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# Highly Pathogenic New World Arenavirus Infection Activates the Pattern Recognition Receptor Protein Kinase R without Attenuating Virus Replication in Human Cells

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**ABSTRACT** The arenavirus family consists of several highly pathogenic viruses, including the Old World (OW) arenavirus Lassa fever virus (LASV) and the New World (NW) Junin virus (JUNV) and Machupo virus (MACV). Host response to infection by these pathogenic arenaviruses is distinct in many aspects. JUNV and MACV infections readily induce an interferon (IFN) response in human cells, while LASV infection usually triggers an undetectable or weak IFN response. JUNV induces an IFN response through RIG-I, suggesting that the host non-self RNA sensor readily detects JUNV viral RNAs (vRNAs) during infection and activates IFN response. Double-stranded-RNA (dsRNA)-activated protein kinase R (PKR) is another host non-self RNA sensor classically known for its vRNA recognition activity. Here we report that infection with NW arenaviruses JUNV and MACV, but not OW LASV, activated PKR, concomitant with elevated phosphorylation of the translation initiation factor $\alpha$ subunit of eukaryotic initiation factor 2 (eIF2$\alpha$). Host protein synthesis was substantially suppressed in MACV- and JUNV-infected cells but was only marginally reduced in LASV-infected cells. Despite the antiviral activity known for PKR against many other viruses, the replication of JUNV and MACV was not impaired but was slightly augmented in wild-type (wt) cells compared to that in PKR-deficient cells, suggesting that PKR or PKR activation did not negatively affect JUNV and MACV infection. Additionally, we found an enhanced IFN response in JUNV- or MACV-infected PKR-deficient cells, which was inversely correlated with virus replication.

**IMPORTANCE** The detection of viral RNA by host non-self RNA sensors, including RIG-I and MDA5, is critical to the initiation of the innate immune response to RNA virus infection. Among pathogenic arenaviruses, the OW LASV usually does not elicit an interferon response. However, the NW arenaviruses JUNV and MACV readily trigger an IFN response in a RIG-I-dependent manner. Here, we demonstrate for the first time that pathogenic NW arenaviruses JUNV and MACV, but not the OW arenavirus LASV, activated the dsRNA-dependent PKR, another host non-self RNA sensor, during infection. Interestingly, the replication of JUNV and MACV was not restricted but was rather slightly augmented in the presence of PKR. Our data provide new evidence for a distinct interplay between host non-self RNA sensors and pathogenic arenaviruses, which also provides insights into the pathogenesis of arenaviruses and may facilitate the design of vaccines and treatments against arenavirus-caused diseases.

**KEYWORDS** PKR, arenavirus, host-pathogen interactions, innate immune response, interferons

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Arenaviruses are enveloped, negative-sense RNA viruses (1). Their bisegmented genomic RNAs encode four viral proteins: the RNA-dependent RNA polymerase L protein, the small zinc finger Z protein, the viral nucleoprotein (NP), and the glycoprotein precursor (GPC). Mammalian arenaviruses are classified into Old World (OW) and New World (NW) arenaviruses (2). These viruses are usually rodent borne and may cause chronic infections in their natural rodent hosts (1). The arenavirus family includes several important human pathogens that cause severe and fatal diseases (1, 3). Among the human-pathogenic arenaviruses, the OW Lassa fever virus (LASV) is clinically the most important arenavirus; it causes Lassa fever (LF) disease in an estimated 300,000 people and 5,000 deaths annually in West Africa (4–6). In addition, the NW arenaviruses Junin virus (JUNV) and Machupo virus (MACV) cause Argentine hemorrhagic fever (AHF) and Bolivian hemorrhagic fever (BHF), respectively, in South America (7–10). Due to the high risk of these pathogenic arenaviruses to the public health and national security, LASV, JUNV, and MACV are classified as category A priority pathogens in the United States. Experiments involving infectious JUNV, MACV, and LASV samples must be performed in high-containment biosafety level 4 (BSL4) facilities.

Although LASV, JUNV, and MACV are all highly pathogenic in humans, their pathogenesis and the host innate immune responses to the infections are different in many aspects (11). This fact has implications in vaccine and treatment development. Severe and fatal LASV infection cases are typically immunosuppressive in nature, characterized by the lack of type I interferon (IFN) induction, proinflammatory response, and T cell activation (12, 13). LASV productively infects macrophages and dendritic cells but fails to activate these cells as well as $CD4^+$ and $CD8^+$ T cells (14, 15), which contributes to the severe LF diseases in fatal cases. An early IFN response has been found to be protective in a lethal LASV nonhuman primate infection model (16). In contrast to the general immunosuppression associated with LASV infection, clinical studies have revealed elevated levels of serum IFN-$\alpha$ (2,000 to 64,000 IU/ml) (17, 18) and proinflammatory cytokines in NW arenavirus JUNV-infected patients, which correlate with the severity and fatality of AHF diseases. MACV infection also induces potent IFN production in a lethal nonhuman primate model (19).

The interactions between the host innate immunity and pathogenic arenaviruses are largely unclear, particularly for pathogenic NW arenaviruses. It has been reported that JUNV infection does not induce IFN and cytokine production in macrophages and monocytes *ex vivo* (20). Nevertheless, we have found that pathogenic NW arenavirus JUNV and MACV infection, but not infection with the OW LASV, elicits a strong IFN response in human cells, including human lung epithelial A549 cells and human primary dendritic cells (21, 22), in agreement with clinical and animal study reports. Others have also reported the activation of IFN response upon JUNV infection in human and murine cells (23–25).

A key step in initiating the IFN response is the recognition of viral RNAs (vRNAs) that often contain unique features detected as pathogen-associated molecular patterns (PAMPs) by host pattern recognition receptors (PRRs) (26–28). RIG-I and MDA5 are examples of PRRs that recognize non-self RNA molecules typically containing double-stranded RNA (dsRNA) structures or a 5′-triphosphate group at the 5′ end. All these non-self RNA patterns are present in arenavirus vRNAs: the arenavirus genomic RNAs form 5′-triphosphate-containing, panhandle structures at the ends; meanwhile, the viral genomic RNAs and viral mRNAs contain the highly structured intergenic sequences (1). We previously reported that JUNV induced an IFN response in a RIG-I-dependent manner (21), suggesting that JUNV vRNAs could be detected by host PRRs during infection.

The double-stranded-RNA-activated protein kinase R (PKR) is another classical host non-self RNA sensor which is ubiquitously expressed at basal levels in cells (29–31). PKR contains two N-terminal dsRNA-binding motifs and a C-terminal kinase domain that is normally inactive. It is widely believed that upon virus infection, the RNA-binding domains of PKR recognize virus-derived dsRNA, directly leading to PKR autophosphorylation and activation (29). The enzymatically active PKR inhibits translation through

