Distinct Biological Phenotypes of Marburg and Ravn Virus Infection in Macaques

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Filoviruses are among the most pathogenic infectious agents known to human, with high destructive potential, as evidenced by the recent Ebola virus epidemic in West Africa. As members of the filovirus family, marburgviruses have caused similar devastating outbreaks, albeit with lower case numbers. In this study we compare the pathogenesis of Ravn virus (RAVV) and Marburg virus (MARV) strains Angola, Musoke, and Ozolin in rhesus and cynomolgus macaques, the 2 nonhuman primate species most commonly used in filovirus research. Our results reveal the most pathogenic MARV strain to be Angola, followed by Musoke, whereas Ozolin is the least pathogenic. We also demonstrate that RAVV is highly pathogenic in cynomolgus macaques but less pathogenic in rhesus macaques. Our results demonstrate a preferential infection of endothelial cells by MARVs; in addition, analysis of tissue samples suggests that lymphocyte and hepatocyte apoptosis might play a role in MARV pathogenicity. This information expands our knowledge about pathogenicity and virulence of marburgviruses.

Keywords. Filovirus; Ravn virus; Marburg virus; nonhuman primates; macaques; pathogenesis.

Marburgvirus is a genus in the family Filoviridae along with the genera *Ebolavirus* and *Cuevavirus* and consists of a single virus species, *Marburg marburgvirus* [1]. Since its discovery in 1967, there have been 11 outbreaks caused by either Marburg virus (MARV) or Ravn virus (RAVV), the 2 representative viruses of the species. MARV caused the largest outbreak in Angola in 2005, resulting in 227 reported deaths and a case fatality rate of approximately 90% [2]. After a relatively short incubation period of 5–9 days, patients infected with MARVs develop Marburg hemorrhagic fever (MHF), which initially presents with nonspecific flulike symptoms, including fever, headache, nausea, and vomiting [3–6]. Additional clinical and biochemical parameters include elevated liver enzyme and serum creatinine levels as well as thrombocytopenia leading to disseminated intravascular coagulation, hypovolemic shock, multiorgan failure, and eventually death [3, 6–8].

MARVs and RAVV can cause life-threatening disease for which there is no licensed vaccine or treatment available; they are classified as biosafety level 4 pathogens. These circumstances make scientific experiments logistically challenging, so relatively little research data are available. The majority of existing studies use MARV Musoke and Angola, the most commonly used and best characterized strains of MARV. Isolated during an outbreak in Kenya in 1980, MARV Musoke became widely used as the prototype MARV for understanding the molecular determinants of virulence as well as for validating vaccines and therapeutics

against MARVs [9]. This changed after isolation of MARV Angola during a large outbreak in 2004–2005. With its high case fatality rate, MARV Angola was postulated to be the more virulent MARV strain and thus became the preferred strain to assess countermeasure efficacy [10]. MARV Ozolin was isolated in 1975 from a traveler in Zimbabwe who became ill and subsequently infected a healthcare worker in South Africa [11]. Since its discovery, no research using this particular MARV isolate has been published to our knowledge, raising questions about its pathogenicity. Similarly, since its discovery in 1987, very few studies have been performed using RAVV. The few studies that do exist are almost exclusively vaccine efficacy experiments involving challenge of cynomolgus macaques with RAVV [12, 13].

In the current study we compared the pathogenicity of RAVV and 3 geographically and temporally distinct MARV strains (Angola, Musoke, and Ozolin) in cynomolgus and rhesus macaques. Based on limited animal numbers, we found that MARV Angola was lethal in both macaque species, whereas no disease and lethality were associated with MARV Ozolin in either species. However, MARV Musoke showed delayed disease progression in rhesus macaques and resulted in only partial lethality in cynomolgus macaques. Interestingly, RAVV was lethal in cynomolgus macaques but did not cause lethal disease in rhesus macaques. Analyzing tissue samples, we discovered a possible role of lymphocyte and hepatocyte apoptosis in MARV pathogenesis, as well as preferential infection of endothelial cells.

