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Virus-related stimuli modulate SARS-CoV-2 entry factor expression in pediatric tonsillar epithelial cells *in vitro*

To the Editor,

Coronavirus disease 2019 (COVID-19), caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has been a worldwide pandemic since late 2019. Most pediatric COVID-19 patients do not develop severe symptoms. However, some have developed serious conditions such as multisystem inflammatory syndrome in children (MIS-C), the etiology of which remains largely unknown.

Two clinical and epidemiological features of MIS-C attracted our attention. 1) Twenty-eight percent of MIS-C patients reportedly exhibited pharyngitis/tonsillitis,[1] which is relatively rare in adults. 2) Newborns and infants, whose tonsils are immature, are less likely to develop MIS-C than older children. These facts imply involvement of tonsillitis in the pathogenesis of MIS-C. A recent multicenter study of pediatric COVID-19 patients found viral co-infection to be a significant risk factor for ICU admission.[2] From that, we hypothesized that tonsillar cells' immune responses to SARS-CoV-2 play critical roles in the development of MIS-C, and that viral co-infection of tonsillar epithelial cells (TEpiC) enhances their surface expression of SARS-CoV-2 entry factors.

To test this hypothesis, we cultured pediatric TEpiC with various microbe-related compounds and then examined their expression of mRNA for SARS-CoV-2 entry factors. Angiotensin-converting enzyme 2 (ACE2) is the main host-cell entry receptor for SARS-CoV-2. Recently, a novel truncated isoform of ACE2 (*d*ACE2) was identified in the respiratory epithelium.[3] Since *d*ACE2 lacks the SARS-CoV-2 binding domain, we used probes specific for full-length ACE2 to detect ACE2 by qPCR (Figure S1).

The clinical and demographic details of the subjects, methods, and statistical analyses are described in the Appendix S1. Isolated TEpiC, human nasal epithelial cells (HNEpC), and normal human bronchial epithelial cells (NHBE) were each cultured in the presence of various toll-like receptor ligands or cytokines. mRNA expression for SARS-CoV-2 entry factors was determined by qPCR.

First, without any stimulation, we compared TEpiC with HNEpC and NHBE for their mRNA expression levels for SARS-CoV-2 entry factors. Furin, one of the proteases responsible for priming coronavirus spike proteins, was highly expressed in TEpiC. However, expression of full-length ACE2, a major entry receptor, and expression of NRP1, which promotes SARS-CoV-2 entry and infection, were significantly lower in TEpiC than in NHBE (Figure 1), suggesting that NHBE are the primary route for SARS-CoV-2 transfection.

