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Original article

Sendai virus V protein decreases nitric oxide production by inhibiting RIG-I signaling in infected RAW264.7 macrophages

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ABSTRACT

Sendai virus V protein is a known antagonist of RIG-I-like receptors (RLRs) RIG-I and MDA5, which activate transcription factors IRF3, leading to activation of ISGF3 and NF-κB. These transcription factors are known activators of inducible NO synthase (iNOS) and increase the production of nitric oxide (NO). By inhibiting ISGF3 and NF-κB, the V protein acts as an indirect negative regulator of iNOS and NO. Here we report that the V gene knockout Sendai virus [SeV V(−)] markedly enhanced iNOS expression and subsequent NO production in infected macrophages compared to wild-type SeV. The knockout of RIG-I in cells inhibited SeV V(−)-induced iNOS expression and subsequent NO production. To understand the underlying mechanism of the V protein-mediated negative regulation of iNOS activation, we transfected HEK293T cells with RIG-I and the RIG-I regulatory protein TRIM25. Our results demonstrated that the V protein inhibited iNOS activation via the RIG-I/TRIM25 pathway. Moreover, the V protein inhibited TRIM25-mediated K63-linked ubiquitination of RIG-I, as well as its CARD-dependent interaction with mitochondrial antiviral signaling (MAVS) molecules. These results suggest that the V protein down-regulates iNOS activation and inhibits NO production by preventing the RIG-I-MAVS interaction, possibly through its effect on the ubiquitination status of RIG-I.

A V gene knockout Sendai virus [SeV V(−)] is attenuated *in vivo* but not *in vitro* [1]. Previous studies have shown that SeV V protein counteracts innate immune responses other than those mediated by interferons [2,3], suggesting that the SeV V protein may play a role in modulating the immune response. This also indicates that the host factors responsible for the early clearance of SeV V(−) *in vivo* might be expressed in cells such macrophages and dendritic cells rather than in the standard cell lines used to model infection studies *in vitro*.

Macrophages are a part of the critical first line of defense of the immune system, especially against respiratory pathogens [4,5]. In response to viral infection, macrophages secrete nitric oxide (NO) as well as large amounts of other cytokines [6]. In addition to its microbicidal function, NO can regulate the activation and level of the innate and adapted immune responses. NO is an early immune response mediator produced in immune cells by nitric oxide synthase (iNOS) in response to pathogenic challenge [7,8]. The expression of the iNOS gene is upregulated either directly by cellular infections [9] or by pro-inflammatory cytokines released by inflammatory cells. The expression of the iNOS gene is regulated by transcription factors NF-κB and IFN-stimulated gene factor 3 (ISGF3) [10], which is activated in response to type I interferon (IFN).

To grow efficiently inside the body, viral pathogens have evolved virulence factors to counter NO-mediated immune response pathways [11–13]. For instance, the SeV C protein was reported to limit the generation of double-stranded (ds) RNA and/or defective interfering (DI) RNA during viral transcription and replication [14,15], thereby limiting activation of RIG-I-like receptor (RLR) pathways that would lead to the activation of transcription factors IRF3 and NF-κB. Inactivation of the IRF3 pathway does not increase production of IFN-β, which in turn would activate ISGF3 via the JAK-

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STAT pathway. This, in turn, keeps prevents iNOS gene expression and limits NO levels [16]. On the other hand, SeV accessory protein V has been shown to inhibit the activation and nuclear translocation of ISGF3 and NF-κB by interacting with RLRs, thereby downregulating IFN-β expression [17–21]. Therefore, we hypothesized that SeV may have evolved the V protein to counter the NO-mediated immune response in the host. In the present study, we studied SeV V protein-mediated iNOS regulation and the plausible mechanism behind the regulation. Our results show that SeV V protein disrupts RIG-I signaling thereby impairing NO production.

1. Materials and methods

1.1. Cells, viruses, and reagents

The ISGF3 reporter cell line RAW-Lucia ISG and the ISGF3 and NF-κB dual-reporter cell line RAW-Dual cells were used as RAW264.7 murine macrophages to monitor the activation of the ISGF3 and NF-κB pathways. RAW-Lucia ISG-knock-out (KO)-RIG-I cells and RAW-Lucia ISG-KO-MDA5 cells were used as RIG-I-deficient and MDA5-deficient RAW264.7 macrophages, respectively. The above listed four cell lines were purchased from InvivoGen (San Diego, CA, USA) and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and 200 μg/ml Zeocin according to the manufacture's instruction. HEK293T cells and Vero cells were cultured in DMEM containing 10% FBS. SeV WT, a cDNA-derived Z strain, mutant SeV V(−), from which V protein had been knocked out [22], and mutant SeV 4C(−), from which all four C proteins had been knocked out [23], were propagated in Vero cells in the presence of 3 μg/ml trypsin. 5′Triphosphate hairpin RNA (3p-hpRNA), which is a specific agonist of RIG-I and does not activate other dsRNA sensors such as TLR3 and MDA5, was purchased from InvivoGen. The cells were transfected with 3p-hpRNA using LyoVec, a cationic lipid-based transfection reagent (InvivoGen).

1.2. ISGF3 luciferase activity and NF-κB secreted alkaline phosphatase activity

RAW-Lucia ISG cells were stably transfected with an inducible reporter construct and expression was detected by measuring the luciferase (Luc) activity. Similarly, RAW-Dual cells were stably transfected with two inducible reporter constructs, and the activation of the ISGF3 and NF-κB pathways was detected by measuring Luc and alkaline phosphatase (SEAP) activity, respectively as per the manufacturer's instructions.

1.3. Plasmid construction

Mammalian expression plasmids encoding cellular or viral proteins were created by insertion of cDNA fragments containing a specific open reading frame (ORF) into a multicloning site downstream of the cytomegalovirus enhancer chicken β-actin hybrid (CAG) promoter of pCA 7 [24,25]. The cDNA fragment for mouse

