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Review

Clinical features and pathobiology of Ebolavirus infection☆

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ABSTRACT

There has clearly been a deluge of international press coverage of the recent outbreak of Ebolavirus in Africa and is partly related to the "fear factor" that comes across when one is confronted with the fact that once infected, not only is the speed of death in a majority of cases rapid but also the images of the cause of death such as bleeding from various orifices gruesome and frightening. The fact that it leads to infection and death of health care providers (10% during the current epidemic) and the visualization of protective gear worn by these individuals to contain such infection adds to this "fear factor". Finally, there is a clear perceived notion that such an agent can be utilized as a bioterrorism agent that adds to the apprehension. Thus, in efforts to gain an objective view of the growing threat Ebolavirus poses to the general public, it is important to provide some basic understanding for the lethality of Ebolavirus infection that is highlighted in Fig. 1. This virus infection first appears to disable the immune system (the very system needed to fight the infection) and subsequently disables the vascular system that leads to blood leakage (hemorrhage), hypotension, drop in blood pressure, followed by shock and death. The virus appears to sequentially infect dendritic cells disabling the interferon system (one of the major host anti-viral immune systems) then macrophages (that trigger the formation of blood clots, release of inflammatory proteins and nitric oxide damaging the lining of blood vessels leading to blood leakage) and finally endothelial cells that contribute to blood leakage. The virus also affects organs such as the liver (that dysregulates the formation of coagulation proteins), the adrenal gland (that destroys the ability of the patient to synthesize steroids and leads to circulation failure and disabling of regulators of blood pressure) and the gastro-intestinal tract (leading to diarrhea). The ability of the virus to disable such major mechanisms in the body facilitates the ability of the virus to replicate in an uncontrolled fashion leading to the rapidity by which the virus can cause lethality. Various laboratories have been working on defining such mechanisms utilizing in vitro culture systems, a variety of animal models including inbred strains of normal and select gene knock out mice, guinea pigs and nonhuman primates that have led to a better understanding of the potential mechanisms involved. There have also been some major advances made in the identification of therapies from the very simple (major supportive type of therapy), to the identification of a number of highly effective chemotherapeutic agents, a variety of highly effective preventive (demonstrating 100% effectiveness in nonhuman primate models) recombinant formulations (adenovirus based, VSV-based, rabies virus based), therapeutic candidate vaccines (cocktail of monoclonal antibodies such as ZMAPP) and alternate approaches (RNAi-based such as TKM-Ebola and antisense based such as AVI-7537) that show great promise and at an unprecedented rate of discovery that speaks well for the scientific research community at large.

1. Introduction

The recent outbreak of Ebolavirus infection in several adjoining countries (Guinea, Liberia, Sierra Leone and Nigeria) in Africa which has still to be contained has resulted so far in deaths of >1552 individuals associated with >3069 recorded cases (as of this writing) and thought to be an underestimate of the actual scope of the epidemic (WHO, Geneva, Switzerland) and recent calculation estimates >20,000 cases in the next 9 months. The high mortality rate (51%) has provided serious concern to public health officials worldwide and has prompted a sense of urgency to develop

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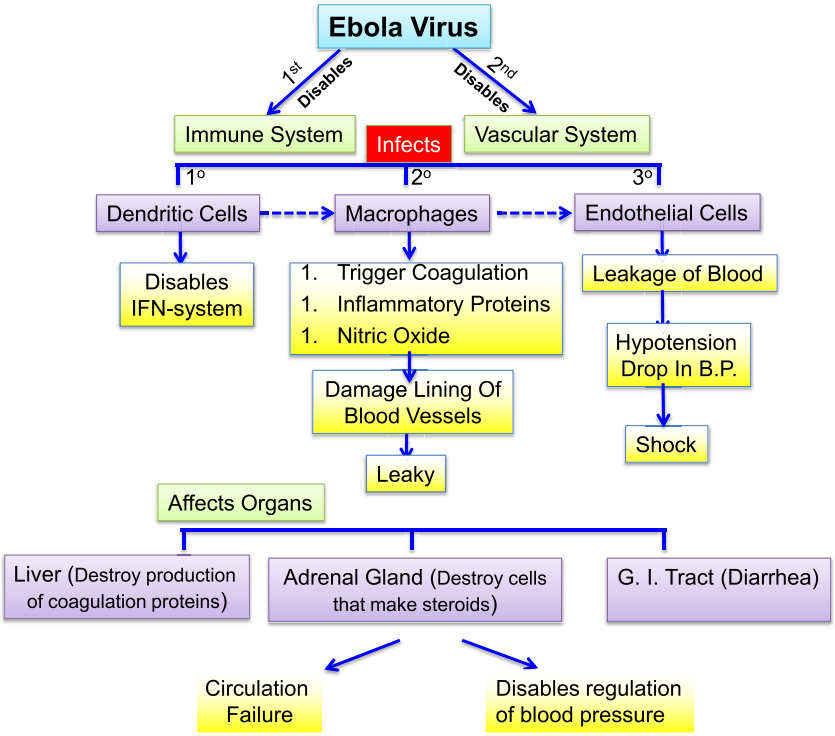

Fig. 1. The cascade of pathological events that results in the rapid severity of Ebolavirus infection.

