A RIG-I–like receptor directs antiviral responses to a bunyavirus and is antagonized by virus-induced blockade of TRIM25-mediated ubiquitination

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The RIG-I–like receptors (RLRs) retinoic acid–inducible gene 1 protein (RIG-I) and melanoma differentiation–associated protein 5 (MDA5) are cytosolic pattern recognition receptors that recognize specific viral RNA products and initiate antiviral innate immunity. Severe fever with thrombocytopenia syndrome virus (SFTSV) is a highly pathogenic member of the Bunyavirales. RIG-I, but not MDA5, has been suggested to sense some bunyavirus infections; however, the roles of RLRs in anti-SFTSV immune responses remain unclear. Here, we show that SFTSV infection induces an antiviral response accompanied by significant induction of antiviral and inflammatory cytokines and that RIG-I plays a main role in this induction by recognizing viral 5′-triphosphorylated RNAs and by signaling via the adapter mitochondrial antiviral signaling protein. Moreover, MDA5 may also sense SFTSV infection and contribute to IFN induction, but to a lesser extent. We further demonstrate that the RLR-mediated anti-SFTSV signaling can be antagonized by SFTSV nonstructural protein (NSs) at the level of RIG-I activation. Protein interaction and MS-based analyses revealed that NSs interacts with the host protein tripartite motif–containing 25 (TRIM25), a critical RIG-I activating ubiquitin E3 ligase, but not with RIG-I or Riplet, another E3 ligase required for RIG-I ubiquitination. NSs specifically trapped TRIM25 into viral inclusion bodies and inhibited TRIM25-mediated RIG-I Lys-63–linked ubiquitination/activation, contributing to suppression of RLR-mediated antiviral signaling at its initial stage. These results provide insights into immune responses to SFTSV infection and clarify a mechanism of the viral immune evasion, which may help inform the development of antiviral therapeutics.

Mammalian hosts have evolved a variety of cellular pattern recognition receptors (PRRs), such as retinoic acid–inducible gene 1 (RIG-I)–like receptors (RLRs), Toll-like receptors (TLRs), and structurally diverse DNA sensors, which sense the presence of pathogen-associated molecular patterns (PAMPs), including viral nucleic acids or other conserved molecular components of invading microbes (1–5). Among the several PRRs, RLRs are broadly expressed in most cell types and can recognize many RNA virus infections (6–8). The RLR family has three members: RIG-I, melanoma differentiation-associated gene 5 (MDA5), and laboratory of genetics and physiology 2 (LGP2). RIG-I and MDA5 consist of two N-terminal caspase recruitment domains (CARDs), a central DECH box ATPase domain, and a C-terminal regulatory/repressor domain (RD), whereas LGP2 lacks CARD-like domains and has been suggested as a regulator for RLR signaling (9–11). RIG-I and MDA5 function as cytosolic receptors for viral RNAs, albeit with different ligand types. Specifically, C-terminal RD of RIG-I preferentially recognizes RNA substrates marked with a 5′-triphosphorylated (5′ppp) end (12–14), whereas MDA5 mainly recognizes long dsRNA or higher-order RNA complexes (reviewed in Ref. 15). Upon ligand binding, the activation of RIG-I and MDA5 further needs Lys-63–linked ubiquitination by E3 ubiquitin ligases, including the tripartite motif protein 25 (TRIM25) and Riplet responsible for RIG-I ubiquitination (16–21) and TRIM65 catalyzing MDA5 ubiquitination (22). Following full activation, RLRs can interact with the downstream adapter, mitochondrial antiviral signaling protein (MAVS, also named VISA/IPS-1/Cardif) (23–26) and then trigger signaling cascades that activate transcription factors, interferon regulatory factor 3 (IRF3) and nuclear factor–κB (NF–κB), resulting in the induction of type I interferons (IFNs), IFN-stimulated genes (ISGs), and inflammatory cytokines and establishment of an antiviral state. Due to the crucial activities of RLRs in recognition and control of many RNA viruses, RLR signaling should be a strong pressure on these viruses, likely leading to the evolution of specific viral evasion strategies (8, 27). Currently, various antagonizing mechanisms of viral pathogens against RLR signaling have been identified. It seems that every critical step of RLR signaling cascades can be virally targeted. Moreover, many viruses have evolved more than one immune evasion strategy for efficient infection and replication in the host (28–32).

Severe fever with thrombocytopenia syndrome (SFTS) is an emerging infectious disease with a mortality rate of 12–30% that is caused by the SFTS virus (SFTSV) belonging to the newly proposed Banyangivirus genus in the Phenuiviridae family, Bunyavirales order (33, 34). SFTSV was first identified in China in 2009 (33, 35, 36) and subsequently found in South Korea (37) and Japan (38). Like other bunyaviruses, the SFTSV genome possesses triplicate single-stranded RNA, characterized with complementary 3′ and 5′ ends, which facilitate the formation of the panhandle structure of each segment (39). The large (L) and medium (M) segments are of negative polarity and encode the RNA-dependent RNA polymerase and
glycoproteins, respectively, whereas the small (S) segment encodes the nucleocapsid protein and the nonstructural protein (NSs) with an ambisense strategy (33). Clinically, SFTSV infection can result in acute inflammatory responses accompanied by abnormal induction of immune and inflammatory cytokines in patients’ sera (40–42); however, it is unknown how SFTSV is sensed by the host cells and hence triggers the immune and inflammatory cytokine responses. Although the host perception and antiviral signaling mechanism against SFTSV infection is unclear, previous studies by us and others have established that the NSs of SFTSV can antagonize RIG-I and MDA5 signaling cascades by targeting the downstream kinases TBK1/IKKe and inhibit type I IFN production, implying that the RLRs might be implicated in host response to SFTSV infection (28, 43–45). In addition, consistently, Yamada et al. (46) reported that the deficiency of MAVS appeared to block IFN-α induction by SFTSV infection in a mouse model. However, the detailed roles of RLRs in host immune and inflammatory responses to SFTSV infection have not been directly investigated.

As the emerging representative of high-pathogenic bunyaviruses, SFTSV is considered as one of the most dangerous pathogens and is included by the World Health Organization in its list of the top priority viruses requiring urgent research and development efforts. The mechanisms underlying the host recognition and immune responses to SFTSV thus particularly merit clarification. In this work, we demonstrate that SFTSV infection triggers rapid immune and inflammatory cytokine responses in human cells, in which indeed, RIG-I plays a major role by recognizing the viral 5’ppp RNAs and signaling through the adaptor MAVS. MDA5 appears to also sense SFTSV infection and induce IFN expression, but to a significantly lesser extent. Next, we show that the RLR antiviral signaling can be antagonized by SFTSV NSs at the level of RIG-I ubiquitination and activation. Previously, Santiago et al. (43) observed possible interactions of NSs with overexpressed TRIM25 and RIG-I in transient transfection assays, which, however, was not validated in the contexts of endogenous proteins or viral infection or further investigated functionally. Here, endogenous TRIM25 (but not RIG-I itself or another E3 ubiquitin ligase, Riplet) was first identified in NSs coprecipitates by MS. Then the specific interaction of NSs with TRIM25 was further validated in various contexts, including those of viral infection. Furthermore, we found that by the specific interaction of NSs with TRIM25, NSs traps TRIM25 into viral inclusion bodies (IBs) and abates TRIM25-mediated Lys-63 ubiquitination and activation of RIG-I. The work not only clarifies the roles of RLRs in cellular recognition of SFTSV and consequent immune and inflammatory responses but also establishes an IB-associated, RIG-I ubiquitination–targeting immune escape mechanism by which SFTSV achieves inhibition of the host antiviral response at the initial stage of RLR signaling.

