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Picornavirus-Induced Airway Mucosa Immune Profile in Asymptomatic Neonates

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Background. Bacterial airway colonization is known to alter the airway mucosa immune response in neonates whereas the impact of viruses is unknown. The objective was therefore to examine the effect of respiratory viruses on the immune signature in the airways of asymptomatic neonates.

Methods. Nasal aspirates from 571 asymptomatic 1-month-old neonates from the Copenhagen Prospective Studies on Asthma in Childhood 2010 birth cohort were investigated for respiratory viruses. Simultaneously, unstimulated airway mucosal lining fluid was obtained and quantified for levels of 20 immune mediators related to type 1, type 2, type 17, and regulatory immune paths. The association between immune mediator levels and viruses was tested by conventional statistics and partial least square discriminant analysis.

Results. Picornaviruses were detected in 58 neonates (10.2%) and other viruses in 10 (1.8%). A general up-regulation of immune mediators was found in the neonates with picornavirus (P < .0001; partial least square discriminant analysis). The association was pronounced for type 1- and type 2-related markers and was unaffected by comprehensive confounder adjustment. Detection of picornavirus and bacteria was associated with an additive general up-regulating effect.

Conclusions. Asymptomatic presence of picornavirus in the neonatal airway is a potent activator of the topical immune response. This is relevant to understanding the immune potentiating effect of early life exposure to viruses.

Keywords. cytokines; chemokines; children; virus; mucosal lining fluid.

Newborn infants are exposed to microbes from the moment of birth, requiring an immediate ability to mount an appropriate immune response against commensal organisms and invading pathogens. The immune cells of the airway mucosa are the first line of defense against invading microorganisms. When activated, they release an armory of cytokines and chemokines [1], as we demonstrated elsewhere in asymptomatic neonates with bacterial airway colonization [2].

It is well established that the presence of viruses can alter the cytokine response ex vivo in peripheral blood mononuclear cells [3, 4], but it is unknown whether the presence of airway viruses in asymptomatic healthy neonates triggers a topical immune response. The aim of the current study was to investigate the in vivo activity of the immature immune system in the airway mucosa of asymptomatic neonates in response to common respiratory viruses. For that purpose, we quantified the topical immune response in the airway mucosal lining fluid of 1-month-old asymptomatic healthy neonates [5] from the population based Copenhagen Prospective Studies on Asthma in Childhood 2010 (COPSAC2010) mother-child cohort in relation to simultaneous detection of airway viruses.

METHODS

The COPSAC2010 Cohort

The COPSAC2010 cohort is an ongoing, prospective, population based clinical mother-child cohort study of 700 children recruited in Zealand, Denmark, during 2009–2010, as described in detail elsewhere [6]. At 1 month of age, the infants were brought to the clinical research unit for sampling of airway mucosal lining fluid and aspirations from nasopharynx and hypopharynx. Each infant was examined by a research pediatrician including assessment of any lower or upper respiratory infection. The assessments were performed at the COPSAC clinical research units (2 clinical research units situated on Zealand, Denmark). In addition, all families kept a day-to-day diary from birth capturing the child’s respiratory symptoms between clinic visits.

Ethics

The study was conducted in accordance with the guiding principles of the Declaration of Helsinki. Approval by the Ethics

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Airway Inflammatory Mediator Assessment in Nasosorption Samples

Unstimulated airway mucosal lining fluid was sampled at 1 month of age with 3 × 15-mm strips of filter-paper (Accuwik Ultra fibrous hydroxylated-polyester sheets; catalog No. SPR0730; Pall Life Sciences), as described elsewhere in detail [2,5]. The filter papers were inserted bilaterally into the anterior part of the inferior turbinate of the nasal cavity. After 2 minutes of absorption, the filter papers were removed and immediately frozen at −80°C. Prior to analyses, the filter papers were thawed and immersed in 300 µL of assay buffer, and subsequently placed in the cup of a tube filter within an Eppendorf tube and centrifuged for 5 minutes in a cooled centrifuge at 16,000 g.