effective chemotherapeutic and pre-/post-exposure vaccines as tools to halt this and future outbreaks of Ebolavirus infection. The current outbreak initiated in Guinea has been identified as being due to an outlier strain of Zaire Ebolavirus [1] based on full genome sequencing and, based on phylogenetic analyses, it is reasoned that a similar virus was the cause of the outbreaks in the Democratic Republic of Congo, Republic of Congo and Gabon [2]. Detailed sequencing studies of 99 Ebolavirus genomes from 78 patients in Sierra Leone show that there have been rapid accumulation of inter- and intra-host variations (with 395 mutations) and suggest that while the initial transmission was zoonotic in origin, all the remaining transmission are likely from human to human [3]. The hammer-headed fruit bat (*Hypsignathus monstrosus*) and the little collared bat (*Myonycteris torquata*) belonging to the family Pteropodidae have been implicated as the most likely reservoirs from where this virus likely emerged [4]. Vectors involved in transmitting the virus from the bat reservoirs to humans continues to be studied and may be distinct for each of the strains of Ebolavirus reasoned to be either nonhuman primates or pigs besides direct transmission via the consumption of bats. Previous epidemiological studies following an outbreak in Gabon revealed that whereas there was a 15.3% sero-prevalence rate of Ebola specific IgG in the general population, individuals residing in forested areas showed a prevalence rate as high as 32.4% [5,6]. This finding suggests that either this prevalence rates are due to periodic epidemics or that there is a continuous exposure of individuals to this virus and requires further study. The purpose of this review is to summarize our current understanding of the make up of the virus, why the viral infection has such high pathogenicity and discuss the many therapeutic and vaccine formulations that are at various stages of preparation, evaluation and testing (Fig. 1).

2. The virus

The Ebolaviruses are enveloped non-segmented negative strand RNA viruses of 19 kb in length belonging to the family Filoviridae. The virus is filamentous and pleomorphic with a mean unit length of 1200 nm. So far, there have been 5 different viral sub-types that have been recognized since the original description in 1976 of the Ebolavirus Zaire (ZEBOV) named after a river in a country now called the Democratic Republic of Congo. The others include the Sudan Ebolavirus (SUDV), the Tai Forest Ebolavirus (TAFV), the Reston Ebolavirus (RESTV) and the Bundibugyo Ebolavirus (BDBV) [7]. Each of these is pathogenic for humans except RESTV that so far has only been shown to be pathogenic for nonhuman primates. Each of these viruses has been sequenced and their evolutionary characteristics reported [8]. The current Ebolavirus shares 97% homology with ZEBOV. The viral genome encodes for a nucleoprotein (NP), glycoprotein (GP), RNA dependent RNA polymerase (L), and four structural proteins termed VP24, VP30, VP35 and VP40. In addition, the Ebolavirus is able to express a truncated soluble form of GP (sGP) through RNA editing (Fig. 2). The role of some of these proteins in viral assembly and packaging has been studied and more recently, a novel viral life cycle modeling system using BSL-2 conditions was utilized to identify a new role for VP24, a system that serves as a template for studying life cycles of other similar BSL-4 restricted viruses [9]. The Ebolavirus is a lipid enveloped virus consisting of a lipid bilayer coat that serves to protect the virus genome, facilitates its entry into host cells. The lipid content of the viral envelope and its heavy glycosylation are reasoned to contribute towards immune evasion (described more in detail below). Detailed studies of its structure, intracellular assembly, interaction with host cell constituents and budding mechanisms are described elsewhere [10].

3. Clinical characteristics

The virus is transmitted by exposure of uninfected individuals that have abrasions in the skin, exposure of their mucosal tissues and/or parental exposure to bodily fluids from an infected individual. The incubation period varies and reasoned to be between 2 and 21 days with an average incubation period of 7–10 days. The

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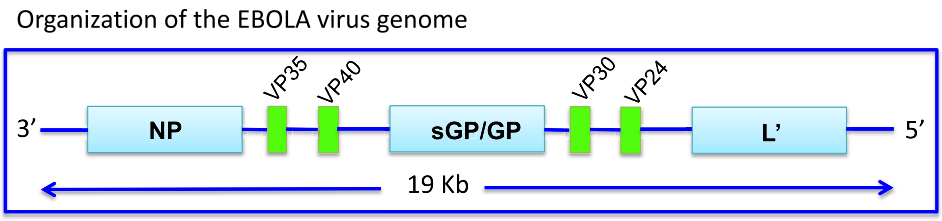

Fig. 2. The basic organization of the Ebolavirus genome.

problem with the diagnosis of Ebola infection is that the initial clinical symptomology is very general consisting of onset of fever, myalgia, and general malaise and sometimes accompanied by chills and often confused with malaria or dengue in tropical climates. This initial period is followed by a period wherein the patient shows flu-like symptoms accompanied by gastro-intestinal symptoms and in severe cases maculo-papulary rash, petichae, conjunctival hemorrhage, epistaxis, melena, hematemesis, shock and encephalopathy. Some of these clinical characteristics have recently been summarized by Fauci [11]. Blood analysis shows evidence of leukopenia (associated with increased lymphoid cell apoptosis), thrombocytopenia, increased levels of aminotransferase, thrombin and partial thromboplastin times accompanied by the detection of fibrin split products indicative of the occurrence of disseminated intravascular coagulation (DIC).

