Heartland virus antagonizes type I and III interferon antiviral signaling by inhibiting phosphorylation and nuclear translocation of STAT2 and STAT1

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Abstract.

Heartland virus (HRTV) is a pathogenic phlebovirus recently identified in the United States and related to severe fever with thrombocytopenia syndrome virus (SFTSV) emerging in Asia. We previously reported that SFTSV disrupts host antiviral responses directed by interferons (IFNs) and their downstream regulators, signal transducer and activator of transcription proteins (STATs). However, whether HRTV infection antagonizes the IFN–STAT signaling axis remains unclear. Here, we show that similar to SFTSV, HRTV also inhibits IFN-\(\alpha\)– and IFN-\(\lambda\)–mediated antiviral responses. As expected, the nonstructural protein (NSs) of HRTV (HNSs) robustly antagonized both type I and III IFN signaling. Protein interaction analyses revealed that a common component downstream of type I and III IFN signaling, STAT2, is the target of HNSs. Of note, the DNA-binding and linker domains of STAT2 were required for an efficient HNSs-STAT2 interaction. Unlike the NSs of SFTSV (SNSs) which blocks both STAT2 and STAT1 nuclear accumulation, HNSs specifically blocked IFN-triggered nuclear translocation.
translocation only of STAT2. However, upon HRTV infection, IFN-induced nuclear translocation of both STAT2 and STAT1 was suppressed, suggesting that STAT1 is an additional HRTV target for IFN antagonism. Consistently, despite HNSs inhibiting phosphorylation only of STAT2 and not STAT1, HRTV infection diminished both STAT2 and STAT1 phosphorylation. These results suggest that HRTV antagonizes IFN antiviral signaling by dampening both STAT2 and STAT1 activities. We propose that HNSs-specific targeting of STAT2 likely plays an important role but is not all the “tactics” of HRTV in its immune evasion.

Introduction

Heartland virus (HRTV) is an emerging pathogenic phlebovirus (Phlebovirus genus, Phenuiviridae family, Bunyavirales order) first isolated from two Missouri farmers hospitalized with severe fever, leucopenia, and thrombocytopenia in 2009 (1). HRTV is the first known autochthonous phlebovirus pathogenic to humans in North America (1,2). As of September 2018, sporadic human cases of HRTV infection have been identified from ten states in the Midwestern and southern United States (3). Based on the virus RNA detection in arthropods, the Lone Star tick (Amblyomma americanum) has been implicated as a vector of HRTV (4), while serological assessment of HRTV exposure showed that many domestic and wild animals likely are potential amplification hosts of the virus (2). HRTV is genetically closely related to the severe fever with thrombocytopenia syndrome virus (SFTSV), another highly pathogenic tick-borne phlebovirus emerging in China (5-7) and the neighboring countries (8,9). SFTSV has been associated with thousands of cases of symptomatic human disease characterized by similar hemorrhagic fever-like clinical manifestations with HRTV infection (6,10). With the emergence of SFTSV and HRTV, more SFTSV/HRTV-related phleboviruses of potential high-pathogenicity have been isolated in recent years around the world (11). It is apparent that SFTSV, HRTV, and other related emerging phleboviruses have posed a severe threat to worldwide human health (12,13). However, there is currently no vaccine or medication available to prevent or treat these virus infections (12). Moreover, it is still poorly understood in terms of the phlebovirus-host interactions (13).

Type I and III interferons (IFNs), as “antiviral IFNs”, are the key components of innate immunity in host defense against invading viral pathogens (14-17). IFN responses upon virus infection comprise two phases, IFN induction and IFN signaling (14,15). In the induction phase, viral
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infection promptly stimulates expression and secretion of type I IFNs (especially IFN-α and IFN-β) and type III IFNs (IFN-λ) (14,15,18,19). In the IFN signaling phase, although type I and III IFNs employ different cell surface receptors, they share a similar downstream Janus kinase (JAK)-signal transducer and activator of transcription (STAT) signaling (14,15,18,19). The binding of type I and III IFNs to their receptors initiates activation of several JAKs, including tyrosine kinase 2 (TYK2), JAK1, and JAK2, leading to the subsequent activation of STAT1 and STAT2 by phosphorylation (14,20). Phosphorylated STAT1 and STAT2 heterodimerize and interact with IFN regulatory factor 9 (IRF9) to form an IFN-stimulated gene factor 3 (ISGF3) transcription complex (14). ISGF3 translocates to the nucleus where they bind to the IFN-stimulated response element (ISRE) of the interferon-stimulated gene (ISG) promoters, resulting in the expression of these antiviral genes and the establishment of host antiviral state (14,21,22).

Although the pathogenesis of the emerging phleboviruses remains elusive, most (if not all) of pathogenic viruses have evolved various countermeasures against host innate immunity and particularly the IFN system, for example encoding a robust IFN-antagonizing protein (23,24). Like all members of the genus Phlebovirus, HRTV and SFTSV have a genome organization which consists of three single-stranded negative-sense RNA segments, designated L, M, and S (13). The L segment encodes the RNA-dependent RNA polymerase, the M segment encodes the glycoproteins, and the S segment encodes the nucleocapsid protein (NP) and a nonstructural protein (NSs) by ambisense strategy (13). The NSs proteins of some phleboviruses likely function as the crucial virulence factor and contribute to the viral pathogenesis by subverting host interferon responses through diverse strategies (24). For instance, our previous studies showed that SFTSV NSs (SNSs) irreversibly hijacks host kinases TBK1/IKKe (25) and transcriptional activators STAT2/STAT1 (26) into SNSs-induced inclusion bodies (IBs), leading to the blockade of IFN induction and downstream signaling, respectively. Furthermore, we found that HRTV NSs (HNSs) interacts with TBK1 and blocks the association of TBK1 with its substrate IRF3, thus inhibiting IFN induction (27). However, it is unclear whether IFN signaling can be counteracted by HRTV infection and the potential role of HNSs therein needs further detailed elucidation as well.