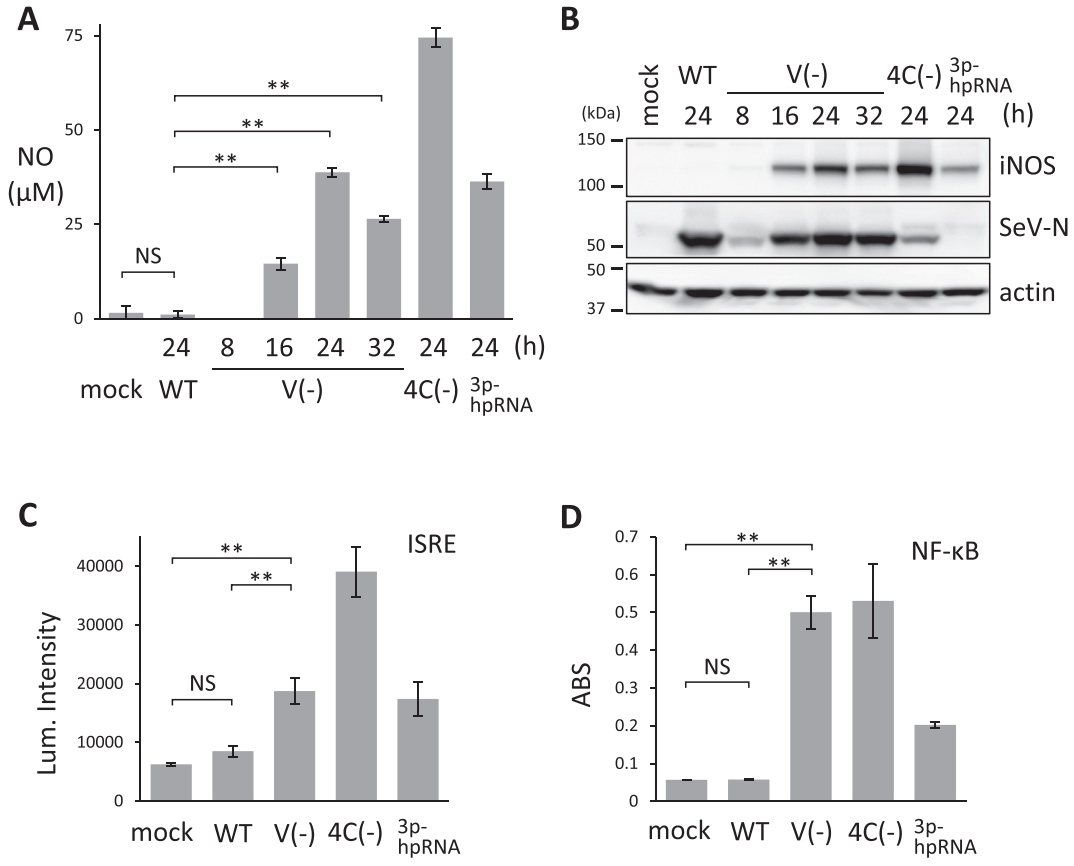

Fig. 1. Activation of NO and iNOS in RAW264.7 cells infected with SeV V(−). (A–D) RAW-Lucia ISG cells in a 96-well plate (A, C), a 24-well plate (B), or RAW-Dual cells in a 96-well plate (D) were infected with the indicated SeV strains at MOI 5 or transfected with 3p-hpRNA (100 ng/ml) as a positive control. Culture media were collected at the indicated time points (A) or at 24 h (C, D) after infection or treatment and assayed for nitrite (A). (B) Cells were also lysed in RIPA buffer at the indicated time points after infection and immunoblotted with anti-iNOS antibody or anti-SeV serum. (C) ISGF3 activity was determined by measuring the Luc activity in culture media from the RAW-Lucia ISG cells. (D) NF-κB activity was evaluated by measuring the SEAP activity in culture media from the RAW-Dual cells. SeV–N, SeV N protein. **: $P < 0.01$ vs. infection with SeV WT at 24 h (Turkey's test).

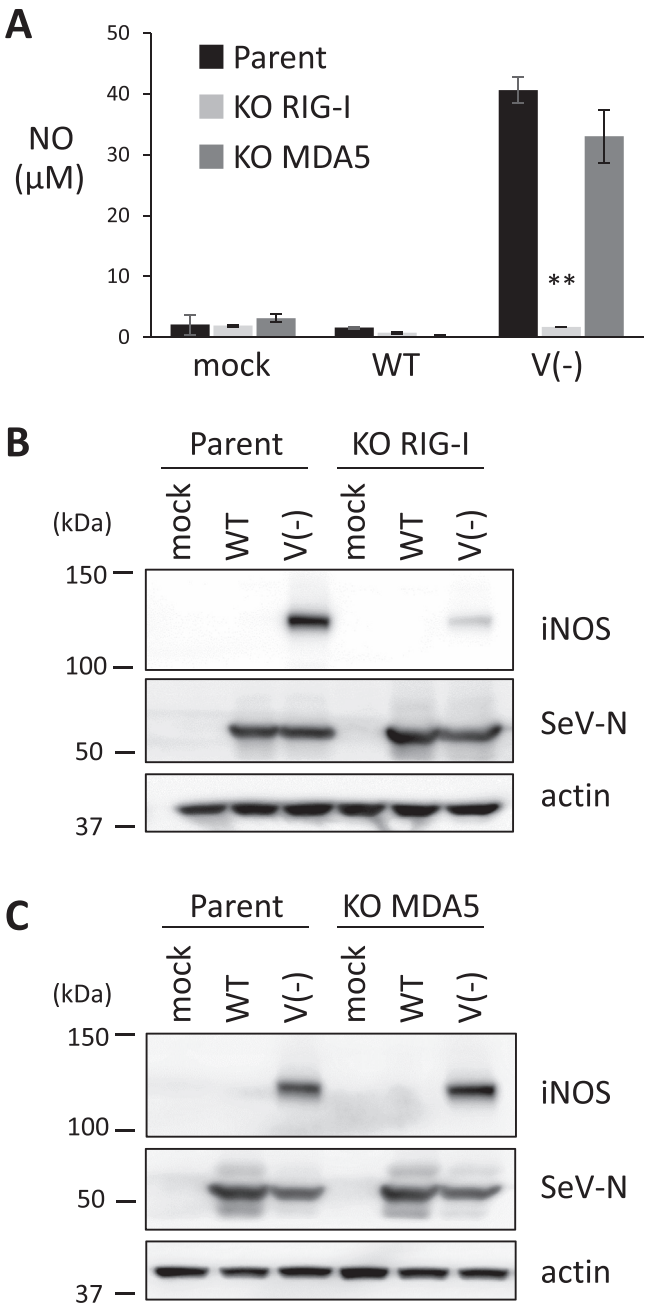

Fig. 2. Role of RIG-I in the regulation of SeV V(−)-induced iNOS expression. Parent-, KO RIG-I-RAW-, or KO MDA5-RAW-Lucia ISG cells in a 96-well plate were infected with the indicated SeV strains at MOI 5. Culture media were collected at 24 h after infection and assayed for nitrite (A). (B, C) Parent-, KO RIG-I-, or KO MDA5-RAW-Lucia ISG cells in a 24-well plate were infected with the indicated SeV strains at MOI 5. Cells were lysed in RIPA buffer at 24 h after infection and immunoblotted with anti-iNOS antibody or anti-SeV serum. **: $P < 0.001$ vs. infection with SeV V(−) in parental cells (Dunnett's test).

RIG-I [1–926 aa] was synthesized by reverse transcribing total RNA isolated from RAW-Lucia ISG cells. The cDNA fragments for mutants of RIG-I protein (RIG-IN [1–230 aa] and RIG-IC [231–926 aa]), were synthesized by PCR using appropriate primers and mouse RIG-I cDNA. The cDNA fragments for RIG-I, RIG-IN, and RIG-IC were introduced into pCA7 with a Flag tag. The cDNA for mouse TRIM25 was purchased from OriGene (Rockville, MD, USA). The cDNA fragment containing the ORF was then introduced into pCA7 with a Myc tag. The mammalian expression plasmid, pCA7 with a V5 tag encoding SeV V protein [1–384 aa], Vn [1–317 aa], or Vc [318–384 aa], P protein, C protein, Nipah virus (NiV) V protein, NiV P protein, measles virus (MeV) V protein, or MeV P protein with V5 tag was described previously [22]. pCA7 with a Myc tag encoding UbK63 was a gift from Y. Kitagawa [26]. The sequences of all the newly created plasmids were confirmed by sequence analysis.

