Pathogenesis of Marburg Hemorrhagic Fever in Cynomolgus Macaques

Lisa E. Hensley,1 Derron A. Alves,2 Joan B. Geisbert,3,4 Elizabeth A. Fritz,3,4 Christopher Reed,1 Tom Larsen,2 and Thomas W. Geisbert3,4

1Virology Division, and 2Pathology Division, US Army Medical Research Institute of Infectious Diseases, Fort Detrick, Maryland; 3Galveston National Laboratory, and 4Department of Microbiology and Immunology, University of Texas Medical Branch, Galveston, Texas

Background. Marburg virus (MARV) infection causes a severe and often fatal hemorrhagic disease in primates; however, little is known about the development of MARV hemorrhagic fever. In this study we evaluated the progression of MARV infection in nonhuman primates.

Methods. Eighteen cynomolgus monkeys were infected with MARV; blood and tissues were examined sequentially over an 8-day period to investigate disease pathogenesis.

Results. Disease caused by MARV in cynomolgus macaques was very similar to disease previously described for Ebola virus–infected macaques. Monocytes, macrophages, Kupffer cells, and dendritic cells (DCs) were identified as the initial targets of MARV infection. Bystander lymphocyte apoptosis occurred at early stages in the disease course in intravascular and extravascular locations. The loss of splenic and lymph node DCs or downregulation of dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) on DCs as early as day 2 and continuing through day 8 after MARV infection was a prominent finding. Evidence of disseminated intravascular coagulation was noted; however, the degree of fibrin deposition in tissues was less prominent than was reported in Ebola-infected macaques.

Conclusions. The sequence of pathogenic events identified in this study provides an understanding of the development of disease processes and also may provide new targets for rational prophylactic and chemotherapeutic interventions.

Over the past 30 years, Marburg virus (MARV) and Ebola virus (EBOV) have caused sporadic outbreaks of usually fatal hemorrhagic disease in central Africa [1, 2]. The higher frequency of EBOV outbreaks along with the higher mortality rates and importation of Reston ebolavirus into the United States in 1989 resulted in more public awareness of EBOV than MARV. However, recent large outbreaks of MARV infection in the Democratic Republic of the Congo [3] and Angola [4] with case fatality rates approaching 90%, and the importation of MARV into the Netherlands [5] and the United States [6] in 2008 by travelers who had visited Uganda, have highlighted the pathogenic potential and public health importance of MARV.

While there are no approved vaccines or post-exposure treatment modalities available for preventing or managing MARV infections, there are at least 5 different vaccine systems that have shown promise in completely protecting nonhuman primates against a lethal MARV challenge [7–11]. Because adequate and well-controlled clinical studies in humans cannot be ethically conducted and as field efficacy studies are not feasible for MARV hemorrhagic fever (HF), licensure of any of these countermeasures must be carried out under the US Food and Drug Administration (FDA) “Animal Efficacy Rule.” This policy was implemented in 2002 and allows for the evaluation of vaccines or therapeutics using data derived from studies carried out in an animal model that accurately reflects the disease observed in humans. Importantly, the animal model must be well

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* L. E. H. and D. A. A. contributed equally to this work.

Correspondence: Thomas W. Geisbert, PhD, University of Texas Medical Branch, Galveston National Laboratory 301 University Blvd, Galveston, Texas 77550-0610 (tom.geisbert@utmb.edu).

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characterized, and the pathophysiology of the disease in the animal must faithfully reproduce disease in humans.

Licensure of any vaccine or antiviral drug against MARV under the FDA Animal Rule will likely require use of a nonhuman primate model. Several nonhuman primate species have been employed to model MARV HF, including squirrel monkeys (Saimiri sciureus), African green monkeys (Chlorocebus aethiops), cynomolgus macaques (Macaca fascicularis), and rhesus macaques (Macaca mulatta) [12–19]. Similar pathologic features of MARV infection have been documented among these species; however, African green monkeys do not present with a macular cutaneous rash that is a characteristic feature of disease in macaques and man [13, 20].

A number of studies have used cynomolgus or rhesus monkeys as a model for MARV HF; however, with few exceptions, previous investigations examined infected animals euthanized when moribund and shed little light on the pathogenesis of MARV infection during times before death. The aim of this study was to characterize the early stages of MARV HF in a relevant nonhuman primate model of human disease.

