



Fibroblastic reticular cell infection by hemorrhagic fever viruses

Viral hemorrhagic fevers (VHFs) often cause high mortality with high infectivity, multiorgan failure, shock and hemorrhagic diathesis. Fibroblastic reticular cells (FRCs) within secondary lymphoid organs provide a supporting scaffold to T-lymphocyte areas. These cells regulate the movement of various immune cells and soluble molecules that promote T-lymphocyte homeostasis. We previously reported Ebola virus infection of FRCs, but ascribed little significance to this finding. Here, we studied infection of FRCs by Ebola, Marburg and Lassa viruses. We demonstrate that FRCs, or the extracellular 'conduit' of the fibroblastic reticulum of nonhuman primates, are targets of Ebola, Marburg and Lassa viruses. Furthermore, we observed that FRC damage correlates temporally and spatially with lymphocyte damage and that FRCs serve as nidi of fibrin deposition. In addition, we show that nonhuman primate FRCs express p75 NGF receptor and tissue transglutaminase. Our data suggest that viral infection of FRCs may be crucial to the immunological dysfunction and coagulopathy characteristic of VHFs. We further propose that p75 NGF receptor and tissue transglutaminase may be involved in FRC-associated dysfunction during the course of infection.

KEYWORDS: dendritic cell ■ Ebola ■ fibroblastic reticular cell ■ hemorrhagic fever ■ Lassa ■ lymphoid organ ■ Marburg ■ virus

Keith E Steele[†],
Arthur O Anderson
& Mansour
Mohamadzadeh¹

[†]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
¹Northwestern Medical School,
Chicago, IL, USA

Diseases caused by the filoviruses Ebola (EBOV) and Marburg (MARV), and the arenavirus Lassa (LASV), are some of the most lethal of the so-called viral hemorrhagic fevers (VHFs), a set of acute, multisystemic illnesses caused by several lipid-enveloped, ssRNA viruses. Key features of VHFs evident in human infections and their applicable animal models include damage to secondary lymphoid organs, widespread coagulopathy, and induction of a 'cytokine storm' that may lead to septic shock [1–11]. Another important feature of VHFs, including EBOV, MARV and LASV, is a shared tropism for monocytic lineages, including macrophages and dendritic cells (DCs) [12–19]. In addition, we previously demonstrated that EBOV shows significant tropism for fibroblastic reticular cells (FRCs) [13,15,20,21].

Architecturally, FRCs are pleiomorphic stromal cells situated in a variety of tissues. They comprise four FRC subsets within the outer lymph node (LN) where they provide a 3D supporting scaffold, define the T- and B-lymphocyte compartments, direct the movement of fluid and cellular constituents, provide homeostatic factors for T lymphocytes and, finally, interact directly with T and B lymphocytes, natural killer (NK) cells and DCs [22–34]. The four described subsets of FRCs include cortical sinus-lining cells, sinus-crossing cells, paracortical FRCs that delineate the so-called 'corridors' through which lymphocytes traffic and a double layer

of pericytes that surround the high endothelial venules (HEVs). FRCs exhibiting similar structure and functions have also been described in the spleen and other lymphoid tissues [35–39]. Consistent with the various locations where FRCs are found and the diverse functions they perform, FRCs express a number of molecules, including IL-7, CCL19, CCL21 [32], CXCL16, CCL2/MCP-1, IL-6 [32,40,41], VCAM-1, ICAM-1, BP-3, PDGF-R α , PDGF-R β , LT β -R, TNF-R1, tissue transglutaminase (TTG), the Erasmus University Rotterdam-thymic reticulum antibody 7 (ER-TR7), fibronectin, Meca-79, vimentin, smooth-muscle actin, desmin and gp38 [23,27,34,42–45]. It was previously reported that FRCs via TTG regulate LN function [44], leading us to include its examination in this study. The specific molecules expressed by FRCs and the particular functions they perform may depend on the species involved, their anatomic location or their state of activation. The concept of FRC function originally suggested by anatomical studies and increasingly supported by recent functional and molecular investigations, is that FRCs are central to the greatly ordered micro-architecture of secondary lymphoid organs, serve multiple roles in promoting the survival of T and B cells, regulate T- and B-cell trafficking, and dictate the movement and presentation of foreign antigens. They may thus play pivotal roles in the host immune response to infectious diseases,

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as shown recently for strains of lymphocytic choriomeningitis virus (LCMV) [46,47]. FRCs may also be involved in preventing coagulopathy. Accordingly, FRCs ensheath reticular fibers [22] composed of types 1 and 3 collagen, basement membrane components and extracellular proteoglycans such as keratan sulfate and perlecan in tissues receiving abundant flow of blood or lymph, each of which is capable of clotting. As long as the collagen fibers remain ensheathed, the FRCs will prevent exposure of the procoagulant molecules of the reticulum to clotting factors present in the blood or lymph. In light of the recent picture of FRC immunobiology that has emerged and in consideration of key features of disease seen in VHF, we provide additional evidence of FRC infection with viruses such as EBOV, MARV and LASV. In the present study, we examined archival pathology tissues from a variety of nonhuman primate (NHP) studies to provide evidence of FRC infection by EBOV, MARV and LASV. Thus, these findings in the context of FRC biology may shed light on the pathogenesis of these deadly viruses.