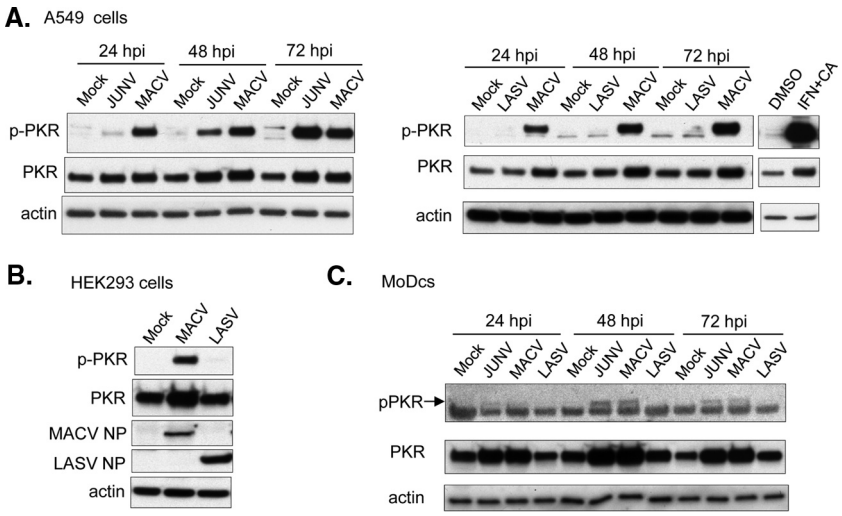


**FIG 1** PKR activation in human cells infected by pathogenic arenaviruses. Human A549 cells (A), HEK293 cells (B), and human monocyte-derived dendritic cells (MoDCs) (C) were mock infected or infected with JUNV, MACV, or LASV at an MOI of 1. Cell lysates were prepared at the indicated times after infection and subjected to Western blotting to detect phosphorylated PKR (p-PKR; T451), PKR, and human $\beta$-actin. For HEK293 cells, protein lysates were prepared at 48 h postinfection. As a positive control for PKR phosphorylation, A549 cells were treated with 1,000 U of IFN-$\alpha$ overnight and then incubated with 0.1 $\mu$M calyculin A for 10 min. The expression levels of MACV NP and LASV NP in HEK293 cells are shown (B). The immunoblotting data for JUNV, MACV, and LASV NP in A549 cells and MoDCs are not shown here and have been reported in our previous study as described in Materials and Methods.

phosphorylation of the translation initiation factor $\alpha$ subunit of eukaryotic initiation factor 2 (eIF2$\alpha$) and also regulates several signaling pathways, including the NF-$\kappa$B pathway (32). PKR expression could be further transcriptionally induced by IFN (28, 29). Here we report that the infection with NW arenaviruses JUNV and MACV, but not OW LASV, readily activated PKR, concomitant with elevated phosphorylation of eIF2$\alpha$. Furthermore, LASV infection did not affect poly(I·C) (pIC)-induced PKR activation, suggesting that LASV evaded PKR detection during infection. Host protein synthesis was substantially reduced in MACV- and JUNV-infected cells but was only slightly affected in LASV-infected cells. The replication of JUNV and MACV was less efficient in PKR-deficient cells, suggesting that PKR may facilitate JUNV and MACV infection. Our data provide new insights into host and arenavirus interactions and highlight the diversity and complexity of host responses to various pathogenic arenaviruses.

## RESULTS

**Infection with highly pathogenic NW arenaviruses MACV and JUNV, but not OW LASV, led to PKR activation in infected human cells.** In our previous studies, we found that the highly pathogenic NW arenaviruses JUNV and MACV, but not the pathogenic OW LASV, induces an IFN response in infected human cells (21, 22). The activation of the IFN response is RIG-I dependent in JUNV-infected cells (21). RIG-I is a host non-self RNA sensor that is activated by recognition of the PAMP often present in viral RNAs. It is highly likely that viral RNAs of NW pathogenic JUNV and MACV are detected by the host non-self RNA sensor RIG-I during infection. We asked whether arenavirus infection could also activate the double-stranded-RNA-dependent protein kinase R (PKR), another conventional host viral RNA sensor. Human lung epithelial A549 cells were infected with JUNV, MACV, or LASV at a multiplicity of infection (MOI) of 1. At 24, 48, and 72 h postinfection (hpi), robust phosphorylation of PKR at T451 was detected by Western blotting assay in MACV-infected cells (Fig. 1A), indicating a strong activation of PKR. PKR activation was also identified in JUNV-infected A549 cells, which was detectable at 24 h after JUNV infection, increased substantially at 48 hpi, and reached at a peak level at 72 hpi. In addition, PKR activation was found in MACV-infected HEK293 cells (Fig. 1B) as well as in primary human monocyte-derived dendritic

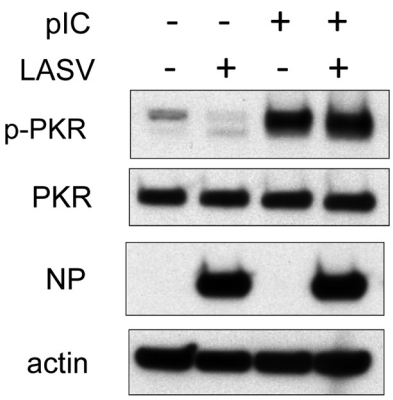


**FIG 2** LASV did not inhibit pIC-mediated PKR activation. A549 cells were infected with LASV for 24 h (MOI = 3) and then transfected with pIC (0.1 $\mu$g/ml) for 6 h. Protein lysates were prepared and subjected to immunoblotting analysis of phosphorylated PKR (p-PKR; T451), PKR, LASV NP, and human $\beta$-actin.

cells (MoDCs) infected with JUNV or MACV (Fig. 1C). The total amount of PKR increased simultaneously with PKR phosphorylation in JUNV- and MACV-infected cells, but to an extent much lower than the level of PKR phosphorylation (Fig. 1). These results indicated that NW arenavirus JUNV and MACV infection steadily activated PKR in various types of human cells. In contrast, PKR activation was not detectable in LASV-infected A549 cells, HEK293 cells, or the primary human monocyte-derived dendritic cells throughout infection (Fig. 1).

**LASV did not inhibit pIC-mediated PKR activation.** Since our data showed that LASV infection did not stimulate PKR activation, it is possible that either LASV inhibited the PKR pathway or LASV vRNAs evaded PKR detection. To address this issue, we examined double-stranded-RNA-induced activation of PKR in LASV-infected cells. Poly(I·C) (pIC), a synthetic analog of double-stranded RNA, was used as a surrogate for viral dsRNA. A549 cells were infected with LASV (MOI = 3.0) for 24 h and then transfected with pIC (0.1 $\mu$g/ml) for 6 h. pIC induced PKR activation to similar degrees in LASV-infected and mock-infected cells (Fig. 2), indicating that LASV could not efficiently block dsRNA-mediated PKR activation.

**Infection by human-pathogenic NW JUNV and MACV resulted in upregulation of eIF2$\alpha$ phosphorylation and suppression of global protein synthesis.** One well-characterized effect of PKR activation is that the enzymatically active PKR phosphorylates the host translation initiation factor 2$\alpha$ (eIF2$\alpha$) and further causes global translation arrest (30, 31). To study the biological significance of PKR activation upon pathogenic NW arenavirus MACV and JUNV infection, we characterized the phosphorylation of eIF2$\alpha$ in virus-infected human cells (MOI = 1) at different time points after infection. Sodium arsenite treatment of cells causes oxidative stress, leading to eIF2$\alpha$ phosphorylation and inhibition of translation. As a positive control, sodium arsenite treatment (250 $\mu$M) remarkably upregulated eIF2$\alpha$ phosphorylation in A549 cells (Fig. 3A). In repeated experiments, the level of phosphorylated eIF2$\alpha$ was elevated considerably in MACV-infected A549 cells at 24, 48, and 72 hpi (Fig. 3A), which was consistent with the kinetics of PKR activation (Fig. 1A). In JUNV-infected cells, the level of phosphorylated eIF2$\alpha$ was similar to that in the mock-infected cells initially at 24 hpi but substantially upregulated at 48 and 72 hpi. The level of phosphorylated eIF2$\alpha$ in LASV-infected A549 cells was comparable to that in mock-infected cells throughout infection (Fig. 3B). MACV infection (MOI = 1) also remarkably upregulated eIF2$\alpha$ phosphorylation in HEK293 cells at 48 hpi (Fig. 3C). A moderate increase in eIF2$\alpha$ phosphorylation was observed in LASV-infected HEK293 cells at the same time point, although PKR activation was not detectable (Fig. 1B).