MATERIALS AND METHODS

Animals and Inoculations
Healthy, adult cynomolgus macaques (Macaca fascicularis; n = 18, 5–10 kg) were used for these studies. Animals were inoculated in the caudal thigh with 1,000 plaque-forming units (PFUs) of MARV (Ci67 strain). The MARV Ci67 strain was kindly provided by Werner Slenczka and Stephan Becker [21] and has the same glycoprotein amino acid sequence as the Popp strain provided by Werner Slenczka and Stephan Becker [21].

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Hematology, Serum Biochemistry, and Coagulation Tests
Hematology and serum biochemistry were performed as previously described using a laser-based hemologic analyzer (Coulter Electronics) and a Piccolo Point-of-Care Blood Analyzer (Abaxis), respectively. Plasma levels of D-dimers and protein C were measured as detailed in other studies [22, 23]. Clotting times (prothrombin time [PT] and activated partial thromboplastin time [aPTT]) were determined using a ThromboScreen (Fisher Diagnostics) according to manufacturer’s directions.

Cytokine/Chemokine Production
Cytokine/chemokine levels in monkey plasma were assayed using a human cytokine multiplex-25 bead-array assay kit (BioSource) for the Bio-Plex 200 System (Bio-Rad) according to manufacturer’s directions. Results were recorded as positive if optical density (OD) values were greater than twice the OD of pre-MARV challenge controls. Cytokines/chemokines assayed included eotaxin, granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon (IFN)–α, IFN-γ, interleukin (IL)–1β, IL–1 receptor antagonist, IL–2, IL–2R, IL–4, IL–5, IL–6, IL–7, IL–8, IL–10, IL–12p40/p70, IL–13, IL–15, IL–17, IFN–γ–inducing protein (IP)–10, monocyte chemoattractant protein (MCP)–1, macrophage inflammatory protein (MIP)–1α, MIP–1β, monokine induced by IFN–γ (MIG), regulated upon activation normal T cell expressed and secreted (RANTES), and tumor necrosis factor (TNF)–α.

Histopathology and Immunohistochemistry
Tissues were collected and immersion-fixed in 10% neutral-buffered formalin and processed for histopathology, phosphotungstic acid hematoxylin (PTAH) staining to demonstrate polymerized fibrin [17, 22–23], and immunohistochemistry as previously described [17]. For immunohistochemistry, a mouse monoclonal antibody against MARV GP (III 5D7 [24]; 1:4,000) was employed. Negative controls included replicate sections exposed to EBOV antibodies and uninfected cynomolgus monkey tissue. Serial sections of spleen and lymph nodes were also immunostained for dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) (CD209), a C-type lectin receptor highly expressed on the surface primate dendritic cells (DCs) as previously described [25].

Automated Cellular Imaging
Sections of spleen and axillary lymph nodes from 2/3 randomly selected animals at days 2–4 and 6–8 post–MARV infection were evaluated for DC-SIGN immunostaining. Specimens obtained from 2 uninfected cynomolgus macaques were used as controls. Sections were scanned at low magnification (×10) for comprehensive image detection as well as color analysis and intensity. After scanning, both color and morphometry-based criteria were developed in order to exclude and/or avoid areas containing tissue and cellular debris and artifacts that could falsely elevate or reduce slide scores. Cells considered negative for DC-SIGN immunostaining did not exhibit dark brown cell membranes. For immunohistochemistry, a mouse monoclonal antibody against MARV GP (III 5D7 [24]; 1:4,000) was employed. Negative controls included replicate sections exposed to EBOV antibodies and uninfected cynomolgus monkey tissue. Serial sections of spleen and lymph nodes were also immunostained for dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) (CD209), a C-type lectin receptor highly expressed on the surface primate dendritic cells (DCs) as previously described [25].

Terminal Deoxynucleotidyl Transferase–Mediated Deoxyuridine Triphosphate Nick End Labeling (TUNEL) Staining
The ApopTag detection kit (S7100 kit, Chemicon) was used to detect DNA fragmentation in cells in spleen, lymph nodes, and liver as previously described [25].
Electron Microscopy
Select tissues for ultrastructural examination were immersion-fixed in 4% formaldehyde plus 1% glutaraldehyde in 0.1 mol/L Millonig’s phosphate buffer and processed for transmission electron microscopy (TEM) as previously described [25].

