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Full Length Article

Differential immune activation profile of SARS-CoV-2 and SARS-CoV infection in human lung and intestinal cells: Implications for treatment with IFN-β and IFN inducer

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\textbf{Objectives:} Respiratory and intestinal tract are two primary target organs of SARS-CoV-2 infection. However, detailed characterization of the host-virus interplay in infected human lung and intestinal epithelial cells is lacking.

\textbf{Methods:} We utilized immunofluorescence assays, flow cytometry, and RT-qPCR to delineate the virological features and the innate immune response of the host cells against SARS-CoV-2 infection in two prototype human cell lines representing the human lung (Calu3) and intestinal (Caco2) epithelium when compared with SARS-CoV.

\textbf{Results:} Lung epithelial cells were significantly more susceptible to SARS-CoV-2 compared to SARS-CoV. However, SARS-CoV-2 infection induced an attenuated pro-inflammatory cytokines/chemokines induction and type I and type II IFN responses. A single dose of 10 U/mL interferon-β (IFNβ) pretreatment potently protected both Calu3 and Caco2 against SARS-CoV-2 infection. Interestingly, SARS-CoV-2 was more sensitive to the pretreatment with IFNβ and IFN inducer than SARS-CoV in Calu3.

\textbf{Conclusions:} Despite robust infection in both human lung and intestinal epithelial cells, SARS-CoV-2 could attenuate the virus-induced pro-inflammatory response and IFN response. Pre-activation of the type I IFN signaling pathway primed a highly efficient antiviral response in the host against SARS-CoV-2 infection, which could serve as a potential therapeutic and prophylactic maneuver to COVID-19 patients.

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Summary

Screening of the clinical specimens from COVID-19 patients showed large quantities of viral shedding in respiratory secretions and stool, which indicate that the respiratory and intestinal tract were primary target organs of SARS-CoV-2 infection. However, detailed characterization of the virus-host interplay in infected human lung and intestinal epithelial cells is lacking. Therefore, we utilized immunofluorescence assays, flow cytometry, and RT-qPCR to delineate the virological features and the innate immune response of the host cells against SARS-CoV-2 infection in two prototype human cell lines representing the human lung (Calu3) and intestinal (Caco2) epithelium when compared with SARS-CoV. Importantly, Calu3 was 100-fold more susceptible to infection by SARS-CoV-2 than SARS-CoV. In contrast, Caco2 supported robust infection of both viruses. Unlike SARS-CoV infection of Calu3, which induced upregulation of nine out of thirteen (69.23%) pro-inflammatory cytokines/chemokines and IFN stimulated genes (ISGs) tested, SARS-CoV-2 only upregulated the expression of IP-10, indicating that SARS-CoV-2 effectively suppresses the innate immune and inflammatory response. Intriguingly, SARS-CoV-2 replication was more susceptible than SARS-CoV to suppression by pretreatment with IFNβ or its inducer, polyinosinic-
polycytidic acid, suggesting type I IFN pathway as a key host antiviral response and should be considered in the therapy or prophylaxis against COVID-19.

Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) was first reported from Wuhan in December 2019. It spread rapidly and has caused over 3.5 million laboratory-confirmed cases with over 0.25 million deaths globally in less than 6 months. SARS-CoV-2 is the seventh human coronaviruses and belongs to Betacoronaviruses, the same genus as the other two highly pathogenic human coronaviruses, severe acute respiratory syndrome coronavirus (SARS-CoV) and Middle East respiratory syndrome coronavirus (MERS-CoV). A prominent feature of SARS-CoV-2 is its unusually high transmissibility among close contacts in family, nursing facility, cruise ship, and also the hospital setting. The estimated basic reproductive number (R0) of SARS-CoV-2 ranges from 1.4 to 6.49 (median 2.79), which is higher than that of SARS-CoV.