MATERIALS AND METHODS

Biosafety and Ethics Statement

All infectious work with MARV and RAVV was performed by using standard operating procedures approved by the

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Rocky Mountain Laboratories (RML) Institutional Biosafety Committee in the maximum containment laboratory at the RML, part of the Division of Intramural Research, National Institute of Allergy and Infectious Diseases (NIAID), National Institutes of Health (NIH). Animal work was performed in strict accordance with the recommendations described in the NIH *Guide for the Care and Use of Laboratory Animals* [14], the Office of Animal Welfare, and the US Department of Agriculture, and it was approved by the RML Animal Care and Use Committee. Procedures were conducted in animals anesthetized with ketamine by trained personnel under the supervision of veterinary staff. All efforts were made to ameliorate animal welfare and minimize animal suffering, in accordance with the Weatherall report on the use of nonhuman primates (NHPs) in research [15].

Animals were housed in adjoining individual primate cages that enabled social interactions, under controlled conditions of humidity, temperature, and light (12-hour light/12-hour dark cycles). Food and water were available ad libitum. Animals were monitored and fed commercial monkey chow, treats, and fruit at least twice a day by trained personnel. Environmental enrichment consisted of commercial toys, music, and video. End point criteria, specified by the clinical score parameters approved by the RML Animal Care and Use Committee, were used to determine when animals were humanely euthanized.

Challenge Viruses

MARV strain Angola (passage 2; GenBank accession No. KY047763.1) [16], MARV strain Musoke (passage 3), MARV strain Ozolin (no passage information; received from the US Army Medical Research Institute for Infectious Diseases; coding sequence matches GenBank entry AY358025.2) and RAVV (passage 4; passage 3 received from the US Army Medical Research Institute for Infectious Diseases; sequence matches GenBank entry NC_024781.1 with only 1 coding mutation at position 7118, resulting in glycine-to-alanine substitution) were propagated on Vero E6 cells, titrated via a focus-forming unit assay using a MARV glycoprotein (GP)-specific antibody (provided by Stephan Becker, Philipps University, Marburg, Germany) on Vero E6 cells, and stored in liquid nitrogen.

Viral Genome Sequences

Virus genome sequences were determined from TRIzol-extracted and purified viral RNA using the Qiagen AllPrep kit, as described by manufacturer. Total RNA libraries were prepared using the TruSeq Stranded Total RNA Kit (Illumina). Final libraries were validated with an Agilent 2100 Bioanalyzer using the DNA 1000 chip and quantified using the KAPA Library Quantification Kit for Illumina Sequencing (Kapa Biosystems). Sample libraries were normalized and pooled for sequencing on the MiSeq System Sequencer (Illumina), using the 2 × 150-base pair MiSeq Reagent Nano Kit (version 2;

Illumina). Reads were trimmed to remove adapter sequence and filtered to remove low-quality sequences, using the FASTX-Toolkit (Hannon Laboratory, Cold Spring Harbor Laboratory). Reads were mapped to reference sequences using Bowtie 2 software and default parameters. Single-nucleotide polymorphisms were detected using SNPEff v3 software (<http://snpeff.sourceforge.net>) and are shown in [Supplementary Table 1](#).

Study Design

Two separate studies following the same experimental outline with either rhesus or cynomolgus macaques are presented here. The animals had been used in previous studies and had no detectable immunity to MARV or RAVV. The first study used 7 male rhesus macaques (weight, 11–14 kg), 3 groups consisting of 2 animals (MARV Angola, animals A1 and A2; MARV Musoke, animals M1 and M2; and RAVV, animals R1 and R2) and 1 group with a single animal (MARV Ozolin, animal O1), which were challenged intramuscularly with 1000 focus-forming-units of the specified virus.