Material & methods

■ Animal studies

We examined histology, immunohistochemistry (IHC), *in situ* hybridization and electron microscopy (EM) samples from NHP studies with EBOV, MARV and LASV in the pathology archives of the United States Army Medical Research Institute for Infectious Diseases (USAMRIID). All studies involved protocols approved by the Institute Animal Care and Use Committee and research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals. Experiments involving animals adhered to principles stated in the Guide for the Care and Use of Laboratory Animals, National Research Council, 1996. USAMRIID is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. The cases selected for analysis were either from viral pathogenesis studies or were infected, untreated controls in several vaccine or efficacy studies. All selected cases included in this report developed clinical signs or gross lesions considered typical of the respective viruses. The EBOV and MARV studies both included cynomolgus and rhesus macaques and African green monkeys inoculated intramuscularly or by aerosol with lethal doses (10–1000 plaque forming units [pfu]) of viruses. Studies with EBOV used the Zaire

strain and studies with MARV used the Ci67 and Angola strains. Infected NHPs were either euthanatized at selected time points postinfection (PI) or died terminally. Some of these studies have been previously reported [13,15,48–51]. In addition, only a single limited study of LASV was available that involved six cynomolgus macaques inoculated with 5×10^3 pfu of the Josiah strain of LASV [HENSLEY L, UNPUBLISHED DATA]. Three of these macaques were euthanatized at day 7 and the other three were euthanatized at days 13, 15 or 17 PI.

■ Histology, immunohistochemistry & electron microscopy

In all cases, tissues were fixed in 10% neutral-buffered formalin for a minimum of 21 days. Subsequently, routine histological processing was performed. Examined tissue sections were stained by hematoxylin and eosin (HE) or by IHC or *in situ* hybridization. IHC was used to identify cells expressing EBOV, MARV or LASV antigens or to detect fibrin II deposition. In addition, we used IHC to identify two particular molecules in FRCs of uninfected NHP tissues, the p75 NGF receptor (NGFR) and TTG. Specific features of the IHC procedures are summarized in TABLE 1 or are described elsewhere [13,51]. Samples for EM were also processed using routine methods as described in these two reports.

Results

■ FRC expression of p75 NGFR & TTG

We observed that FRCs in multiple species of NHPs were positive for p75 NGFR, a member of the TNF receptor (TNFR) family [52]. On the basis of their morphology and location, the p75 NGFR-specific antibody-stained cells were identified as FRCs in the LN (FIGURE 1A), including sinus-lining cells, sinus-crossing cells, HEV pericytes and FRCs forming corridors in the cortex. Thus, all four subtypes of FRCs described previously were positive for this marker [23,27]. In addition, we observed p75 NGFR-positive cells morphologically similar to FRCs in the periarterial lymphoid sheath (PALS) of the spleen (FIGURE 1B), and in the tonsils and gut-associated lymphoid tissues (GALT) (FIGURE 1C). In these areas, the stained FRCs outlined lymphocyte-filled spaces that correlate with the corridors in LNs as previously described [27]. Our findings are also consistent with the reports of the presence of FRCs or FRC-like cells in secondary lymphoid tissues other than the LNs [35,36,53–56]. The p75 NGFR antibody also stained fibroblast-like cells

Table 1. Summary of immunohistochemical methods.

Antigen	mAb clone (supplier)	IHC method	Pretreatment	Antibody dilution
P75 NGFR, human	NGFR5 (Abcam, Cambridge, MA, USA)	IPO, IFA	ARTE	1:500 (IPO); 1:100 (IFA)
Tissue transglutaminase, guinea pig	CUB 7402 (Thermo Fisher Scientific, Fremont, CA, USA)	IPO	ARTE	1:2000
Fibrin II, human (B β 15–42)	T2G1 (Accurate Chemical, Westbury, NY, USA)	IPO	ARTE	1:100
EBOV, GP/VP40*	X-FB05-BA11/B-MD04-BD07-AE11 (USAMRIID, USA)	IPO, AP	PK	1:5000–1:8000
MARV, NP/unknown* (reacts with Ci67)	X-BB06-BB01/X-AA05-BC03 (USAMRIID, USA)	IPO	PK	1:1200
MARV (reacts with Angola)	MBGIII-507 (Hensley L, USAMRIID, USA)	IPO	ARTE	1:750
LASV, Gp1	L52–121–22-BA02 (C. Rossi, USAMRIID)	IPO	ARTE	1:15000

*EBOV immunostain performed using a cocktail of antibodies to GP and VP40 and MARV immunostain for Ci67 performed using a cocktail of antibodies to NP and an uncharacterized antigen.
AP: Alkaline phosphatase method; ARTE: Antigen retrieval, EBOV: Ebola virus; GP: Glycoprotein; IFA: Immunofluorescent antibody; IHC: Immunohistochemistry; IPO: Immunoperoxidase method (Envision System, DAKO Cytomation, Carpinteria, CA, USA); LASV: Lassa virus; mAb: Monoclonal antibody (all antibodies were mAbs, IgG, class); MARV: Marburg virus; NGFR: NGF receptor; NP: Nucleoprotein; PK: Proteinase K; USAMRIID: United States Army Medical Research Institute for Infectious Diseases; VP40: Viral protein 40 kDa.

in the lamina propria of the gut and the stroma of other parenchymal tissues of naive NHPs, as well as pericytes in various tissues (FIGURE 1C). Interestingly, these cells are unlike the FRCs of the lymphoid tissues in that histologically they did not appear to form corridors, nor did they necessarily associated with lymphocytes. Importantly, these p75 NGFR-positive cells, similar to the p75 NGFR-expressing FRCs of lymphoid tissues, were consistent targets of filoviruses [13,15,20,49,57].