Clinical symptoms of COVID-19 generally resemble those observed in SARS, ranging from relatively mild common cold-like illness such as cough, fever, myalgia and fatigue, to severe dyspnea, chest pain, multifocal peripheral ground-glass pneumonia, acute respiratory distress syndrome (ARDS), and multiorgan dysfunction syndrome. Unlike SARS-CoV, a significant proportion of COVID-19 cases were mildly symptomatic or asymptomatic. Moreover, prolonged viral shedding in high titer was detected in the nasopharyngeal and oropharyngeal swabs from the asymptomatic individuals, which might have facilitated the efficient transmission of SARS-CoV-2 in immune naive population worldwide. Apart from infection in the airway mucosa and the pulmonary alveoli, the gastrointestinal tract is also susceptible to highly pathogenic human coronaviruses, including SARS-CoV and MERS-CoV. Besides, viral RNA could also be detected in the intestinal tissues of SARS-CoV-2-infected hamsters. Interestingly, SARS-CoV-2 appeared to replicate more efficiently in the human bronchus and lung than that of SARS-CoV. In contrast, while diarrhea was the most common extrapulmonary clinical manifestation of SARS and was reported in up to 130 (20%) of 647 SARS patients, it was uncommon in COVID-19 (42/1099, 3.8%). Suggesting less enteric involvement of SARS-CoV-2 than SARS-CoV. Despite these differential virological and clinical observations between SARS and COVID-19, the underlying mechanisms of these differences remain largely unexplored. We previously reported that Calu3 and Caco2 both supported robust replication of SARS-CoV-2 among nine human cell lines tested. In this study, we further investigated the efficacy of infection, innate immune, and inflammatory response induced by SARS-CoV-2 in Calu3 and Caco2 as prototype cell lines that mimic virus infection in the human lung and the intestinal tract, respectively. Using SARS-CoV as a control, our results revealed substantial differences in the infection and host innate immune response between these two highly pathogenic human coronaviruses, thus providing scientific basis for the therapeutic or prophylactic treatment for COVID-19 patients.

Material and methods

Virus and cells

SARS-CoV-2 HKU-001a (GenBank accession number: MT230940) was isolated from the nasopharyngeal aspirate specimen of a clinical patient with laboratory-confirmed COVID-19 as we previously described. SARS-CoV GZ50 (GenBank accession number: AV304495) was a 2003 isolate archived in the Department of Microbiology at the University of Hong Kong. Both strains were propagated in VeroE6 cells. Viral titers from supernatants were assessed in VeroE6 with plaque assays. Experiments involving live viruses were performed in accredited biosafety level-3 (BSL-3) laboratory. VeroE6 and Caco2 (ATCC) were cultured with the minimal essential medium (MEM) supplemented with 1% P/S, and 10% or 20% fetal bovine serum (FBS), respectively. Calu3 was cultured with Dulbecco’s minimal essential medium F12 (DMEM/F12) supplemented with 20% fetal bovine serum (FBS), 1% penicillin/streptomycin (P/S). At the point of virus inoculation, FBS in the culture medium was removed. All cell lines used in this study was tested negative for mycoplasma contamination.

RNA extraction and quantitative reverse transcription polymerase chain reaction (RT-qPCR)

Infected cells and supernatants were lysed at 2, 24, 48, 72 and 120 h post-infection (hpi) and extracted with QIAamp viral RNA mini kit (QIAGEN) or QIAeasny mini kit (QIAGEN) according to manufacturer’s instructions. For virus replication kinetics assays, the extracted RNA was quantified with the one-step QuantiNova Probe RT-PCR kit (QIAGEN). For profiling the host genes, the extracted RNA was reverse transcribed with Transcriptor First Strand cDNA Synthesis Kit (Roche). Gene expression was quantified by qPCR with LightCycler 480 SYBR Green I Master (Roche) and normalized to mock-infected controls or GAPDH gene expression as we previously described. Primer sequences used in this study were listed in supplementary Table 1.

Infectivity assay

To determine the infectivity of SARS-CoV-2 and SARS-CoV, Calu3 and Caco2 cells were infected with SARS-CoV-2 or SARS-CoV at 0.002, 0.02, 0.2, 2 and 20 MOI. Cells were fixed with 10% neutral-buffered formalin solution at 24 hpi for immunofluorescence staining to visualize viral antigen expression.

Immunofluorescence staining and imaging

Cells were fixed with 10% neutral-buffered formalin solution at the designated time points. Viral antigen expression was detected with an in-house polyclonal rabbit anti-serum against the nucleocapsid protein of SARS-CoV but also cross-react with that of the SARS-CoV-2. The Alexa Fluor 488 goat anti-rabbit antibody was obtained from ThermoFisher Scientific. Nuclei were stained with Prolong antifade mountant with DAPI (ThermoFisher Scientific). Images were obtained with Olympus BX53 fluorescence microscope. Relative fluorescence units of the fluorescence images were quantified with ImageJ.

