

Fibroblastic reticular cells and their role in viral hemorrhagic fevers

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Keith E Steele[†],
Arthur O Anderson
and Mansour
Mohamadzadeh

[†]Author for correspondence
Division of Pathology, US Army
Medical Research Institute of
Infectious Diseases, 1425 Porter
Street, Frederick, MD 21702,
USA

Tel.: +1 301 619 1015

Fax: +1 301 619 4627

keith.steele1@us.army.mil

Viral hemorrhagic fevers (VHFs) caused by Ebola, Marburg and Lassa viruses often manifest as multiple organ dysfunction and hemorrhagic shock with high mortality. These viruses target numerous cell types, including monocytes and dendritic cells, which are primary early targets that mediate critical pathogenetic processes. This review focuses on fibroblastic reticular cells (FRCs), another prevalent infected cell type that is known as a key regulator of circulatory and immune functions. Viral infection of FRCs could have debilitating effects in secondary lymphoid organs and various other tissues. FRCs may also contribute to the spread of these deadly viruses throughout the body. Here, we review the salient features of these VHFs and the biology of FRCs, emphasizing the potential role of these cells in VHFs and the rapid deterioration of immune and hemovascular systems that are characteristic of such acute infections.

KEYWORDS: Ebola virus • fibroblastic reticular cell • Lassa virus • Marburg virus • viral hemorrhagic fever

The viral hemorrhagic fevers (VHFs) are a group of acute, multisystemic illnesses caused by several lipid-enveloped, single-stranded RNA viruses. Prominent among the causative VHF viruses are the filoviruses Ebola virus (EBOV) and Marburg virus (MARV), and the arenavirus Lassa virus (LASV). All three of these pathogens require working with biosafety level-4 conditions. Outbreaks of EBOV and MARV are sporadic and typically cause no more than a few hundred human cases; however, the mortality rate can reach 90% of infected individuals [1–3]. The filoviruses are also of great concern as agents of biowarfare or bioterrorism [3]. In contrast to the filoviruses, LASV causes many thousands of cases each year, but has a much lower mortality rate [4]. In human cases involving these three viruses and in experimentally infected animals, key pathological changes may include lymphoid damage, coagulopathy and induction of a so-called ‘cytokine storm’ [5–14]. Another important finding is the tropism of EBOV, MARV and LASV for monocytes, macrophages and dendritic cells (DCs) [15–22], a feature also shared by some other VHF viruses. We previously showed that EBOV demonstrates strong tropism for the fibroblastic reticular cell (FRC) [16,18,23,24], a cell type whose biological functions have become the subject of increasing interest. Recently, we

extended this finding to also include FRC targeting by LASV and MARV. Based on the emerging paradigm of FRC function that has resulted from our research studies and others, we believe that FRC infection may contribute in important ways to the pathogenesis of EBOV in particular, and possibly other VHFs as well. We thus focus on the immunoregulatory and hemovascular roles that these cells could serve in the pathogenesis of VHFs.

Filoviruses

The viral family *Filoviridae* is named for the filamentous structure of its members MARV and EBOV [25]. It is further divided taxonomically into two genera (*Marburgvirus* and *Ebolavirus*) containing one species of MARV and four species of EBOV [26]. Furthermore, there are natural variants in each species, for example, strains of MARV that diverge by as much as 22% in the amino acid sequences of their glycoprotein (GP) molecules [27,28]. The deadliest species of EBOV, known as *Zaire ebolavirus*, is the most completely studied. Both MARV and EBOV are negative-stranded, nonsegmented viruses, with genomes of 19 kb that encode seven proteins. The genome is ordered as: 3′-leader, nucleoprotein, virion protein (VP)35, VP40, GP, VP30, VP24, L protein and 5′-trailer. Among MARV

and EBOV proteins, there is some sequence homology between individual proteins of the two viruses, but immunological cross-reactivity has yet to be documented [29]. However, the structures of these viral proteins seem to be similar and, furthermore, homologs can functionally substitute among filoviral species [30,31]. It has been previously demonstrated that GP can be translated in several forms based on the RNA editing in EBOV (but not MARV) followed by post-translational cleavage events and abundant *N*-linked and *O*-linked glycosylation [32–35].

Filovirus diseases

Filovirus infections in humans often present with initial symptoms that are readily mistaken for many other diseases. Despite the labeling of MARV and EBOV as 'VHFs', patients afflicted by these and other viruses so-called do not uniformly show overt hemorrhage, even in advanced stages, and many of the symptoms may differ among patients [36]. Initial symptoms, including sudden headache and muscle ache, are followed hours later by fever, nausea and vomiting, and watery diarrhea. During the peak of the infection, many patients have exhibited confusion and mental instability. On days 5–8 of infection, a distinctive nonitching, maculopapular rash typically develops, progressing from the face to the trunk and extremities. Disease progression has further been characterized by an early lymphopenia, followed by an elevated number of atypical leukocytes. The liver is a key target organ of the filoviruses and peak elevations in liver enzymes frequently occur within 6–9 days. Subsequently, 30–50% of infected individuals show hemorrhagic symptoms. Infected patients often succumb due to cardiovascular failure or cerebral coma within 8–16 days of infection. In the Kikwit EBOV outbreak of 1995, patients who survived the infection showed nonspecific fever, asthenia, diarrhea, headaches, myalgia, arthralgia, vomiting and abdominal pain. Overall, bleeding signs were observed in less than 45% of the infected patients [37]. Furthermore, coagulopathies have long been observed as a major feature of MARV and EBOV infection in humans and animal models, such as nonhuman primates (NHPs) [10,12,13,38–41]. Coagulation deficits usually occur late in the course of the disease and correlate with the onset of liver damage [40,42,43]. It has also been shown that therapeutic intervention targeted at the coagulopathy was somewhat useful in an experimental NHP model of EBOV infection [11].