1.4. Measurement of nitric oxide production

Levels of nitrite, a stable degradation product of NO, were determined in culture media by the Griess reaction and measured using a microplate reader as described previously [27]. Sodium nitrite diluted in complete culture medium was used as the standard.

1.5. Immunoprecipitation assay

HEK293T cells were plated in a 6-well plate and transfected with plasmids encoding RIG-IN (300 ng/well), TRIM25 (100 ng/well), and UbK63 (600 ng/well), along with the plasmids encoding viral protein (P, V, Vn, Vc [SeV], P, V [NiV], or P, V [MeV]) (1000 ng/well). At 24 h post-transfection, the cells were lysed in 400 μl of lysis buffer (50 mM Tris–HCl [pH 7.4], 150 mM NaCl, 1% Triton X-100) containing protease inhibitor cocktail (Nacalai Tesque, Kyoto, Japan). Subsequently, cell lysates were incubated with anti-Flag or anti-V5 mouse monoclonal antibody-coated Protein G-magnetic agarose beads (MBL, Aichi, Japan) at 4 °C for 2 h. Beads were washed five times with the lysis buffer, and proteins were eluted from the beads by boiling in Laemmli sample buffer (Nacalai Tesque). Eluted proteins were analyzed by IB analysis.

1.6. Immunoblot analysis

Samples were boiled with Laemmli sample buffer (Nacalai Tesque). Proteins were resolved by SDS-polyacrylamide gel (7.5% or 10–20%) electrophoresis (FUJIFILM Wako, Osaka, Japan) and transferred onto polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA). The membrane was blocked with Blocking buffer (Nacalai Tesque) for 30–60 min, followed by incubation with the following antibodies at room temperature for 1 h: anti-iNOS rabbit antibody (GTX130246; GeneTex, Irvine, CA, US), anti-SeV rabbit serum [28], anti-Flag antibody (PM020; MBL), anti-V5 antibody (PM003; MBL), anti-Myc antibody (562; MBL), anti-MAVS antibody (#24930; Cell Signaling Technology, Danvers, MA, US), or anti-actin antibody (Santa Cruz, Dallas, TX, USA). Subsequently, the membrane was incubated with horseradish peroxidase-conjugated anti-mouse, anti-rabbit, or anti-goat IgG secondary antibodies (Santa Cruz) at room temperature for 30 min. Immunoreactive bands were visualized by enhanced chemiluminescence using the substrate (PerkinElmer, Waltham, MA, USA) and a chemiluminescence imaging system (Vilber Lourmat, Collégien, France or GE Healthcare).

1.7. Reporter gene assay

HEK293T cells were plated in a 24-well plate and transfected with the following plasmids in triplicate: iNOS promoter-driven Firefly luciferase (Fluc) reporter [90 ng/well] [16,29], ISRE-responsive Fluc reporter (TaKaRa Bio, Shiga, Japan) [90 ng/well], or an NF-κB-driven Fluc reporter (TaKaRa Bio) [90 ng/well] plasmid, and Renilla luciferase (Rluc) pRL-TK control vector (Promega, Madison, WI, USA) [10 ng/well], along with plasmids encoding RIG-I signaling molecules (RIG-IN [10 ng/well] and/or TRIM25 [10 ng/well]), and the plasmids encoding the viral proteins (P, C, V, or V mutants [200 ng/well]), using polyethyleneimine (PEI)

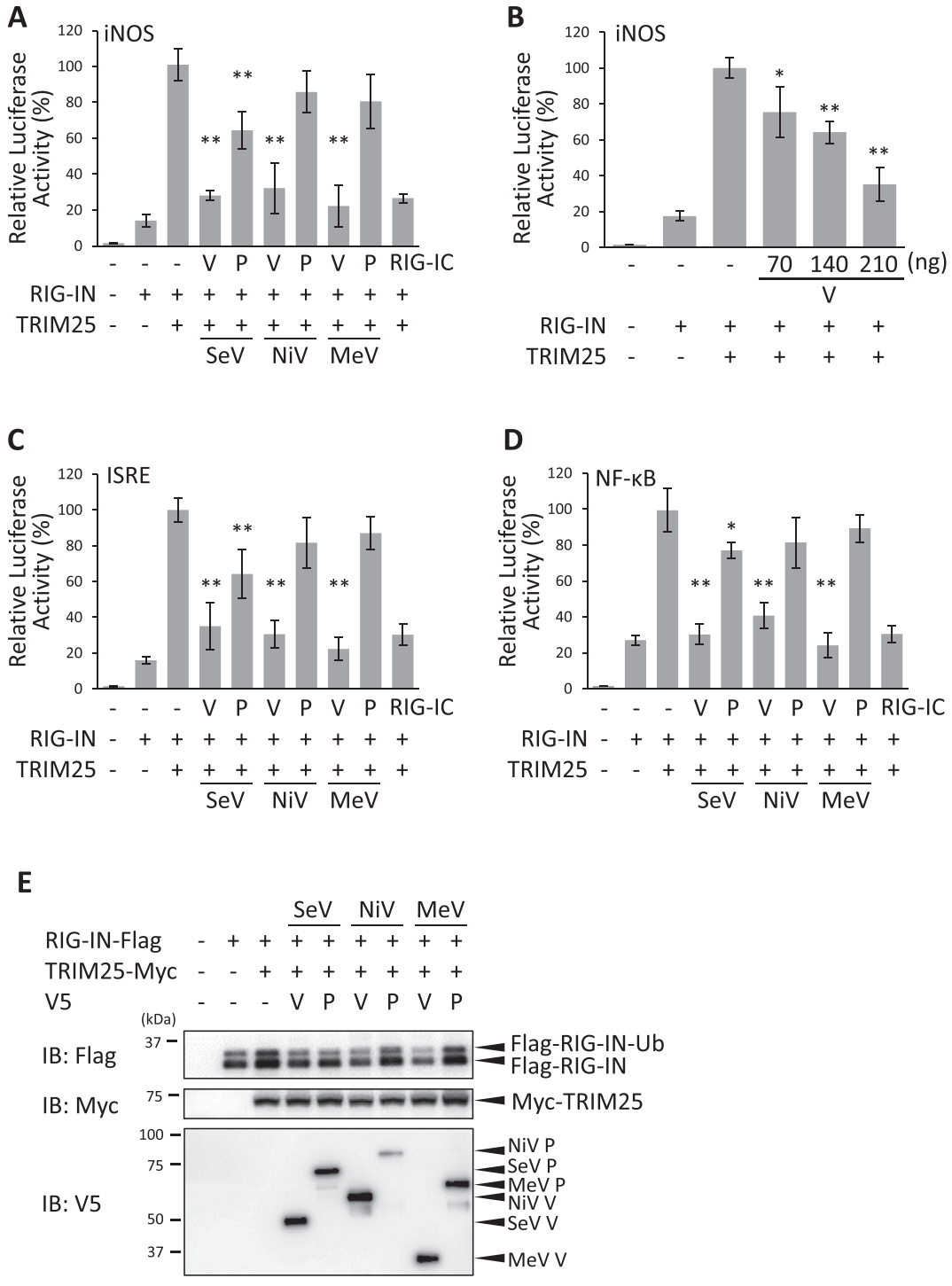

Fig. 3. Effect of SeV V protein on RIG-I/TRIM25-mediated iNOS activation. (A–C) An iNOS promoter-driven (A), ISRE promoter-driven (B), or NF-κB-dependent (C) Fluc reporter plasmid was transfected into HEK293T cells, along with the internal control, pRL-TK, and the indicated plasmids. At 24 h post-transfection, Fluc and Rluc activities were measured. Relative luciferase activity was calculated as the ratio of Fluc activity to Rluc activity. *: $P < 0.05$, **: $P < 0.01$ vs. transfection with empty vector (Dunnett's test). (E) HEK293T cells were transfected with the indicated plasmids. At 24 h post-transfection, the cells were lysed in lysis buffer. (E) The cell lysates were subjected to IB with anti-Flag, anti-Myc, or anti-V5 antibody. NiV, Nipah virus; MeV, measles virus.

