such as dendritic cell-specific ICAM-3-grabbing non-integrin (DC-SIGN) (Simmons et al. 2003) and liver/lymph node SIGN (L-SIGN) (Alvarez et al. 2002). DCs of the epidermis and mucosal epithelium do not express these molecules, but Langerhans cells in the skin and CD141⁺ DCs in mucosal epithelium do express the C-type lectin langerin (Merad et al. 2008). In fact, studies in monkeys and pigs have indicated that DC-SIGN⁺ cells are scarce in the dermis and the lamina propria or submucosa in the steady state (Schwartz et al. 2002; Huang et al. 2009), suggesting that other cell types may be targets for early EBOV replication. Initiating EBOV infection may depend on attachment to target cells via TIM-1 and TIM-4, which are highly expressed in mucosal epithelia (Rhein et al. 2016; Kondratowicz et al. 2011). Initial virus amplification could then lead to inflammation and infiltration of a high number of myeloid cells expressing DC-SIGN and other described EBOV attachment factors, like triggering receptor expressed on myeloid cells 1 (TREM-1) expressed by neutrophils (Mohamadzadeh et al. 2006), and human macrophage C-type lectin specific for galactose/N-acetylgalactosamine (hMGL) expressed by macrophages (Takada et al. 2004). The elucidation of the initial steps by which EBOV establishes productive infection in a host organism is highly needed to understand the mechanisms by which the virus disseminates from the initial site of entry to the body, and perhaps to design medical countermeasures aimed at preventing virus spread.

3 Virus Dissemination and Initiation of EBOV-Specific Immunity

As mentioned above, DCs and macrophages are early targets of EBOV infection. Due to the migratory potential of DCs, these immune cells may participate in disseminating EBOV from the initial points of entry to the draining lymph nodes

(Geisbert et al. 2003a). This strategy is commonly used by other viruses, including SARS coronavirus (Liu et al. 2015), Toscana virus (Cusi et al. 2016), and measles virus (Mesman et al. 2012), for dissemination in the host. However, perhaps due to the lack of suitable *in vivo* models for kinetic studies of EBOV, the involvement of DCs in EBOV dissemination has not been experimentally addressed. In any case, the specific subsets of cells responsible for EBOV dissemination remain to be identified. It is plausible that tissue-resident DCs or inflammatory DCs derived from infiltrating monocytes are important for EBOV dissemination. Both myeloid DC populations are migratory and can transport a variety of antigens from inflamed tissues to the draining lymph nodes (Leon et al. 2007; Ersland et al. 2010). Conversely, macrophages and neutrophils are less likely to participate in EBOV dissemination due to their low mobility and nonproductive infection, respectively (Mohamadzadeh et al. 2006).

Recent studies have demonstrated that human EVD is associated with loss of peripheral blood monocytes, in particular nonclassical CD16⁺ monocytes (Ludtke et al. 2016) which have been proposed as the main antiviral monocyte subset (Cros et al. 2010). Even though this study did not demonstrate direct infection of CD16⁺ by EBOV, it raised the possibility that this cell subset could be involved in virus dissemination. In fact, CD16⁺ monocytes are also called patrolling monocytes due to their ability to attach to endothelial cells in a LFA-1-dependent manner and to extravasate into inflamed tissues where they differentiate into inflammatory DCs and macrophages (Cros et al. 2010; Auffray et al. 2007). This hypothesis is also substantiated by a previous study that demonstrated that EBOV particles can attach to monocytes and enter these cells only when the monocyte differentiation program has started, that is, during their differentiation into DCs and macrophages in inflamed tissues (Martinez et al. 2013).

The identification of the DC subsets specifically involved in filovirus dissemination is a highly relevant topic of study, because the function of DCs can be enhanced or inhibited, *ex vivo* or *in vivo*, by antigen delivery or use of molecules and antibodies. Therefore, DCs are putative immunotherapeutic targets for post-exposure EVD treatment (Klechevsky and Banchereau 2013). For example, the ligand of the DC co-stimulatory molecule CD40 (sCD40L) is commonly used to enhance DC-mediated antigen presentation (Kornbluth and Bot 2012), and previous studies have demonstrated a correlation between circulating levels of sCD40L and survival after SUDV infection (McElroy et al. 2014a, b). In addition, poor activation profiles of circulating antigen-presenting cells have been correlated with severe EVD (Ludtke et al. 2016). These findings provide a rationale for the use of DC enhancers as immunotherapy candidates in filovirus disease.