Current status of vaccines & treatments

Despite some success in animal models, it remains the case that no licensed vaccines or specific treatments for either MARV or EBOV are available [29,36,44–46]. The most common protein employed for various experimental vaccine platforms is GP. It shows immune potency evidenced by promising animal survival rates, including NHPs infected with either MARV or EBOV [42,47–50]. In lower animal models, such as rodents, additional filovirus antigens have also induced protection, as discussed previously [29,46,51]. Nonetheless, numerous vaccine platforms show protection in NHPs, mice, and guinea pigs infected with MARV or EBOV [29,42,46–48,50–55]. In therapeutic studies, partial and thus far only prophylactic success has been achieved in NHPs by direct attack upon viral genomes

with antisense compounds [56], and as noted above by treating one of the symptoms, coagulopathy [11]. As with vaccines, additional therapeutic strategies have proven effective in mice or guinea pigs but are not yet proven in NHPs; these include treatment with virus-specific antibodies [42,57–59], interferons [60] and other compounds [61].

Immunity & the hurdles

Clearance of microbial infections depends on properly regulated inflammatory, coinhibitory and costimulatory signals induced by component cells of the innate immune system. In this context, DCs loaded with immunogenic peptides along with critical costimulatory molecules and inflammatory cytokines such as IL-12 activate CD4⁺ T cells that in turn induce optimal CD8⁺ T-cell activation [62]. This regulated inflammatory and costimulatory dialog among the immune cells then determines the quality of an efficient immune response that leads to complete microbial clearance. However, microbes subvert such a regulated immunity by utilizing various strategies to prevail over the host defense. Accordingly, within days, filoviruses initiate a severe acute infection that manipulates various host cellular and molecular pathways through which innate and T-cell-mediated immunity will be profoundly dampened, resulting in unbalanced and dysfunctional immunity that permits disease progression that can culminate in death [62]. For example, the most complete evaluations of human EBOV disease in recent years centered, in part, upon analyses of the differences between individuals who survived disease and those who succumbed. Key features exhibited in patients for whom the infection would ultimately prove fatal included a failure to develop adaptive immunity and the lack of induction of several cytokines of early innate immune responses, whereas survivors had detectable virus-specific IgM along with transient upregulation of primary inflammatory cytokines such as IL-6, IL-1 β , TNF- α and other cytokines [5–7,63]. Furthermore, it has been found that monocytes, macrophages and DCs, but not lymphocytes, are among the first to become infected with filoviruses in NHPs [18,23,41]. In addition, it has been observed that productively infected monocytes and DCs respond quite differently to filoviral infection. In particular, EBOV-infected monocytes produced TNF- α [17], whereas DCs were functionally paralyzed, typified by an absence of both cytokine production and DC maturation [64,65]. The effects upon DCs were shown to be associated with the capacity of VP35 of either MARV or EBOV to ablate IFN- α production by DCs, and moreover were shown to result in a diminished DC function to induce T-cell activation [64]. Finally, whether as a cause or consequence of immune dysregulation and inflammation, lymphocyte apoptosis occurs intravascularly in humans [6,7] and in lymphoid organs in NHPs during acute filoviral infection [66]. This immunological feature is still under intensive scrutiny.

Arenaviruses

The family *Arenaviridae* includes the rodent-borne, causative agents of several VHFs, such as the old-world virus LASV and several new-world viruses including Junin, Machupo and Guanarito viruses.

It also includes the prototype lymphocytic choriomeningitis virus (LCMV), although by convention it is not included among the VHF. As opposed to many other VHFs that occur as sporadic outbreaks, LASV is highly prevalent in west Africa, affecting as many as 300,000 individuals each year [4]. The case–fatality rate for Lassa fever is typically around 1–2% of infected individuals overall, although the mortality rate may be much higher in some outbreaks or among hospitalized patients and pregnant women [2,4,67]. There are known genetic and serological variants of LASV that may help account for differences in mortality rate among various outbreaks. Mortality rates associated with the new-world arenaviruses are often much higher than with LASV. An outbreak of Guanarito virus caused 33% mortality [68] and an outbreak of Machupo virus resulted in 41% mortality [69]. The numbers of humans affected by the new-world arenaviruses, however, is much less than with LASV, therefore LASV is typically considered to be of greater concern. It is also worth pointing out that new-world arenaviruses are judged to be potential biological warfare agents [69].

LASV disease & pathogenesis

The clinical presentation of Lassa fever is typically one of febrile, influenza-like illness [70]. Some patients may exhibit pharyngitis, cardiopulmonary signs, facial edema or neurological manifestations. Multifocal hepatic necrosis is the main histological lesion, and interstitial pneumonia and edema in multiple organs have also been described [71,72]. Obvious hemorrhage and coagulopathy are infrequently manifested in LASV, even in terminal cases [70], although fibrin deposition in the marginal zone of the spleen has been reported [73]. Edema, rather than overt hemorrhage, seems to be more typical of human cases of LASV [2,70]. As is true of the filoviruses, LASV targets cells of monocytic lineage and DCs [65,74]. Similar to EBOV, LASV infection of human monocyte-derived DCs failed to induce production of proinflammatory cytokines [15,65]. Unlike the filoviruses, however, LASV infection of macrophages did not result in upregulation of TNF- α [75]. Similar to the filoviruses, there are no approved vaccines to protect humans against LASV [67].

A number of NHP and guinea pig models of LASV have been described that typify features of the human disease [76]. It is worth noting that the LASV models have not been as extensively characterized as have the animal models of filoviruses. Nonetheless, as with the filoviruses, the liver is a major target of LASV in NHPs, producing elevated hepatocellular enzymes and resulting in hepatic necrosis [76,77]. Interstitial pneumonia is also a common finding in NHPs infected with LASV, and in the guinea pig model, interstitial pneumonia is actually the major histological lesion [78]. Lymphoid depletion is another important features of LASV in animal models. In the marmoset model, the loss of lymphocytes has been shown to involve both T and B lymphocytes [76].