Phosphorylation of translation initiation factor eIF2$\alpha$ leads to suppression of global translation. The conventional approach to study the rate of host translation involves the use of radioisotopes to metabolically label cultured cells. To facilitate studies in the

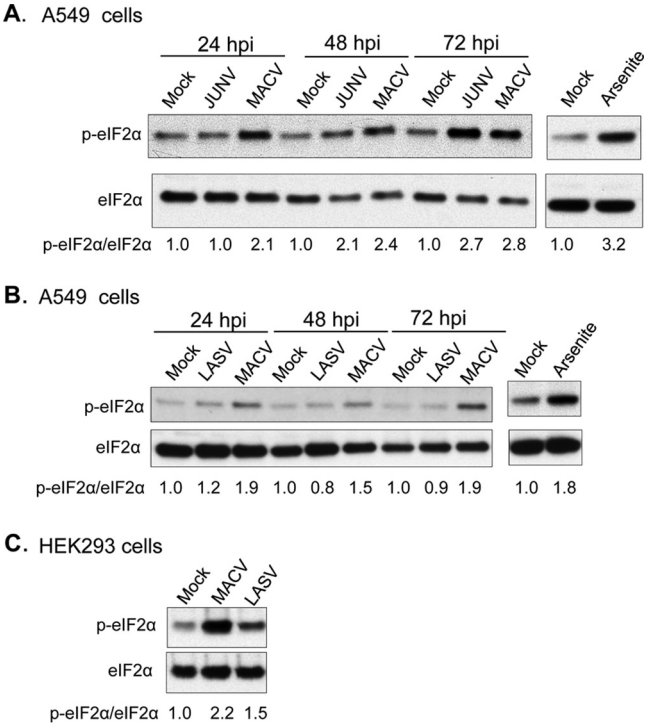


**FIG 3** Upregulation of eIF2$\alpha$ phosphorylation in cells infected with pathogenic NW JUNV and MACV. Human A549 cells were mock infected or infected with pathogenic NW arenaviruses JUNV and MACV (A) or infected with OW arenavirus LASV and MACV (B) at an MOI of 1. Cell lysates were prepared at the indicated times after infection. HEK293 cells were mock infected or infected with MACV or LASV at an MOI of 1 for 48 h (C). The positive controls for eIF2$\alpha$ phosphorylation were prepared by incubating cells with medium containing 250 $\mu$M sodium arsenite or prewarmed growth medium (mock treatment) for 1 h at 37°C and 5% $CO_2$. Protein samples were analyzed by Western blotting to detect phosphorylated eIF2$\alpha$ (Ser51). The same blots were then stripped and reprobed to detect total eIF2$\alpha$. Densitometry measurement of protein levels was performed, and the ratio of the phosphorylated form versus total eIF2$\alpha$ is shown.

high-containment (BSL4) facility, we utilized the surface sensing of translation (SUnSET) nonradioisotope protein labeling technique (33) to investigate the influence of pathogenic arenavirus infection on protein synthesis. The SUnSET method has been used in many studies (34–37); in this method, puromycin is used at a low concentration (10 $\mu$g/ml) for labeling newly synthesized protein without affecting host protein translation (33). Puromycylated nascent polypeptides are then immunodetected by a puromycin-specific monoclonal antibody. The puromycin-labeled polypeptides were readily detected with this assay (Fig. 4, Pur + Mock), while no signals could be detected in the absence of puromycin (Fig. 4, Pur −). In the presence of cycloheximide (CHX; 500 $\mu$M), a potent translation inhibitor, the signal of labeled proteins diminished drastically (Fig. 4, Pur + CHX), demonstrating that only newly synthesized polypeptides were detected by the SUnSET assay. pIC transfection (2 $\mu$g/ml) for 6 h led to shutdown of translation in A549 cells, concomitant with strong PKR activation and eIF2$\alpha$ phosphorylation (Fig. 4). In JUNV- and MACV-infected A549 cells (MOI = 3), the translation level was substantially decreased, by almost 60% and 80%, respectively, at 48 hpi, demonstrating a strong translational inhibition during infection. In comparison, there was only a slight reduction in translation in LASV-infected cells, consistent with the marginal upregulation of eIF2$\alpha$ phosphorylation (Fig. 4). This result clearly showed that pathogenic NW arenaviruses JUNV and MACV caused substantial translation arrest in infected A549 cells; meanwhile, the highly pathogenic OW arenavirus LASV only slightly affected protein synthesis.

**Pathogenic arenavirus replication in PKR-deficient cells.** PKR is known for its antiviral activity (30, 31). Since infection with the pathogenic NW arenaviruses JUNV and

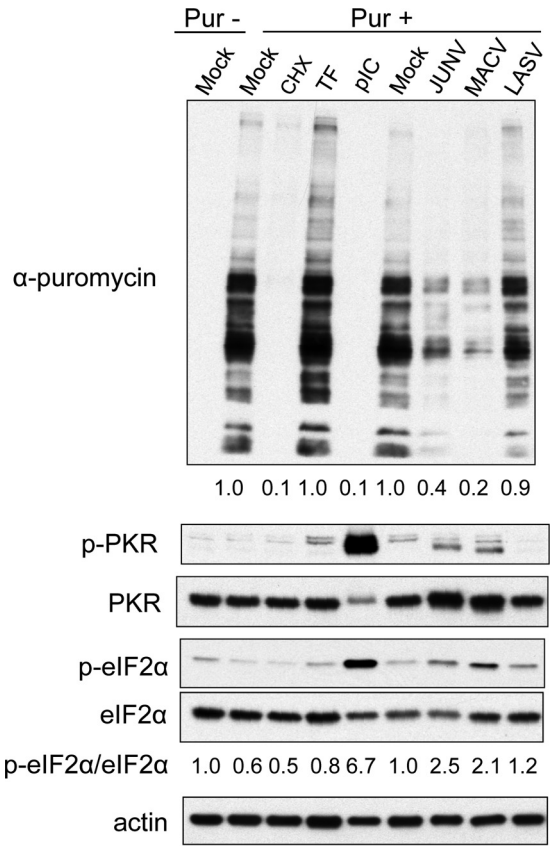


**FIG 4** Protein synthesis in cells infected with pathogenic arenavirus. A nonradioisotope protein labeling technique (SUnSET) was utilized to monitor nascent polypeptide synthesis in arenavirus-infected A549 cells. Cells were incubated with a low concentration of puromycin (10 $\mu$g/ml). Puromycylated, nascent polypeptides were immunodetected by a puromycin-specific monoclonal antibody. In the absence of puromycin (Pur $-$), no newly synthesized protein was detected. Treatment with cycloheximide (500 $\mu$M), a potent translation inhibitor, drastically diminished the signal of detected proteins (Pur $+$ CHX). Cells were mock transfected with Lipofectamine 2000 (TF) or transfected with pIC (2 $\mu$g/ml) for 6 h. A549 cells were mock infected or infected with JUNV, MACV, or LASV at an MOI of 3 for 48 h, and the translation status was analyzed. The immunoblotting results for p-PKR, PKR, p-eIF2$\alpha$, eIF2$\alpha$, and human $\beta$-actin are shown. Relative translation level and p-eIF2$\alpha$/eIF2$\alpha$ ratio are indicated.

