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Recombinant Marburg viruses containing mutations in the IID region of VP35 prevent inhibition of Host immune responses☆

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Abstract

Previous in vitro studies have demonstrated that Ebola and Marburg virus (EBOV and MARV) VP35 antagonize the host cell immune response. Moreover, specific mutations in the IFN inhibitory domain (IID) of EBOV and MARV VP35 that abrogate their interaction with virus-derived dsRNA, lack the ability to inhibit the host immune response. To investigate the role of MARV VP35 in the context of infectious virus, we used our reverse genetics system to generate two recombinant MARVs carrying specific mutations in the IID region of VP35. Our data show that wild-type and mutant viruses grow to similar titers in interferon deficient cells, but exhibit attenuated growth in interferon-competent cells. Furthermore, in contrast to wild-type virus, both MARV mutants were unable to inhibit expression of various antiviral genes. The MARV VP35 mutants exhibit similar phenotypes to those previously described for EBOV, suggesting the existence of a shared immune-modulatory strategy between filoviruses.

Keywords

Filovirus; Marburg virus; Bat virus; VP35; IFN-antagonism; Central basic; Patch; Immune-modulatory; Ebola virus

Introduction

Filoviruses (*order Mononegavirales*) are enveloped viruses with a single-strand RNA genome of negative-sense polarity (Feldmann et al., 2013). The viral genome is approximately 19 kb and encodes 7 genes: NP, VP35, VP40, GP, VP30, VP24, and L. These genes are separated by 6 intragenic untranslated regions and transcribed in sequential order from the 3′ to the 5′ end of the viral genome (Fig. 1A). Several viruses of the *Filoviridae* family, such as Ebola (EBOV), Sudan, Bundibugyo, Marburg (MARV), and Ravn viruses, cause sporadic outbreaks of viral hemorrhagic fevers (VHFs) in sub-Saharan Africa with high case fatality rates (Albarino et al., 2013a; Hartman et al., 2010; Leroy et al., 2011). Three other filoviruses, Tai Forest virus, Reston virus, and Lloviu virus, have not caused

☆The fidings and conclusions in this report are ours and do not necessarily represent the views of the Centers for Disease Control and Prevention.

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known human fatalities to date (Feldmann et al., 2013; Leroy et al., 2011; Negredo et al., 2011).

A critical component of filovirus replication is the ability of virus-encoded proteins, such as the well-studied VP35 protein, to modulate the host immune response [see review in (Ramanan et al., 2011) and Fig. 1B]. The VP35 protein from EBOV and MARV, has been shown in protein expression studies to inhibit production of IFNβ by binding to dsRNA through a central basic patch (CBP) in the IFN inhibitory domains (IID), and block recognition of pathogen associated molecular patterns (PAMPs) by retinoic-acid inducible gene I (RIG-I)-like receptor (RLR) pathways (Bale et al., 2012; Bale et al., 2013; Leung et al., 2009; Leung et al., 2010; Ramanan et al., 2011; Zinzula et al., 2012). EBOV VP35 also inhibits the induction of RIG-I by PACT (Luthra et al., 2013) and dampens RIG-I signaling by interacting with the TBK-1/IKKε kinase and inhibiting IRF-3/7 phosphorylation (Cardenas et al., 2006; Hartman et al., 2006; Hartman et al., 2004; Prins et al., 2009). Moreover, EBOV VP35 was also shown to inhibit RNA silencing by interacting with two dsRNA-binding proteins, TRBP and PACT (Fabozzi et al., 2011; Zhu et al., 2012). In accordance with these findings, recombinant EBOV carrying specific mutations in the CBP region of VP35 showed an attenuated phenotype in mice and guinea pigs (Hartman et al., 2008; Prins et al., 2010a). In addition other filovirus proteins, such as VP24 and sGP from EBOV, and VP40 from MARV and Reston virus (Fig.1B) inhibit the host immune response by different mechanisms (Basler et al., 2004; Mateo et al., 2011; Mateo et al., 2010; Mohan et al., 2012; Reid et al., 2006; Reid et al., 2007; Valmas and Basler, 2011; Valmas et al., 2010).

In this report, we investigated the immune modulatory function of VP35 in MARV replication by engineering specific lesions designed to abrogate dsRNA PAMP recognition, and measure the effects of these mutations in the context of infectious virus. The mutant MARVs were generated using a recently developed reverse genetics system (Albarino et al., 2013b) derived from a MARV bat isolate and their phenotype and characterized in interferon-competent cells.