Fibroblastic reticular cells

The complex 3D architecture of secondary lymphoid organs (SLOs), and the functional role of the mesenchymal components that make up the supportive structure of these organs, have been largely overlooked until recently. One component of lymphoid

architecture, the FRC, has become the subject of increased interest. Although many of the morphological and ultrastructural features of FRCs were characterized previously [79–86], only recently have the functional and molecular aspects of these cells been investigated in detail. In particular, recent studies have focused on the role of FRCs in T-lymphocyte homeostasis and trafficking within secondary lymphoid tissues. Additional studies have illustrated the interactions between FRCs and other immune cells, as well as the variety of immunoregulatory molecules expressed by FRCs. Thus, they may play critical roles in the host immune response to infectious diseases.

FRCs are pleiomorphic stromal cells (FIGURE 1A & 2) that, together with extracellular molecules that they produce, form a 3D supporting scaffold in the outer lymph node (LN) and analogous areas of other peripheral lymphoid tissues. FRCs structurally and functionally define the T- and B-lymphocyte compartments of lymphoid tissues, regulate the movement of fluid and cellular constituents through them, and interact directly with T and B lymphocytes, mononuclear phagocytes, natural killer (NK) cells and DCs. Four different FRC subsets have been described, based on their location in the cortex of the LNs. These include FRCs that line cortical and paracortical sinuses, those that ensheath reticular fibers that cross the sinuses, FRCs that ensheath reticular fibers projecting from the sinuses to the high endothelial venules (HEVs), and a double layer of FRC pericytes that surround the HEVs [80,83]. Individual subsets of FRCs vary not only in their location, but also in their cell morphology [83] and in their expression of specific cell-surface molecules [87]. The FRC network thus defines key compartments of the LN evident at the histological or ultrastructural levels. One such compartment is composed of the perivenular ‘channels’ (PVCs), both the inner PVC that is the potential space between the abluminal side of the endothelium of HEVs and the inner of the two layers of pericytes and the outer PVC that lies between the inner and outer layers of FRC pericytes. A second compartment is the so-called ‘corridor’, a fluid containing paracortical space that measures 10–25 μm in diameter and contains transiting lymphocytes. These corridors are bounded by FRCs that interconnect via thin cytoplasmic processes. The third important compartment is the ‘conduit’ [79], the extracellular reticulum containing collagen fibers and extracellular matrix (ECM) components ensheathed by the same FRCs that also line the corridors. The conduit is best appreciated at the ultrastructural level. A similar FRC conduit network has been shown to exist in the spleen [88,89].

In histological sections of normal LN using routine stains, the FRCs are evident upon careful observation as small, flattened to elongate cells or stellate cells with delicate cytoplasmic processes [90]. Those that line sinuses and surround the HEVs are typically flattened, while those that ensheath reticular fibers tend to be elongate or stellate. The FRCs are frequently visualized by using one or more immunohistochemical markers. Classically, the FRCs are demonstrated by labeling of the reticular fibers upon which they lie using histochemical ‘reticulin’ stains [16,83,91], by immunolabeling of matrix components such as laminin, tenascin or keratan sulfate [91], or by labeling of the FRC conduit by injecting tracer molecules that are taken up by the conduit [80,92]. A key marker of

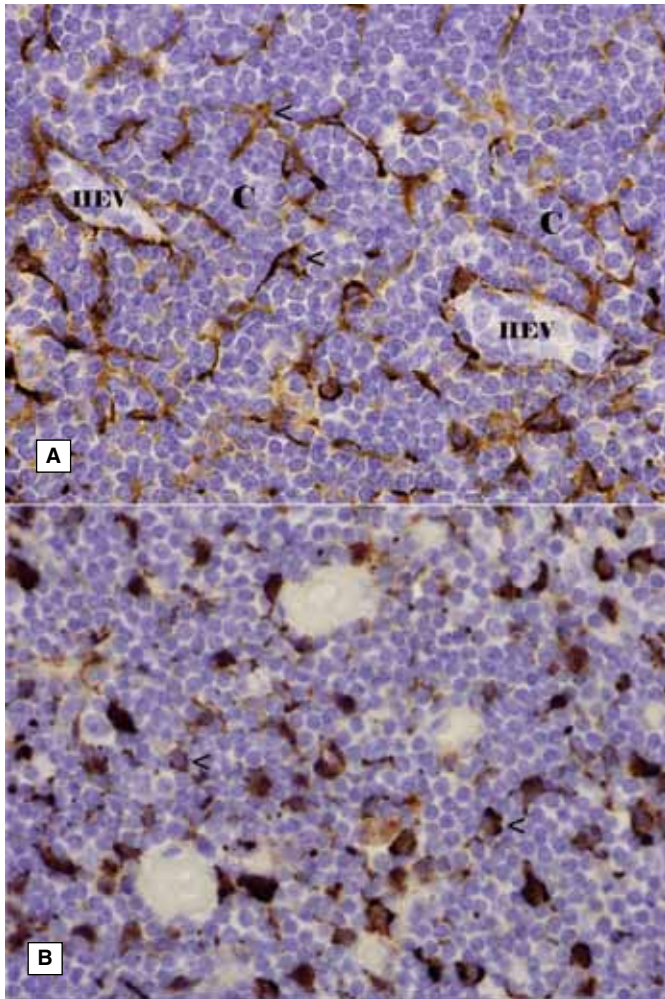


Figure 1. Fibroblastic reticular cells (FRCs) in the normal and Ebola virus-infected lymph node. (A) FRCs in the paracortex of a lymph node from a normal cynomolgus macaque express tissue transglutaminase. Note how branching triangular to stellate FRCs (arrowheads) define the corridors within which are transiting lymphocytes. Furthermore, note the presence of flat FRCs surrounding the HEV. **(B)** Many degenerate or dead FRCs are evident in the paracortex of a lymph node from a cynomolgus macaque infected with Ebola virus Zaire and euthanized on day 7 postinfection. Remaining viable FRCs have attenuated cytoplasmic processes (arrowheads). Note the lack of identifiable corridors (compared with **A**). Immunohistochemical labeling using a monoclonal antibody against tissue transglutaminase (Therm Fisher Scientific, Fremont, CA, USA), hematoxylin counterstain. C: Corridor; HEV: High endothelial venule.