hydrochloride (MW 40,000) (#24765; Polysciences Inc., Washington, PA, USA). An equal amount of DNA was used for transfections by adjusting with an appropriate amount of pCA 7 empty plasmid. The cells were lysed at 24 h post-transfection, and the relative Luc activity was determined using the dual-Luc reporter assay system (Promega).

1.8. Statistical analysis

Two sets of values were compared using Student's t-test, while the Dunnett test or Tukey's test was used for comparing three or more sets of values. Differences with $P < 0.05$ were considered statistically significant.

2. Results

2.1. SeV V(−) activated iNOS to induce NO production

To determine the effect of SeV V protein on host NO production, we infected RAW-Lucia ISG cells with the SeV V(−) strain. SeV V(−) induced NO production rapidly 24 h post-infection (pi), whereas SeV WT elicited a minimal response (Fig. 1A). The expression of iNOS was also upregulated (Fig. 1B). Infection with SeV 4C(−) and transfection with 3p-hpRNA, well-known activators of RLR pathways, clearly increased NO production and iNOS expression. Similar results were obtained in RAW-Dual cells. To ascertain the effect of the V protein on the modulation of ISGF3 and the NF-κB pathways, we measured the Luc activity in RAW-Lucia ISG cells infected with SeV V(−) and the SEAP activity in RAW-Dual cells infected with SeV V(−). Our results show that SeV V(−) significantly increased Luc and SEAP activities as compared to SeV WT, which is similar to the effects of SeV 4C(−) and 3p-hpRNA (Fig. 1C and D), indicating activation of ISGF3 and NF-κB pathways by the SeV V(−) strain. These results suggest that SeV V(−) activates the ISGF3 and NF-κB pathways and indirectly regulates NO production via RLR pathways in RAW264.7 macrophages.

2.2. RIG-I is required for SeV V(−)-induced NO production

To investigate the role of RLRs in SeV V(−)-induced production of NO, we infected RAW-Lucia ISG-KO-RIG-I cells or RAW-Lucia ISG-KO-MDA5 cells with SeV V(−). Our results showed that SeV V(−)-mediated NO activation was blocked in RIG-I knockout cells. However, in RAW-Lucia ISG-KO-MDA5 cells, the activation of NO induced by SeV V(−) was similar to that of the parent cell line (Fig. 2A). Similar results were obtained for iNOS expression (Fig. 2B–C). These results indicate that RIG-I, but not MDA5, is required for SeV V(−)-induced production of NO. NS: not significant.

2.3. SeV V protein inhibited TRIM25/RIG-I signaling leading to the activation of iNOS

Results presented in the preceding sections suggested that SeV V protein negatively regulates iNOS expression and NO production by inhibiting the RIG-I signaling pathway. To determine whether SeV V protein blocks the RIG-I signaling pathway, we reconstituted the iNOS signaling pathway in HEK293T cells. In this reconstituted system, TRIM25, which is a ubiquitin E3 ligase that catalyzes K63-linked polyubiquitination of RIG-I [30,31], was transfected into HEK293T cells along with RIG-IN, which consists of the N-terminal CARD domain and is constantly active in RIG-I signaling [30]. The activity was measured by the iNOS-responsive Fluc reporter plasmid as described previously [16,21]. RIG-IC (consisting of the C-terminal helicase and regulatory domains) acts as a dominant negative inhibitor [30], and therefore controls for the RIG-I specificity of iNOS promoter activation. As shown in Fig. 3A, the transfection of TRIM25 along with RIG-IN significantly activated the iNOS promoter when compared with RIG-IN transfection alone. However, this activation was suppressed when cells were transfected with RIG-IC, indicating that the signaling is RIG-I specific. This suppression was also seen with SeV V or P, as well as NiV V or MeV V, co-transfection. This confirmed that the V protein-mediated iNOS suppression is RIG-I specific. This was further confirmed by the V protein-mediated dose-dependent suppression of Luc activity (Fig. 3B). These results demonstrated that the V protein inhibits the activation of iNOS by preventing the activation of the RIG-I/TRIM25 signaling pathway. Since the iNOS promoter is an ISRE-responsive (ISGF3-driven) and NF-κB-driven promoter, similar experiments were performed using ISRE-responsive and NF-κB-driven Fluc plasmids. As shown in Fig. 3C and D, SeV V protein suppressed RIG-I/TRIM25-induced ISRE and NF-κB activation, similar to NiV V or MeV V, whereas SeV P protein showed only moderate inhibition in contrast to NiV or MeV P. To monitor the expression profiles of the proteins, we performed IB analysis using anti-Flag, anti-Myc, and anti-V5 antibodies. As previously reported [32], ectopically expressed Flag-tagged RIG-IN (Flag-RIG-IN) was robustly ubiquitinated, and the ubiquitination was enhanced by co-expressing Myc-tagged TRIM25 (Myc-TRIM25) (Fig. 3E). Thus, the minimal ubiquitination by RIG-IN alone was catalyzed endogenous TRIM25. When SeV V was co-expressed, RIG-IN showed loss of this ubiquitination as was seen with NiV V or MeV V [21], indicating that SeV V inhibits RIG-IN ubiquitination. On the other hand, SeV P moderately suppressed RIG-IN ubiquitination, unlike NiV and MeV P. Collectively, these results demonstrate that V inhibits the activation of iNOS by preventing activation of the RIG-I/TRIM25 pathways, which would lead to activation of both the ISGF3 and NF-κB pathways, through interfering with TRIM25 ubiquitination of RIG-I.