Results and discussion

To characterize relative MARV VP35 immune modulatory activities in our system, we compared the abilities of EBOV VP35 and MARV VP35, VP24, and VP40 to inhibit IFNβ production induced by Sendai virus (SeV). For this purpose, HEK-293 T cells were co-transfected with Pol lI expression plasmids encoding each of the viral proteins (EBOV-VP35, MARV-VP35, MARV-VP24, or MARV-VP40), an IFNβ promoter-driven firefly luciferase (FLuc) reporter plasmid, and a *Renilla* luciferase (RLuc) transfection control plasmid. 24 h post-transfection (hpt), cells were induced by SeV infection, and luciferase activity was measured one day post infection (dpi). As shown in Fig. 1C, MARV VP35 inhibits IFNβ promoter activity to a greater extent than either MARV VP24 or VP40, while EBOV VP35 showed the most robust effect, as previously reported (Bale et al., 2012). Our results with MARV VP40 on the IFNβ promoter are consistent with those previously described for this protein (Valmas and Basler, 2011; Valmas et al., 2010) while the effect of MARV VP24 is consistent with the well characterized role of EBOV VP24 as an IFN

antagonist (Mateo et al., 2011; Mateo et al., 2010; Reid et al., 2006; Reid et al., 2007). Although a direct role as IFN antagonist has not been reported for MARV VP24, two recent reports describe an association of this protein with the interference of the inflammatory response (Edwards et al., 2014; Page et al., 2014). As shown in Fig. 2A and by others (Bale et al., 2012; Hartman et al., 2004; Prins et al., 2010b; Ramanan et al., 2012), the IFN inhibitory domain (IID) located in the C-terminal region of VP35 is strongly conserved among filoviruses. Previous reports showed that mutations in R312 of EBOV VP35, and in the equivalent position of MARV VP35 (R301), abrogate RNA binding and IFN suppression. In contrast, mutations in R305 of EBOV VP35 or R294 in MARV VP35 only moderately suppress these abilities (Bale et al., 2012; Bale et al., 2013; Hartman et al., 2006; Hartman et al., 2004; Leung et al., 2009; Leung et al., 2010; Ramanan et al., 2012). Moreover, recombinant EBOV with mutations in R305 and R312 of VP35 exhibited different in vitro and in vivo phenotypes when compared to WT virus (Hartman et al., 2008; Hartman et al., 2006).

In order to further characterize the immune modulatory activity of MARV VP35, we introduced two specific aminoacids changes, R294A and R301A, into the WT expression plasmid, and tested the activity of the IFNβ promoter-driven FLuc as described above. As shown in Fig. 2B, WT MARV VP35 exhibits the most robust inhibition of IFNβ promoter activity. Both mutated forms of VP35 were less effective, particularly R301A. To confirm that the observed phenotypes were due to the introduced mutations and not differences in expression levels, we used Western blot analysis to quantitate the amounts of WT and mutated forms of VP35 produced in transfected cells. When compared to actin levels, there are no substantial changes in R294A and R301A protein expression levels that could be linked to the decreased ability to inhibit the IFNβ promoter (Fig. 2C).

Based on the pronounced inhibitory effect of MARV VP35 on IFNβ production in human 293 T cells compared to the other MARV proteins (Fig. 1C), we wanted to further characterize the role of VP35 in dampening host cell PAMP recognition in the context of MARV infection. Before attempting the rescue of VP35 mutant MARV, we wanted to confirm that R294A and R301A mutations in VP35 would had deleterious effects on the MARV minigenome system. To do so, WT and mutant MARV VP35 expression plasmids were co-transfected with MARV NP, VP30, and L plasmids, along with the MARV minigenome expressing *Gaussia* Luciferase (gLuc) (Uebelhoer et al., 2014). Consistent with previous reports for EBOV VP35 (Hartman et al., 2006), the MARV VP35 mutations had no deleterious effects on the minigenome activity (Fig. 2D).

Using the MARV reverse genetics system (Albarino et al., 2013b; Uebelhoer et al., 2014), we generated WT recombinant MARV (rMARV) and two mutant viruses, rMARV-294 and rMARV-301, carrying R294A or R301A amino acid changes in VP35 respectively (Fig. 3A). Co-transfection of transcription plasmids containing the full-length genomes and support plasmids allowed the rescue of recombinant viruses in BHK cells. Viral stocks were further propagated by two passages in Vero-E6 cells, and growth kinetics were examined in Vero-E6 cells. As shown in Fig. 3B, rMARV, rMARV294, and rMARV-301 all exhibited similar growth kinetics in Vero-E6 cells, supporting our minigenome findings that, similar to EBOV, these mutations do not deleteriously affect the function of VP35 in MARV RNA replication.