The detailed pathogenesis of the disease is not well understood. Studies in nonhuman primates have shown that EBOV replicates in monocytes, macrophages, and dendritic cells; however, in situ hybridization and electron microscopy have also shown the presence of virus in endothelial cells, fibroblasts, hepatocytes, and adrenal cells. The virus disseminates to lymph nodes, the liver, and the spleen. There is a significant inflammatory response and significant lymphoid cell apoptosis (most likely due to release of TNF-α), which leads to lymphopenia and seems to be a marker of poor prognosis. Inhibition of the type I interferon response seems to be one of the most important aspect in the pathogenesis of Ebola. Thus, the virus disables the innate immune response but also the acquired humoral and cellular responses (detailed below) that lead to uncontrolled viral replication and dissemination (see Fig. 3). One of the major outcomes of such dissemination is the dysregulation of the coagulation cascade and production of proinflammatory cytokines by macrophages that leads to shock and multiorgan failure during the terminal phase.

4. Laboratory diagnosis

Being a BSL-4 agent, confirmed clinical laboratory diagnosis of viremia during the acute phase is only possible in developed countries where such facilities exist. The assays that can be utilized are based on the stage of the disease. During acute disease the assays include a) virus isolation using Vero or Vero E6 cell lines, b) RT-PCR and real time quantitative PCR assays with appropriate false negative and false positive controls, c) antigen capture ELISA, and d) IgM ELISA. Later during the course of disease the tests that can be utilized include a) IgM and IgG ELISA using authentic viral antigens, and in the case of death, autopsy tissues can be utilized for a) antigen detection using immunostaining techniques, b) immunohistochemical aided detection of Ebola antigen [12], and c) in-situ hybridization techniques for the detection of viral RNA. The details of each of these techniques have been summarized [13]. The ELISA based assay has been standardized by the CDC for the detection of Ebolavirus specific antibodies. The assay has high sensitivity and has been shown to be capable of detecting antibodies in the sera of humans exposed 10 years previously to Ebola. A cell-based plaque assay and an end point titration assay (TCID50) have also been developed to detect and quantitate filoviruses for use in pre-clinical studies [14,15].

5. Pathogenic mechanisms

The clinical outcome of Ebolavirus infection as stated above is varied in humans. In a minority of the cases the infection results in a transient flu like symptoms associated with mild coagulopathy, thrombocytopenia and leukocytosis and full recovery and in the majority of the infected population who develop severe illness followed by hemorrhage, DIC, shock and death. The reasons for such varied outcome are not clear at present but assumed to be

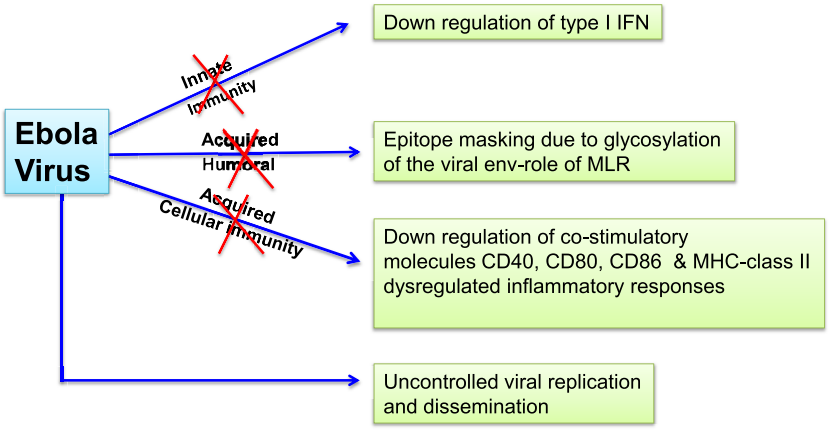

Fig. 3. The 3 arms of the immune system that are disabled allowing for the uncontrolled replication of Ebolaviruses.

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secondary to the level of viral replication and the corresponding robustness and rapidity by which the dysfunctional innate immune responses are reversed and the rapidity and robustness by which the adaptive immune responses, in particular, neutralizing antibodies are generated in the host. As one can imagine the early innate immune responses to Ebolavirus infection are difficult to study in humans for obvious reasons. Thus, the study of the innate immune responses is best characterized using animal models of Ebolavirus infection, notably nonhuman primates and these studies can also only be performed in laboratories that have BSL-4 facilities. The fact that Ebolavirus infection of nonhuman primates leads to essentially similar clinical manifestations as humans infected with Ebolaviruses, it is safe to assume that the findings in the nonhuman primates have direct implications to humans. The general findings of Ebolavirus infection are that the virus primarily targets cells of the monocytes/macrophage and dendritic cell lineages and subsequently endothelial cells [16]. The studies below are structured to provide a summary of what we know about the effect of Ebolavirus and its various components on in vitro tissue culture cell lines, followed by the characterization of animal models and lastly of results performed in humans.