In this study, we demonstrate that HRTV infection can block both type I and III IFN antiviral signaling. Furthermore, the
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mechanism underlying HRTV manipulation of type I and III IFN signaling was unraveled by detailed comparative studies in the context of HRTV infection and transient expression of potential IFN-inhibiting protein, HNSs. Finally, a function and mechanism model for IFN antagonism by HRTV as well as HNSs was proposed in comparison with SFTSV and SNSs.

Results

Similar to SFTSV, HRTV potently suppresses type I IFN-directed antiviral signaling and ISG expression.

To determine whether HRTV infection interferes with type I IFN signaling, we firstly examined the impact of HRTV infection (with SFTSV infection serving as a positive control) on IFN-α-triggered ISRE promoter activation by dual-luciferase reporter assay (DLR). As shown in Figure 1A, in mock-infected cells, ISRE promoter was expectably activated by the addition of IFN-α; however, following HRTV infection, the activation of ISRE promoter was evidently abolished, indicating the robust type I IFN-inhibiting capacity of HRTV. Meanwhile, in accordance with the previous report (26), a similar inhibitory effect on ISRE promoter activation was observed in the context of SFTSV infection (Figure 1A). Furthermore, we detected IFN-α-driven expression of several representative ISGs, including oligoadenylate synthetase 1 (OAS1), myxovirus-resistance A (MxA), ISG15, and ISG56, by real-time quantitative PCR (qPCR). Consistently, the infection with HRTV as well as SFTSV greatly blocked the ISG induction by IFN-α (Figure 1B), confirming the type I IFN-antagonistic activity of HRTV and meanwhile suggesting the functional conservativeness of these emerging phleboviruses in type I IFN antagonism.

HNSs, but not HNP, antagonizes IFN-α-elicited antiviral signaling and ISG expression.

It has been established that SNSs can disable both IFN induction and signaling (25,26,28-30). In addition, a previous study by us has showed that HNSs suppresses the production of type I IFNs (27). Considering the homology of HNSs with SNSs (~ 60% identity in amino acid sequence) (1), we hypothesized that HNSs may also perturb type I IFN signaling like SNSs. To test the hypothesis, we assessed the effect of HNSs on IFN-α-induced ISRE promoter activation by DLR assay. As shown in Figure 2A, similar to SNSs, HNSs efficiently inhibited the activation of ISRE promoter by IFN-α in a dosage-dependent manner; however, by contrast, HNP expression did not exhibit such inhibitory effect, suggesting the specific inhibition of IFN-α-mediated ISRE
activation by HNSs. Next, we examined the influence of HNSs expression on IFN-α-induced expression of ISGs. Real-time qPCR analyses showed that HNSs expression by transient transfection significantly weakens the induction of ISGs by IFN-α (Figure 2B), confirming the suppression of type I IFN signaling pathway by HNSs. Together, these results validate the role of HNSs as an antagonist of type I IFN antiviral signaling.

HRTV infection and HNSs transient expression both can inhibit type III IFN signaling.

Type III IFNs are the most recently described “antiviral IFNs” which are structurally similar to members of the interleukin-10 (IL-10) family but functionally similar to type I IFNs (19). Type III and I IFNs signal through distinct receptor complexes, but they downstream drive the similar JAK-STAT signaling and largely overlapping ISG expression (14). Thus, we next investigated whether HRTV and its HNSs perturb type III IFN signaling. As presented in Figure 3A, IFN-λ-induced activation of ISRE promoter was reduced by the infection of HRTV as well as SFTSV. It should be noted here that viral infections themselves appear to trigger a slight activation of ISRE promoter (Figure 3A, left) and the viral inhibitory effects on IFN-λ signaling were manifested more evidently by calculating the fold activation of ISRE over the corresponding untreated groups (Figure 3A, right). Furthermore, ISRE promoter activation by IFN-λ was likewise impaired in cells transfected with the plasmids expressing HNSs or SNSs (Figure 3B), suggesting that HNSs, like SNSs, is also an antagonist of type III IFN signaling.

HNSs targeting of STAT2.

The suppression of both type I and III IFN signaling by the viral NSs proteins suggests that their cellular target(s) is(are) likely shared by the two IFN signaling cascades. Indeed, our previous studies showed that STAT2 and STAT1, the common transcription activators downstream of type I and III IFN signaling, are the targets of SNSs (26). Thus, we examined whether HRTV and HNSs also target the STATs. Firstly, the interaction of S-tagged HNSs (HNSs-S) with STAT2 and STAT1 was evaluated by S-tag pulldown (S-pulldown) assays. As indicated in Figure 4A, STAT2 was strongly co-precipitated by HNSs (but not HNP), while only a faint signal of STAT1 could be detected in the HNSs co-precipitates, even with a long-time exposure. Then, a reciprocal protein-interaction S-pulldown assay was performed in the context of HRTV infection. Consistently, HNSs (but not the other viral
Inhibition of antiviral IFN signaling by HRTV proteins) could be high efficiently enriched in the S-tagged STAT2 (but not STAT1) co-precipitates in HRTV-infected cells (Figure 4B), indicating the strong and specific interaction between HNSs and STAT2. Furthermore, the interaction of endogenous STATs with HNSs was determined in the context of HRTV infection as well. As shown in the co-immunoprecipitation (Co-IP) assay, endogenous STAT2 but not STAT1 was co-precipitated with HNSs (Figure 4C), further confirming the specific HNSs-STAT2 interaction. Next, the subcellular colocalization of HNSs and STATs was analyzed by immunofluorescence assay (IFA). In line with the findings of the protein-interaction assays, HNSs was obviously colocalized with STAT2 in the cytoplasm (Figure 4D), whereas no comparable colocalization between HNSs and STAT1 was observed (Figure 4E). These results validated the specific targeting of STAT2 by HNSs.