To examine the immune modulatory role of VP35, we used rMARV, rMARV-294, and rMARV-301 to infect HEK-293 T cells stably transfected with an IFNβ promoter-driven FLuc reporter plasmid (293 T-IFNβ-FF). Infected cells were lysed 3 dpi, and luciferase activity was measured. Mock and rMARV-infected cells showed comparable levels of luciferase activity in (Fig. 3C). In contrast, cells infected with rMARV-294 showed slightly higher IFNβ promoter-driven FLuc activity, while those infected with rMARV-301 showed the highest relative FLuc activity. These results suggest that rMARV-294 and rMARV-301 viruses differ in their ability to modulate IFNβ activity.

We next compared the growth kinetics of WT and mutant rMARV in three different immuno-competent cell lines: Huh7 and A549 human-derived cell lines, and CD14+ macrophages, a primary target of viral replication during the course of primate infections. As shown in Fig. 4A, WT and mutant viruses exhibited similar growth kinetics in Huh7. In contrast, WT rMARV had a slight growth advantage over the mutant viruses in A549 cells and a 1–2 log advantage in CD14+ human macrophages. The different phenotypes of these recombinant viruses are consistent with previous reports of EBOV carrying the equivalent mutations in VP35 when tested in immune-competent cells, such as human macrophages (Hartman et al., 2006).

We suspect that the reduced growth of mutant viruses in CD14+ macrophages is due to their impaired ability to control the innate antiviral response during the course of infection. In order to test this hypothesis, we infected CD14+ macrophages with WT and mutant viruses and analyzed the expression of selected genes involved in the innate antiviral response. As shown in Fig. 4B, nearly all of the antiviral genes tested were over-expressed in macrophages infected by the mutants compared to those infected by WT virus. Infection by MARV-301 caused the greatest induction of innate immune genes and was also the mutant that was the most attenuated in CD14+ macrophages, thereby linking attenuated growth to the inability to antagonize innate immune responses. Consistent with this hypothesis, we found Huh7 and A549 cells to exhibit very minor changes in the expression level of these same antiviral genes (data not shown), when infected with wild-type or mutant viruses.

Conclusions

In this report we describe the successful generation of bat-derived recombinant MARV carrying mutations in VP35. Using these mutant MARVs, we demonstrated the ability of MARV VP35 to modulate expression of host immune response genes during infection of immune-competent cells. These mutant viruses lack the ability to suppress the innate antiviral response, resulting in high expression levels of several critical antiviral genes that correlate with impaired growth in human macrophages. Based on comparison to the growth phenotypes of analogous EBOV VP35 mutants with defects in dsRNA PAMP recognition (Hartman et al., 2006), these data suggest shared immune-modulatory strategies between genera within the family *Filoviridae*. It should be noted that MARV VP35, VP40, and VP24 likely function together as immune modulators. Indeed, the role of VP40 has been well characterized for MARV (Valmas and Basler, 2011; Valmas et al., 2010), while the role of VP24 as an IFN antagonist has been only reported for EBOV (Edwards et al., 2014; Mateo

et al., 2011; Mateo et al., 2010; Page et al., 2014; Reid et al., 2006; Reid et al., 2007), and will warrant further investigation.

Materials and methods

Cell culture and biosafety

All work with WT or recombinant MARV was performed in a biosafety level 4 (BSL-4) facility. BSR-T7/5, BHK21, and Vero-E6 cells were propagated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum (FBS) and penicillin-streptomycin (Pen-Strep), as recommended by the manufacturer (Invitrogen). BSR-T7/5 cells were a generous gift from K. Conzelmann (Buchholz et al., 1999). CD14+ macrophages were purified and seeded as previously described (Albarino et al., 2013b; McElroy and Nichol, 2012)

Plasmid construction

The construction of support plasmids, gLuc minigenome, and full-length clones corresponding to the full-length genome of 371Bat MARV (GenBank FJ750958) has been described before (Albarino et al., 2013b; Uebelhoer et al., 2014). Complete details on the construction of support expression plasmids and plasmids encoding a full-length clone carrying mutations in the C-terminal region of VP35 are available upon request. Briefly, the CGC codon at position 3,824 and the CGG codon at position 3,845 were changed to GCA in order to introduce the R294A and R301A amino acid changes in the VP35 protein.