6. In vitro studies

Earlier studies performed on autopsy tissues of confirmed Ebolavirus infected patients using immunohistochemical techniques indicated the presence of Ebolavirus primarily within endothelial cells, mononuclear phagocytic cells, to some degree within fibroblasts and within hepatic sinusoids [12]. It has since been shown that while initial infection targets macrophages and dendritic cells, gradually the virus is able to gain entry into endothelial cells and hepatocytes coinciding temporally with the gradual development of symptoms associated with dysfunction of the monocyte/macrophage/dendritic cell lineage (fever, TNF-α, cytokine storms, etc.) to the coagulopathy (complement activation, endothelial cell dysfunction and vascular leakage) and the failure of the liver (hepatocellular necrosis and failure to synthesize coagulation factors and DIC). With regards to cell lineage susceptibility, it is of interest to note that there has been some controversy as to whether monocytes can be readily infected with Ebolavirus. Studies by Martinez et al. [17] addressed this issue and showed that while primary monocytes are refractory to Ebolavirus infection, they do bind to monocytes and following in vitro differentiation into macrophages become infected as do differentiated dendritic cells. These in vitro cultured monocytes down regulate the expression of viral entry restriction factors such as interferon-inducible trans-membrane proteins and concurrently up regulate the expression of factors that are critical for Ebolavirus entry such as cathepsin B and NPC1 (which have been targeted for therapeutic intervention) and thus provide a molecular basis for the infection of the monocytoid cell lineage. Details of the role of the various viral proteins in the life cycle of the virus once it gains entry into the cells have also been studied. Thus it is clear the viral envelope glycoprotein (GP) is responsible for both receptor binding and fusion of the viral envelope with the host cell membrane [18,19]. The Ebolavirus ENV is heavily glycosylated that includes both N- and O-linked glycans which is reasoned to serve as a shield against host immune attack and thus contributes to the ability of the virus to escape immune effector mechanisms. These glycans also lead to the generation of antibodies against highly variable and disposable regions of the ENV and are non-neutralizing. The sites of major glycosylation have been localized to the middle third of the ENV GP. This region is also referred to as the mucin-like region (MLR) [20,21]. The viral ENV GP is cleaved into 2 subunits by host cell origin proteases such as furin resulting in GP1 (that is primarily involved in MLR facilitated binding to the putative host cell receptors) and GP2 that facilitates the assembly of the GP as a trimer. It has recently been suggested that cysteine proteases such as cathepsins B and L (cysteine proteases) promote the fusion of the viral GP with the host cell membrane [22,23]. Since the wild type Ebolavirus is highly pathogenic and working with it requires BSL-4 facilities, various recombinants have been prepared that contain Ebolavirus ENV GP in the form of pseudotyped viruses. Such pseudotyped viruses provide a valuable tool to study the biological role of the various individual components of the Ebolavirus that can be studied under BSL-2 facilities.

As stated above, Ebolaviruses have been shown to target monocytoid/macrophage, dendritic cells, endothelial cells and hepatocytes which is a wide range of cell lineages and thus the specific mechanisms (receptors) utilized by the virus to gain entry into such cell types have been difficult to define. Among the receptors implicated utilizing pseudotyped Ebolavirus GP containing viruses as tools are the GPI-anchored cell surface expressed folate receptor-alpha, members of the tyrosine receptor kinases (Axl, Dtk and Mer), the T-cell immunoglobulin and mucin domain (Tim-1), and more recently the cholesterol transporter protein Niemann-Pick C1 (NPC1). The Ebolavirus ENV MLR rich in glycosylated residues continues to be viewed as playing an important role in viral infection of endothelial cells, hepatocytes and the monocytoid cell lineages and is thought to involve membrane anchored C-type lectins that can serve as attachment factors rather than specific receptors [24].

7. Viral escape from host anti-viral mechanisms

As described briefly above, the heavy glycosylation of the viral ENV GP serves to shield the cell free virus from access to potential virus neutralizing antibodies (epitope shielding). As a matter of fact, such glycans promote the generation of antibodies against the more variable and dispensable regions of the GP and to a large extent these antibodies are non-neutralizing and is reminiscent of the problems associated with antibodies generated against HIV-1 [25]. In addition, the shedding of soluble viral glycoproteins is yet another way that the virus utilizes to misdirect humoral anti-viral mechanisms. These sGP that represent approximately 70% of the transcripts encoded by the ENV GP thus serve as “sinks” or “decoys” and are likely also responsible for binding of the much needed neutralizing antibodies. Once the virus gains entry into host cells, in addition, there are a number of mechanisms the virus has developed to counteract naturally occurring host anti-viral mechanisms. Thus, the Ebolavirus primarily antagonizes both the Interferon alpha and beta host responses [16] utilizing various components of the virus. Thus as seen in Fig. 3, the viral VP24 desensitizes cells to the effects of IFN-α, -β and -γ by blocking the homo-dimerization of JAK-1 and hetero-dimerization of TYK-2 that leads to preventing the nuclear localization of these transcription factors and thus decreasing/inhibiting the transcription of Interferon Stimulating genes (ISG's). The VP35 has been shown to have multiple inhibitory effects that include the inhibition of the phosphorylation of IRF-3, the inactivation of IRF-7, the inhibition of activation of IFN inducible dsRNA and Dicer dependent protein kinase R. In addition, VP35 binds dsRNA and sequesters its recognition by RIG-1, inhibits the upregulation of a number of co-stimulatory molecules such as CD40, CD80, CD86 and MHC-class II and the maturation of dendritic cells. It is important to note, however, that the viral GP is not sufficient to cause virulence.

The above findings in concert therefore suggest that the Ebolavirus has a number of physical and biological mechanisms to evade host innate and acquired humoral and cellular immune responses that likely contributes to uncontrolled virus replication and

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promotes rapid dissemination that is the cause of the degree of pathogenicity that has so far been recorded (see Fig. 4).