**Results**

**SFTSV infection induces a rapid antiviral and inflammatory response**

Currently, how host cells recognize SFTSV infection and mediate immune and inflammatory responses is unclear. To gain insights into the antiviral and inflammatory pathways triggered by SFTSV, we first investigated type I IFN, ISG, and inflammatory cytokine responses in SFTSV-infected cells. As monitored by real-time quantitative RT-PCR (qRT-PCR), along with SFTSV replication in HEK293 cells, a prompt and substantial expression of IFN-β mRNA could be observed as early as 3 h postinfection (hpi) (Fig. 1, A and B). Similarly, the mRNA expression of ISG56 (also known as IFTI1), a notable antiviral ISG (47), could be quickly induced 3 hpi and peaked at 6 hpi in the infected cells (Fig. 1C). The mRNA expression of several other antiviral ISGs, including 2′-5′-oligoadenylate synthetase 2 (OAS2), myxoma resistance protein 1 (MxA), and ISG15, was also elevated at the early stage of SFTSV infection (the first 12 h of infection) and could be further increased and peaked at 24 hpi (Fig. 1C), exhibiting a somewhat different behavior from the expression of ISG56. Consistently, notable induction of the antiviral ISGs upon SFTSV infection could be validated at protein levels by Western blotting (WB) analyses (Fig. S1A). These observations suggest that host cells can rapidly sense SFTSV infection and mount a specific antiviral IFN and ISG response.

Clinical studies reported that a panel of inflammatory cytokines, including interleukin-8 (IL-8), IFN-γ–inducible protein 10 (IP-10), regulated on activation normal T cell–expressed and secreted (RANTES), and tumor necrosis factor α (TNFα), is highly induced in SFTSV-infected human sera (40–42). To investigate whether SFTSV infection can directly result in cytokine eruption, we tested the expression levels of the representative cytokines and chemokines in SFTSV-infected cells. As shown in Fig. 1D, the mRNA levels of IL-8, RANTES, TNFα, and IP-10 were notably elevated as early as 3 hpi and continuously increased to peak values at 24–36 hpi in SFTSV-infected HEK293 cells (Fig. 1D). Furthermore, secretion of the cytokines induced by the virus infection was evidently detected by ELISA (Fig. S1B). These findings indicate that SFTSV infection provokes a rapid and substantial inflammatory reaction. Consistently, substantial induction of IFN-β, ISGs, and inflammatory cytokines was also detected in A549 and THP-1 cells (Fig. S2). Additionally, we observed that infections with higher SFTSV multiplicities of infection (MOI) were associated with increased immune and inflammatory responses (Fig. 1, E and F). Together, these results suggested that SFTSV infection can be rapidly recognized by the host cells and thus trigger distinct antiviral and inflammatory responses.

**RIG-I and MDA5 expression is up-regulated by SFTSV infection**

RLRs, RIG-I and MDA5, are expressed broadly in most cell types, including all of the cells used in the present study. Moreover, RIG-I has been shown to play a critical role in host immune response to several bunyaviruses. Thus, we began to explore the roles of RLRs in the context of SFTSV infection. First, the mRNA and protein expression of RIG-I and MDA5 during SFTSV infection was analyzed by qRT-PCR and WB analyses, respectively. As shown in Fig. 2A, following SFTSV infection, mRNA expression of RIG-I and MDA5 could be quickly enhanced. Correspondingly, protein expression of RIG-I and MDA5 was also rapidly elevated to remarkably higher
levels 12 hpi (SFTSV-infected cells versus the mock group) (Fig. 2B). In comparison, no obvious induction was observed in the expression of MAVS, the adaptor downstream of RIG-I and MDA5 (Fig. 2, A and B). Consistently, the specific induction of RIG-I and MDA5 also could be detected in SFTSV-infected A549 and THP-1 cells (Fig. S3). These data indicate the substantial induction of RIG-I and MDA5 in response to SFTSV infection, providing details and references for further characterizing the roles of these RLRs in SFTSV infection.

MAVS is a key mediator in the antiviral and inflammatory responses to SFTSV infection

MAVS is an essential component of the RLR antiviral signaling by acting as the common adaptor of RIG-I and MDA5 (23–26). To clarify the role of RLR-MAVS signaling in SFTSV infection, we examined the effects of MAVS knockdown by RNAi on SFTSV-triggered immune and inflammatory response. As shown in the reporter gene assays, SFTSV infection resulted in pronounced activation of IFN-β and NF-κB promoters;
however, knockdown of MAVS to around a half-level appeared to completely block SFTSV-stimulated IFN-β promoter activation and substantially suppressed NF-κB promoter activation (Fig. 3, A and B), manifesting a vital role of MAVS in the cellular response to SFTSV. Correspondingly, down-regulation of MAVS expression also significantly inhibited SFTSV infection–induced mRNA expression of IFN-β and representative inflammatory cytokines, IL-8 and TNFα, as indicated by the qRT-PCR analyses (Fig. 3C). Note that inhibition of cytokine induction by MAVS knockdown was likely underestimated in qRT-PCR analyses based on shRNA plasmid transfection, because a portion of cells were invalidly transfected and had no shRNA efficient expression, whereas reporter gene assays reflect the effects on only the successfully transfected cells, thus likely better exhibiting the influence of knockdown on the cellular responses. These results reveal the pivotal role of MAVS in SFTSV-triggered immune and inflammatory responses. As shown in Fig. 4 (A and B), specific shRNAs significantly knocked down the mRNA and protein levels of RIG-I and MDA5 in both mock- and SFTSV-infected cells. In agreement with the results of MAVS knockdown, the RNAi of RIG-I nearly abolished SFTSV-induced IFN-β promoter activation (Fig. 4C) and evidently impaired SFTSV-stimulated NF-κB promoter response (Fig. 4D). Meanwhile, MDA5 knockdown also decreased IFN-β promoter activation to a lesser extent (Fig. 4C) but, in comparison, did not substantially affect NF-κB promoter response (Fig. 4D). Furthermore, RIG-I knockdown significantly inhibited the induction of cytokines (especially IFN-β) by SFTSV (Fig. 4E), whereas down-regulation of MDA5 exhibited seemingly weaker influence on the virus-stimulated cytokine expression (Fig. 4F). These observations reveal the involvement of RLRs in host perception of SFTSV infection and particularly the primary role of RIG-I in SFTSV-induced immune and inflammatory responses at least in HEK293 cells.

A549 cells that can be efficiently infected by bunyaviruses harbor various cytosolic and membrane-anchoring PRRs, including both RLRs and TLRs, whereas HEK293 cells express cytosolic PRRs, including RLRs but not TLRs (48). To further evaluate the significance of RLRs as the PRRs of SFTSV, we investigated the effects of RIG-I and MDA-5 knockdown on cellular responses to SFTSV infection in A549 cells. As
transfection efficiency of A549 is very low, lentiviral vectors encoding shRNAs were used here instead of transient transfection of plasmids. As shown in Fig. 4 (G–J), SFTSV infection strongly triggered induction of RLRs, IFN-β, and TNFα in A549 cells. Consistent with the results from HEK293, the induction of IFN-β and TNFα by SFTSV infection was mostly deprived by RIG-I knockdown (Fig. 4I), indicating the primary role of RIG-I in cellular response to SFTSV, even in the presence of TLRs. MDA5 down-regulation also seemed to partially impair IFN-β expression (to an obviously lesser extent compared with that resulted from RIG-I knockdown) but did not result in significant change of SFTSV-elicited TNFα induction (Fig. 4J).

Considering the limited knockdown efficiency of RNAi, we further generated RIG-I knockout (KO) and MDA5-KO HEK293 cells by the CRISPR-Cas9 system (Fig. 5A) to further characterize RLR-mediated immune responses against SFTSV infection. Consistently, in WT HEK293 cells (control), SFTSV infection resulted in evident up-regulation of RIG-I and MDA5 (Fig. 5A). However, in RIG-I-KO cells, the up-regulation of MDA5 upon SFTSV infection was depleted, whereas the RIG-I expression induced by SFTSV was slightly or moderately affected in MDA5-KO cells (Fig. 5A). In accordance with the result, RIG-I KO almost eliminated SFTSV-stimulated production of cytokine mRNAs and proteins (Fig. 5, B and C), whereas MDA5 KO also impaired the immune responses induced by SFTSV, but to a lesser extent. Collectively, these results clearly establish that RLRs are involved in cellular recognition of SFTSV infection, and in particular, RIG-I plays a major role in SFTSV infection–stimulated immune and inflammatory responses.