Another key driver of EBOV dissemination may be the cytokine microenvironment, since these innate immune signaling molecules play an important role in recruiting myeloid cells, which are putative EBOV targets, to sites of inflammation. A considerable body of research exists on cytokine and chemokine responses during EVD. Despite some conflicts, in general, these data correlate fatal outcomes

during EVD with high concentrations of pro-inflammatory cytokines (e.g., IL-6), pro-inflammatory chemokines (e.g., IP-10), and anti-inflammatory cytokines (IL-1RA and IL-10), overall suggesting a general dysregulation in the expression of these key immune signaling molecules (Hutchinson and Rollin 2007; Gupta et al. 2012; Wauquier et al. 2010; Baize et al. 2002; Villinger et al. 1999). An inability to control viral replication is likely leading to continued innate immune stimulation. Data from asymptomatic human cases have shown an even greater magnitude of cytokine and chemokine upregulation, followed by rapid downregulation of this response in association with control of viral replication (Leroy et al. 2000, 2001), suggesting that cytokine/chemokine dysregulation is a consequence of uncontrolled viral replication rather than a primary mediator of pathogenesis.

Type I interferons (IFN-I) are key antiviral cytokines, and perhaps one of the more conflicting aspects of comparing EBOV experimental and clinical human data is the role of IFN-I in EBOV immunity and pathogenesis. Importantly, IFN-I not only induces an antiviral state in infected and bystander cells during early virus infection, but also is a key modulator in the transition between innate and adaptive immunity. By enhancing natural killer cell function, antigen presentation by DCs, and expansion of effector T cells, IFN-I bridges natural and acquired antiviral immunity (see (McNab et al. 2015) for a review), so elucidating its functions during human EVD is highly relevant to understanding disease pathogenesis. IFN is critical in protecting laboratory mice from EBOV (Brannan et al. 2015; Bray et al. 2002), but data are somewhat conflicting in nonhuman primates (NHPs) and humans (McElroy et al. 2016; Smith et al. 2013; Villinger et al. 1999; Yen et al. 2011). Higher levels of IFN-alpha were associated with fatal EVD cases (Villinger et al. 1999), but higher IFN-beta was associated with less severe EVD (McElroy et al. 2016) and IFN-beta administration prolonged survival in nonhuman primates (Smith et al. 2013). However, as IFN responses are highly dynamic, drawing conclusions regarding human pathogenesis is difficult. For example, EVD survivors may mount early and robust IFN responses that keep viral replication at bay, while patients who succumb to EVD may display higher IFN levels later on due to increased viral replication and inflammation.

Interestingly, recent studies have shown that patients who survive and patients who succumb to EVD both show robust T cell activation (McElroy et al. 2015a; Ruibal et al. 2016). Since DCs are the only antigen-presenting cells capable of priming naïve T cells (Banchereau and Steinman 1998), these results suggest at least two possibilities. On one hand, infected DCs may retain their capacity to initiate T cell-specific responses, as has been shown in other viral infections (Wahid et al. 2005; Rivera and McGuire 2005; Kvale et al. 2006). On the other hand, some DC subsets may be spared from infection and thus able to prime EBOV-specific T cells. The generation of this EBOV-specific adaptive immunity is the topic of the next two sections.

4 Adaptive Immunity: Human Antibody Responses

While innate immune responses may play a chief role in controlling early EBOV replication in humans, the current model identifies the character (though not necessarily the magnitude) of adaptive immunity as the main factor driving viral clearance and recovery. Both humoral and cellular immunity seem to be required for EBOV clearance in humans, a hypothesis strengthened by the finding that EVD patients mount robust adaptive immune responses (McElroy et al. 2015a; Ruibal et al. 2016) with high numbers of circulating plasmablasts and EBOV-specific T cells.

The more difficult task is assessing whether adaptive immune responses mark substantial differences between fatal and surviving patients. Initial studies supported the idea that early development of IgM and isotype switching to IgG correlated with positive outcome. Indeed, a high percentage of patients with fatal outcomes do not seem to develop IgM (Ksiazek et al. 1999). These field studies are also in agreement with findings in patients evacuated into Europe or the US for medical treatment during the recent West African EVD outbreak. Surviving EVD patients mounted early IgM responses and showed upregulation of serum IgG over the course of the disease, which was correlated with viral clearance (Kreuels et al. 2014; Wolf et al. 2015). Conversely, deficient or diminished IgM and IgG responses have been reported in fatal cases of both EVD and MVD (van Paassen et al. 2012; Baize et al. 1999).

