SUPPLEMENT ARTICLE

Importin-α7 Is Involved in the Formation of Ebola Virus Inclusion Bodies but Is Not Essential for Pathogenicity in Mice

Gülsah Gabriel,[1,2,a] Friederike Feldmann,[3] Rudolph Reimer,[1] Swantje Thiele,[1] Meike Fischer,[1] Enno Hartmann,[2] Michael Bader,[2,4] Hideki Ebihara,[5] Thomas Hoenen,[5] and Heinz Feldmann[5,a]

[1]Viral Zoonoses and Adaptation, Heinrich Pette Institute, Leibniz Institute for Experimental Virology, Hamburg, and [2]Center for Structural and Cellular Biology in Medicine, Institute of Biology, University of Lübeck, Germany; [3]Rocky Mountain Veterinary Branch, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Hamilton, Montana; [4]Max Delbrück Center for Molecular Medicine, Berlin, Germany; and [5]Laboratory of Virology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Hamilton, Montana

Ebola virus (EBOV) protein 24 antagonizes the host interferon (IFN) response by hijacking select nuclear importin-α isoforms. Thereby, it blocks STAT1-mediated IFN-α/β and IFN-γ synthesis. However, owing to the lack of importin-α knockout animal models in the past, their role in EBOV pathogenesis remained largely unknown. Here, we demonstrate that importin-α7 is involved in the formation of EBOV inclusion bodies and replication. However, deletion of the gene encoding importin-α7 was not sufficient to increase survival rates among mice infected with EBOV.

Keywords. Ebola virus; importin-α; inclusion bodies; pathogenicity; VP24; interferon; STAT1.

Ebola virus (EBOV; species *Zaire ebolavirus*, genus *Ebolavirus*, family *Filoviridae*) can cause severe Ebola hemorrhagic fever (EHF) in humans and nonhuman primates. Currently, we are experiencing the largest outbreak of EBOV infection, which started in late 2013 and has already affected multiple countries in West Africa. To date, approximately 26 000 cases with >11 000 deaths have been reported [1]. Thus, it is mandatory to understand the molecular determinants involved in EBOV pathogenicity. Several viral determinants are known to contribute to EBOV pathogenicity, such as the glycoprotein (GP), viral protein 24 (VP24), and VP35 [2–8]. However, the role of cellular factors involved in EBOV pathogenicity remains largely unknown [9].

Importin-α isoforms belong to the best-described cellular interaction partners of EBOV VP24, which is a key viral pathogenicity determinant. VP24 interacts with and sequesters human importin-α5, importin-α6, and importin-α7 isoforms (also known as KPNA1, KPNA5, and KPNA6, respectively) in the cytoplasm [10, 11], thus inhibiting importin-α–mediated interaction and transport of tyrosine-phosphorylated STAT1 (PY-STAT1) into the nucleus. This blockage of interferon α/β (IFN-α/β)– and IFN-γ–induced nuclear accumulation of PY-STAT1 leads to the inhibition of IFN-induced gene expression. The direct competition between VP24 and PY-STAT1 for importin-α binding allows VP24 to promote viral escape from the host innate immune response. This inhibition of the cell-intrinsic innate immune response renders EBOV-infected cells nonresponsive to IFN-α/β or IFN-γ treatment [12]. Interestingly, this VP24/importin-α interaction was observed with several members of the genus *Ebolavirus* [10]. Marburg virus (MARV; species *Lake Victoria marburgvirus*, genus *Marburgvirus*, family *Filoviridae*), on the other hand, has evolved mechanisms to counteract IFN signaling that differ from those for EBOV [13]. MARV infection does not detectably block STAT1 nuclear import but instead inhibits phosphorylation of STAT1 and STAT2. Surprisingly, this function was not associated with MARV VP24 but with the MARV matrix protein VP40. It was shown that expression of MARV VP40 was sufficient to block IFN signaling, probably by targeting the Janus kinase family kinase 1

[a]G. G. and H. F. contributed equally to this work.

Correspondence: Gülsah Gabriel, PhD, Heinrich-Pette-Institute, Leibniz Institute for Experimental Virology, Martinistr. 52, 20251 Hamburg (guesah.gabriel@hpi.uni-hamburg.de).