2.4. SeV V protein inhibited TRIM25-mediated RIG-I ubiquitination and the downstream RIG-I-MAVS protein interaction

TRIM25 is a ubiquitin E3 ligase that catalyzes K63-linked polyubiquitination of RIG-I [30,31]. This ubiquitination promotes RIG-I

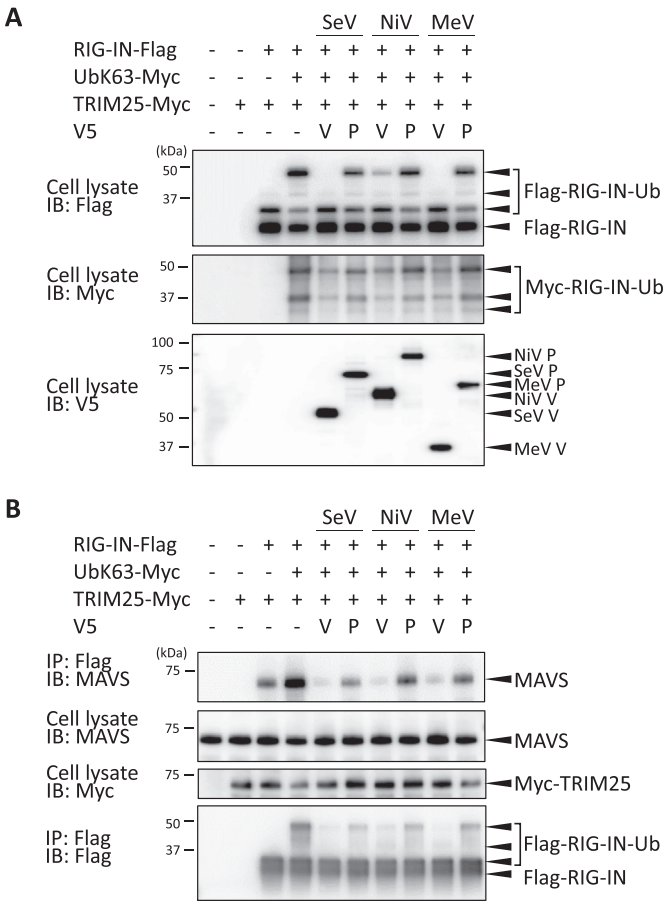

Fig. 4. Effect of SeV V protein on RIG-I ubiquitination and subsequent RIG-I-MAVS interaction by TRIM25. HEK293T cells were transfected with the indicated plasmids. At 24 h post-transfection, the cells were lysed in lysis buffer. (A) The cell lysates were subjected to IB with anti-Flag, anti-Myc, or anti-V5 antibody. (B) The cell lysates were subjected to IP with anti-Flag, followed by IB with anti-MAVS antibody. The cell lysates for IP were also subjected to IB with anti-MAVS, anti-Myc, or anti-Flag antibody. NiV, Nipah virus. MeV, measles virus.

oligomerization and facilitates RIG-I-MAVS binding, thereby leading to the activation of the genes downstream of type I IFN and other cytokines. Next, we thus studied the role of SeV V protein in blocking TRIM25-mediated K63-linked polyubiquitination of RIG-I and the downstream RIG-I-MAVS protein interaction. Flag-RIG-IN and Myc-TRIM25 transfected into HEK293T cells with or without Myc-tagged UbK63 (Myc-UbK63). First, the ubiquitination level of RIG-IN was analyzed by probing the IB with anti-Flag or anti-Myc antibody. As shown in Fig. 4A, a ladder of K63-linked polyubiquitinated RIG-IN bands was observed in cells co-transfected with Myc-TRIM25 and Myc-UbK63, but this was not observed in the absence of Myc-UbK63 (Fig. 3E). However, the ubiquitination was markedly suppressed when SeV V was co-transfected, similar to the results with NiV or MeV V co-transfection. On the other hand, SeV P moderately suppressed RIG-IN ubiquitination but no such effect was observed with NiV P or MeV P.

Next, to detect the interaction between RIG-IN and the MAVS protein, the cell lysate was subjected to immunoprecipitation (IP) with anti-Flag antibody followed by IB analysis with MAVS antibody. When TRIM25 was co-expressed with RIG-IN, the interaction between RIG-IN and MAVS was enhanced, which correlated with RIG-IN ubiquitination (Fig. 4B). However, when SeV V was co-transfected, the interaction was significantly inhibited. Similar results were observed when NiV V or MeV V were co-transfected. By contrast, SeV P moderately suppressed the interaction between RIG-IN and MAVS, unlike NiV and MeV P. These results also correlated with the results shown in Fig. 4A. Therefore, SeV V blocks the RIG-I MAVS protein interaction, correlating with the inhibition of TRIM25-mediated RIG-I ubiquitination.

2.5. SeV V protein interacted with both RIG-I and TRIM25 to inhibit RIG-I/TRIM25-mediated iNOS activation

Our results indicate that unlike NiV or MeV, SeV P has a moderate inhibitory effect on the RIG-I/TRIM25 signaling pathway. SeV P and V proteins share 317 amino acid residues at the amino terminus (Vn region), whereas the V protein has 67 V-specific residues at the carboxyl terminal (Vc region), suggesting that the SeV P/V common region (aa 1–317) may be responsible for the inhibitory effect. To map the functional region of the V protein responsible for its inhibitory effect, we examined the effect of truncated V mutants, Vn (1–317 aa) and Vc (318–384 aa) (Fig. 5A) on the RIG-I signaling pathway. A reporter assay using an iNOS reporter showed that while Vc lost its inhibitory ability upon introducing mutations, Vn exhibited moderate inhibition (Fig. 5B). These results were similar to those obtained with the P protein. IB analysis showed that Vc lost its inhibitory effect on RIG-IN and MAVS interaction as well, whereas Vn exhibited moderate inhibition (Fig. 5C). This inhibition correlated with the inhibition of TRIM25-mediated RIG-I ubiquitination. Furthermore, to investigate the interaction of V with RIG-IN or TRIM25, the cell lysates were subjected to IP with anti-V5 antibody followed by IB analysis with anti-Flag or anti-Myc antibody. Flag-RIG-IN co-immunoprecipitated with V5-tagged V (V5-V), consistent with a previous report for RIG-IN Ref. [21]. On the other hand, lesser amounts co-immunoprecipitated with V5-tagged Vn (V5-Vn) but not with V5-tagged Vc (V5-Vc). Similar results were obtained with Myc-TRIM25. These results signified the importance of the V interaction with both RIG-IN and TRIM25 in the inhibition of RIG-I-mediated iNOS activation and showed that both the N- and C-terminal regions are required for the inhibition of the RIG-I signaling pathway.

3. Discussion

Earlier studies have evaluated the signaling pathways responsible for the production of NO during viral infection [33,34]. The viral constituents that activate toll-like receptor (TLR) pathways have been reported to regulate NO production [8]. In addition to viral nucleic acids, components of the viral envelope are also known to modulate NO production.

In this study, we expanded on previous findings that SeV V protein is a negative regulator of IFN-β production and demonstrate that SeV V protein inhibits iNOS activation leading to decreased NO production. However, unlike the earlier published studies [33,34], our results show that RIG-I is an important player in V protein-mediated iNOS regulation. To our knowledge, this is the first study to indicate a relationship between the RIG-I signaling pathway and iNOS activation.

SeV V(−) induced a high level of NO expression in mouse RAW264.7 macrophages compared with SeV WT (Fig. 1A). The knockout of RIG-I in cells inhibited SeV V(−)-induced NO production (Fig. 2A). Since SeV V(−) can replicate in RIG-I KO mice as efficiently as SeV WT [3], the inhibitory activity of NO by SeV V might partly explain why the V(−) virus is attenuated *in vivo*.