The DNA-binding domain (DBD) and linker domain (LD) of STAT2 are required for the efficient HNSs-STAT2 interaction.

To gain insight of the region(s) within STAT2 required for the HNSs-STAT2 interaction, we assayed the ability of a series of N-terminal-truncated STAT2 mutants (Figure 5A) to co-precipitate with HNSs by S-pulldown assay. Expression of all the STAT2 mutants by transfection was readily detected, while some differences in the expression levels were still observed (Figure 5B). To better assess the protein interactions, band intensities of full-length or truncated STAT2 in WB analyses of the lysate inputs and HNSs-S pulldown products (Figure 5B) were respectively measured and the pulldown ratios of STAT2 and its mutants (pulldown over input) were then calculated and compared. As shown in Figure 5B and C, STAT2 mutants lacking the N-terminal (NTD) and coiled-coil (CCD) domains retained the capability of strongly interacting with HNSs; however, the deletion of DBD resulted in a substantial reduction of the pulldown ratio and further deletion of LD almost abolished the interaction with HNSs (Figure 5C). Taken together, these data suggest that the middle regions, DBD and LD, within STAT2 are likely important for the efficient HNSs-STAT2 interaction.

Different from SNSs disruption of both STAT2 and STAT1 nuclear accumulation, HNSs specifically blocks IFN-induced nuclear translocation of STAT2, but not STAT1.

Given the potent interaction of HNSs with STAT2, we investigated whether HNSs can affect IFN-induced STAT2 nuclear translocation which is the essential step for
the antiviral signal transduction. HEK293 cells transfected with viral protein expression plasmids were left untreated (Figure 6A and C) or treated with IFN-α (Figure 6B and D) and then fixed to visualize the expression and localization of the viral proteins and endogenous STAT1 and STAT2 by IFA. In accordance with the previous reports (25,27), SNSs was located in cytoplasmic SNSs IBs, while HNSs was distributed diffusely in cytoplasm (Figure 6). Furthermore, in SNSs-expressing cells, both of STAT2 and STAT1 were relocated into SNSs IBs and their nuclear accumulation induced by IFN-α was obviously suppressed; however, HNSs expression could specifically block the nuclear translocation of STAT2, but not STAT1 (Figure 6), reflecting a difference between the two viral NSs proteins. Meanwhile, HNP did not notably influence the nuclear translocation of either STAT2 or STAT1 (Figure 6B and D), in line with the results that HNP cannot inhibit IFN-α-triggered ISRE promoter activation (Figure 2A) or co-precipitate the STATs (Figure 4A). Additionally, similar results regarding the effects of the viral proteins on STAT nuclear translocation were obtained from experiments under the treatment of IFN-λ (data not shown). Collectively, these data indicate that HNSs only can block STAT2 nuclear translocation induced by type I and III IFNs, different from SNSs-mediated inhibition of both STAT1 and STAT2 nuclear accumulation.

**HRTV infection interferes with IFN-induced nuclear translocation of both STAT2 and STAT1.**

We next investigated whether IFN-triggered STAT nuclear translocation can be affected in the context of HRTV infection exactly as that mediated by HNSs expression alone with transient transfection. HEK293 cells infected with HRTV were left untreated or treated with IFN-α or IFN-λ for 30 min, followed by fixation for IFA. As shown in Figure 7A, B, and C, IFN-α or IFN-λ treatment resulted in STAT2 and STAT1 accumulation into nucleus in uninfected cells, whereas in the HRTV-infected cells, the nuclear translocation of STAT2 was nearly abolished (Figure 7B, C, and D), consistent with the observation in the cells transiently expressing HNSs. However, to our surprise, the nuclear translocation of STAT1 appeared to be also diminished by HRTV infection (Figure 7B, C, and E), different from the inhibitory ability of HNSs confined to STAT2 nuclear translocation (Figure 6). To further confirm HRTV inhibition of STAT1 nuclear translocation, nuclear proteins were extracted by cellular fractionation. WB analyses of the subcellular fractions further showed that nuclear translocation of both
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STAT1 and STAT2 induced by IFN-α or IFN-λ was inhibited by HRTV infection (Supporting data, Figure S1). These findings suggest that HRTV infection represses the nuclear translocation of not only STAT2 but also STAT1, likely together leading to the viral antagonism of type I and III IFN signaling.

**Differential inhibition of STAT2 and STAT1 phosphorylation by HNSs transient expression and HRTV infection.** Tyrosine phosphorylation of STAT2 and STAT1 represents the activation of the transcription factors and is the prerequisite for their nuclear accumulation to stimulate ISG transcription (14,15). Thus, we tested the effects of HNSs transient expression and HRTV infection on IFN-induced STAT phosphorylation, respectively. In transient transfection-based experiment, HNSs expression resulted in an evident inhibition of IFN-α-elicited STAT2 phosphorylation but in contrast did not exhibited any noticeable influence on the phosphorylation of STAT1 (Figure 8A), confirming the confined targeting of STAT2 by HNSs. Meanwhile, as expected, HNP had no significant effect on the activation of either STAT (Figure 8A). Importantly, in the context of HRTV infection, the phosphorylation of STAT2 stimulated by IFN-α was nearly undetectable (Figure 8B), in accordance with the robust inhibitory activity of HNSs to STAT2 activation. Further, intriguingly, a substantial decrease of IFN-α-triggered STAT1 phosphorylation was also resulted from HRTV infection (Figure 8B), consistent with the suppression of STAT1 nuclear translocation by HRTV infection. Additionally, HRTV infection, similarly, could disable both STAT2 and STAT1 phosphorylation induced by IFN-λ as well (data not shown). Type II IFN, IFN-γ, can also trigger the phosphorylation of STAT1 that further forms an activated homodimer (i.e. IFN-γ activation factor, GAF) rather than ISGF3 via different receptors and JAK-STAT signaling (20). To gain further insights into HRTV inhibition of STAT1 activation, we also tested the effect of HRTV infection and HNSs transient expression on IFN-γ-induced STAT1 phosphorylation. Interestingly, neither HNSs transient expression (Figure 8C) nor HRTV infection (Figure 8D) detectably impaired IFN-γ-elicited STAT1 phosphorylation, revealing that HRTV infection specifically inhibits STAT1 phosphorylation and activation directed by the antiviral type I and III IFNs.