However, limited field data also indicate survivors who did not develop IgG, as well as patients who died after developing detectable circulating anti-EBOV antibodies (Onyango et al. 2007). In addition, limited clinical data obtained from the SUDV-caused EVD outbreak in Gulu, Uganda, did not reveal significant differences between the humoral responses in fatal and nonfatal EVD, with very late expression of IgG in both groups that was unrelated with viral clearance (Towner et al. 2004). As in many other aspects of EVD immunology, the kinetics of antibody responses in a statistically relevant cohort of acute-stage patients with defined outcomes must be studied.

Antibodies play many roles during the immune response to pathogens, including neutralization and antibody-dependent killing of virus-infected cells by targeting them to Fc receptor bearing cells (ADCC) or complement (CMC). Neutralizing antibodies (N_{AB}) probably play a small role in recovery from acute EVD, since in many survivors N_{AB} are not detectable until weeks or even months after recovery (Luczkowiak et al. 2016; Sobarzo et al. 2012). This is a puzzling and as yet unexplained finding. One of the plausible hypotheses is that disrupting lymphoid architecture during acute EVD infection may compromise germinal center formation and B cell affinity maturation, a feature that has been observed during Lassa fever (Carrion et al. 2007). However, this hypothesis does not reconcile easily with the levels of circulating plasmablasts in patients in the acute stage of illness (McElroy et al. 2015a), or with the limited focal necrosis observed in human biopsy samples of lymphoid tissues (Martines et al. 2015). One interesting possibility is

that the long filament shape of filovirus virions may require a highly diverse repertoire of N_{AB} for effective neutralization. In fact, studies have found greater B cell clonality in EVD survivors than in individuals with B cell memory against HIV-1 or influenza A virus (Bornholdt et al. 2016b). Still, this cannot be the whole story, as similar delays in N_{AB} production have been described in other viral hemorrhagic fevers (e.g., Lassa fever). Perhaps more importantly, delayed N_{AB} production strongly suggests long-term virus or antigen persistence, which is in agreement with duration of post-EVD sequelae (see below).

Nevertheless, long-term survivors develop effective N_{AB} , mainly directed against several epitopes of the ebolaviral $GP_{1,2}$ (Bornholdt et al. 2016a, b; Misasi et al. 2016; Corti et al. 2016). Many of the described N_{AB} isolated from survivors are directed against the $GP_{1,2}$ glycan cap as well as against the region bridging GP1 and GP2, which seem to be epitopes amenable for antibody-based therapeutics like ZMapp (Murin et al. 2014). Importantly, a number of studies in surviving patients have highlighted the presence of naturally occurring N_{AB} with cross-reactivity against other ebolaviruses and even against MARV (Olal et al. 2012; Misasi et al. 2016; Bornholdt et al. 2016a). These findings strongly suggest that antibody-mediated immune memory may provide long-term protection against secondary infection with filoviruses, and may have important implications for public health measures (e.g., recruiting survivors as caregivers in future outbreaks).

Currently, the relative importance of N_{AB} versus other antibody-mediated mechanisms, such as ADCC and CMC, is unclear, even though most protective antibodies probably act through both neutralization and ADCC/CMC activation (Schmaljohn and Lewis 2016). Antibodies with both neutralizing and ADCC capacity have been detected in EVD survivors more than a decade after recovery (Corti et al. 2016), and ADCC is probably an important mechanistic feature of ZMapp (Olinger et al. 2012). In addition to dissecting whether or not neutralization and ADCC specifically contribute to EVD immunity, it is important to determine the kinetics of antibody-mediated immunity from acute infection to long-term recovery. Lack of information on EVD antibody kinetics and EBOV-specific quantitative activity is probably largely responsible for the lack of protective effect demonstrated by convalescent plasma therapy (van Griensven et al. 2016). This therapeutic strategy has putative applicability for field outbreak conditions, but requires characterization of the virus specific activity in the product as well as optimization to ensure transfer of sufficient quantities of protective antibodies.

5 Adaptive Immunity: Human T Cell Responses

T cells, particularly CD8 T cells, are essential for clearance of acute viral infections. Naïve T cells react to stimulation with pathogen-specific peptides by massively expanding, differentiating into effector cells and migrating to peripheral infection sites for elimination of infected cells (see (Zhang and Bevan 2011) for a review). Because naïve CD8 T cells can be activated only by DCs (Banchereau and

Steinman 1998), the initial assumption was that EBOV-induced DC inactivation would in turn result in poor T cell priming and overall inability of the host to eliminate infection. This hypothesis was substantiated by early studies demonstrating that, despite being spared from infection, many T cells underwent apoptosis during human EVD (Baize et al. 1999; Wauquier et al. 2010). While this observation is still valid, data gathered mainly during the recent West African outbreak have suggested that lymphocytes in general display very dynamic kinetics during EVD, which may include early proliferation followed by lymphopenia (Kreuels et al. 2014; Wolf et al. 2015). Similar dynamics occur during other systemic viral infections and during pro-inflammatory disorders such as sepsis (Luan et al. 2015), suggesting that perhaps lymphopenia is not a differential characteristic of EVD.