To reveal the molecular mechanism by which SeV V protein inhibits iNOS and NO production, we analyzed the effect of the V protein on iNOS promoter activation in RIG-I/TRIM25-reconstituted HEK293T cells. Our results showed that the overexpression of the V protein inhibited RIG-I/TRIM25 signaling and downstream iNOS activation (Fig. 3). This demonstrated that the V protein targeted the RIG-I/TRIM25-mediated iNOS signaling pathway and could do so without requiring other viral proteins. Moreover, it inhibited TRIM25-mediated ubiquitination of RIG-I and its CARD-dependent interaction with MAVS (Fig. 4A and B). Further, the binding of the V protein with both RIG-I and TRIM25 was correlated with its ability to block the iNOS signaling pathway (Fig. 5B, C, and D). Therefore, our study demonstrates that the SeV V protein negatively regulates RIG-I signaling by interacting with RIG-I and TRIM25, leading to iNOS inhibition. An earlier study had also shown that paramyxovirus V proteins, such as NiV and MeV, inhibited RIG-I/TRIM25-mediated IFN-β activation by interacting with both RIG-I and TRIM25 [21]. However, it did not show that SeV V inhibited the RIG-I/TRIM25-signaling pathway. Our study demonstrated that in addition to inhibiting iNOS via RIG-I/TRIM25, SeV V protein also inhibited the RIG-I/TRIM25-mediated activation of ISRE and NF-κB.

Overall, our results confirm the earlier findings by Sánchez-Aparicio et al. [21] who reported similar results with NiV V, although they used RIG-IN and TRIM25 of human origin. The importance of the SeV interaction with both RIG-IN and TRIM25 was corroborated by using the V mutants (Fig. 5B and C). However, some conflicting results were observed as to the functional domains of the V protein. Sánchez-Aparicio et al. [21] showed that the C-terminal domain of NiV V (NiV Vc) interacted with RIG-IN and TRIM25, although they did not provide any direct evidence for the NiV Vc-mediated inhibition of RIG-I/TRIM25. The V protein in SeV, NiV, or MeV is synthesized from an additional mRNA, which is transcribed from the P gene by insertion of a pseudo-templated G residue at the specific editing site. As a result, the amino terminus (Vn region) of the P and V proteins from SeV, NiV, and MeV share 317, 407, or 230 common amino acid residues, respectively, whereas each V protein has a V-specific 67-, 49-, or 69-residue carboxyl terminus (Vc region). In our study, while SeV P protein moderately inhibited RIG-IN/TRIM25-mediated signaling (Fig. 4A and B), no such inhibition was shown with the P proteins from NiV or MeV, indicating that the inhibition by SeV V requires both the Vn and Vc regions, while in NiV and MeV, the inhibition is mediated in

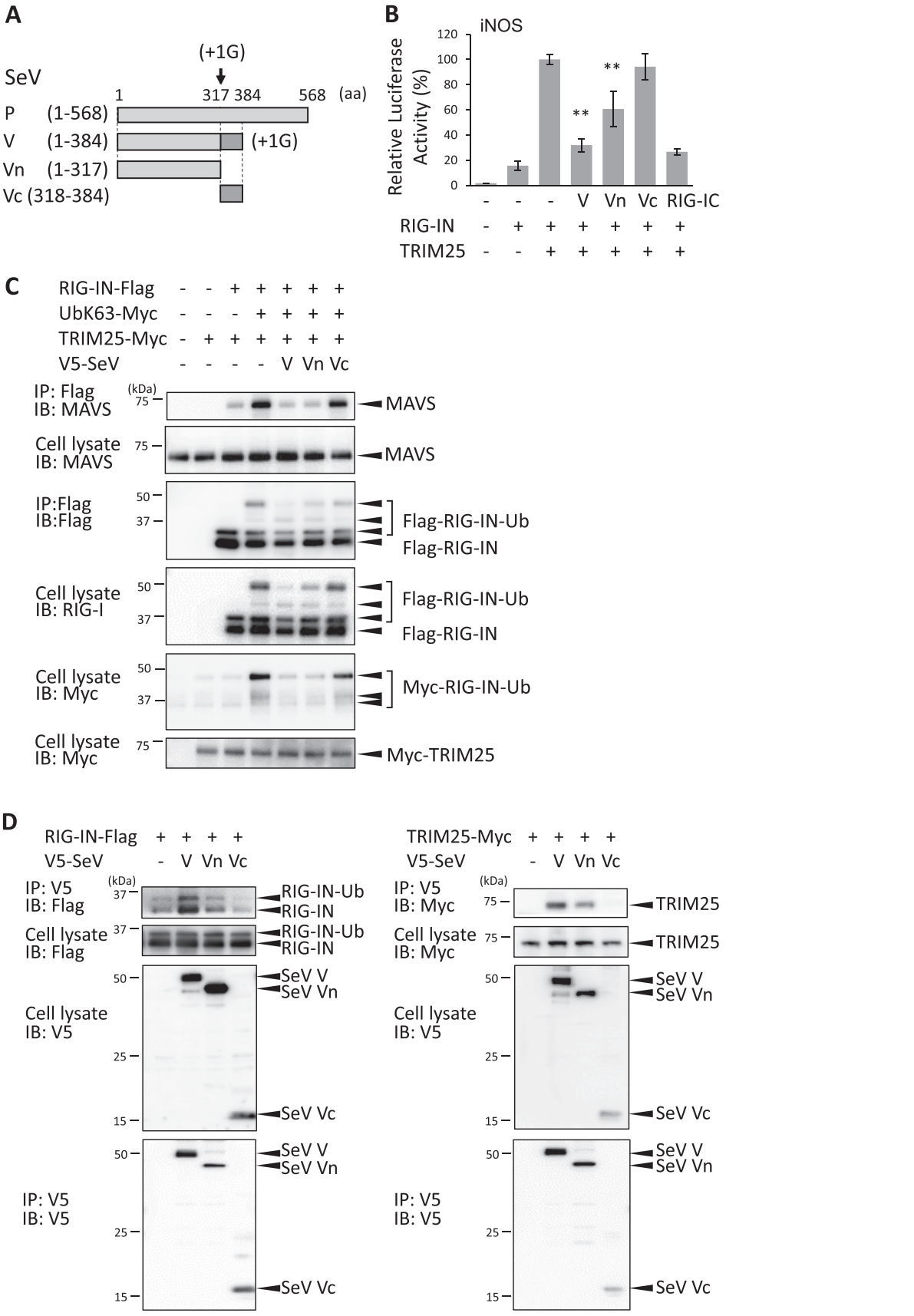

Fig. 5. Requirement of the protein V binding with RIG-I- and TRIM25 for the inhibition of RIG-I/TRIM25-mediated iNOS activation. (A) Schematic diagram of the SeV mutants Vn (1–317 aa) and Vc (318–384 aa). (B) HEK293T cells were transfected with the indicated plasmids, an iNOS promoter-driven reporter plasmid, and pRL-TK. At 24 h post-transfection, Fluc and Rluc activities were measured. Relative luciferase activity was calculated as the ratio of Fluc activity to Rluc activity. **: $P < 0.01$ vs. transfection with empty vector (Dunnett's [1] test). (C) HEK293T cells were transfected with the indicated plasmids. IP and IB were performed as described for Fig. 4A and B. (D) HEK293T cells were transfected with the indicated plasmids. At 24 h post-transfection, the cell lysates were subjected to IP with anti-V5 antibody, followed by IB with anti-Flag or anti-Myc antibody. The cell lysates for IP were also subjected to IB with anti-Flag, anti-Myc, or anti-V5 antibody.

