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Pediatric nasal epithelial cells are less permissive to SARS-CoV-2 replication compared to adult cells

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At a Glance Commentary

Scientific Knowledge on the Subject

There is now a growing body of evidence that children are less susceptible to SARS-CoV-2 infection compared to adults and if infected, children are more likely to develop an asymptomatic infection. The reasons for this remain unclear. In particular, the role of the pediatric nasal epithelium, the primary point of viral entry into the human host, in this differential susceptibility has yet to be investigated.

What This Study Adds to the Field

Our study indicates that pediatric nasal epithelial cells produce a more vigorous anti-viral and pro-inflammatory response to SARS-CoV-2 compared to adult cells. This is associated with reduced SARS-CoV-2, but not influenza virus, replication in pediatric epithelial cells. We also show that on a protein level SARS-CoV-2 receptor expression on nasal epithelial cells is not significantly different between children and adults. These data provide an important insight into pediatric infections with SARS-CoV-2.

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Abstract:

Rationale: Young children (typically those <10 years old) are less susceptible to SARS-CoV-2 infection and symptoms compared to adults. However, the mechanisms that underlie these age-dependent differences remain to be determined and could inform future therapeutics for adults.

Objective: To contrast the infection dynamics of SARS-CoV-2 in primary nasal epithelial cells from adults and children.

Methods: Viral replication was quantified by plaque assay. The cellular transcriptome of infected and uninfected cells was assessed by RNA-seq. ACE2 and TMPRSS2 protein expression were quantified by Western Blot.

Measurements and Main Results: We report significantly higher SARS-CoV-2 replication in adult compared to pediatric nasal epithelial cells. This was restricted to SARS-CoV-2 infection, as the same phenomenon was not observed with influenza virus infection. The differentiational SARS-CoV-2 replication dynamics were associated with an elevated type I and III interferon response, and a more pronounced inflammatory response in pediatric cells. No significant difference between the two age groups was observed in the protein levels of ACE2 and TMPRSS2.

Conclusions: Our data suggest that the innate immune response of pediatric nasal epithelial cells, and not differential receptor expression, may contribute to the reported reduced SARS-COV-2 infection and symptoms reported amongst children.

Keywords: SARS-CoV-2; pediatric nasal epithelial cells; anti-viral response; innate immune response; susceptibility to SARS-CoV-2 infection

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Introduction

SARS-CoV-2 (the causative agent of COVID-19) causes a broad range of clinical symptoms, ranging from asymptomatic infection to potentially fatal acute respiratory distress syndrome (ARDS). Children typically experience mild symptoms of COVID-19 when compared to adults (1). However, there is now a growing body of evidence that children may also be less susceptible to SARS-CoV-2 infection. Specifically, a low rate of pediatric SARS-CoV-2 infections has been observed in multiple countries including China (2), Italy (3), the U.S.A (4), Spain (5, 6) and Poland (6, 7). However, this may be underrepresented since SARS-CoV-2 infections in children are frequently asymptomatic and may therefore go undetected in screening studies of symptomatic individuals (8). To overcome this potential confounder, an Icelandic study tested 5069 healthy adults and 433 healthy children (or those with only a mild cold) for SARS-CoV-2 (9). Whilst more adults were tested than children, positive infection in children was lower compared to adults (9). These data are in line with those from Vo', Italy (10). Here, all age groups were homogenously sampled, yet no children tested positive for SARS-CoV-2 infection, despite the fact that at least 13 of these children lived together with infected family members (10). Similarly, in a meta-analysis of SARS-CoV-2 household transmission clusters, children were significantly less likely to contract SARS-CoV-2 from infected family members compared to adult members of the household (11). These findings have been echoed in multiple single site studies where, both within and outside of households, the infection rate of SARS-CoV-2 amongst children <10 years old is significantly lower than that of adults (12). Reduced SARS-CoV-2 infection and transmission is also observed in juvenile ferrets compared to their older counterparts (13).

The reasons for less frequent SARS-CoV-2 infection and symptoms in children remain unclear and may be influenced by a multitude of factors. Pre-existing immunity to SARS-CoV-2 (likely derived from seasonal coronaviruses) may offer some form of cross-protection from

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infection in children (14). SARS-CoV-2 spike glycoprotein reactive antibodies in SARS-CoV-2 uninfected individuals are more prevalent amongst children and adolescents (14). It is also striking to note that this differential susceptibility is somewhat unique to SARS-CoV-2. For example, children have been identified as having the highest influenza virus infection rate of any age group and pay a major role in the spread of the virus (15).

It is possible that pediatric nasal epithelial cells (NECs) (the initial target of SARS-CoV-2 infection) are fundamentally different from those of adults. Gene expression studies from the nasal epithelium of healthy individuals suggests that the SARS-CoV-2 receptor, ACE2, is expressed at lower levels in the nasal cavity of children compared to adults (16). However, this has yet to be validated on a protein level. Moreover, this does not appear to be the case in all patient cohorts (17, 18). Following binding of the SARS-CoV-2 spike (S) protein to ACE2, the host surface protease TMPRSS2 cleaves the full-length S protein at its S2' site, facilitating the fusion of viral and cellular membranes to deliver the viral RNA into the cytosol (19). Low levels of TMPRSS2 trigger viral entry via a less efficient pathway, that is dependent on endosomes and cathepsin mediated spike cleavage (20). The nasal cavities of children express less TMPRSS2 than those of adults, which may explain the less frequent pediatric infections with SARS-CoV-2 (21). However, this has also yet to be confirmed at a protein level.