A substantial difference between earlier studies and those carried out in the context of the recent outbreak has been the application of multiparametric flow cytometry, which has allowed for the first time collection of phenotypic and functional information from single cells in unprecedented detail. These studies have revealed that, in fact, EVD is characterized by massive T cell activation rather than inhibition in both surviving and fatal cases. Co-expression of activation markers such as CD38 and HLA-DR, as well as proliferation markers like Ki-67, were detected in a significant percentage of CD4 and CD8 T cells in EVD patients (McElroy et al. 2015a; Ruibal et al. 2016) and were comparable with the magnitude of activation observed in other acute infections or after vaccination (Lindgren et al. 2011; Miller et al. 2008). Of note, since co-expression of CD38 and HLA-DR is correlated with engagement of the T cell receptor (Appay et al. 2002), these findings strongly suggest that proper T cell priming by antigen-presenting cells occurs during EVD in humans. Additionally, these findings were comparable between patients receiving experimental therapy (McElroy et al. 2015a) and those who received supportive care in the field (Ruibal et al. 2016), indicating that robust T cell activation is a characteristic of EVD unrelated to treatment.

A paramount question, therefore, is why robust T cell activation does not lead to viral clearance during EVD. To some extent, this lack of T cell effectiveness may be related to defects in negative immune checkpoints, namely the molecular mechanisms that control the transition from activation to immune homeostasis and that are essential for autoimmune control (Buchbinder and Desai 2016). Two such mechanisms are triggered by the T cell co-inhibitor molecules, programmed cell death-1 (PD-1) and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4). An earlier review already hypothesized that T cell dysfunction during filovirus infection could be related to high expression of PD-1 and CTLA-4 in T cells (Mohamadzadeh et al. 2007), which leads to a nonfunctional but reversible status termed T cell exhaustion (Wherry 2011). Studies from the recent West African EVD outbreak found that peripheral blood T cells from EVD patients expressed high levels of PD-1 and CTLA-4 (McElroy et al. 2015a; Ruibal et al. 2016), which were significantly higher in fatal cases (Ruibal et al. 2016). As a follow-up to these observational studies, determining the correlation between high expression of T cell inhibitory molecules and T cell function and apoptosis will be important. Determining this correlation will most likely require relevant in vivo models that can reproduce this T cell

phenotype. Utilizing immunotherapeutic approaches to block PD-1 and CTLA-4 function during postexposure filovirus infection treatment may provide an interesting opportunity. Several therapeutic products are licensed extensively to block PD-1 and CTLA-4 in several types of cancer, thereby restoring T cell function (see (Sharma and Allison 2015) for a review).

Another important and related question is whether broad and polyfunctional T cell responses lead to increased disease manifestations, like in hantavirus (cardio) pulmonary syndrome (Terajima and Ennis 2011), or to decreased susceptibility, as in dengue virus 1–4 infections (Weiskopf et al. 2013). To answer this question, an exhaustive analysis of EBOV T cell immunodominance in humans must be performed, which is still not available. Previous evidence shows that the viral nucleoprotein (NP) drives most of the CD8 T cell response (McElroy et al. 2015a; Sundar et al. 2007; Wilson and Hart 2001). This finding is consistent with the observation that HLA alleles recognizing conserved filovirus NP epitopes provide protection against SUDV infection (Sanchez et al. 2007b). Additional studies of HLA association with EVD outcomes in a statistically significant cohort of patients are highly needed to strengthen these initial observations. The finding that NP drives most of the CD8 T cell response also has significant implications for vaccine design and may explain, at least to some extent, why most GP_{1,2}-based vaccines induce poor T cell immunity (Agnandji et al. 2016; Ewer et al. 2016; Zhu et al. 2015).