Flow cytometry

To quantitatively compare the amount of antigen expression in cells infected by SARS-CoV-2 and SARS-CoV, flow cytometry was performed with Calu3 and Caco2 infected at 0.1, 1 and 10 MOI as previously described with slight modifications. Briefly, cells were detached with EDTA-trypsin, fixed with neutral-buffered 10% formalin solution, permeabilized with 0.1% Triton-X-100 in PBS, and nucleocapsid protein was immunolabeled for flow cytometry analysis with a LSR Fortessa analyzer (BD). Data obtained were analyzed with FlowJo 7.6.5 software (FlowJo LLC.).

IFN and IFN inducer pretreatment assay

To investigate the sensitivity of SARS-CoV-2 to IFN and IFN inducer, Calu3 and Caco2 cells were pretreated with 0, 10, 50, 100,
500, or 1000 U/mL IFN-α (R&D Systems), -β (Abcam) or -γ (Abcam) and polyinosinic-polyribocytidylic acid (poly(I:C)) (Invivogen) for 18 h prior to infection. After pretreatment, cells were inoculated with SARS-CoV-2 at a MOI of 2 and were fixed with 10% neutral-buffered formalin solution at 24 hpi for immunofluorescence staining. Cell lysates and supernatants were harvested at 24 and 48 hpi for viral load quantification with one-step RT-qPCR. Mock-infected cell lysates were harvested for RT-qPCR analysis at the point of infection to determine the expression levels of ISGs and SARS-CoV-2 entry-related host factors.

**Statistical analysis**

All data obtained were analyzed with GraphPad Prism 7.0 software. Statistical analyses for two groups were determined using unpaired Student’s t-test. Statistical analyses among three or more groups were computed with one-way or two-way ANOVA. Two-tailed p-value < 0.05 was considered statistically significant.

**Results**

**Lung epithelial cells were more susceptible to SARS-CoV-2 infection**

To explore the differential tropism of SARS-CoV-2 and SARS-CoV, we compared the virus susceptibility in Calu3 and Caco2 using MOIs ranging from 0.002 to 20. The efficacy of infection was visualized by the detection of viral nucleocapsid (N) protein upon immunofluorescence staining. Supporting our findings on the replication kinetics profile (Fig. S1), the infectivity of SARS-CoV-2 in Calu3 and Caco2 were similarly efficient, as indicated by the comparable expression level of viral nucleocapsid protein at 0.02 MOI or above (Fig. 1, left panels). However, for SARS-CoV infection, drastic differences in infection efficiency were observed between the two cell lines. Particularly, SARS-CoV is substantially less capable of infecting Calu3, which was indicated by the low abundance of virus antigen detected at 2 MOI and was further reduced to almost non-detectable level at 0.2 MOIs or below (Fig. 1, right panels). Notably, Calu3 infected with 20 MOI of SARS-CoV has a similar nucleocapsid protein expression level as those infected with only 0.2 MOI of SARS-CoV-2, thus amounting to approximately 100-fold difference in protein expression between the two viruses in this cell type (Fig. 1 and Fig. S2). Since ACE2 and TMPRSS2 were reported to be essential for SARS-CoV-2 entry, we profiled the expression of these two genes in Calu3 and Caco2. In general, the expression of ACE2 in Calu3 was around 1-to-4 folds higher than Caco2 while the expression of TMPRSS2 was approximately 4-to-64 folds more abundant in Caco2 than Calu3 (Fig. S3). Next, we further quantitatively investigated the differential infection of SARS-CoV-2 and SARS-CoV in Calu3 and Caco2 with flow cytometry. Similarly, SARS-CoV-2 infected Calu3 significantly more efficiently than SARS-CoV, as indicated by the consistently higher percentages of N positive cells across three different MOIs examined (Fig. 2A and 2C, nucleocapsid positive cells 1.32% vs 0.75%; 2.06% vs 0.81%; 4.65% vs 1.08%) as well as the significantly higher mean fluorescence intensities (Fig. 2D). In Caco2 cells, SARS-CoV infection was more efficient than SARS-CoV-2 at 0.1 MOI (nucleocapsid positive cells 8.98% vs 5.83%, p < 0.05) but the difference in the infection rate decreased as MOIs increased to 1 or above (Fig. 2B, E and F). Overall, our data suggested that SARS-CoV-2 infected human lung epithelial cells more efficiently than SARS-CoV, while SARS-CoV infected human intestinal cells more efficiently than that of SARS-CoV-2.