8. Innate immune responses

It is generally known that the innate immune responses are the primary host defense mechanisms against pathogenic microbial exposure. These innate immune responses include a highly sophisticated set of pathways that is endowed with a unique ability to distinguish self from non-self. The recognition of non-self leads to the unleashing and coordinated orchestration of molecules to counteract the invading microbe. The relative success of such initial innate immune responses leads to the generation of host mediated humoral and cellular immune responses that limit and in most cases eliminate the invading microbe. Several microbes, however, such as the Ebolaviruses, have developed a variety of mechanisms to subvert these innate immune functions. Notably, the Ebolaviruses not only counteract the type one IFN system but in concert also lead to the synthesis of large amounts of relatively pro-inflammatory cytokines for an extended period of time that together contribute to immune dysfunction and facilitate uncontrolled viral replication. Thus, while transient innate immune responses in the form of cytokines are beneficial to the host, the same essential spectrum of cytokines lead to dysregulation of homeostatic mechanisms, destruction of host tissues and lead to uncontrolled microbial replication. The viral proteins of the Ebolavirus that subvert host innate immune responses are briefly described above (under viral escape mechanisms). It is important to note that one of the major innate immune responses that is counteracted by the virus is the interferon system in which the EBOV has been shown (at least using tissue culture systems) to inhibit the synthesis of proteins that could serve as barriers against Ebolaviruses. Thus, the synthesis of host cell IFN-inducible transmembrane proteins 1–3, tetherin and other virus restricting molecules are inhibited by Ebolavirus proteins [7]. Several studies have been performed using guinea pigs, immune-compromised mice and nonhuman primates to study either the effects of Ebolavirus infection or the testing of potential vaccines with the use of recombinant constructs and various inbred strains of gene knock out mice [26–30]. While the studies in small animal models are quite informative, this review will focus primarily on what we have learnt so far from studies of humans and nonhuman primates who have survived Ebolavirus infection as compared to those who died following infection.

The infection of monocytes and macrophages leads to increased synthesis of TNF-α that induces fever and contributes to lymphoid cell apoptosis (giving rise to lymphopenia characteristic of Ebolavirus infection) and marked inhibition of interferon α/β. Such monocyte/macrophage infection also leads to the release a variety of pro-inflammatory proteins that include IL-1, IL-6, IL-8, IL-15, IL-16, the chemokines MIP-1 alpha and beta, MCP-1, M-CSF, MIF, IP-10, eotaxin to name a few. It is important to note that essentially the same occurs in patients that recover from Ebolavirus infection but it is transient and in these patients the levels are 5–1000 times less than those that proceed to lethal infection. In addition, the levels seen in patients with SUDV as compared with EBOV are much lower and it is reasoned that the SUDV is attenuated. There is a report of the importance of NK cells in protection against Ebolavirus in a murine model in which depletion of NK cells abolished protection [31].

9. Acquired immune responses

Most of our understanding of the relative importance of the protective role of humoral and cellular immune effector mechanisms with regards to Ebolavirus infection have been generated by the use of a variety of vaccine platforms in mice, guinea pigs and nonhuman primates. The murine model allows for the delineation of the role of specific cell lineages such as the $CD4^+$ T cells, $CD8^+$ T cells, NK cells and B cells with the use of cell lineage specific knock out strains of mice. The results using such KO strains of mice have shown that mice deficient in $CD4^+$ T cells and B cells survived infection whereas those deficient in $CD8^+$ T cells did not survive infection highlighting the role of cytotoxic T cells in protection against Ebolavirus [26]. These findings of a major role of cytotoxic T cells were supported by studies in mice and nonhuman primates (NHP). In mice, the adoptive transfer of $CD8^+$ T cells from immunized mice to naïve mice afforded protection against lethal challenge with Ebolavirus [32]. In the case of NHP, passive transfer of high titers of anti-viral IgG failed to protect 3/4 monkeys and depletion of $CD8^+$ T cells prior to challenge in monkeys immunized using an Adenovirus serotype 5 (Ad5) abolished protection in 4/5

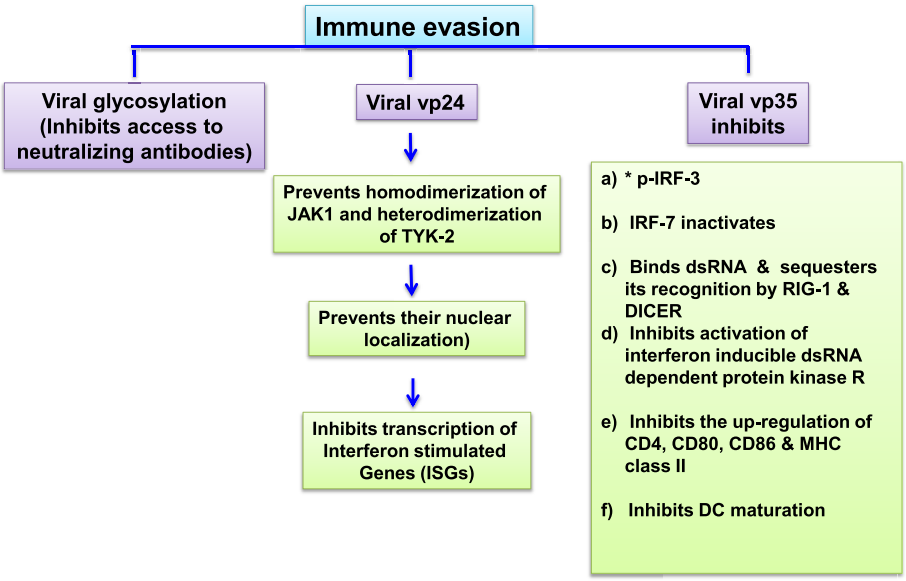

Fig. 4. The potential mechanisms by which the various components of the Ebolaviruses evade host innate and acquired immune systems.