The samples were analyzed in 2 batches for levels of interleukin 12p70, CXCL10 (interferon-γ-induced protein 10), interferon γ, tumor necrosis factor (TNF) α, CCL4 (macrophage inflammatory protein-β), CCL2 (monocyte chemoattractant protein [MCP]–1), CCL11 (eotaxin-1), CCL13 (MCP-4), interleukin 4 (IL-4), interleukin 5 (IL-5), interleukin 13 (IL-13), CCL26 (eotaxin-3), CCL17 (thymus and activation regulated chemokine), CCL22 (macrophage-derived chemokine), interleukin 17A (IL-17A), interleukin 1β (IL-1β), CXCL8 (interleukin 8), transforming growth factor β1 (TGF–β1), interleukin 10 (IL-10), and interleukin 2 (IL-2). The sensitivities were ≤1 pg/mL for all cytokines and 1–50 pg/mL for chemokines, as described elsewhere [5,7]. The lower limit of detection was set as the mean signal from blanks plus 3 standard deviations.

Selection of the measured cytokines and chemokines was decided a priori to represent mediators associated with different types of immune responses that we grouped into type 1 (T-helper [Th] 1/CD8+/natural killer /innate lymphoid cell [ILC] 1), type 2 (Th2, eosinophils, ILC2), type 17 (Th17, neutrophils, ILC3), and regulatory type responses [5,8,9]. This was based on the present understandings of which cell types mainly produce the given mediators and/or are affected by the mediators.

Detection of Airway Viruses in Nasopharyngeal Aspirates

Nasopharyngeal sampling was performed at age 1 month and done after the sampling of mucosal lining fluid. The samples were collected via one of the nostrils and diluted in 1 mL of isotonic saline. Specimens were frozen and stored at −80°C until shipment to Imperial College, London, United Kingdom, for RNA extraction and further analysis with reverse-transcriptase polymerase chain reaction (PCR).

After extraction the RNA was reverse-transcribed to produce complementary DNA representative of all RNA species in the original clinical sample [10]. This complementary DNA was then used in a panel of PCR assays specific for respiratory syncytial viruses (RSVs) A and B [11], influenza A (H1 and H3) and B [12], and picornaviruses [13]. Rhinoviruses were differentiated from enteroviruses by means of restriction enzyme digestion of the PCR product from all picornavirus-positive tests with BglII [13] and subsequent gel electrophoresis.

Statistics

Data were log-transformed before analyses to obtain normally distributed residuals of the mediator levels. Probabilistic principal component analysis of the mediator levels was used to select among a list of candidate covariates (batch of mucosal lining fluid, season of sampling, location of sampling, pathogenic airway bacteria, older siblings, maternal antibiotics consumption, smoking in the third trimester, and influenza virus). Using multiple linear regression analysis with the first principal component as the response variable, significant predictors (α = 0.05) were selected among the potential covariates and included as covariates in the analyses. Furthermore, based on our previous studies [3,8], a maternal history of asthma, allergy, or eczema and detection of any of the pathogenic airway bacteria Streptococcus pneumoniae, Haemophilus influenzae, or Moraxella catarrhalis were also included as covariates in all statistical models.

The univariate associations between mediator levels and presence of any of the respiratory viruses were analyzed using analysis of variance, with the transformed mediator levels as the outcome variables and presence of viruses as well as possible confounders as the explanatory variables. Results were reported as geometric mean ratios (GMR) of the mean mediator levels, for neonates with a virus detected versus no virus detection with 95% confidence intervals (CIs). For the association between bacteria and viruses, asymptotic CIs were calculated.

In addition to the univariate analysis, partial least square (PLS) discriminant analysis (PLS-DA) was used to unravel the cytokine-to-cytokine covariance structure relevant for differentiating between the children with and those without picornavirus. PLS-DA is a multivariate discrimination method that is especially powerful when the descriptive information is correlated. PLS regression was used to investigate the difference in patterns of mediator levels associated with virus. As a first step, mediator variables were imputed using probabilistic principal component analysis. The first latent PLS component was tested for any association with the viruses detected, using permutation test adjusted for the identified covariates and an analysis of variance with viruses and covariates as explanatory variables and the first latent component as outcome. Analyses were carried out using SAS (version 9.3; SAS Institute) and MATLAB R2013a (version 8.1.0.604; MathWorks) software.

RESULTS

Baseline

Complete information about nasopharyngeal samples for viral detection and immune mediator assessments were available for
82% (n = 571) of the neonates in the cohort after exclusion of neonates with symptoms of airway infection on the day of sampling (see Figure 1 for more details). A dropout analysis of baseline characteristics was performed comparing the 571 children included in the analyses and the 129 excluded children (Supplementary Table 1). The 2 groups were identical except for a higher household income (P = .02) and a lower gestational age (P = .004) among the excluded children.

