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<td>Author(s)</td>
<td>Chaudhary, V; Zhang, S; Yuen, KS; Li, C; Lui, PY; Fung, SY; Wang, PH; Chan, CP; Li, D; Kok, KH; Liang, M; Jin, D</td>
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Suppression of type I and type III interferon signalling by NSs protein of severe fever-with-thrombocytopenia syndrome virus through inhibition of STAT1 phosphorylation and activation

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Running title: Suppression of interferon signalling by SFTSV NSs protein

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Summary

Severe fever-with-thrombocytopenia syndrome virus (SFTSV) is an emerging tick-borne pathogen causing significant morbidity and mortality in Asia. NSs protein of SFTSV is known to perturb type I interferon (IFN) induction and signalling, but the mechanism remains to be fully understood. Here, we showed the suppression of both type I and type III IFN signalling by SFTSV NSs protein mediated through inhibition of STAT1 phosphorylation and activation. Infection with live SFTSV or expression of NSs potently suppressed IFN-stimulated genes but not NF-κB activation. NSs was capable of counteracting the activity of IFN-α1, IFN-β, IFN-λ1 and IFN-λ1. Mechanistically, NSs associated with STAT1 and STAT2, mitigated IFN-β-induced phosphorylation of STAT1 at serine 727, and reduced the expression and activity of STAT1 protein in IFN-β-treated cells, resulting in the inhibition of STAT1 and STAT2 recruitment to IFN-stimulated promoters. Taken together, SFTSV NSs protein is an IFN antagonist that suppresses phosphorylation and activation of STAT1.

Keywords: severe fever-with-thrombocytopenia syndrome virus (SFTSV); phlebovirus; NSs protein; STAT1; STAT2; interferon signalling
Severe fever-with-thrombocytopenia syndrome virus (SFTSV) is a novel phlebovirus first isolated in 2009 in China, where it causes a tick-borne zoonosis in humans and domestic animals (Yu et al., 2011; Niu et al., 2013). Human patients are presented with acute fever, thrombocytopenia, leukocytopenia as well as gastrointestinal and joint symptoms. In a small subset of severe cases the disease progresses rapidly to multiorgan failure, hemorrhage and death, with the case fatality rate ranging from 2% to 15% (Liu et al., 2014; Li, 2015). Whereas the majority of patients are farmers who live in wooded upland areas and work in the fields, a few clusters of cases and human-to-human transmission through direct contact with blood or respiratory secretions have also been reported (Bao et al., 2011; Gai et al., 2012). Human infection of SFTSV has been identified retrospectively in Korea and Japan (Kim et al., 2013; Takahashi et al., 2014). SFTSV and related viruses have also been isolated in ticks collected in Korea and Australia (Yun et al., 2014; Wang et al., 2014). Furthermore, Heartland virus, another tick-borne phlebovirus sharing 60-73% amino acid sequence homology with SFTSV, has been shown to be etiologically associated with an SFTS-like severe disease in US (McMullan et al., 2012; Muehlenbachs et al., 2014). Thus, SFTSV and closely related human pathogens are distributed widely.

SFTSV belongs to the Phlebovirus genus of the Bunyaviridae family. Similar to other bunyaviruses, SFTSV contains a tripartite single-stranded RNA genome of negative sense (Walter & Barr, 2011). Whereas the L segment encodes viral polymerase, the M segment codes for envelope glycoproteins Gc and Gn. Nucleocapsid and nonstructural protein NSs are expressed in opposite directions from the ambisense S segment. Characterization of viral virulence factors might shed light on the mechanism of severe diseases caused by SFTSV. As a major virulence factor, NSs proteins from many
bunyaviruses are capable of antagonizing interferon (IFN) response. Among them NSs protein from Rift Valley fever virus (RVFV) is one of the most extensively studied (Ikegami & Makino, 2011). However, the mechanism of NSs-mediated immune evasion varies from one to another bunyavirus (Walter & Barr, 2011).

