Stimulation of dysregulated IFN- β responses by aberrant SARS-CoV-2 small viral RNAs

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Abstract

Patients with severe COVID-19 exhibit a cytokine storm characterized by greatly elevated levels of cytokines during worsening disease. Despite this, the interferon (IFN) response is delayed, contributing to disease progression. Here, we report that SARS-CoV-2 generates excessive amounts of small viral RNAs (svRNAs) encoding exact 5' ends of positive-sense genes in human cells, whereas significantly fewer similar svRNAs are produced by endemic human coronaviruses (OC43 and 229E). SARS-CoV-2 5' end svRNAs are RIG-I agonists associated with IFN-beta expression in later stages of infection. The first 60-nt ends bearing duplex structures and 5'-triphosphates are responsible for immune-stimulation. The 5' end svRNAs were also produced during infection *ex vivo* and *in vivo*. The delta variant retains the robust 5' end svRNA production of the parental strain, whereas omicron (BA.1 and BA.2) produces little of these erroneous svRNAs. We propose that RIG-I activation by accumulated 5' end svRNAs overcomes the initial IFN antagonistic ability of viral proteins and contributes to drive late over-exuberant IFN production leading to the development of severe COVID-19 and suggest that evolutionary modification of SARS-CoV-2 5' end svRNA production may correlate with the reduced disease severity likely seen with omicron (BA.1 and BA.2).

Introduction

The coronavirus disease 2019 (COVID-19) pandemic, caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), is an ongoing global health threat. Although the delta variant (B.1.617.2) dominated previous variants and spread to >180 countries by September 2021 ([WHO, 2021](#)), the newly emerging omicron variants (B.1.1.529.1/BA.1 and B.1.1.529.2/BA.2) are outcompeting delta and quickly becoming the dominant lineage globally as of February 2022 ([WHO, 2022](#)). Patients with severe COVID-19 exhibit a hyperinflammatory response, referred to as a “cytokine storm” ([Pedersen and Ho, 2020](#)), which is characterized by excessive levels of cytokines including interleukin (IL)-6 and a variety of different interferons (IFNs) ([Lamers et al., 2020](#); [Lee et al., 2020](#); [Mathew et al., 2020](#); [Sposito et al., 2021](#); [Zhou et al., 2020](#)).

Like most viral RNAs, coronavirus RNA is detected by host RNA-sensors, cytosolic retinoic acid-inducible gene-I (RIG-I)-like receptors including RIG-I and melanoma differentiation-associated protein 5 (MDA-5) ([Li et al., 2010](#); [Sa Ribero et al., 2020](#); [Thorne et al., 2021](#)). Upon activation, RIG-I and MDA-5 transduce signaling cascades, leading to the activation of interferon regulatory factor 3 (IRF3) and NF- κ B that are required for type 1 IFN and pro-inflammatory cytokine production, respectively. During SARS-CoV-2 infection, both RIG-I and MDA5 reportedly sense SARS-CoV-2 RNAs to activate innate immune responses ([Kouwaki et al., 2021](#); [Thorne et al., 2021](#)), although studies suggest that it is MDA5 that predominantly governs the innate immune response to SARS-CoV-2 ([Rebendenne et al., 2021](#); [Yin et al., 2021](#)). However, it is not well understood which viral RNA species are sensed by these molecules on infection. Additionally, longitudinal analyses revealed that SARS-CoV-2 does elicit an IFN response, but this is delayed ([Lucas et al., 2020](#)), suspected to contribute to disease progression ([Huang et al.,](#)

2020; Jose and Manuel, 2020; Mehta et al., 2020). However, the viral mechanism driving the characteristic signature of delayed IFN induction in COVID-19 is poorly understood.

Abortive RNA production has been understood to be a result of RNA transcription by RNA polymerase of host machinery and RNA viruses (double-stranded RNA viruses and single-stranded RNA viruses with both polarities) (Li et al., 2008; Perez et al., 2010; Shim et al., 2002; Te Velhuis et al., 2018; Zhong et al., 2000). RNA viruses are known to exhibit high replication error rates during their reproduction and thereby produce abortive leader RNAs. It was suggested that these are recognized by RIG-I, thereby inducing an innate immune response (Oh et al., 2016; Plumet et al., 2007; Te Velhuis et al., 2018). In particular, the emergent RNA virus SARS-CoV-2 is now in a transitional period moving from bats to human hosts (Zhou et al., 2021) and supposedly faces challenges in hijacking the replication machinery in human cells (Bashor et al., 2021; Benedetti et al., 2020). Thus, SARS-CoV-2 replication in humans may produce erroneous RNAs in large amounts, which may trigger dysregulated host cell transcriptomic and immune responses, including the cytokine storm as seen with other highly pathogenic viruses such as some avian influenza virus strains (Te Velhuis et al., 2018). Although other human coronaviruses (HCoVs) also originated from non-human CoVs, they have already established stable virus lineages in humans over long adaptation periods. These considerations motivated us to hypothesize that the distinct production of aberrant viral RNAs among different CoVs may represent one factor responsible for the different outcomes in terms of immune responses and disease severity. Interplay between aberrant viral RNAs and COVID-19 pathogenesis in this context remains to be elucidated. Here, we applied deep sequencing, focusing on the small RNAs (<200-nt) produced by SARS-CoV-2 in comparison with two endemic HCoVs (229E and OC43) that cause common colds and more mildly symptomatic respiratory disease. We report that SARS-CoV-2 infection results in the generation of excessive amounts of aberrant small RNAs that act as RIG-I ligands to induce the delayed and over-exuberant IFN- β responses observed in severe COVID-19.

Results

SARS-CoV-2 replicates efficiently in cultured cells at levels similar to HCoVs.

We initially determined the kinetics of replication of HCoV-OC43, HCoV-229E and SARS-CoV-2 parental strain (hereafter referred to as SARS-CoV-2) in different cell lines. This was necessary because there is no single cell line available that is permissive to all the three CoVs. We therefore used human cell lines (HCT9, MRC5 and Calu-3 cells) and infected HCT8 cells with HCoV-OC43, MRC5 cells with HCoV-229E and Calu-3 cells with SARS-CoV-2 at a multiplicity of infection (MOI) of 0.001. The CoVs are generally propagated at different temperatures, namely 33°C for HCoV-OC43 and HCoV-229E and 37°C for SARS-CoV-2. We therefore monitored the replication kinetics at 33°C for HCoV-OC43 and HCoV-229E and at both 33°C and 37°C for SARS-CoV-2 to exclude any potential bias due to different culture temperatures. All CoVs efficiently produced viral progeny, reaching almost 100% infection rates in the host cell populations 72 or 96 hours post-infection (hpi) at either temperature (**Figure 1A**) with peak titers at the corresponding times (**Figure 1B**). We also tested non-human primate Vero cells in this study, which are permissive to HCoV-OC43 and SARS-CoV-2 infections, monitored in the same manner. HCoV-OC43

achieved 100% infection up to 96 hpi at 33°C (**Figure S1A**), whereas SARS-CoV-2 did so up to 72 hpi at 33°C or 48 hpi at 37°C, with peak titers at the corresponding times (**Figure S1B**). These data show that all CoVs completed a full replication cycle in these cells at either tested temperature and efficiently promulgated infection throughout the whole cell population with slightly different kinetics.

SARS-CoV-2 infection elicits a delayed IFN response in human cells.

Poly (I:C) robustly stimulated IFN- β and IL-6 secretion from HCT8, MRC5 and Calu-3 cells at 24 h post-transfection (**Figure S2A-B**), which correlated with the rapid induction of RIG-I expression (**Figure S2C**). These data confirm the presence of substantial intact antiviral signaling machinery in these established human cell lines.

We then infected these human cells with CoVs under the same conditions as above and measured the kinetics of IFN- β and IL-6 expression post-infection. The different CoVs evoked distinct antiviral responses in these cells (**Figure 1C-D**). HCoV-OC43 induced little IFN- β and IL-6 production over the whole time course, implying the intact antagonistic ability of the host antiviral response ([de Wit et al., 2016](#)). HCoV-229E also suppressed IFN- β production throughout the course of infection but IL-6 expression gradually increased over time. Notably, however, SARS-CoV-2 evoked both IFN- β and IL-6 expression at a later stage of infection, explained by its ability to dampen early IFN- β induction and reflecting a delayed IFN- β response. Delayed IFN- β induction was specific to SARS-CoV-2 infection regardless of culture temperature, and correlated with the induction of RIG-I which was observed only in SARS-CoV-2-infected cells (**Figure 1E**). These data prompted us to identify the IFN- β agonist(s) active in SARS-CoV-2 infection. To this end, we focused on viral RNA species produced during SARS-CoV-2 replication in infected cells.