In addition to differential receptor expression, pediatric and adult NECs may also mount fundamentally different innate immune response to SARS-CoV-2. The induction of type I and III interferon (IFNs) in the respiratory tract is essential for protection against viral disease (22). SARS-CoV-2 is a poor inducer of the type I and III interferon response (23), and the significance of interferons in controlling SARS-CoV-2 infection is best demonstrated by the fact that the presence of neutralising anti-type I IFNs antibodies increases the risk of severe COVID-19 (24). Recent RNA sequencing of the whole epithelium from pediatric and adult proximal airways suggest that there is a higher expression of genes associated with interferon

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alpha and gamma responses in children compared to adults (25). However, whether this results in reduced replication of SARS-CoV-2 in the nasal epithelium of children remains to be determined.

Here, we use primary NECs, differentiated at an air-liquid interface, to investigate differential infection kinetics and antiviral responses to SARS-CoV-2 infection in children and adults.

Methods

Cell collection and ethics statement

Primary NECs were collected from healthy individuals by placing a sterile nasal mucosal curette (Arlington Scientific Inc., USA) in the mid-inferior portion of the inferior turbinate. This study was approved by the University of Queensland's Human Research Ethics Committee (2020001742). Additional detail is provided in the online supplement.

Cell culture

African green monkey kidney epithelial Vero cells and Madin-Dkarby Canine Kidney (MDCK) cells were obtained from American Type Culture Collection (ATCC; Virginia, USA). Primary NECs were expanded in PneumaCult EX Plus media (STEMCELL Technologies Inc, Canada) and seeded on 6.5 mm transwell polyester membranes with 0.4um pores (Corning Costar, USA) and were maintained with PneumaCult ALI S Medium (26) for at least 3 weeks until fully differentiated. Additional detail is provided in the online supplement.

Viral stocks and viral infection

SARS-CoV-2 isolate hCoV-19/Australia/QLD02/2020 was provided by Queensland Health Forensic & Scientific Services, Queensland Department of Health. A/Auckland/4/2009 (Auckland/09; H1N1) stocks were prepared in embryonated chicken eggs as previously

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described (27). Fully differentiated adult and pediatric NECs were infected with mock (PBS), SARS-CoV-2 or Auckland/09 at a multiplicity of infection (MOI) of 3 for each virus. Additional detail is provided in the online supplement.

Quantification of infectious virus

SARS-CoV-2 titres in cell culture supernatants were determined by plaque assay on Vero cells, as described previously (28). Influenza virus titers in cell culture supernatants were determined by plaque assay on MDCK cells, as described previously (29).

Histology

Fixed cells on a transwell membrane were routine processed and embedded in paraffin, sectioned at 5µm and subsequently stained with hematoxylin and eosin (H&E). Sections were assessed for cellular morphology by a veterinary pathologist (H.B.) blinded to the experimental design.

RNA extraction and quantitative Reverse Transcription PCR (qRT-PCR)

RNA was extracted from NECs and transcribed into cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's instructions. Real time PCR was performed with SYBER Green (Invitrogen) and gene expression was normalized relative to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression. Additional detail is provided in the online supplement.

Quantification of NP, ACE2 and TMPRSS2 protein expression

For comparison of protein expression in respond to pediatric versus adult NECs infection with SARS-CoV-2 during the viral replication, we performed western blot and immunolabeling. Additional detail is provided in the online supplement.

RNA Sequencing analysis

Deferentially expressed genes (DEGs) were identified using DESeq2 (30) in R. The R package fast gene set enrichment analysis (FGSEA) (31) was used for gene set enrichment analysis using

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hallmark gene sets (version 7.2) (32). Gene Ontology enrichment analysis was performed using the R package GOseq. Additional detail is provided in the online supplement.

Statistical analysis

Unless otherwise indicated, outliers of continual variables were removed using ROUT's test (Q = 1%). Data were tested for normality using the Shapiro-Wilk test. Where data were normally distributed, data was analyzed using an unpaired two-tailed student's t-test. Where data were not normally distributed, data was analyzed using a Mann-Whitney U test. Significance was set at $p<0.05$.