the FRC itself in the mouse is the Erasmus University Rotterdam-thymic reticulum antibody 7 (ER-TR7) [93–95]. However, ER-TR7 does not label FRCs of some other species, including humans. Other reported markers of FRCs include fibronectin, Meca-79, vimentin, smooth-muscle actin, desmin and gp38 [80,83,92,96,97]. We found that FRCs in multiple species of NHPs also express the p75 NGF receptor (p75NGFr) [98], a member of the TNF receptor family [99]. The expression of particular FRC markers may be influenced by the species involved, the anatomic subset and the

activation state of the FRCs. Another aspect of FRCs important to their function is that they are highly polarized. While the basal side is intimately associated with ECM molecules, the apical surface of FRCs neither expresses nor binds ECM molecules except fibronectin [92]. The apical surface does, however, display integral membrane proteins, such as VCAM-1, or tethered proteins, such as hyaluronate [91,100,101].

A variety of additional molecules are expressed by FRCs. For example, mouse FRCs express the chemokine CXCL16 [102]. Cultured FRCs have also been shown to express CCL2/MCP-1, CCL19 and CCL21, as well as cytokines such as IL-6, IL-7 and IL-33 [87,103,104]. Indeed, FRCs were shown to be the main source of IL-7, CCL19 and CCL21 in SLOs [87]. Additionally, LN FRCs express the adhesion molecules VCAM-1, ICAM-1, as well as BP-3, PDGF-R α , PDGF-R β , LT β -R, TNF-R1 and tissue transglutaminase (TTG). Such mediators have the potential to affect various aspects of the immune response, in particular T-lymphocyte recruitment and homeostasis. For example, CXCL16 was shown to mediate the adherence of CD8⁺ T cells to FRCs via CXCR6 in a TNF-dependent fashion [102]. Both CCL19 and CCL21 contribute to adhesion of lymphocytes to HEV by binding to CCR7 [105], and conceivably they mediate adhesion to FRC as well. CCL21 has also been shown to direct the movement of naive CD4⁺ and CD8⁺ T cells into SLOs [106]. Further, IL-7 and CCL19 are critical factors that promote T-cell survival [87,107,108]. Mature DCs upregulate CCR7 and are also responsive to CCL19 and CCL21 [109]. The important role of FRCs in lymphocyte survival was shown *in vitro*, whereby isolated FRCs were able to maintain the viability of naive T cells, but other LN stromal or hematopoietic cell types did not [87]. It is also important that the role of FRCs in promoting lymphocyte survival solely affected T cells; they did not promote B-cell survival. However, the upregulation of TTG in FRCs seen in humans with reactive LNs associated with germinal center formation and also in cultured FRCs that were stimulated with IL-4 suggests an alternative role for FRCs in the regulation of B-cell responses [110]. We have observed that FRCs in the NHP LN and spleen strongly express TTG [98], as shown in **FIGURE 1**. The diverse array of molecules reported to be expressed by FRCs is summarized in **FIGURE 2**.

Moreover, a study in mice using intravital microscopy and other methods showed that FRCs actively control the access of naive T lymphocytes to the LN paracortex and also regulate T-cell migration within the paracortex [93]. Importantly, it has been shown that T cells crawl through the corridors by interacting with FRCs or their processes, turning back towards the paracortex at the T-cell/B-cell border. This study showed that B cells transiting the paracortex also do so by interacting with FRCs; however, unlike T cells, they continue into the follicular area where they then associate with follicular DCs. Interestingly, follicular DCs have been shown to express gp38 and the fibroblast antigen AS02, which is evidence of their possible fibroblastic reticular origin [87,111]. T lymphocytes in the spleen similarly traffic along FRCs of the marginal zone ‘bridging channels’ and in the periarteriolar lymphoid sheath (PALS) [112]. NK cells also appear to traffic along FRCs in the spleen [113]. Several studies have illustrated the