As noted above, it has been reported recently that TTG may be an important means through which FRCs affect LN regulatory function [44]. We observed that FRCs in secondary lymphoid tissues of naive NHPs strongly expressed TTG. Indeed, they were the major cell type expressing TTG in the NHP LNs (FIGURE 1D). All four FRC subtypes were positive for TTG. Moderate-to-strong expression of TTG was evident in endothelial cells and many cells residing in germinal centers of the follicles, possibly representing follicular DCs. In the spleen, TTG expression was pronounced in FRCs of the PALS and the marginal zone and moderate-to-strong expression was typical of endothelial cells and cells in germinal centers and the red pulp (data not shown).

■ FRC infection by EBOV

We previously reported that EBOV infects FRCs of NHPs, guinea pigs and mice, especially FRCs of LNs, but also FRCs of tonsils and GALT [13,15,20,21]. In the present study, we re-examined tissues from previously reported NHP studies and from additional NHPs

infected with EBOV and confirmed that FRC infection was a consistent finding in a variety of lymphoid tissues of NHPs that were euthanized in the terminal stage of EBOV infection, typically from 6 to 8 days PI. In particular, EBOV-infected FRC pericytes associated with HEVs (FIGURE 2A), FRCs in the paracortex of LNs, as well as those in the tonsils and GALT, consistent with previous reports. [13,15]. In addition, we observed that FRCs of the splenic marginal zone were commonly infected. However, sinus-lining and sinus-crossing cells of LNs and FRCs of the splenic white pulp were less frequently infected with EBOV. Infected FRCs in terminally infected NHPs were often degenerate (FIGURE 2B) and were associated with lymphocytolysis and a general disruption of the lymphoid architecture. In addition to FRCs, EBOV antigen was also observed in additional cell types reported to be targets of this virus, in particular cells morphologically consistent with macrophages and DCs [13,15,20,21].

In a serial pathogenesis study of EBOV-infected NHPs, infection of FRCs was previously demonstrated to be an early event [15]. Using IHC and *in situ* hybridization to identify viral infection, infected FRCs were present as early as day 3 PI in the splenic marginal zone and day 4 in LNs and the splenic PALS. Our current studies show that although in most lymphoid tissues, infected FRCs were outnumbered by macrophages containing EBOV antigen, in some LNs the FRCs were the predominant infected cells (data not shown). By EM, we also observed that virus particles were present within the collagen fibers of the so-called

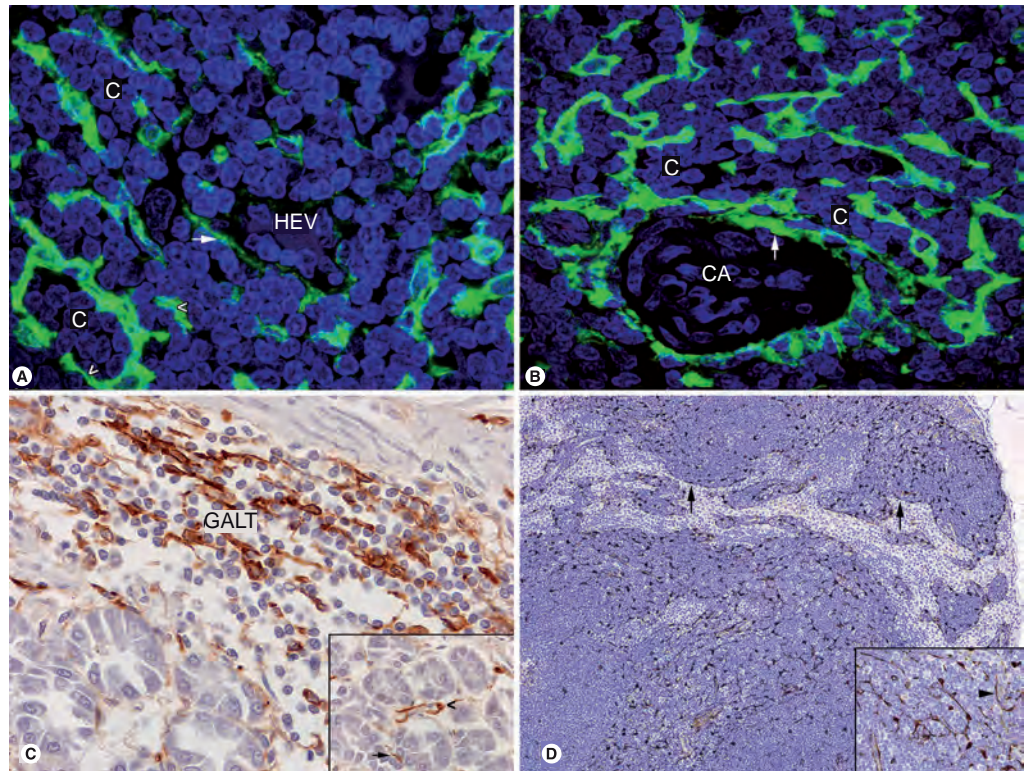


Figure 1. Fibroblastic reticular cells in normal nonhuman primate secondary lymphoid organs.