SARS-CoV-2-derived small viral RNA species include IFN stimuli.

We purified total RNAs from human cells mock-infected or infected with the different CoVs. The RNAs were separated into a large RNA (lRNA) fraction (>200-nt) and a small RNA (sRNA) fraction (<200-nt), which were then transfected into human 293T cells or differentiated THP-1 cells to assess their IFN- β stimulatory ability (**Figure S3A**). Neither RNA fraction from mock- or HCoV-OC43-infected cells stimulated IFN- β production above basal levels in the transfected cells (**Figure S3B**). The sRNA fractions from HCoV-229E-infected cells also failed to increase IFN- β levels, although the lRNA fraction raised them substantially above baseline. In contrast, both RNA fractions from SARS-CoV-2-infected cells stimulated IFN- β production to a similar degree as Poly (I:C). These data show that both sRNAs and lRNAs produced by SARS-CoV-2 are IFN- β stimulatory. Depletion of cytoplasmic RNA-sensors (RIG-I and MDA-5) reduced the inflammatory response after infection, suggesting that RNA sensing is a key driver of SARS-CoV-2-induced innate immune activation (**Figure 1F-G**). Because the IFN- β stimulatory activity of the small viral RNA (svRNA) fraction was unique to SARS-CoV-2 and likely to be associated with the increased IFN-

b response during infection, we then focused on identifying SARS-CoV-2-derived sRNAs that evoke IFN- β production.

SARS-CoV-2 produces excessive amounts of immune-stimulatory svRNAs in infected cells.

To investigate which svRNA species are involved in IFN- β production during CoV replication, we harvested sRNAs (<200-nt) and lRNAs (>200-nt) from CoV-infected cells shortly before viral titers had plateaued at which time no cytopathic effects were apparent (**Figure 1A-B**). These were then subjected to RNA-sequencing (RNA-seq), as established by others ([Te Velthuis et al., 2018](#)). The sRNA preparations included a step whereby all 5' monophosphate RNAs were first removed by incubation with XRN-1, followed by RppH treatment to enable viral RNAs bearing 5' PPP to be ligated onto a sequence adapter (**Figure 2A**). The advantage of the protocol is that it permits the efficient recovery of svRNAs bearing a 5' PPP from RNA libraries and reduces cellular RNAs bearing other 5' modifications, such as a 5' P or a cap, despite also recovering common RNA breakdown products derived from hydrolysis during RNA isolation. We performed two comparative RNAseq analyses, one for human cells (HCT8, MRC5 and Calu-3) and the other for non-human primate cells (Vero).

The sRNA-seq approach provided a high output, similarly for all samples, in the order of 10 million total reads per sample (**Table S1**). Upon mapping the reads to the human and non-human primate genomes, we found similar read counts for host genes from all the infected cells. We then mapped the reads to the CoV genomes and compared the read counts between them. Total virus reads from SARS-CoV-2 were more abundant than from the HCoVs both in human and Vero cells, up to 27-fold and 4-fold more respectively, despite svRNAs comprising <3% of these libraries. The lRNA-seq also provided in the order of 10 million total reads per sample similarly for all samples (**Table S2**). However, the relative abundance of virus reads from the lRNA library was less prominent for SARS-CoV-2 than for HCoVs, with up to 9-fold and 2-fold more in human cells and Vero cells, respectively. This implies that SARS-CoV-2 produces svRNAs in greater amounts during RNA synthesis than the HCoVs. Next, we separated the virus reads from the sRNA library by strand specificity and discovered that most reads from all the CoV svRNAs mapped to positive-sense RNA, with >99% occupancy (**Figure 2B**). These reads were mapped across the entire CoV genome (**Figure 2C**).

To characterize the CoV-derived svRNAs, their fragments were categorized into three classes (**Figure 3A**). First, svRNA fragments present inwards of the 5' untranslated region (UTR) (here termed "5' UTR svRNAs"). Second, svRNA fragments containing the leader sequence and transcriptional regulatory sequence (TRS) precisely jumping to the start sites of the major N, M and S open reading frames (ORFs) (here termed "N/M/S svRNAs"). Third, svRNA fragments harboring the leader sequences and TRS fused to other minor ORFs and all fragments that excluded the 5' UTR (here termed "other svRNAs"). Notably, counts for the 5' UTR svRNA fragments were much higher in SARS-CoV-2 than in the HCoVs, up to 44-fold and 9.6-fold more in human and Vero cells, respectively (**Figure 3B and Table S3**). Patterns of the svRNA classes were dependent on the CoV species but independent of host cell type. Thus, 5' UTR svRNAs

represented a major fraction of the SARS-CoV-2 svRNAs, but less so in the HCoV svRNAs, where N ORF svRNAs were abundant fractions (**Figure 3C**). Nonetheless, a group formed by 5' UTR svRNAs and N/M/S ORF svRNAs, which constitute most of the 5' end-containing svRNA species, were always in the majority (about >54% occupancies) in the svRNAs of all these CoVs.

The coverage of svRNAs at 5' end genomes was then further elucidated. Most strikingly, the high abundance of SARS-CoV-2 svRNAs started from the precise 5' end (here termed "5' end svRNAs") (**Figure 4A-E, left panels**). In contrast, substantially less coverage at the 5' end was seen with the HCoVs, despite the svRNAs mostly harboring exact 5' termini. The peak sizes of the 5' UTR svRNA fragments were around 60 to 80-nt (SARS-CoV-2), bimodal at 50 to 80-nt and 140 to 150-nt (HCoV-OC43) and relatively broad at 50 to 80-nt (HCoV-229E) (**Figure 4A-E, right panels**). Concordantly, the svRNA fragments with the highest counts had the precise 5' ends of the CoVs genomes with the first 63-nt for SARS-CoV-2, the first 72-nt for HCoV-OC43, and the first 53-nt for HCoV-229E, with the fragments identical in human and Vero cells (**Figure 4F**). Collectively, these data suggest that svRNA production is specific to the CoV species but not to host cell type, and that svRNAs, particularly 5' end svRNAs, are produced in extremely large amounts by SARS-CoV-2 relative to HCoVs.

Interestingly, svRNAs formed by both 5' and 3' ends of CoVs genomes (termed "5' UTR-3' UTR svRNAs") were also present in the sRNA libraries, at levels higher in SARS-CoV-2 (**Figure S4A**), and with a peak size of 81-85-nt (**Figure S4B**). However, these were all present at a level >1,000-fold lower than the levels of the 5' end svRNAs. The identities of the 5' UTR-3' UTR svRNAs were verified by PCR, gel isolation and Sanger-sequencing using complementary primers to both termini (**Figure S4C-D**). Moreover, representative 5' UTR-3' UTR svRNAs of different lengths induced IFN- β secretion from transfected 293 cells and the 5' PPP end was essential for this stimulatory activity (**Figure S4E-G**). However, given the paucity of their production, they are hypothesized to make a much lower contribution to IFN- β activation in SARS-CoV-2-infected cells, compared to the 5' end svRNAs.

The first 60-nt sequence of the SARS-CoV-2 5' end svRNAs bearing 5' PPP and with a duplex structure is responsible for their immune-stimulatory ability.

To determine the exact sequence of the 5' end svRNA that is involved in IFN and cytokine production during SARS-CoV-2 replication, *in vitro* transcribed (IVT) RNAs corresponding to the first 40, 60, 80 and 100-nt of the 5' end of the SARS-CoV-2 genome (**Figure 5A**) were transfected into 293T cells or into differentiated THP-1 cells, and IFN- β and IL-6 secretion was quantified. IVT RNAs originating from the 60, 80 and 100-nt 5' ends induced markedly high IFN- β and IL-6 production (**Figure 5B**), whereas the 40-nt RNA had only a slight effect, correlating with the RIG-I response in cells transfected with IVT RNAs of the corresponding lengths (**Figure 5C**). Calf intestine alkaline phosphatase (CIP) treatment or capping with a Cap 1 analog resulted in a complete loss of the immune-stimulatory activity, indicating dependence on 5' PPP for stimulating host antiviral signaling. We also hypothesized that the 5' end svRNAs would form high order RNA structures (**Figure 5D**), recognized by the J2 antibody that detects duplex RNAs. Indeed,

we found that the J2 antibody recognized IVT RNAs of >60-nt in length (**Figure 5E-F**) that formed highly structured epitopes as expected, contributing to the high IFN- β and IL-6 stimulatory activity (**Figure 5B**). These data show that induction of IFNs and cytokines by SARS-CoV-2 5' end svRNAs can be attributed to the first 60-nt sequence that bears 5' PPP and to the corresponding secondary structure.