MACV readily activated PKR, we further studied the potential impact of PKR activation on virus replication. We established a stable A549 cell line in which the PKR gene expression is deficient (PKR-deficient A549 cells) by utilizing the CRIPSPR/Cas9 gene editing technology to disrupt the PKR gene. The expression of PKR protein was below the detection level in these PKR-deficient A549 cells (Fig. 5A, Mock). Wild-type (wt) and PKR-deficient A549 cells were infected with JUNV, MACV, and LASV at MOI of 3 (Fig. 5A). In MACV-infected PKR-deficient A549 cells, the upregulation of eIF2$\alpha$ phosphorylation was ablated at 24 hpi and diminished at 48 hpi. In the case of JUNV infection, no upregulation of eIF2$\alpha$ phosphorylation was identified at 24 hpi in either wt A549 cells or PKR-deficient A549 cells. Only a low level of PKR activation was observed in JUNV-infected wt A549 cells at the same time point. At 48 h after JUNV infection, the level of the phosphorylated eIF2$\alpha$ was considerably elevated in wt A549 cells, coinciding with the PKR activation. In comparison, the upregulation of eIF2$\alpha$ phosphorylation abated in JUNV-infected PKR-deficient A549 cells at 48 hpi. LASV infection did not upregulate eIF2$\alpha$ phosphorylation in either wild-type or PKR-deficient A549 cells at 24 and 48 hpi (Fig. 5A). These results demonstrate the role of PKR in upregulation of eIF2$\alpha$ phosphorylation during JUNV and MACV infection and confirmed the functionality of PKR gene disruption in the PKR-deficient A549 cell line.

We also investigated the potential effect of PKR on arenavirus infection. Interestingly, JUNV and MACV NP levels were not decreased but instead were relatively higher

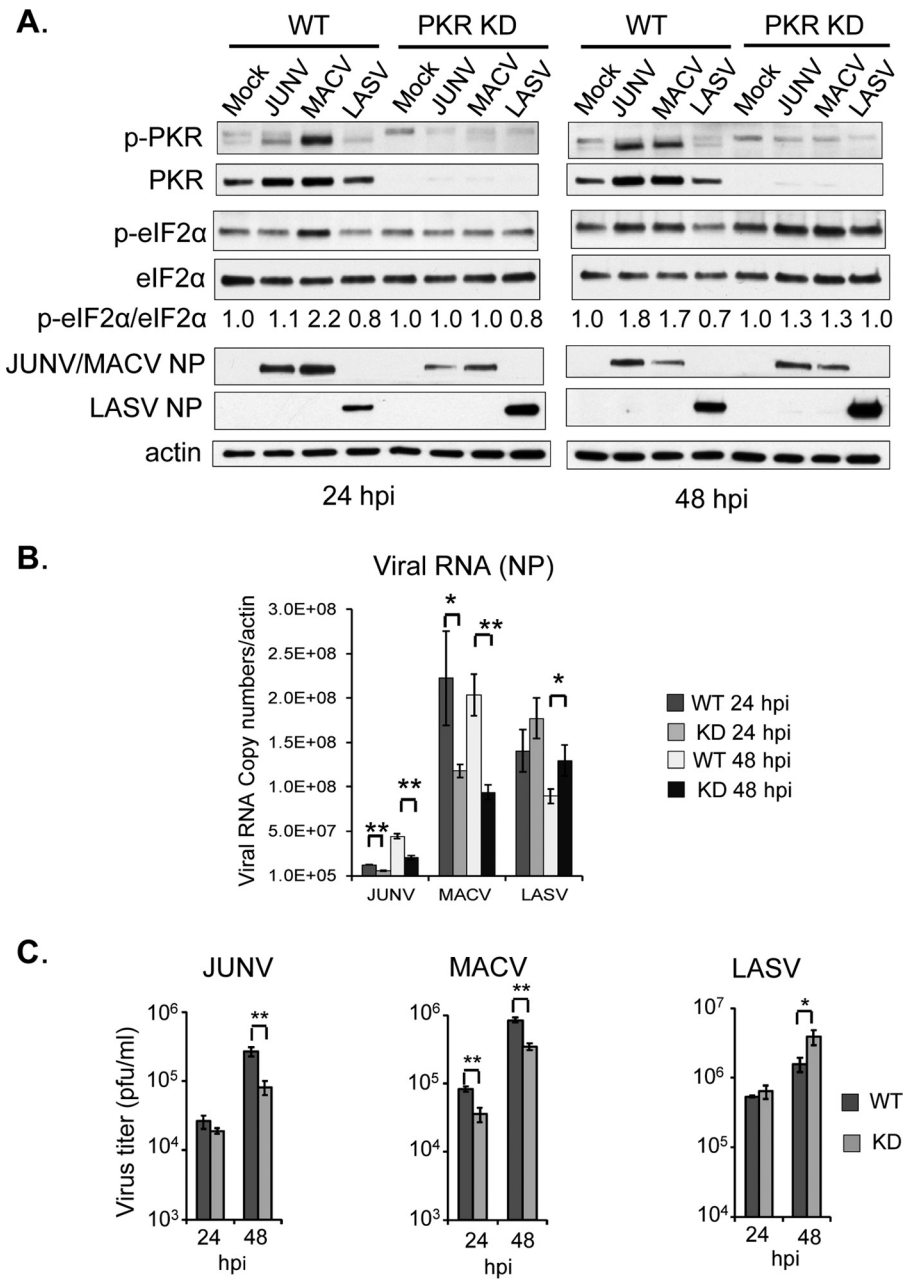


**FIG 5** Arenavirus infection in wt and PKR-deficient A549 cells (MOI = 3). PKR-deficient A549 cells were constructed by the CRISPR-Cas9 gene editing technology. wt A549 cells and PKR-deficient A549 cells (PKR-KD) were mock infected or infected with pathogenic NW JUNV or MACV or OW LASV at an MOI of 3. (A) At 24 and 48 hpi, cellular protein lysates were prepared and subjected to Western blotting for p-PKR, PKR, p-eIF2$\alpha$, eIF2$\alpha$, viral NPs, and human $\beta$-actin. (B) Real-time RT-qPCR analysis was performed to quantitate the copy numbers of viral RNA (NP gene region) at the same time points. Data were normalized to the levels of the housekeeping gene for human $\beta$-actin and are presented as the means ± SEM from three experiments. (C) Virus titers were determined at the indicated time points and are presented as the means ± SEM from three experiments. Student's *t* test was performed for statistical analysis (*, $P < 0.05$; **, $P < 0.01$).

in wt A549 cells than in PKR-deficient cells at 24 hpi (Fig. 5) in repeated experiments. Consistently, the viral RNA levels for JUNV and MACV were relatively higher in wt A549 cells than in PKR-deficient cells at 24 and 48 hpi as measured by real-time reverse transcription-quantitative PCR (RT-qPCR) (Fig. 5B). The titers of JUNV and MACV were also marginally higher in wt A549 cells than in PKR-deficient cells (Fig. 5C). These results indicate that despite the antiviral activity of PKR known for many other viruses, the