Furthermore, we examined the respective influence of individual expression of the other viral proteins (GP and RdRp) or combinatorial co-expression of all the HRTV proteins on IFN-α-triggered STAT1 activation. As shown in Figure 8E, like
HNSs and HNP (Figure 8A), HRTV GP or RdRp did not affect IFN-α-induced STAT1 phosphorylation when transiently expressed individually; moreover, combined co-expression of HRTV proteins did not detectably inhibit STAT1 activation either. These data indicate that HRTV does not encode any viral protein as the direct antagonist against STAT1. Meanwhile, it is also demonstrated that HNSs (like SNSs) is the specific STAT2 antagonist, as in the absence of HNSs, individual expression or combinatorial co-expression of the other HRTV proteins (HNP, GP, and RdRp) could not impair STAT2 activation (Figure 8A and E).

Altogether, these results establish that HNSs (but not the other viral proteins) exclusively abates STAT2 activation, acting as a type I and III IFN antagonist. Furthermore, although HRTV does not encode any viral protein as a direct antagonist against STAT1, in the context of infection, HRTV can abolish type I and III IFN activation of both STAT2 and STAT1 (but not IFN-γ activation of STAT1), likely leading to a specific and more rigorous disruption of the antiviral type I and III IFN signaling cascades.

Discussion.
Emerging tick-borne phleboviruses, represented by SFTSV and HRTV, are challenging public health (12,13). There is urgent need to understand the phleboviral pathogenesis and virus-host interactions for facilitating antiviral drug and vaccine development. Previously, several studies by us and others have investigated the complex immune evasion strategies employed by SFTSV (25,26,28-30). Therein, we found that SNSs induces viral IB formation and sequesters STAT2 and STAT1 into the IBs, blocking both STAT2 and STAT1 nuclear translocation (25,26). Meanwhile, the phosphorylation of STAT2 (but not STAT1) was specifically hampered by SNSs (26). Consistent effects on STAT2 and STAT1 were observed in the context of SFTSV infection (26). Here, we demonstrated that HNSs has the conservative function in the antagonism of type I and III IFN signaling, but unlike SNSs, HNSs can only target the STAT2 actions. Furthermore, HRTV infection blocks the phosphorylation and nuclear accumulation of both STAT2 and STAT1 and hence robustly subverts type I and III IFN antiviral signaling, revealing that not only STAT2 but also STAT1 is the viral target for the immune evasion. The anti-IFN strategies of HRTV and HNSs were summarized in Figure 9, in comparison with those of SFTSV and SNSs.

We previously showed that HRTV can subvert IFN induction through HNSs blocking of TBK1-IRF3 interaction and
Inhibition of antiviral IFN signaling by HRTV signaling (27). Together with the present findings here, we demonstrate an overall view for HRTV disruption of the antiviral IFN system, including IFN induction and signaling. During the current study, based on transient transfection of HNSs expression plasmid, Rezelj et al. showed that HNSs transient expression exhibited an inhibitory effect on the IFN-β (another type I IFN)-STAT2 signaling (31), which is in line with our parallel and expanded findings that HNSs, but not HNP, disables IFN-α-STAT2 signaling and hence antiviral ISG expression. Importantly, viral antagonism of type I IFN signaling is not only revealed here with further significant characterization and mechanistic investigation but also detailely explored in the context of HRTV infection. Intriguingly, we demonstrate that divergent with HNSs transient expression, HRTV infection impairs the phosphorylation and nuclear translocation of not only STAT2 but also STAT1, indicating that HNSs targeting of STAT2 is not the sole mechanism for HRTV antagonism of IFN signaling and diminished STAT1 activities should contribute to the viral immune evasion as well. The most recently discovered type III IFNs engage their tissue specifically distributed receptors which differ from the broadly expressed type I IFN receptors (19). Thus, type III IFNs preferentially direct localized antiviral response, especially at barrier surfaces and mucosal sites (including liver and intestine epithelium) which coincidently may be involved in these phleboviral infections and the pathogenesis of the resultant hemorrhagic fever-like diseases (19,20,32-35). In this study, we found that HNSs can also suppress type III IFN signaling by impeding STAT2 actions. Furthermore, in the context of viral infection, HRTV disrupted type III IFN signaling by targeting the activities of both STAT2 and STAT1. These findings imply that type III IFN response may have an important role in host restriction to HRTV infection as well, further shedding light on the interactions of HRTV with innate immune system and the viral pathogenesis.

Indeed, Bosco-Lauth et al. reported that Ag129 mice lacking functional IFN receptors (but not immunocompetent animals) are highly susceptible to HRTV challenge and inoculation with even low dosages of HRTV resulted in severe illness and death (36), manifesting the critical role of IFN system in host defense against HRTV infection. Additionally, Westover et al. recently found that HRTV causes only moderate illness in STAT2 knockout hamsters and most of the infected animals can recover, indicating that in addition to STAT2, other component(s) of the IFN system can also offer significant protection against HRTV infection (37). Here, the...
identification of STAT1 as an additional target of HRTV suggests the potential role of STAT1 therein. Further knockout of STAT1 may render the animals more susceptible and is merited to be tested for developing better animal models of HRTV infection and for further characterizing the role of STAT1 in anti-HRTV immunity in vivo.