Innate immune response is triggered by the sensing of pathogen-associated molecular patterns by host pattern recognition receptors (Habjan & Pichlmair, 2015). Subsequent activation of the signalling cascades leads to the induction of type I and type III IFNs by transcription factors IRF3 and IRF7. The binding of type I and type III IFNs to their receptors results in auto-phosphorylation and activation of the receptor-associated kinases TYK2 and JAK1, which regulate the activation of STAT1 and STAT2. Together with IRF9, homodimers or heterodimers of phosphorylated STAT1 and STAT2 form the ISGF3 complex, which translocates into the nucleus, binds to specific IFN-stimulated response elements (ISREs) present in the promoters of IFN-stimulated genes (ISGs), and activates their transcription (Schneider et al., 2014). Viruses have evolved various IFN antagonists to counteract IFN induction and signalling at all steps (Randall & Goodbourn, 2008; Hoffmann et al., 2015).

During the course of SFTSV infection in humans, IFNs are almost undetectable in the blood (Qu et al., 2012), indicating the suppression of IFN production. Consistent with this, SFTSV NSs protein has been shown to suppress type I IFN production through the interaction with RIG-I, TRIM25 as well as IRF3 kinases TBK1 and IKKe, leading to their sequestration in virus-induced cytoplasmic subdomains separated from mitochondria (Ning et al., 2014; Qu et al., 2012; Santiago et al., 2014; Wu et al., 2014).
In addition, SFTSV NSs has recently been found to perturb type I IFN signalling by interacting with STAT2 and thus retaining STAT1 and STAT2 in the cytoplasm (Ning et al., 2015). However, mechanistic details of NSs-induced suppression of IFN production and signalling remain controversial and thus merit further investigations.

Although SFTSV NSs has been shown to inhibit both NF-κB and IRF3 transcription factors (Qu et al., 2012), we noted the upregulated expression of many NF-κB-regulated cytokines such as interleukin 6 (IL6), IL8 and tumour necrosis factor α (TNF-α) in humans and primates infected with SFTSV (Sun et al., 2012; Deng et al., 2012; Jin et al., 2015). To resolve this discrepancy, we compared the impact of NSs expression on Sendai virus-induced activation of IFN-β promoter and canonical κB elements. Dual luciferase assays were performed (Chan et al., 2010; Kok et al., 2011), with reporter constructs driven respectively by IFN-β promoter (IFNβ-Luc) and by five tandem copies of canonical κB element (κB-Luc). It is known that the activation of IFN-β promoter by Sendai virus is mediated primarily through IRF3 (Lin et al., 1998). Notably, VP35 from Ebola virus and NSs from RVFV, which are well characterized suppressors of type I IFN induction (Billecocq et al., 2004; Cardenas et al., 2006; Ikegami et al., 2009; Kalveram et al., 2013), were capable of impeding Sendai virus-induced activation of IFN-β promoter in our assay (Fig. 1a, bars 4 and 5 versus 2). As expected, VP35 and IkBα super-repressor also ablated NF-κB activation (Fig. 1b, bars 4 and 5 versus 2). In contrast, SFTSV NSs suppressed the activation of IFN-β promoter by Sendai virus (Fig. 1a, bar 3 versus 2), but had no influence on its activation of NF-κB (Fig. 1b, bar 3 versus 2). A similar pattern was also observed when we used MAVS to induce the activation of IFN-β promoter and NF-κB (Fig. 1b, d). In addition, SFTSV
NSs had no inhibitory effect on Sendai virus- or TNF-α-induced activation of NF-κB in HeLa or HepG2 cells (Fig. 1e, f). Thus, SFTSV NSs preferentially suppressed IRF3 but not NF-κB activity in our setting. These results were at odds with a previous report (Qu et al., 2012) but might be more compatible with the overproduction of NF-κB-induced cytokines in SFTSV-infected cells (Jin et al., 2015). Further studies are required to determine whether NF-κB is activated by SFTSV. On the other hand, the inability of SFTSV NSs to suppress NF-κB activation also implicated that it unlikely suppressed general transcription or translation. Thus, its suppression of type I IFN production is specific.