The Journal of Infectious Diseases® 2015;212:S316–321

DOI: 10.1093/infdis/jiv240

that mediates STAT1 and STAT2 phosphorylation [13]. Thus, it is believed that suppression of the IFN-α/β– and IFN-γ–mediated antiviral response allows sustained filovirus replication, which further promotes viral disease progression and severity [14]. However, owing to the absence of animal models lacking certain importin-α isoforms, the contribution of these cellular host factors to EBOV pathogenicity remains unknown.

Recently, importin-α7 knockout ($\alpha7^{-/-}$) mice became available [15], which allowed us to examine the involvement of importin-α7 in EBOV replication and pathogenesis. We used the mouse-adapted EBOV (MA-EBOV) Mayinga strain [16], which is closely related to the EBOV Makona strain currently circulating in West Africa.

MATERIALS AND METHODS

Biosafety and Ethics Statement

All work with infectious EBOV was performed in the Maximum Containment Laboratory of the Rocky Mountain Laboratories (RML) according to standard operation protocols (SOP) approved by the institutional biosafety committee. All animal work was conducted in compliance with the guidelines of and under a protocol approved by the RML Institutional Animal Care and Use Committee. The RML facility is fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International and has an approved Office of Laboratory Animal Welfare assurance number.

Animal Experiments

Generation of importin-α7 knockout ($\alpha7^{-/-}$) mice in the C57BL/6 genetic background was described before [15]. Mice were bred at the animal facilities of the Max Delbrück Center for Molecular Medicine, in Berlin, and the Heinrich Pette Institute, Leibniz Institute for Experimental Virology, in Hamburg, Germany. To assess MA-EBOV pathogenicity in wild type (WT) and $\alpha7^{-/-}$ mice, groups of 16–25 animals were infected intraperitoneally with 1×, 10×, and 100× mouse lethal dose that can produce death in 50% of the animals (MLD_{50}). Weight loss and survival were monitored for 14 and 28 days after infection, respectively, as described before [4]. To assess virus replication in mice, groups of 4–6 animals were euthanized on day 4 after infection, and spleen, liver, and blood samples were collected for virologic and pathologic analyses. Virus titers were determined as 50% tissue culture infective doses on Vero E6 cells as described before [4].

Immunohistochemical Analyses

To assess viral pathology in the infected organs, the spleens from 4–6 infected WT and $\alpha7^{-/-}$ mice were fixed and inactivated in 10% formalin according to SOP. Subsequently, samples were removed from high containment for immunohistochemical staining. Briefly, formalin-fixed tissues were embedded in paraffin and thin sections were stained using a monoclonal anti-VP40 antibody as described before [9]. Tissues were counterstained with hematoxylin, as previously described [17]. Tissue sections were then analyzed in a blinded fashion by the pathologists at the Heinrich Pette Institute, Leibniz Institute for Experimental Virology.

Transmission Electron Microscopy

Tissue samples were fixed and inactivated with 2.5% glutaraldehyde according to approved SOP. Subsequently, samples were removed from high containment, washed in phosphate-buffered saline, and postfixed with 1% osmium tetroxide. For better structure preservation, specimens were treated with 1% gallic acid after osmification, as described elsewhere [18]. Thereafter, samples were stained with 1% uranyl acetate in double-distilled water, dehydrated in graded ethanol, and embedded in Epon. Ultrathin sections were prepared on a Leica Ultracut UCT microtome. The images were acquired with a FEI Eagle 4 k camera on a Tecnai G20 microscope operated at 80 kV.

Statistical Analysis

The statistical significance of obtained data was calculated with GraphPad Prism 5 (v5.03), using the unpaired, 2-tailed Student *t* test or, when all values in one group were identical (eg, 0), the 1-sample, 2-tailed Student *t* test. Statistical significance was defined as a P value of $<.05$.