The second study used 8 male cynomolgus macaques (weight, 3.5–5.5 kg), with 2 animals per group (MARV Angola, animals A3 and A4; MARV Musoke, animals M3 and M4; MARV Ozolin, animals O2 and O3; and RAVV, animals R3 and R4), also challenged intramuscularly with 1000 focus-forming-units of the specified virus. Clinical examinations were performed, and blood samples were collected on days 3, 6, 9, 12, and 21 days after infection or on the day of euthanasia for animals that reached the clinical end point. The animals were observed at least twice daily for clinical signs of disease and were humanely euthanized when clinical signs indicated terminal disease on the basis of preestablished end points or on day 21 (study end point).

Viremia and Tissue Virus Loads

For determination of virus loads in blood and tissue samples, Vero E6 cells were used, and the median tissue culture infectious dose (TCID₅₀) was calculated for each sample, as described elsewhere [17].

Clinical Chemistry

The concentrations of alanine aminotransferase, alkaline phosphatase, serum urea nitrogen (SUN), and creatinine from serum samples were determined using the Piccolo Xpress Chemistry Analyzer and Piccolo General Chemistry 13 Panel discs (Abaxis).

Enzyme-Linked Immunosorbent Assay and Serum Cytokine Levels

Postchallenge serum samples were inactivated by gamma-irradiation (5 Mrad) before removal from the biosafety level 4 laboratory, following Institutional Biosafety Committee–approved standard operating procedures. Total MARV GP-specific immunoglobulin G (IgG) responses were measured by means of enzyme-linked immunosorbent assay, using a 2-fold dilution

series starting at 1:100 with MARV GPΔTM as an antigen (IBT BioServices), as described elsewhere [18]. Samples were deemed positive for MARV GP-specific IgG when the optical density value was higher than the mean value for negative serum plus 3 standard deviations.

Serum cytokine levels were determined by diluting the serum samples 1:1 in serum matrix for analysis using the Milliplex Non-Human Primate Magnetic Bead Panel (Millipore), according to the manufacturer's instructions. Concentrations of interferon γ , interleukin 6, monocyte chemoattractant protein 1, macrophage inflammatory protein 1 β , tumor necrosis factor α (TNF- α), and interleukin 15 were determined for all samples using the Bio-Plex 200 System (BioRad Laboratories).

Histology, Immunohistochemistry, and In Situ Hybridization

All tissues were fixed in 10% neutral-buffered formalin, removed from the biosafety level 4 laboratory according to approved standard operating procedures, processed, and embedded in paraffin. Slides were cut in 5- μ m sections and stained with hematoxylin-eosin. The tissues were evaluated and scored for pathologic damage. MARV and RAVV antigen was detected using immunohistochemistry (IHC) staining performed by the Ventana Discovery Ultra machine protocol, using a rabbit polyclonal antibody directed against the MARV nucleoprotein (provided by Ayato Takada, Hokkaido University, Sapporo, Japan). MARV and RAVV genomic RNA was detected using in situ hybridization probes targeting the nucleoprotein, designed by ACD Bio using their RNAscope protocol. Because of the genetic difference between RAVV and the MARV strains, a separate probe was designed for the RAVV-infected tissues.

Slides were analyzed on the Ventana Discovery Ultra machine using their RNAscope VS 2.5 assay. Active caspase 3 was detected using IHC staining performed by the Ventana Discovery Ultra machine protocol with a rabbit polyclonal antibody (Promega), and the number of positive cells was determined by scanning the slides at 4 $\times$ magnification, using the Aperio AT2 scanner and running a positive pixel analysis.

RESULTS

MHF in MARV Angola- and MARV Musoke-Infected Macaques

In both the rhesus and cynomolgus macaques infected with MARV Angola or MARV Musoke, clinical signs developed that were indicative of severe MHF. All of the rhesus macaques infected with MARV Angola or MARV Musoke reached the clinical end point and were humanely euthanized, as did the MARV Angola-infected cynomolgus macaques; in contrast, only 1 of the 2 MARV Musoke-infected cynomolgus macaques (M4) reached the clinical end point (Figure 1A and 1B). During the critical phase of the study (days 6–9) all MARV Angola- and MARV Musoke-infected animals that died of disease demonstrated viremia levels between 10^6 and 10^9

TCID₅₀/mL (Figure 1C and 1D). These animals also had viral titers $\geq 10^5$ TCID₅₀/g in key target tissues of MARV infection (Supplementary Figure 1A), similar to previously published data [16].

