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SUPPLEMENT ARTICLE

An Antiviral Role for TRIM14 in Ebola Virus Infection

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Ebola virus (EBOV) is a highly pathogenic virus that encodes 7 multifunctional structural proteins. Multiple host factors have been reported to interact with the EBOV proteins. Here, we found that tripartite motif-containing 14 (TRIM14), an interferon-stimulated gene that mediates cellular signaling pathways associated with type I interferon and inflammatory cytokine production, interacts with EBOV nucleoprotein to enhance interferon-β (IFN-β) and nuclear factor-κB (NF-κB) promotor activation. Moreover, TRIM14 overexpression reduced viral replication in an infectious but biologically contained EBOVΔVP30 system by approximately 10-fold without affecting viral protein expression. Furthermore, TRM14-deficient mice were more susceptible to mouse-adapted EBOV infection than wild-type mice. Our data suggest that TRIM14 is a host factor with anti-EBOV activity that limits EBOV pathogenesis.

Keywords. NF-κB; TRIM14; Ebola virus; interferon.

Ebola virus (EBOV), a member of the Filoviridae family, has been associated with recent outbreaks in West Africa [1] and the Democratic Republic of the Congo [2, 3]. The virion contains a single-stranded, negative-sense RNA genome that complexes with the viral nucleoprotein (NP) and structural proteins VP35, VP30, and L, to form the ribonucleocapsid complex, which is incorporated into an enveloped particle. Once EBOV virions enter the host cells via viral membrane fusion, viral genomes are released into the cytoplasm for transcription followed by replication. In the cytoplasm, cellular RNA sensors of viral pathogens such as RIG-I and MDA5 helicases detect viral double-stranded RNA (dsRNA) produced during replication [4]. RIG-I recognition of viral dsRNA leads to the activation of downstream signaling pathways including the nuclear factor-κB (NF-κB) signaling pathway that induces the production of cytokines including type I interferons (IFNs) and inflammatory cytokines, resulting in an antiviral state [5, 6].

To counteract host responses to infection, EBOV encodes at least 2 proteins, VP35 and VP24, that antagonize RIG-I and IFN signaling, respectively [7]. VP35 suppresses IFN production by antagonizing RIG-I–mediated signaling by disrupting (1) the binding of RIG-I with the viral dsRNA [8], (2) the interaction between RIG-I and PACT [9], and (3) dendritic cell maturation [10, 11]. To antagonize the signaling pathway triggered by IFNs, EBOV VP24 prevents the translocation of STAT-1 to the nucleus via its interaction with the nuclear import protein karyopherin-α1 [12–14], resulting in reduced expression of interferon-stimulated genes (ISGs), many of which have antiviral activity [15].

Members of the tripartite motif-containing (TRIM) protein family are involved in a broad range of biological functions and often associated with innate immunity [16]. Many TRIM proteins are ISGs induced by type I and type II IFNs, including TRIM14 [17–19]. TRIM14 is expressed in many human tissues such as brain, thymus, spleen, lung, liver, bone marrow, and the gastrointestinal tract [20], and these tissues are often targets of EBOV infection [21, 22]. TRIM14 mediates RIG-I signaling through its interaction with mitochondrial antiviral signaling protein (MAVS) [20, 23]. Knock-out (KO) of TRIM14 results in the disruption of RIG-I signaling resulting in reduced IFN production, and results in reduced IFN-mediated antiviral activity, potentially through an interaction with TRIM14 and IFN signaling [18, 23]. TRIM14 KO mice are more susceptible to herpes simplex virus type 1 infection, in part due to effects on IFN production [24]. For influenza A virus (IAV) and hepatitis C virus (HCV) infections, TRIM14 has been shown to inhibit virus replication through a type I IFN and NF-κB-independent manner [19, 25, 26]. Overexpression of TRIM14 can activate NF-κB signaling [19, 20, 24], potentially via a noncanonical NF-κB pathway independent of RIG-I-MAVS signaling [27].

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Given the reports of the involvement of TRIM14 in these other viral infections, we investigated the role of TRIM14 in EBOV infection. Here, we report that EBOV NP interacts with TRIM14 resulting in an increase in TRIM14-mediated IFN-β and NF-κB promoter activity. In cell culture, TRIM14 reduced viral replication without affecting viral protein expression, while TRIM14 KO mice were more susceptible to EBOV infection.