RESULTS

Importin-α7 Promotes MA-EBOV Replication in Mice

To assess whether importin-α7 plays a role in MA-EBOV replication in mice, we infected WT and $\alpha7^{-/-}$ mice [15] with 1×, 10×, and 100× MLD_{50} (Figure 1*A*–*C*). Control mice were mock infected with Dulbecco's modified Eagle's medium. On day 4 after infection, we collected spleen, liver, and blood specimens from infected or uninfected control groups and determined virus titers. None of the mock-infected control groups were infected (data not shown). At a low infection dose of 1× MLD_{50}, none of the MA-EBOV–infected $\alpha7^{-/-}$ mice showed virus titers in spleen and liver, in contrast to WT mice (Figure 1*A*). Virus titers were significantly reduced in the blood of $\alpha7^{-/-}$ mice infected with 1× MLD_{50}, compared with WT mice (Figure 1*A*). Upon infection with 10× MLD_{50}, virus titers were generally reduced in $\alpha7^{-/-}$ mice, compared with WT mice, albeit not significantly (Figure 1*B*). At the highest infection dose (100× MLD_{50}), no differences in organ and blood titers were observed among the infected groups (Figure 1*C*). Thus, importin-α7 seems to be involved in efficient MA-EBOV replication in mice.

Importin-α7 Plays a Role in the Formation of Viral Inclusion Bodies

We then analyzed the effect of importin-α7 on organ pathology in MA-EBOV–infected mice. Therefore, thin sections of spleens, as one of the major site of EBOV replication [19, 20], collected