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monkeys [33]. These studies highlighting a major role for $CD8^+$ T cells in mediating protection against Ebolavirus were followed by a series of studies that highlighted a major role for humoral immunity, and the importance of cellular versus humoral immunity continues to be a subject of debate. Thus, one such study showed that monkeys infected with Marburg or Ebolavirus when administered IgG from monkeys that survived Ebolavirus infection led to protection of these monkeys as compared to the monkeys that were not administered the IgG led to the conclusion that post-exposure therapy with IgG was important [34]. These studies were soon followed by studies in mice that highlighted a role of antibody mediated protection that was contradictory to the studies previously performed [35]. It is likely that differences in the route and dose of infection could be the basis for such distinct results. Subsequent analysis of survivors versus non-survivors of Ebolavirus infection of guinea pigs and NHP appeared to show a strong correlation between levels of GP specific IgG and survival. In vivo depletion of $CD4^+$ T cells, $CD20^+$ B cells or $CD8^+$ T cells before and during vaccination basically reached a similar conclusion in that monkeys depleted of $CD8^+$ T cells survived infection whereas those depleted of $CD4^+$ T cells or B cells failed to survive. A study of survivors versus non-survivors of human Ebolavirus infection appeared to document a role for both humoral and cellular mechanisms as did a large scale epidemiological study of 4349 adults and 362 children in Gabon [36,37].

Thus, while a definite requirement of humoral immunity against Ebolavirus can be made, the requirement for cellular immunity has been a subject of considerable debate. However, it seems logical to assume that whereas appropriate virus neutralizing humoral antiviral immunity can readily neutralize cell free virus. It is difficult to envision the clearance of virus infected cells by humoral mechanisms including ADCC associated mechanisms. It seems therefore reasonable to conclude that both virus specific humoral and cellular mechanisms are required for clearance of viral infection and the former more important during acute infection to halt the progress of viral spread and the latter more important to eliminate any viral infected cells that could continue to serve as a source of virus.

10. Use of transcriptomic and kinome analyses in Ebolavirus infection

There have been several studies that have utilized genome profiling to compile a list of genes that could be potentially associated with protection as compared with those that lead to lethal disease. One such recent study used samples from NHP who were anti-coagulant induced survivors of lethal Ebolavirus infection and compared the transcriptomal profiles of the monkeys that did not survive and untreated control monkeys [38]. A set of 20 genes were identified that were highly predictive of survivors versus non-survivors. In addition, this study also identified a larger set of 238 genes that were correlated with disease outcome and treatment. Among survival associated genes were a subset of genes that were transcriptionally regulated by 1) CCAAT/enhancer binding protein alpha, 2) p53, 3) megakaryoblastic leukemia-1, and 4) myocardium protein 2. These findings should serve as a foundation to begin to ferret out those specific genes that need to be a focus for vaccine efficacy studies. Another study aimed at identifying markers of host responses during Ebolavirus infection involved kinome analysis [39]. The temporal analysis was aimed at identifying markers associated with early, intermediate and late host response during Ebolavirus infection. The results of these studies showed that TGF-β signaling and secretion were upregulated and inhibition of kinases involved in TGF-β inhibited virus replication. The cellular markers associated with upregulation of TGF-β included upregulation of matrix metalloproteinase 9, N-cadherin and fibronectin associated with reductions of E-cadherin and claudin-1. These findings suggest an important role for epithelial cell barrier breakdown and modulation as an initial step in the pathogenicity of Ebolavirus infection and points to identifying agents that can inhibit such initial steps for the prevention of the pathology that results in Ebolavirus infection.

11. Chemotherapeutic strategies against Ebolaviruses

Although considerable advances have already been made and have identified a variety of vaccine formulations, it is important to summarize what we know about chemotherapeutic agents that can be utilized to prevent and/or treat Ebolavirus infection. Among the agents so far identified are a) recombinant human activated protein C [40], b) recombinant nematode anticoagulant protein c2 (rNAPc2) [41], c) two small molecule therapeutics that are anti-sense phosphorodiamidate morpholino oligomers (PMOs AVI-6002 and AVI-6003) and lipid nanoparticle small interfering RNA (LNP-siRNA:TKM-Ebola) that have been approved for Phase I clinical trials by the FDA [42], d) a broad spectrum nucleoside analog BCX4430 that shows inhibition against a wide variety of viruses including Ebolavirus in vitro and post-exposure inhibition in vivo in monkeys [43] being developed in association with the US Army, e) a broad spectrum anti-viral small molecule that inhibits the entry of a wide variety of viruses including Ebolavirus by targeting the cathepsin L cleavage of the viral GP, that is required by the virus to fuse with the host cell membrane [44], f) the identification of a pyrazinecarboximide derivative T-705 (favipiravir) that was shown to inhibit EBOV replication 4 log units in vitro with an IC90 of 110 μM and clear virus infection within 4 days in vivo when administered at a dose of 300 mg/kg daily initiated at day 6 post-infection [45], g) the potential of new compounds such as FGI-103, FGI-104, FGI-106, dUY11, and LJ-001 for the treatment of filoviruses that include Ebolaviruses [46], and h) a variety of newly developed drugs that have the potential to target Ebolavirus VP35 and VP40 [10,47]. Thus, as one can gather there are a variety of chemotherapeutic agents that are being tested and are at various stages of development even though as widely known the market for such drugs is limited.