METHODS

Cells

Human embryonic kidney (HEK)-293 T cells and HEK-293 T cells stably expressing EBOV VP30 (HEK-293 T VP30 cells, established in a similar manner as Vero VP30 cells [28]) were grown in high-glucose Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, and antibiotics, and were maintained at 37 °C and 5% CO_2. Cells were routinely tested for mycoplasma on a monthly basis.

Viruses

EBOV expressing green fluorescent protein (EBOVΔVP30-GFP) or *Renilla* luciferase (EBOVΔVP30-luc) instead of the viral VP30 gene were propagated in Vero VP30 cells (African green monkey kidney-derived Vero cells stably expressing EBOV VP30) [28]. The use of EBOVΔVP30 viruses in biosafety level 2 (BSL-2) containment at the University of Wisconsin is approved by the Institutional Biosafety Committee, the National Institutes of Health (NIH), and the Centers for Disease Control and Prevention. Mouse-adapted EBOV (MA-EBOV) was grown and tittered on Vero E6 cells, and sequence confirmed [29].

Infection and Assessment of Viral Titers

EBOVΔVP30-luc infection

HEK-293 T VP30 cells were seeded into a 96-well plate (2.0×10^4 cells/well) and then transfected in triplicate with pCAGGS protein expression vectors for TRIM14 or GFP, or empty vector (70 ng/well) by using EndoFectin Max (GeneCopoeia). Twenty-four hours later, cells were infected with EBOVΔVP30-luc at a multiplicity of infection (MOI) of 0.05. Virus-driven *Renilla* luciferase activity was measured on days 1–3 postinfection using EnduRen Live Cell Substrate (Promega) according to the manufacturer's instructions.

EBOVΔVP30-GFP infection

HEK-293 T VP30 cells were seeded into a 12-well plate (3.0×10^5 cells/well) and then transfected in duplicate with pCAGGS expression vectors for TRIM14 or GFP, or empty vector (700 ng/well) by using EndoFectin Max (GeneCopoeia). Twenty-four hours later, cells were infected with EBOVΔVP30-GFP at an MOI of 0.5 or 0.05. Supernatants were harvested on day 3 postinfection, and viral titers were determined by use of focus-forming assay as previously described [28].

Coimmunoprecipitation Assay

The TRIM14 gene was N-terminally FLAG tagged and inserted into the pCAGGS protein expression vector. HEK-293 T cells were transfected with the FLAG-TRIM14 vector along with protein expression vectors (pCAGGS) for EBOV NP, VP35, and VP24 by using TransIT-LT1 (Mirus). Two days after transfection, the cells were washed once with phosphate-buffered saline and then treated for 30 minutes with lysis buffer (1% NP40, 50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 0.25% sodium deoxycholate) containing protease inhibitor cocktail (Sigma) to prepare whole-cell extracts. Cell extracts were mixed with preconjugated anti-FLAG M2 magnetic beads (Sigma) and incubated on a rotator at 4°C overnight. The next day, the magnetic beads were washed 3 times with lysis buffer. The bound proteins were eluted with excess FLAG peptide (Sigma) and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by immunoblotting with the indicated antibodies.

Western Blot Analysis

Cell extracts and immunoprecipitated samples were mixed with an equal volume of 2× Laemmli sample buffer (Bio-Rad) containing 5% β-mercaptoethanol and then incubated at 95°C for 10 minutes. Samples were resolved by SDS-PAGE through a 4%–20% SDS-polyacrylamide gel (Life Technologies). After electrophoresis, the proteins were transferred onto polyvinylidene fluoride membranes (Life Technologies) and blocked for 1 hour at room temperature with 5% skim milk/ Tris-buffered saline solution containing 0.05% Tween-20 (Sigma). Membranes were incubated overnight at 4°C with primary antibodies against EBOV NP (1:1000, R5071), EBOV VP35 (1:1000, cl. 1), EBOV VP40 (1:1000, cl. 6), EBOV VP30 (1:1000, PA5-112031, Invitrogen), EBOV VP24 (1:1000, cl. 21-5.2.5), TRIM14 (1:1000, HPA053217; Sigma), α-tubulin (1:1000, ab7291; Abcam), and FLAG tag (M2; Sigma). Membranes were then incubated with a secondary antibody coupled to horseradish peroxidase for 1 hour. Bound antibody was detected with SuperSignal Pico or Dura chemiluminescence reagent (Thermo Fisher Scientific) on an Amersham ImageQuant 800 imager (GE Healthcare Life Sciences).