Virus Isolation
Infectious MARV in ethylenediaminetetraacetic acid plasma and in portions of liver, spleen, lung, kidney, adrenal gland, pancreas, heart, testis, brain, femoral bone marrow, and mandibular, mesenteric, axillary, and inguinal lymph nodes was assayed by counting plaques on Vero cell monolayers as previously described [17, 25].

RESULTS

Clinical Illness
Clinical illness was unremarkable until day 5, when 2/9 remaining animals had cutaneous rashes involving the axilla and/or groin and were anorexic. By day 6, all 9 remaining animals were anorexic, and 5/9 animals given physical examinations had fevers (defined as temperature over 102°F). These 5 febrile animals and 2 additional animals had cutaneous rashes involving the axilla and/or groin. By day 7, all 6 remaining monkeys had macular cutaneous rashes on the arms and groins, and on 2 of these animals, the rash also involved the thorax, proximal limbs, and face, particularly on the peri orbital area and forehead. Persistent bleeding at the venipuncture site was also noted in one of these 2 animals, and another animal had bleeding of the gums. All 6 remaining animals on day 7 were anorexic and showed signs of moderate to severe depression, 3/6 were recumbent, and 2/6 had diarrhea. By day 8, all 3 remaining monkeys had characteristic macular cutaneous rashes, were anorexic, showed approximately 10% dehydration (as evaluated subjectively by skin-fold retraction), and remained sitting in a hunched-over position; all showed signs of severe depression and were recumbent.

Hematology, Clinical Chemistry, and Coagulation
Total and differential white blood cell (WBC) counts showed a developing leukocytosis due to an increased neutrophilia (Figure 1). By day 7, the mean WBC count (27.7 K/UL) was 2.3 times baseline values. Neutrophils ranged from approximately 52% of the leukocyte population on day 1 to 84% on days 5 and 6 (an approximate 1.6-fold increase; Figure 1), with a marked increase in immature neutrophils (band cells). Concurrently, monocytes declined from approximately 6% on day 1 to 1% on day 6. There was a concomitant lymphopenia as lymphocytes dropped from approximately 35% of the leukocyte population on day 1 to <10% on day 6 and then appeared to slightly rebound to just less than 16% on day 8 (Figure 1). Although still within normal reference range, circulating platelet numbers declined from a mean of 323 × 10^3/mm^3 (range 100–700 × 10^3/mm^3) on day 1 to 194 × 10^3/mm^3 on day 5 (Figure 1). However, by day 8, platelet numbers returned to and exceeded day 1 levels (mean 446 × 10^3/mm^3). Development of fibrin degradation products (D-dimers) showed rapid increases of 35-fold by day 5 and 45-fold by day 7 (Figure 1). Plasma levels of protein C remained unchanged through day 7 but decreased substantially (50%) at day 8 (Figure 1). Clotting times increased slightly by day 6 from baseline means of 15.9 seconds for PT and 27.8 seconds to aPTT to 29.3 seconds and 36.6 seconds, respectively; levels increased further by day 8 to 37.6 seconds for PT and 48.1 seconds for aPTT (Figure 1).

Early serum enzyme levels were unremarkable, but many were elevated during the late stages of disease. On day 6, aspartate aminotransferase rose sharply (mean 617 IU/L), as did alanine aminotransferase (mean 178 IU/L; Figure 2). Alkaline phosphatase levels were increased nearly 4-fold over baseline values by day 6, while gamma-glutamyltransferase levels increased approximately 2.5-fold over baseline by day 7 (data not shown). Blood urea nitrogen levels remained generally within normal limits through day 7 and increased 2-fold over baseline on day 8 (Figure 2). Serum creatinine levels also remained within normal limits through day 7 and increased 3-fold over baseline on day 8 (Figure 2).