In contrast, MARV Ozolin infection in both macaque species resulted in survival with minimal clinical disease (Figure 1A and 1B). Animals began to show very mild clinical signs of illness around day 7 after infection, including mild fever, abnormal or hunched posture, and mild to moderate anorexia, but they quickly recovered and survived. These animals (except animal O1, which had no viremia), showed viral titers in the blood that peaked at about 10^3 TCID₅₀/mL by day 6 after infection followed by rapid clearance of viremia (Figure 1C and 1D). By day 21, all 3 animals had seroconverted to infection with MARV GP IgG-specific titers $\geq 1:25\,600$ (Supplementary Figure 1B).

By day 9 after infection increased levels of the liver enzymes alkaline phosphatase and alanine aminotransferase were evident in all nonsurvivors (Supplementary Figure 1C and 1E) indicative of hepatic damage and dysfunction, as were increases in SUN and serum creatinine, indicative of prerenal azotemia (Supplementary Figure 2A and 2C), findings similar to those in previous studies [7, 19–21]. In contrast, the survivors, including all MARV Ozolin-infected and 1 MARV Musoke-infected macaque (M3), demonstrated liver enzyme levels that were only mildly elevated at about day 6–9 after infection (Supplementary Figure 1D and 1F), with no other clinical chemistry or hematologic abnormalities noted (Supplementary Figure 2B and 2D).

Cytokine analysis revealed up-regulation of the proinflammatory cytokines interferon γ , interleukin 6, monocyte chemoattractant protein 1, and TNF- α in nonsurvivors, indicative of cytokine storm (Supplementary Figure 3A, 3C, 3E, and 3G), similar to findings from other MARV Angola studies in NHPs [16, 19]. Survivors, however, showed only a mild, early up-regulation of these cytokines, followed by down-regulation (Supplementary Figure 3B, 3D, and 3F); in addition, no changes in TNF- α levels were detected (data not shown).

MARV pathology presented primarily in the liver, spleen, and lymph nodes [22]. Histologically, all the MARV Angola- and MARV Musoke-infected macaques that died of infection demonstrated pathologic findings typical of MHF, as described elsewhere [7, 19, 20, 22] with moderate to severe, acute, multifocal hepatocellular necrosis in the liver (Supplementary Figure 4A); however, only subacute hepatitis was noted in animal M2 (Supplementary Figure 4B). In contrast, the MARV Ozolin-infected macaques and the MARV Musoke-infected survivor had no disease demonstrated in the liver (Supplementary Figure 4C).

The spleens from the MARV Angola- and MARV Musoke-infected nonsurvivors demonstrated moderate to severe fibrin deposition, necrosis, and hemorrhage of the red pulp of the spleen, as well as moderate to severe lymphoid necrosis with

Figure 1. Survival and viremia of Marburg virus (MARV)-infected macaques. *A, B*, Survival curves for rhesus (*A*) and cynomolgus (*B*) macaques infected with MARV strain Angola, Musoke, or Ozolin or Ravn virus (RAVV). *C, D*, Virus titers in the blood of rhesus (*C*) and cynomolgus (*D*) macaques infected with MARV Angola, Musoke, or Ozolin or RAVV. Abbreviations: Cyno, cynomolgus; TCID₅₀, median tissue culture infectious dose.

occasional marginal zone hemorrhage in the white pulp (Supplementary Figure 4D). Again, the MARV Ozolin-infected macaques and the MARV Musoke-infected survivor demonstrated no disease in the spleen (Supplementary Figure 4E). The MARV Angola- and MARV Musoke-infected nonsurvivors also demonstrated mild to severe follicular necrosis of the mediastinal and mesenteric lymph nodes (Supplementary Figure 4F), but no significant disease was seen in the adrenal glands or urinary bladder (Supplementary Figure 5A and 5B).