Reporter Gene Assay

HEK-293 T cells were seeded in a 96-well plate and transfected in triplicate with pCAGGS-GFP (30 ng), pCAGGS-NP or its deletion mutants (30 ng; [15, 30]), pCAGGS-TRIM14 (5–30 ng), or empty vector, together with an NF-κB promoter reporter vector (10 ng; Promega) or an IFN-β promoter reporter vector (10 ng; p125-luc vector containing the full-length

IFN-β promoter upstream of the firefly luciferase gene, kindly provided by T. Fujita, Kyoto University). A *Renilla* luciferase expression vector (0.2 ng) was also transfected for luciferase data normalization. The total amount of transfected DNA (60–70 ng/well) was kept constant by adding empty pCAGGS vector. Twenty-four hours after transfection, cells were lysed in 50 μL of Glo-lysis buffer (Promega) and analyzed for firefly and *Renilla* luciferase activities by using the dual-luciferase reporter assay system (Promega) according to the manufacturer's instructions.

Animal Ethics and Biosafety

All infectious work with MA-EBOV was performed in the maximum containment laboratories at the Rocky Mountain Laboratories (RML), Division of Intramural Research, National Institute of Allergy and Infectious Diseases, NIH. RML is an institution accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. All procedures followed standard operating procedures approved by the RML Institutional Biosafety Committee. Mouse work was performed in strict accordance with the recommendations described in the Guide for the Care and Use of Laboratory Animals of the National Institute of Health, the Office of Animal Welfare and the Animal Welfare Act, United States Department of Agriculture. The study was approved by the RML Animal Care and Use Committee). Procedures were conducted in mice anesthetized by trained personnel under the supervision of veterinary staff. All efforts were made to ameliorate animal welfare and minimize animal suffering; food and water were available ad libitum.

Mouse Study

TRIM14 KO mice (C57BL/6J backbone) were designed and generated as recently described [31]. The mice were maintained as homozygotes with no apparent defects in development, growth, and fecundity. Both wild-type (WT) C57BL/6J (Jackson Laboratories) and TRIM14 KO female mice were housed in microisolator cages and allowed to acclimate for 7 days in BSL-4 containment prior to the experiment.

Mice were anesthetized with isoflurane and infected intraperitoneally with 100 focus-forming units of MA-EBOV. Animals were monitored for clinical signs of illness and weighed daily for 14 days. Study end point was 21 days after infection. Separate groups of mice were euthanized on days 3 and 6 after infection and blood, liver, and spleen samples were collected for virus titration. Titrations were performed as previously described [32].

Statistical Analysis

All statical analysis was performed using GraphPad Prism (version 9.3.1). Unless otherwise noted, the Student 2-tailed, paired, and unpaired *t* test was used to assess statistical differences between samples. A P value of <0.05 was considered significant.

RESULTS

EBOV NP, but not VP35 or VP24, Interacts With TRIM14

We first examined whether TRIM14 interacts with EBOV viral proteins. We focused on potential interactions of TRIM14 with EBOV VP35 and VP24 given the roles of these viral proteins in signal transduction pathways (ie, RIG-I and JAK-STAT signaling [7]). In addition, because IAV NP interacts with TRIM14 [26], we also determined whether TRIM14 interacts with EBOV NP. To examine potential interactions, we coexpressed an N-terminally FLAG-tagged TRIM14 (FLAG-TRIM14) with EBOV NP, VP35, or VP24 in HEK-293 T cells and performed a coimmunoprecipitation (co-IP) assay with an anti-FLAG antibody, followed by western blotting with anti-NP, VP35, or VP24 antibodies. Similar to the interaction between TRIM14 and IAV NP [26], we observed an interaction between TRIM14 and EBOV NP (Figure 1*A*). However, we did not detect an interaction between TRIM14 and EBOV proteins VP35 and VP24 (Figure 1*B* and 1*C*).