12. Vaccine efforts against Ebolaviruses

12.1. The target population

While considerable efforts have been made and continue to be made for the formulation and testing of vaccines against Ebolavirus, it is important to have some clarity as to the intended target population because it will clearly differ in the type of vaccine being formulated. Thus, one can envision the following different schema:

a) A vaccine to target populations where Ebolavirus has shown to cause outbreaks. However, this is a daunting task given the large geographical areas where such outbreaks have been recorded and the logistics in providing such a vaccine to large numbers of people in relatively rural settings. The maintenance of a cold chain will clearly be an issue and a credible laboratory that can execute such a massive effort.
b) A vaccine that targets a population in which an outbreak has already been recorded thus limiting the spread of the infection within and outside the affected population. This would also include indigenous and foreign health care workers that have to go into such populations to contain the epidemic. Such a vaccine would have to induce rapid immunity (one shot) for it to be effective. In addition, if a vector based vaccine is being formulated, immunity against the vector needs to be considered.

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c) A vaccine that targets health care providers and the military population that are tasked to enter such geographical areas. In this case, the vaccine has not only to provide long lasting immunity but also be sufficiently broad to be effective against multiple Ebolaviruses. The finding that only homologous vaccines were shown to be effective for Ebolaviruses, highlight this concern [48]. The cold chain in this case would not be an issue.
d) A vaccine that targets the intermediate hosts of the Ebolavirus such as NHP and pigs for instance. The challenges here would be to have the resources to target wild populations and that the vaccine would have to be of a nature that is incorporated in the natural foods that are eaten by the intermediate hosts and thus the stability of such a formulation would have to be considered. In addition, it would be important to make sure that such a vaccine does not adversely affect other wildlife population.

Thus, as noted above, the types of vaccine platforms being developed have to take into account the above factors for the potential candidate vaccines to be effective.

12.2. Types of vaccines

In general, there are 3 types of vaccines. These include live-attenuated (which is not feasible in the case of agents such as Ebolaviruses for the potential of reversion), killed or inactivated (induce mostly short term immunity) and subunit vaccines (including recombinant vaccines, which are to a large extent the major forms being studied). There are also so called “therapeutic” vaccines and these include antibodies that are either produced in animals (both polyvalent forms and monoclonal antibody forms) or sera/immunoglobulins from individuals who have survived from infection. These therapeutic vaccines are administered to patients post-infection and are thus termed “passive immunizations” since they are acquired from animals or other humans as compared with “active immunizations” which is the case when individuals are immunized prior to infection and are by definition preventive vaccines. The vaccines against Ebolavirus that have been studies so far have been previously discussed [49] and include:

12.2.1. Preventive including subunit vaccine formulations

Initial attempts at identifying a vaccine against Ebolavirus involved the use of formalin treated and heat inactivated preparations that were tested in guinea pigs and mice but were not found to be very effective. An irradiated vaccine that was shown to be 100% protective in mice, however, was shown to be ineffective in NHP even though such a vaccine induced high titers of antibody responses that included neutralizing antibodies [50]. Attempts to utilize a variety of replicons such as Venezuelan equine encephalitis virus (VEEV) and Kunjin virus showed some promise but once again either failed to show efficacy in NHP or have yet to prove effective. DNA based vaccines have also shown a high degree of protection in mice and guinea pigs but require multiple immunizations. The use of recombinant vaccines has had the greatest success rate specially the adenovirus based recombinant Ebolavirus vaccines. An adenovirus based recombinant Ebolavirus vaccine that included the env regions of both the ZEBOV and SUDV showed protection against both Ebolaviruses after a single vaccination and furthermore when combined with a DNA based vaccine + the same adenovirus recombinant vaccine showed cross protection against not only ZEBOV and SUDV but also BEBOV [51,52]. One of the drawbacks with the use of adenovirus based recombinant vaccines is the fact that several human populations have existing immunity against adenoviruses type 5 which is the type used in the vaccine formulations and as such the dominant response induced using such a vaccine is against the adenovirus failing to induce significant response in these populations against the recombinant protein. The use of different adenoviruses has been one approach to overcome this problem. Recently, a VSVΔG based preparation that included the linkage region between GP1 and 2 of ZEBOV that includes the furin site and an internal fusion loop (termed MFL) was utilized as an immunogen and found to be highly effective in inducing high levels of antibody responses in mice and the specificity of the antibodies generated were localized to this very same region denoting the importance of this fragment in eliciting Ebolavirus neutralizing antibodies. Furthermore a combination of the MFL fragment with a rVSV based bivalent vaccine expressing Sudan EBOLA GP (SGP) elicited immunity against both the Zaire and Sudan GP [53]. These constructs, however, need further testing in NHPs. A number of Ebolavirus GP prepared using recombinant technology have also been tested but have yet to reach the level of efficacy to be tested in NHP so far. On the other hand, Ebolavirus VLP's have shown a higher degree of efficacy in both mice and NHP [54] and the use of baculovirus systems to produce large amounts of such VLP's make these preparations feasible to push forward for further testing and evaluation in NHP [55]. Genetically engineered Ebolaviruses such that the recombinant can only replicate once have also been prepared and tested. Thus a VP30 deleted Ebolavirus was prepared which was shown to infect cells limited to a single replication cycle. This virus preparation was found to be immunogenic and shown to protect 100% of the mice and of importance shown to have no potential for recombination [56]. Such a construct is also considered valuable for future testing.