Results

Pediatric nasal epithelial cells are phenotypically different to adult nasal epithelial cells

To investigate the role of NECs in SARS-CoV-2 infection, adult and pediatric NECs were differentiated at an air-liquid interface. The phenotype and transcriptome of these cells at baseline (prior to infection) was then assessed. Adult NECs grew as a pseudostratified columnar epithelium with scattered goblet cells and ciliated epithelial cells (Figure 1A). Pediatric NECs also grew as a pseudostratified columnar epithelium with ciliated epithelial cells and goblet cells. However, scattered cells with pyknotic nucleus and condensed cytoplasm were also observed, leaving pseudocysts in the epithelium (Figure 1A). This is potentially indicative of higher cell turn-over and metabolic rate in the pediatric epithelial cells (33, 34). To characterize the transcriptional difference between these cells, RNASeq was performed. Principle component analysis suggested that pediatric and adult NECs formed distinct groups based on their transcriptome (Figure 1B). DEGs of naive pediatric NECs compared to adult cells were identified and listed in Table E1 in the online data supplement (Fig. 1C). Numerous gene signatures associated with metabolism (e.g., Ox Phos, MTORC1, glycolysis) and cell

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replication (e.g., MYC targetsand DNA repair) were upregulated in pediatric cells, consistent with the observed morphology. Moreover, relative to adult cells, pediatric epithelial cells upregulated genes associated with pro-inflammatory pathways such as tumor necrosis factor alpha (TNF-α) signaling via NFkB, Complement, IFN alpha response and IFN gamma response (Figure 1D and Figure E1 in the online data supplement). Together, these data indicate that even in the absence of a viral infection, pediatric NECs *in vitro* have a different and more pro-inflammatory transcriptome compared to adult NECs.