Necropsy Findings
No significant gross lesions were observed in animals euthanized at day 2. At days 3 and 4, there was minimal enlargement of one or more lymph nodes in all animals examined. Mild pyloric (2/6), ileocecal (2/6), and urinary bladder mucosal reddening (1/6) was observed by day 4. By day 6, overt axillary and inguinal lymphadenopathy (2/3) and congestion (3/3) were present. The mandibular lymph nodes were also enlarged in all 3 animals euthanized at this time point. Additional findings at day 6 included enlarged and congested spleens (3/3); enlarged, congested, or pale yellow friable livers (2/3); and pyloric and proximal duodenal mucosal congestion (2/3). By days 7 and 8, a cutaneous petechial rash was observed in most animals (5/6). Similar hepatic (6/6) and splenic (5/6) changes like those observed at day 6 were also present. Likewise, variable congestion and enlargement of the inguinal, axillary, and to a lesser extent, mandibular and mesenteric lymph nodes were also suggestive of MARV infection.

Virus Titers in Blood and Tissues
Onset of plasma viremia occurred in 3/9 animals on day 3 and in all animals (6/6) by day 4. Peak viremia (mean 8.2 log_{10} PFU/mL) occurred on day 8 (Figure 3). Infectious virus was first detected in tissues on day 3 in liver, spleen, axillary lymph node, inguinal lymph node, and mesenteric lymph node—suggesting that these organs are early sites of viral replication—and on day 4 in mandibular lymph node, kidney, adrenal gland, lung, pancreas, heart, testis, and bone marrow (Figure 3). Mean organ...
titers increased progressively and reached their highest levels (5.5–9.2 log_{10} PFU/g) on day 8. The highest titers were documented in liver, followed by spleen, adrenal gland, and various lymph nodes.

Analysis of Cytokines/Chemokines in Circulation

No detectable increases in plasma/sera levels of cytokines/chemokines were observed at early (days 1–3) or mid stages (days 4–5) of the disease course. At late stages of disease (days 6–8), increased levels of IFN-α, IL-6, MIP-1α, MIP-1β, MCP-1, and eotaxin were observed (Figure 4). Increased levels of IFN-β, IFN-γ, IL-1R, IL-2R, IL-8, IL-12 p40/p70, IL-13, and TNF-α were also observed at late stages of disease (data not shown). Increased levels of IL-1β, IL-4, IL-5, IL-7, IL-10, IL-15, IL-17, IP-10, GM-CSF, MIG, and RANTES were not detected in any animal at any time point.

Histology, Immunohistochemistry, and Ultrastructure

The principal histopathologic and immunohistochemical findings and their association with viral isolation are presented in Table 1. Temporal results of MARV-infected cells demonstrated in primary target tissues by immunohistochemistry are shown in Table 2.
Lymphoid Tissues

**Lymph Nodes.** Viral antigen was not detected in the lymph nodes until day 4, when, focally, free antigen was present in a mantle zone in a mesenteric lymph node (1/3). At day 6, sinus histiocytosis and edema were seen in some lymph nodes in the 3 animals euthanized at this time point. Few MARV-positive sinus histiocytes and free, granular antigen were detected in the medullary, subcapsular, and to a lesser extent, cortical sinuses. There was an increase in the numbers of TUNEL-positive lymphocytes and cellular debris in follicular centers and marginal zones.

By days 7 and 8, increased numbers of MARV-positive cells were observed in depleted paracortical areas. Follicular (germinal center) lymphocytolysis varying in severity with few tingible body macrophages (TBMs) was consistently observed in one or more lymph nodes. The depletion of small lymphocytes, often surrounding congested high endothelial venules (HEVs), hemorrhage, scattered TBMs, and/or lymphoblasts and plasmablasts were also frequent findings. Immunohistochemistry for MARV antigen revealed strong, free, occasionally linear immunostaining within the medullary and subcapsular sinuses; granular to linear immunostaining in mantle zones surrounding and within lymphocytolytic germinal centers; strong MARV staining associated with HEV endothelium; and occasional immunoreactive TBMs. A noticeable increase in TUNEL-positive lymphocytes and scattered TBMs with cytoplasmic TUNEL-positive debris was present especially in areas of lymphoid depletion and lymphocytolysis and within medullary and subcapsular sinuses. Ultrastructurally, the nodal architecture was clearly disrupted with cellular and necrotic debris, extravasated erythrocytes, and apoptotic lymphocytes. Free intravascular virions with small amounts of fibrin were noted particularly at day 8.

**Spleen.** Focally, free MARV antigen was observed in the red pulp of one animal at day 3. By day 4, free MARV antigen and rare MARV-positive parenchymal cells with DC morphology (Figure 5A) were localized to marginal zones, perilymphoid red pulp, and to a lesser extent, red pulp cords. A slight increase in TUNEL-positive lymphocytes in the red pulp and follicular marginal zones was observed.