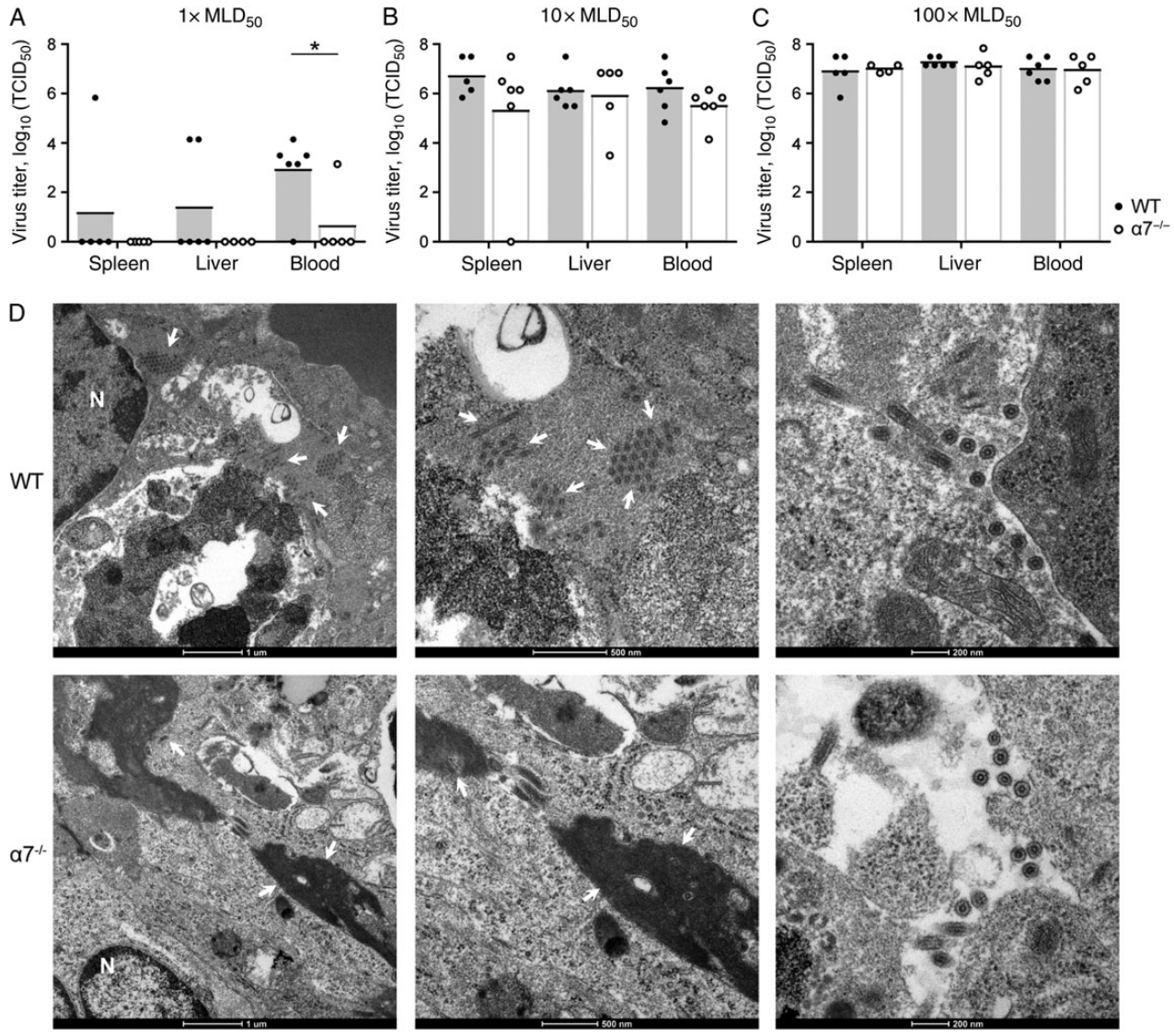

Figure 1. Ebola virus replication and visualization of inclusion bodies in wild-type (WT) and importin-α7–deficient ($\alpha7^{-/-}$) mice. *A–C*, WT and $\alpha7^{-/-}$ mice were intraperitoneally infected with 1× mouse lethal dose that can produce death in 50% of the animals (MLD_{50}; *A*; n = 4–6), 10× MLD_{50} (*B*; n = 5–6), or 100× MLD_{50} (*C*; n = 5–6) of the mouse-adapted Ebola virus (MA-EBOV) strain as described before [4]. Virus titers were measured by determining the 50% tissue culture infective dose ($TCID_{50}$) on day 4 after infection in the spleen, liver, and blood specimens from infected mice [4]. No virus was detected in control groups, which were mock treated with Dulbecco's modified Eagle's medium (data not shown). Each data point represents an individual organ sample per animal. Additionally, means of the logarithmic virus titers are shown (n = 4–6; $*P < .05$). *D*, Transmission electron microscopy of spleen sections for WT mice (upper panel) and $\alpha7^{-/-}$ mice (lower panel) infected with 10× MLD_{50} (n = 4) of the MA-EBOV strain. The left panel shows an overview of the infected cell, the middle panel shows magnification of inclusion bodies, and the right panel shows magnifications of virus particles. Inclusion bodies are indicated with arrows. Abbreviation: N, nucleus.

from experimental groups on day 4 after infection were stained immunohistochemically for the presence of VP40, an abundant EBOV protein [19]. The presence of viral antigen was predominantly detected in macrophages (Supplementary Figure 1). The number of viral antigen–positive cells was generally reduced in $\alpha7^{-/-}$ mice, compared with WT mice that were infected with 10 or even 100× MLD_{50}. Interestingly, dense granular structures were observed at higher magnification in macrophages of WT mice but not in macrophages in infected $\alpha7^{-/-}$ mice. These granular structures resembled inclusion bodies found in tissues infected with EBOV or MARV [21, 22] and have been identified as a site of EBOV replication [23]. The reduced formation of these granular structures, most likely representing inclusion bodies, further correlates with the generally reduced presence of viral antigen in splenic macrophages of MA-EBOV–infected $\alpha7^{-/-}$ mice.

Next, we assessed whether the granular structures present in WT splenic macrophages represent EBOV inclusion bodies

[23]. Therefore, we performed transmission electron microscopy of murine spleen sections infected with 10× MLD_{50} of MA-EBOV on day 4 after infection (Figure 1*D*). After analyzing large areas of spleen sections, we could visualize the symmetrical formation of these structures, indicating inclusion bodies in WT tissues (Figure 1*D*). In contrast, such symmetrical structures were rarely detected in tissues of $\alpha7^{-/-}$ mice, and instead, mostly large and homogenous dense structures were present (Figure 1*D*). In both WT and $\alpha7^{-/-}$ spleen tissues, viral particles were detected (Figure 1*D*). Thus, our findings show that importin-α7 is involved in efficient formation of inclusion bodies.

Deletion of the Gene Encoding Importin-α7 Does Not Increase Survival in Mice

Finally, we addressed whether importin-α7 plays a role in MA-EBOV pathogenicity. Therefore, we infected WT and $\alpha7^{-/-}$ mice with 1×, 10×, or 100× MLD_{50} of MA-EBOV and monitored weight loss and survival for 14 and 28 days after infection, respectively. With all infection doses tested, weight loss in $\alpha7^{-/-}$ mice was slightly less than that in WT mice, although the difference was not statistically significant (Figure 2). Survival rates did not differ between WT and $\alpha7^{-/-}$ mice in any of the infected groups independently of the infection dose. These data show