We next examined the effect of SFTSV NSs protein on type I and type III IFN signalling using a luciferase reporter driven by ISREs (ISRE-Luc). ISRE-dependent reporter expression was potently induced in HEK293 cells treated with IFN-β, IFN-λ1 or IFN-λ2. This activity was largely ablated when SFTSV NSs was expressed (Fig. 1g, h and Fig. S1a; bar 3 versus 2). The suppressive activity of SFTSV NSs was more pronounced than that of RVFV NSs (Fig. 1g, h and Fig. S1a; bar 3 versus 4). In light of this, we went on to verify the suppression of IFN signalling in SFTSV-infected cells. We chose THP-1 cells, which were further induced to differentiate into macrophages, for the infection experiment, because macrophages are highly responsive to IFN treatment and they are also thought to play an important role in SFTSV pathogenesis (Jin et al., 2012). mRNA expression of five selected ISGs, namely MX1, OAS1, ISG15, ISG56 and STAT1, was assessed by RT-qPCR as described (Tang et al., 2013; Yuen et al., 2015). Primer sequences are presented in the supplementary material. All five ISGs were strongly induced by IFN-α1 and SFTSV individually (Fig. 1i-l and Fig. S1b, bars
2 and 3 versus 1). However, the steady-state mRNA levels of the ISGs did not increase further but decreased in IFN-α1-treated and SFTSV-infected cells (Fig. 1i-l and Fig. S1b, bar 4 versus 2). Similar results were also obtained from IFN-α1-treated SFTSV-infected HEK293T cells (Fig. 1m, n and Fig. S1c), indicating that the effect was not cell type-specific. Although SFTSV replication was inhibited by IFN-α1, reasonably high copy numbers of viral RNA were still detected in IFN-α1-treated THP-1 cells (Fig. S2). The inability of the remaining SFTSV to augment or at least maintain the ISG-inducing activity of IFN-α1 suggested that SFTSV might antagonize IFN-α1. In contrast to previous findings (Ning et al., 2014), in our setting SFTSV was capable of inducing ISG expression more substantially in THP-1 and HEK293T cells. It remains to be determined whether the use of different SFTSV strains in the two studies might account for the different results.

In addition to representative ISGs, we also examined the expression of two proinflammatory cytokines IL8 and CCL5 in infected cells. Both IL8 and CCL5 are well-characterized NF-κB target genes (Kunsch & Rosen, 1993; Wickremasinghe et al., 2004). Their mRNA expression levels remained unchanged in SFTSV-infected THP-1 cells stimulated with lipopolysaccharide (LPS), a strong activator of NF-κB (Fig. 1o, p). Furthermore, expression of SFTSV NSs did not affect phorbol ester-induced nuclear translocation of p65 subunit of NF-κB in HeLa cells (Fig. 2a, panel 2; NSs-expressing versus NSs-non-expressing cells). Thus, our results from luciferase assays (Fig. 1b, d, e, f), RT-qPCR (Fig. 1o, p) and confocal staining (Fig. 2a) consistently indicated no suppression of NF-κB activation by SFTSV NSs.
The suppression of type I and III IFN signalling by SFTSV NSs protein prompted us to investigate further whether it might affect the stability and function of STAT1 and STAT2. Although SFTSV NSs has recently been shown to interact with STAT2 but not STAT1 (Ning et al., 2015), we would like to re-examine this issue in our experimental setting. We noted that STAT1, STAT2 and an active form of STAT1 phosphorylated at S727 appeared in the nucleus of IFN-β-treated HeLa cells. However, only weak and cytoplasmic staining of STAT1 and STAT2 was observed in NSs-expressing cells (Fig. 2b, c, panel 2, NSs-expressing versus NSs-non-expressing cells). In addition, nuclear staining of STAT1 phosphorylated at S727 was not seen in the presence of NSs (Fig. 2d). This suggested that NSs might exert an inhibitory effect on STAT1 and STAT2 activation. To shed further light on this, co-immunoprecipitation was performed as described (Ng et al., 2011; Tang et al., 2014). STAT1 and STAT2 were detected in the NSs-containing immunoprecipitate (Fig. 3a, lane 2 versus 1). Although NSs might indirectly interact with STAT2 through STAT1, our results were also consistent with the model that NSs could interact with both STAT1 and STAT2.