SARS-CoV-2 launched an attenuated IFN and pro-inflammatory response

Next, we asked whether SARS-CoV-2 and SARS-CoV would activate the IFN and pro-inflammatory response in Calu3 and Caco2 cells. To this end, we evaluated the expression of five pro-inflammatory cytokines/chemokines and a panel of eight genes associated with type I or type II IFN response. To our surprise, despite robust SARS-CoV-2 replication in Caco2, only IP-10 was significantly upregulated among the five cytokines/chemokines tested (Fig. 3B, p < 0.01). Besides, SARS-CoV also upregulated only IP-10 to a similar magnitude as that induced by SARS-CoV-2 in Caco2 (Fig. 3B, p < 0.01). However, drastic differences in the expression profile of the proinflammatory cytokines/chemokines in Calu3 were observed between the two viruses. Unlike SARS-CoV, which significantly upregulated the expression of TNFα, IL-8, IP-10 and RANTES, none of these proinflammatory genes were substantially induced by SARS-CoV-2 infection in Calu3 across five time points examined (Fig. 3A). Consistent with expression profiles of the proinflammatory cytokines/chemokines, the eight genes tested for type I and type II IFN response were not significantly activated by either SARS-CoV-2 or SARS-CoV in Caco2. Interestingly, SARS-CoV-2 infection induced the upregulation of 62.5% (five out of eight) of the IFNs or ISGs tested in Calu3, including IFNα and IFNβ (type I IFN), IFNγ (type II IFN), IFIT1 and IFITM3 (Fig. 4A). On the contrary, both type I and type II IFN response were attenuated upon SARS-CoV-2 infection in Calu3 (Fig. 4A). In summary, our results demonstrated that the virus-induced innate immune response was largely diminished in the intestinal epithelial cells upon the infection of both SARS-CoV-2 and SARS-CoV. However, substantial differences were observed between the two cell lines. Importantly, SARS-CoV-2 induced a significantly attenuated innate immune response in lung epithelial cells compared to SARS-CoV despite more robust virus infection and propagation.

SARS-CoV-2 was more sensitive to IFN-β and IFN inducer than SARS-CoV

Since type I IFN response, which was reported to be critical for host defense against various viral infections, were not significantly activated by SARS-CoV-2, we hypothesized that pre-activation of IFN signaling pathway to prime the antiviral response of the host might be highly beneficial to restricting SARS-CoV-2 infection. We tested this hypothesis by pretreating Calu3 and Caco2 cell lines with IFNβ (type I IFN) at increasing concentrations of 0, 100, 500 and 1000 U/mL. We first verified the potency of IFNβ by examining the expression levels of five representative IFN-stimulated genes (ISGs). Expectedly, all five ISGs were efficiently upregulated by IFNβ pretreatment (Fig. S4). Next, we investigated the antiviral potency of IFNβ and found that a single dose of 100 U/mL IFNβ pretreatment prior to SARS-CoV-2 infection reduced around 70% of virus production in Calu3 with the inhibitory effect lasted at least until 48 hpi (Fig. 5A and 5B). Under the same IFNβ pretreatment, we observed a less than 25% reduction in virus production in Calu3 infected with SARS-CoV (Fig. 5A and 5B). Consistently, IFNβ pretreatment was more potent against SARS-CoV-2 than SARS-CoV in Caco2 (Fig. 5C and 5D). We further reduced the dosage of IFNβ pretreatment to more physiologically feasible levels and the results showed that the IFNβ pretreatment effectively suppressed the expression of viral antigen at a concentration as low as 10 U/mL (Fig. 5E). ACE2 was recently identified as a potential ISC, which might compromise the effectiveness of IFNβ treatment. By evaluating the expression of ACE2 upon IFNβ treatment, our data showed that IFNβ treatment did not upregulate ACE2 expression.
Discussion