5′-Triphosphate RNAs produced in SFTSV-infected cells are the primary PAMPs triggering anti-SFTSV immune and inflammatory signaling

Phleboviral RNAs, including genome segments, antigenome segments, and RNA replication intermediates, are supposed to contain a 5′-triphasate group (49, 50). Alkaline phosphatase that removes terminal phosphate groups from RNA and 5′-polyphosphatase that converts 5′ppp RNA to 5′-monophosphorylated RNA are commonly used to evaluate the contribution of 5′ppp RNAs to elicit RIG-I immune signaling (49–51). As RIG-I functions as a major PRR in recognition of SFTSV infection...
Interplay of SFTSV infection and RIG-I signaling

(A) Relative RIG-I mRNA level

(B) Relative MDA5 mRNA level

(C) Relative pIFN-β-luc activity

(D) Relative pNF-κB-luc activity

(E) Relative IFN-β mRNA level

(F) Relative IL-8 mRNA level

(G) Relative TRIF mRNA level

(H) Relative RIG-I mRNA level

(I) Relative IFN-β mRNA level

(J) Relative TNF-α mRNA level
infection, we next analyzed the role of 5’ppp RNAs potentially generated by SFTSV infection in triggering antiviral signaling. HEK293 cells were mock-infected or infected with SFTSV (MOI = 5) for 24 h. Total RNAs were then extracted from the cells and treated with alkaline phosphatase or 5’-polyphosphatase. After repurification, the RNA samples were used for the following stimulation of cells and reporter gene assays. As shown in Fig. 6, RNAs from mock-infected cells did not affect IFN-β or NF-κB promoter activities (compared with the control without RNA transfection), whereas SFTSV infection–generated RNAs can strongly activate IFN-β and NF-κB promoters. However, treatment with either alkaline phosphatase or 5’-polyphosphatase eliminated most of the IFN-β and NF-κB promoter activation (Fig. 6, A and B). These data demonstrate that 5’ppp RNAs yielded by SFTSV infection likely are predominant immune stimulators in SFTSV-triggered host responses, which is in consistent with the role of RIG-I as the primary SFTSV-recognizing PRR.

Roles of RIG-I and MDA5 in host restriction to SFTSV infection

As the RLRs, particularly RIG-I, play significant roles in immune and inflammatory responses to SFTSV infection, we further tested the respective effects of RIG-I and MDA5 knockdown on SFTSV replication. HEK293 cells were first transfected with the RNAi plasmids against RIG-I and MDA5. At 48 h posttransfection, cells were infected with SFTSV (MOI = 0.1) for 8 or 16 h, followed by RNA extraction and qRT-PCR for determining the relative levels of viral S-segment RNA. As shown in Fig. 7 (A and B), SFTSV S RNA replication was noticeably increased in RIG-I knockdown cells (∼2.2–3.5-folds at 8 hpi and ∼3.1–3.7-fold at 16 hpi, compared with the control groups at the corresponding time points). In comparison, MDA5 knockdown appeared to slightly promote viral RNA replication only at 16 hpi (relative S RNA levels: control, 72.7; MDA5 shRNA2#, 104.5; and MDA5 shRNA3#, 103.1) despite no statistically significant differences. Furthermore, the replication of SFTSV in RIG-I-KO and MDA5-KO cells was also monitored (Fig. 7C). SFTSV S-, L-, and M-segment RNA replication was significantly increased in RIG-I-KO cells (∼8-fold compared with the control WT cells) and MDA5-KO cells (∼2–4.5-fold). Consistently, analyses of growth curves by virus titration showed that virus propagation was remarkably enhanced in RIG-I-KO cells at 18 and 28 hpi, and meanwhile MDA5 KO noticeably promoted SFTSV RNA replication, albeit to a lesser extent (Fig. 7D). These results suggest that RLRs (especially RIG-I) act as significant host restriction factors of SFTSV replication, due to their critical roles in recognition of SFTSV and the resulting antiviral immune responses.

**Figure 4. Roles of RIG-I and MDA5 in SFTSV-triggered antiviral and inflammatory responses.** A and B, down-regulation of RIG-I or MDA5 by RNAi in mock- or SFTSV-infected HEK293 cells. Cells were transfected with the indicated shRNA plasmids and infected with SFTSV (MOI = 5) or mock-infected at 36 h posttransfection. At 12 hpi, the relative RIG-I or MDA5 mRNA levels were quantified by qRT-PCR, and protein levels were also detected by WB. C and D, effects of RIG-I or MDA5 knockdown on SFTSV-induced NF-κB and IFN-β promoter activation. HEK293 cells were transfected with the indicated shRNA plasmids, along with the reporter plasmids for IFN-β or NF-κB promoter and pRL-TK. Thirty-six hours posttransfection, cells were mock-infected or infected with SFTSV (MOI = 5) for 16 h and then delivered to luciferase activity measurement. E and F, effects of RIG-I or MDA5 knockdown on SFTSV-stimulated mRNA expression of IFN-β and inflammatory cytokines. HEK293 cells were transfected with the indicated shRNA plasmid, followed by SFTSV infection (MOI = 5). At 12 hpi, relative mRNA levels of the indicated genes were analyzed by qRT-PCR. G–I, effects of RIG-I or MDA5 knockdown by viral vector–delivered shRNAs in mock- or SFTSV-infected A549 cells. A549 cells were transfected with lentiviruses encoding the indicated shRNAs and then infected with SFTSV (MOI = 5) or mock-infected at 36 h posttransduction. At 12 hpi, mRNA and protein levels of the indicated genes were analyzed by qRT-PCR and WB, respectively. Data are presented as means ± S.D. (error bars), n = 4 for reporter gene assays, and n = 3 for qRT-PCR. *p < 0.05, **p < 0.01, ***p < 0.001; NS, not significant.

**Figure 5. Interplay of SFTSV infection and RIG-I signaling**

After several hours postinfection, the viral NSs protein can be expressed and gradually accumulated to high abundance (45). Previous studies by us and others have established that SFTSV NSs can inhibit RIG-I and MDA5 antiviral signaling by targeting host kinases TBK1/IKKe (28, 44). Additionally, Santiago et al. (43) observed the possible interactions of NSs with overexpressed TRIM25 and RIG-I in transient transfection assays. Given the important roles of RLRs in cellular anti-SFTSV response as suggested here, we further explored the potential cellular targets of NSs in RLR signaling pathway by protein interaction analyses and MS. HEK293T cells were transfected with the plasmid expressing S-tagged NSs (NSs-S) or the control vector. Cellular proteins potentially interacting with NSs were extracted by S-tag pulldown assays (S-pulldown) and analyzed by LC–MS/MS. Interestingly, in addition to TBK1, endogenous TRIM25 (but not RIG-I) was identified specifically in the NSs coprecipitates by MS (Fig. 8, A and B). To further validate the mass spectrometric results, we also examined the protein interaction with S-pulldown and WB analyses. As shown in Fig. 8C, endogenous TRIM25 as well as TBK1 (but not RIG-I) was detected specifically in the NSs pulldown products. Furthermore, we confirmed the specific interaction between NSs and cellular endogenous TRIM25 in the context of SFTSV infection by co-immunoprecipitation (co-IP) assays (Fig. 8D). In contrast, no noticeable interaction of NSs with endogenous RIG-I was detected even under conditions where the input level of RIG-I was much higher in SFTSV-infected cells than mock-infected control because of the up-regulation of RIG-I expression by SFTSV infection (Figs. 2 and 8D). Collectively, these data determine that besides TBK1, TRIM25 (but not RIG-I itself) is an additional target of SFTSV NSs in the RIG-I signaling pathway.

Riplet is another important E3 ubiquitin ligase for RIG-I activation (17, 19, 21, 52). To further characterize the specificity of NSs targeting of TRIM25, we further analyzed the potential targeting of Riplet by NSs. First, unlike TRIM25, Riplet was not identified in the NSs coprecipitates by the MS. Consistently, the interaction of NSs with TRIM25 but not Riplet is also validated in pulldown and WB analysis (Fig. S4A). These analyses further demonstrate the specific targeting of TRIM25 by NSs.