NP Enhances TRIM14-Mediated NF-κB and IFN-β Promotor Activation

Next, we investigated the effect of NP on TRIM14-induced activation of IFN-β and NF-κB promotor activities. First, HEK-293 cells were transfected with an NF-κB promoter-driven luciferase vector along with protein expression vectors for TRIM14, GFP (control), or EBOV NP. In the absence of TRIM14, EBOV NP did not increase NF-κB promoter-driven luciferase expression compared to the GPF control, as expected (Figure 2*A*). In contrast, ectopic expression of TRIM14 induced NF-κB promoter-driven luciferase expression, as reported by others [19, 20, 24]; this induction by TRIM14 was enhanced by the coexpression of EBOV NP (approximately 60-fold increase) compared with the GFP control (approximately 20-fold increase) (Figure 2*A*). Ectopic expression of TRIM14 also induced IFN-β promoter-driven luciferase expression in transfected HEK-293 T cells (Figure 2*B*), as reported by others [20, 24]. However, the induction of luciferase expression when EBOV NP was coexpressed with TRIM14 was not as robust as that observed with the NF-κB promoter (approximately 10-fold increase; Figure 2*A* and 2*B*).

To identify what region of EBOV NP modulates TRIM14-mediated signaling, we repeated the assay using a series of NP deletion mutants. EBOV NP has a core domain (amino acid [aa] 1–405) that consists of an N-terminal lobe (N-lobe; aa 39–240) and a C-terminal lobe (C-lobe; aa 241–405) that are important for EBOV NP oligomerization and RNA encapsidation [30, 33, 34]. The C-terminal tail (aa 641–739) of EBOV NP is required for NP-driven inclusion body formation and viral

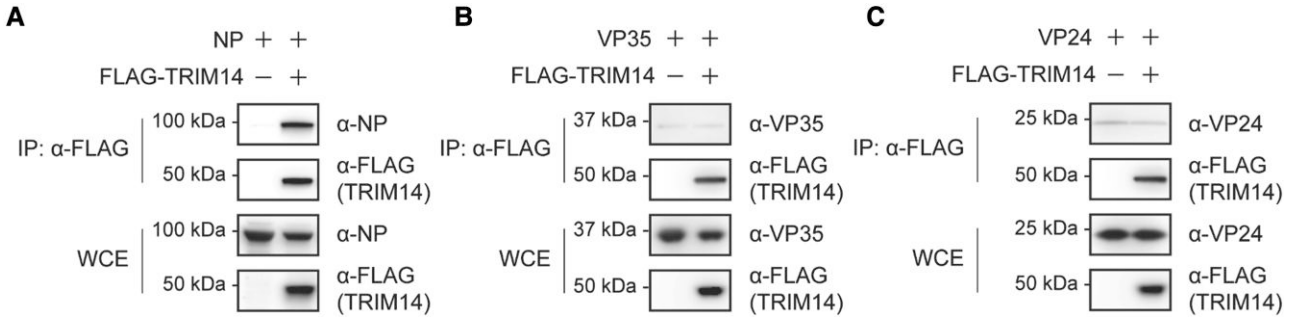

Figure 1. NP interacts with TRIM14. The interaction between TRIM14 and NP (*A*), VP35 (*B*), or VP24 (*C*) was assessed in HEK-293 T cells transfected with the indicated combination of expression vectors. Cell lysates were immunoprecipitated with anti-FLAG antibody and then immunoblotted. Data are representative of 2 or 3 independent experiments.