A number of other replication competent constructs have been evaluated as potential vaccine candidates against Ebolavirus. These have included the VSV constructs, whose GP was deleted and the EBOV GP inserted. Such a VSV/ΔG/GP was shown to induce immunity at a 3 log lower level than the Ad5 based recombinant Ebola vaccine and of importance found to demonstrate 100% protection in mice and NHP [57] and show no adverse effect upon testing in 100 NHP, underscoring its safety. Furthermore this construct was shown not to cause any detectable pathological effects in 4/6 SHIV infected rhesus macaques providing testimony as to its relative safety even in immune-compromised hosts. The 2 monkeys that developed disease had the lowest CD4 counts and considered severely immune-compromised [58]. Studies using similar VSV constructs that contained more than one species of Ebolaviruses and a Marburg virus were also found to be highly effective vaccines. Of great interest is the finding that such formulations can serve also as therapeutic vaccines in addition to preventive vaccines specially if administered shortly following infection in NHP [59]. The common human respiratory pathogen termed Human parainfluenza virus 3 (HPIV3) has also been successfully utilized to prepare and test a recombinant Ebolavirus that can be used to vaccinate via the respiratory route. While such a construct showed 100% protection following a single vaccination in guinea pigs, it required 2 vaccinations to induce protective immunity in NHP [60]. However, one of the potential limitations of such a construct is that similar to the Adenovirus constructs, humans have immunity to the parainfluenza virus that could potentially interfere in the generation of high titer immunity. The rabies virus has also been successfully utilized to prepare and test recombinant Ebolavirus vaccine. This is particularly interesting because the rabies vaccine utilized for these studies is already being used as a wildlife vaccine in Europe. The ZEBOV GP was inserted between the N and P gene of the rabies virus and this construct (BNSPΔG-GP) includes the rabies G and such a preparation was shown to be safe and protect 100% of mice

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following challenge with ZEBOV [61]. Further studies using such a construct are in progress. There have also been attempts to use CMV as a platform to prepare and test an Ebolavirus vaccine but this needs considerable further work before it is accepted as a viable vaccine candidate.

12.2.2. Therapeutic vaccines

There has always been some confusion on the meaning of the term "vaccine". A vaccine in its strict sense is supposed to be preventive in nature and is administered to otherwise healthy individuals to INDUCE protective immunity in the vaccinated individual against the target of the vaccine. However, there are instances in which certain agents particularly sera or purified immunoglobulins from the sera of either animals who were immunized against the pathogen or individual who were previously exposed and survived the infection that when administered to individuals who are already infected can lead to protection from the pathogen (passive immunization). In addition, in select cases monoclonal antibodies containing high titers of pathogen neutralizing activity have also been utilized and all these strategies have given rise to the term "therapeutic vaccination" to denote the administration of a vaccine that has therapeutic efficacy when administered post-infection. As described above, there have been a number of chemotherapeutic strategies that have been tested as candidates for therapy post-exposure some of which indeed do show some promise (see above). The most effective strategies, however, has been the use of select monoclonal antibodies that have a high neutralizing potential such as the ones against epitopes of the Ebolavirus. Thus cocktails of monoclonal antibodies termed MB-003 (clones c13C6, h13F6 and c6D8) [62] that were chimaeric denoted by c and human denoted by 'h' and the cocktail termed ZMAb (consisting of murine monoclonal antibody clones m1H3, m2G4 and m4G7) [63] have been shown to be quite effective in Ebolavirus infected NHP when administered 24–72 h post-EBOV exposure. A follow up study was conducted in which various combinations of the monoclonal antibodies that were first made chimeric (consisting of human constant regions and the CDR from the mouse monoclonal antibodies) were tested in mice, guinea pigs and the ones showing highest efficacy advanced to NHP studies. These led to the derivation of ZMAPP (c13C6 + c2G4 + c4G7) that were each produced in large quantities in the tobacco plant (*Nicotiana benthamiana*) and then made into a cocktail and has recently been shown to be effective in Ebolavirus infected NHP as long as 5 days post-exposure [64]. Thus, for the first time a highly effective therapeutic vaccine formulation has been identified.

The above studies clearly establish the fact that a number of effective preventive vaccine formulations against Ebolaviruses exist that are highly effective in the NHP and that need to be advanced to clinical trials. The recombinant adenovirus based vaccine formulation appear to be the most promising so far and have been shown to be safe in Phase I human clinical trials. The VSV based vaccines are also highly protective in NHP and are potentially the only vaccine that shows post-exposure protection. Some of these formulations should be considered for limiting spread of the virus by the vaccination of wildlife in specific geographical locations known to have outbreaks. In addition, the successful use of a cocktail of monoclonal antibodies as therapeutic tools paves the way for larger scale clinical testing of such a therapeutic vaccine formulation. Several important questions remain to be answered and these include the duration of immunity conferred by such vaccines, the mechanisms by which these vaccines confer immunity and the correlates of protective immunity and the breadth of cross protection against the several Ebolavirus species.

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