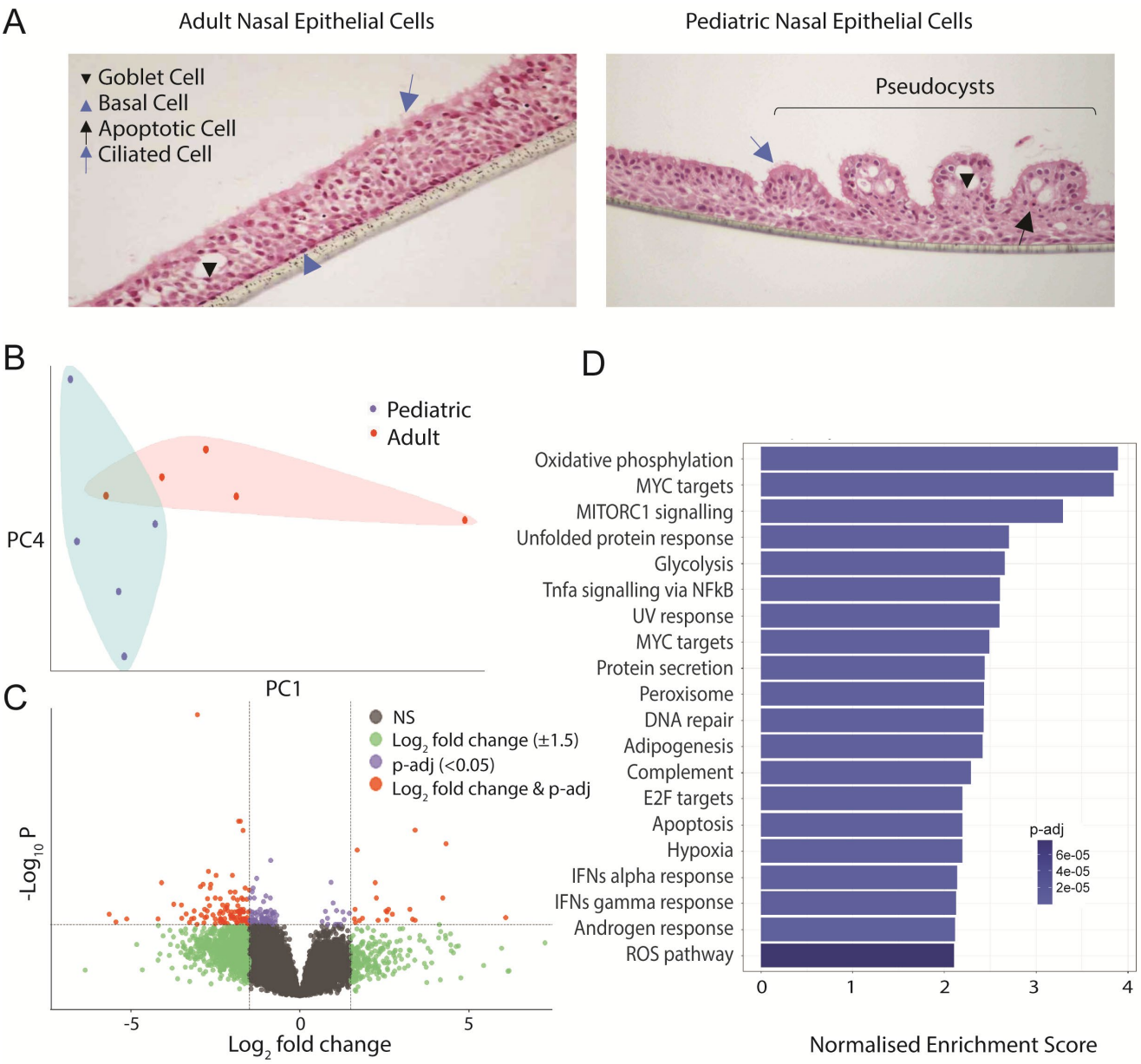

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Figure 1. Pediatric nasal epithelial cells are phenotypically different to adult nasal epithelial cells. A) Representative Hematoxylin & Eosin stained sections of pediatric and adult NECs culture differentiated at an air-liquid interface (representative of 2 adult and 3 pediatric donors). **B)** Principal component analysis for the global transcriptional response of naive pediatric and adult NECs**.** Data points represent different donors (N=10). **C)** Volcano plot illustrating differentially expressed genes (DEGs) of naive pediatric NECs compared to adult cells. DEGs statistically different between the two patient groups with a fold change of >1.5 are shown in orange. DEGs statistically different between two groups with a fold change of <1.5 are shown in purple. DEGs not statistically different between two groups with a fold change of >1.5 are shown in green. NS = not significant. **D)** Gene enrichment pathway analysis were performed on the DEGs of naive pediatric NECs compared to adult cells by using gene set enrichment analysis (GSEA) to interrogate the Hallmarks dataset from MSIgDB. Top 20 pathways shown indicate those upregulated in pediatric samples relative to those of adults (see Figure E1 for full list). For pathways with a false discovery rate (FDR) <0.05, normalized enrichment scores are shown.

Pediatric and adult nasal epithelial cells produce similar amounts of ACE2 and TMPRSS2 protein

Previous mRNA expression studies suggest that pediatric NECs express lower levels of ACE2 and TMPRSS2 compared to their adult counterparts (16, 21). However, these findings remains controversial (18). Indeed, our RNASeq data showed that there was no significant difference the expression level of ACE2 and TMPRSS2 (p value > 0.05; data not shown). To investigate the levels of ACE2 and TMPRSS2 protein between adult and pediatric NECs Western blots were performed on uninfected cells (Figure 2). Whilst some donor-to-donor variation was observed there was no significant difference in the protein levels of ACE2 and TMPRSS2 between adult and pediatric donors ($p = 0.2$ and $p = 0.5$ respectively).

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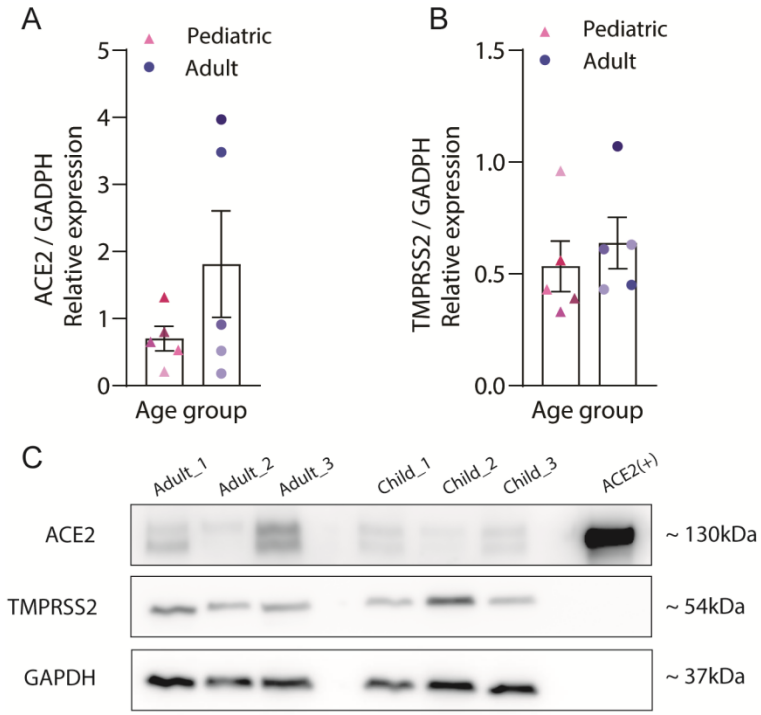

Figure 2. Pediatric nasal epithelial cells do not express lower levels of ACE2 and TMPRSS2 protein relative to adult nasal epithelial cells. A) Relative ACE2 protein levels compared to GAPDH in pediatric and adult NECs. **B)** Relative TMPRSS2 protein levels compared to GAPDH in pediatric and adult NECs. Each data point represents a different donor. Mean ± SEM is shown. **C)** Representative western blot of NECs from three adult and three pediatric donors blotted for ACE2, TMPRSS2 and GAPDH.

Pediatric nasal epithelial cells are less permissive to SARS-CoV-2 replication

Given the enhanced inflammatory response of nasal epithelial cells, and despite the absence of differential ACE2 and TMPRSS2 levels, we next sought to determine if pediatric epithelial cells were less susceptible than adult cells to SARS-COV-2 replication. Strikingly, significantly reduced SARS-CoV-2 replication was observed in pediatric NECs at 24 and 72 h.p.i (Figure 3A). Reduced western blot signal for SARS-CoV-2 NP was also observed in pediatric NECs at 72 hours post-infection, although this did not reach significance ($p = 0.07$; Figure 3B and C, Figure E2 in the online data supplement).To determine if decreased viral replication was specific to SARS-CoV-2, these experiments were repeated using influenza A virus, which is known to be highly transmissible amongst children (15, 35). Strikingly, there was a trend

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towards *increased* influenza A virus replication in pediatric NECs compared to adult cells at 24-, 48- and 72-hours post-infection (Figure 3D).