6 Electrolyte Imbalances

The degree to which electrolyte abnormalities contribute to EVD pathogenesis was not appreciated in earlier outbreak responses because real-time serum electrolyte data were not available. The ability to acquire these measurements in patients during the West African EVD outbreak, as well as the degree of profuse watery diarrhea that was reported, have brought to the forefront the severity of electrolyte imbalances in EVD and the impact electrolytes could have on patient outcome. Such data were first collected during the SUDV outbreak in Gulu, Uganda, in 2000–2001, during which elevated BUN/Cr levels and hypocalcemia were associated with severe disease and fatal outcomes (Rollin et al. 2007). Data from African cohorts (Hunt et al. 2015) combined with data from repatriated patients who were cared for in developing nations (Uyeki et al. 2016b) have revealed potassium abnormalities, hyponatremia, hypomagnesemia, and hypocalcemia. Some of these alterations may be related to acute renal injury that is also common among severely ill patients; others might be related to volume and electrolyte imbalance secondary to profuse watery diarrhea. The clinical consequences of electrolyte imbalances could include cardiac arrhythmias, seizures, or coma. Indeed, 41% of the repatriated patients exhibited arrhythmia or electrocardiographic changes, one patient had seizures, and three were in a coma (Uyeki et al. 2016b). Electrolyte levels are easily measured blood chemistry parameters that can be

corrected with electrolyte and fluid administration. Such measures may have contributed to improved outcomes during the recent outbreak, as one EVD treatment center that incorporated these data into patient management had a case fatality rate of only 37%, significantly lower than the 50 and 74% rates reported from two other treatment centers in Sierra Leone (Hunt et al. 2015; Schieffelin et al. 2014; Lanini et al. 2015).

7 Endothelial Dysfunction

Multiple lines of evidence have suggested that the endothelium is dysfunctional during EVD. While endothelial cells are directly infected, they do not show significant cytopathic effect, and endothelial infection is thought to occur during the terminal phase of the illness (Martines et al. 2015; Geisbert et al. 2003c). The overall dysfunction of the endothelium is thought to be an indirect effect of pro-inflammatory cytokines like TNF-alpha (Villinger et al. 1999; Feldmann et al. 1996), or other molecules, like nitric oxide, that increase the permeability of the endothelium during inflammation (Sanchez et al. 2004). Increased levels of several pro-inflammatory cytokines and chemokines are associated with EVD-related deaths (Baize et al. 2002; Wauquier et al. 2010; Gupta et al. 2012; Hutchinson and Rollin 2007; McElroy et al. 2014a, b). Infected antigen-presenting cells, such as macrophages, DCs, or monocytes are the presumed source of these cytokines (Feldmann et al. 1996; Gupta et al. 2001), and these cytokines lead to endothelial activation.

Increased vascular permeability due to loosening of the endothelial barrier is a normal and necessary physiologic function that allows cells and biomolecules to reach sites of inflammation, but widespread activation in many inflammatory diseases results in fluid movement that can be detrimental to the host. Clinical and laboratory findings in EVD, including tachypnea (with or without pulmonary edema), hypotension, oliguria, tachycardia, impaired distal perfusion, hypoalbuminemia, and hemoconcentration, are consistent with fluid extravasation into extravascular spaces secondary to increased vascular permeability (Rollin et al. 2007; Uyeki et al. 2016b; Hunt et al. 2015; Chertow et al. 2014). This constellation of clinical findings is thought ultimately to lead to hypovolemic shock in fatal cases.

In recent years, additional evidence that dysfunctional endothelia contribute to the disease process include the findings of increased levels of sICAM, thrombomodulin, PE-CAM, and P-selectin in patients with severe or fatal disease (McElroy et al. 2014a, b). All of these biomarkers, when released into the plasma, indicate an activated endothelium and/or breakdown of endothelial intercellular junctions. An activated endothelium is both pro-inflammatory and pro-coagulant, and likely contributes both to the ongoing inflammatory response that characterizes severe EVD and to the coagulopathy that has been observed in some patients (discussed in more detail below).

Also noteworthy is the sometimes conflicting body of evidence implicating the viral glycoprotein (GP_{1,2}) in endothelial dysfunction. The EBOV *GP* gene coding region produces two proteins based on a transcriptional editing site, the soluble GP (sGP) and the full-length structural GP_{1,2} (Sanchez et al. 1996). The full-length GP_{1,2} produced by pseudotyped retrovirus or virion-like particles (VLPs) can bind to and activate endothelial cells, leading to increased endothelial permeability (Wahl-Jensen et al. 2005; Yang et al. 1998). sGP has been detected in the plasma of infected individuals (Sanchez et al. 1999), and, in fact, inhibits TNF-mediated increases in vascular permeability *in vitro*, perhaps suggesting a compensatory mechanism to control virus-induced inflammation. A third form of the protein, known as shed GP, is shed from the surface of infected cells *in vitro* and increases permeability of cultured endothelial cells (Escudero-Perez et al. 2014). While shed GP was detected in infected guinea pigs, it has not yet been detected *in vivo* in humans. Finally, overexpression of GP_{1,2} in explanted human, porcine, or NHP blood vessels leads to increased endothelial permeability and endothelial cytotoxicity mediated by the mucin domain of the protein (Yang et al. 2000). The relevance of this finding to EVD is unclear, since endothelial cells are infected long after endothelial function has already been compromised, and do not show cytopathic effects when infected *in vitro* (Geisbert et al. 2003c). Taken together, these data suggest that the various forms of EBOV GP may modulate endothelial function, but the precise role of the protein in human EVD pathogenesis is unclear.