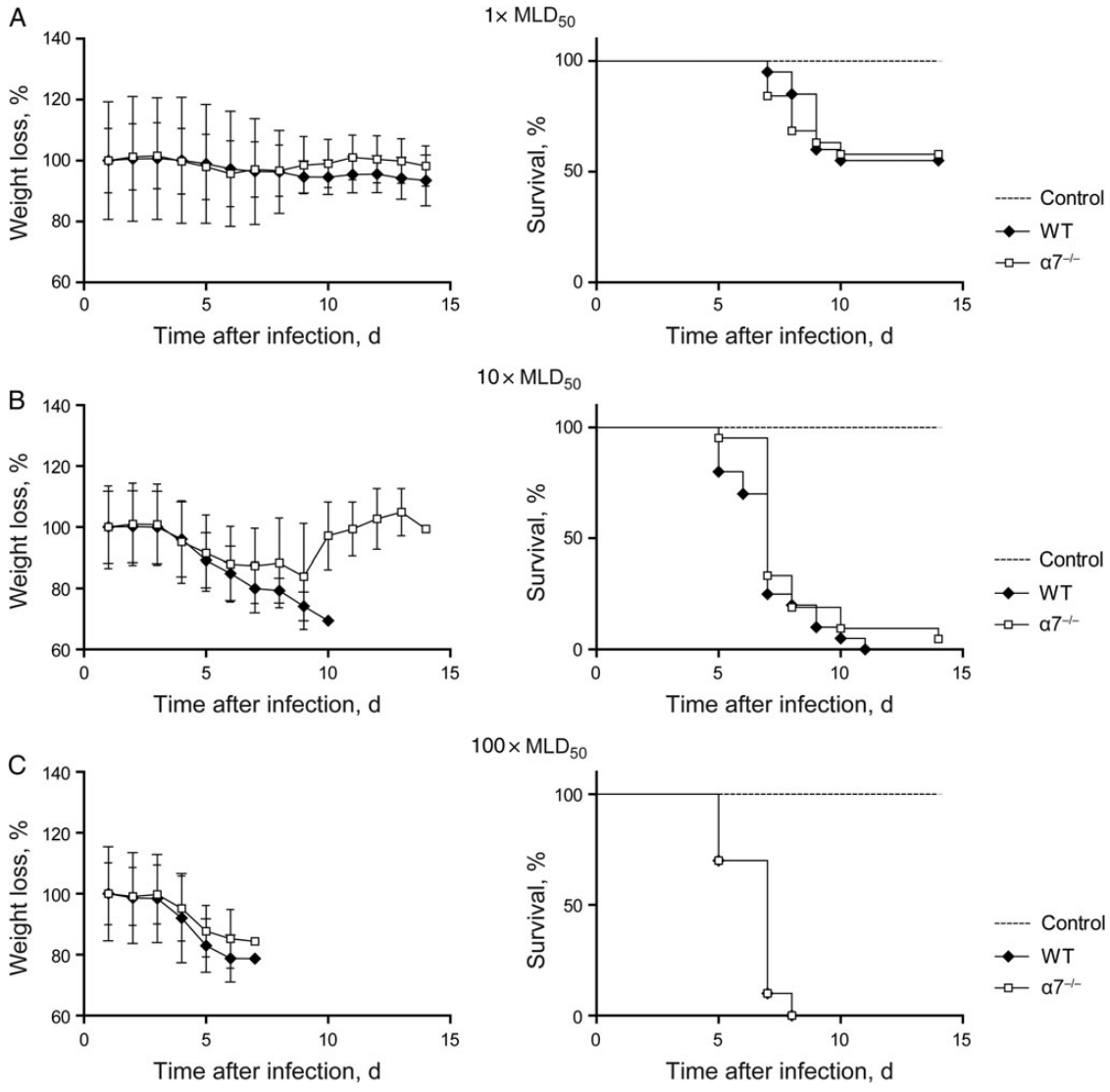

Figure 2. Pathogenicity of Ebola virus in wild-type (WT) and importin-α7–deficient ($\alpha7^{-/-}$) mice. *A–C*, WT mice (filled square) and $\alpha7^{-/-}$ mice (open square) were intraperitoneally infected with 1× mouse lethal dose that can produce death in 50% of the animals (MLD_{50}; *A*; n = 13–19), 10× MLD_{50} (*B*; n = 13–21), or 100× MLD_{50} (*C*; *n* = 10) of the MA-EBOV strain as described before [4]. Animals were monitored daily for weight loss (14 days) and survival (28 days). Control groups that were mock infected with Dulbecco's modified Eagle's medium did not show any weight loss (data not shown) or pathological alterations between WT and $\alpha7^{-/-}$ mice (Supplementary Figure 1), and all animals survived (gray line). Data are mean values ± SD for weight loss.

that importin-α7 is not essential for MA-EBOV pathogenicity in mice.