Minigenome expression

A ~75% confluent monolayer of BSR-T7/5 cells grown in 12-well plates was transfected with 1.5 μg of MARV gLuc minigenome plasmid, 0.5 μg pC-L, 0.5 μg pC-NP, 0.05 μg pC-VP30, and 0.05 μg pC-VP35 WT or mutant plasmids. Plasmid pC-L was replaced by empty pCAGGS in the control. Aliquots of 5–10 μL of supernatant were collected 2–4 days post-transfection (dpt) to measure gLuc activity, as described before (Uebelhoer et al., 2014).

Rescue of infectious viruses

Rescue of recombinant viruses was performed in BHK21 cells as described previously (Albarino et al., 2013b). Briefly, a ~70% confluent monolayer of BHK21 cells grown in 12-well plates was transfected with 1.5 μg pMARV (expressing the full-length MARV genome in viral-complementary sense), 0.5 μg pC-L, 0.5 μg pC-NP, 0.05 μg pC-VP35, 0.05 μg pC-VP30, and 0.5 μg of pC-T7. Supernatants from the transfected cells were harvested 4 dpt, clarified by low-speed centrifugation, and passaged twice in Vero-E6 cells. The rescued viruses were sequenced to completion and no unexpected changes were noted. The complete sequence of recombinant wild-type and mutant viruses were deposited in GenBank (KP117261, KP117260, KP117259).

Virus titration and growth curves

To characterize the growth kinetics of recombinant viruses, 2×10^6 Huh7 and A549 cells, or 1×10^5 CD14+ macrophages were infected with WT or mutant viruses at multiplicity of

infection (moi) of 0.02. Virus was allowed to adsorb for 1 h with gentle rocking. After adsorption, monolayers were washed three times with PBS to eliminate any residual virus. Aliquots of 200 μL of the supernatant were taken daily, and viral titers were obtained by a tissue culture infective dose 50 ($TCID_{50}$) assay, as described before (Uebelhoer et al., 2014).

IFNβ promoter assay in SeV infected cells

Subconfluent monolayers of HEK-293 cells were co-transfected with pCAGGS plasmids encoding various viral proteins, the p125-luc FLuc reporter plasmid, and a plasmid constitutively expressing the RLuc (pRL-TK). The IFNβ FLuc reporter plasmid p125-luc, kindly provided by T. Fujita (Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan), expresses FLuc under the control of the murine IFN-β promoter region (Yoneyama et al., 1996). The pRL-TK plasmid (Promega) contains a Herpes simplex virus thymidine kinase (HSV TK) promoter-driven encoding RLuc, used to control for transfection efficiency. 24 hpt, cells were induced with 200 hemagglutinin (HA) units/mL of SeV. Infected cells were lysed 1 dpi, and luciferase activity was measured using the Dual-Luciferase reporter assay system according to the manufacturer's instructions (Promega). FLuc activity was normalized to the RLuc value for each individual sample, and the fold-induction values were obtained by comparing infected cells with uninfected cells. Experiments were done in triplicate, and values are expressed as percent (%) activity relative to empty vector control set at 100% induction ± SD.

IFNβ promoter assay in MARV-infected cells

293 T-IFNβ-FF cells (Baum and Garcia-Sastre, 2011), a kind gift from Adolfo García-Sastre (Icahn School of Medicine at Mount Sinai Hospital), were cultured in DMEM supplemented with 10% FBS, 1% L-glutamine, and 1% Pen-Strep. Cells were plated in low-serum media (2% FBS) to sub-confluent levels in 48-well plates, and infected with rMARV, rMARV-294, and rMARV-301 (moi=0.5). At 3 dpi, cells were lysed, and luciferase activity was measured using the LARII reagent component of the Dual-Luciferase reporter assay system, according to the manufacturer's instructions (Promega). Activity is expressed as average relative luciferase units (RLU) of 8 replicates ± SD.

Gene expression array

Sub-confluent monolayers of Huh7, A549 cells, or CD14+ selected human macrophages grown in 24-well plates were mock-infected or infected with rMARV, rMARV-294, or rMARV-301 (moi=2) in duplicate. 24 hpi, supernatants were removed, and cells were lysed for total RNA purification following previously described protocols (McMullan et al., 2012). 2 μg of total RNA was subjected to an extensive DNAse digestion following the manufacturer's protocol (RNeasy, Qiagen), and subsequently used in a qRT-PCR reaction using SuperScript III Platinum SYBR Green One-Step qRT-PCR and following the manufacturer's protocol (Invitrogen). Gene expression profiles were obtained using a commercially available array (Antiviral Response PCR Array, Qiagen/SABiosciences).