8 Coagulopathy

The moniker “viral hemorrhagic fever” was applied to EBOV EVD during the first outbreak identified in 1976, and was appropriate because 78% of fatal cases had hemorrhagic manifestations, mostly melena (Ebola haemorrhagic fever in Zaire 1976/1978). This 1976 outbreak was unique, because the route of virus transmission was via injection in approximately one-third of the 288 patients, and this mode of entry could have contributed to the manifestations and severity of disease (the authors note that all patients who were infected by injection died). Notably, a concurrent outbreak of SUDV-caused EVD also had high frequencies (71%) of hemorrhagic manifestations. However, in several of the larger subsequent outbreaks where appropriate data were available, significantly fewer patients had hemorrhagic manifestations of disease: 41% (EBOV 1995), 30% (SUDV 2000), and 47% (BDBV 2007) (Okware et al. 2002; MacNeil et al. 2010; Bwaka et al. 1999). Additionally, in these three outbreaks, no association was observed between bleeding and death, arguing against the commonly held belief that hemorrhage equates to a fatal outcome. Furthermore, in the Western African outbreak, hemorrhagic manifestations were rarely reported; fewer than 15% of all patients from Liberia and Sierra Leone had any bleeding symptom recorded (Chertow et al. 2014; Schieffelin et al. 2014; Lado et al. 2015; Yan et al. 2015; Dallatomasina et al. 2015; Li et al. 2016; Qin et al. 2015), but two reports from a single center in Guinea

reported bleeding in 51 and 26% of patients (Barry et al. 2014; Bah et al. 2015). Perhaps reported differences in hemorrhage frequency are related to genetic or nutritional factors that cannot be controlled for in observational reports. Regardless, hemorrhaging can occur during EVD, but is not the most prominent feature. In contrast, hemorrhage does seem to be a common (50–80% of patients) feature of MVD, based upon limited data from the two largest outbreaks to date (Colebunders et al. 2007; Roddy et al. 2010).

Hemorrhaging is a clinical sign that can be secondary to multiple types of hematologic disorders. In the simplest terms, two general categories of hematologic disorders manifest clinically as bleeding: low levels of platelets and coagulation factor deficiencies (Hunt 2014). Platelet counts have not been routinely measured in patients with EVD, but in one study of 150 patients with EVD during the West African outbreak, platelet counts were not especially low, ranging $119\text{--}247 \times 10^9/\text{L}$ (normal range is $150\text{--}300$) (Hunt et al. 2015). Interestingly, the same phenomenon was noted years ago in the NHP model, and while absolute platelet counts were not very low, platelet function was severely affected as a result of *in vivo* activation and degranulation (Fisher-Hoch et al. 1985). No measurements of platelet function have been reported to date in humans. However, elevated levels of sCD40L were observed in surviving patients with EVD caused by SUDV (McElroy et al. 2014a, b). Since platelets are the major source of sCD40L in the bloodstream (Henn et al. 2001), this finding suggests platelet activation in humans during EVD. These data suggest a process that consumes platelet functional activity in severe or fatal EVD; this process would be consistent with the finding that in lethal NHP studies of EVD, sCD40L levels are elevated initially, but decline to undetectable at the time of death (Ebihara et al. 2011).

MARV might be a bit different, since in the original report of the first outbreak in Europe in 1967, most patients had severe thrombocytopenia, sometimes less than $10 \times 10^9/\text{L}$ (Martini 1973), coincident with significant hemorrhage in about half of the patients. Finally, the type of bleeding often described in EVD (and MVD) patients—epistaxis, conjunctival hemorrhages, bleeding into the GI tract and from the oral cavity—is mostly mucosal in nature, consistent with loss of platelet numbers or function.

The second general category of hematologic disorders that manifest as bleeding is deficiency in coagulation factors. Coagulation factors are quantitated clinically by measuring partial thromboplastin time (PTT) and prothrombin time (PT) to evaluate the intrinsic and extrinsic coagulation pathways. Unfortunately, these measurements have only been reported in case studies, and provide no consensus regarding the levels of PT and PTT during EVD. This is a clear information gap that needs to be addressed. An early report on MARV states that PTT and PT were measured in 10 patients, but the values obtained did not explain the observed severity of the hemorrhaging (Martini 1973).