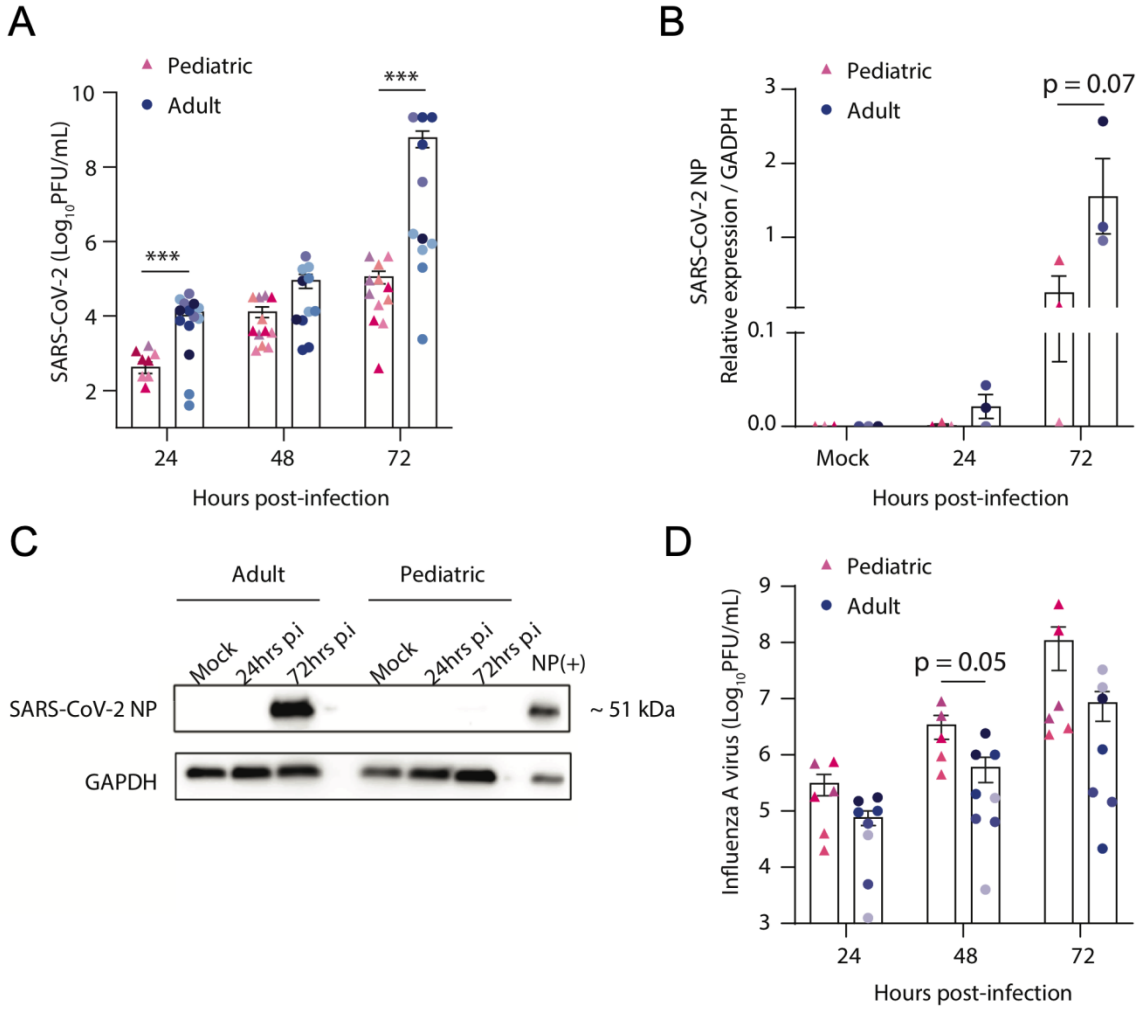

Figure 2. Pediatric and adult nasal epithelial cells produce similar amounts of ACE2 and TMPRSS2 proteins. A) Relative ACE2 protein levels compared to GAPDH in pediatric and adult NECs (as determine by densitometry). **B)** Relative SARS-CoV-2 NP levels compared to GAPDH in pediatric and adult NECs. Each data point represents a different donor (as determined by densitometry). **C)** Representative western blot of adult and pediatric donor blotted for SARS-CoV-2 NP at various timepoints post-infection. All of the western blot results (N= 3 adults and n = 3 children) for SARS-CoV-2 NP is shown as Figure E2 in the online data supplement. **D)** Plaque forming units (PFU) of influenza A virus from the apical surface of NECs at various timepoints post-infection. Matching colours indicate samples derived from the same donor. Mean ± SEM is shown. $p < 0.05$*, $p < 0.01$**, $p < 0.001$***.