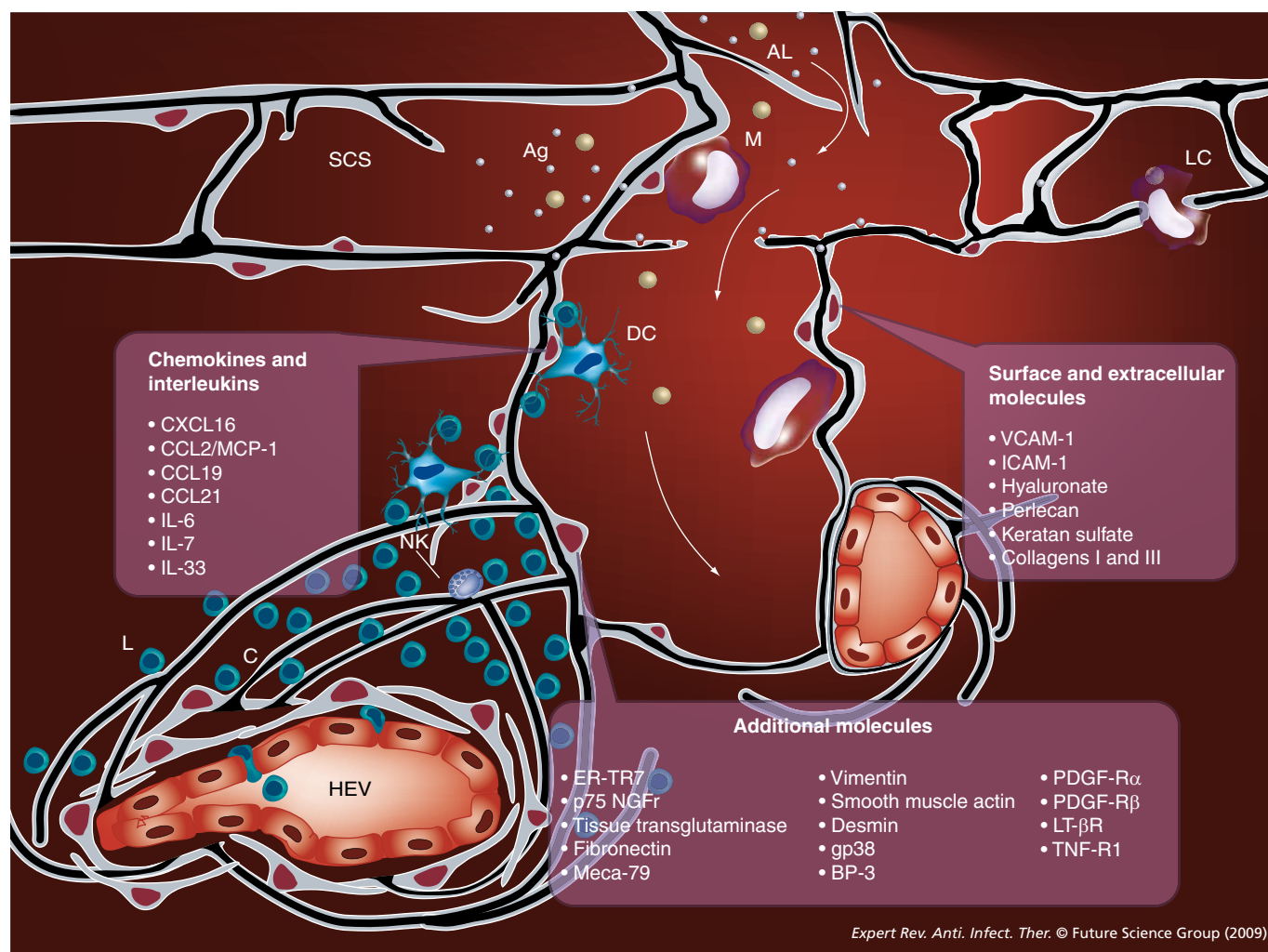


Figure 2. Architecture of the fibroblastic reticulum and molecules expressed by fibroblastic reticular cells. Fibroblastic reticular cell locations and other anatomical features typical of the outer lymph node as discussed in the text are shown. In addition, fibroblastic reticular cells have been reported to express a variety of molecules related to immune function and additional functions, as listed. Furthermore, low-molecular-weight Ags (small spheres) are selectively conducted through the 'conduits' (black lines ensheathed by fibroblastic reticular cells), whereas high-molecular-weight Ags (larger spheres) passively diffuse through the sinuses or are processed by macrophages and LCs.

AL; Afferent lymph; Ag; Antigen; BP-3; Binding protein 3; C; Corridor; CCL; Chemokine ligand; CXCL; Chemokine (C-X-C motif) ligand; DC; Dendritic cell; ER-TR7; Erasmus University Rotterdam-thymic reticulum antibody 7; gp38; Glycoprotein, 38 kDa; HEV; High endothelial venule; ICAM; Intercellular adhesion molecule; L; Lymphocyte; LC; Langerhans cell; LT- β R; Lymphotoxin β receptor; MCP; Monocyte chemoattractant protein; Meca79; Monoclonal antibody designate Meca79; NK; Natural killer cell; NGFr; Nerve growth factor receptor; PDGF-R; Platelet-derived growth factor receptor; SCS; Subcapsular sinus; TNF-R; Tumor necrosis factor receptor; VCAM; Vascular cell adhesion molecule.

relationship between resident DCs and FRCs [84,85,87,91–93,114,115]. Resident DCs in the paracortex maintain a close relationship with conduit-lining FRCs and also contact the conduit itself in specialized regions. This association with FRCs facilitates the encounter between DCs and T cells as the T cells transit along the fibroblastic reticulum. Furthermore, FRCs are critical in directing the movement of soluble molecules within the LNs. The FRCs that line sinuses and ensheath conduits are tightly associated, providing an effective barrier against the passive movement of molecules into the parenchyma. Studies have shown, however, that low-molecular-weight (low-MW) soluble antigens entering LNs via the afferent lymph are captured by FRCs and conveyed to the conduit core by

transcytosis, and are then rapidly transported to the HEV [80,84,114]. Conversely, high-MW antigens drain to the efferent lymph via the sinuses. Such low-MW molecules capable of transport via the FRC conduit include cytokines, chemokines and other inflammatory factors [80,92,105,114,116]. This kind of rapid transport system can serve multiple functions. First, the FRC conduit can deliver molecules to the HEV necessary to alter lymphocyte recruitment. Second, as shown recently, conduit-associated DCs can take up and process soluble antigens transported via the FRC conduit [92]. This provides an additional means of antigen presentation to T lymphocytes, distinct from that which occurs via migrating, epidermal Langerhans' cells that capture antigen peripherally, then process

and carry it to LNs [92,117,118]. Not only do these two means of antigen delivery and presentation differ temporally, the FRC conduit being the faster of the two, they also differ in the induction of the T-cell responses [92,118]. A similar FRC conduit system that regulates the movement of soluble molecules in the spleen has also been described [89]. Finally, it is also worth noting that the tight sheath around the conduit normally provided by FRCs forms an effective barrier that prevents coagulation factors in the blood and lymph from making contact with the collagen fibers in the conduit core, thus preventing coagulation and depletion of clotting factors.

In recently reported studies, FRCs of mice were shown to be strong targets of LCMV [94,106,119]. In particular, FRCs were targeted by strains WE and CL-13, considered to have intermediate-to-chronic replication kinetics, whereas the acute Armstrong strain of LCMV infected few FRCs. Interestingly, LCMV strain WE causes lethal hemorrhagic fever in rhesus macaques [120,121], in contrast to the avirulent Armstrong strain. Infection of mouse FRCs by both the WE and CL-13 strains led to their destruction by CD8⁺ T cells, resulting in disruption of SLO integrity, loss of FRC conduit function, and the ability of mice to respond to secondary antigens. These recent studies have contributed to our understanding of the effect of FRC damage on the immune system, as we discuss later. It is also worth noting that murine cytomegalovirus has been reported as the most recent addition to viruses that target FRCs [122].