DIC is often seen in critically ill patients, especially those with sepsis, and involves both low platelet counts and coagulation factor deficiencies. The bleeding seen in patients with EVD is often reported as due to DIC, although whether the criteria for DIC are met is unknown because the necessary laboratory tests are not

routinely available. DIC laboratory features include thrombocytopenia, elevated fibrin split products, prolonged PT, and consumption of fibrinogen (Levi et al. 2009). As noted above, the level of thrombocytopenia seen in EVD patients rarely meets the criteria to assign a DIC score, but elevated fibrin split products (such as D-dimer) have been measured retrospectively, are elevated in EVD patients, and are associated with fatal outcomes (Rollin et al. 2007). PT measurements are normal in the few available case reports (Sueblinvong et al. 2015), and fibrinogen levels were not associated with outcome or hemorrhagic manifestations (McElroy et al. 2014a, b, 2016). Measurement of DIC markers is clearly an area that requires additional study for clarification.

Less conventional evaluations of factors involved in coagulation pathways have also been conducted. Thrombomodulin, a protein expressed on endothelial cells, has anticoagulant properties in the microenvironment of the cell surface. When present in the plasma, thrombomodulin can act more globally, as shown in one family with a genetic deficiency that results in elevated levels of free plasma thrombomodulin in association with a bleeding disorder (Langdown et al. 2014). Endothelial cells also release thrombomodulin when they become activated. Elevated plasma levels of thrombomodulin were associated with both hemorrhage and death in SUDV patients, and with more severe disease in a cohort of EVD patients (McElroy et al. 2014a, b, 2016), suggesting that loss of this protein from the endothelial surface exacerbates both endothelial dysfunction and coagulopathy during EVD. Additionally, tissue factor, which is implicated in coagulopathy observed in NHPs (Geisbert et al. 2003b), was also elevated in patients with severe EVD (McElroy et al. 2016). Von Willebrand factor (vWF), a protein that is present in both platelets and endothelial cells and mediates interactions between platelets and the damaged endothelium, was elevated both in SUDV-infected patients with hemorrhage and in pediatric SUDV-infected patients with fatal outcomes. It was also elevated in EBOV-infected patients with severe disease (McElroy et al. 2015b, 2016).

A complex interplay of activated endothelial cells, activated platelets, inflammation, and coagulopathy is clearly at work during EVD. How intervening in any one aspect of the network impacts human disease is still unknown. It would be invaluable to determine the effects on EVD outcome of readily available clinical products that affect aspects of these processes. Some compounds of interest are statins, which stabilize the endothelium; soluble GPIIb/IIIa, which inhibits the interaction between platelets and vWF; and sCD40L, which appears to be consumed during severe disease.

9 The Roles of Co-infections, Co-morbidities, and Age

One key and largely unaddressed question is the role of co-infections and co-morbidities in EVD pathogenesis. Especially relevant to patient populations in the affected African nations are the possible contributions of malnutrition and

malarial co-infection in the disease process. Malnutrition is prevalent in the regions affected by EVD (Wirth et al. 2016), and long-standing malnutrition leads to defects in both innate and adaptive cellular immune responses (Schaible and Kaufmann 2007). Malnutrition may contribute to the high case fatality rates observed during EVD outbreaks in Africa as compared to filoviral infections in patients repatriated to the US and Europe (Uyeki et al. 2016b; Martini 1973).

Malaria co-infection is likely to increase EVD-related mortality, although this has not been rigorously evaluated, a study of the effects of various antimalarial drugs has been conducted during the West African outbreak. Antimalarial drugs were routinely given to EVD-positive patients at the ETC in Foya, Liberia; during a time of artesunate-lumefantrine shortage, artesunate-amodiaquine was prescribed. While the amodiaquine preparation was associated with improved survival in malaria-negative patients, interestingly suggesting a direct antiviral effect, this effect was lost in the malaria-positive patients, suggesting that malaria and EVD co-infection lead to worse outcomes even when malaria is treated (Gignoux et al. 2016).

Also of potential consequence are co-infections with HIV or other hepatotropic viruses. HIV co-infection has only been examined in one study; during the SUDV outbreak in Gulu, 18% of the tested patients were HIV-1-positive by antibody testing. No differences in EVD outcome were observed based upon HIV status in this study (McElroy et al. 2014a, b), but no CD4 counts were obtained, so it is possible that all cases were newly acquired and the patients were not yet immune-compromised enough for HIV-1 infection to influence EVD outcome. One study evaluated publicly available next-generation sequencing data, and using a cohort of 49 patients, posited that co-infection with GB virus C (a common, clinically innocuous pegivirus infection) results in improved outcomes during EVD infection (Lauck et al. 2015). The results were somewhat confounded by the fact that age is a major determinant of both GB virus C infection and outcome during EVD.