Within the lymph nodes of all nonsurvivors, antigen was found primarily within sinus and follicular macrophages and macrophages in the spleen (Supplementary Figure 5C). In addition, abundant amounts of antigen were found in the liver, primarily in endothelial cells and hepatocytes (Figure 2A). However, only 2 animals had antigen demonstrated within the sinus (animals M2 and O2) or follicular (O2) macrophages of the mesenteric lymph node (Supplementary Figure 5D). In situ hybridization was performed to detect viral genomic RNA by designing probes with sequence complementary to sections of the MARV RNA genomes; this method identified large amounts of MARV genomic RNA, found primarily in endothelial cells and hepatocytes of the livers in all the nonsurvivors (Figure 2B) except animal M2.

Interestingly, despite extensive hepatocellular necrosis (Figure 2C), reticulin silver staining of the livers of terminally ill

animals revealed no change to the reticulin network (Figure 2D). Hepatic necrosis, with the subsequent loss of hepatocytes, generally results in collapse of hepatic reticulin fibers. In this case, the maintenance of reticulin architecture suggests that hepatocellular necrosis is multifocal and random, leaving enough intact hepatocytes to maintain the reticulin architecture.

Different Effects of RAVV Infection in Rhesus and Cynomolgus Macaques

An unexpected result of this comparative pathology study was the difference observed between macaque species after RAVV infection. Despite seroconversion in all animals, as shown by enzyme-linked immunosorbent assay (Supplementary Figure 1B), the rhesus macaques (R1 and R2) exhibited very mild illness, but the cynomolgus macaques (R3 and R4) had severe and fatal disease. The cynomolgus macaques presented with classic signs of MHE, including elevated liver enzyme levels (Supplementary Figure 1C and 1E) as well as elevated serum creatinine and SUN concentrations (Supplementary Figure 2A and 2C). For the 2 rhesus macaques, viremia was detected only in animal R1, at about 10^3 TCID₅₀/mL by day 6 after infection, and viral titers were undetectable for R2 (Figure 1C).

In contrast to the rhesus macaques, the 2 cynomolgus macaques demonstrated viral blood titers peaking at about 10^7 TCID₅₀/mL or higher at the time of euthanasia, on day 9 after infection (Figure 1D). No virus was detected in any of the tissue samples

Figure 2. Histology, immunohistochemistry, and in situ hybridization of liver samples from a macaque (animal A2) infected with Marburg virus (MARV), strain Angola. A, Hematoxylin-eosin staining shows necrosis (arrow). B, Reticulin silver staining shows reticulin fibers (arrow). C, D, Infected endothelial cells (arrows) demonstrated by immunohistochemistry staining (C) and in situ hybridization (D). All images are 200× original magnification.

on day 21 in the rhesus macaques, but titers $>10^6$ TCID₅₀/g were detected in the cynomolgus macaques at the time of euthanasia (Supplementary Figure 1A). This difference is consistent with evidence of a cytokine storm in the cynomolgus macaques but only a mild increase in proinflammatory cytokines in the rhesus macaques around day 8, with levels that quickly dropped to baseline by the end of the study (Supplementary Figure 6A–6D).

At histology, liver samples from the 2 rhesus macaques exhibited no to very mild hepatocellular necrosis (Supplementary Figure 7A) with no viral antigen detectable with IHC staining (Supplementary Figure 7B). However, the liver samples from the 2 cynomolgus macaques exhibited marked, acute hepatocellular necrosis (Supplementary Figure 7C), with antigen detectable within endothelial cells and hepatocytes at the final stage of disease (Supplementary Figure 7D). Spleen samples from the 2 rhesus macaques (Figure 3A), along with the mediastinal lymph node, adrenal gland, mesenteric lymph node, and urinary bladder, showed no significant pathology (Supplementary Figure 8A and 8B) and no detectable viral antigen (Figure 3B), except in the sinus macrophages of the mesenteric lymph node in 1 animal (R1) (Supplementary Figure 8C). In contrast, spleen samples from the 2 cynomolgus macaques exhibited

mild to severe fibrin accumulation and hemorrhage of the red pulp, as well as mild to marked lymphoid necrosis of the white pulp with marginal zone hemorrhage (Figure 3C) and some mild follicular necrosis in both the mediastinal and mesenteric lymph nodes of animal R4 (Supplementary Figure 8D), with antigen detected primarily within follicular and sinus macrophages (Figure 3D).