Virus was detected in 12% (n = 68) of the 571 included neonates, 85% (n = 58) of these being picornavirus, 4% (n = 3) RSV, and 10% (n = 7) influenza virus. No children had >1 virus detected. Of the 58 picornaviruses, 81% (n = 47) were rhinovirus and 14% (n = 8) were “other picornavirus”; in the remaining 5% (n = 3), no further classification was possible. Because of the very low number of samples positive for influenza virus and RSV, we restricted the analyses to the effect of picornaviruses.

The mean age at sampling in the included children was 32 days (standard deviation, 5.4 days); 51% (292) were boys. Baseline characteristics are depicted in Table 1. The variables significantly associated with having picornavirus (older siblings, maternal smoking in the third trimester, and maternal consumption of antibiotics in the third trimester) were further tested in a multivariable backward selection analysis for association with the immune mediator levels. Older siblings and maternal smoking in the third trimester were found to affect the level of immune mediators and were included as covariates in the final models. Using backward selection with the first principal component as the response variable, sampling site, sampling season,

**Table 1. Baseline Characteristics of Children With Picornavirus Versus Controls**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>With Picornavirus (n = 58)</th>
<th>Controls (n = 513)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>History of maternal asthma, allergy, or eczema</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>White race</td>
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<td></td>
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<tr>
<td>High income (&gt;130 000 euro/year)</td>
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<td></td>
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</tr>
<tr>
<td>Maternal consumption of antibiotics in 3rd trimester</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maternal smoking in 3rd trimester</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Young gestational age (&lt;37 wk)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low Apgar score (&lt;7 at 1 min)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cesarean delivery</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Older siblings</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exclusively breastfeeding at 1 month of age</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Furred pets in the home</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Significant difference (P ≤ .05). P values were determined with χ² test, except when otherwise noted.

b Fisher exact test.

>1 units/ wk (more than 1 beer/1 glass of wine/2 cl of spirits per week).
and batch of immune mediator analysis were found to affect the immune mediator level and were included as covariates in the models, along with a maternal history of asthma, allergy, or eczema and detection of any of the pathogenic airway bacteria *S. pneumoniae*, *H. influenzae*, or *M. catarrhalis*.

Effect of Picornavirus on Airway Immune Mediator Profiles

We found a uniform up-regulation of all immune mediator levels in the children with picornavirus, compared with those without picornavirus. For 19 of 20 immune mediators the GMR was significantly elevated (GMR range, 1.15–7.22; 95% CI, .93–10.52) (Table 2 and Figure 2A). The picornavirus-driven immune response was most pronounced for CXCL10, CCL4, and TNF-α, associated with type 1–related responses, and for IL-1β, involved in type 17–associated responses via expansion of Th17 cells when produced by dendritic cells (Table 2). Adjusting the analyses did not modify the associations substantially (Table 2 and Figure 2B).

Neonates with bacterial airway colonization had an overall elevated GMR of the measured mediators (Supplementary Figure 1); however, the up-regulation was quantitatively smaller than the effect of picornavirus. Coexistence of picornavirus and bacterial colonization resulted in an additively increased level of all the immune mediators but with no evidence of interaction between bacteria and picornavirus (*P* = .91), solely suggesting an additive and not a synergistic effect (data not shown). The neonates with symptoms of an airway infection on the day mucosal lining fluid was sampled (n = 44) were studied further, and their levels of immune mediators were clearly elevated compared with the neonates with no sign of an airway infection on the day of sampling.

The daily diary cards were further investigated for presence of troublesome lung symptoms. Apart from the 44 with symptomatic airway infection, we found that another 15 neonates had coughing, wheezing, and/or breathlessness 1 week before and/or after the sampling day. Excluding these children from analysis did not modify our findings (data not shown).

The conventional statistical approach was accompanied by a multivariate data-driven PLS-DA to reveal the profiles of coregulated airway immune mediators. In the loading plot (Figure 3A), the immune mediators were all clustered in the first component, suggesting a strong intercorrelation between the immune mediator levels. In the score plot (Figure 3B), where each dot represented a single child, a separation was found in the first component between picornavirus-positive versus picornavirus-negative children, underscoring that the presence of picornavirus in the nasopharynx of an asymptomatic infant is immune stimulatory. In support of this observation, the PLS regression analysis showed a highly significant up-regulation of the immune mediators in children with picornavirus (confounder adjusted *P* < 1 × 10^{-5}).

By inspecting the second component, we observed that picornavirus-positive infants generally expressed higher cytokine and chemokine levels (Figure 3B), which was primarily driven by...