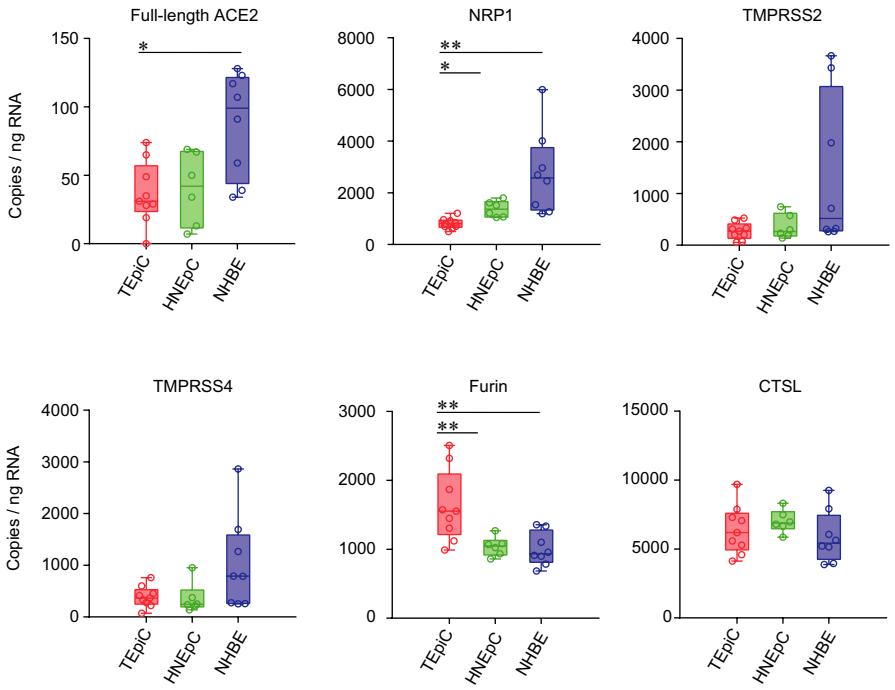

FIGURE 1 Expression levels of mRNA for SARS-CoV-2 entry factors in tonsillar, nasal, and bronchial epithelial cells at steady state. All the cells were cryopreserved one time, and mRNA expression was examined by qPCR. $^{*}p < .05$, $^{**}p < .01$ (Kruskal–Wallis test followed by Mann–Whitney *U*-test). Full-length ACE2, full-length isoform angiotensin-converting enzyme 2; NRP1, neuropilin-1; TMPRSS2, transmembrane protease serine 2; TMPRSS4, transmembrane protease serine 4; CTSL, cathepsin L; TEpiC, tonsillar epithelial cells; HNEpC, human nasal epithelial cells; and NHBE, human bronchial epithelial cells

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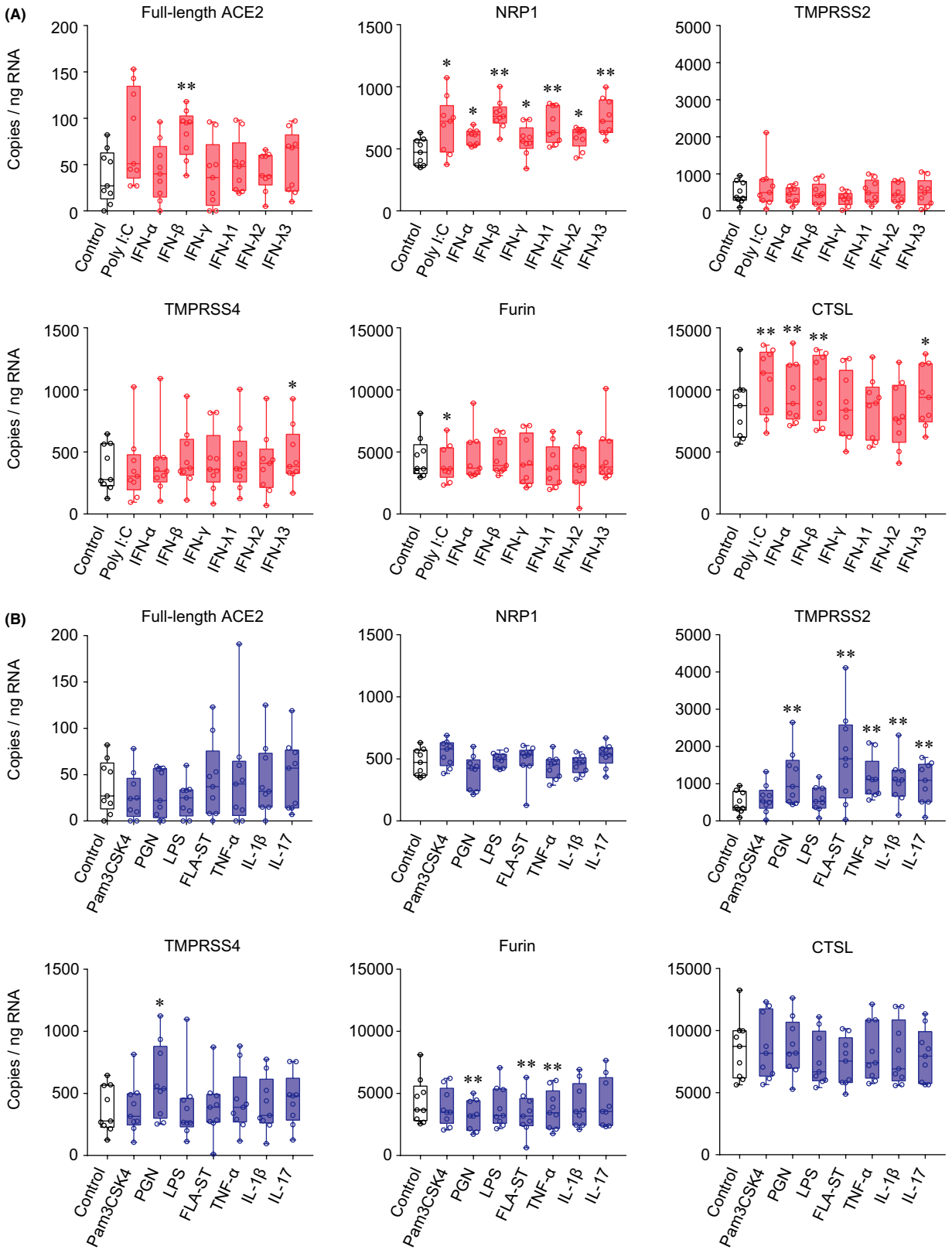