We next asked whether exclusively SARS-CoV-2 5' end sequences had IFN stimulatory ability, and not those from other coronaviruses. For this, IVT RNAs corresponding to HCoV-OC43 and HCoV-229E 5' ends with the same lengths as tested for SARS-CoV-2 were transfected into cells, and IFN- β secretion was quantified. The two HCoV IVT RNAs induced IFN- β production maximally at 60-nt in length, although the peak levels were different among the three CoVs (**Figure S5A**). These data suggest that the IFN-stimulatory ability of SARS-CoV-2 5' end svRNAs largely depends on the high quantity of material produced and not on the sequence of the 5' end region. The 5' end sequences of CoV species have the highest homology among the genomes ([Madhugiri et al., 2014](#)) and, indeed, the two HCoV 5' ends would be expected to form secondary structures similar to the SARS-CoV-2 5' end (**Figure S5B**), supporting this notion.

5' end svRNAs are recognized by cytosolic RIG-1 but not MDA5.

SARS-CoV-2 5' end svRNAs produced in the cytoplasm are thought to be recognized by host RNA-sensors. To determine the role of cytosolic RIG-I and MDA-5 in svRNA sensing during SARS-CoV-2 infection, we transfected IVT RNA corresponding to SARS-CoV-2 5' end svRNAs into 293T RIG-I- or MDA-5-knockdown cells. Silencing was shown to be effective in that specific siRNAs reduced the levels of RIG-1 and MDA-5 mRNAs by 90% compared to mock-transfected cells (**Figure 5G**). RIG-I silencing reduced IFN- β production to background levels on IVT RNA stimulation, whereas knocking down MDA-5 had little effect (**Figure 5H**). Furthermore, we sequenced svRNAs co-purified with RIG-I from Calu-3 cells infected with SARS-CoV-2 and found that RIG-I largely recognized the 5' end svRNAs (**Figure 5I-K**). Moreover, this signature was similar to that seen in the cytoplasmic fraction of the infected cells, where the svRNAs mostly start from the exact 5' end of the genome, on average being 53-nt in length and with positive polarity. These data document that RIG-I is the primary cytosolic sensor of 5' end svRNAs originating from SARS-CoV-2 to trigger IFN and cytokine responses.

SARS-CoV-2 5' end svRNAs accumulate in cells at later times after infection *in vitro* and *ex vivo*.

To determine 5' end svRNA biogenesis in infected cells, the kinetics of SARS-CoV-2 5' end svRNA production were studied in Calu-3 cells under the same conditions as in **Figure 1**. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was performed using a stem-loop RT primer specific for the most common svRNA having 63-nt (**Figure 6A**). Specificity of the primer set was confirmed using IVT RNA templates with different 5' end lengths (**Figure 6B**). This showed that the SAR-CoV-2 63-nt-

specific primer set could detect <3% of 43-nt and 265-nt 5' end svRNAs, with minimal detection at <10% of the 150-nt counterpart. These data indicated that the stem-loop qRT-PCR were able to distinguish different lengths of 5' end-containing RNAs (i.e. 5' end svRNA vs. genomic/subgenomic RNAs). The levels of svRNAs were normalized to levels of snRNA-U6, and values were expressed relative to the earliest sampling time (8 hpi). As expected, only minimal amounts of the 5' end svRNAs were detected at 8 hpi (**Figure 6C**), in agreement with the lack of significant IFN- β secretion at this time. The svRNAs progressively accumulated up to 96 hpi at 33°C and 72 hpi at 37°C, with a >5 log increase compared to levels at 8 hpi. These data suggest that 5' end svRNAs arise at low levels early in the viral life cycle and accumulate in infected cells over time, reaching high levels at later times after infection.

To further define the profile of SARS-CoV-2 5' end svRNA production *ex vivo*, reconstituted human nasal epithelia were infected with SARS-CoV-2 or HCoV-OC43 at an MOI of 0.1 (**Figure 6D**). The kinetics of production of progeny viruses and 5' end svRNAs and expression profiles of IFN- β and IL-6 were compared between the two viruses. SARS-CoV-2 and HCoV-2 showed similar replication kinetics in the reconstituted human epithelia, yielding >4 log FFU/ml at 96 hpi (**Figure 6E**). In contrast, there was a clear difference between them in terms of 5' end svRNA production; thus, SARS-CoV-2 accumulated to a high degree at 96 hpi, whereas HCoV-OC43 produced only marginal amounts throughout the culture period (**Figure 6F**). This findings are in accord with the marked elevation of the IFN- β level at 96 hpi (**Figure 6G**) and to some extent with the gradual increase in the IL-6 level during at 48 and 96 hpi (**Figure 6H**). These *ex vivo* data further support the interpretation that SARS-CoV-2 abortive svRNAs accumulate excessively during replication in human airway epithelia, compared with endemic HCoVs.

Stoichiometric balance of SARS-CoV-2 IFN antagonists and 5' end svRNAs determines the host IFN response.

SARS-CoV-2 encodes numerous accessory proteins (e.g. ORF6) that act as IFN antagonists ([Kimura et al., 2021](#); [Lei et al., 2020](#)). These are initially translated from the viral genomic RNA after virus entry to swiftly inhibit type 1 IFN production. In contrast, as shown in **Figure 6C**, svRNAs accumulate in infected cells at later times after infection. We thus hypothesized that IFN activation mediated by accumulated 5' end svRNAs overcomes the antagonistic ability of the accessory proteins and drives later IFN production. To elucidate counteractions between the IFN antagonists and 5' end svRNA agonists in host cells, we stimulated 293T cells ectopically expressing SARS-CoV-2 ORF6 with IVT 5' end svRNAs from SARS-CoV-2. ORF6 dose-dependently suppressed IFN induction mediated by IVT 5' end svRNAs (**Figure 6I**). Thus, these data demonstrate that the stoichiometric balance between IFN antagonists and IFN-stimulatory 5' end svRNAs determines the IFN response in host cells, consistent with our hypothesis.

Exosomes released from SARS-CoV-2-infected cells contain 5' end svRNAs.

The SARS-CoV-2 packaging signal generator is estimated to reside within an internal region encompassing nsp15 and nsp16 (Syed et al., 2021), indicating that 5' end svRNAs are unlikely to be packaged into released virions as defective interfering particles. Instead, exosomes are recognized as a key vehicle to transfer various genetic materials including small RNAs including miRNAs (Valadi et al., 2007). We thus hypothesized that excessive SARS-CoV-2 5' end svRNAs are released extracellularly via exosomes. To determine whether SARS-CoV-2-infected cells secrete exosomes carrying 5' end svRNAs, exosomes were isolated from supernatants of SARS-CoV-2-infected Calu-3 cells at 72 hpi. To separate exosomes from free virions, isolated exosomes were immunoprecipitated using an antibody against CD63, a selective marker of exosomes. Total RNAs from purified exosomes were then separated into sRNA (<200-nt) and lRNA (>200-nt) fractions and sRNA fraction was assessed by RNA-seq (Figure S6A). Immunoblotting confirmed the specific enrichment of exosomes and exclusion of virions in these samples (Figure S6B). We found that a substantial proportion of sRNAs from such exosomes mapped to SARS-CoV-2, especially to the positive-sense genomic material (Table S4). These svRNAs were the exact 5' end genome and had similar signatures of 5' end svRNAs as found in cell lysates (Figure S6C-E). These data indicate that IFN-stimulatory 5' end svRNAs can be released from infected cells, although their contributions to any deterioration of immune responsiveness remain unknown.

The SARS-CoV-2 delta variant produces 5' end svRNAs in infected cells at levels similar to the parental virus.