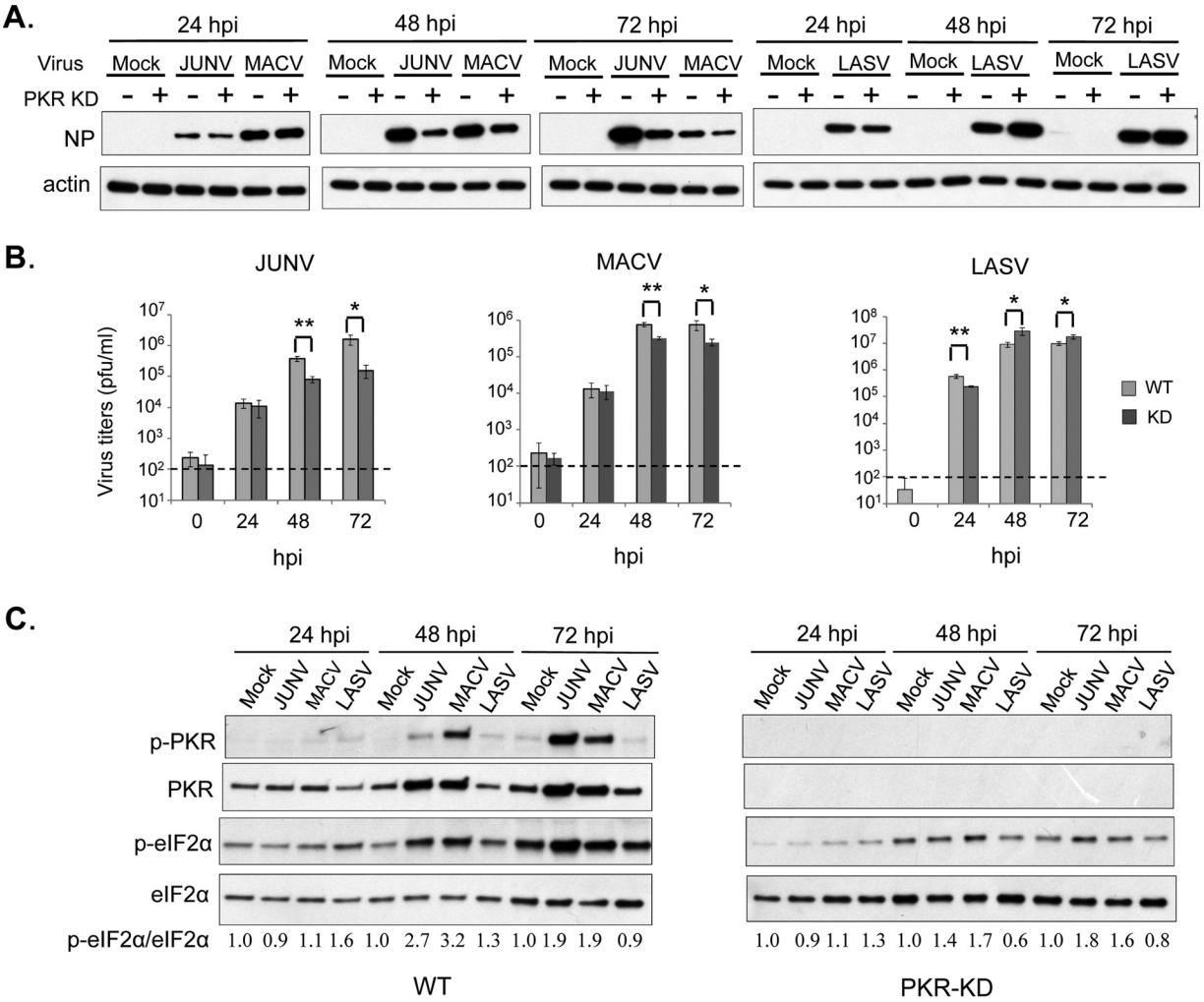


**FIG 6** Arenavirus infection in wt and PKR-deficient A549 cells (MOI = 0.3). wt A549 cells and PKR-deficient A549 cells were mock infected or infected with pathogenic NW JUNV or MACV or OW LASV at an MOI of 0.3. (A) At 24, 48, and 72 hpi, cellular protein lysates were prepared and subjected to Western blotting for viral NPs and human $\beta$-actin. (B) Virus titers were determined at the indicated time points and are presented as the means ± SEM from three experiments. Dashed lines indicate the detection limit of the plaque assay. Student's *t* test was performed for statistical analysis (*, $P < 0.05$; **, $P < 0.01$). (C) Protein samples were analyzed by Western blotting to detect p-PKR and p-eIF2$\alpha$ (Ser51). The same blots were then stripped and reprobed to detect total PKR and eIF2$\alpha$. Densitometry measurement of protein levels was performed, and the ratio of the phosphorylated form versus total eIF2$\alpha$ is shown.

replication of JUNV and MACV was not impaired but appeared to be more efficient in wt A549 cells than in PKR-deficient cells. In comparison, the replication of OW LASV was slightly augmented in PKR-deficient A549 cells, as indicated by the relatively higher levels of NP (Fig. 5A) and vRNA (Fig. 5B) and higher virus titers (Fig. 5C) in PKR-deficient cells than in A549 cells.

To further confirm our results, we repeated the arenavirus infection experiment at a lower MOI (0.3). Augmented NP expression was observed in wt A549 cells compared to that in PKR-deficient A549 cells at 48 and 72 h after JUNV and MACV infection (Fig. 6A), which was consistent with the moderately higher virus titers, at the same time points, in wt A549 cells than in PKR-deficient A549 cells (Fig. 6B). In comparison, LASV NP expression and virus titers were relatively higher in PKR-deficient cells than in wt A549 cells at 48 and 72 h after infection (Fig. 6A and B). The LASV titer and NP expression were lower at 24 hpi in PKR-deficient cells than in wt cells. At 24 h after JUNV and MACV infection, no upregulation of phosphorylated PKR (p-PKR) or p-eIF2$\alpha$ was identified in A549 cells compared with mock-infected A549 cells (Fig. 6C); meanwhile, the NP levels for both viruses were similar in A549 cells and PKR-deficient cells (Fig. 6A).

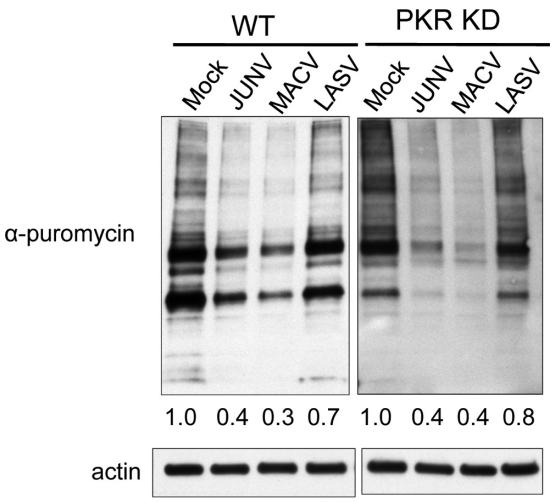


**FIG 7** Protein synthesis in pathogenic-arenavirus-infected PKR-deficient cells. wt A549 cells or PKR-deficient A549 cells were mock infected or infected with JUNV, MACV, or LASV at an MOI of 3 for 48 h. The translation status was analyzed by the SUnSET nonradioisotope protein labeling technique. The relative translation level was quantitated by densitometry analysis and is indicated.

Upregulation of p-PKR and p-eIF2$\alpha$ was readily detected starting from 48 to 72 h after JUNV and MACV infection in A549 cells (Fig. 6C), concomitantly with higher NP expression in wt A549 cells than in PKR-deficient cells for JUNV and MACV (Fig. 6A). These data further support the notion that PKR activation did not interfere with JUNV and MACV replication. A transient and modest increase of p-eIF2$\alpha$ was identified in LASV-infected cells at 24 hpi, which was diminished afterwards (Fig. 6C). Our results also demonstrate decreased eIF2$\alpha$ phosphorylation in PKR-deficient cells compared with that in wt A549 cells at 48 h after JUNV and MACV infection, suggesting at least a partial role of PKR in eIF2$\alpha$ phosphorylation.

Inhibition of protein translation occurred in concomitance with PKR activation and eIF2$\alpha$ phosphorylation during NW arenavirus JUNV and MACV infections. To study the contribution of PKR activation and eIF2$\alpha$ phosphorylation to translation inhibition, next we characterized the host translation in PKR-deficient A549 cells and wt A549 cells after arenavirus infection (MOI = 3). Interestingly, profound levels of inhibition of protein translation were still observed in JUNV- or MACV-infected PKR-deficient A549 cells ($\sim$60% reduction), to an extent similar to that in wt A549 cells ($\sim$60 to 70% reduction) (Fig. 7). These data suggest that it is highly likely that another molecular mechanism(s) might also contribute to the translation suppression during JUNV and MACV infection.