In the present study, we found that HNSs specifically inhibits IFN-induced phosphorylation and nuclear translocation of STAT2 but not STAT1. Interestingly, Ho et al. previously reported that unphosphorylated STAT2 appeared to retain a fraction of phosphorylated STAT1 in cytoplasm by the formation of a hemiphosphorylated STAT1-STAT2 heterodimer (38), while we observed the blockade of STAT2 nuclear translocation and simultaneously likely unaffected nuclear translocation of STAT1 triggered by IFN-α in cells transiently expressing HNSs (Figure 6), suggesting that the unphosphorylated STAT2 here might fail to encumber STAT1 nuclear accumulation. A simple explanation is that HNSs likely hindered the hemiphosphorylated STAT1-STAT2 heterodimer formation by the robust HNSs-STAT2 interaction. Otherwise, molecular number of STAT1 may be far more than that of STAT2 in HEK293 cells like several cell lines tested by Ho et al. (38) and thus the influence of STAT2 on STAT1 was hardly observable. Furthermore, the phenomenon (IFN-triggered nuclear translocation of STAT2 was blocked but that of STAT1 was intact) resulted from HNSs transient expression was not similarly observed in the context of actual viral infection, as HRTV infection can inhibit the phosphorylation and nuclear accumulation of not only STAT2 but also STAT1. In contrast with the unique participation of STAT2 in type I and III IFN antiviral circuits, STAT1 is engaged in multiple signaling cascades triggered by various cellular factors such as some interleukins and growth factors, besides the whole IFN family (39). Accordingly, the targeting of STAT1 by HRTV can not only enhance the destruction of type I and III IFN antiviral responses but may also lead to a wider perturbation of other cell signaling pathways involving STAT1 (although we excluded the viral interference with IFN-γ-triggered STAT1 activation), which is worthy of future study to further expand our knowledge of HRTV-host interactions. Although we observed a weak HNSs-STAT1 co-precipitation, HNSs, unlike SNSs, cannot or is insufficient to affect STAT1 function. Additionally, we did not detect the interaction of STAT1 with the other HRTV proteins (Figure 4B) and HRTV does not encode any viral protein as the STAT1-activation antagonist (Figure 8E). Therefore,
the viral further detailed strategy underlying HRTV targeting of the STAT1 axis still remains to be clarified. In addition to encoding a direct IFN antagonist protein, some viruses can hijack the function of cellular suppressor of cytokine signaling (SOCS) proteins which attenuate IFN-STAT signaling in a negative feedback loop (16,40-43), leading to the viral immune evasion from IFN responses. It will be interesting to address whether similar indirect strategies are employed by HRTV.

HNSs and SNSs share ~60% identity in amino acid sequence (1). SNSs is localized in itself induced IBs (25), whereas HNSs cannot mediate IB formation and distributes diffusely in cytoplasm (27,31). Although they have conservative IFN signaling-antagonizing function, in addition to the distinct subcellular localization, the two NSs proteins exhibit some other remarkable differences in their detailed activities. As aforementioned, SNSs can interfere with the nuclear translocation of both STAT2 and STAT1 via hijacking the two transcription factors into viral IBs (26), whereas HNSs specifically blocks STAT2 nuclear translocation through disabling STAT2 phosphorylation and activation. Additionally, by contrast with the involvement of STAT2 DBD within the SNSs-STAT2 interaction (26), both DBD and LD are likely required for the efficient targeting of STAT2 by HNSs, revealing one more difference between the two NSs. These findings highlight the function conservativeness and mechanism divergence of these homologous viral nonstructural proteins. Currently, reverse genetic system for HRTV has not been established. Comparison of HNSs deficient viruses yielded by reverse genetics with the wildtype HRTV will further unravel the significance of HNSs in viral infection and pathogenesis. Anyway, identification of these NSs proteins as robust IFN antagonists and hence the potential viral virulence factors may advance the design of attenuated vaccines and antiviral drugs by targeting NSs actions in future.

At present, reported hospitalized and fatal cases of HRTV disease are relatively fewer (3), compared to those caused by SFTSV infection. It is sometimes considered that HRTV may be less virulent than SFTSV. However, our findings suggest that HRTV and SFTSV, as well as their NSs proteins, appear to have potent and comparable capacity to destroy type I and III IFN antiviral responses (including both IFN induction and signaling), albeit by partly divergent strategies. The pathogenic risk of HRTV may need reassessment. Recently, several other new phleboviruses genetically related to HRTV and SFTSV were successively discovered worldwide (11,44-46). Together with HRTV and SFTSV, these
clustered emerging viruses have been designated as the SFTSV/HRTV group (11). Further comparative studies on these phleboviruses and their NSs proteins will provide clues to the molecular basis of the conservativeness and variance and promote the understanding of viral pathogenicity and virus-host interactions in evolutionary perspectives, thus likely benefiting the future development of specific or broad-spectrum antiviral therapies.

Materials and Methods

Cells and viruses

HEK293 cells were cultured in minimum essential medium (MEM; GIBCO) with 10% fetal bovine serum (FBS; GIBCO) at 37°C in a 5% CO2 atmosphere. Vero and Huh7 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM; GIBCO) supplemented with 10% FBS. HRTV (strain MO-4) was obtained from World Reference Center for Emerging Viruses and Arboviruses (WRCEVA), the University of Texas Medical Branch (UTMB). The proliferation of HRTV was performed in Vero cells in a biosafety level 3 laboratory. SFTSV was propagated and handled as previously described (25-27).