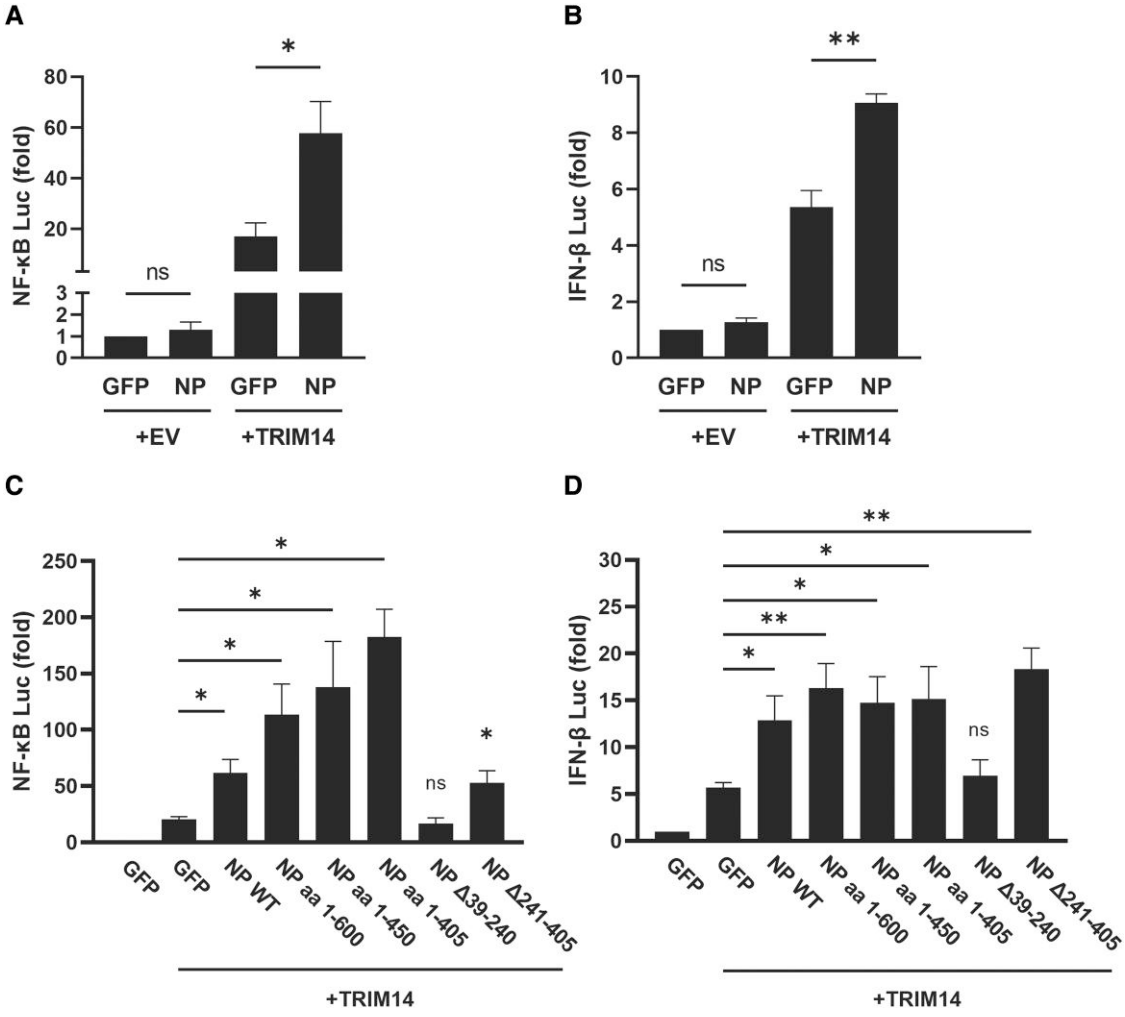

Figure 2. NP enhances TRIM14-mediated NF-κB and IFN-β promotor activation. Relative luciferase activity in HEK-293 T cells after transfection for 24 h with NF-κB protomer vector (*A* and *C*) or IFN-β promoter vector (*B* and *D*) together with the indicated combination of expression vectors and an internal *Renilla* luciferase control vector. Data are presented as means of fold changes (the GFP control was set to 1) ± SD of 3 independent experiments. Statistical significance was determined by 1-way ANOVA with Tukey multiple comparisons (*A* and *B*) and with Dunnett multiple comparisons (*C* and *D*). * Indicates a statistically significant difference (*$P < .05$, **$P < .01$) from the GFP control.

genome incorporation into virus particles [35]. We found that EBOV NP deletion mutants containing a core domain (aa 1–600, aa 1–450, and aa 1–405 constructs) were able to enhance both TRIM14-induced NF-κB and IFN-β promoter activities, comparable to or more potent than the full-length WT (aa 1–739), suggesting that the C-terminal approximately 300 amino acids are not necessary for the enhancement of the TRIM14-mediated activation (Figure 2*C* and 2*D*). Furthermore, while the EBOV NP deletion mutant lacking the N-lobe (Δ39–240) lost its ability to enhance TRIM14-mediated activation, the deletion mutant lacking the C-lobe (Δ241–405) retained enhancing activity, suggesting that the N-lobe is required for the enhancement (Figure 2*C* and 2*D*).