DISCUSSION

Here, we show that importin-α7, previously proposed to function as a potential EBOV pathogenicity factor [10, 11], is not essential for MA-EBOV pathogenicity in mice. However, importin-α7 seems to play a role in the formation of viral inclusion bodies, which are a known site of EBOV replication. Macrophages play a pivotal role in viral dissemination. EBOV spreads from the initial infection site to regional lymph nodes via monocytes, macrophages, and dendritic cells and probably through the lymphatic system. The virus can then further spread via the blood to the liver and spleen [20]. Thus, the impaired inclusion body formation, particularly in macrophages, might be reflected in significantly reduced blood titers early in infection. Moreover, extensive virus replication was still observed later in infection, albeit with reduced formation of inclusion bodies. Thus, one might speculate whether additional sites of replication exist within the host cell that do not involve inclusion bodies. Moreover, it would be interesting to address in future studies whether importin-α7 might also function as a cytoplasmic chaperone, as has been reported for nonclassical importins [24], and whether this function might be used by viruses for the formation of replication complexes, similar to inclusion bodies.

In contrast to EBOV infection, importin-α7, but not importin-α4 or -α5, was described to be a major determinant of influenza virus pathogenicity in mice [15]. Therefore, one might speculate whether importin-α isoforms other than importin-α7 might contribute to EBOV pathogenicity. Interestingly, VP24 has previously been shown to interact with importin-α5 and importin-α6 [10, 11]. However, since there is no homologue of human importin-α6 in mice and human importin-α6 is only expressed in testes [25], the focus should be on importin-α5, which is one of the major transport proteins of STAT-1 [26]. Future studies should address the impact of a VP24/importin-α5 interaction on EBOV pathogenicity by using the corresponding knockout mice.

Recently, it was shown that WT mouse strains generally used in experimental studies, such as BALB/c or C57BL/6 (used here), do not represent the best animal disease model for Ebola infection. In these mice, hallmark symptoms of the human disease, such as coagulation abnormalities, are not present. However, certain recombinant, inbred (CC-RIX) mice from the Collaborative Cross panel have been shown to exhibit a disease similar to human EHF [9] and might serve as a more suitable small-animal disease model to study the role of individual importin-α isoforms in EBOV pathogenesis.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (http://jid.oxfordjournals.org). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

Acknowledgments. We thank Carola Schneider, Gundula Pilnitz-Stolze, Gökhan Arman-Kalcek, and Annette Preuss, for excellent technical assistance; and the staff of the Rocky Mountain Veterinary Branch, National Institute of Allergy and Infectious Diseases (NIAID), National Institutes of Health (NIH), for their help with animal care.

Financial support. This work was supported by the Emmy-Noether Research Programme of the German Research Foundation (grant GA 1575/1-1 to G. G.), the Free and Hanseatic City of Hamburg, the German Federal Ministry of Health, and the Intramural Research Program of NIAID, NIH.

Potential conflicts of interest. All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