(A) Fibroblastic reticular cells (FRCs) in the paracortex of a lymph node (LN) from a macaque express low-affinity p75 NGF receptor (NGFR) in this confocal microscopic image. Note how branching FRCs define the corridors within which are transiting lymphocytes (blue nuclei). Also note the presence of flat FRC processes surrounding the HEV (pericytes – arrow) and the thin, linear, unlabeled areas corresponding to the extracellular ‘conduits’ (arrowhead). (B) The periarteriolar lymphoid sheath of the spleen shows architecture similar to the LN paracortex, including p75 NGFR + branching FRCs outlining the corridors and flattened pericytes (arrow). (C) p75 NGFR-labeled FRCs in the GALT are similar to those in the spleen and LNs. Inset: Note the presence of p75 NGFR-positive pericytes around a capillary (arrowhead) and individual fibroblastic cells in the lamina propria (arrow). (D) Transglutaminase expression is evident in numerous FRCs of a LN, including those lining sinuses (arrows), surrounding HEVs, and forming corridors. Inset: higher magnification showing the detail of tissue transglutaminase-expressing FRCs forming conduits and surrounding HEVs (arrow). (A,B) Immunofluorescence labeling using a monoclonal antibody against p75 NGFR (DAKO Cytomation, Carpinteria, CA, USA); 4',6-diamidino-2-phenylindole (DAPI) counterstain. (C,D) Immunoperoxidase stain using the p75 NGFR antibody (C) and a monoclonal antibody to guinea pig transglutaminase II (D), clone CUB 7402 (Thermo Fisher Scientific, Fremont, CA, USA); hematoxylin counterstain.

C: Corridor; CA: Central arteriole; GALT: Gut-associated lymphoid tissue; HEV: High endothelial venule.

conduit core [22], whereupon the surrounding FRCs were not infected (FIGURE 2C). This finding suggests that EBOV may migrate through the conduit from afferent lymph in a fashion that could facilitate rapid viral transport to the paracortex and the HEVs. We further observed that FRCs succumbed to infection early, as degenerate immunolabeled FRCs were evident histologically by day 4 PI, shortly after infection. At later timepoints, increased numbers of infected and degenerate FRCs appeared to correlate with the onset of lymphocytolysis. In a previous report, ultrastructural evidence of degeneration of EBOV-infected FRCs in terminal-stage NHPs varied from damage of mitochondria and other organelles to

increased autophagosomes and dissolution of nuclear and plasma membranes [13]. Related to those findings, our current studies show that ultrastructural damage to FRCs by EBOV also results in the presence of conduit components (e.g., collagen bundles) free of their FRC sheath (FIGURE 2D); and by inference loss of cell surface moieties (e.g., costimulatory molecules such as hyaluronate-CD44) needed to prevent apoptosis of activated T or B cells.

■ FRC infection by Lassa virus

In a pilot study of LASV-infected cynomolgus macaques, we observed that FRCs were consistently infected with LASV in a variety of lymphoid tissues using IHC to detect virus antigen