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Pediatric nasal epithelial cells mount a strong anti-viral response to SARS-CoV-2

To gain a further insight into the anti-viral response of pediatric NECs to SARS-CoV-2, RNA Seq was performed on adult and pediatric cells 72 hours post-SARS-CoV-2 infection. A global analysis of the transcriptome of infected cells (independent of donors' age) suggested that infection was associated with the expression of interferon stimulated genes (ISGs) (Online data supplement Figure E3 and Table E3). However, there was no clear clustering of infected and uninfected cells by PCA (Figure E3). In contrast, when DGEs (Table E4 in the online data supplement.) in infected cells was analyzed by controlling for the effect of the age group, adult and pediatric cells formed distinct clusters by PCA (Figure 4A). Relative to infected adult cells, infected pediatric cells upregulated numerous pathways associated with the inflammatory response, including complement and inflammatory response (Figure 4B & C). Strikingly, gene ontology (GO) enrichment analysis (Figure 4D) demonstrated a strong interferon response in infected pediatric cells, with GO terms such as 'viral process', 'type I interferon signaling', 'response to virus', 'regulation of defense response to virus', 'negative regulation of viral genome replication', 'defense response to virus' and 'cellular response to interferon alpha' being significantly upregulated in infected pediatric epithelial cells. None of these GO terms were identified amongst top differentially expressed GO terms in adult cells infected with SARS-CoV-2 (Online data supplement Figure E5).

To determine if this interferon response was specific to SARS-CoV-2 infection, the expression of three ISGs (*IFIT1, CXCL10, ISG15*; identified as DEG in RNA Seq analysis) was assessed in pediatric and adult NECs infected with either SARS-CoV-2 or influenza virus by qPCR (Figure 4E). Globally, there was a reduced IFN response to SARS-CoV-2 compared to influenza virus, in line with previous reports (36) (Figure 4E). Consistent with our RNA-seq data, IFIT1 and ISG15 were significantly upregulated in SARS-CoV-2 infected pediatric NECs compared to adult cells at 48 hours post-infection. Akin to the situation with SARS-CoV-2,

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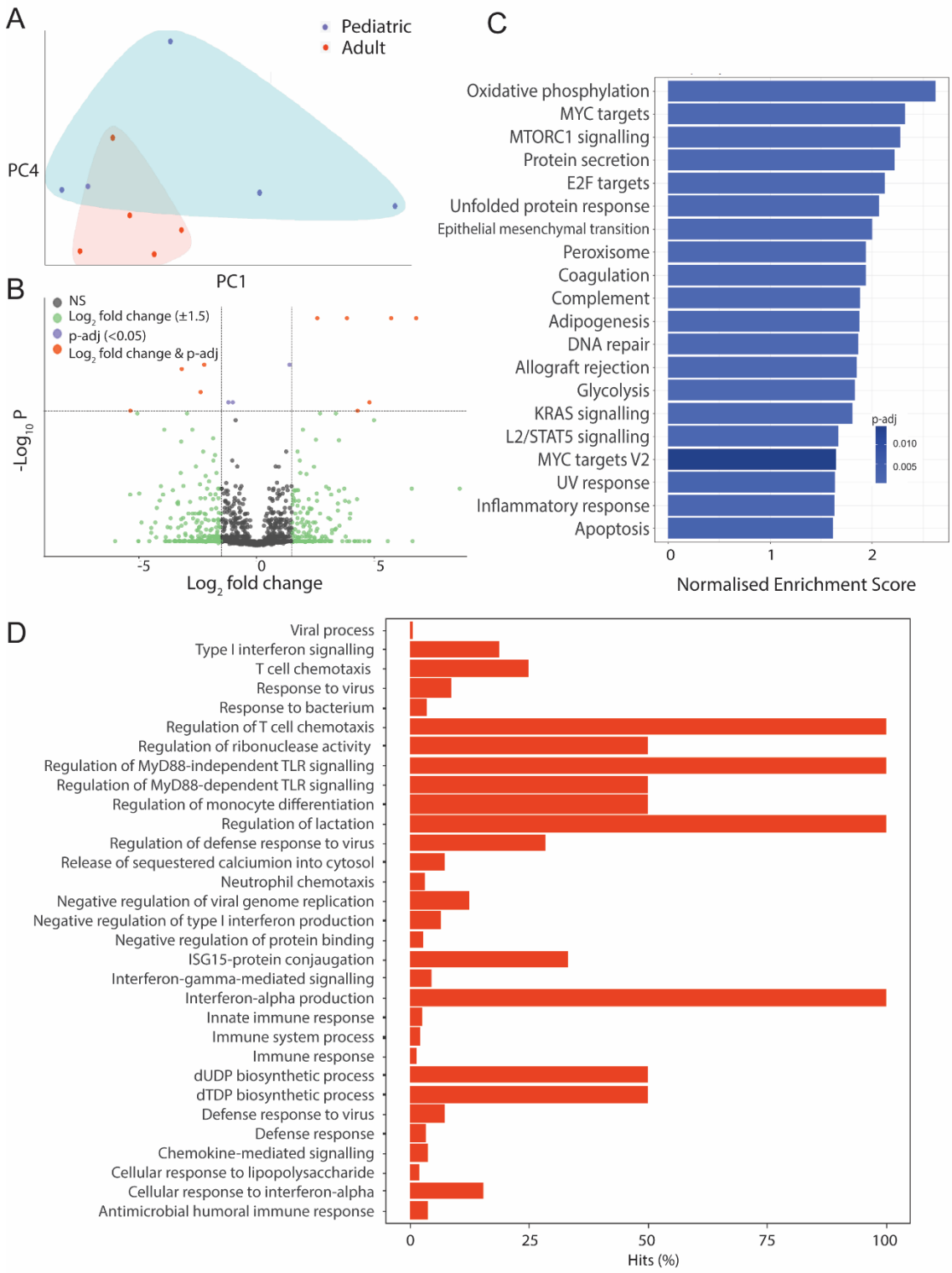
pediatric epithelial cells infected with influenza A virus had increased expression of CXCL10 (24- and 48-hours post-infection) and ISG15 (24 hours post-infection).