Infection of FRCs by hemorrhagic fever viruses

Multiple studies in our laboratory have provided evidence of FRC targeting by EBOV [16,18,23,24]. In the serial sacrifice studies among these involving mice, guinea pigs and macaques, FRCs were observed to be infected at the same time, or just after, infection of macrophages [18,23]. In other animal studies of EBOV at our laboratory and elsewhere, it is likely that infection of FRCs *per se* was not specifically investigated so the exact timing of FRC infection probably requires further investigation. Our recent study reported that FRCs of serially sacrificed NHPs are also targets of MARV and LASV [98]. These combined studies showed that infection of FRCs by EBOV in particular is an early event and rapidly leads to FRC degeneration [16,18,23,24,98]. FRC infection and damage also appeared to correlate with the onset of lymphocytolysis and a general disruption of lymphoid architecture, caused disruption of lymphoid corridors (FIGURE 1B) and resulted in unsheathed conduit elements (e.g., collagen) left exposed to soluble constituents. We also showed that FRCs are targets of LASV in a limited study of NHPs. However, LASV infection appeared much less cytopathic to FRCs than EBOV, similar to the lack of direct cytopathic effect upon FRCs exhibited by the related arenavirus LCMV [94,119]. Also unlike EBOV, LASV infection of FRCs was not associated with lymphocytolysis in the few macaques we examined, in apparent contrast to other reports [70,76]. Inherent in these studies is the notion that FRC infection adds to the viral load and possibly also serves as a means of viral dissemination within lymphoid tissues. Multiple studies with MARV-infected NHPs and guinea pigs indicated that infection of FRCs was an infrequent and late event with this virus. With MARV, viral antigens were more typically associated with FRC conduits than with the cells themselves [98].

Another key finding of our studies relevant to the pathogenesis of VHF was the distinct deposition of fibrin upon FRCs in the spleens of NHPs infected with EBOV, LASV and MARV. Fibrin deposited especially along FRCs of the marginal zone, as well as the PALS, in a pattern indicating *in situ* formation. In addition, we observed fibrin in thin, linear extracellular profiles in FRC locations, possibly representing deposition along exposed collagen of the FRC conduits owing to damage of the FRC ensheathment. We further observed that fibrin deposition on FRCs might be the result of either virus-induced damage or indirect FRC activation. While our combined studies have begun to show that FRCs are targets of these VHF viruses and sustain pathological changes, either as the result of direct infection or by indirect means, much work remains to fully demonstrate the extent of FRC infection and the exact consequences of infection or activation. The studies involving FRC infection by LCMV reported recently [94,106,119] exemplify in some ways the kinds of studies still needed for EBOV, MARV, LASV and possibly other VHF agents. However, such investigations will be challenging given the safety and security requirements and scientific difficulties of performing animal studies with these lethal viruses in maximum biocontainment. Especially needed, in our opinion, are in-depth and well-timed serial sacrifice studies to characterize changes in FRCs throughout the course of infection by VHF viruses.

Potential role of FRCs in the pathogenesis of VHF

In recent years, a picture of FRC form and function has emerged, which provides a much better understanding of the role this cell type plays in multiple biological processes. Here we consider the current state of knowledge regarding FRCs in the context of our understanding of VHFs in order to highlight possible mechanisms by which FRCs may contribute to the complex pathogenesis of these multisystemic infections. In particular, we focus our attention on four essential aspects of FRC function: their structural role in the architecture of secondary lymphoid tissues, their contribution to the homeostasis of T lymphocytes, their contribution to other aspects of innate and acquired immunity, and their contribution to hemovascular dysfunction, including coagulopathy and edema formation.

As reviewed earlier, various studies have demonstrated the structural contribution of FRCs to lymphoid architecture, their ability to physically interact with a variety of immune cells and their role in specifically directing and controlling lymphocyte trafficking and acting as a conduit for the movement of cytokines and chemokines. Thus, damage to FRCs has the potential to alter critical aspects of the immune response to infectious agents. This could affect the ability to mount an effective immune response to the primary pathogen that caused the damage, or could result in more generalized immunosuppression with decreased ability to respond to secondary antigens, in a manner recently attributed to FRC damage by LCMV infection [94,119] and addressed previously. In our own studies using three specific VHF viruses, we observed physical changes in FRCs that ranged from simple attenuation of cells and loss of cytoplasmic processes to outright

degeneration and loss of cells (FIGURE 1B). The more severe changes were seen in particular in NHPs infected with EBOV. In either case, physical alteration of FRCs resulted in disruption of the so-called 'corridors' through which immune cells pass. We have also provided evidence of physical damage to the FRC 'conduit' [98]. Therefore, it follows that physical changes to FRCs may alter the immune processes that involve the movement of cells and soluble molecules through the fibroblastic reticulum, in accordance with the extent and type of FRC damage. For example, it has been shown that T and B lymphocytes crawl along the surface of FRCs of secondary lymphoid tissues in order to transit to domains where necessary immune processes occur [93]. It is therefore reasonable that T and B lymphocytes would be subject to undirected movement whenever they encounter areas of FRC damage, resulting in delayed or otherwise ineffectual immune interactions. Other studies have shown that NK cells also traffic along FRCs [113], and that resident DCs physically interact with FRCs [84,85,87,91–93,114,115]. Therefore, FRC damage might also alter immune interactions involving these key cell types. It is also worth considering that less severe FRC changes, such as reduced expression of surface molecules necessary for specific interactions with immune cells, could contribute to immune dysfunction. Examples of molecules expressed by FRCs that might apply in this sense include CXCL16, ICAM-1 and VCAM-1. We are not aware, however, of any studies demonstrating such molecular changes in FRCs owing to infection by VHF viruses. Finally, because the movement of soluble molecules through SLOs also requires a structurally intact FRC conduit [80,92,105,114,116], FRC damage in a manner we have shown for EBOV [98] could affect this critical function too.