**Enhanced IFN response in PKR-deficient cells during JUNV and MACV infection.** Our results indicate that PKR did not interfere with JUNV and MACV replication. Hepatitis C virus suppresses interferon-stimulated gene (ISG) expression by inducing PKR activation (38). It is conceivable that PKR might be beneficial to JUNV and MACV replication by attenuating the IFN response. We characterized IFN and ISG gene expression after pathogenic arenavirus infection in wt and PKR-deficient A549 cells (Fig. 8). The IFN-$\beta$ mRNA level was increased approximately 7-fold in PKR-deficient cells over that in wt A549 cells at 24 h after JUNV and MACV infection (Fig. 8A). At the same time point, noticeably higher levels of ISG expression (ISG15, OAS1, and RIG-I) were also identified in PKR-deficient cells than in wt A549 cells during JUNV and MACV infection (Fig. 8B). These results clearly demonstrate an enhanced IFN response in JUNV- and MACV-infected PKR-deficient cells, which inversely correlates to the level of viral replication (Fig. 5A).

## DISCUSSION

In this study, we found that pathogenic NW arenavirus JUNV and MACV infections activated PKR, another host pattern recognition receptor that recognizes non-self RNA.

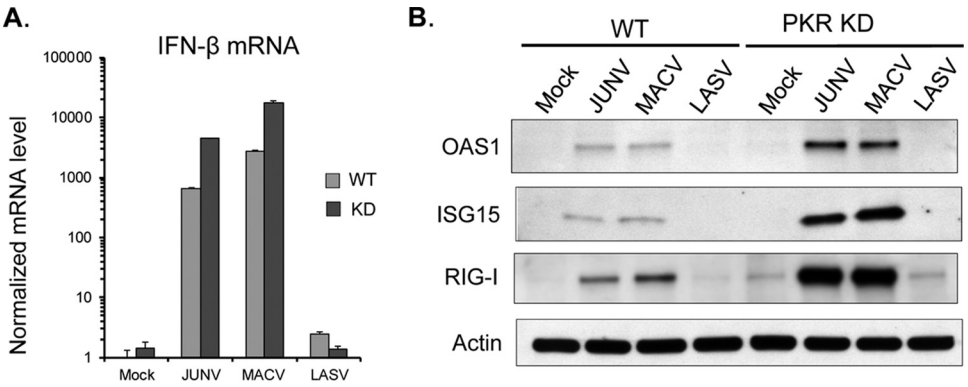


**FIG 8** IFN response in pathogenic-arenavirus-infected PKR-deficient cells. wt A549 cells and PKR-deficient A549 cells were mock infected or infected with pathogenic NW JUNV, MACV, or OW LASV at an MOI of 3. (A) Real-time RT-qPCR analysis was performed to quantitate IFN-$\beta$ mRNA at 24 hpi. Data were normalized to the levels of the human housekeeping $\beta$-actin gene and are presented as the means $\pm$ SEM from three experiments. (B) Western blotting data for ISG proteins (OAS1, ISG15, and RIG-I) and human $\beta$-actin at 24 hpi.

In comparison, pathogenic OW arenavirus LASV neither stimulated nor interfered with PKR activation. PKR is known for its antiviral activity against many other viruses. Interestingly, our data indicate that PKR did not suppress but might facilitate JUNV and MACV RNA synthesis. PKR activation resulted in elevated phosphorylation of the translation initiation factor eIF2$\alpha$ in JUNV- and MACV-infected cells. Host protein synthesis was substantially suppressed in MACV- and JUNV-infected cells but only slightly decreased in LASV-infected cells. Our data also demonstrate an enhanced IFN response in PKR-deficient cells upon NW JUNV and MACV infections.

The host innate immune response to pathogenic arenaviruses, especially to the pathogenic NW arenaviruses, is largely not understood. This is partly due to the high biosafety risk associated with these viruses, which necessitates the requirement for a high-containment (BSL4) facility to perform infection experiments. We previously reported that pathogenic NW arenavirus JUNV and MACV infections, in contrast to the OW LASV, readily elicit an IFN response in human cells (21), which is consistent with clinical and animal study data. A key step in initiation of host innate immune response, including IFN response, is the recognition of viral RNAs by host non-self RNA sensors. Viral RNAs often contain unique features that could be detected as PAMPs by PRRs, such as RIG-I, MDA5, and PKR. The vRNAs of arenavirus contain a 5′-triphosphate-containing panhandle structures compromising the 5′ and 3′ ends of genomic RNAs and the highly structured intergenic regions. All these structures are potential targets for RIG-I and PKR recognition. Arenavirus RNA synthesis occurs in the viral replication-transcription complex (RTC). A study with the nonpathogenic arenavirus Tacaribe virus and a vaccine strain of JUNV has revealed that arenavirus RTCs are compartmentalized and associated with virus-induced, NP-containing, discrete cytosolic membrane structures (39). The viral genomic and antigenomic RNAs are mainly identified in RTCs, where the viral mRNAs are scant and presumably exported for translation. Based on this model, it is possible that some of the arenavirus mRNAs might be exposed to PRRs when being exported from the compartmentalized RTCs to cytosolic ribosomes and rough endoplasmic reticulum for translation. The apparent independence of viral translation from cellular translation in cells infected with JUNV and MACV suggests that viral translation may also occur within specialized domains that may not be physically associated with the RTC itself. JUNV infection stimulates the IFN response in a RIG-I-dependent manner (21), indicating that JUNV vRNAs are recognized by host PRRs in infected cells and subsequently induce the signal transduction through the IFN signaling cascade and the expression of IFN and ISGs. PKR is another classical host non-self RNA sensor. Importantly, it is activated directly after dsRNA binding and autophosphorylation. Our data clearly show that pathogenic NW arenaviruses JUNV and MACV, but not OW LASV, readily activated the PKR response in various cell types (Fig. 1). These

results demonstrate that the vRNAs of JUNV and MACV are detected by PRRs such as PKR and RIG-I during infection. LASV apparently did not interfere with pIC-mediated PKR activation (Fig. 2), suggesting that it is less likely that LASV vRNAs were efficiently recognized by PKR during infection; otherwise there would have been PKR activation in LASV-infected cells. It is likely that LASV vRNAs evade PRR recognition through a mechanism yet to be identified. We could not rule out the possibility that a partial inhibition of PKR activation by LASV might be detected at a lower concentration of pIC. Future studies are warranted to address the basis of LASV evasion of PKR recognition.

The impact of PKR activation on pathogenic arenavirus infection was investigated. PKR activation has been known as one of the early host innate immune response limiting infection by many other viruses (28, 29). Therefore, many viruses utilize a variety of approaches to counteract PKR to optimize their replication (30). We observed PKR activation in JUNV- or MACV-infected cells (Fig. 1 and 2), which might be detrimental to JUNV and MACV infection. Interestingly, the presence of PKR did not seem to negatively affect JUNV and MACV infection but rather facilitated the viral replication marginally as suggested by the relatively higher levels of viral RNA and NP and the slightly higher levels of virus titer in wt cells than in PKR-deficient A549 cells (Fig. 5 and 6).