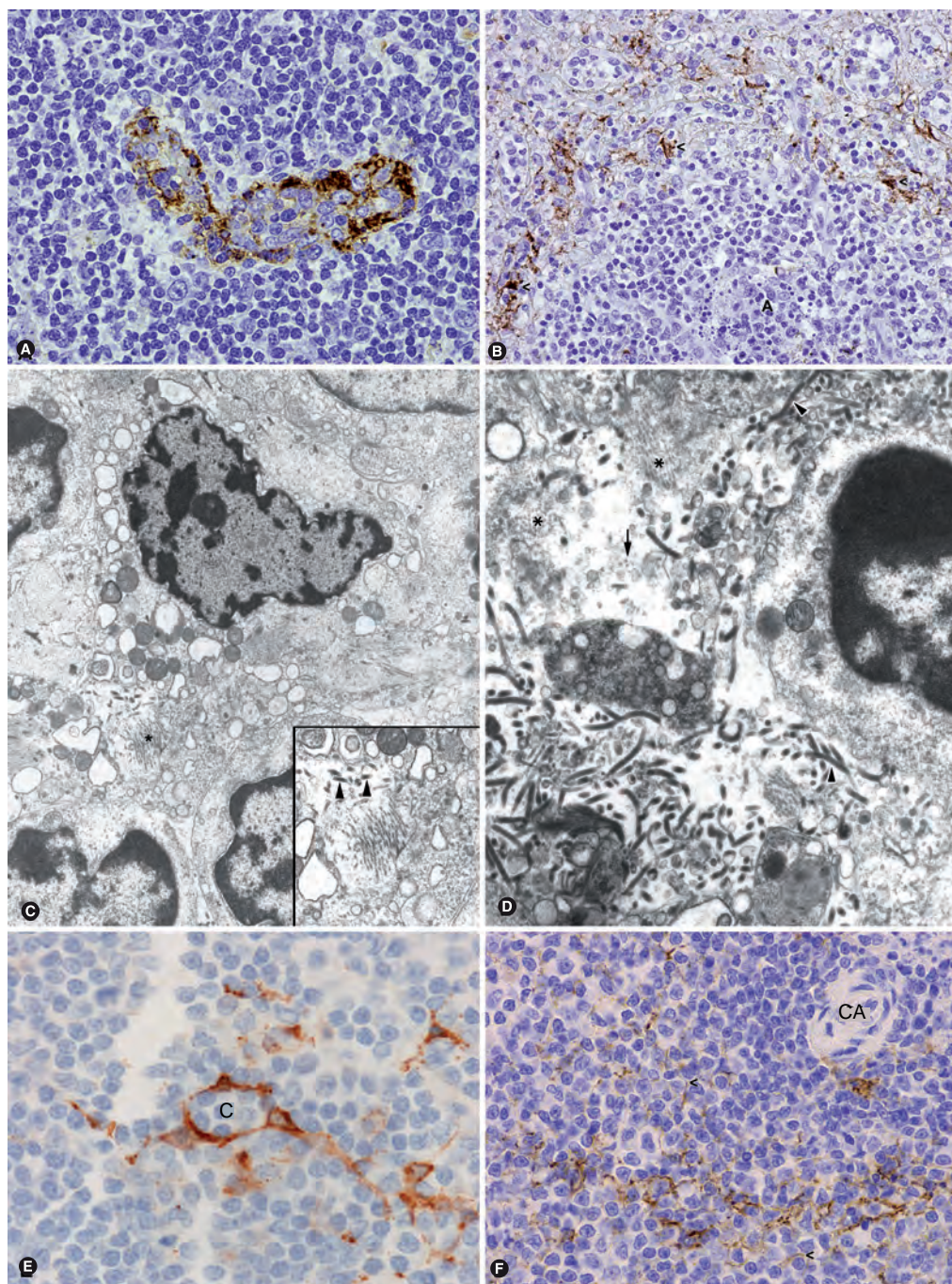


Figure 2. Infection of fibroblastic reticular cells by Ebola, Lassa and Marburg viruses. (A) Ebola virus (EBOV) antigen indicates infection of several fibroblastic reticular cell (FRC) pericytes of the high endothelial venule (HEV) in the lymph node paracortex of a macaque inoculated with EBOV Zaire and euthanized at day 8 postinfection (PI). **(B)** EBOV infection is evident in several FRCs of the marginal zone of the spleen of a macaque inoculated with EBOV Zaire and euthanized at day 7 PI. Most of these infected FRCs are degenerate, typified by shrunken cells lacking structural detail (arrowhead). Note the presence of a focus of apoptotic lymphocytes in the periarteriolar lymphoid sheath. **(C)** Electron micrograph (EM) showing EBOV within the collagen fibers of the conduit core (*), but in the absence of infected FRCs that ensheath the conduits. Inset: Higher magnification of the affected area shows the detail of the collagen fibers of the conduit and nearby EBOV particles (arrowhead). Also note some swollen mitochondria in the cytoplasm of the FRC and the presence of an uninfected lymphocyte. **(D)** EM showing disruption of the fibroblastic reticulum and the presence of exposed collagen bundles (*) and individual collagen fibers (arrow). Numerous EBOV particles are present (arrowheads). **(E)** Lassa virus (LASV) infection is evident in several FRCs of the paracortex of a lymph node from a macaque inoculated with LASV Josiah and euthanized at day 7 PI. Infected FRCs are nondegenerate and outline a corridor containing lymphocytes. **(F)** Marburg virus (MARV) antigen is present as numerous thin linear profiles (arrowhead) in the splenic periarteriolar lymphoid sheath of a macaque inoculated with MARV Angola and euthanized at day 9 PI. This linear staining pattern suggests antigen is present within the FRC 'conduit' rather than indicating infection of the FRCs themselves. **(A,B,E,F)** Immunoperoxidase stain using combined monoclonal antibodies to EBOV glycoprotein (GP) and viral protein 40 kDa proteins **(A,B)**, a monoclonal antibody to LASV GP1 **(E)** and a monoclonal antibody to MARV GP **(F)**; hematoxylin counterstain. C: Corridor; CA: Central arteriole.

in cells (FIGURE 2E). As with EBOV, LASV-infected FRCs included those surrounding HEVs, and also FRCs lining sinuses and corridors in the paracortex of LNs. Infected FRCs were also seen in corresponding areas of the GALT, and in the marginal zone and PALS of the spleen. Furthermore, similar to EBOV, FRCs in the marginal zone of the spleen were strongly infected by LASV. LASV antigen was also observed in cells of lymphoid tissues that resembled macrophages and DCs. The apparent consequences of FRC infection by LASV, however, were less severe than those seen with EBOV infection. Whereas FRCs were consistently infected with LASV at day 7, only one of the three NHPs euthanized on days 13 to 17 showed LASV-infected FRCs. Interestingly, viral antigens were abundant in other locations of these three terminally ill NHPs, including the liver, which indicates that LASV-infected FRC in particular appeared to be a transient process. Furthermore, the majority of LASV-infected FRCs seemed morphologically intact and lymphocytolysis was not a prominent feature of the lymphoid tissues of these macaques. Indeed, it is noteworthy that lymphoid hyperplasia was a prominent finding in all the LASV-infected cases, both at day 7 and the end stage of infection (data not shown). Given the few animals included in this study, a more complete study of LASV-infected NHPs with additional time points may be required to cement these observations of LASV-infected FRC. Nonetheless, the differences between EBOV and LASV observed in the present studies highlight a potentially critical distinction between these two VHFs, one that could provide the basis for additional studies necessary to elucidate the specific mechanisms of immune dysregulation that could be induced during the course of infection by these viruses.