Another important role of FRCs relevant to this discussion is their contribution to the homeostasis of T lymphocytes. This is a point of particular relevance to the pathogenesis of VHFs, as lymphocyte destruction is often a key feature of these diseases [6,10,16,66,76,123,124]. Lymphocyte destruction with the filoviruses has been attributed to so-called 'bystander apoptosis', with a number of factors possibly involved. We propose that alteration of FRCs in the course of filovirus infection might contribute to lymphocyte damage in ways not yet shown, but that could involve homeostatic factors produced by FRCs. Importantly, IL-7 and CCL19 are specific factors known to promote T-cell survival and FRCs have been shown to be the main source of IL-7 and CCL19 in SLOs [87,107,108]. It is noteworthy that FRC damage subsequent to LCMV infection of mice correlates with reduced splenic expression of CCL19 and IL-7 as well as reduced levels of the chemokine CCL21 [106,119]. With regard to the potential role that reduced levels of these molecules might play in the immune dysfunction that occurs with filoviruses and other VHFs, two points are worth considering. First is the exact nature and extent to which FRC damage occurs with each of the VHF viruses we have studied and second is the degree to which such damage might reduce available levels of these factors. As previously stated, we have not fully quantified the extent of FRC infection by EBOV, MARV and LASV, nor have we or others demonstrated alteration of T-lymphocyte homeostatic factors in FRCs during

the course of these infections. Another consideration is the general significance of T-lymphocyte homeostatic factors relative to acute virus infections, such as the filoviruses. In other words, even if reduced levels of IL-7 and CCL19 do occur as a result of FRC damage due to EBOV, MARV or LASV, it can be argued that this would have limited consequence for T lymphocyte homeostasis during the course of viral infections that can kill their hosts within a matter of days, as opposed to more chronic viral infections such as LCMV. On the other hand, reduced levels of IL-7 and CCL19, combined with alterations in other immune factors such as reduced costimulatory molecules, might contribute to T-lymphocyte destruction in substantial ways.

Fibroblastic reticular cells could also influence other aspects of immune function in VHFs. For example, it was recently shown that FRCs constitutively express IL-33 [103], an IL-1-like cytokine that activates primary inflammatory cytokines, Th2 lymphocytes and mast cells. The role that this molecule, which is associated with Th2 protective immunity against parasite infection, may play in regard to an acutely lethal virus remains to be explored. However, its effect on mast cells, whose products are known to affect hemodynamic function and coagulation, could have direct relevance to the pathogenesis of VHFs given that hemodynamic dysfunction and coagulopathy are defining features of the most severe manifestations of these conditions. Another role ascribed to IL-33 is its ability to serve as an 'alarmin' – that is, an endogenous signal to the immune system of a possible infection. In this regard, it is intriguing that IL-33 is expressed by FRCs, which stand at the interface with numerous immune system cells and also encounter soluble molecules entering the LNs via passage through the FRC conduits. An additional example is provided by reports of chemokine production by FRCs. FRC production of CXCL16, CCL2/MCP-1, CCL19 and CCL21 [87,102,104,106] implies that alteration of their production in the course of viral infection could inhibit T-lymphocyte recruitment in some VHFs.

Also worth noting is the possible role of FRCs with regard to coagulopathy and vascular dysfunction. As previously mentioned, FRCs ensheath reticular fibers of LNs and spleen that are composed of types I and III collagen fibers along with additional ECM molecules. Because fibrillar collagens can induce clotting when exposed to factors present in blood and lymph, the tight ensheathment of conduit collagens is an important feature of normal lymphoid architecture. However, as we have shown, this ensheathment is disrupted as the result of damage to FRCs caused by EBOV [98], raising the possibility that clotting occurs along damaged reticulum. Our studies also demonstrated that FRCs themselves serve as *nidi* for fibrin deposition in the spleens of NHPs infected with EBOV, MARV and LASV. One possible mechanism for this phenomenon is the strong expression of TTG by FRCs. TTG, similar to its better known relative transglutaminase XIII, has been shown to promote coagulation [125,126]. Therefore, the promotion of coagulation in VHFs could involve two FRC-related phenomena, which combined could contribute to the procoagulant state in addition to that which has previously been shown to be mediated by overexpression of tissue factor by monocytes and macrophages [12]. Finally, viral damage of the FRC pericytes that

surround and support blood vessels may also result in the development of tissue edema, another common finding in VHFs and a particular feature of Lassa fever. Pericytes, whether those qualifying as FRCs in lymphoid tissues or related cells in a variety of other organs, including those lining sinusoids in the liver and adrenal glands, play an important role in maintaining vascular integrity, especially with respect to small caliber vessels [127]. Thus, damage to these cells caused by virus infection eventually will cause the vessel wall to break down as the endothelium loses its ability to contain the blood flowing through its lumen.

Conclusion

In summary, it is implied that various events, whether early innate immune responses, events at the junction between innate and adaptive immunity, or terminal multisystemic dysfunction, are pivotal in determining the outcome of VHFs. In humans and animal models, it appears the normal crosstalk between cells of innate and adaptive immunity is subverted by EBOV, MARV, and LASV, leading to a delayed adaptive immune response, unimpeded viral replication, and even an exacerbation of disease, as monocytes and neutrophils are continuously triggered to release cytokines. In addition to vaccines and specific antiviral therapies, attention has turned toward treatments that may tilt early events in favor of the adaptive immunity ultimately required for viral clearance. However, once the viral burden is increased and disease is overt, it is also desirable to mitigate the onslaught of inflammation and dysfunctional coagulation until adaptive immunity develops and viral replication is reversed. Thus, a coordinated approach that seeks to understand both early and late host-pathogen relationships is needed in order to devise medical

interventions for filoviruses and other viral diseases that share pathogenetic mechanisms. To that end, it is now clear that FRCs are an important cellular target of EBOV, MARV and LASV, although the exact manifestation of FRC damage caused by each virus appears to vary. This knowledge, combined with the current understanding of FRC structure and function, permits reasonable speculation about ways that FRC dysfunction could play critical roles in the pathogenesis of these viruses. Furthermore, it is conceivable that FRC infection by other viruses, related or unrelated to VHF, may have been overlooked, and that FRC dysfunction could play an important role in additional viral diseases.