A special mention must be made that early data regarding the effect of age on EBOV susceptibility (Dowell 1996) and outcome (Mupere et al. 2001; McElroy et al. 2014a, b) have been repeatedly observed in large cohorts during the West African outbreak (Team et al. 2015; Faye et al. 2015; Li et al. 2016; Schieffelin et al. 2014; Bower et al. 2016). Case fatality rates are high in children under 5, lowest in school-aged children, reaching a nadir around puberty, and increase again to peak in the elderly. This phenomenon has been seen in other infectious diseases in children, and suggests perhaps that school-aged children are in the perfect immunologic window of life, with a fully mature and functioning immune system without the alterations that occur secondary to the influence of sex hormones. To date, only one study has examined pediatric patients for laboratory evidence of this protective effect in EVD; this study demonstrated that pediatric patients have viral loads similar to adult patients (McElroy et al. 2014a, b) and thus do not appear to control the viremia better. However, higher levels of RANTES, a T cell chemokine, were associated with pediatric survival, an association not seen in adults. Thus, stronger immune responses in pediatric patients might contribute to better outcomes, but this remains to be proven definitively and will require additional research efforts.

10 Post-EVD Syndrome

Perhaps one of the most striking findings during the Western African EVD outbreak has been the identification of severe sequelae in EVD survivors long after recovery. These sequelae have important implications both for medical treatment and for public health. In 1975, one year before the identification of EBOV in Zaire, MARV was successfully isolated from the ocular fluid of a convalescent patient with uveitis (Gear et al. 1975). A later follow-up study described arthralgia, myalgia, and abdominal pain as common sequelae in EVD survivors of the Kikwit outbreak (Rowe et al. 1999). Similar findings in individuals long after recovery from BDBV infection (Clark et al. 2015) suggest that post-recovery sequelae may be common in filovirus infections.

To date, EBOV RNA has been detected in semen, ocular fluid, cerebrospinal fluid, breast milk, and other body fluids in EVD survivors for several weeks or even months after discharge (Green et al. 2016; Chughtai et al. 2016). Moreover, infectious virus has been isolated from semen (Uyeki et al. 2016a), ocular fluid (Varkey et al. 2015), saliva, and breast milk (Bausch et al. 2007), and epidemiological evidence of sexual EBOV transmission has been established (Mate et al. 2015). An important task will be determining the pathogenic potential of virus isolates from semen compared to those of blood from the same patient. Because sexual transmission seems to be uncommon (based on the large numbers of male survivors and few sexual transmission events), virus isolates from semen may be less infectious, either due to attenuating mutations or inactivation by EBOV-specific antibodies secreted at the mucosal surface. In general, the pathogenic features of EVD sequelae and their putative physiological mechanisms are poorly understood.

Importantly, many of the symptoms reported by EVD survivors, as well as some of the observed signs like uveitis and skin desquamation, suggest an inflammatory syndrome. Indeed, immune activation persists after the acute phase of EVD (McElroy et al. 2015a; Rowe et al. 1999), strongly suggesting continuous immune stimulation and postinfection autoimmunity. These hypotheses still need to be experimentally tested, but are consistent with virus persistence in immunoprivileged sites. Alternatively, sustained inflammation could be due to deposition of immune complexes in the joints or to viral antigen persistence, as seen commonly in alphavirus infections and influenza A virus infection respectively (Hoarau et al. 2010; Tamburini et al. 2014; Tappe et al. 2016).

Since some, but not all, EVD survivors suffer sequelae, the factors leading to post-EVD syndrome must be determined. Co-morbidities and co-infections are likely contributing to sequelae development, particularly those involving immune phenomena like bystander T cell activation (Fujinami et al. 2006). Also, a positive correlation has been described between viremia levels during the acute phase of EVD and increased risk of sequelae (Mattia et al. 2016). These findings suggest that during infection, EBOV may be confined to immunoprivileged sites by treatment or by the host immune response, leading to viral persistence, and that this

phenomenon would be favored by high viral loads. Consistent with this hypothesis, one surviving patient with very high virus loads had virus persistence even within the central nervous system, followed by virus reactivation and meningoencephalitis (Jacobs et al. 2016).

In the future, the causes and molecular mechanisms of post-EVD syndrome must be determined. In particular, assessing whether reactivation of infectious virus is involved in sequelae development is a key. Due to the magnitude of the West African outbreak and the high number of surviving individuals, this is an important issue that needs to be addressed to establish adequate medical countermeasures and public health policies.

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