TRIM14 Overexpression Suppresses EBOVΔVP30 Infection

Next, we examined the effect of TRIM14 on EBOV infection with our EBOVΔVP30 system where the EBOVΔVP30 can replication in VP30-expressing cells [28]. First, we monitored EBOV-driven luciferase activity by using a virus that expresses *Renilla* luciferase instead of the VP30 gene (EBOVΔVP30-luc). HEK-293 T cells that stably express VP30 (HEK-293 T VP30 cells) were transfected with empty control vector or expression vectors for GFP or TRIM14 and then, 24 hours later, were infected with EBOVΔVP30-luc at an MOI of 0.05. Luciferase activity was measured every 24 hours after infection for 3 days. At the earlier time points, there was no significant difference in luciferase expression between TRIM14-transfected cells and cells transfected with either the empty vector or the GFP control ($P = .147$; on day 2). However, on day 3 postinfection, TRIM14 overexpression significantly reduced viral-driven luciferase expression by more than 60% compared to the GFP control cells ($P = .0078$; Figure 3*A*).

Next, we examined the effect of TRIM14 overexpression on viral infection. HEK-293 T VP30 cells were infected with

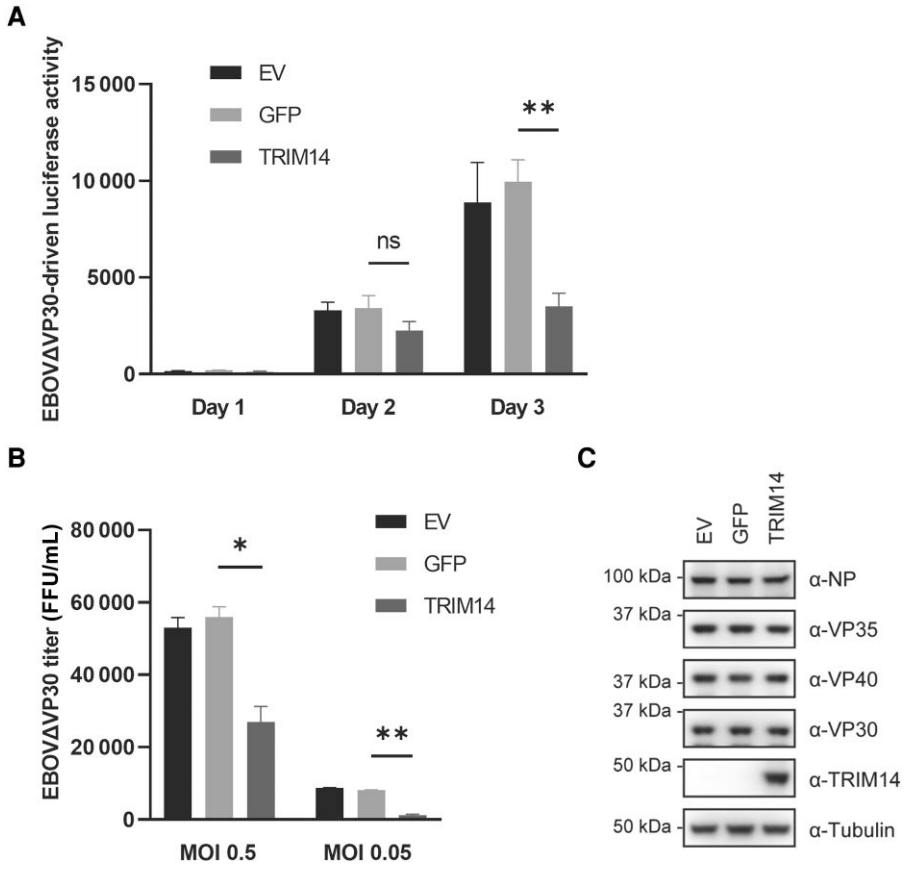

Figure 3. Ectopic expression of TRIM14 suppresses EBOVΔVP30 infection. *A*, EBOVΔVP30-driven *Renilla* luciferase activity in HEK-293 T VP30 cells. Cells were transfected with control EV or expression vectors for GFP or TRIM14 for 24 h prior to infection with EBOVΔVP30-luc at an MOI of 0.05. Virus-driven *Renilla* luciferase activity was measured every 24 h after infection for 3 days. Data are presented as means ± SD of 3 independent experiments. *B*, Titers of EBOVΔVP30-GFP from HEK-293 T VP30 cells after ectopic expression of TRIM14. Cells were transfected with the indicated expression vectors for 24 h prior to infection with EBOVΔVP30-GFP at an MOI of 0.5 or 0.05. Virus titers were then measured on day 3 postinfection. Data are presented as means ± SD of 2 independent experiments. * Indicates a statistically significant difference (*$P < .05$, **$P < .01$) from the GFP control. *C*, Viral protein expression in HEK-293 T VP30 cells transfected with the indicated expression vectors for 24 h prior to infection with EBOVΔVP30-GFP at an MOI of 0.5. At 24 h postinfection, cells were lysed and the indicated protein expression levels were analyzed by immunoblotting.