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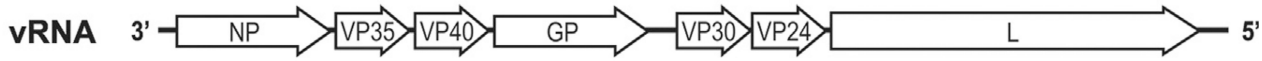
A



B

IFN production	• **VP35 (EBOV and MARV) binds dsRNA and blocks RNA recognition by RIG-I** (Bale et al 2012a, 2012b; Bale et al 2013; Zinzula et al 2012; Leung et al 2012; Ramanan et al 2012)
	• **VP35 (EBOV) inhibits PACT-induced RIG-I** (Luthra et al 2013)
	• **VP35 (EBOV) interacts with TBK-1/IKKε and inhibit IRF3/7 phosphorylation and nuclear translocation** (Cardenas et al 2006, Prins et al 2009)
IFN signaling	• **VP40 (MARV) blocks phosphorylation of JAK and inhibits STAT activation** (Valmas et al 2010, 2011)
	• **VP24 (EBOV) inhibits nuclear transport of P-STAT by binding KPN-α** (Mateo et al 2010; Reid et al 2006, 2007)
other	• **VP35 (EBOV) inhibits RNA silencing** (Fabozzi et al 2011; Zhu et al 2012)
	• **VP24 (EBOV) interfere inflammatory response** (Edwards et al 2014; Page et al 2012)
	• **GP (EBOV) blocks tetherin budding restriction** (Kaletsky et al 2009)
	• **sGP (EBOV) as possible immune decoy** (Ito et al 2001; Mohan et al, 2012)

C

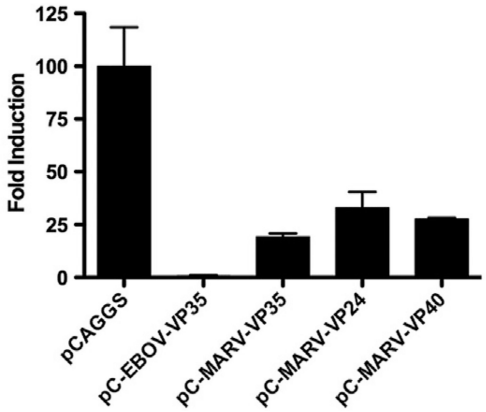


Fig. 1.

(A) Basic organization of the MARV genome. The 7 ORFs are depicted in the viral complementary sense (5′ to 3′). (B) Filovirus proteins involved in immune evasion. The immune modulator functions of filovirus proteins are divided into three broad classes; citations are included. (C) IFNβ promoter assay in SeV infected cells. HEK-293 cells were co-transfected with expression plasmids encoding EBOV VP35, MARV VP35, MARV VP24, or MARV VP40, along with p125-luc reporter plasmid expressing firefly luciferase (FLuc), and pRL-TK control plasmid expressing *Renilla* luciferase (RLuc). Cells were infected with SeV 24 hpt, and luciferase activity was measured 1 dpi. FLuc activity was

normalized to RLuc, and the fold-increase in induction was determined by comparing infected cells with uninfected cells.