Delta variants reportedly have a higher replication efficiency in human airway epithelia (Mlcochova et al., 2021), indicating a better fitness in human cells. Thus, we investigated whether their fitness in humans had resulted in a reduced production of erroneous svRNAs in infected human cells. The delta strain (termed Delta) efficiently infected Calu-3 cells and produced larger amounts of viral progeny with more rapid replication kinetics than the parental strain (Figure S7A-B). This correlated with slightly faster IFN- β expression induced by the delta strain at 48 hpi at 33°C or 72 hpi at 37°C (Figure S7C-D). RIG-I expression was also induced by infection with Delta (Figure S7E).

sRNA-seq generated a high output of reads per sample (Table S5) and showed that svRNAs from Delta were more abundant than from the HCoVs, despite the svRNAs being represented to only a minor degree in the library (<0.63%). Again, a large majority of the Delta reads mapped to positive-sense RNA with the 5' end having the highest coverage (Figure S7F-G). The Delta 5' end svRNAs were the major svRNA categories mostly having the exact 5' end (Figure S7H) and produced at levels similar to the parental strain (Table S6). svRNA fragment patterns were identical to the parental strain with a peak size of 63-nt (Figure S7I). qRT-PCR using the above-mentioned looped RT primers revealed that 5' end svRNAs accumulated in infected human cells with slightly faster and higher kinetics than parental strain (Figure S7J). These data show that Delta has evolved in humans without correcting the production of erroneous svRNAs in human cells.

The SARS-CoV-2 Omicron variants BA.1 and BA.2 produce less 5' end svRNAs in infected cells than the parental virus.

As of February 2022, omicron variants have emerged as the latest lineage with multiple mutations and reportedly have a greater transmissibility at the same time as reduced pathogenicity ([WHO, 2022](#)). While the original Omicron lineage BA.1 has become dominant in many countries, BA.2 has been detected in at least 67 countries and has become dominant in several of them. We investigated whether these variants produce less aberrant 5' end svRNAs in human cells. Compared with the parental strain, the Omicron variants BA.1 and BA.2 (termed BA.1 and BA.2) replicated less well in Calu-3 cells as reported previously ([Yamasoba et al., 2022](#)), with the most drastic difference observed at 33°C rather than 37°C (**Figure 7A**). IFN- β expression was markedly attenuated, although finally achieving a substantial level at 120 hpi at 37°C but not 33°C (**Figure 7B**), whereas IL-6 expression was less reduced in Omicron-infected cells (**Figure 7C**). These correlated with only a modest accumulation of 5' end svRNAs from BA.1 and BA.2 (**Figure 7D**). Additionally, in Vero cells, Omicron (BA.1 and BA.2) again accumulated markedly less 5' end svRNAs and exhibited less IFN and cytokine responses compared to the parental strain and Delta, even though Omicron and Delta had similarly attenuated replication kinetics (**Figure 7E-F**), similar to a prior report ([Shuai et al., 2022](#)). These data show that Omicron (BA.1 and BA.2) is less immune-stimulatory, associated with lower production of aberrant svRNAs in infected cells.

SARS-CoV-2 produces 5' end svRNAs during virus replication in hamsters.

To address whether 5' end svRNAs are produced on SARS-CoV-2 infection *in vivo*, an established hamster infection model was used (**Figure 7G**). Infection in this species does not result in severe symptoms or ARDS, but represents a reproducible COVID-19 model with moderate pneumonia and inflammatory infiltrates in the lung ([Higuchi et al., 2021](#); [Winkler et al., 2021](#)). We infected hamsters with SARS-CoV-2 parental, Delta and BA.1 Omicron strains at the same inoculation titers and sampled lungs and serum at 5 dpi. Viral titers were measured in the lungs and 5' end svRNA levels in the lungs and sera by stem-loop RT-PCR for the 63-nt 5' end svRNAs described above. All infected hamsters exhibited sufficiently high virus titers in the lungs with >5 log TCID₅₀/g tissue (**Figure 7H**), although Delta had a slightly lower titer and Omicron had a moderately lower titer than the parental strain, consistent with prior reports for Omicron BA. 1 ([Halfmann et al., 2022](#)).

The 5' end svRNAs were present in all infected lung at levels of >8 log copies/100 ng RNA in all hamsters infected with parental and Delta strains (**Figure 7I**). In marked contrast, these were below the level of detection in lungs infected with Omicron. These data are consistent with the results from cultured human and Vero cells. Taken together, they indicate that SARS-CoV-2 parental virus and Delta clearly produce 5' end svRNAs during viral replication in target cells *in vivo*, but their production is markedly diminished in Omicron-infected cells.

We also assessed whether 5' end svRNAs were released into the sera of SARS-CoV-2-infected hamsters, but the amounts, if any, were below the level of detection in all individuals (**Figure 7J**). This is probably due to the mild-to-moderate symptomatic model of SARS-CoV-2 infection in hamsters. Thus, our study has a limitation with regard to this issue and further studies using clinical serum samples from patients with severe COVID-19 are needed to validate the release of 5' end svRNAs into sera and to carefully document its association with disease aggravation.

Discussion

Here, we found that SARS-CoV-2 generates significantly higher levels of aberrant svRNAs that originate from the exact 5' ends in infected human and Vero cells than do HCoV-OC43 and HCoV-229E. The 5' end svRNAs activated RIG-I and led to high expression of IFN- β and IL-6, both of which can contribute to the hyperinflammatory responses commonly observed in severe COVID-19 (Pedersen and Ho, 2020; Suryawanshi et al., 2021). Most studies investigating this issue have used total RNA libraries for RNA-seq to map viral RNA transcripts, where sRNAs species have rarely been captured. In contrast, RNA preparation technique we opted for here, that was previously established by others (Te Velthuis et al., 2018), included a step whereby only sRNAs (<200-nt) bearing 5' PPP were enriched, which enabled us to capture unexpectedly large amounts of svRNAs bearing 5' PPP by RNA-seq. Our study showed substantial utility of this method to faithfully detect 5' termini of viral RNA genomes. Also, this is, to the best of our knowledge, the first report showing that aberrant svRNAs, produced during SARS-CoV-2 replication, can be associated with exacerbated immune responses in severe COVID-19.

Our analyses resulted in the discovery of small RNAs derived from SARS-CoV-2. There is growing evidence that support the generation of small RNAs originating from RNA viruses including influenza virus (Perez et al., 2010), HCV, Dengue virus, West Nile virus (Parameswaran et al., 2010) and SARS-CoV-1 (Morales et al., 2017). Most svRNAs, recovered both from SARS-CoV-2-infected cells themselves and the exosomes that they release, were the precise 5' end of the positive-sense genome under uncapped conditions. The strand specificity indicated that they were synthesized on an antigenomic intermediate template during replication. One could envisage that the svRNAs were RNA degradation products. However, we found that small numbers of svRNAs with random sizes mapped equally across the entire genome of the CoVs investigated here, implying that these random svRNAs are viral RNA breakdown products and support the notion that the 5' end svRNAs were produced during viral replication. The production of similar 5' terminal viral RNAs was first described during influenza virus replication (Perez et al., 2010; Umbach et al., 2010) despite their shorter length (22-27-nt). The influenza svRNAs correspond to the exact 5' end of the RNA genome and are supposedly involved in transcription-to-replication switching, but not IFN induction. In contrast, our results suggest that SARS-CoV-2 5' end svRNAs can drive robust immune responses. This may be explained by the distinct length spectrum of the 5' end svRNAs (average 25-nt in influenza viruses vs. 65-nt in SARS-CoV-2). Indeed, whereas 5' end svRNAs of SARS-CoV-2 ≥ 63 -nt in length effectively activated the IFN response, shorter 43-nt 5' end svRNAs had a lesser ability to do so (**Figure 5B**). Another difference is that CoVs replicate in the cytoplasm, whereas influenza viruses replicate in the nucleus, so that cytosolic RNA sensors barely have access to intracellular influenza

svRNAs. Many details regarding the biogenesis and function of CoV 5' end svRNAs in the virus life cycle remain to be determined. However, interestingly, SARS-CoV-2 5' end svRNAs mostly end just before the TRS (**Figure 4A-B**), which is actively implicated in the discontinued transcription that is characteristic of CoVs. Furthermore, despite being quantitatively lower, qualitatively similar signatures of 5' end svRNAs were also recovered from cells infected with HCoV-C43 or HCoV-229E (**Figure 4C-E**). Taken together, these findings suggest that these 5' end svRNAs may have certain functional roles in the CoV life cycle, but the high level of their production is abortive in SARS-CoV-2 replication. Unfortunately, our current data can not completely exclude the possibility that CoV 5' end svRNAs are degraded by-products of replication. Nonetheless, regardless of how they are generated, the presence of abundant cytoplasmic SARS-CoV 5' end svRNAs is, at least partially, associated with host immune activation in a late stage of the infection.