**NSs captures TRIM25, but not RIG-I or Riplet, into viral IB “jails”**

SFTSV NSs expression can induce the formation of cytoplasmic inclusion bodies (IBs), where NSs itself localizes (28, 45).
Previously, we proposed that NSs IBs are likely virus-built “jails” hijacking some crucial host factors and interfering with the corresponding biological processes through spatial isolation (28, 45). Given the interaction of NSs with TRIM25, we next examined whether NSs expression can change the subcellular localization of TRIM25. First, HeLa cells were transfected with the plasmids expressing FLAG-tagged TRIM25 or RIG-I, together with an NSs expression plasmid (without any tag) or the control vector, and fixed for immunofluorescence assays at 24 h posttransfection. In our studies, we observed that GFP and its derivants (often used as expression tags) appear to be colocalized with SFTSV NSs to some extent by themselves (data not shown), and thus the small tag (FLAG) was used here to avoid the influence of a large expression tag like GFP. As shown in

Figure 5. Effects of RIG-I and MDA5 KO by CRISPR-Cas9 on SFTSV-triggered antiviral and inflammatory responses. A, WB analysis of RIG-I- or MDA5-KO HEK293 cells with or without SFTSV infection. Two cell clones with RIG-I or MDA5 KO were respectively generated by CRISPR/Cas9-mediated gene editing and infected with SFTSV or mock-infected. At 24 hpi, the indicated protein expression was monitored by WB. Data are representative of two independent experiments with similar results. B and C, effects of RIG-I or MDA5 KO on SFTSV-stimulated cytokine expression. RIG-I- or MDA5-KO cells or the control cells were infected with SFTSV or mock-infected. At 24 hpi, relative mRNA levels (B) and secreted protein levels (C) of the indicated cytokines were analyzed by qRT-PCR and ELISA, respectively. Data are presented as means ± S.D. (error bars), n = 3. ***, p < 0.001.
Figure 6. Characterization of the main immunostimulatory RNAs produced in SFTSV-infected cells. HEK293 cells were mock-infected or infected with SFTSV (MOI = 5) for 24 h. Total RNAs were then extracted by TRIzol and delivered to treatment with alkaline phosphatase or 5'-polyphosphatase or mock treatment. After repurification with TRIzol LS, 100 ng of the indicated RNA samples were then transfected into HEK293 cells that had been transfected with the reporter plasmids for IFN-β (A) or NF-κB (B) promoter and pRL-TK, 24 h prior to RNA transfection. Control, no RNA transfection. Sixteen hours later, cells were delivered to luciferase activity measurement. Data are presented as means ± S.D. (error bars), n = 4.

Figure 7. Roles of RIG-I and MDA5 in the host restriction to SFTSV infection. A and B, HEK293 cells were transfected with the indicated RNAi plasmids. Forty-eight hours posttransfection, cells were infected with SFTSV at MOI of 0.1. At the indicated time points (8 or 16 hpi), viral S-segment RNA levels were analyzed by qRT-PCR. C and D, RIG-I- or MDA5-KO cells or the control were respectively infected with SFTSV at an MOI of 0.1. At 16 hpi, viral S, L, and M RNA levels were analyzed by qRT-PCR (C), and titers of infectious viruses released in supernatants at the indicated time points were also determined by TCID50 (D). Data are presented as means ± S.D. (error bars), n = 3. *, p < 0.05; **, p < 0.01; ***, p < 0.001.
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**A.** TRIM25 sequence (accession, NP_005073):

MAECLCPCEISSLSCILPEKFQPTCGLCGSNLNETWAVQSPYLCPQCRAYQARPQLHKNTVLNVCVVEQFIQADLAREPAPDVWTPPARASAPSPNAQKADHCLKEAKVKTLCVMAAESQCEHLQPFDSPAQPQHPLQPYPDRLRKKCSQHQNLREFFCEHSECICLCHVEHKTCSAPSLSQASADLEATLHKLTVYMQINGASRALDDYVRNQQDVYRMTANKVEQOQQETYEMKLALLDASETSTRKKEEKKVNNKDFDYYQLLKKKCEEOQQLKEEQSLTKRDEDIFLEKA SKLQGISTKPVYPEVELHHLKIGHOSTDLKNEKLKCGIGRIQEEPTSSGIIPGEPHDPASTHKSTRPVKSKVEEKSSKPPPVPALPSKLEPTGAEPEQVLMLKQA GLEAAAKATSHSNPSLSTKAVLFEELAKSRPELLEYIYKVIILDYNTAHNKVALLSCTYTVSAEAMPOQNYRPHPQRTFYSQCVIGLHCYKGYHIIWELVQLKN NFCVGIGCYGSNRQQPSLEGLRANSWCVWFTNKJSAWHNNVKTLPSTKATRGVGLNCHDGFVIFFAVADKVLHMYKFRYDFTEALYPAPWFSAGA TLSICSPK

Representative peptide: **ALLDASETSTR**

**B.** TBK1 sequence (accession, NP_037386):

MQTSNSHLWLSDLQGATANTVRPRHIKTGDFAKYEENNTISLRLPDPVQEMRFEPWVLKKLMNKVNKLFAIIEETTRTHRKVLIHMEPCPGCSLVTYVLEEPSNAY GLPESEFLVLDVVGEMNHLRENQVHVHDRKPNHMRIGEDGQSVVYKLTDGFARLELEDEDFVSLYGETTEYLHPMYNERAVLRKDHQKCYGAINTVVALWSIG VTFHYAHTGSLFERPRFEPGRNKEVMVYKIGHTGKPGSAISGGQKAEHGPIDWSDGMVPVSCSSLRGLQVLTLTPVLANILEADEQKCGWDQFFAEUSDLLHRMVHIF SLQQMTHAHLYISHYNTATIFIEELYKVQTKISSNQQELIEYGRVLLEPGRFLAQHPKITEENPIFVVSREPLNTIGLIEYIKSLPKVHPYRDLDGADMAKATGV VCYACRASISSLQYELMRIGRWELIKLDYNETVHKKTEVLDFCRNIEKTKVYKELMKINLEAELGIEISDHTKLLRLSSQGTTIETSQODISRSPG SGLADAWAQIJEQTHIPDKRNVNKLQVLLLNCMTIEYIYYFKDKAERLLAYNEEQHQRDFQKLYYHATKAMHTFTDECYKKEAFLKSEXIRKLHLRKLQIL LSITNQCFIDEESVKEQYETYNELQETLQPKMTFASGKJHTMTPYPSSNLTVEMTTLGMKKLKEEEMGYKLAEHNLHRLFGLSMDGGALRVDCI

Representative peptide: **GLQVTLTPVLANILEADEQK**

**C.** Lysate input

| Protein | Lysate input |
|---------|--------------|
| TRIM25  | 72 kDa       |
| TBK1    | 95 kDa       |
| RIG-I   | 95 kDa       |
| NSs     | 35 kDa       |

**D.** Lysate input

| Protein | Lysate input |
|---------|--------------|
| TRIM25  | 72 kDa       |
| TBK1    | 95 kDa       |
| RIG-I   | 130 kDa      |
| NSs     | 35 kDa       |

**IP: anti-NSs**

| Protein | Lysate input |
|---------|--------------|
| TRIM25  | 72 kDa       |
| TBK1    | 95 kDa       |
| RIG-I   | 130 kDa      |
| NSs     | 35 kDa       |

β-actin 45 kDa
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NSs inhibits TRIM25-mediated Lys-63–linked ubiquitination of the RIG-I CARDs

TRIM25 plays an important role in RIG-I activation and signaling (16, 31, 53–56). NSs targeting of TRIM25 may further reflect the importance of TRIM25–RIG-I signaling for host antiviral response against SFTSV infection. Indeed, as shown in Fig. S5, TRIM25 knockdown seemed to enhance SFTSV RNA replication, and consistent with the knockdown efficiency, the TRIM25-targeting shRNA1# appeared to promote the viral replication more evidently than the shRNA3# (Fig. S5), supporting the role of TRIM25 in host restriction of the viral infection.