FRC infection by MARV

In contrast to EBOV and LASV, data from NHPs infected with MARV indicate that infection of FRCs was an infrequent and late event. Analyzing selected tissues from a serial sacrifice study in NHPs [48], we observed that few if any infected FRCs were present in spleen and LNs of macaques euthanized in the early and middle stages of MARV infection. Nonetheless, MARV antigens were present infrequently in FRC locations of the lymphoid tissues of NHPs euthanized late in the course of the disease or terminally ill when the viral burden was high (FIGURE 2F). However, MARV antigen was not readily evident in the cytoplasm of FRCs,

as was typical for EBOV- and LASV-infected FRCs, and MARV-infected macrophages and DCs. Instead, MARV antigen appeared as thin linear profiles, suggesting the uptake of viral antigens by the FRC conduit rather than infection of FRCs *per se*. To support these findings of limited MARV FRC infection in NHPs, we then examined numerous immunostained lymphoid tissues of MARV-infected guinea pigs from a serial sacrifice pathogenesis study [REED D ET AL., MANUSCRIPT IN PREPARATION]. These tissues confirmed that FRC infection by MARV in that species was also a rare event (data not shown).

■ Fibroblastic reticular cells & coagulopathy

Multiple lines of reasoning led us to investigate the possibility that FRCs might contribute to coagulopathy. First, coagulopathy is a key feature of VHF. Second, FRCs are present in the locations where they may readily come in contact with blood constituents during pathological conditions, especially in the marginal zone of the spleen, where the process of filtration of blood begins. Finally, FRCs ensheath the collagen bundles of reticular fibers and prevent direct exposure of collagen to blood and lymph. Therefore, disruption of this FRC barrier could lead to coagulation through the action of tissue factor.

We thus examined fibrin deposition by IHC in the spleens of NHPs infected with all three viruses. Fibrin II, the product of thrombin-induced cleavage of fibrinopeptides A and B from the fibrinogen molecule, was evident in the spleens of NHPs infected with all three viruses (FIGURE 3). In particular, fibrin II was often localized primarily in an FRC pattern in the marginal zone and occasionally in a similar manner in the PALS. In these locations, the fibrin frequently decorated the cell bodies and cell processes of FRCs. This appearance was in clear contrast to the amorphous, extracellular fibrin clots that were also present in the splenic red pulp or which filled the lumens of some vessels. The pattern of fibrin deposition on FRCs indicates that fibrin monomer or multimers may be formed *in situ*. In addition to the fibrin staining of FRCs, we also observed fibrin in the spleens of virus-infected animals as thin, linear extracellular profiles in resident FRCs, consistent with localization along FRC conduits.

Interestingly, the presence of deposited fibrin associated with FRCs of MARV-infected NHPs was somewhat surprising in light of our observation that MARV-infected FRCs appeared to be uncommon. We further noted, however,