By day 6, MARV antigen was present in all 3 animals in similar locations as seen at day 4. Circulating immunoreactive mononuclear cells were rare and unequivocally observed in one animal; immunoreactive parenchymal cells could not be clearly identified. Occasional circulating monocytes and red pulp macrophages with either cytoplasmic MARV inclusions or budding virions were seen ultrastructurally (Figure 5B). Mild lymphoid depletion and lymphocytolysis, mostly affecting periarteriolar lymphoid sheaths (PALS), and red pulp necrosis with numerous apoptotic lymphocytes, bodies, and debris confirmed by TUNEL staining (Figure 5C and 5D) and TEM (Figure 5E) were present in 2 animals. PTAH demonstrated infrequent deposition of polymerized fibrin in the red pulp and marginal zones.

At days 7 and 8, the splenic white and red pulp histologic changes increased in severity and had become more widespread. Lymphocytolytic PALS and follicular germinal centers consisted of numerous TUNEL-positive lymphocytes and apoptotic bodies with few or no TBMs. Red pulp necrosis and apoptosis with diffuse sinus congestion and increased circulating mononuclear and polymorphonuclear cells were also
observed. Polymerized fibrin detected by PTAH was occasion-
ally present in the red pulp and marginal zones of day 8 ani-
mals (Figure 5C). MARV immunostaining in the remain-
ing 4 animals at days 7 and 8 was similar to that observed at
day 6 but slightly more intense, especially adjacent to lym-
phocytolytic PALS. Splenic lymphocytes were consistently
MARV immunonegative.

Liver. Few to low numbers of TUNEL-positive hepatocytes
were present primarily in periportal areas at day 3 (1/3) and day 4
(3/3). Immunohistochemical evidence of hepatic MARV infec-
tion was not observed until day 4, when occasional MARV-
positive spindled sinusoidal lining cells, morphologically con-
sistent with sinusoidal Kupffer cells, were seen (1/3). Of note,
TUNEL-positive sinusoidal lining cells with similar histo-
morphologic appearance were observed in 2/3 animals.

Between days 6 and 8, MARV antigen staining, associated
with Kupffer cells, sinusoidal lining cells, and hepatocyte
membrane surfaces, increased in intensity and became more
diffuse as the infection progressed. TEM confirmed these find-
ings. Although still present in portal areas, TUNEL-positive
hepatocytes were distributed multifocally throughout the he-
patic parenchyma at day 6. Histologically, the severity of hepatic
changes varied between animals. The most noteworthy lesions
were individual hepatocellular necrosis with eosinophilic pleo-
morphic intracytoplasmic inclusions, scattered necrotic cellular
debris, and mild portal lymphoplasmacytic and neutrophilic

**Figure 3.** Plasma and organ titers. Mean infectivity of cynomolgus monkey plasma and tissue homogenates (10% wt/vol) inoculated with Marburg virus. Axil, axillary; FBoneMar, femoral bone marrow; ing, inguinal; LN, lymph node; mand, mandibular; mes, mesenteric.
inflammation. TUNEL-positive lymphocytes and free TUNEL-positive debris were present occasionally in sinusoids and congested vessels. At days 7 and 8, the histologic lesions had progressed, and the remaining hepatocytes were markedly swollen with cytoplasmic vacuolization histologically consistent with hepatocellular lipoidal degeneration.

Fibrin deposition was not a significant finding in the livers of any monkeys when examined by routine histology or PTAH.

**Other Tissues**

Results for other tissues analyzed were consistent with results previously reported for EBOV-infected macaques [25]. Histopathology and immunohistochemical findings for adrenal gland and kidney are shown in Table 1, while cells in adrenal gland positive for MARV are shown in Table 2.