TRIM25 is likely involved in RIG-I activation by facilitating RIG-I Lys-63–linked ubiquitination (16, 55). Next, therefore, we investigated the possible effect of NSs expression on RIG-I Lys-63–linked ubiquitination. As shown in Fig. S6, NSs expression indeed could evidently inhibit the Lys-63–linked ubiquitination of RIG-I. Additionally, in line with the inevitable stimulation of RIG-I signaling upon SFTSV infection, the viral infection inevitably led to an enhancement of the RIG-I ubiquitination. Likewise, the ubiquitination in the context of viral infection appeared to be strongly blocked by NSs expression (Fig. S6), consistent with the specific spatial sequestration of TRIM25 by NSs.

TRIM25-mediated Lys-63–linked ubiquitination of RIG-I is located in the N-terminal CARD domains (16, 56). Thus, we further tested the effect of NSs expression on TRIM25-mediated Lys-63 ubiquitination of RIG-I CARD domains. HEK293 cells were cotransfected with the plasmids expressing the N-terminal CARD domains of RIG-I fused with an S-tag (S-RIG-IN) and TRIM25 with HA (TRIM25-HA), along with the indicate doses of NSs expression plasmid. The S-RIG-IN was then extracted from cell lysates by an S-pulldown assay for subsequent analysis of ubiquitination levels (31, 56). As indicated in Fig. 10, TRIM25 overexpression significantly enhanced ubiquitination of RIG-I CARDs that was further confirmed to be Lys-63–linked using the specific antibody. However, interestingly, NSs expression remarkably diminished RIG-I CARD ubiquitination by TRIM25 in a dose-dependent manner (Fig. 10), further confirming the ability of NSs to impede TRIM25-mediated RIG-I ubiquitination and activation.

Effects of NSs mutants with important motifs disrupted on TRIM25–RIG-I signaling

Previously, two NSs motifs, PXXP (amino acid residues 66–69) and 21V/23L (two conservative residues valine and leucine at positions 21 and 23, respectively), had been identified to be important for NSs function as an antagonist of IFN induction by us and others (28, 57). To gain more insights into NSs activities to interfere with RLR signaling, we further investigated the effects of disruption of these motifs on NSs targeting of RLR-signaling molecules and NSs inhibition of SFTSV-triggered immune responses. Consistent with previous studies (57), disruption of these motifs by substitution of the conservative amino acids by alanines seriously reduced the level of the protein expressed by transient transfection of expression plasmids (Fig. 11A) (data not shown). To properly compare the activities of the mutants and WT NSs, the transfection dosages of mutant expression plasmids had to be higher, and meanwhile an expression gradient of WT NSs was set, as done in the previous studies (57). As shown in Fig. 11A, interaction of the NSs mutant 66/69A with endogenous TBK1 was largely weakened, and replacement of 21V/23L by alanines (i.e., the mutant 21/23A) completely abolished the interaction with TBK1 (Fig. 11A).

Meanwhile, interestingly, the two mutants both retained notable interactions with endogenous TRIM25 (Fig. 11A), and moreover, as shown in the following reporter gene assays, both of the mutants exhibited reduced but statistically significant inhibitory activities against SFTSV infection–triggered IFN-β induction (Fig. 11B). Furthermore, we found that the two mutants also retained the capacity to hinder TRIM25-mediated Lys-63 ubiquitination of RIG-I CARDs (Fig. 11C), consistent with their activities to interact with TRIM25 and inhibit immune responses. These data manifest the significance of not only the targeting of TBK1 but also the targeting of TRIM25 for NSs antagonism of anti-SFTSV immune responses and provide further insights into NSs functioning.

Figure 8. NSs interacts with endogenous TRIM25, but not RIG-I. A and B, identification of potential NSs targets in RIG-I signaling pathway by LC–MS/MS. HEK293T cells were transfected with the S-tagged NSs (NSs-S) expression plasmid or the control vector. At 24 h posttransfection, cells were lysed for S-pulldown assays. NSs coprecipitates or the control samples were respectively subjected to in-gel trypsin digestion and then LC–MS/MS analyses. Endogenous TRIM25 as well as TBK1 (but not RIG-I) is identified specifically in NSs coprecipitates (but not in the control samples). Tandem spectra of representative peptides (identified with >99% confidence) of TRIM25 (A) and TBK1 (B) are shown, respectively. C, validation of the NSs interaction with endogenous TRIM25 by S-tag pulldown (S-pulldown) and WB assays. HEK293 cells were transfected with the empty control plasmid (vector) or the plasmid expressing S-tagged NSs (NSs-S). At 48 h posttransfection, cells were lysed for S-pulldown. The cell lysates and NSs coprecipitates were respectively analyzed by WB using antibodies against the indicated proteins. D, validation of endogenous TRIM25–NSs interaction in the context of SFTSV infection by co-IP. HEK293 cells were mock-infected or infected with SFTSV (MOI = 5). At 48 hpi, cell lysate supernatants pretreated with preimmune serum and protein A/G-agarose were delivered to the co-IP assay using NSs-specific antiserum. Cell lysates and NSs immunoprecipitates were analyzed by WB using antibodies against the indicated proteins. Data in C and D are representative of three independent experiments with similar results. See also Fig. S4A.
Interplay of SFTSV infection and RIG-I signaling

Figure 9. NSs captures TRIM25 into viral IBs. A, HeLa cells were transfected with the plasmids expressing FLAG-TRIM25 or FLAG-RIG-I, along with NSs expression plasmid or the empty vector. Twenty-four hours later, the localization of FLAG-tagged proteins (red) and NSs (green) was visualized by confocal microscopy, following immunofluorescence staining with the anti-FLAG or anti-NSs antibodies. B, THP1-deprived macrophages were infected with SFTSV or mock-infected. At 24 hpi, the localization of endogenous TRIM25 (red) and NSs (green) was visualized by confocal microscopy after immunofluorescence staining with antibodies against the indicated proteins. Nuclei stained with Hoechst are shown in blue. Scale bars, 11 μm.

Discussion

As a highly pathogenic bunyavirus that can be transmitted by multiple routes, including tick bites and human-to-human contacts, SFTSV has posed a severe threat to human health (33, 58–60). However, there is currently no specific anti-SFTSV drug or vaccine available. Moreover, knowledge of SFTSV-host interactions and viral pathogenesis is still quite limited, hindering the development of medical countermeasures against SFTSV infection. The present study shows that SFTSV can trigger an immune and inflammatory response in which RLR-MAVS signaling (especially RIG-I-MAVS signaling) plays significant roles by sensing PAMPs produced by viral infection (mainly 5’ppp RNAs) and inducing type I IFN, ISG, and inflammatory cytokine expression. As the PRRs recognizing SFTSV, RIG-I, and MDA5 are up-regulated by SFTSV infection at their mRNA and protein levels, moreover, the primary PRR against SFTSV, RIG-I, can significantly attenuate SFTSV replication, acting as a host restriction factor of SFTSV. Furthermore, RLR-mediated antiviral signaling, in turn, can be counteracted by the NSs of SFTSV at the RIG-I activation stage. In addition to TBK1, the E3 ubiquitin ligase TRIM25 is also targeted by SFTSV NSs. By the specific NSs-TRIM25 interaction, NSs hijacks cellular endogenous TRIM25 (but not RIG-I or Riplet) into viral IB “jail” and blocks TRIM25-mediated activation of RIG-I by Lys-63 ubiquitination, achieving inhibition of RLR antiviral signaling at the level of the PRR (i.e. RIG-I) (Fig. 12). The study not only presents an important mechanism by which host cells mount immune and inflammatory responses against SFTSV infection but also unravels a corresponding immune evasion strategy, shedding light on the viral pathogenesis and
Interplay of SFTSV infection and RIG-I signaling

SFTSV-host interactions and thus likely benefiting the future development of antiviral therapy.