Since other viral IFN-antagonizing proteins such as simian virus 5 V protein are known to induce ubiquitination and degradation of STAT1 (Precious et al., 2005), we asked whether SFTSV NSs might also affect the steady-state levels of STAT1. Western blot analysis of whole cell extracts was carried out as described (Chin et al., 2005; Chun et al., 2013) and the results indicated that NSs had no influence on STAT1 protein stability ambiently in HEK293 cells (Fig. 3b, lane 2 versus 1). However, when STAT1 was activated by IFN-β, the steady-state amounts of STAT1 in NSs-expressing cells detected over a time course of 24 hours were diminished consistently (Fig. 3b, lane 4 versus 3, lane 6 versus 5, and lane 8 versus 7). The inhibitory effect of NSs was not
seen when cells were treated with actinomycin D, an inhibitor of RNA polymerase II (Fig. 3c, lane 5 versus 4). These results suggested that NSs-induced inhibition likely occurs at the level of STAT1 transcription. As previously shown by others (Wong et al., 2002) and in Fig. 11, n, STAT1 is an ISG. The abrogation of the inhibitory effect of NSs on STAT1 expression in IFN-β-treated cells by actinomycin D suggested that NSs might suppress type I IFN-induced activation of STAT1 transcription.

Exactly how SFTSV NSs modulates phosphorylation and activation of STAT1 remains elusive. IFNs induce STAT1 phosphorylation at two major sites Y701 and S727. Both modifications are required for full activation of STAT1 (Wen et al., 1995; Takaoka et al., 1999). To determine the impact of NSs expression on STAT1 phosphorylation, Western blotting was performed with phospho-specific antibodies. Whereas NSs had no influence on Y701 phosphorylation of STAT1, it exerted a suppressive effect on S727 phosphorylation (Fig. 3d, lane 3 versus 2). To shed light on where this inhibition by NSs might occur, we collected and analyzed the cytosolic and nuclear fractions. NSs and STAT1 were detected in both fractions. In addition, STAT1 with phospho-S727 was also found in reduced levels in both the cytosol and the nucleus (Fig. 3d, lane 6 versus 5, and lane 9 versus 8). Although a primarily cytoplasmic staining of NSs was observed in HeLa cells (Fig. 2), we cannot rule out that a subset of NSs might enter the nucleus. Biochemical fractionation is a more sensitive method. On the other hand, the nuclear localization of NSs might not be observed by confocal microscopy when cytoplasmic NSs is more prominent. Considered together with the absence of STAT1 with phospho-S727 in NSs-expressing HeLa cells (Fig. 2d), our results were generally compatible with the notion that NSs might suppress STAT1 phosphorylation at S727 in both the cytoplasm and the nucleus. These data do not support the model in which
NSs functions solely to sequester STAT1 and STAT2 in the cytoplasm (Ning et al., 2015).

Whether SFTSV NSs affects STAT1 and STAT2 recruitment to the ISREs in ISG promoters has not yet been characterized. To address this, we performed chromatin immunoprecipitation (ChIP) assay as described (Tang et al., 2014). Primers for qPCR analysis of ISREs in IFI6 and ADAR1 promoters are presented in the supplementary material. IFI6 and ADAR1 are two representative ISGs (Samuel, 2011; Schneider et al., 2014). They were chosen in the ChIP-qPCR assay only for technical reasons. We observed that IFN-β-induced recruitment of STAT1 and STAT2 to the ISREs in both IFI6 and ADAR1 promoters was impeded in NSs-expressing HEK293 cells (Fig. S3a, b, bar 5 versus 3, and bar 6 versus 4). Consistent with this, mRNA levels of IFI6 and ADAR1 were dampened in the presence of NSs (Fig. S3c, d, bar 3 versus 2). Hence, SFTSV NSs inhibits IFI6 and ADAR1 expression by preventing the recruitment of STAT1 and STAT2 proteins to their promoters.