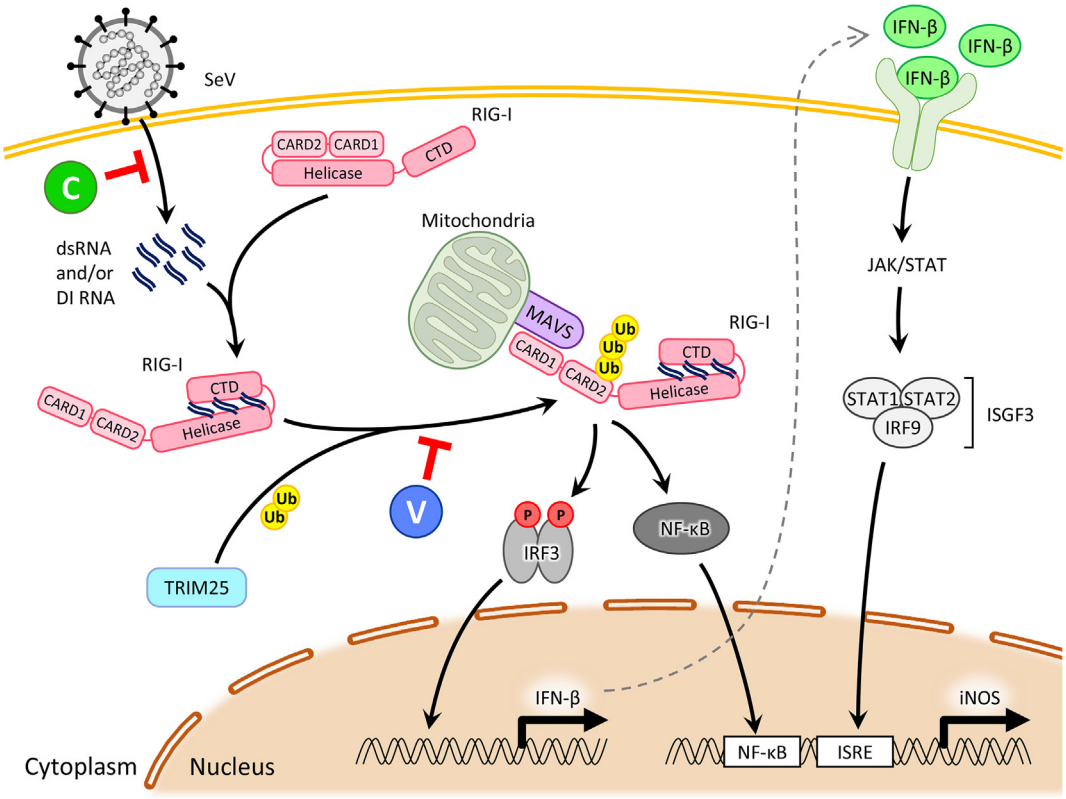

Fig. 6. Schematic representation of RIG-I activation during SeV infection. C and V interfere with distinct steps in RIG-I activation and thereby prevent RIG-I from antagonizing the production of NO. The effect of V protein on RIG-I activation was assessed in this study. The assessments were performed at their corresponding steps indicated in the schematic of the RIG-I activation pathway. (A) C presumably antagonizes RIG-I activation by limiting the generation of dsRNA and/or DI RNA during viral transcription and replication [14,15]. (B) V has previously been determined to interact with RIG-I and TRIM25 [21]. The data presented here support this observation (Fig. 5D) and indicates that V also inhibits the TRIM25-mediated ubiquitination of the N-terminal CARD domain of RIG-IN and the subsequent CARD-dependent interaction of RIG-IN with MAVS, thereby preventing the activation of the RIG-I signaling pathway (Figs. 4B and 5C). CTD, C-terminal domain. The concept for RIG-I activation is referenced from published work [35].

a Vn-independent manner. Our results are consistent with a previous report showing that RIG-I did not interact with the SeV Vc region [19]. Because we could not observe the inhibition of iNOS activation by the interaction of SeV Vc protein with RIG-I and TRIM25, it would be of great interest to determine the functional regions involved in V-RIG-I/TRIM25 interactions in these paramyxoviruses, including those involving the MeV V protein.

It was initially surprising that SeV V did not downregulate the expression of iNOS during SeV 4C(−) infection. SeV 4C(−) produces large amounts of dsRNA during viral transcription and replication [14,15], thereby strongly activating RLR signaling. Although 4C(−) has an intact V gene, its V protein cannot completely inhibit the strong activation of the RLR signaling and expression of iNOS. In contrast, SeV WT produces only a small amount of dsRNAs, thus only slightly activating the RLR signaling, and its V protein can inhibit the activation of the RLR signaling and expression of iNOS [16]. Conversely, since the SeV V protein is an antagonist of RLR, SeV V(−) stimulates the RLR signaling pathway despite the intact C gene [17–21]. Although V and C proteins both antagonize RLRs, they have distinct targets. A schematic of the RLR (especially RIG-I) activation during SeV infection, showing the specific steps at which V and C suppress the activation of RIG-I, is illustrated in Fig. 6.

Our findings raise the critical question of whether the NO antagonism of SeV-mediated inhibition of iNOS and the concomitant decrease in NO production contributes to its virulence and pathogenicity. Although NO has not shown significant inhibitory effects on SeV replication [16], it is involved in both innate and adaptive immune responses [7,8]. We preliminarily investigated the effects of macrophage depletion on SeV V(−) replication and pathogenesis. Depletion of airway macrophages by clodronate-loaded liposomes enhanced viral replication and pathogenesis *in vivo* (unpublished data). Therefore, we infected RAW264.7 macrophages with SeV V(−) to examine the impact on macrophage function. Although there was no difference in cytotoxicity in RAW264.7 macrophages, we found a significant increase in NO production between the SeV V(−) and SeV WT groups. Therefore, compromising NO production may be one strategy evolved by these viruses to enhance their pathogenicity.

In summary, our study reveals that SeV protein V suppresses NO induction in macrophages. Additionally, the V protein blocks the RIG-I signaling pathway by inhibiting the TRIM25-mediated ubiquitination of RIG-I and its CARD-dependent interaction with MAVS.

Declaration of Competing Interest

The authors declare no conflicts of interest.

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References

[1] Kato A, Kiyotani K, Sakai Y, Yoshida T, Nagai Y. The paramyxovirus, Sendai virus, V protein encodes a luxury function required for viral pathogenesis. EMBO J 1997;16:578–87.

[2] Kiyotani K, Sakaguchi T, Kato A, Nagai Y, Yoshida T. Paramyxovirus Sendai virus V protein counteracts innate virus clearance through IRF-3 activation, but not via interferon, in mice. Virol 2007;359:82–91.

[3] Irie T, Kiyotani K, Igarashi T, Yoshida A, Sakaguchi T. Inhibition of interferon regulatory factor 3 activation by paramyxovirus V protein. J Virol 2012;86:7136–45.

[4] Cline TD, Beck D, Bianchini E. Influenza virus replication in macrophages: balancing protection and pathogenesis. J Gen Virol 2017;98:2401–12.