Bunyavirus is a large group of RNA viruses containing hundreds of members. Although RIG-I and MDA5 are shown to be involved in host immune response to a significant number of RNA viruses, only several bunyaviruses (including Hantaan virus, La Cross-e virus, Rift Valley fever virus, and Crimean-Congo hemorrhagic fever virus) have been investigated with regard to their cellular recognition mechanism by specific PRRs, including RLRs (49–51, 61). Moreover, RIG-I, but not MDA5, has been suggested as the PPR sensing these bunyaviruses (49–51). Thus, further investigation of immune responses to medically important bunyaviruses is urgently needed, and SFTSV, as the emerging highly pathogenic representative, is obviously a most valuable research model. The present study shows that RIG-I indeed is the major PPR recognizing SFTSV, and consistently, 5’ppp RNAs (the PAMPs triggering RIG-I) produced in SFTSV-infected cells are likely the main immunostimulator in SFTSV-induced antiviral and inflammatory responses. Additionally, we found that MDA5 also appears to be able to recognize SFTSV infection and contribute to the host response to SFTSV, indicating that SFTSV infection may lead to production of some PAMPs sensed by MDA5 as well, like branched dsRNAs or higher-order RNA complexes. Further, the findings may reflect subtle difference in host usage of PRRs to sense various bunyaviruses. Detailed characterization of the viral RNA products during different bunyaviral infections will further expand the limited knowledge of molecular mechanisms underlying host recognition of bunyaviruses.

In addition to RLRs, several TLRs can also recognize some RNA virus infections (62–66), whereas compared with the broad expression of RLRs in most cells, TLR expression exhibits tissue specificity (66, 67). The present study shows that RIG-I likely plays a major role in recognition of SFTSV infection and the resultant immune responses even in A549 cells that harbor various cytosolic and membrane-anchoring PRRs, including both RLRs and TLRs, although the involvement of specific TLRs cannot be ruled out. In parallel with the present study, Yamada et al. (46) reported that the deficiency of IPS-1 (i.e. MAVS) but not MYD88 (adapter of several TLRs) abolished the production of IFN-α triggered by SFTSV infection in bone marrow–derived dendritic cells isolated from mouse models, supporting our conclusion that RLR-MAVS signaling likely plays the major role in anti-SFTSV immune response. However, the observations from MAVS- or MYD88-deficient experiments alone cannot adequately reflect the roles of specific TLRs and RLRs, as these adaptors are involved in multiple signaling pathways. Therefore, it will be necessary to further verify the in vivo effects of PRRs (particularly RIG-I) on SFTSV-induced immune and inflammatory reaction in animal infection models with specific PRR deficiency.

Although RLRs can sense SFTSV infection and trigger anti-SFTSV responses, this highly pathogenic virus can still replicate in many cells to some substantial extent, reflecting the viral potential to counteract the antiviral responses. Previous work by us and others demonstrated that SFTSV NSs inhibits RLR signaling by hijacking TBK1/IKKe into viral IBs (28, 44). Furthermore, we showed that IFN-provoked JAK-STAT signaling downstream of RLR-mediated IFN expression is also disrupted by SFTSV through NSs targeting of STATs (45, 68). Here, considering the critical role of RIG-I in host recognition and restriction of SFTSV, we further examined the potential viral mechanisms for antagonizing RIG-I signaling cascades. Consequently, TRIM25 (but not RIG-I itself and another critical E3 ligase for RIG-I activation, Riplet) is determined as an additional remarkable NSs target in RIG-I signaling pathway. Due to the crucial function in RIG-I activation, TRIM25 in turn has been suggested as a vulnerable target of several other viruses, including influenza A virus, dengue virus, severe acute respiratory syndrome coronavirus, human papillomavirus, herpesvirus, and respiratory syncytial virus very recently (31, 69–72). Previously, Santiago et al. (43) also showed that SFTSV NSs appeared to interact with overexpressed TRIM25 and RIG-I through transient transfection assays of protein expression plasmids; however, the protein interactions were not further analyzed in the contexts of endogenous expression or viral infection, and the possible biological effects on TRIM25–RIG-I signaling were not unraveled. Here, we

**Figure 10.** NSs inhibits TRIM25-mediated RIG-I Lys-63 ubiquitination. HEK293T cells were transfected with the indicated dosages of NSs expression plasmid, along with plasmids encoding S-tagged RIG-I-2CARD (i.e. S-RIG-IN) and FLAG-TRIM25 or the corresponding vector plasmids. At 48 h posttransfection, cells were lysed for the S-pulldown assay. Cell lysates and S-pulldown products were respectively subjected to WB for monitoring the protein expression or RIG-IN ubiquitination with the indicated antibodies. Data are representative of two independent experiments with similar results.
systematically clarified the specific interaction of NSs with endogenous TRIM25 but not RIG-I by multiple experimental methods and in the contexts of both transfection and SFTSV infection. Moreover, we established that NSs specifically arrests TRIM25 (but not RIG-I) into viral IBs where NSs and the E3 ubiquitin ligase exhibit a notable colocalization, consistent with the observed specific protein interaction. Importantly, it was finally shown that NSs attenuates TRIM25-mediated Lys-63 ubiquitination of RIG-I CARDs by the specific interaction of NSs with TRIM25 and the consequent sequestration of TRIM25 into NSs IBs, resulting in suppression of the antiviral signaling at an early stage of RIG-I activation. These data present a novel viral IB-associated strategy of immune evasion by targeting the TRIM25–RIG-I signalosome and enrich the understanding of the roles of SFTSV IBs as a virally built “jail” that likely hijacks some host factors and interferes with corresponding biological processes. In addition to the key role in RIG-I activation, TRIM25 is also implicated in other intracellular signaling pathways, such as regulating the antiviral actions of zinc finger antiviral protein (ZAP) and MAVS (73–76). It will be interesting to further investigate the effects of SFTSV NSs on other TRIM25-involved biological processes.

We previously demonstrated that disruption of the N-terminal PXXP motif largely impaired NSs inhibition of Sendai virus–stimulated immune responses (28). Recently, Moriyama et al. (57) further reported that replacement of 21V/23L by alanines abolished NSs antagonism to MAVS overexpression–driven IFN-β promoter activation. In the present study, we found that both of the two mutants 66/69A and 21/23A retained interactions with TRIM25 into NSs IBs, resulting in suppression of the antiviral signaling at an early stage of RIG-I activation. These data present a novel viral IB-associated strategy of immune evasion by targeting the TRIM25–RIG-I signalosome and enrich the understanding of the roles of SFTSV IBs as a virally built “jail” that likely hijacks some host factors and interferes with corresponding biological processes. In addition to the key role in RIG-I activation, TRIM25 is also implicated in other intracellular signaling pathways, such as regulating the antiviral actions of zinc finger antiviral protein (ZAP) and MAVS (73–76). It will be interesting to further investigate the effects of SFTSV NSs on other TRIM25-involved biological processes.

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Figure 11. Activities of NSs mutants to interact with TRIM25 and interfere with TRIM25–RIG-I antiviral signaling. A, interactions of NSs or NSs mutants with endogenous TBK1 or TRIM25. HEK293 cells were transfected with the control plasmid or the indicated dosages of plasmids expressing S-tagged NSs or mutants (66/69A or 21/23A). At 48 h posttransfection, cells were lysed for S-pulldown. The cell lysates and pulldown products were then analyzed by WB using antibodies against the indicated proteins. B, effects of NSs mutants on SFTSV-induced IFN-β promoter activation. Cells were transfected with the plasmids encoding NSs (1, 5, or 20 ng) or the indicated mutants (100 ng), along with the IFN-β promoter reporter plasmid and pRL-TK. Twenty-four hours posttransfection, cells were infected with SFTSV (MOI = 5) for 16 h and then delivered to luciferase activity measurement. Dotted line, the ordinate value 1 for reference. Data are presented as means ± S.D. (error bars), n ≥ 3. ***, p < 0.001. C, NSs mutants 66/69A and 21/23A retain the activity to interfere with TRIM25-mediated RIG-I Lys-63 ubiquitination. Cells were transfected with the indicated dosages of NSs or NSs mutant expression plasmid, along with the plasmids encoding S-RIG-IN and TRIM25-HA. At 48 h posttransfection, cells were lysed for S-pulldown. Cell lysates and S-pulldown products were respectively subjected to WB analysis. WB results are representative of two independent experimental replicates.
mechanistic explanation, the mutants were shown to maintain noticeable inhibitory activities against TRIM25-mediated RIG-I ubiquitination. These results establish the link of NSs targeting of TRIM25 as well as TBK1 with its antagonistic actions against cellular anti-SFTSV responses, presenting further detailed insights into NSs functioning. As reported previously, replacements of the two motifs evidently impaired IB formation \((28, 57)\), which likely resulted from the amino acid mutations themselves and/or the resultant protein instability and much lower abundances produced in cells. Therefore, the detailed mechanisms employed by the mutants and WT NSs for inhibition of TRIM25 function may have subtle differences. NSs hijacks TRIM25 into viral IBs, thus likely depriving TRIM25 of the chance to participate in RIG-I signaling transduction, whereas the mutants block TRIM25 actions, possibly due to steric hindrance \((77)\) caused by their interactions with TRIM25. Identification of additional NSs mutants like those specifically retaining the ability to interact with TBK1 but not TRIM25 and further characterization of the critical protein motifs or domains involved in NSs-TRIM25 interaction will be useful to better understand the protein interactions and the influences on molecular function.