### Table 2. Median Values of Immune Mediators and Association Between Immune Mediators in Children With Picornavirus and Controls

<table>
<thead>
<tr>
<th>Mediator</th>
<th>Children With Picornavirus</th>
<th>Controls</th>
<th>Crude Ratio (95% CI)</th>
<th>P Value</th>
<th>Adjusted* Ratio (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-12p70</td>
<td>6.74 (2.82–13.01)</td>
<td>4.91 (2.07–9.34)</td>
<td>2.54 (1.18–3.42)</td>
<td>&lt;.0001</td>
<td>2.16 (1.61–2.88)</td>
</tr>
<tr>
<td>CXCL10</td>
<td>682.13 (270.8–3664.14)</td>
<td>487.0 (205.24–1570.18)</td>
<td>7.22 (4.95–10.52)</td>
<td>&lt;.0001</td>
<td>6.52 (4.83–9.40)</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>8.37 (3.65–20.5)</td>
<td>4.76 (1.84–14.09)</td>
<td>3.96 (2.65–5.62)</td>
<td>&lt;.0001</td>
<td>3.47 (2.45–4.91)</td>
</tr>
<tr>
<td>TNF-α</td>
<td>60.99 (13.49–182.57)</td>
<td>27.13 (10.2–79.92)</td>
<td>2.34 (1.52–3.62)</td>
<td>&lt;.0001</td>
<td>2.06 (1.53–2.73)</td>
</tr>
<tr>
<td>CCL4</td>
<td>235.2 (76.04–1337.23)</td>
<td>149.95 (53.3–497.88)</td>
<td>1.65 (1.16–2.35)</td>
<td>&lt;.0001</td>
<td>1.46 (1.14–1.90)</td>
</tr>
<tr>
<td>CCL2</td>
<td>188.79 (89.44–229.15)</td>
<td>141.34 (77.65–256.92)</td>
<td>1.34 (0.98–1.84)</td>
<td>&lt;.0001</td>
<td>1.22 (0.96–1.57)</td>
</tr>
<tr>
<td>CCL13</td>
<td>21.66 (12.17–26.58)</td>
<td>16.52 (11.28–25.17)</td>
<td>1.31 (0.98–1.74)</td>
<td>&lt;.0001</td>
<td>1.23 (0.97–1.56)</td>
</tr>
<tr>
<td>IL-4</td>
<td>2.85 (1.07–6.96)</td>
<td>2.16 (0.68–4.7)</td>
<td>1.35 (0.93–1.93)</td>
<td>&lt;.0001</td>
<td>1.24 (0.97–1.60)</td>
</tr>
<tr>
<td>IL-5</td>
<td>4.45 (1.82–8.5)</td>
<td>2.82 (1.01–5.9)</td>
<td>1.56 (1.14–2.08)</td>
<td>&lt;.0001</td>
<td>1.43 (1.07–1.90)</td>
</tr>
<tr>
<td>IL-13</td>
<td>20.5 (9.01–35.07)</td>
<td>15.8 (7.84–29.73)</td>
<td>1.31 (0.94–1.80)</td>
<td>&lt;.0001</td>
<td>1.23 (0.95–1.58)</td>
</tr>
<tr>
<td>CCL11</td>
<td>114.52 (64.17–196.1)</td>
<td>83.65 (51.64–138.31)</td>
<td>1.38 (0.99–1.98)</td>
<td>&lt;.0001</td>
<td>1.24 (0.95–1.58)</td>
</tr>
<tr>
<td>CCL22</td>
<td>101.81 (34.08–152.31)</td>
<td>71.95 (18.53–153.05)</td>
<td>1.42 (1.01–1.97)</td>
<td>&lt;.0001</td>
<td>1.31 (0.98–1.73)</td>
</tr>
<tr>
<td>CCL17</td>
<td>16.72 (9.23–28.48)</td>
<td>16.25 (9.84–24.82)</td>
<td>1.06 (0.78–1.46)</td>
<td>&lt;.0001</td>
<td>1.00 (0.78–1.26)</td>
</tr>
<tr>
<td>CCL22</td>
<td>80.48 (47.51–228.63)</td>
<td>72.6 (43.27–179.52)</td>
<td>1.19 (0.86–1.63)</td>
<td>&lt;.0001</td>
<td>1.07 (0.81–1.40)</td>
</tr>
<tr>
<td>IL-17</td>
<td>2.46 (0.78–8.72)</td>
<td>1.66 (0.43–5.54)</td>
<td>1.54 (1.22–1.94)</td>
<td>&lt;.0001</td>
<td>1.46 (1.18–1.72)</td>
</tr>
<tr>
<td>IL-18</td>
<td>332.51 (35.79–1418.34)</td>
<td>98.88 (22.72–518.16)</td>
<td>6.71 (4.00–11.27)</td>
<td>&lt;.0001</td>
<td>5.83 (3.58–9.20)</td>
</tr>
<tr>
<td>CXCL8</td>
<td>5482.55 (2256.12–17229.22)</td>
<td>4202.98 (1426.18–7586.25)</td>
<td>2.25 (1.58–3.22)</td>
<td>&lt;.0001</td>
<td>1.99 (1.46–2.73)</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>61.93 (38.56–84.49)</td>
<td>51.79 (36.48–69.27)</td>
<td>1.29 (1.18–1.43)</td>
<td>&lt;.0001</td>
<td>1.25 (1.08–1.43)</td>
</tr>
<tr>
<td>IL-10</td>
<td>33.19 (13.14–70.71)</td>
<td>21.15 (8.95–45.22)</td>
<td>1.54 (1.24–1.92)</td>
<td>&lt;.0001</td>
<td>1.46 (1.18–1.72)</td>
</tr>
<tr>
<td>IL-2</td>
<td>25.66 (9.96–58.88)</td>
<td>20.18 (9.52–39.75)</td>
<td>1.29 (1.18–1.43)</td>
<td>&lt;.0001</td>
<td>1.25 (1.08–1.43)</td>
</tr>
</tbody>
</table>