Plasmids

The firefly luciferase reporter plasmid for ISRE and *Renilla* luciferase control plasmid (pRL-TK) were kindly provided by Dr. Hong-Bing Shu (Wuhan University, China) (47-49). HA-tagged full-length or truncated STAT2 expression plasmids and the plasmids encoding HA- or S-tagged HNSs, HNP, or SNSs were described previously (25-27). Expression plasmids for S-tagged STAT1 and STAT2 were constructed by standard molecular biological approaches.

Antibodies

Primary antibodies used in this study included mouse antibodies to HA-tag (H3663; Sigma-Aldrich), β-actin (60008; Proteintech), glyceraldehyde 3-phosphate dehydrogenase (GAPDH, 60004-1-lg; Proteintech), STAT1 (sc-464; Santa Cruz Biotechnology, SCBT), or STAT2 (sc-514193; SCBT), and rabbit antibodies to S-tag (ab18588; Abcam), histone deacetylase 1 (HDAC1, 10197-1-AP; Proteintech), STAT1 (9172S; Cell Signaling Technology, CST), phospho-STAT1 (9167S; CST), STAT2 (sc22816; SCBT), or phospho-STAT2 (sc21689; SCBT). Rabbit antisera against the viral proteins (NSs, NP, GP, or RdRp) were generated by serial vaccination of the corresponding viral proteins prepared in *Escherichia coli*. Secondary antibodies used in immunofluorescence assays included Alexa Fluor 488-conjugated goat anti-rabbit (ab150077) or anti-mouse (ab150113) IgG (H+L) and Alexa Fluor 647-conjugated goat
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Reporter gene assay

Reporter gene assays were performed in HEK293 cells seeded on 24-well plates. Cells were cotransfected with 100 ng ISRE reporter plasmid and 20 ng pRL-TK plasmid using Lipofectamine 3000 (Invitrogen). Twelve hours posttransfection, HEK293 cells were mock-infected or infected with HRTV or SFTSV at a multiplicity of infection (MOI) of 5. At 24 hpi, cells were stimulated with IFN-α2b (PBL Biomedical Inc.) or IFN-λ2 (Peprotech) or left unstimulated for 18 h, followed by the measurement of luciferase activities with a dual-luciferase reporter (DLR) assay kit (Promega). The firefly luciferase activities were normalized on the basis of Renilla luciferase activities to exhibit the relative luciferase activities (Rel. Luc. Act.). Fold activation of ISRE was calculated by further normalization to the untreated controls. For the reporter gene assays with viral protein transient expression by transfection, HEK293 cells were cotransfected with 100 ng ISRE reporter plasmid, 20 ng pRL-TK, and indicated amounts of HNSs, SNSs, or HNP expression plasmids. Total amounts of DNA transfected per well were constant by the addition of the empty control plasmids, correspondingly. After 24 h, cells were left untreated or treated with IFN-α or IFN-λ for 18 h before the detection of luciferase activities.

Quantitative real-time PCR

The relative mRNA levels of indicated genes were analyzed by the $2^{\Delta\Delta CT}$ method with quantitative real-time PCR as previously described (26).

Protein-protein interaction analysis

S-pulldown assays were used for protein interaction analysis as previously described (25-27). The principle of S-pulldown assay is to utilize the high-specific and strong affinity of S-tag with S-protein coupled on agarose beads to precipitate the S-tagged protein and its interacting proteins. In a nutshell, transfected or infected cells were lysed in a lysis buffer (25 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, and 1% Triton X-100) supplemented with a cocktail protease inhibitor (Roche) on ice for 15 min. Following centrifugation, supernatants of the cell lysates then were mixed with the S-protein agarose slurry (Merck Novagen) by rotating incubation for 4 h at 4°C. After extensively washing the beads, bound proteins were eluted with 1× SDS sample buffer by boiling for 5 min, followed by SDS-PAGE and Western blot analyses.

For the validation of endogenous STAT2-HNSs interaction in the context of HRTV infection, co-immunoprecipitation
Inhibition of antiviral IFN signaling by HRTV (Co-IP) assay was performed. HEK293 cells (~$5 \times 10^7$) were infected with HRTV (MOI = 5) or mock infected and lysed in the lysis buffer at 36 hpi. Subsequently, the lysate supernatants were firstly pretreated with preimmune serum and protein A/G-Plus agarose (SCBT). After centrifugation, the pretreated supernatants were incubated with the HNSs-specific antiserum at 4°C for 1h, and then mixed with the protein A/G agarose at 4°C overnight. After extensive washes, the immunoprecipitates were delivered to Western blot analyses.

**Immunofluorescence and confocal microscopy**

Immunofluorescence assays coupled with confocal microscopy were used to monitor protein expression and localization as described previously (25-27,50). Briefly, transfected or infected cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PFA-PBS) and then permeabilized with 0.5% Triton X-100-PBS. After blocking with 2.5% bovine serum albumin (BSA) (Biosharp) and 2.5% normal goat serum (Jackson ImmunoResearch) in PBS, cells were stained successively with the primary antibodies for the target proteins and the corresponding fluorescently-labeled secondary antibodies. For visualization of nuclei, cells were stained with Hoechst 33258 (Beyotime). Images were obtained and analyzed with a Nikon Ti confocal microscope and the matched Volocity software (PerkinElmer).

**Cellular fractionation and Western blot analysis**

Nuclear extracts were prepared by cellular fractionation with a nucleus/cytoplasm fractionation kit (Beyotime biotechnology) according to the manufacturer’s instructions, followed by Western blot analysis. Western blot was performed as previously described (27,50). In brief, protein samples were subjected to SDS-PAGE and then transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore). After blocking with 5% skim milk in Tris-buffered saline-Tween 20 (TBST), the PVDF membranes were incubated with primary antibodies and then the corresponding secondary antibodies conjugated with horseradish peroxidase (HRP) (Sigma-Aldrich) in 1% BSA-TBST. Protein bands were detected with an enhanced chemiluminescence (ECL) kit (Thermo Fisher).