EBOVΔVP30-GFP at MOI of 0.5 or 0.05 24 hours after transfection. On day 3 postinfection, TRIM14 overexpression reduced virus growth compared to the GFP control ($P = .0151$; at MOI of 0.5; Figure 3*B*). This inhibitory effect by TRIM14 was more apparent when the cells were infected at the lower MOI (0.05), resulting in 85% inhibition ($P = .0015$). Despite this reduction in virus titers, viral protein expression levels were not affected by TRIM14 overexpression (Figure 3*C*), suggesting that TRIM14 may suppress EBOV infection in a way mediating interferon and cytokine expression rather than directly inhibiting viral protein expression.

TRIM14 KO Mice Are More Susceptible to EBOV Infection

Lastly, we investigated the impact of TRIM14 on EBOV infection in mice. WT C57BL/6J mice (n = 6, 6–8-week-old females) and TRIM14 KO mice (n = 12) were infected with a sublethal dose (100 focus-forming units) of MA-EBOV. Mice were monitored daily for weight loss and survival over 14 days. Both groups of mice showed similar, gradual body weight loss of approximately 15% of their original weight on day 0 over the disease course (Figure 4*A*). The survival rate of WT mice was 83% with the loss of 1 mouse on day 6 after infection (Figure 4*B*). In contrast, only 25% of the TRIM14 KO mice (3/12) survived infection with MA-EBOV, suggesting that the absence of TRIM14 increased these mice's susceptibility to EBOV infection.

With a separate group of WT and TRIM14 KO mice (n = 5 mice/group), we measured virus titers in the liver, spleen, and blood on days 3 and 6 after infection. While virus titers in the livers and spleens of the mice in both groups were similar on day 3 after infection, there was a 100-fold increase in virus titers in the blood of TRIM14 KO mice compared with WT mice ($P = .0397$; Figure 4*C*). On day 6 after infection, virus titers in all samples were similar in both groups of mice (Figure 4*C*).