In replicating influenza A virus, small aberrant viral RNAs containing both the 5' and 3' ends of viral RNAs were shown to activate RIG-I ([Te Velthuis et al., 2018](#)); erroneous or dysregulated replication by avian influenza virus causes high production of these aberrant RNAs, underlying the high IFN and cytokine inductions in mammals. Similarly, SARS-CoV-2 probably has not completed human adaptation, whereas endemic HCoVs are fully human-adapted viruses. Consistent with its poorer human adaptation, highly abundant svRNAs were present in human/Vero cells or reconstituted human airway epithelia infected with SARS-CoV-2 compared to those infected with HCoV-OC43 and/or HCoV-229E. We thus propose that 5' end svRNAs might be generally produced by dysregulated replication/transcription of newly-emerging CoVs in humans.

CoVs replicate in the cytoplasm of host cells, wherein cytosolic RNA-sensors, including RIG-I and MDA-5, are mostly responsible for monitoring viral RNAs. RIG-I preferentially recognizes short structured RNAs with 5' PPP ends, whereas MDA-5 ligands are much less well-characterized and are presumed to be long structured RNAs, with no requirement for 5' PPP ([Chazal et al., 2018](#)). Thus, SARS-CoV-2 5' end svRNAs that possess 5' PPP and duplex structures are ideal RIG-I ligands and, consistent with this, they activate RIG-I-dependent signaling pathways. In addition to the avian influenza svRNAs ([Te Velthuis et al., 2018](#)), RIG-I was also shown to sense leader-containing short RNAs of other positive-sense RNA viruses such as flaviviruses ([Chazal et al., 2018](#)), where RIG-I recognized the 5' PPP ends of nascent transcripts before capping, inducing IFN secretion. Both RIG-I and MDA5 mediate innate immune responses during SARS-CoV-2 infection ([Kouwaki et al., 2021](#); [Thorne et al., 2021](#)), although MDA5 is regarded as a central mediator of IFN production ([Rebendenne et al., 2021](#); [Yin et al., 2021](#)). These results support the notion that SARS-CoV-2 5' end svRNAs are RIG-I ligands, and likely contribute to excessive immune responses.

CoVs have developed diverse strategies to counteract IFN responses, in particular the type 1 IFN pathway ([Konno et al., 2020](#); [Li et al., 2020a](#); [Sa Ribero et al., 2020](#)). Numerous nonstructural proteins and accessory ORF proteins from various different CoVs were shown to prevent type 1 IFN induction and its downstream STAT1 signaling pathway in human cells ([Frieman et al., 2007](#); [King and Sprent, 2021](#); [Xia et al., 2020](#)). After infection by SARS-CoV-2, viral positive-sense genomic RNA facilitates rapid translation of these proteins, which in turn antagonizes viral RNA-induced RIG-I-IFN signaling. The antagonistic capability of CoV proteins was shown to be more selective for type 1 IFN signaling than NF- κ B signaling

during CoV infections ([Konno et al., 2020](#); [Lei et al., 2020](#); [Sa Ribero et al., 2020](#)). This may promote activation of RIG-I-NF- κ B signaling, leading to release of other cytokines in the absence of IFN responses at early disease stages, as seen with HCoV-229E infection in the present study. Unlike the endemic HCoVs, SARS-CoV-2 infection results in the accumulation of 5' end svRNAs and reaches high levels at later stages of disease; the threshold for RIG-I activation can then be achieved by overcoming the antagonistic ability of viral defense proteins, which in turn drives exuberant IFN production and multiple ISGs, reported to be associated with the immunopathogenesis of COVID-19 ([Huang et al., 2020](#); [Li et al., 2020b](#)). Therefore, it is feasible that the stoichiometric balance between antagonistic viral proteins and agonistic 5' end svRNAs would at least to some extent be associated with the disease development of COVID-19 and delayed IFN- β activation. Our data also demonstrate that Delta has not corrected the erroneous svRNA production that the parent virus exhibited, whereas the Omicron variant (BA.1 and BA.2) may have done so, implying an association with a decreased immune stimulatory signature. However, SARS-CoV-2 evolutionary modification of 5' svRNA production requires further in-depth monitoring to confirm its association with COVID-19 disease progression.

The 5' end sequences of CoVs have the highest similarity among any part of their genomes ([Madhugiri et al., 2014](#)). RNA-based antisense therapy is now widely used. Our studies thus extend the understanding of SARS-CoV-2 immunopathology and shed light on the design of drug targets against COVID-19 and future emerging CoV variants of concern.

Declarations

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AUTHOR CONTRIBUTIONS

Y.A. and Y.W. performed the *in vitro* experiments. I.Y., N.N., and N. K. performed the RNA-seq analysis. T.O. and T. S. performed the hamster infection experiments. Y.A., I.Y., T.O., A.I., N.N., N. K., T.D., T.O., T.N., K.M., D.O., and Y.W. interpreted the results. Y.A., and Y.W. conceptualized the study and designed the experiments. Y.A., and Y.W. wrote the manuscript. All authors reviewed and proofread the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests exist.

Methods

STAR METHODS

Detailed methods are provided in the online version of this paper and include the following:

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RESOURCE AVAILABILITY

Lead Contact

Further information and requests for resource and reagent should be directed to and will be fulfilled by the Lead Contact, Yohei Watanabe (nabe@koto.kpu-m.ac.jp).

Materials Availability

All requests regenerated in this study are listed in the Key Resource Tables and are available from the Lead Contact with a completed Materials and Transfer Agreement.

Data and code availability

The results presented in the study are available from the lead contact upon request.

Experiment model and subject details

Hamster

The hamster experiments were approved by the Institutional Committee of Laboratory Animal Experimentation of Research Institute for Microbial Diseases, Osaka University (R02-08-0). All efforts were made during the study to minimize animal suffering and to reduce the number of animals used in the experiments.

Cell culture

293T cells (human embryonic kidney cell line) and Vero cells (African green monkey kidney cell line) were obtained from the RIKEN BioResource Center Cell Bank. Calu-3 cells (human bronchial epithelial cell line), MRC5 cells (human fetal lung fibroblast cell line) and HCT8 cells (human rectal adenocarcinoma cell line) were obtained from the American Type Culture Collection (ATCC). THP-1 cells and Vero/TMPRSS2 cells ([Matsuyama et al., 2020](#)) were obtained from the Japanese Collection of Research Bioresources Cell Bank. 293T, Calu-3 and MRC5 cells were maintained in Dulbecco's modified Eagle's Medium (DMEM) containing 10% fetal calf serum (FCS). HCT8 and THP-1 cells were maintained in RPMI-1640 medium with 10% FCS. Vero cells and Vero/TMPRSS2 cells were maintained in DMEM containing 10% FCS. 1 mg/ml G418 (Invivogen) was added to the growth medium for Vero/TMPRSS2 cells. Human *ex vivo* reconstituted nasal epithelia was purchased from Epithelix and maintained at an air-liquid interface with provided culture medium, according to the manufacture's instructions.

METHODS DETAILS

Viruses

The parental strain JPN/TY/WK-521/2020 (LC522975), the Delta (B.1.617.2 lineage) strain Japan/TY11-927/2021 (EPI_ISL_2158617), the Omicron (B.1.1.529.1 lineage/BA.1) strain Japan/TY38-873/2021 (EPI_ISL_7418017) and the Omicron (B.1.1.529.2/BA.2) strain Japan/TY40-385/2022 (EPI_ISL_9595859) of SARS-CoV-2 were kindly provided by the National Institute of Infectious Diseases, Tokyo Japan. HCoV-OC43 (ATCC VR-1558) and HCoV-229E (ATCC VR-740) were obtained from the ATCC. SARS-CoV-2 was propagated once in Vero/TMPRSS2 cells in DMEM-F12 containing 0.2% bovine serum albumin (BSA) at 37°C. HCoV-OC43 and HCoV-229E were propagated once in HCT8 cells and MRC5 cells respectively in DMEM-F12 containing 0.2% BSA at 33°C. All viral stocks used in this study were prepared by limiting dilutions of the provided original stocks to eliminate potential inclusion of defective interfering particles in the stocks. Virus titration was performed by measuring focus-forming units (FFU) in focus-forming assays or the median tissue culture infective dose (TCID₅₀) method on Vero cells (SARS-CoV-2 and HCoV-

OC43) and MRC5 cells (HCoV-229E) as described previously ([Arai et al., 2020a](#); [Arai et al., 2019](#); [Watanabe et al., 2018](#)).