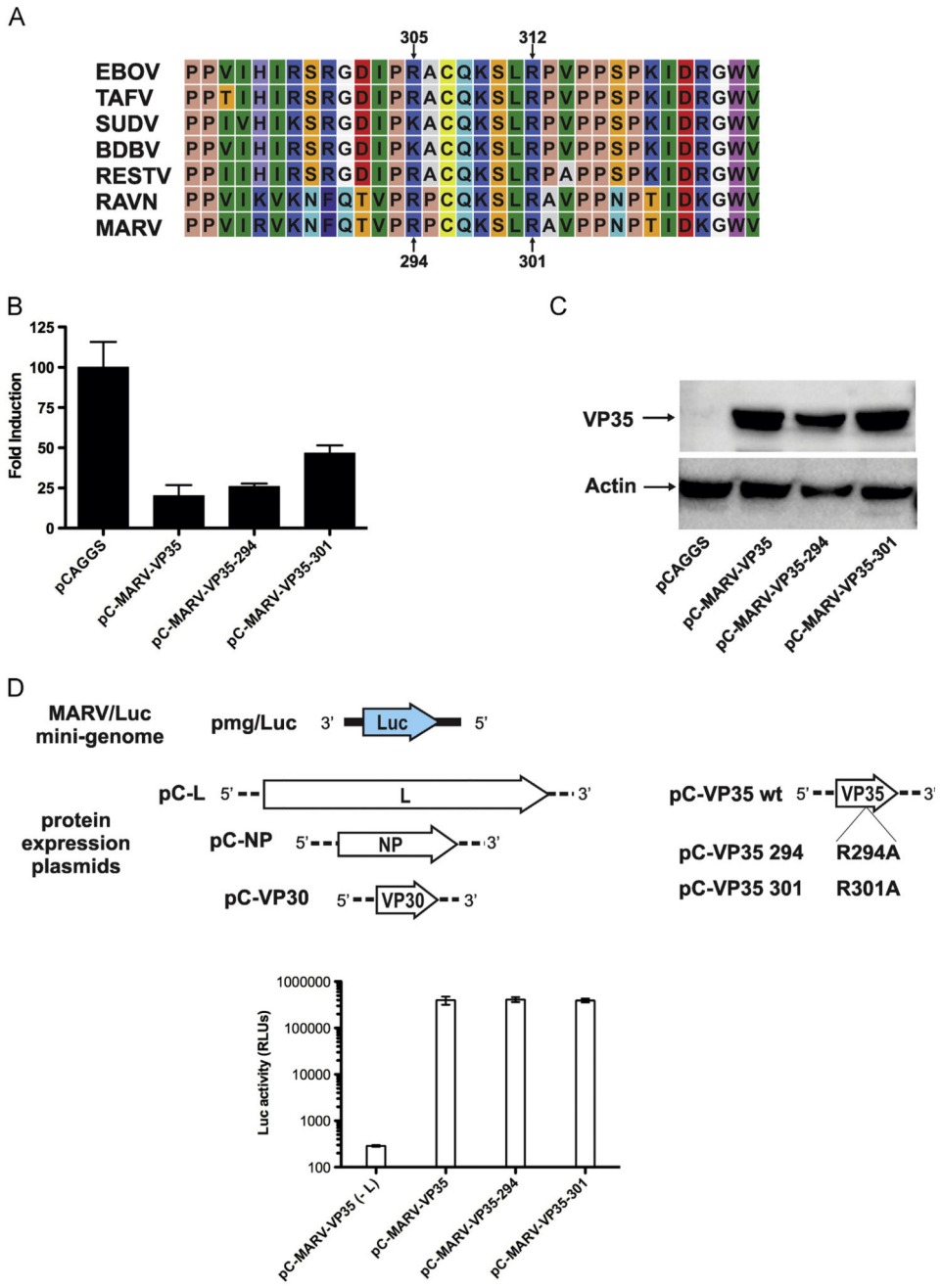


Fig. 2.

(A) Sequence conservation in central basic patch (CBP) of filovirus VP35 IFN-inhibiting domain (IID). Partial amino acid sequences were aligned to show the conservation of IID. The two amino acid residues modified in EBOV in a previous report (Hartman et al. 2006), and in MARV in this current report are indicated with arrows. VP35 sequences were derived from accession numbers AF086833, NC_004161, FJ217162, NC_014373, NC_006432, DQ447649, NC_001608, and FJ750958. (B) IFNβ promoter assay. HEK-293 cells were co-transfected with expression plasmids encoding WT MARV VP35, or the mutant proteins VP35-294 or VP35-301, Cells were infected with SeV 24 hpt, and luciferase activity was

measured as described in Fig.1C. (C) Expression of WT and mutant VP35. HEK-293 cells were transfected as described above, and protein lysates were immunostained with antibodies against MARV (upper panel) or actin (lower panel). (D) Minigenome replication. Schematic of MARV Luc minigenome system. The structure of plasmids coding the minigenome (Uebelhoer et al., 2014) is shown with 371Bat 5' and 3' untranslated regions (black line), and support plasmids expressing viral proteins (dashed line indicates pCAGGS plasmid sequence). Amino acid substitutions to alanine in VP35 are indicated for pC-VP35-294 and pC-VP35-301 expression plasmids. To assess minigenome replication BSR-T7/5 cells co-transfected with plasmids encoding the MARV mini-genome and with support plasmids expressing viral proteins pC-NP, pC-VP35, pC-VP30, and pC-L. In the indicated lanes, pCVP35 was swapped for pC-VP35-294, pC-VP35-301, or pC-L was swapped for pCAGGS as a no polymerase control. Data are representative of three independent experiments, with mean and standard error of the mean (SEM) of luciferase expression from three wells displayed for each time point in relative light units (RLUs). (B).