Despite the differential tissue-specific virological and clinical observations between SARS and COVID-19, the underlying mechanisms of the differences remain largely unexplored. Using Calu3 and Caco2 as two prototype cell lines to mimic SARS-CoV-2 infection of the human lung and intestinal epithelium, we demonstrated several key differences between SARS-CoV-2 and SARS-CoV in infection efficiency, host innate immune response, as well as the sensitivity to IFN pretreatment. First, we showed that SARS-CoV-2 infectivity was more robust than SARS-CoV in Calu3. Since one of the most prominent features of SARS-CoV-2 is its high person-to-person transmissibility and infectability,\(^2,5,8\) our findings provided possible explanations by addressing the important question of whether the infectivity of SARS-CoV-2 differs with that of SARS-CoV in human lung epithelial cells. In contrast, SARS-CoV infected intestinal epithelial cells more efficiently, which was particularly appreciable at lower MOIs. Our findings corroborated with the clinical observations that although both COVID-19 and SARS patients could shed viral RNA through the gastrointestinal tract,\(^31,32\) diarrhea was five-folds more frequently observed in SARS (20%) than in COVID-19 (3.8%) patients.

Viruses can employ a variety of mechanisms to suppress the host innate immune system,\(^33-35\) thus evading from crucial antiviral responses, like the IFN signaling pathways, to facilitate virus propagation and dissemination. Here, our study demonstrated that SARS-CoV-2 infection induced attenuated pro-inflammatory cytokines/chemokines and IFN responses in both Calu3 and Caco2 cells, despite robust virus infection and propagation. Importantly,
Fig. 2. Quantification of the differential infectivity of SARS-CoV-2 and SARS-CoV. Calu3 and Caco2 were infected with SARS-CoV-2 or SARS-CoV at 0.1, 1 and 10 MOI. Cells were detached with EDTA-trypsin at 24 hpi, fixed with neutral-buffered 10% formalin and immunolabeled with an in-house rabbit antiserum against the SARS-CoV N protein but also cross-react with SARS-CoV-2 N protein. (A and B) Representative pseudocolor dot plots showing the infection rate of SARS-CoV-2 and SARS-CoV in Calu3 (A) and Caco2 cells (B). (C and E) Quantification of the percentage of nucleocapsid protein positive Calu3 (C) and Caco2 (E) cells. (D and F) Quantification of mean fluorescence intensity in the infected Calu3 (D) and Caco2 (F) cells. The results represented the mean and standard deviation of three independent experiments. Statistical significance between two groups at each time point was performed with unpaired Student’s t-test. *, ** and *** represented p value <0.05, <0.01 and <0.001, respectively.
these attenuated host responses in the presence of robust virus replication at the primary infection sites may contribute to the high proportion of mild symptomatic or asymptomatic infections in COVID-19 patients, and explain the mild and insidious course of COVID-19 till late deterioration.

Type I IFN response is known to be important for restricting virus replication before adequate adaptive immunity is mounted.\(^\text{36}\) To facilitate efficient virus propagation, coronaviruses encode multiple structural and nonstructural viral proteins\(^\text{37-39}\) to antagonize the IFN signaling pathway upon infection. Our data showed that SARS-CoV-2 launched an attenuated innate immune response despite efficient virus replication, suggesting the virus can also efficiently modulate IFN signaling. Early administration of type I IFN was associated with efficient virus clearance and alleviated disease severity in SARS-CoV-infected mice and SARS patients.\(^\text{40-42}\)

Therefore, we investigated the therapeutic potential of type I IFN by priming the host with IFN\(\beta\) pretreatment and poly(I:C) as an upstream stimulator. Our results demonstrated that compared to SARS-CoV, SARS-CoV-2 was more sensitive to IFN\(\beta\) and poly(I:C) pretreatment, indicating treatment with IFN\(\beta\) in COVID-19 patients could be more effective and beneficial than that in SARS patients. Importantly, several lines of evidence suggested the potential use

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**Fig. 3.** Pro-inflammatory response against SARS-CoV-2 and SARS-CoV infection.