Viral infection of cells in cultures

Human cells and Vero were infected with CoVs at a multiplicity of infection (MOI) of 0.001. After 1 h of incubation at 37°C, the cells were washed twice with phosphate-buffered saline (PBS), maintained in DMEM-F-12 medium containing 0.2% BSA, and incubated at 33°C or 37°C.

Immunostaining and confocal microscopy

Cells infected with CoVs were fixed at the indicated times post-infection with 4% paraformaldehyde in PBS for 15 min followed by permeabilization with 0.2% Triton-X for 20 min at room temperature. The infected cells were stained with rabbit antibodies against SARS-CoV-2 NP (GENETEX), HCoV-OC43 NP and HCoV-229E NP (Sino Biological) for 1 h at 37°C, followed by incubation with a secondary antibody conjugated with Alexa Fluor-488 (Invitrogen) at 37°C for 1 h. To confirm the IVT RNA secondary structures, 293T cells were transfected with IVT RNAs and fixed 24 h later, followed by permeabilization as described above. The cells were stained with mouse anti-dsRNA J2 antibody (SCICONS) for 1 h at 37°C, followed by incubation with Alexa Fluor-488 secondary antibody at 37°C for 1 h. Hoechst 33342 (Invitrogen) was used for the counterstaining of nuclei. Immunofluorescence images were captured using an FV3000 confocal laser scanning microscope (OLYMPUS). Foci for dsRNA were quantified using cellSens imaging software (OLYMPUS) from eight randomly selected image fields.

ELISA

The amounts of IFN- β and IL-6 in cell-culture supernatants were quantified using Quantikine kits (R&D Systems), according to the manufacturer's instructions. Optical density at 450 nm was measured with an SH-9000 lab microplate reader (Corona Electric).

Immunoblotting

Cell lysis and immunoblot analysis were performed as described previously ([Arai et al., 2020b](#); [Watanabe et al., 2018](#)). An Amersham Imager 680 (GE Healthcare) was used for chemiluminescence detection. The band intensities were quantified by Amersham Imager 680 Analysis software (GE Healthcare).

Total RNA transfection

Total RNAs were extracted from Vero cells mock-infected or infected with SARS-CoV-2 using miRNeasy Mini kits (QIAGEN), fractionated into small (<200-nt) RNAs and large (>200-nt) RNAs according to the manufacturer's instructions, and transfected into 293T cells and THP-1 cells using Transit-mRNA (Mirus). Proper fractionation of small and large RNAs was verified by the 2100 Bioanalyzer system (Agilent) before use. Poly (I:C) was included as a positive control. At 24 h post-transfection, cell culture supernatants were collected to measure IFN- β and IL-6 levels by ELISA as described above.

RNA fractionation from CoV-infected cells and enzyme treatment for small RNA sequencing

Total RNAs from CoV-infected cells were isolated shortly before the times when titers plateaued at which times cytopathic effects were yet not apparent (72 hpi for CoVs in human/Vero cells at 33°C except 48 hpi for SARS-CoV-2 in Vero cells and at 37°C, 48 hpi and 24 hpi for SARS-CoV-2 in Calu-3 cells and Vero cells, respectively). Total RNAs were extracted and fractionated into small and large RNAs as described above and the small RNA fraction was then treated as described previously ([Te Velthuis et al., 2018](#)) with some modifications. Briefly, the small RNA fraction was treated with XRN-1 (New England BioLabs) in NEB buffer 2 and incubated at 37°C for 1 h to digest host-derived miRNAs harboring 5' P. XRN-1 was inactivated by incubating at 70°C for 10 min. 5' PPP RNAs derived from viral RNAs were converted to monophosphorylated RNAs by RppH treatment at 37°C for 1 h. The enzyme-treated small RNAs were purified using RNA Clean & Concentrator™-25 (ZYMO RESEARCH). The prepared RNAs were applied to RNA-sequencing as described below.

Small RNA sequencing

A miRNA library was constructed using the NEBNext Multiplex Small RNA Library Prep Set for Illumina (NEB) following the manufacturer's instructions. For this, 0.05 ng of RNA was reversed transcribed into cDNA after ligation of the multiplex 3' SR Adaptor, hybridization of the reverse transcription primer, and ligation of the multiplex 5' SR Adaptor. The RNA library was then amplified by 20 PCR cycles using Illumina compatible index primers. The amplified library was resolved on a 2% E-Gel EX agarose gel (Thermo Fisher). DNA fragments corresponding to approximately 150-350 bp (small RNA inserts plus 3' and 5' adaptors) were recovered using QIAquick Gel Extraction Kits (QIAGEN). The library was quantified by Qubit fluorometer (Thermo Fisher) and sequenced on the Illumina NovaSeq 6000 platform using paired end reads (100 bp). The sequencing generated >1,000,000 raw reads from the sample. Adaptor sequences were removed from the raw sequencing reads using the Cutadapt program. The trimmed reads were mapped to the SARS-CoV-2 parental strain genome (LC522975) or the Delta strain (EPI_ISL_2158617) using HISAT2 version 2.1.0 (options: `-pen-noncansplice 0 -no-temp-splicesite -no-softclip -pen-canintronlen G,0,0 -pen-noncanintronlen G,0,0`). The trimmed reads were also mapped to the

host genome (human reference genome sequence (hg19) or *Chlorocebus sabaeus* strain WHO RCB 10-87 unplaced genomic scaffold, Vero_WHO_p1.0 scaffold-1, whole genome shotgun sequence (NW_023666033)) using HISAT2 version 2.1.0 (option: `–no-softclip`).

Large RNA-sequencing

Full-length cDNA was generated using a SMART-Seq HT Kit (Takara Bio) according to the manufacturer's instructions. An Illumina library was prepared using a Nextera DNA Library Preparation Kit (Illumina, San Diego, CA) according to the SMARTer kit instructions. Sequencing was performed using an Illumina NovaSeq 6000 sequencer (Illumina) in the 100-base paired-end mode. Illumina RTA3 v3.4.4 software was used for base calling. Primer sequences were removed from the raw sequencing reads using the Cutadapt program. The trimmed reads were mapped to the SARS-CoV-2 genome of the parental strain (LC522975) or the delta strain (EPI_ISL_2158617) using HISAT2 version 2.1.0 (options: `–pen-noncansplice 0 –no-temp-splicesite –no-softclip –pen-canintronlen G,0,0 –pen-noncanintronlen G,0,0`).

In vitro production of RNA fragments

DNA fragments corresponding to the first 40, 60, 80 and 100-nt in the 5' UTR of SARS-CoV-2, HCoV-OC43 and HCoV-229E genomes were amplified by PCR using specific forward primers containing the T7 promoter sequence (ATTGTAATACGACTCACTATAGGG) and a *Hind III* restriction site, and specific reverse primers containing an *Xba I* restriction site. Amplified fragments were digested with *Hind III* and *Xba I* and cloned into enzyme-digested pUC18. Plasmid DNAs were linearized with *Xba I*, purified with QIAquick PCR Purification Kits (QIAGEN) and used as templates for T7 *in vitro*-transcription using the T7 RiboMAX™ Express Large Scale RNA Production System (PROMEGA). IVT RNAs were gel-purified and then further purified with miRNeasy mini kits. For preparation of dephosphorylated RNAs, IVT RNAs were treated with CIP for 1 h at 37°C and the treated RNAs were then purified with RNA Clean & Concentrator™-25 (ZYMO RESEARCH). Ribo m7G Cap Analog (PROMEGA) was used to synthesize Cap-0 RNA transcripts. Cap-0 RNAs were then converted to Cap-1 RNAs with mRNA Cap 2'-O-Methyltransferase (NEB) and purified with RNA Clean & Concentrator™-25. IVT RNAs were transfected into 293T cells and PMA-stimulated THP-1 cells using Transit-mRNA (Mirus). At 24 h post-transfection, cell culture supernatants were collected to measure IFN- β and IL-6 levels by ELISA as described above.

siRNA transfection

siRNA for RIG-I (GACUAGUAAUGCUGGUGUAUU with dTdT overhangs) was chemically synthesized by a gene synthesis service (Fasmac). Silencer™ Select Pre-Designed siRNA was used for MDA5 silencing (s34499; Thermo Fisher). siRNAs were transfected using Transit-TKO (Mirus) and incubated for 48 h. To

validate silencing, total RNAs were isolated from siRNA-transfected cells using RNeasy Mini Kits (QIAGEN) and qPCR was performed for RIG-I (Hs01061436_m1; Thermo Fisher), MDA5 (Hs00223420_m1; Thermo Fisher) and 18s ribosomal RNA (Hs99999901_S1; Thermo Fisher). Data were normalized to the expression levels of 18S ribosomal RNA for each sample and the DDCT method was used for the relative value quantification.