Further characterization suggests that the apparently beneficial effect of PKR on JUNV and MACV replication seems to be related to the attenuated IFN response through PKR activation (Fig. 8). This observation is not unique to JUNV and MACV. Hepatitis C virus preferentially suppresses interferon-stimulated-gene translation by inducing PKR activation (38). Upon depletion of PKR in IFN-treated cells, the expression of ISG proteins and the antiviral effects of IFN are both enhanced, resulting in impaired HCV replication. In addition, influenza virus with deletion of the NS1 gene (Flu-delNS1) induces the type I IFN response through the RIG-I pathway. PKR has been found to not be critical to the RIG-I-dependent IFN response (40). In PKR-null mouse embryonic fibroblasts (MEFs) and PKR-deficient A549 cells, Flu-delNS1 infection stimulates stronger IFN-$\beta$ mRNA expression than that in wild-type MEFs and A549 cells (40). Similar to the Flu-delNS1 virus, JUNV and MACV trigger the type I IFN response in a RIG-I-dependent manner. Importantly, enhanced IFN-$\beta$ mRNA expression was also observed in PKR-deficient cells during JUNV and MACV infection (Fig. 8). Collectively, these data support the idea that PKR may facilitate the replication of some viruses by negatively regulating the IFN response.

PKR appears to be dispensable for the translation suppression in JUNV- and MACV-infected cells, as the extents of translation inhibition were similar in PKR-deficient cells and wt A549 cells (Fig. 7). Activated PKR phosphorylates the translation initiation factor eIF2$\alpha$ and renders eIF2$\alpha$ as having a high affinity for the GTP exchange factor eIF2B, leading to the sequestration of eIF2B and the prevention of ternary complex eIF2-GTP-$^{Met}$tRNA$_i$ regeneration (31). eIF2-GTP-$^{Met}$tRNA$_i$ formation and eIF4E activity regulation are two major rate-limiting steps in translation initiation (41). We observed PKR activation and upregulation of eIF2$\alpha$ phosphorylation in JUNV- and MACV-infected cells but not in LASV-infected cells, which coincided with the translation suppression in JUNV- and MACV-infected cells but not in LASV-infected cells (Fig. 3 and 4). In JUNV-infected or MACV-infected PKR-deficient cells, the upregulation of eIF2$\alpha$ phosphorylation was diminished or reduced relative to that in wt A549 cells (Fig. 5), suggesting a partial contribution of PKR to eIF2$\alpha$ phosphorylation. Nevertheless, our data show similar degrees of translation inhibition in PKR-deficient and wt A549 cells during JUNV and MACV infection (Fig. 7). Similarly, PKR-independent translation suppression has also been identified for Sindbis virus (42) and Chikungunya virus (43).

It is possible that a mechanism(s) other than PKR activation which might be complementary to or more dominant than the PKR pathway during JUNV and MACV infection is also involved in translation arrest. Protein kinase R-like endoplasmic reticulum kinase (PERK), another kinase for eIF2$\alpha$ phosphorylation (41), could be activated and contribute to eIF2$\alpha$ phosphorylation and host translation inhibition during JUNV and MACV infection. PERK activation might also contribute to the moderate upregu-

lation of eIF2$\alpha$ phosphorylation in LASV-infected HEK293 cells in the absence of PKR activation (Fig. 3). On the other hand, we found induction of OAS1 expression in both wt and PKR-deficient cells during JUNV and MACV infection (Fig. 8B). Activation of the 2′,5′-oligoadenylate synthetase/RNase L pathway is known to suppress host protein synthesis (29, 44). Activations of the PKR, PERK, and OAS/RNase L pathways are not necessarily exclusive to each other in translation suppression. Further studies are required to determine the potential contribution of the PERK pathway and OAS/RNase L pathway to arenavirus-induced host translation inhibition.

Arenavirus might interfere with eIF4E to affect host translation. Regulation of eIF4E activity is another major rate-limiting step in translation initiation (41). The Z protein of the prototypic arenavirus lymphocytic choriomeningitis virus (LCMV) has been shown to interact with the translation initiation factor eIF4E, affect the binding of eIF4E with the $m^7$ cap structure on host mRNA, and cause translation suppression (45, 46). LASV Z protein has also been shown to inhibit translation of a reporter gene in an *in vitro* translation assay (46). These data might explain the slight reduction in translation in LASV-infected cells (Fig. 4). Whether the Z proteins of JUNV and MACV might also affect eIF4E function or suppress host translation remains unclear. Interestingly, there is evidence suggesting that inhibition of eIF4E does not impair JUNV protein synthesis (47). The NP of NW arenavirus JUNV has been proposed to replace eIF4E to initiate translation from viral mRNA (47). However, in the case of NW Tacaribe virus, although depletion of eIF4E has a moderate impact on virus-like mRNA translation, the translation efficiency of viral mRNA is independent of NP (48). It has also been reported that eIF4E is involved in LCMV protein synthesis and that the LCMV NP less likely replaces eIF4E in the eIF4F complex (49).

While our manuscript was in preparation, King et al. reported that infection with the Candid#1 vaccine strain of JUNV activated the PKR response in infected cells and that small interfering RNA (siRNA)-mediated knockdown of PKR had no impact on viral propagation (50). Furthermore, JUNV NP is shown to interact with PKR in infected cells, suggesting that JUNV NP might locally inhibit PKR activity, thereby facilitating viral protein synthesis. Their observations made with the vaccine strain of JUNV are generally consistent with our data with the pathogenic strains of JUNV and MACV. Interestingly, it seems that the Candid#1 infection neither upregulates eIF2$\alpha$ phosphorylation through an unknown mechanism nor leads to translation suppression. These results suggest a phenotypic difference in the impact of virus infection on protein synthesis between the vaccine and pathogenic strains of JUNV, which remains to be investigated in future studies. Another potential way to explain the observed differences is that we prepared and used recombinant virus stocks that are likely less heterogeneous than the vaccine Candid#1 strain used in the aforementioned study (50).

In summary, our studies provide new evidence showing distinct host responses to NW arenaviruses JUNV and MACV and to OW arenavirus LASV, although all these arenaviruses cause severe or lethal diseases in humans. A better understanding of virus-host interactions and their pathogenesis will facilitate the development of vaccines and therapeutics against infection by these highly pathogenic viruses.

## MATERIALS AND METHODS

**Viruses and cells.** Recombinant JUNV (Romero strain), MACV (Carvallo strain), and LASV (Josiah strain) stock viruses were prepared and propagated by infecting Vero cells (American Tissue Culture Collection [ATCC], Manassas, VA) at an MOI of 0.001 (51, 52). Supernatants were harvested at day 4 postinfection and clarified by centrifugation and filtration through filters (0.45-$\mu$m pore size) to remove cell debris. Virus stocks were further purified by using Ultra 100K filter devices (Ultralcel 100K; molecular weight cutoff, 100,000; Amicon, Millipore). All infection work with pathogenic arenaviruses was performed at the BSL4 facilities in Galveston National Laboratory in the University of Texas Medical Branch (UTMB) in accordance with institutional health and safety guidelines and federal regulations.

Human lung epithelial A549 cells (ATCC CCL-185) and HEK293 cells (ATCC CRL-1573) were purchased from the ATCC and maintained in Dulbecco modified Eagle medium (DMEM) containing 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml of streptomycin, and 100 U/ml of penicillin. Human primary monocyte-derived dendritic cells (MoDCs) were prepared as described previously (22). Briefly, human blood buffy coats containing peripheral blood mononuclear cells (PBMCs) from three healthy anonymous donors were obtained from the UTMB Blood Center under an institutional review board (IRB)

exemption. After centrifugation on Histopaque 1083 (Sigma-Aldrich, St. Louis, MO), monocytes were positively selected with CD14-specific microbeads (human CD14 microbeads; Miltenyi Biotec Inc., San Diego, CA) and cultured in RPMI 1640 differentiation medium containing 10% heat-inactivated FBS, 100 U/ml of streptomycin, 100 U/ml of penicillin, 2 mM glutamine, 50 ng/ml of human interleukin 4 (IL-4), and 250 ng/ml of human granulocyte-macrophage colony-stimulating factor (GM-CSF; R&D Systems, Inc., Minneapolis, MN). Cells were incubated at 37°C under 5% $CO_2$ for 6 days. On days 2 and 4, half of the medium was removed and replaced with fresh differentiation medium.