Several salient points concerning SFTSV NSs-dependent perturbation of IFN production and signalling emerged in our study. First, we provided evidence for differential modulation of IRF3 and NF-κB by NSs (Fig. 1a-f, i-p and Fig. 2). Second, we characterized the suppression of type III IFN signalling by NSs (Fig. 1h). Third, we demonstrated the interaction of NSs with STAT1 (Fig. 3a), the inhibition of IFN-β-induced STAT1 expression and phosphorylation at S727 but not Y701 by NSs (Fig. 2d and Fig. 3b-d). Finally, we documented the reduced recruitment of STAT1 and STAT2 to the IRSEs in ISG promoters in NSs-expressing cells (Fig. S3). Because NSs-deficient
viruses might be developed as attenuated SFTSV vaccines and IFNs could be tested as antivirals against SFTSV infection, our work also has implications in the design and development of SFTSV vaccines and antivirals.
Acknowledgements

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Wong, L. H., Sim, H., Chatterjee-Kishore, M., Hatzinisiriou, I., Devenish, R. J., Stark, G. & Ralph, S. J. (2002). Isolation and characterization of a human STAT1


**Figure Legends**

**Fig 1.** SFTSV NSs suppresses IFN induction and signalling. (a-h) HEK293 cells expressing the indicated proteins and reporter were infected with 100 hemagglutinating units/ml of Sendai virus (SENV; a, b, e), transfected with 200 ng/ml of MAVS expression vector (c, d) or treated with TNF-α (10 ng/ml, f), IFN-β (1000 U/ml purchased from PBL; g) or IFN-λ1 (100 ng/ml from Peprotech; h) for 24 hours. NSs from Hb29 strain of SFTSV was cloned into pVR1012 expression vector and 200 ng/ml of this plasmid was used to transfecit cells. Dual luciferase assay was performed. Results represent means ± SD (n = 3). S-NSs: SFTSV NSs. VP35: Ebola virus VP35. R-NSs: RVFV NSs. IκB-sr: IκBα super-repressor with S→A mutations at positions 32 and 36 (EMD Millipore). The difference between groups 2 and 3 in b was statistically not significant (n.s.) by Student’s t test (p = 0.22). (i-p) SFTSV suppression of IFN-α1 signalling. THP-1 cells were treated with 100 nM of phorbol 12-myristate 12-acetate for 72 hours to induce the differentiation into macrophages. HEK293T cells and induced THP-1 cells were then either mock-infected or infected with Hb29 strain of SFTSV at 1000 TCID₅₀/ml. At 24 hours post infection, cells were either mock-treated or treated with 10 ng/ml of IFN-α1 or 100 ng/ml of LPS for an additional 16 hours. mRNA levels of the indicated ISGs were measured by RT-qPCR. Results represent means ± SD (n = 3). Differences between selected groups were highlighted with asterisks and statistically assessed by Student’s t test. The p values are 0.018 (i), 0.0009 (j), 0.040 (k), 0.008 (l), 0.025 (m), 0.003 (n), 0.49 (o) and 0.39 (p).

**Fig 2.** Influence of SFTSV NSs on NF-κB and STAT1 activation. (a) HeLa cells were transfected with an expression vector for HA-tagged NSs protein for 48 hours. Cells
were stimulated with 40 nM of phorbol 12-myristate 12-acetate for 30 minutes and then stained for HA and p65. The NSs- (green) and p65-specific (red) fluorescent signals were merged in panel 3. (b-d) HA-tagged NSs was expressed in HeLa cells for 48 hours. Cells were treated with IFN-β (1000 U/ml) for 30 minutes and then stained for HA (green) and STAT1, STAT2 or phospho-S727 STAT1 (red). Nuclear morphology (blue) was visualized with 4',6-diamidine-2'-phenylindole dihydrochloride (DAPI). Different fluorescent signals were merged in panel 4. Arrows point to NSs-expressing cells, whereas NSs-non-expressing cells in the same field are highlighted by arrowheads. Bar, 20 μm.