**Statistical analysis**

Data were presented as mean ± SD. Statistical significance was determined by Student’s t-test using IBM SPSS software. $P$ values <0.05 were considered statistically significant.
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Conflict of interest

The authors declare that they have no conflicts of interest with the contents of this article.
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**Footnotes**

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Abbreviations: HRTV, Heartland virus; SFTSV, severe fever with thrombocytopenia syndrome virus; IFN, interferon; STAT, signal transducer and activator of transcription protein; NP, nucleocapsid protein; NSs, nonstructural protein encoded by S segment; HNSs, HRTV NSs; SNSs, SFTSV NSs; CCD, coiled-coil domain; DBD, DNA-binding domain; LD, linker domain; NTD, N-terminal domain; SH2, Src-homology domain-2; TAD, transactivation domain; DLR, dual-luciferase reporter; hpi, hours postinfection; IBs, inclusion bodies; IFA, immunofluorescence assay; IFNAR, IFN-α receptor; IFNLR, IFN-λ receptor; IL-10, interleukin-10; IL-10R, IL-10 receptor; IRF, IFN regulatory factor; ISG, IFN-stimulated gene; ISGF3, IFN-stimulated gene factor 3; ISRE, IFN-stimulated response element; JAK, Janus kinase; MOI, multiplicity of infection; MxA, myxovirus-resistance A; OAS1, oligoadenylate synthetase 1; Rel. Luc. Act., relative luciferase activity; SOCS, suppressor of cytokine signaling; S-pulldown, S-tag pulldown; TYK2, Tyrosine kinase 2; WB, Western blot.
Figure 1. Suppression of type I IFN signaling by HRTV infection. (A) HRTV infection inhibits IFN-α-driven activation of ISRE promoter. HEK293 cells were cotransfected with an ISRE reporter plasmid and an internal control plasmid (pRL-TK). At 12 h posttransfection, cells were mock infected or infected with HRTV (MOI = 5) or SFTSV (MOI = 5), respectively. Twenty-four hours postinfection (hpi), cells were left untreated or treated with IFN-α (1000 U/ml) for 18 h, followed by the measurement of luciferase activities. Relative luciferase activity (Rel. Luc. Act.) and fold activation of ISRE are respectively shown. (B) HRTV infection blocks IFN-α-induced ISG expression. HEK293 cells were mock infected or infected with HRTV (MOI = 5) or SFTSV (MOI = 5). At 24 hpi, cells were left untreated or treated with IFN-α (1000 U/ml) for 10 h, followed by the detection of ISG mRNA expression by real-time qPCR. Graphs show mean ± standard deviation (SD), \( n = 3 \).
Figure 2. HNSs, but not HNP, is an antagonist of type I IFN signaling. (A) HNSs, but not HNP, inhibits IFN-α-stimulated activation of ISRE promoter. HEK293 cells were transfected with the indicated amounts of viral protein expression plasmids, together with the reporter plasmids. At 24 h posttransfection, cells were left untreated or treated with IFN-α for 18 h before the measurement of luciferase activities. Meanwhile, viral protein expression levels were monitored by Western blot (WB). (B) HNSs suppresses IFN-α-triggered ISG expression. HEK293 cells transfected with the HNSs expression plasmid or the empty control plasmid (vector) were left untreated or treated with IFN-α for 10 h, followed by the analyses of the indicated ISG mRNA expression through real-time qPCR. Graphs show mean ± SD (n = 3). *, P < 0.05; **, P < 0.01.
**Figure 3.** Suppression of type III IFN signaling by HRTV infection and HNSs expression. (A) HRTV infection interferes with IFN-λ-stimulated ISRE activation. Huh7 cells transfected with the reporter plasmids were mock infected or infected with HRTV or SFTSV for 24 h. Cells were then stimulated with IFN-λ (100ng/ml) for 18 h or left unstimulated, before the measurement of luciferase activities. (B) HNSs functions as an antagonist of type III IFN signaling. Huh7 cells were transfected with the reporter plasmids, along with the vector plasmid or the plasmids encoding SNSs or HNSs. At 24 h posttransfection, cells were left untreated or treated with IFN-λ (100ng/ml) for 18 h, followed by the detection of luciferase activities. Graphs show mean ± SD, n = 3. *, P < 0.05; **, P < 0.01.
Figure 4. Interaction and colocalization of HNSs with STAT2. (A) HEK293 cells were transfected with the plasmids encoding S-tagged HNP (HNP-S) or HNSs (HNSs-S) or the vector plasmid. At 36 h posttransfection, cells were harvested for S-pulldown assay. S-pulldown products and cell lysates were subjected to WB analyses with the indicated antibodies. (B) HEK293 cells were transfected with the
plasmids expressing S-tagged STAT2 (STAT2-S) or STAT1 (STAT1-S) or the control vector. At 12 h posttransfection, cells were mock infected or infected with HRTV (MOI=5) for 36 h and then lysed for S-pulldown assay and WB analyses. Therein, GP expression was monitored with the detection of GN (the N-terminal region of GP) using anti-GN antibody. (C) Mock- or HRTV-infected HEK293 cells were lysed for co-immunoprecipitation (Co-IP) assay at 36 hpi. The lysate supernatants were firstly pretreated with preimmune serum and protein A/G agarose and then used for Co-IP with the HNSs-specific antiserum. Immunoprecipitates (IP) and cell lysates were delivered to WB analysis using the indicated antibodies. (D and E) HEK293 cells were transfected with the plasmids expressing the indicated protein or the control vector. At 36 h posttransfection, cells were fixed for IFA to visualize the indicated proteins (HNSs in green and STAT2/STAT1 in red) by confocal microscopy. Nuclei were stained with Hoechst as shown in blue.