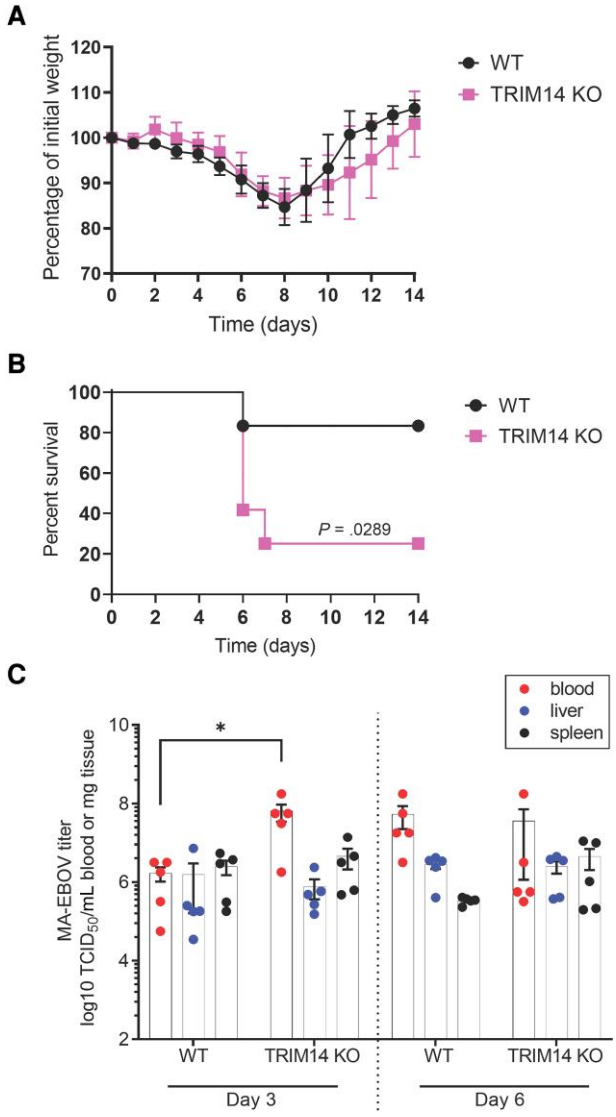

Figure 4. TRIM14 KO mice are more susceptible to EBOV infection. Weight changes (*A*) and survival (*B*) of WT mice (n = 6) or TRIM14 KO mice (n = 12) infected with MA-EBOV. Data are presented as means ± SD. *C*, Infectious virus titers in the blood, liver, and spleen of MA-EBOV-infected WT (n = 5) and TRIM14 KO (n = 5 each) mice on days 3 and 6 after infection. Symbols indicate data from individual animals. Geometric mean and geometric SD are presented. Statistical significance as determined by Mann-Whitney test is indicated; * $P < 0.05$.

DISCUSSION

TRIM14 is known to be involved in the life cycle of many viruses [18, 19, 24–26]. This IFN-induced host factor inhibits HBV replication through its interaction with viral regulatory protein HBx [18] and inhibits HCV replication by binding to and inducing degradation of viral nonstructural protein NS5A [19]. IAV NP interacts with TRIM14 resulting in the degradation of IAV NP and the restriction of IAV replication independently of IFN and NF-κB responses [26].

Here, we demonstrated that TRIM14 is an antiviral host factor in the context of EBOV infection. While TRIM14 interacted with EBOV NP but not with other viral proteins (VP35 and VP24) we tested (Figure 1), we did not observe degradation of NP or any other EBOV proteins in infected cells when TRIM14 was overexpressed (Figure 3*C*).

Although TRIM14 overexpression inhibited viral growth, the reduction in virus titers was relatively modest, less than 10-fold (Figure 3*A* and 3*B*). This degree of reduction in virus titers might explain why TRIM14 was not a positive hit in our previous ISG library screen [15]. NP overexpression enhanced TRIM14-mediated NF-κB and IFN-β promoter activation, which required an NP core domain, specifically the N-lobe (Figure 2), suggesting that TRIM14 may more likely be involved in cytokine responses combatting viral infection, as reported elsewhere [20, 24]. More detailed mechanistic studies on

how the N-lobe of NP enhances TRIM14-mediated signaling are warranted to reveal the role of the NP-TRIM14 interaction in EBOV infection.

We also demonstrated that the loss of TRIM14 in KO mice resulted in increased susceptibility to EBOV infection in this animal model (Figure 4). Despite increased susceptibility, virus replication in tissues (spleen and liver) was similar between WT and TRIM14 KO mice on both days 3 and 6 post-infection (Figure 4C) although the virus titers in the blood of TRIM14 KO mice were 100-fold higher than those in WT mice ($P = .0397$) on day 3 after infection. These data suggest that the virus may be more broadly disseminated to other tissues in the absence of TRIM14 during the early stage of infection due to impaired host responses mediated by TRIM14 rather than increased virus replication in each organ. Of note, it was recently reported that TRIM14 KO mice are also more susceptibility to severe acute respiratory syndrome coronavirus (SARS-CoV) and SARS-CoV-2 infection [31], suggesting a potential for TRIM14 to have broad-spectrum antiviral activity.

Our data suggest that TRIM14 is an important inhibitory ISG with anti-EBOV activity. Understanding the mechanism of viral antagonism along with the mechanism of action of TRIM14 and other ISGs with antiviral activity against filoviruses may lead to the development of therapeutic countermeasures.

Notes

Author contributions. M. K. and P. J. H. designed the study. M. K., P. J. H., and A. M. performed the experiments, data collection, and data analysis. L. B. T. and M. S. D. generated and provided TRIM14 KO mice. P. J. H. supervised the project. P. J. H., H. F., and Y. K. acquired funding. M. K. wrote the original draft. P. J. H. edited the manuscript. All authors reviewed the manuscript.

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