Expert commentary

The pathogenesis of VHF caused by filoviruses and several other RNA viruses is complicated, involving infection of multiple cell types, multisystem dysfunction and a diverse set of host factors. The role of the monocytic lineage, including infection of monocytes and DCs, as well as the host factors elaborated by these cells, have been shown to affect key aspects of the progression of VHF; nonetheless, the complex host-pathogen interactions involved in these diseases remain to be fully explored. Accordingly, it is necessary to investigate other aspects of the pathogenesis of VHFs in order to identify avenues for the development of improved medical countermeasures. In this regard, we have shown FRCs to be targets of EBOV, MARV and LASV, and shown that FRC infection or indirect FRC alteration correlates with lymphoid tissue damage and the localized deposition of fibrin. FRCs provide the architectural backbone of secondary lymphoid tissues; direct the trafficking of DCs, NK cells, mononuclear phagocytes and T/B lymphocytes; and also produce homeostatic and

Key issues

- Ebola virus (EBOV), Marburg virus (MARV) and Lassa virus (LASV) are important agents of viral hemorrhagic fever (VHF), manifesting as lethal multisystemic dysfunction, shock and/or hemorrhagic diathesis in the most severe cases.
- Key pathogenetic features of these viruses include targeting of liver and lymphoid tissues, tropism for mononuclear phagocytes and dendritic cells and indirect lymphocyte destruction.
- Immune dysfunction in infected humans and animals, especially with EBOV, is characterized by suppression of innate and T-cell immunity, bystander lymphocyte apoptosis, and the elaboration of several inflammatory mediators that contribute to a 'cytokine storm'.
- Fibroblastic reticular cells (FRCs) constitute a critical 3D supporting structure to T-lymphocyte areas of secondary lymphoid organs, composed of interconnected cells that line lymphoid sinuses, surround high endothelial venules, delineate 'corridors' and ensheath the extracellular reticular 'conduit'. Analogous cells in other tissues, such as liver and the adrenal glands, have similar form and function.
- Key FRC functions include directing the movement of T and B lymphocytes, mononuclear phagocytes, dendritic cells and natural killer cells; the movement of soluble molecules; the elaboration of factors that promote T-lymphocyte homeostasis; and the prevention of coagulation.
- FRCs express a variety of molecules such as ER-TR7, fibronectin, Meca-79, vimentin, smooth-muscle actin, desmin, gp38, p75 NGF receptor, VCAM-1, ICAM-1, hyaluronate, CXCL16, CCL2/MCP-1, CCL19, CCL21, IL-6, IL-7, IL-33, BP-3, PDGF-R α , PDGF-R β , LT β -R, TNF-R1 and tissue transglutaminase.
- FRCs are known targets of EBOV, MARV and LASV, as well as some strains of lymphocytic choriomeningitis virus and murine cytomegalovirus, sustaining different degrees of damage by each virus.
- FRC damage by EBOV is particularly severe, manifested overtly by FRC attenuation and death, disruption of the FRC conduit and exposure of extracellular collagen, disturbance of FRC corridors and the deposition of fibrin.
- Direct targeting of FRCs by EBOV, MARV and LASV or their indirect alteration could potentially contribute to multiple pathogenetic features of these viruses, including immune suppression, lymphocyte damage, coagulopathy and the development of tissue edema.
- Additional studies needed to more fully characterize the consequences of FRC targeting by EBOV, MARV and LASV will be challenging for these lethal biosafety-level-4 pathogens.

other soluble factors central to T-lymphocyte function. FRCs and related cells also are involved in maintaining hemovascular integrity. Whether due to direct viral infection or indirect alteration during the course of infection by hemorrhagic fever viruses, FRCs thus have the potential to contribute to the pathogenesis of VHF in critical ways.

Five-year view

Future studies should confirm the role of FRCs in the progression of viral infection with filoviruses and other hemorrhagic fever viruses and the contribution of FRCs as a host factor to the development of VHF. Such studies should reveal opportunities to cultivate medical interventions needed for these severe infectious diseases, in particular regarding a means of effectively managing the host immune response and controlling the development of coagulopathy and hemorrhagic diathesis. We further suspect that additional viruses will be shown to target FRCs and that FRCs will be demonstrated to be key immune cells that affect the pathogenesis of these additional viruses as well.

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Affiliations

- Keith E Steele, DVM, PhD, Diplomate ACVP
Colonel, US Army, Chief, Division of Pathology, US Army Medical Research Institute of Infectious Diseases, 1425 Porter Street, Frederick, MD 21702, USA
Tel.: +1 301 619 1015
Fax: +1 301 619 4627
keith.steele1@us.army.mil
- Arthur O Anderson, MD
Director, Office of Human Use and Ethics, Research Integrity Officer US Army Medical Research Institute of Infectious Diseases, 1425 Porter Street, Fort Detrick, Frederick, MD 21702-5011, USA
Tel.: +1 301 619 4723
Fax: +1 301 619 1250
arthur.anderson2@amedd.army.mil
- Mansour Mohamadzadeh, PhD
Associate Professor of Medicine & Immunology-Microbiology Department of Medicine Northwestern, Feinberg School of Medicine, 303 East Chicago, Searle 10:526, Chicago, IL 60611, USA
Tel.: +1 312 503 2693
Fax: +1 312 502 8253
m.zadeh@northwestern.edu