Fig. 3. Interaction of SFTSV NSs with STAT1 and STAT2. (a) Immunoprecipitation (IP). HEK293 cells were either mock-transfected or transfected with an expression vector for HA-tagged NSs. Cell lysates were collected and subjected to precipitation with mouse anti-HA antibody. Precipitates and cell lysates (input) were analyzed by Western blotting with antibodies against the indicated proteins. All blots were exposed for 3 minutes. Rabbit polyclonal anti-STAT1 and anti-STAT2 antibodies were purchased from Santa-Cruz. Similar results were also obtained for V5-tagged NSs. (b, c) Steady-state levels of STAT1 protein in NSs-expressing HEK293 cells treated with IFN-β (1000 U/ml). Relative levels of STAT1 protein normalized to β-actin (STAT1/β-actin) are determined by densitometry and indicated below the panel. Some cells were treated with of 5 μg/ml of actinomycin D (ActD) for 6 hours before harvest. (d) NSs inhibits STAT1 phosphorylation at S727. HEK293 cells were treated with IFN-β (1000 U/ml) for 24 hours. Whole cell extracts (WCE) as well as cytosolic and nuclear fractions were prepared and probed for the indicated proteins. Rabbit polyclonal
phospho-specific antibodies recognizing phospho-Y701 (Y701p) and phospho-S727 (S727p) of STAT1 were purchased from Cell Signalling. Cell fractionation was performed as described (Schreiber et al., 1989). Relative levels of STAT1 S727p protein normalized to total STAT1 and either glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or lamin C (rel. S727p) are indicated below the panels.
Figure 1
Figure 3
Table S1. Primers used in this study

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Fig S1. SFTSV NSs suppresses IFN-λ2 signalling and ISG56 induction. (a) Suppression of IFN-λ2 signalling by SFTSV-NSs. HEK293 cells transfected with ISRE-Luc reporter were treated with IFN-λ2 (100 ng/ml from PBL) for 24 hours. Dual luciferase assay was performed. Results represent means ± SD (n = 3). S-NSs: SFTSV NSs. R-NSs: RVFV NSs. (b, c) Phorbol 12-myristate 12-acetate-induced THP-1 cells and HEK293T cells were mock-infected or infected with SFTSV. Cells were then mock-treated or treated with 10 ng/ml of IFN-α1 at 24 hours post infection. Levels of ISG56 mRNA were analysed by RT-qPCR. Results represent means ± SD (n = 3). Differences between the selected groups were highlighted with asterisks and statistically assessed by Student’s t test. The p values are 0.005 (b) and 0.007 (c).

Fig. S2. RT-qPCR analysis of SFTSV RNA segments. Copy numbers of L, M and S segments in infected and IFN-α1-treated THP-1 cells were calculated as described (Jin et al., 2012).
Fig. S3. SFTSV NSs inhibits ISG expression by impeding STAT1 and STAT2 recruitment. (a, b) STAT1 and STAT2 recruitment to the ISREs in IFI6 and ADAR1 promoters in IFN-β-treated HEK293 cells was blunted when NSs was overexpressed. ChIP was performed with anti-STAT1 and anti-STAT2. The STAT-bound ISREs were analyzed by qPCR. Expression of SFTSV N protein had no influence on STAT recruitment (data not shown). AU: arbitrary unit. (c, d) Verification of mRNA expression. Levels of IFI6 and ADAR1 mRNA in NSs-expressing and IFN-β-treated HEK293 cells were analyzed by RT-qPCR. The difference between bars 2 and 3 in d (p = 0.0027; highlighted with **) was statistically significant by Student’s t test.