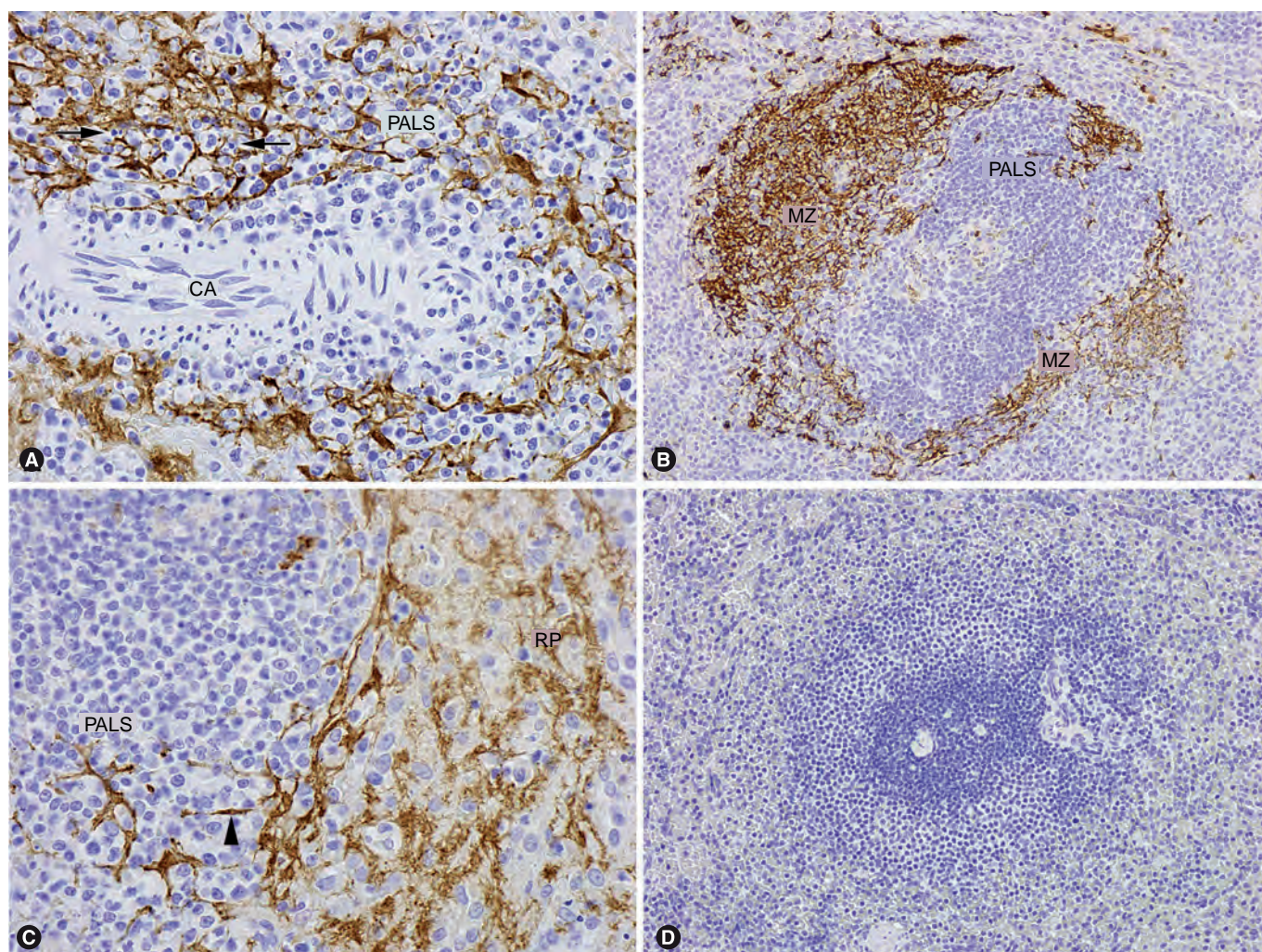


Figure 3. Fibrin deposition along splenic fibroblastic reticular cells of Ebola, Lassa and Marburg viruses. Fibrin is deposited in a pattern that recapitulates the location of fibroblastic reticular cells (FRCs) in the PALS and MZ of the spleens of macaques inoculated with Ebola virus Zaire (**A**, day 10 postinfection [PI]), Lassa virus Josiah (**B**, day 7 PI) and Marburg virus strain Ci67 (**C**, day 10 PI). In (**A**), note that a semblance of corridor architecture is maintained, but some corridors are collapsed and contain apoptotic lymphocytes (arrows). In (**C**), compare the smooth, FRC fibrin localization (arrowhead) to the amorphous clumps of fibrin in the red pulp. (**D**) Negative control, a splenic section from an uninfected (normal) macaque identically immunostained; the FRCs and other white and red pulp elements present are uniformly negative. Immunoperoxidase stain using a mouse monoclonal antibody to the N-terminal CNBr fragment of human fibrin II (B β 15–42; clone T2G1, Accurate Chemical, NY, USA), hematoxylin counterstain. CA: Central arteriole; MZ: Marginal zone; PALS: Periarteriolar lymphoid sheath; RP: Red pulp.

that fibrin was deposited both in FRC locations of EBOV- and LASV-infected animals where virus-infected FRCs were dominant in adjacent tissue sections, and additionally in FRC locations where these viruses were not present. We thus interpreted these data to indicate that fibrin deposition on FRCs may result as a consequence of virus-induced injury, at least for EBOV and LASV, but also independent of viral infection by all three viruses. Presumably, fibrin deposition resulting from FRC activation could involve TTG, which we showed is expressed in FRCs of NHPs. We subsequently interpreted the extracellular linear fibrin profiles to possibly represent fibrin deposition upon collagen in the FRC

conduit, consistent with our observation that EBOV resulted in FRC damage and exposed collagen bundles. In conclusion, FRC alterations during virus infection may lead to coagulopathy by two related means.

Discussion

The recently published studies that show the ability of FRCs to directly control immune cell trafficking and contribute to lymphocyte homeostasis, are particularly interesting in light of our previous and present findings that FRCs are targets of EBOV, MARV and LASV. Thus, infection of FRCs by these viruses may play a pivotal role in the immune dysregulation

described for VHF. Such immune dysregulation could follow along the lines of the immunosuppression associated with loss of secondary lymphoid organ structural integrity caused by LCMV infection of FRCs [46,47], or it could be mediated by other factors such as cytokines, adhesion molecules and hyaluronate typically expressed and released by FRCs involved in immune cell trafficking [24,26,32,40,41,44,58]. Nonetheless, further studies are required to dissect these possibilities. To date, the differences we noted in FRC infection between the three viruses present the opportunity for fruitful comparative studies. For example, it is particularly interesting that the greater damage inflicted on FRCs by EBOV correlated with abundant lymphocytolysis, whereas the lesser damage evident in FRCs infected with LASV was associated with preservation or even proliferation of lymphocytes. These findings suggest that more severe FRC damage caused by EBOV may contribute directly to lymphocyte loss. The matter of FRC damage by MARV, as described above, seems to be less certain.