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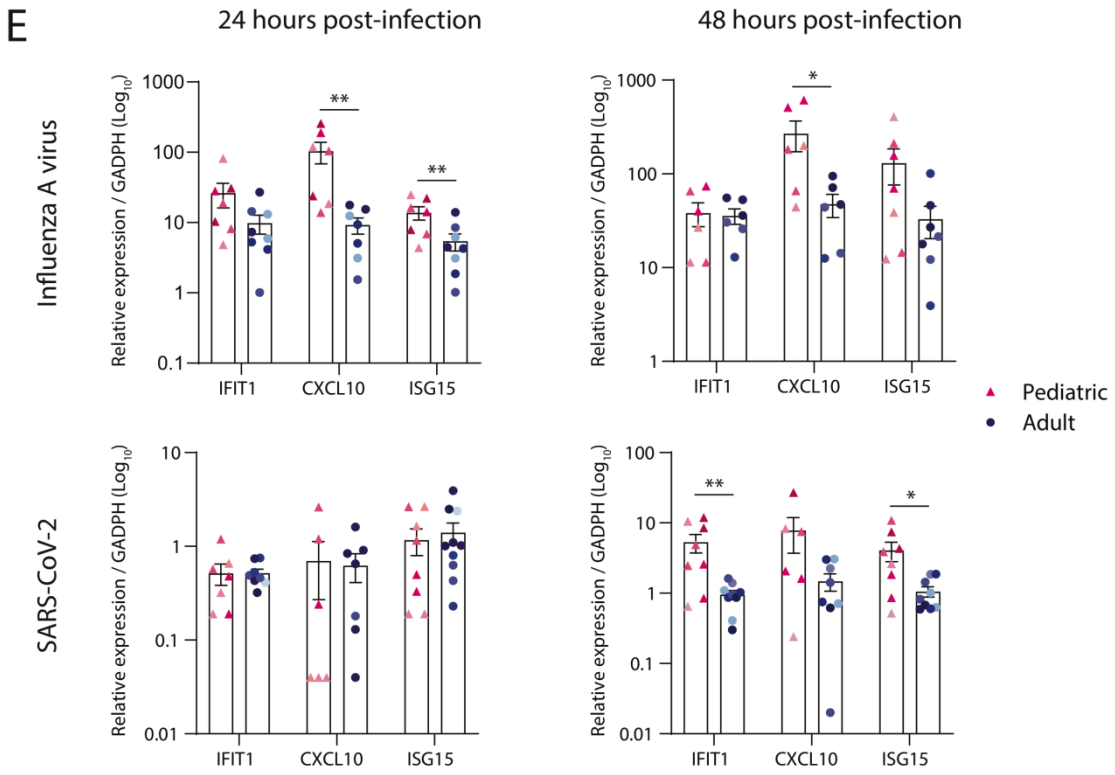

Figure 4. Pediatric nasal epithelial cells mount a strong anti-viral response to SARS-CoV-2. A) Principal component analysis for the global transcriptional response from SARS-CoV-2 infected pediatric and adult NECs (72 hours post-infection). **B)** Volcano plot illustrating differentially expressed gens (DEGs) of pediatric NECs relative to adult cells at 72 hours post-infection. DEGs statistically different between the two patient groups with a fold change of >1.5 are shown in orange. DEGs statistically different between the two patient groups with a fold change of <1.5 are shown in purple. DEGs not statistically different between the two patient groups with a fold change of >1.5 are shown in green. NS = not significant. **C)** Gene enrichment pathway analysis of DEGs of SARS-CoV-2 infected pediatric NECs (72 hours post-infection) compared to adult cells was performed by using gene set enrichment analysis (GSEA) to interrogate the Hallmarks dataset from MSIgDB. For pathways with adjusted p value <0.05, normalized enrichment scores are shown. Top 20 pathways shown indicate those upregulated in pediatric samples relative to those of adults (see Figure E4 for full list). **D)** Gene ontology (GO) analysis of DEGs in pediatric NECs were displayed by the bar chart. The bars of significantly GO enriched results were marked in red, x-axis reflects the gene count hits as a percentage over genes in a given GO category; y-axis reflects different GO terms. **E)** Relative expression of genes identified as DE in SARS-CoV-2 infected pediatric cells by RNA-Seq in influenza virus and SARS-CoV-2 infected NECs. Matching colours indicate samples derived from the same donor. Gene expression (fold change) is calculated using the ΔΔCt method relative to GAPDH expression. Mean ± SEM is shown. p <0.05*, p <0.01**, p <0.001***.

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Discussion

Large clinical data sets and systematic reviews suggest that children are less often infected with SARS-CoV-2 than adults (11, 37-39). However, the mechanisms driving these observations have been unclear. Here, we have provided the first evidence that the innate immune response of pediatric nasal epithelium may play an important role in reducing the susceptibility of children to SARS-CoV-2 infection.