Target Cell Infection and Macrophage Apoptosis

Preferential hepatocyte infection is typically seen in the livers of NHPs infected with Ebola virus (EBOV) during the terminal stage of illness [22]. In contrast, in all the liver sections from terminally ill MARV- or RAVV-infected animals, viral antigen and viral genomic RNA, detected with IHC staining and in situ hybridization, respectively, were present in almost all endothelial cells of the sinusoids (Figures 2A and 2B and Supplementary Figure 7D). This diffuse distribution of viral antigen and RNA is typically not observed during EBOV infection, which tends to occur in discrete foci with patches of infected and uninfected hepatocytes [22]. In the current study, however, the MARV Angola-infected animals and 1 RAVV-infected cynomolgus macaque (R4) had an even distribution of infection, covering almost all the hepatocytes

Figure 3. Histology and immunohistochemistry (IHC) of macaques infected with Marburg virus (MARV), strain Angola, or Ravn virus (RAVV). *A, B*, Spleen samples from an RAVV-infected rhesus macaque (animal R2), as shown with hematoxylin-eosin (HE) (*A*) and IHC (*B*) staining. *C, D*, Spleen samples from an RAVV-infected cynomolgus macaque (animal R4), as shown with HE (*C*) (Fi, fibrin; MZH, marginal zone hemorrhage; N, necrosis) and IHC (*D*) staining. *E, F*, IHC staining for active caspase 3 in spleen samples from a MARV Angola-infected rhesus macaque (*E*; animal A2) and an RAVV-infected cynomolgus macaque (*F*; animal R4). All images are 200× original magnification.

and endothelial cells present in the sections visualized (Figure 2C and 2D and Supplementary Figure 7D).

IHC staining against active caspase 3 (Figure 3E and 3F), indicative of apoptotic progression, demonstrated that the number of positive lymphocytes (and hepatocytes in the liver) was significantly above baseline for the MARV Angola-, MARV Musoke-, and RAVV-infected nonsurvivors in both liver and spleen sections, with the exception of the liver in animal M2

and the spleen in animal R3, an RAVV-infected cynomolgus macaque (Supplementary Figure 6E and 6F). Levels of positive lymphocytes were either at or only slightly above baseline in the MARV Ozolin-infected animals, as well as in the RAVV-infected rhesus macaques and the MARV Musoke-infected survivor, except in the spleen. Baseline levels of apoptosis were determined by IHC staining of liver and spleen sections from uninfected rhesus and cynomolgus macaque.

DISCUSSION

Here we present a limited comparative pathology study that describes the pathophysiologic effects of infection with varied strains of MARV and RAVV in 2 macaque species that represent the most commonly used animal models for preclinical evaluation of countermeasures in the filovirus field. The objective of the current study was to provide initial data on comparative pathology of MARV and RAVV infection in macaque models. We recognize the small group sizes as a limitation, resulting in the inability to perform statistical analysis. Nevertheless, our study provides new information on the pathogenesis of genetically distinct MARVs and RAVV.

Although most of the published molecular studies on MARVs have been performed with the prototype strain MARV Musoke, MARV Angola appears favored for countermeasure development, because it seems to be the most virulent strain in humans and NHPs. However, the pathogenicity of most MARV strains is poorly studied. The current findings supports MARV Angola as the most pathogenic isolate in NHPs, followed by MARV Musoke and MARV Ozolin. The disease caused by MARV Ozolin infection in macaques was surprisingly mild. This finding seems in contrast to the 3 human cases, with severe disease resulting in 1 death and 2 recoveries [11]. One explanation could be the passage history of this isolate, which is unknown to us, possibly resulting in attenuation. This has been described for MARV Angola, with passaging in cells resulting in a single amino acid substitution at position 12 (leucine-to-serine substitution) in the GP signal peptide that probably led to tissue culture adaptation and decreased in vivo virulence [23]. The MARV Ozolin strain used in our study also encodes a leucine at this position, but other sequence differences may account for the lower pathogenicity of this strain, something that could be confirmed in the future through reverse genetics–engineered virus mutants.