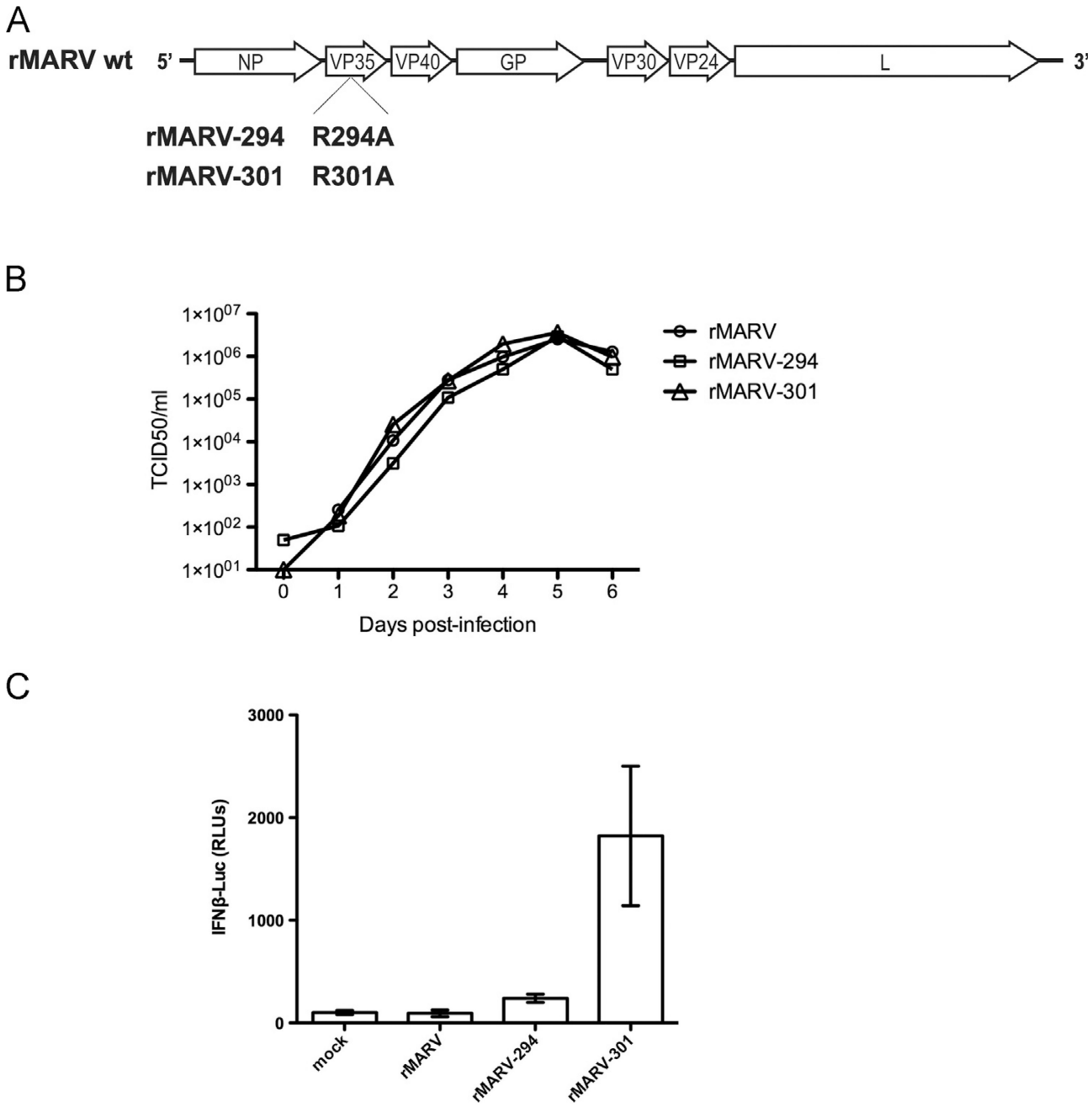


Fig. 3.

Rescue and characterization of recombinant WT and mutant MARV viruses. (A) Schematic representation of recombinant MARV genomes. Full-length MARV complementary genome is depicted with the amino acid substitutions in VP35 indicated for rMARV-294 and rMARV-301 mutants viruses. WT and mutant recombinant viruses were rescued in BHK-21 cells and passaged twice in Vero-E6 cells as described before (Albarino et al., 2013b). (B) Growth kinetics in Vero-E6 cells. Cells were infected with rMARV, rMARV-294, or rMARV-301 mutants viruses (moi=0.02). After 1 h adsorption followed by three PBS washes, supernatants were collected at the indicated time points. Viral titers were measured

by tissue culture infective dose 50 ($TCID_{50}$) in Vero-E6 cells as described before (Uebelhoer et al. 2014). (C) IFN-β promoter assay in MARV infected cells. HEK-293 cells stably transfected with an IFN-β promoter-driven FLuc reporter plasmid (293 T-IFNβ-FF) were infected with rMARV, rMARV-294, or rMARV-301. Cells were lysed 3 dpi, and luciferase activity was measured.