Previous studies have suggested that the reduced susceptibility of children to SARS-CoV-2 infection is due to reduced expression of ACE2 and TMPRSS2 mRNA. Specifically, it has been hypothesized that the lower level of ACE2 and TMPRSS2 in pediatric upper airways epithelial cells limits viral infectivity in children (16), although this has remained somewhat controversial (17, 18). In the present study we showed that ACE2 and TMPRSS2 protein expression level were not modulated with age. These data suggest that mRNA expression does not always correlate to protein expression and it is important to quantify protein level expression across a broader number of studies.

Despite similar expression of ACE2 and TMPRSS2, we observed a significant reduced replication decrease of SARS-CoV-2 in pediatric nasal cells compared to those of adults. Given that the nasal epithelium is the first site of SARS-CoV-2 infection these data are consistent with the reduced number of SARS-CoV-2 infected children recorded in epidemiological studies (40, 41). There have been previous suggestions that nasopharyngeal SARS-CoV-2 titers in children and adolescents are equivalent to those of adults (42, 43). However, reanalysis of the aforementioned studies have shown that young children (<10 years old) did indeed have a significantly lower viral load (44), or that the comparison was being performed between children in the first 2 days of symptoms and hospitalized adults with severe disease (45) or that the dataset included few children younger than 16 years (46). Other such studies (47) have been questioned in relation to the unusually low cycle threshold

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(Ct) values recorded (48, 49). Moreover, decreased viral replication in pediatric epithelial cells is consistent with experimental studies in ferrets where aged ferrets showed higher viral load and longer nasal virus shedding (50). Therefore, it is currently difficult to ascertain if there is or is not an age-dependent difference in SARS-CoV-2 nasal titres in infected individuals.

Consistent with reduced SARS-CoV-2 replication in the nasal epithelium of children, pediatric epithelial cells had a more pronounced pro-inflammatory response (compared to adult cells) prior to and following a SARS-CoV-2 infection. In particular, a pronounced interferon response and the expression of ISGs was detected in infected pediatric, but not adult NECs. Increased ISG expression, and the subsequent anti-viral response may contribute to the reduced viral replication observed in pediatric cells. These findings are consistent with those of Maughan et al, who analysed transcriptional profile of airway (tracheobronchial) epithelium and observed upregulated type I and II IFNs associated genes expression levels in children (25). Similarly, in the nasal fluid of children and adults presenting to the Emergency Department with SARS-CoV-2 there were significantly higher levels of IFN-a2 in the fluid derived from children. Increased interferon signaling was also recorded in the nasopharyngeal transcriptome of children compared to that of adults (17). The question remains as to why pediatric epithelial cells mount a stronger inflammatory and anti-viral response to SARS-CoV-2 compared to adult cells. This may represent an adaptation to the increased antigenic challenge observed in childhood. Alternatively, it is possible that increased antigenic exposure in childhood 'trains' nasal epithelia in children to mount a stronger pro-inflammatory response to any antigenic challenge. Finally, we cannot exclude the possibility that any metabolic differences between pediatric and NECs (as potentially suggested by the different morphologies of the cells) could alter gene expression. It is also important to recognize that these data may not be applicable to

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all patient populations as previous studies of children and adults hospitalized with COVID-19 did not find an age dependent difference in the interferon response (51).

A pronounced pro-inflammatory and anti-viral response in pediatric cells was not restricted to SARS-CoV-2 infection and a similar result was observed following influenza virus infection. However, influenza virus replicated equally as well (if not better) in pediatric cells compared to adults. These data are consistent with the high sensitivity of children to influenza virus infection (35). These data are also seemingly discordant with the increased inflammatory response of pediatric cells. However, SARS-CoV-2 is highly sensitive to interferon treatment, more so than influenza A virus (23, 52). Therefore, we speculate that whilst the differential interferon response between children and adults is sufficient to inhibit SARS-CoV-2 replication, it is not sufficient to inhibit influenza virus replication.

These data potentially have important clinical implications. Specifically, the use of recombinant IFNs as a treatment in SARS has been identified as a promising therapeutic candidate (53). The data presented here support this notion. Further investigation as to the clinical efficacy of interferon therapy in COVID-19 is warranted.

Finally, it is important to recognize the limitations of this study. Due to the difficulties associated with obtaining NECs from children only a limited number of donors could be used. However, as donors were not selected on susceptibility to respiratory viral infection their responses should be broadly representative of other healthy children. Furthermore, our data focused on the role of nasal epithelial cells in age-dependent differences in SARS-CoV-2 infection. However, there can be a number of other mechanisms to explain the reduced susceptibility of children to SARS-CoV-2 infection that were not measured in the present study. For example, children and adolescents have much higher titres of preexisting antibodies to SARS-CoV-2 compared to adults (14, 54). This study is unable to ascertain if this plays a more significant role than the nasal epithelium in protecting children from infection *in vivo*. Finally,

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it is important to recognize that these in *vitro* studies were performed with an early SARS-CoV-2 isolate and therefore did not represent the viral mutations that are currently circulating in the community. This remains an area of ongoing research in our laboratory.

In sum, the data presented here suggest that the nasal epithelium of children is distinct and that it may afford children some level of protection from SARS-CoV-2 infection.

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