**ACIS Results: Evaluation of Dendritic Cells in Spleen and Axillary Lymph Node**

DC-SIGN was evaluated in the spleen from 2/3 MARV-infected macaques at each time point to further characterize MARV infection (Figure 5G–5I). ACIS analysis quantifiably showed a decrease in DC-SIGN immunohistochemical staining at days 2 through 6 (2.07%, 1.14%, 2.03%, and 1.64%, respectively) when compared with spleens from uninfected cynomolgus macaques (6.76%). By days 7 and 8, splenic DC-SIGN immunostaining decreased markedly (0.53% and 0.41%, respectively). We also evaluated DC-SIGN immunostaining in the axillary lymph nodes in one randomly chosen animal at each time point. The highest percentage of DC-SIGN cell membrane staining in the axillary lymph node was observed at day 4 (5.32%). By day 8, ACIS analysis showed almost a 5-fold decrease (1.07%) in DC-SIGN immunostaining when compared with day 4.

**DISCUSSION**

Early studies from small outbreaks of MARV HF as well as experimental studies in laboratory animals have shown that MARV has a predilection for liver and lymphoid tissues and that disseminated intravascular coagulation (DIC) is an important feature of disease [12–19, 26–30]. Findings from our current study in MARV-infected cynomolgus macaques showed similar observations at terminal stages of disease. Importantly,
the current study provides new information during the entire course of infection that further elucidates underlying disease mechanisms and will help guide human clinical studies.

Evaluation of tissues by conventional histology, immunohistochemistry, and TEM demonstrated a regular pattern of MARV infection in cynomolgus monkeys consistent with that

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**NOTE.** Data are presented as tissue viral titer (log_{10} plaque-forming units [PFU]/g tissue), histopathologic severity (1 = minimal, 5 = severe), and immunohistochemical result (+/-). < indicates viral titer below the detectable limit of 1.7 log_{10} PFU/g tissue.

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**NOTE.** + indicates that Marburg virus (MARV) antigen-positive cells were rarely detected; + + indicates that MARV-positive cells were occasionally detected; + + + indicates that MARV-positive cells were frequently detected; and – indicates that no MARV-positive cells were detected.
previously shown for EBOV infection in cynomolgus macaques [22, 25]. MARV infection began with monocytes/macrophages and DCs in the lymphoid tissues as well as Kupffer cells and cells lining the sinusoids in liver, progressed to infection of parenchymal cells in liver and adrenal gland and HEV in lymphoid tissues, and finally spread to endothelial cells in a variety of tissues. Evidence of increased levels of apoptosis of lymphocytes was detected as early as 2 days after MARV infection.

Similar to EBOV infections, primate MARV infections are characterized by a dysregulation of the host immune response. In this study, we observed increased circulating levels of proinflammatory cytokines/chemokines including IL-6, IL-8, MCP-1, MIP-1α, MIP-1β, IL-1 receptor, IL-2 receptor, eotaxin, IL-12, IL-13, and TNF-α at late stages (days 6–8) of disease. However, the proinflammatory response seen in the MARV-infected macaques occurred later in the disease course than previously reported for EBOV infections [25].

Similar to changes noted in the proinflammatory response at end stages of disease, changes in coagulation were also noted at late stages of disease. Increases in circulating levels of D-dimers and fibrin deposition in tissues were all noted at late stages of disease. While these observations show that DIC is a clear feature
of MARV infection of cynomolgus monkeys, the development of these coagulation defects was slower and the severity was considerably less than what have previously been reported in EBOV-infected macaques [22].

In contrast to what has been observed in EBOV-infected cynomolgus macaques [25], DCs may be early targets of MARV infection; however, they may not be sustained cellular targets of MARV. Although our ACIS results are not statistically significant, these findings suggest either loss of splenic and lymph node DC or downregulation of DC-SIGN on DC as early as day 2 and continuing through day 8 after MARV infection. Similar findings were observed in MARV-Angola–infected macaques challenged by aerosol [19]. The reduction in DC-SIGN staining indicating possible DC impairment or loss may be one of the most critical events during MARV infections. We are uncertain whether this reduction in staining is a result of cell death, interference caused by MARV virions or proteins binding to DC-SIGN, or other factors possibly triggered by MARV infection such as increased levels of C-reactive protein or reduced levels of complement C3, both of which have been shown to reduce DC expression of DC-SIGN and impair DC function [31, 32].

The sequence of morphological, cytologic, virologic, serological, and inflammatory change following MARV in cynomolgus monkeys creates a useful model in the study of MARV HF and provides a basic understanding of the disease model and pathogenesis. This understanding is critical for characterizing the MARV nonhuman primate model and identifying gaps in knowledge as well as identifying critical pathways for validation with limited available human data.

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**References**