In each experiment, cells were treated with the eukaryotic protein synthesis inhibitor cycloheximide (catalog number 0970; R&D) at 500 $\mu$M for 5 min at 37°C and 5% $CO_2$. The positive controls for eIF2$\alpha$ phosphorylation were prepared by incubating cells with medium containing 250 $\mu$M sodium arsenite (Fisher; certified grade) or prewarmed regular medium (mock treatment) for 1 h at 37°C and 5% $CO_2$. The positive controls for the phosphorylated form of PKR were prepared by treatment of cells with 1,000 U of IFN-$\alpha$ (Intron A; Schering Corporation, NJ) overnight and then with 0.1 $\mu$M calyculin A (C5552; Sigma) for 10 min. Low-molecular-weight poly(I·C) (pIC; tlrl-picw; InvivoGen) was transfected with Lipofectamine 2000 reagent (Invitrogen) per the manufacturer's protocol.

**Construction of PKR-deficient A549 cells by the CRISPR-Cas9 gene editing technology.** To construct PKR-deficient A549 cells, the PKR clustered regularly interspaced short palindromic repeat (CRISPR)/Cas9 knockout (KO) plasmid (sc-400177; Santa Cruz), which consists of a pool of 3 plasmids, each encoding the Cas9 nuclease and a target-specific 20-nucleotide (nt) guide RNA, was transfected into A549 cells grown on a 6-well plate by using Lipofectamine 2000 (Invitrogen). The PKR HDR plasmid (sc-400177-HDR; Santa Cruz) was cotransfected to replace the cleaved PKR gene on human chromosome DNA with a red fluorescent protein (RFP)-encoding gene and a drug selection gene. Two days after transfection, cells were selected by 1 $\mu$g/ml of puromycin-containing medium for 1 week. Surviving cells were cloned by limiting dilution and amplification. The disruption of PKR expression in PKR-deficient A549 cells was validated by immunoblotting detection of the PKR protein.

**Detection of protein synthesis by SUnSET.** A nonradioisotope labeling method, surface sensing of translation (SUnSET), was used to monitor protein synthesis in cells treated with puromycin (33). For each assay, medium was removed from cultured cells. Cells were washed twice with phosphate-buffered saline (PBS) and then treated with prewarmed medium containing 10 $\mu$g/ml of puromycin (ant-pr-1; InvivoGen) and incubated for 10 min at 37°C and 5% $CO_2$. The medium of cultured cells was removed. Cells were washed with PBS. Then cell lysates were harvested by using 2× SDS-PAGE buffer (Bio-Rad) containing protease inhibitor cocktail for mammalian cells (P8340; Sigma) and phosphatase inhibitor cocktails (P5726 and P0044; Sigma) and then incubated at 95°C for 5 min. Puromycin-incorporated, nascent polypeptides were detected by immunoblotting using a monoclonal antipuromycin antibody (clone 12D10; Millipore).

**RNA extraction and real-time RT-qPCR.** RNA purification was performed as described previously (53, 54). Briefly, RNA lysates were prepared from infected cells with TRIzol reagent (Life Technology). Samples were further purified by using an RNeasy Micro or RNeasy minikit (Qiagen) according to the manufacturer's instructions. To avoid potential host chromosome DNA contamination, RNA samples were treated with DNase I (Qiagen). cDNA synthesis was carried out using the iScript Advanced cDNA synthesis kit (Bio-Rad, CA) for real-time reverse transcription-quantitative PCR (RT-qPCR). Real-time PCR amplification was performed with SsoAdvanced Universal SYBR green Supermix (Bio-Rad) on a CFX96 real-time PCR detection system (Bio-Rad). Threshold cycle ($C_T$) values of targeting genes were normalized to the average $C_T$ values of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or $\beta$-actin housekeeping gene. Thirty nanograms of total RNA was used for each qPCR assay. The following primers targeting the NP regions of viral RNAs were used: JUNV NP forward primer (5′-GGCAGTAAGCCGATCAC GTA), JUNV NP reverse primer (5′-TCGACATTGAAGGACCAGCC), MACV NP forward primer (5′-GCCCTTC AATGTCAAGCCAC), MACV NP reverse primer (5′-GACCGAGACAACCCGAGAAA), LASV NP forward primer (5′-GAAGGGCCTGGGAAAACACT), and LASV NP reverse primer (5′-AGGTAAGCCCAGCGGTAAAC). The copy numbers of viral RNAs in each sample were determined by using plasmid DNA templates that contained $10^2$ to $10^9$ copies of corresponding viral NP genes as standards. Quantitated virus RNA copy numbers were further normalized by the levels of the host $\beta$-actin gene. Validated qPCR primers for detection of human GAPDH and actin genes were purchased from Bio-Rad. The PCR results were analyzed by using CFX Manager software (Bio-Rad).

**WB assay and antibodies.** A Western blotting (WB) assay was performed as described previously (51). Cell protein lysates were prepared with 2× Laemmli SDS-PAGE sample buffer containing protease inhibitor cocktail for mammalian cells and phosphatase inhibitor cocktails. Equal loadings of protein among different samples were ensured by Coomassie G250 staining (Bio-Safe Coomassie; Bio-Rad) after SDS-PAGE. Protein samples were resolved on 4 to 20% SDS-PAGE gels and transferred to a polyvinylidene difluoride (PVDF) membrane using a Mini Trans-Blot electrophoretic transfer cell apparatus (Bio-Rad). The membranes were incubated with primary antibodies overnight at 4°C and with appropriate secondary antibodies for 1 h at room temperature. Proteins were visualized with ECL-2 Western blotting detection reagents (Thermo Scientific) according to the manufacturer's instructions. Primary antibodies used for WB analysis were monoclonal antipuromycin antibody (clone 12D10; Millipore), rabbit anti-PKR antibody (number 3072S; Cell Signaling), rabbit anti-PKR phosphor (T451) antibody (ab81303; Abcam), rabbit anti-phospho-eIF2$\alpha$ antibody Ser51 (number 9721S; Cell Signaling), rabbit anti-eIF2$\alpha$ antibody (number 9722S; Cell Signaling), rabbit anti-ISG15 antibody (number 2743; Cell Signaling), rabbit anti-RIGI antibody (number 3743; Cell Signaling), rabbit anti-OAS1 antibody (number 14498; Cell Signaling), and goat anti-human $\beta$-actin antibody (sc-1616; Santa Cruz Biotechnology). JUNV NP was detected with a monoclonal mouse anti-JUNV NP antibody (NA05-AG12; BEI), which also detects MACV NP. Mouse ascetic

fluid against LASV was provided by Robert Tesh (UTMB). Secondary antibodies horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (115-035-146; Jackson Immunology), HRP-conjugated goat anti-rabbit IgG (Cell Signaling), and HRP-conjugated donkey anti-goat IgG (sc-2020; Santa Cruz) were used. After the detection of phosphorylated forms of PKR and eIF2$\alpha$, the same blots were stripped with Restore Plus Western blot stripping buffer (Thermo Scientific) according to the manufacturer's protocol and reprobed with anti-PKR and anti-eIF2$\alpha$ antibodies, respectively. Equal loadings of protein samples were also confirmed by immunodetection of $\beta$-actin. Densitometry analysis of protein levels obtained in the WB assay was performed by using AlphaEase software (AlphaInnotech). The immunoblotting data for JUNV, MACV, and LASV NP in A549 cells are not shown here but have been reported in our previous study (22). The immunoblotting data for JUNV, MACV, and LASV NP in MoDCs are likewise not shown here but have been reported in the same study (22).

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