(A) Calu3 and (B) Caco2 cells were infected with SARS-CoV-2 or SARS-CoV at 2 MOI and harvested for RNA extraction at 2, 24, 48, 72 and 120 hpi. The expression of pro-inflammatory mediators was determined by RT-qPCR and normalized to mock infected controls. The results represented the mean and standard deviation of three independent experiments. Statistical findings within each group across different time points were performed with two-way ANOVA and were indicated by *, ** and *** representing p value < 0.05, < 0.01 and < 0.001, respectively. Statistical significances between the two viruses were reported in p value numbers.
Fig. 4. IFN response against SARS-CoV-2 and SARS-CoV infection.
(A) Calu3 and (B) Caco2 cells were infected with SARS-CoV-2 or SARS-CoV at 2 MOI and harvested for RNA extraction at 2, 24, 48, 72 and 120 hpi. The expression of IFNs and IFN-stimulated genes was determined by RT-qPCR and normalized to mock infected controls. The results represented the mean and standard deviation of three independent experiments. Statistical significance between two groups across different time points were performed with two-way ANOVA. Statistical findings across different time points were indicated by *, ** and ***, representing p value <0.05, <0.01 and <0.001, respectively. Statistical significances between the two infection groups were reported in p value numbers.
Fig. 5. Sensitivity of SARS-CoV-2 to pre-activation of the IFNβ signaling pathway.

Calu3 and Caco2 cells were pretreated with a single dose of human recombinant IFNβ prior to infection of SARS-CoV-2 or SARS-CoV at MOI of 2. (A and B) Viral titer in lysates (A) and supernatants (B) of IFNβ-pretreated Calu3. (C and D) Viral titer in lysates (C) and supernatants (D) of IFNβ-pretreated Caco2. (E) SARS-CoV-2 nucleocapsid protein expression in IFNβ-pretreated Calu3 and Caco2 at 48hpi. Statistical significance between the two viruses across different concentrations of IFNβ pretreatment at each time point. (F and G) ACE2 expression upon IFNβ pretreatment. Statistical significance across different concentrations of IFNβ pretreatment was performed with one-way ANOVA. (H and I) Viral titer in lysates and supernatants of poly(I:C)-pretreated Calu3. (J and K) Viral titer in lysates and supernatants of poly(I:C)-pretreated Caco2. Statistical significance between poly(I:C) pretreatment was performed with unpaired Student’s t-test. The results represented the mean and standard deviation of three independent experiments. n.s. indicated not statistically significant (p>0.05). *, ** and *** represented p value < 0.05, <0.01 and <0.001, respectively.
of IFNβ as a treatment strategy for COVID-19 infection. First, treatment with IFNβ or its inducer may jump-start the host immune system as the innate immune response by IFN and proinflammatory cytokines are markedly suppressed by SARS-CoV-2 in both model cell lines and in ex vivo human lung tissue explants. Second, IFNβ did not increase the expression of SARS-CoV-2 receptor ACE2 in our lung and intestinal model cell lines, which was recently suggested to be an IFN-stimulated gene. Third, IFNβ was shown to decrease virus-induced lung fibrosis in a mouse model, which might improve outcomes of patients with COVID-19 complicated by acute respiratory distress syndrome. Forth, synergistic effects were reported for leukocytic IFNα with ribavirin, and IFNβ with ribavirin. Finally, IFNβ exhibited potent in vitro and/or in vivo antiviral activity against SARS-CoV and MERS-CoV. These findings formed the basis of a recent randomized clinical treatment trial which showed that the triple combination of antiviral therapy with IFNβ–1b, lopinavir–ritonavir, and ribavirin is safe and highly effective in shortening the duration of virus shedding, decreasing cytokine responses, alleviating symptoms, and facilitating the discharge of patients with mild to moderate COVID-19.

Our study has a few limitations. First, the findings are based on human cell lines and not on ex vivo human tissue explants. But the latter is very difficult to maintain and the tissue explants rapidly deteriorate within 72 h. Second, the findings were not yet validated in suitable animal models. However, our finding on the importance of IFNβ was in part verified by a recent clinical trial using IFNβ–1b in human, which was expedited by the rapid progression of this pandemic. Further studies on the role of the first line of host defense against this sneaky SARS-CoV-2 are warranted.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jinf.2020.07.016.

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