Following the identification of SFTSV, another novel tick-borne bunyavirus, named Heartland virus (HRTV), which is genetically closely related to SFTSV and causes a severe febrile illness similar to SFTS, was reported in the United States in 2012 \((78)\). In recent years, other new SFTSV/HRTV-related bunyaviruses \((79–82)\), including Guertu virus (GTV) reported by our laboratory \((82)\), were successively isolated from animals and ticks around the world. Currently, little is known about the interactions of these bunyaviruses with their hosts. Interestingly, we recently demonstrated that similar to SFTSV NSs, the NSs proteins of HRTV and GTV can also target the host kinase TBK1/IKKe and hence inhibition of the kinase-IRF3 signaling. NSs can also specifically arrest TRIM25 (but not RIG-I) into viral IBs and deprive Lys-63 ubiquitination of RIG-I CARD domains, blocking the antiviral signaling at an early stage. TBK1, TANK-binding kinase 1; IKKe, inhibitor of NF-κB kinase.
viruses. Particularly, it will be worthwhile to further analyze the roles of RLRs in recognition of SFTSV-related viruses and determine whether NSs proteins of these viruses conservatively target TRIM25-mediated RIG-I ubiquitination and activation.

**Experimental procedures**

**Cell and virus**

HEK293T and A549 cells were cultured in Dulbecco’s modified Eagle’s medium (Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco). THP-1 cells were maintained in RPMI 1640 medium supplemented with 10% FBS. HEK293 and HeLa cells were grown in Eagle’s minimum essential medium supplemented with 10% FBS. All of the cell lines were purchased from ATCC. SFTSV was expanded in Vero cells in a biosafety level 3 laboratory as described previously (28, 45). Viruses titers were determined by the 50% tissue culture infectious dose (TCID50) method (77).

**Plasmid and lentiviral construct**

Firefly luciferase reporter plasmids for NF-κB and IFN-β promoters, Renilla luciferase control plasmid (pRL-TK), and the FLAG-RIG-I expression plasmid were kindly provided by Dr. Hong-Bing Shu (Wuhan University, China) (28, 84, 85). The human RIG-I, MDA5, and MAVS RNAi constructs were made by cloning double-stranded oligonucleotides corresponding to specific target sequences into the shRNA-expressing vector pSuper.retro (OligoEngine) or lentiviral pLKO.1 (Sigma-Aldrich), HA tag (Sigma-Aldrich). The PrimeScript RT reagent kit with gDNA Eraser and SYBR Green Realtime PCR Master Mix were purchased from TAKARA. Hoechst 33258 (Beyotime), phosphorol 12-myristate 13-acetate (Sigma–Aldrich), S-protein agarose (MBL), and ELISA kits for human IFN-β, IL-8, TNFα, RANTES, and IP-10 (Beijing 4A Biotech) were purchased from the indicated manufacturers.

**Antibody and reagent**

Primary antibodies were purchased from the indicated manufacturers as follows: mouse mAbs against FLAG (Sigma–Aldrich), HA tag (Sigma–Aldrich), β-actin (Beyotime), and MAVS (Proteintech); rabbit mAbs against RIG-I (Abcam), ubiquitin (linkage-specific K63) (Abcam), and TRIM25 (Abcam); and polyclonal antibodies to S tag (Abcam), TBK1 (Santa Cruz Biotechnology, Inc.), MDA5 (AbClonal), Riplet (Proteintech), MxA (Proteintech), ISG15 (Proteintech), and ISG56 (Proteintech). Rabbit- and mouse-derived anti-NSs antisera were raised against NSs protein purified from *Escherichia coli*. Fluorescence-labeled secondary antibodies were used were Alexa Fluor 488 goat anti-mouse IgG (Abcam), Alexa Fluor 647 goat anti-mouse IgG (Abcam), Alexa Fluor 488 goat anti-rabbit IgG (Abcam), and Alexa Fluor 647 goat anti-rabbit IgG (Abcam). Horseradish peroxidase–labeled goat anti-rabbit and anti-mouse IgG antibodies were purchased from Sigma–Aldrich. The PrimeScript RT reagent kit with gDNA Eraser and SYBR Green Realtime PCR Master Mix were purchased from TAKARA. Hoehcht 33258 (Beyotime), phosphorol 12-myristate 13-acetate (Sigma–Aldrich), S-protein agarose (MBL), and ELISA kits for human IFN-β, IL-8, TNFα, RANTES, and IP-10 (Beijing 4A Biotech) were purchased from the indicated manufacturers.

**Generation of the RIG-I- and MDA5-KO cell lines using the CRISPR/Cas9 system**

Gene-specific guide sequences were designed with online CRISPR Design Tools (RRID:SCR_018710) and cloned into sgRNA scaffold of pSpCas9 (BB)-2A-Puro (PX459) V2.0 vector (Addgene plasmid 62988) (87). The indicated guide sequences are shown in Table 2. The constructed plasmids encoding both Cas9 and sgRNA were then transfected into HEK293 cells with lipofectamine 3000 (Invitrogen). At 24 h posttransfection, cells were selected by puromycin (4 μg/ml) for 4–7 days and then delivered to isolation of clonal cell lines by clonal-density dilution. The selected clone cells were identified by gene sequencing and then confirmed by WB analyses.

**qRT-PCR**

The mRNA levels of the indicated genes were assessed by qRT-PCR analyses as described previously (28, 77, 88). Briefly, total RNA was extracted from cells using TRIzol reagent (Life Technologies) and then subjected to gDNA erasure and RT. Reverse-transcribed cDNA was used as template in quantitative PCRs containing SYBR Green Realtime PCR Master Mix and 0.4 μM forward and reverse primers (Table 3). The real-time quantitative PCRs were run on ABI StepOnePlus™ (Applied Biosystems) using the standard cycling conditions. Relative mRNA levels were calculated by the comparative CT method (2−ΔΔCT method) with the mRNA level of GAPDH as an internal control and were shown as relative -fold change by normalizing to the control samples.

**Reporter gene assay**

HEK293 cells seeded into 96-well plates were transfected in quadruplicate with a total amount of 150 ng of mixed