Figure 5. Mapping of the STAT2 domains required for the efficient HNSs-STAT2 interaction. (A) Domain organization of full-length or N-terminal truncated STAT2 C-terminally fused with HA-tag. NTD, N-terminal domain; CCD, coiled-coil domain; DBD, DNA-binding domain; LD, linker domain; SH2, Src-homology domain-2; TAD, transactivation domain; pY, tyrosine (690) phosphorylation site. The HA-tagged truncated STAT2 proteins were named T1-HA, T2-HA, T3-HA, T4-HA, and T5-HA, respectively. (B) HEK293 cells were transfected with the HNSs-S expression plasmid and the plasmids encoding full-length or truncated STAT2 proteins or the corresponding control vectors, as indicated. At 48 h posttransfection, interactions of HNSs with the full-length or truncated STAT2 proteins were analyzed with S-pulldown assay, followed by WB with the indicated antibodies. (C) Band intensities of full-length or truncated STAT2 proteins in (B) were respectively measured by ImageJ software. To calculate the pulldown ratio of full-length or truncated STAT2, band intensities of the proteins coprecipitated with HNSs were then normalized to the corresponding band intensities in lysate input. The relative pulldown ratio of full-length STAT2 was set to 1, for reference. N.A., not analyzed.
Figure 6. HNSs specifically inhibits nuclear translocation of STAT2 (but not STAT1), in contrast to SNSs inhibition of both STAT2 and STAT1 nuclear accumulation. HEK293 cells were transfected with the HNSs, SNSs, or HNP expression plasmids. At 24 h posttransfection, cells were left untreated (A and C) or treated with IFN-α (1000 U/ml) (B and D) for 30 min. After fixation, subcellular localization of the indicated viral proteins and STAT2 (A and B) or STAT1 (C and D) was visualized by IFA and confocal microscopy. Nuclei were stained with Hoechst.
Figure 7. HRTV infection interferes with type I and III IFN-triggered nuclear translocation of both STAT2 and STAT1. HEK293 cells infected with HRTV (MOI = 1) or mock infected were left untreated (A) or treated with IFN-α (1000U/ml) (B) or IFN-λ (100ng/ml) (C) for 30 min at 24 hpi. After fixation, IFA was performed to visualize the subcellular localization of HNSs and STAT2 or STAT1 under confocal microscope. Nuclei were stained with Hoechst. (D and E) Cells mock-infected or infected with HRTV (HNSs-positive) from the experiments of (B) and (C) were respectively scored for STAT2 (D) or STAT1 (E) nuclear accumulation. For each group, approximately 100 cells were counted. Percentages of the cells with noticeable STAT2 or STAT1 nuclear accumulation were calculated, respectively. Data are presented as mean ± SD, n = 3. See also supporting data, Figure S1.
Figure 8. Differential inhibition of STAT2 and STAT1 phosphorylation by HNSs transient expression and HRTV infection. (A) Transient expression of HNSs exclusively inhibits phosphorylation of STAT2 but not STAT1. HEK293 cells were transfected with the HNSs or HNP expression plasmids or the control vector. At 36 h posttransfection, cells were left untreated or treated with IFN-α (1000 U/ml) for 30 min. Protein levels were assessed by WB analysis with antibodies against the indicated proteins. (B) HRTV infection suppresses the phosphorylation of both STAT2 and STAT1. HEK293 cells were mock infected or infected with HRTV for 36 h and subsequently treated with IFN-α (1000 U/ml) for 30 min or left untreated.
untreated. Protein levels were then analyzed by WB as in (A). (C and D) Neither HNSs transient expression nor HRTV infection affects IFN-γ-induced STAT1 activation. Transfected (C) or infected (D) HEK293 cells were treated with IFN-γ (50ng/ml; Peprotech) for 30 min and then subjected to WB analysis. (E) Effects of individual or combinatorial expression of HRTV proteins on STAT activation. HEK293 cells were transfected with the plasmids as indicated (500 ng for each viral protein expression plasmid). Total transfection amount of plasmids was kept constant by the corresponding addition of the vector. At 36 h posttransfection, cells were treated with IFN-α as in (A) and then delivered to WB analysis.
Figure 9. Model for the disruption of type I and III IFN signaling by HRTV and HNSs, in comparison with SFTSV and SNSs. Type I and III IFNs use two different cell surface receptor complexes (broadly expressed IFNAR1/IFNAR2 for type I IFNs and tissue specifically distributed IFNLRI/IL-10R2 for type III IFNs) and then direct the similar JAK-STAT antiviral signaling. Firstly, binding of type I and III IFNs to their respective receptors initiates the activation of JAKs. Then, the JAKs activate STAT1 and STAT2 by phosphorylation, resulting in nuclear translocation of the transcription factors and subsequent induction of hundreds of antiviral ISGs. SFTSV infection or SNSs expression by transient transfection can inhibit the phosphorylation of STAT2 and the nuclear translocation of both STAT2 and STAT1, yielding the similar inhibitory effects on STAT2 and STAT1 actions. In comparison, although like SNSs, HNSs also functions as an antagonist of type I and III IFN signaling, it exclusively blocks the activities of
STAT2. Consistently, STAT2 phosphorylation and nuclear accumulation can be impeded in the context of HRTV infection. However, interestingly, HRTV infection also can significantly suppress STAT1 activation and nuclear translocation, albeit to a lesser degree, as an additional strategy for the immune evasion. Together, the blockade of both STAT2 and STAT1 actions by HRTV likely leads to a more radical subversion of type I and III IFN signaling. These findings highlight the function conservativeness and mechanism differentiation of these related emerging viruses and their NSs proteins. IFNAR, IFN-α receptor; IFNLR, IFN-λ receptor; IL-10R, IL-10 receptor.
Heartland virus antagonizes type I and III interferon antiviral signaling by inhibiting phosphorylation and nuclear translocation of STAT2 and STAT1
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