Stem-loop qRT-PCR

Total RNA was extracted from virus-infected cells at the indicated time points, or from lungs and sera of hamsters 5 dpi using miRNeasy mini kits. RNA was reverse transcribed using TaqMan MicroRNA Reverse Transcription Kits (Applied Biosystems) and qPCR was performed using TaqMan Fast Advanced Master Mix (Applied Biosystems) according to the manufacturer's instructions. Stem-loop RT primer, primers and probes for qPCR were designed by Custom TaqMan Small RNA Assays (Applied Biosystems) based on the representative sequences of 5' end svRNA from SARS-CoV-2 (ATTAAAGGTTTATACCTTCCCAGGTAACAAACCAACCAACTTTTCGATCTCTTGTTAGATCTGTT) and HCoV-OC43 (ATTGTGAGCGATTTGCGTGCGTGCATCCCGTTTCACTGATCTCTTGTTAGATCTTTTGTAACTAACTTT). The specificity of the SARS-CoV-2 63-nt specific stem-loop qRT-PCR was tested using corresponding 5' end svRNA templates with different 5' end lengths (43, 63, 150 and 265-nt) that were transcribed *in vitro*, followed by gel-purification as described above. For Calu-3 cell samples, TaqMan MicroRNA Assays for snRNA-U6 (001973) were used as controls for normalization of small RNAs from cells and the relative expression was calculated by the DDCT method using the 8 hpi samples as a reference with a set value of one relative unit. For hamster samples, 100 ng total RNA were used for qRT-PCR.

Virus infection of *ex vivo* reconstituted human nasal epithelia

The MucilAir system is a reconstituted human nasal epithelium, consisting of ciliated, goblet and basal cells. Cultures were maintained under air/liquid interface (ALI) conditions in transwells with 700 µL MucilAir medium in the basal compartment. Prior to viral infection, the apical surface was washed twice with 200 µl MucilAir medium (20 min at 37°C) to remove mucus. Cells were infected with SARS-CoV-2 or HCoV-C43 on the apical side at an MOI of 0.1 in 150 µl MucilAir medium for 1.5 h at 37°C. Viral inoculum was removed and cells were washed twice with MucilAir medium (20 min at 37°C) before continuing culture for 24, 48 and 96 h. Apical supernatants were harvested by adding 200 µl MucilAir medium on the apical side and incubating for 20 min at 37°C prior to collection, after which viral titers were determined by FFU assays. Intracellular RNA of each well was harvested using miRNeasy mini kits and 5' end svRNA levels were quantified by stem-loop qRT-PCR as described above. For SARS-CoV-2, a stem-loop primer specific for the most common 63-nt 5' end svRNA was used, whereas for HCoV-OC43, a stem-loop primer

specific for the most common 72-nt 5' end svRNA was used. Basal medium was harvested to measure IFN- β and IL-6 production levels as described above.

RNA immunoprecipitation (RIP) assay

The RIP assay was conducted using the kit for microRNA (MBL) according to the manufacturer's protocol. Calu-3 cells infected with SARS-CoV-2 at 72 hpi were lysed with lysis buffer and pre-cleared with Protein G Dynabeads (Thermo Fisher). The supernatants were incubated with Protein G immobilized with anti-RIG-I antibody (#3743; Cell Signaling) with gentle rotation for 3 h at 4°C. The beads were then washed $\times 3$ with wash buffer. For RNA-seq, the sRNA fraction was extracted from the total RNA bound to the beads using RNeasy Mini Kits (QIAGEN).

IFN- β antagonism assay

A pcXN2 plasmid expressing SARS-CoV-2 ORF6 fused with a Flag-tag at the 3' terminus (0, 50, 100, 150 or 300 ng per well) was transfected into 293T cells (in a 24-well plate) with Transit-LT1 Reagent (Mirus). At 24 h post-transfection, cells were stimulated with 2.5×10^{11} copies of IVT-RNA corresponding to the first 60-nt of the 5' end from the SARS-CoV-2 genome using Transit-mRNA (Mirus). At 48 h post transfection, the cells and culture supernatants were harvested for Western blotting and ELISA to determine ORF6 expression and IFN- β production.

Isolation of exosomes

Culture supernatants from SARS-CoV-2-infected cells were collected and centrifuged at $3000 \times g$ for 15 min to remove cell debris and then filtered through a $0.45 \mu\text{m}$ filter. Then, 90 ml of filtered supernatant was concentrated to a final volume of 1 ml using Amicon Ultra-4 Centrifugal Filter Units with Ultracel-100 membranes (Millipore). The concentrated culture supernatants were mixed with ExoQuick-TC (System Bioscience). Exosomes in the concentrates were immunoprecipitated using anti-CD63 antibody-conjugated Dynabeads Protein G (Thermo Fisher) according to the manufacturer's instructions. Total RNAs were extracted from exosomal samples using miRNeasy mini kits and investigated by next generation sequencing as described above. The concentrated exosomal samples and immunoprecipitated exosomal samples were resolved by SDS-PAGE and transferred onto a polyvinylidene difluoride membrane (Millipore). The S protein of SARS-CoV-2 and exosome marker CD63 were detected with specific primary antibodies and HRP-conjugated secondary antibodies. The Amersham ECL Select Western Blotting Reagent was used for band visualization. The band intensities were quantified by Amersham Imager 680 Analysis Software (GE Healthcare).

Hamster infection model

Four-week-old female Syrian hamsters were purchased from SLC Japan. Under mixed anesthesia (medetomidine-butorphanol-midazolam), the animals were inoculated intranasally with 1.0×10^6 plaque-forming units SARS-CoV-2 (in 60 μ l) as described previously ([Higuchi et al., 2021](#)). On day 5 post-infection, all animals were euthanized and lungs and sera were collected for viral titration and/or stem-loop qRT-PCR.

QUANTIFICATION AND STATISTICAL ANALYSIS

Data analyses were performed using GraphPad Prism Version 6 software (GraphPad Software). Statistically significant differences between virus pairs were determined by ANOVA with Tukey's multiple comparison test. Data are presented as the means $\pm$ SD.

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Figures

Figure 1. SARS-CoV2 stimulates IFN expression in the late stages of infection.

(A and B) Replication kinetics of SARS-CoV-2, HCoV-229E and HCoV-OC43 in human cells. Cells were infected with CoVs at an MOI of 0.001 and incubated at 33°C (for HCoV-OC43 and HCoV-229E) and both at 33°C and 37°C (for SARS-CoV-2). (A) Confocal microscopy images of CoV-infected human cells. (B) At the indicated times post-infection, cells were stained with anti-viral NP antibodies (green). Nuclei were stained with Hoechst 33342 (blue). Progeny virus titers were measured by focus forming assays at the indicated times post-infection. (C and D) IFN-β (C) and IL-6 (D) released from CoV-infected human cells. At the indicated times post-infection, the supernatants were harvested for ELISA. (E) Immunoblot analysis of RIG-I in lysates from CoV-infected human cells at the indicated times post-infection. Representative images from three independent experiments are shown. Quantification of band intensity is expressed relative to 24 h post-infection. (F) RNAi mediated depletion of RIG-I and MDA-5. The expression levels of RIG-I and MDA-5 for mock was set to 100%. (G) IFN-β released from siRNA-transfected cells. At 72 h (for MRC5 cells and Calu-3 cells at 33°C) or 48 h (for Calu-3 cells at 37°C) post-infection, the supernatants were harvested for ELISA. Each data point is the mean ± SD from three independent experiments. Statistically significant differences compared to mock transfected cells are shown as **P < 0.01 (ns = not significant).