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**Table 1**

| Target          | RNAi plasmid | Target sequence (sense) |
|-----------------|--------------|-------------------------|
| Human RIG-I     | shRIG-I-1#   | CAGAGAATCTTGAGGATATA    |
|                 | shRIG-I-2#   | AATTCACTCGAGAATGTCA     |
|                 | shRIG-I-3#   | GGAAGGCTGGATGTACTTT    |
| Human MDA5      | shMDA5-1#    | CAGAAGGGAAAGCGGTTAT     |
|                 | shMDA5-2#    | AAGGTGTTCCGACTATCA      |
| Human MAVS      | shMAVS-1#    | GCAAGAAGGTTTCACACCATT   |
|                 | shMAVS-2#    | GACAGACCTAAAGGTTAT      |
| Human TRIM25    | shTRIM25-1#  | GAGTTGATACGACGATGATA    |
|                 | shTRIM25-2#  | GAAGCTGACCAAAGGCTTATA   |
|                 | shTRIM25-3#  | ATCTTCTGGCTGTGACAATATA  |
| EGFP            | Control shRNA| GCCACACGTTCTATATCAT      |
expression plasmids per well (18.5 ng of reporter plasmid for NF-κB or IFN-β promoter, 3.7 ng of pRL-TK plasmid, and 129.6 ng of shRNA expression plasmid) using Lipofectamine 3000. At 36 h posttransfection, cells were infected with SFTSV (MOI = 5) or mock-infected, followed by luciferase activity measurement at the indicated time points postinfection using a Dual-Luciferase reporter (DLR) assay kit (Promega). Relative luciferase activities are shown by normalizing the Firefly luciferase activity to Renilla luciferase activity, or -fold activation over the untreated control was further calculated. For the analysis of immunostimulatory RNA produced by SFTSV infection, total RNA was extracted from mock-infected or SFTSV-infected (MOI = 5) cells using TRIzol reagent at 24 hpi and then digested with alkaline phosphatase (FastAP, Thermo Fisher Scientific) for 10 min or 5 h-polyphosphatase (Lucigen), followed by digestion with 5% nonfat milk in TBS, the membrane was probed with 0.5% Triton X-100. After blocking with 5% BSA (Bio-sharp), cells were incubated with the indicated primary antibodies overnight at 4 °C and fluorescence-labeled secondary antibodies for 1 h at room temperature. Nuclei were stained with Hoechst 33258. For the analysis of endogenous TRIM25 localization in the context of SFTSV infection, THP-1–derived macrophages prepared by 48-h treatment of THP-1 cells with phorbol 12-myristate 13-acetate (5 ng/ml) were used (90), as they exhibited high expression level and good immunostaining of endogenous TRIM25 as well as tightly adherent growth. Images were taken and analyzed on a Nikon Ti confocal microscope with the Volocity software (PerkinElmer Life Sciences).

**Pulldown, co-immunoprecipitation, and MS**

S-tag pulldown assay (S-pulldown) was used to analyze the interactions of S-tagged NSs with cellular endogenous proteins or gather S-tagged RIG-IN for ubiquitination detection as described previously (16, 28, 56). Briefly, at the indicated time points posttransfection, cells were lysed with a lysis buffer (25 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, and 1% Triton X-100) containing protease inhibitor mixture (Roche Applied Science). After centrifugation, supernatants of the cell lysates were incubated with the S-protein agarose slurry (Merck Novagen) for 4 °C for 4 h. After extensive washing, the precipitated proteins were eluted from the beads by boiling in 1× SDS-loading buffer and then subjected to SDS-PAGE and Western blotting or MS. For mass spectrometric analysis, NSs-coprecipitated products or control samples were respectively subjected to in-gel digestion with trypsin (68, 91). The trypptic peptides were then analyzed by LC–MS/MS using a nano-LC–equipped TripleTOF 5600 system (AB SCIEX). Raw tandem spectra were searched against the Unified Protein Database (UniProt) with ProteinPilot software 5.0 (AB SCIEX). The obtained data were based on a false discovery rate of ≤1% confidence for protein identification. Tandem spectra of representative peptides (identified with >99% confidence) were selectively shown. In co-IP experiments, mock- or SFTSV-infected HEK 293 cells (~5 × 10⁵) were lysed with the lysis buffer as described above. The supernatants were first pretreated with preimmune serum and protein A/G PLUS-agarose (Santa Cruz Biotechnology) at 4 °C for 2 h. After centrifugation, the precleared samples were then incubated with anti-NSs antisera and protein A/G PLUS-agarose at 4 °C overnight. After extensive washing, immunoprecipitates were delivered to SDS-PAGE and Western blotting analysis.

**Western blotting analysis**

The protein levels of the indicated genes were assessed by WB analyses as described previously (92). Briefly, protein samples were first separated by SDS-PAGE and then transferred to polyvinylidene difluoride membrane (Millipore). After blocking with 5% nonfat milk in TBS, the membrane was probed with

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**Table 2**

List of sgRNA target sequences for gene editing

| Target     | sgRNA              | Target sequence                                      |
|------------|--------------------|------------------------------------------------------|
| Human RIG-I| RIG-I-g1           | AACACAAGGGGCGCCCAGATTG (sense)                      |
| Human MDA5 | MDA5-g1            | ATACGGCAATACCTTCCTCTCTGG (anti-sense)                |
|            | MDA5-g2            | TCTAGGCAATACCTTCCTCTCTGG (anti-sense)                |

**Table 3**

List of primers for real-time quantitative PCR

| Primer name | Primer sequence (5′→3′) |
|-------------|-------------------------|
| RIG-1-F     | GGCTTCAGAGATGGCAGAA     |
| RIG-1-R     | AGTGCTTGCGTCGAGTGT      |
| GAPDH-F     | GGACAGGGGGAGGTTTCTTG    |
| GAPDH-R     | GGAAGCTTGAGTTCAAGGAG    |
| TRIM25-F    | TCTTCTCTGGAGAATCTTG     |
| TRIM25-R    | TGTCTCTCTGTAGCTCTCA     |
| MxA-F       | GATCCAGGCAGTGGGAGGAC    |
| MxA-R       | GGAGCTGACAGAAGCCGAG     |
| OAAS-F      | CAGGTTAGATGCGGAGAGCG    |
| OAAS-R      | TCTTGGTGGAGAAATCAAGG    |
| SFTSV L segment-F | TGGTGGAGGGAGGAGGAGG   |
| SFTSV L segment-R | GTCGAGACGCGAGCAGAGG   |
| SFTSV M segment-F | TGTCTACGAGGACTGCTCA   |
| SFTSV M segment-R | AATTCGCTCTGCTGCAGTCT  |
| SFTSV S segment-F | TGGTGGAGGGAGGAGGAGG   |
| SFTSV S segment-R | GTCGAGACGCGAGCAGAGG   |
| IP-10-F     | AGAACACCTCGCTGATACGAG   |
| IP-10-R     | GAATTCGAGCTGCTGATACGAG  |
| SFTSV-L segment-F | TGTCTACGAGGACTGCTCA   |
| SFTSV-L segment-R | AATTCGCTCTGCTGCAGTCT  |
| TRIM25-F    | CGATCGATGCAAGCAGAGA    |
| TRIM25-R    | GTCGAGACGCGAGCAGAGG   |
| GAPDH-F     | AAGCAGCAGCAGGAGGAGGAC   |
| GAPDH-R     | GTCGAGACGCGAGCAGAGG   |

*F, forward primer; R, reverse primer.*
Interplay of SFTSV infection and RIG-I signaling

primary antibodies and then corresponding horseradish peroxidase–conjugated secondary antibodies. Extensive washing with TBS-T (TBS supplemented with 0.1% Tween 20, pH 7.4) was performed between each step. Protein signals were detected by an enhanced chemiluminescence (ECL) kit (Thermo Fisher Scientific).

Statistical analysis

Statistical analyses were performed by GraphPad Prism 5 using the analysis of variance method followed by Dunnett’s test or nonpaired Student t test. All results are expressed as mean ± S.D. p < 0.05 was considered statistically significant.

Data availability

All data are contained within the article.

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Abbreviations—The abbreviations used are: PRR, pattern recognition receptor; RIG-I, retinoic acid–inducible gene 1; RLR, RIG-I–like receptor; TLR, Toll-like receptor; PAMP, pathogen-associated molecular pattern; CARD, N-terminal caspase recruitment domain; RD, regulatory/repressor domain; MAVS, mitochondrial antiviral signaling protein; IFN, interferon; ISG, IFN-stimulated gene; SFTS, severe fever with thrombocytopenia syndrome; SFTSV, SFTS virus; L, large; M, medium; S, small; NSs, SFTSV nonstructural protein; IB, inclusion body; qRT-PCR, quantitative RT-PCR; hpi, hours postinfection; WB, Western blotting; IL, interleukin; RANTES, regulated on activation normal T cell–expressed and secreted; TNF, tumor necrosis factor; MOI, multiplicity of infection; KO, knockout; 5′-PPP, 5′-triphosphorylated; JAK, Janus kinase; STAT, signal transducer and activator of transcription; HRTV, Heartland virus; GTV, Guertu virus; FBS, fetal bovine serum; TCID50, 50% tissue culture infectious dose; sgRNA, single-guide RNA; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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