[5] Vangeti S, Yu M, Smed-Sörensen A. Respiratory mononuclear phagocytes in human influenza A virus infection: their role in immune protection and as targets of the virus. Front Immunol 2018;9:1–11.

[6] Vareille M, Kieninger E, Edwards MR, Regamey N. The airway epithelium: soldier in the fight against respiratory viruses. Clin Microbiol Rev 2011;24:210–29.

[7] Uehara EU, Shida BS, de Brito CA. Role of nitric oxide in immune responses against viruses: beyond microbicidal activity. Inflamm Res 2015;64:845–52.

[8] Abdul-Cader MS, Amarasinghe A, Abdul-Careem MF. Activation of toll-like receptor signaling pathways leading to nitric oxide-mediated antiviral responses. Arch Virol 2016;161:2075–86.

[9] Reiss CS, Komatsu T. Does nitric oxide play a critical role in viral infections ? J Virol 1998;72:4547–51.

[10] Farlik M, Reutterer B, Schindler C, Greten F, Vogl C, Müller M, et al. Nonconventional initiation complex assembly by STAT and NF-κB transcription factors regulates nitric oxide synthase expression. Immunity 2010;33:25–34.

[11] Bi Z, Reiss CS. Inhibition of vesicular stomatitis virus infection by nitric oxide. J Virol 1995;69:2208–13.

[12] Rimmelzwaan GF, Baars MMJW, de Lijster P, Fouchier RAM, Osterhaus ADME. Inhibition of influenza virus replication by nitric oxide. J Virol 1999;73:8880–3.

[13] Stark JM, Khan AM, Chiappetta CL, Xue H, Alcorn JL, Colasurdo GN. Immune and functional role of nitric oxide in a mouse model of respiratory syncytial virus infection. J Infect Dis 2005;191:387–95.

[14] Takeuchi K, Komatsu T, Kitagawa Y, Sada K, Gotoh B. Sendai virus C protein plays a role in restricting PKR activation by limiting the generation of intracellular double-stranded RNA. J Virol 2008;82:10102–10.

[15] Sánchez-aparicio MT, Garcin D, Rice CT, Kolakofsky D, García-sastre A. Loss of Sendai virus C protein leads to accumulation of RIG-I immunostimulatory defective interfering RNA. J Gen Virol 2017;98:1282–93.

[16] Odkhuu E, Komatsu T, Koide N, Naiki Y, Takeuchi K, Tanaka Y, et al. Sendai virus C protein limits NO production in infected RAW264.7 macrophages. Innate Immun 2018;24:430–8.

[17] Andrejeva J, Childs KS, Young DF, Carlos TS, Stock N, Goodbourn S, et al. The V proteins of paramyxoviruses bind the IFN-inducible RNA helicase, mda-5, and inhibit its activation of the IFN-beta promoter. Proc Natl Acad Sci U S A 2004;101:17264–9.

[18] Childs KS, Andrejeva J, Randall RE, Goodbourn S. Mechanism of mda-5 inhibition by paramyxovirus V proteins. J Virol 2009;83:1465–73.

[19] Sakaguchi T, Irie T, Kuwayama M, Ueno T, Yoshida A, Kawabata R. Analysis of interaction of Sendai virus V protein and melanoma differentiation-associated gene 5. Microbiol Immunol 2011;55:760–7.

[20] Childs K, Randall R, Goodbourn S. Paramyxovirus V proteins interact with the RNA helicase LGP2 to inhibit RIG-I-dependent interferon induction. J Virol 2012;86:3411–21.

[21] Sánchez-Aparicio MT, Feinman LJ, García-Sastre A, Shaw ML. Paramyxovirus V proteins interact with the RIG-I/TRIM25 regulatory complex and inhibit RIG-I signaling. J Virol 2018;92. pii:e01960-17.

[22] Komatsu T, Tanaka Y, Kitagawa Y, Koide N, Naiki Y, Morita N, et al. Sendai virus V protein inhibits the secretion of interleukin-1β by preventing NLRP3 inflammasome assembly. J Virol 2018;92:1–15.

[23] Kurotani A, Kiyotani K, Kato A, Shioda T, Sakai Y, Mizumoto K, et al. Sendai virus C proteins are categorically nonessential gene products but silencing their expression severely impairs viral replication and pathogenesis. Gene Cell 1998;3:111–24.

[24] Niwa H, Yamamura K, Miyazaki J. Efficient selection for high expression transfectants with a novel eukaryotic vector. Gene 1991;108:193–9.

[25] Takeda M, Ohno S, Seki F, Nakatsu Y, Tahara M, Yanagi Y. Long untranslated regions of the measles virus M and F genes control virus replication and cytopathogenicity. J Virol 2005;79:14346–54.

[26] Kitagawa Y, Yamaguchi M, Zhou M, Nishio M, Itoh M, Gotoh B. Human parainfluenza virus type 2 V protein inhibits TRAF6-mediated ubiquitination of IRF7 to prevent TLR7- and TLR9-dependent interferon induction. J Virol 2013;87:7966–76.

[27] Odkhuu E, Komatsu T, Naiki Y, Koide N, Yokochi T. Sendai virus C protein inhibits lipopolysaccharide-induced nitric oxide production through impairing interferon-β signaling. Int Immunopharm 2014;23:267–72.

[28] Komatsu T, Takeuchi K, Yokoo J, Gotoh B. C and V proteins of Sendai virus target signaling pathways leading to IRF-3 activation for the negative regulation of interferon-β production. Virol 2004;325:137–48.

[29] Takahashi K, Sugiyama T, Tokoro S, Neri P, Mori H. Inhibition of interferon-γ-induced nitric oxide production by 10-hydroxy-trans-2-decenoic acid through inhibition of interferon regulatory factor-8 induction. Cell Immunol 2012;273:73–8.

[30] Yoneyama M, Kikuchi M, Natsukawa T, Shinobu N, Imaizumi T, Miyagishi M, et al. The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses. Nat Immunol 2004;5:730–7.

[31] Patel JR, Jain A, Chou YY, Baum A, Ha T, García-Sastre A. ATPase-driven oligomerization of RIG-I on RNA allows optimal activation of type-I interferon. EMBO Rep 2013;14:780–7.

[32] Chiang C, Pauli EK, Biryukov J, Feister KF, Meng M, White EA, et al. The human papillomavirus E6 oncoprotein targets USP15 and TRIM25 to suppress RIG-I-mediated innate immune signaling. J Virol 2018;92:1–15.

[33] Zolini GP, Lima GK, Lucinda N, Silva MA, Dias MF, Pessoa NL, et al. Defense against HSV-1 in a murine model is mediated by iNOS and orchestrated by the activation of TLR2 and TLR9 in trigeminal ganglia. J Neuroinflammation 2014;11:20.

[34] Phipps S, Lam CE, Mahalingam S, Newhouse M, Ramirez R, Rosenberg HF, et al. Eosinophils contribute to innate antiviral immunity and promote clearance of respiratory syncytial virus. Blood 2007;110:1578–86.

[35] Jureka AS, Kleinpeter AB, Tipper JL, Harrod KS, Petit CM. The influenza NS1 protein modulates RIG-I activation via a strain-specific direct interaction with the second CARD of RIG-I. J Biol Chem 2020;295:1153–64.