The role that soluble factors specifically induced by filovirus infections of monocytes and macrophages play in coagulopathy has previously been emphasized [1,8–10,59]. Accordingly, our findings that FRCs in the splenic marginal zone and PALS were associated with enhanced fibrin deposition in NHPs infected with EBOV, MARV and LASV indicate that FRCs may also contribute to coagulopathy. The exact mechanisms by which these cells might contribute to coagulopathy remain to be further investigated. Nonetheless, our findings suggest that both damaged FRCs and activated FRCs may play a prominent role in fibrin deposition. The presence of exposed collagen in the fibroblastic reticulum as observed with EBOV infection is also a potentially important finding, because blood clots when directly exposed to collagen molecules. In addition, the greater damage to FRCs seen with EBOV may explain the more common finding of coagulopathy and hemorrhage seen in EBOV infected patients. Finally, viral damage of the special FRCs surrounding and supporting blood vessels that we refer to as pericytes could also contribute to the development of tissue edema, which is another common feature in VHFs.

Our observations that FRCs of NHPs express p75 NGFR and TTG are, to our knowledge, novel findings. These molecules are included in this report to illustrate the morphologic features of FRCs in NHPs and because previous studies have shown they could be relevant to VHF

pathogenesis. Accordingly, the notion that p75 NGFR might represent a mechanism by which the nervous system regulates immune responses via FRCs seems to be an intriguing possibility. Such a possibility is supported by previous studies demonstrating that nerve fibers course through the stroma to supply the spleen, the cortex/paracortex of LNs and the GALT, where they end among lymphocytes in these areas [22,55,60]. It also follows upon previous studies contributing to the developing concept of a so-called 'neuroimmune' system in which NGFs and their receptors play critical roles in immune function [61–65]. TTG is a ubiquitous, multifunctional enzyme implicated in a variety of normal and pathological processes, including apoptosis of lymphocytes and other cell types, control of endothelial cell tight junctions and cell adhesion, activation of proinflammatory cytokines, promoting sepsis, and control of localized clotting [66–69]. TTG has previously been shown to play a role in the phenotypic regulation of human LN reticular stromal cells [44]; however, the function of TTG in FRCs remains to be fully explored. Further studies are required to show any direct mechanistic relationship between p75 NGFR and TTG and the pathogenesis of VHF. In particular, changes in p75 NGFR and/or TTG expression by FRCs during the course of VHFs are areas that need to be addressed.

Conclusion

Dysfunctions of multiple body systems and a variety of viral and host factors contribute to the complicated pathogenesis of VHFs. Such dysfunctions have their most severe manifestations in the diseases caused by filovirus infections. The role of mononuclear phagocytes and DCs and their products, in particular, appear to play dominant roles in the severe disease characteristic of VHFs. However, other factors may also be critical to the pathogenesis of these conditions. It is, therefore, likely that a systems approach looking at both early and late virus–host events is required to provide the basis for developing improved medical interventions and new therapeutic options for filoviruses and other viral diseases that evoke similar pathogenetic events. Accordingly, evidence of FRC infection by EBOV, MARV and LASV, in the context of increased knowledge of this multifunctional cell provided by previous reports, has led us to conclude that FRCs may play a significant role in key aspects of VHFs. In particular, immune dysfunction and fibrin deposition as the result of lethal or sublethal

FRC damage appear to be key features of these viruses relevant to the pathogenesis of VHFs. Additional studies are required to further explore the nature of FRC infection by these viruses. In particular, an understanding of the molecular basis of FRC-mediated pathogenetic factors is required. Among other factors, p75 NGFR and TTG are molecules expressed by FRCs that require additional investigation to understand the dynamics of FRC function in health and disease.

Future perspective

We have demonstrated that FRCs are targets of EBOV, MARV and LASV, which suggests that FRCs may play a critical role in lymphoid damage and clotting in infected NHPs and, conceivably, humans as well. Based on knowledge of FRC biology highlighting their multiple roles influencing the immune system and their role in localized coagulopathy, these cells have the potential to affect multiple pathological processes in the course of VHF. Results from future studies will confirm that FRCs are key contributors to the pathogenesis of VHFs and elucidate pathogenetic mechanisms providing avenues for the development of medical countermeasures for these severe infectious diseases.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

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Executive summary

- Viral hemorrhagic fevers (VHFs) often cause high mortality with high infectivity, multiorgan failure, shock and hemorrhagic diathesis.
- Ebola (EBOV), Marburg (MARV) and Lassa (LASV) viruses are prominent VHFs for which nonhuman primates (NHPs) are key animal models used to understand pathogenesis and to develop medical countermeasures.
- Fibroblastic reticular cells (FRCs) populate secondary lymphoid organs, provide a supporting scaffold to T-lymphocyte areas, regulate the movement of various immune cells and soluble molecules and promote T-lymphocyte homeostasis.
- FRCs prevent coagulopathy by ensheathing collagen fibers in the reticulum.
- FRCs are targets of EBOV, MARV and LASV, sustaining different degrees of damage by each virus.
- FRC damage by EBOV in particular correlates with lymphocyte damage.
- FRCs represent nidi for deposition of fibrin in the spleen of NHPs infected by EBOV, MARV and LASV.
- FRC damage in the course of NHP infection by EBOV, MARV and LASV may contribute in multiple ways to the pathogenesis of these VHFs, in particular with regard to immune dysfunction and coagulopathy.
- p75 NGF and tissue transglutaminase are novel factors expressed by NHP FRCs that could also contribute to the pathogenesis of VHFs.

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