References

1. World Health Organization (WHO). Ebola situation report, 6 May 2015. Geneva, Switzerland: WHO, **2015**.
2. Basler CF, Mikulasova A, Martinez-Sobrido L, et al. The Ebola virus VP35 protein inhibits activation of interferon regulatory factor 3. J Virol **2003**; 77:7945–56.
3. Basler CF, Wang X, Muhlberger E, et al. The Ebola virus VP35 protein functions as a type I IFN antagonist. Proc Natl Acad Sci U S A **2000**; 97:12289–94.
4. Ebihara H, Takada A, Kobasa D, et al. Molecular determinants of Ebola virus virulence in mice. PLoS Pathog **2006**; 2:e73.
5. Feldmann H, Volchkov VE, Volchkova VA, Klenk HD. The glycoproteins of Marburg and Ebola virus and their potential roles in pathogenesis. Arch Virol **1999**; 15:159–69.
6. Feldmann H, Volchkov VE, Volchkova VA, Stroher U, Klenk HD. Biosynthesis and role of filoviral glycoproteins. J Gen Virol **2001**; 82:2839–48.
7. Volchkov VE, Chepurnov AA, Volchkova VA, Ternovoj VA, Klenk HD. Molecular characterization of guinea pig-adapted variants of Ebola virus. Virology **2000**; 277:147–55.
8. Wahl-Jensen VM, Afanasieva TA, Seebach J, Stroher U, Feldmann H, Schnittler HJ. Effects of Ebola virus glycoproteins on endothelial cell activation and barrier function. J Virol **2005**; 79:10442–50.
9. Rasmussen AL, Okumura A, Ferris MT, et al. Host genetic diversity enables Ebola hemorrhagic fever pathogenesis and resistance. Science **2014**; 346:987–91.
10. Reid SP, Valmas C, Martinez O, Sanchez FM, Basler CF. Ebola virus VP24 proteins inhibit the interaction of NPI-1 subfamily karyopherin alpha proteins with activated STAT1. J Virol **2007**; 81:13469–77.
11. Reid SP, Leung LW, Hartman AL, et al. Ebola virus VP24 binds karyopherin alpha1 and blocks STAT1 nuclear accumulation. J Virol **2006**; 80:5156–67.
12. Xu W, Edwards MR, Borek DM, et al. Ebola virus VP24 targets a unique NLS binding site on karyopherin alpha 5 to selectively compete with nuclear import of phosphorylated STAT1. Cell Host Microbe **2014**; 16:187–200.
13. Valmas C, Grosch MN, Schumann M, et al. Marburg virus evades interferon responses by a mechanism distinct from Ebola virus. PLoS Pathog **2010**; 6:e1000721.
14. Basler CF, Amarasinghe GK. Evasion of interferon responses by Ebola and Marburg viruses. J Interferon Cytokine Res **2009**; 29:511–20.
15. Gabriel G, Klingel K, Otte A, et al. Differential use of importin-alpha isoforms governs cell tropism and host adaptation of influenza virus. Nat Commun **2011**; 2:156.

16. Bray M, Davis K, Geisbert T, Schmaljohn C, Huggins J. A mouse model for evaluation of prophylaxis and therapy of Ebola hemorrhagic fever. J Infect Dis **1998**; 178:651–61.
17. Otte A, Sauter M, Alleva L, Baumgarte S, Klingel K, Gabriel G. Differential host determinants contribute to the pathogenesis of 2009 pandemic H1N1 and human H5N1 influenza A viruses in experimental mouse models. Am J Pathol **2011**; 179:230–9.
18. Simionescu N, Simionescu M. Galloylglucoses of low molecular weight as mordant in electron microscopy. I. Procedure, and evidence for mordanting effect. J Cell Biol **1976**; 70:608–21.
19. Geisbert TW, Young HA, Jahrling PB, et al. Pathogenesis of Ebola hemorrhagic fever in primate models: evidence that hemorrhage is not a direct effect of virus-induced cytolysis of endothelial cells. Am J Pathol **2003**; 163:2371–82.
20. Feldmann H, Geisbert TW. Ebola haemorrhagic fever. Lancet **2011**; 377:849–62.
21. Martini G, Siegert R. Marburg virus disease. New York: Springer Verlag, **1971**.
22. Murphy F. Pathology of Ebola infection. In: Pattyn SR, ed. Ebola virus haemorrhagic fever. Amsterdam, the Netherlands: Elsevier, **1978**; 53–71.
23. Hoenen T, Shabman RS, Groseth A, et al. Inclusion bodies are a site of ebolavirus replication. J Virol **2012**; 86:11779–88.
24. Jakel S, Mingot JM, Schwarzmaier P, Hartmann E, Gorlich D. Importins fulfil a dual function as nuclear import receptors and cytoplasmic chaperones for exposed basic domains. EMBO J **2002**; 21:377–86.
25. Kohler M, Ansieau S, Prehn S, Leutz A, Haller H, Hartmann E. Cloning of two novel human importin-alpha subunits and analysis of the expression pattern of the importin-alpha protein family. FEBS Lett **1997**; 417:104–8.
26. Fielhaber JA, Tan J, Joung KB, et al. Regulation of karyopherin alpha1 and nuclear import by mammalian target of rapamycin. J Biol Chem **2012**; 287:14325–35.