Figure 1

See figure for legend.

Figure 2. Most CoV svRNAs map to positive-strand genomes with the highest coverage at the 5' UTRs.

(A) Schematic of RNA preparation and RNA-seq in this study. The RNA preparation method was previously established by others (Te Velthuis et al., 2018). (B) Summary of RNA reads mapped to CoV genomes. (C) sRNA-seq reads mapped to the viral genomes of CoVs. Reads were strand-specifically mapped to positive-sense (+) RNAs or negative sense (-) RNAs. Read counts were quantified for each nucleotide of the genome.

Figure 2

See figure for legend.

Figure 3. SARS-CoV-2 dominantly produces 5' UTR svRNAs in infected human and Vero cells.
 (A) Schematic of svRNA fragments produced by CoVs. The svRNAs are divided into three groups: 1) svRNA fragments containing only the 5' UTRs (termed 5' UTR svRNAs); 2) svRNA fragments including the leader sequence and TRS jumping to the exact start of major N/M/S ORFs (termed N/M/S ORF coding svRNAs); 3) svRNA fragments harboring leader sequence and TRS fused to other ORFs and all fragments excluding the 5' UTR (termed other svRNAs). TRS = transcriptional regulatory sequence. (B) Counts of svRNA fragments produced in CoV-infected cells. (C) Fraction of svRNA fragments produced in CoV-infected cells. The numbers (B) and fractions (C) of each svRNA category in the svRNA population of each infection are shown, according to the color legend in the figure.

Figure 3
 See figure for legend.

Figure 4. SARS-CoV-2 produces excessive amounts of 5' UTR svRNAs encoding the precise 5' end with the representative first 63-nt sequence. (A-E, left panels) Read maps of 5' UTR svRNAs produced by CoVs. Reads were mapped to positive sense RNA. Read counts were quantified for each nucleotide. (A-E, right panels). Size distribution of 5' UTR svRNAs. (F) 5' UTR svRNA fragments with the highest counts in CoV-infected cells.

Figure 4

See figure for legend.

Figure 5. The first 60-nt of 5' end svRNAs with 5' PPP and a duplex structure act as RIG-I agonists. (A) Sequence of 5' UTR svRNAs of SARS-CoV-2 used in this study. (B) Induction of IFN- β and IL-6 secretion from 293T cells and differentiated THP-1 cells by transfection of IVT 5' UTR svRNAs from SARS-CoV-2. Cells cultured in 24-well plates were stimulated by transfection of 40, 60, 80 and 100-nt IVT 5' UTR svRNAs without/with CIP treatment or capping (Cap1). At 24 h after stimulation, the supernatants were harvested for ELISA. (C) Immunoblot analysis of RIG-I in lysates from IVT 5' UTR svRNA-stimulated human cells 24 h post-transfection. Representative images from three independent experiments are shown. (D) Secondary structure of 100-nt IVT 5' UTR svRNA of SARS-CoV-2, predicted by RNAfold with default parameters. SL indicates stem-loop. (E and F) Accumulation of an epitope recognized by the J2 antibody in 293T cells transfected with IVT 5' UTR svRNA of SARS-CoV-2. At 24 h post-transfection, cells were stained with the J2 anti-dsRNA antibody (green). Nuclei were stained with Hoechst 33342 (blue). (E) Representative images of dsRNA foci visualized by the J2 antibody. (F) dsRNA foci were quantified using Olympus imaging software from eight randomly selected image fields. (G and H) 293T cells were mock-transfected or transfected with siRNAs against RIG-I (siRIG-I) and MDA-5 (siMDA-5). (G) Levels of RIG-I and MDA-5 mRNA in cells at 24 h post-transfection were measured by real-time RT-qPCR and are expressed relative to mock-transfected cells. (H) Induction of IFN- β secretion from 293T wild-type or RIG-I/MDA-5 knockdown cells by stimulation with IVT 5' end svRNAs of SARS-CoV-2. At 24 h after mock-transfection or transfection with siRIG-I or siMDA-5, 293T cells were stimulated with IVT 5' UTR of SARS-CoV-2. The supernatants were harvested for ELISA 24 h after stimulation. IFN- β levels are expressed relative to wild-type cells. Each data point is the mean $\pm$ SD from three independent experiments. Statistically significant differences compared to 40-nt IVT svRNA-stimulated cells (B and H) or mock-transfected cells (G and H) are shown as $P < 0.01$ (ns = not significant). (I-K) sRNA-seq analysis of sRNAs recognized by RIG-I. Calu-3 cells were infected with SARS-CoV-2 as indicated in the legend to Figure 1. At 72 hpi, sRNA fractions were extracted from total RNAs immunoprecipitated with anti-RIG-I antibody. These immunoprecipitates were evaluated by sRNA-seq. (I) sRNA-seq reads mapped to the SARS-CoV-2 genome. Reads were strand-specifically mapped to the positive-sense (+) RNAs or negative-sense (-) RNAs. Read counts were quantified for each nucleotide of the genome. (J) Read map of 5' UTR svRNAs from RIG-I. Reads were mapped to the positive sense RNA. Read counts were quantified for each nucleotide. (K) Size distribution of 5' UTR svRNAs from RIG-I. A representative svRNA fragment with the highest count is also shown.

Figure 5

See figure for legend.

Figure 6. SARS-CoV-2 5' end svRNAs accumulate in cells at late stage of infection *in vitro* and *ex vivo*.

Schematic of stem-loop RT-qPCR detection of SARS-CoV-2 5' end svRNA. cDNA was generated using stem-loop RT primer specific for the representative 5' end svRNA with 63-nt. The qPCR was performed with forward and reverse primers that targeted the 5' end region of svRNA and part of the stem-looped RT primer respectively, as well as the TaqMan MGB probe. (B) Verification of specificity of the stem-loop primers for 5' end svRNA of SARS-CoV-2. The specificity of the SARS-CoV-2 63-nt specific primer set was verified using SARS-CoV-2 5' end svRNA templates with different lengths that were prepared by *in vitro* transcription. Stem-loop qRT-PCR was performed with high dose (10¹⁰ copies) and low dose (10⁶ copies) of each template RNA. The specificity of the primer set is shown with respect to the percent of the corresponding 63-nt RNA template. (C) RT-qPCR quantification of 5' end svRNA levels at 8, 24, 48, 72, 96 and 120 hpi in SARS-CoV-2-infected Calu-3 cells at 33°C or 37°C as indicated in the legend to Figure 1. Levels of 5' end svRNAs were related to those at 8 hpi, as calculated by the $\Delta\Delta C_t$ method using snRNA-U6 as an endogenous control. (D-H) Production of SARS-CoV-2 and HCoV-OC43 5' end svRNA in *ex vivo* human nasal epithelia reconstituted at the air-liquid interface. (D) Schematic of the experiment. SARS-CoV-2 or HCoV-OC43 were inoculated onto reconstituted human nasal epithelia at an MOI of 0.1, and cultured for 24, 48 and 96 hpi. For collection at the apical side, medium was added for 20 min at 37°C to elute the virus. (E) Virus replication kinetics. Titers of progeny viruses released into apical supernatants were measured by focus-forming assays at the indicated times post-infection. (F) RT-qPCR quantification of intracellular 5' end svRNA levels at the indicated times post-infection. Levels of 5' end svRNAs were related to those at 8 hpi as described above. (G and H) IFN-β (G) and IL-6 (H) released from virus-infected cells. At the indicated times post-infection, basal medium was harvested for ELISA. (I) Counteraction between SARS-CoV-2 ORF6 and 5' end svRNAs. 293T cells were transfected with four amounts of plasmid expressing SARS-CoV-2 ORF6 fused with a Flag-tag. At 24h post-transfection, the cells were stimulated with 63-nt IVT 5' end svRNAs from SARS-CoV-2. At 48 h post-transfection, cells and supernatants were harvested for Western blotting and ELISA. For Western blotting one representative result of three independent experiments is shown. For ELISA, the value of the IVT-RNA-stimulated and empty-plasmid-transfected cells was set to 100%. Statistically significant differences compared to values at 8 hpi (C and F), 24 hpi (G and H) and mock-transfected cells (I) are shown as *P < 0.01.

Figure 6

See figure for legend.

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