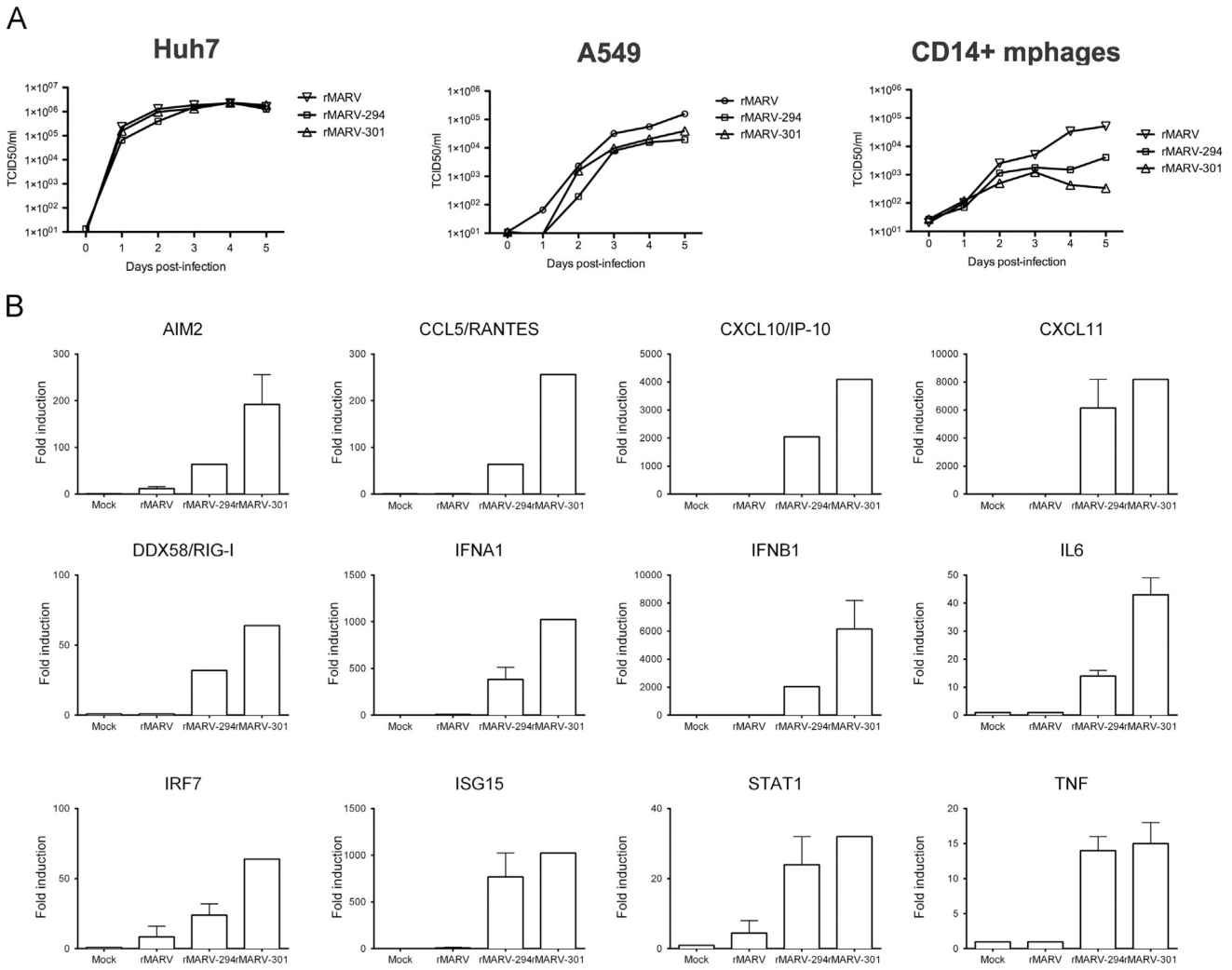


Fig. 4.

A. Growth kinetics of WT and VP35 mutants in IFN-competent cells. Huh7, A549 and human macrophages were infected with WT rMARV, rMARV-294, or rMARV-301, and viral titers were determined by $TCID_{50}$ assay in Vero-E6 cells. Substantial changes in growth kinetics were noticed only in CD14+ macrophages. B. Expression of antiviral genes in cells infected with WT or mutant MARV. Selected genes corresponding to four groups associated with different receptors and their signaling pathways (type-I IFN, Nod-like receptor, toll-like receptor, and RIG-I-like receptor) with important fold regulation values (cut-off= 10) are shown.