FIGURE 2 Effects of pathogen-related stimuli on the expression levels of mRNA for SARS-CoV-2 entry factors in tonsillar epithelial cells (TEpiC). TEpiC ($n = 9$: three from tonsillar hypertrophy, three from recurrent tonsillitis, and three from PFAPA) were stimulated for 24 h with such virus-related factors as Poly I:C and interferons (A), and such bacterial-related factors as toll-like receptor ligands and cytokines (B). Each stimulated sample was compared to the non-stimulated control sample. *$p < .05$, **$p < .01$ (Wilcoxon's signed-rank test). Full-length ACE2, full-length isoform angiotensin-converting enzyme 2; NRP1, neuropilin-1; TMPRSS2, transmembrane protease serine 2; TMPRSS4, transmembrane protease serine 4; CTSL, cathepsin L; PGN, peptidoglycan; LPS, lipopolysaccharide; and FLA-ST, flagellin

Next, we investigated the effects of virus-related compounds on the expression of these genes in TEpiC. Exposure of TEpiC to interferon-beta (IFN-β) significantly increased expression of mRNA for full-length ACE2, almost to the steady-state level in NHBE (Figure 2A). NRP1 and CTSL mRNA expression increased significantly in response to Poly I:C and IFNs (Figure 2A). On the contrary, bacteria-related compounds and proinflammatory cytokines significantly enhanced TMPRSS2 and 4, but decreased furin expression. Expression of full-length ACE2 and NRP1 remained unchanged (Figure 2B). Furin was significantly downregulated by Poly I:C and bacteria-related compounds, but its expression level was still several thousand copies and probably sufficient to function, suggesting that such effects do not greatly alter the mechanism of transfection. These results suggest that SARS-CoV-2 can bind to and infect TEpiC in the presence of viral co-infection, but not bacterial co-infection or at steady state. A recent study revealed that the effect of interferon on full-length ACE2 is cell-specific,[4] and TEpiC are probably susceptible to this effect.

The pathophysiology of MIS-C was recently described as autoreactivity secondary to SARS-CoV-2.[5] Since SARS-CoV-2 can reportedly cause autoimmune reactions,[6] direct infection of the tonsils by SARS-CoV-2 may be the initial step leading to an autoimmune reaction. Limitations of this study are non-use of tonsils from MIS-C patients and live viruses, and insufficient protein data.

In conclusion, we demonstrated *in vitro* that pediatric TEpiC become susceptible to SARS-CoV-2 infection in the presence of viral co-infection. Further studies are needed to investigate whether SARS-CoV-2 directly infects tonsils via TEpiC and causes immune dysregulation or autoimmune reactions, leading to MIS-C.

KEYWORDS

full-length ACE2, SARS-CoV-2 entry factor, tonsillar epithelial cells

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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