Rhesus and cynomolgus macaques differ by only 0.4% in their genetic material [24], so it is surprising that we observe such a difference in disease after RAVV infection. However, a similar observation has recently been published for Crimean Congo hemorrhagic fever virus infection in macaques [25]. Infection of rhesus macaques with this virus resulted in no overt disease, whereas cynomolgus macaques exhibited a spectrum from mild to severe and lethal disease representing human Crimean Congo hemorrhagic fever [25]. In addition, EBOV infection in rhesus macaques has been described with a prolonged time to death compared with cynomolgus macaques (8 vs 6 days, respectively) [26]. Therefore, the difference in disease and pathogenicity between these 2 macaque species offers an opportunity to study the underlying molecular determinants and decipher mechanisms involved in pathogenesis. However, the lack of severe disease and lethality after RAVV infection in rhesus macaques (in animals R1 and R2) is in contrast to published findings in which

infection with a lower passage of RAVV caused uniform lethality in rhesus macaques [27, 28]. The RAVV isolate used in our study shows a glycine-to-alanine change in the GP at position 393 (base pair 7118) (Supplementary Table 1). Future studies need to show whether this mutation is responsible for the lower pathogenicity.

Bystander lymphocyte apoptosis is a hallmark of EBOV infection and has also been reported as a pathologic feature of MARV infections [7, 16, 20, 29]. Liver and spleen sections from all animals were analyzed to determine the role of lymphocyte apoptosis in MARV pathogenesis. The numbers of cells expressing active caspase 3, an initiator of apoptotic progression, were significantly above baseline levels in the spleen sections from all animals that died of MARV infection (Supplementary Figure 6F). This is further supported by the lack of infected lymphocytes in areas of heavy follicular damage seen with IHC staining in the lymph nodes and spleens (Figure 3D and Supplementary Figure 5C and 5D) similar to what has been reported before [19, 22, 30] as the result of bystander lymphocyte apoptosis [6]. Interestingly, the numbers of positive cells in the liver were also significantly above baseline for all animals that died of MARV infection (Supplementary Figure 6E). Taken together, these observations suggest that, along with virus-induced liver cell damage, apoptotic events may contribute to the pathologic mechanism of MARV and RAVV infections.

Macrophages and dendritic cells have been shown to be the early and initial targets of EBOV infections, but little is known regarding the primary target cells in different organs after MARV infection [20, 31]. Severe filovirus infections have been shown to cause substantial vascular dysfunction, which can result in hypovolemic shock [32, 33]. Particularly in Ebola hemorrhagic fever, it has been suggested that this dysfunction is most likely caused by a virus-induced cytokine storm rather than by physical destruction of endothelial cells through virus replication [34]. However, the widespread infection of endothelial cells in liver sections from animals lethally infected with MARVs suggests that physical destruction of these cells may play a role in MARV pathogenesis.

As member of the family *Filoviridae*, MARV and RAVV represent a possible emerging threat to global public health similar to EBOV; therefore, countermeasure development is of the utmost importance. The current study expands our limited knowledge about the pathogenesis of different MARVs and RAVV. Future studies are needed to better define the pathogenesis of MARV and RAVV infections in macaques.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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Author contributions. H. F. and A. M. designed the experiments described in the manuscript. F. F., H. F., and A. M. performed the animal experiments. V. V. N., R. R., F. F., D. L., T. T., and A. M. processed the samples. V. V. N., D. P. S., H. F., and A. M. analyzed the data and wrote the manuscript. All authors approved the manuscript.

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