Abbreviations: CI, confidence interval; IFN, interferon; IL-1β, interleukin 1β; IL-2, interleukin 2; IL-4, interleukin 4; IL-5, interleukin 5; IL-10, interleukin 10; IL-12p70, interleukin 12; IL-13, interleukin 13; IL-17, interleukin 17; IQR, interquartile range; TGF, transforming growth factor; TNF, tumor necrosis factor.

* Ratio adjusted for maternal history of asthma, allergy, or eczema, older siblings in the home, pathogenic airway bacteria, method of virus sampling, sampling site, season of sampling, batch of immune mediator analyses and maternal smoking in the third trimester.

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the type 1–related immune mediators CXCL10, CCL4, and TNF-α (Figure 3A). This is indicative of a particular association between these type 1–associated immune mediators and picornavirus, compared with the remaining investigated mediators, suggesting that picornavirus-positive children have a higher type 1–associated immune response.

**DISCUSSION**

**Principal Findings**

Presence of picornavirus in the airways of asymptomatic neonates affects the upper airway mucosal immune signature, with an increased release of type 1– and type 2–associated immune mediators but dominated by type 1–associated mediators of importance for clearance of intracellular pathogens. Our findings suggest that presence of picornavirus even in asymptomatic neonates promotes early topical airway immune activation and that the expected type 1–based immune enhancement by viruses is already evident in exposed newborns. The concomitant rise in type 2 immune mediators may be an underlying marker accounting for progression to asthma and allergic sensitization.

**Strengths and Limitations**

A major strength of the current study is the mucosal lining fluid sampling method, providing direct biomarker data on levels of immune mediators in vivo in the target organ of respiratory
Figure 3. Airway mucosal immune profiles. A partial least square discriminate analysis model was used to cluster immune mediators based on presence or absence of picornavirus at time of mediator sampling. A, Loading plot showing loadings of the 20 immune mediators. The cytokines are marked and colored according to their function. B, Score plot, in which each point corresponds to a single child. The distribution of picornavirus cases versus the rest is shown as ellipses. Differences are registered between the blue circle, representing controls, and the red circle, representing children with picornavirus. The loading plot shows that the children with picornavirus obtain higher values on component 1, which reflect the overall cytokine level. In the second component, the children with picornavirus are skewed toward type 1 cytokines production. Abbreviations: IFN, interferon; IL-1β, interleukin 1β; IL-2, interleukin 2; IL-4, interleukin 4; IL-5, interleukin 5; IL-10, interleukin 10; IL-12p70, interleukin 12p70; IL-13, interleukin 13; IL-17, interleukin 17; TGF, transforming growth factor; TNF, tumor necrosis factor; Treg, regulatory T cells.
viruses. Viral airway infections are well known to cause a type 1–oriented immune response as assessed in vitro in peripheral blood mononuclear cells [3, 4, 14]. However, it is not fully understood how this extrapolates to the in vivo situation in the airway mucosa, where the signaling between various cell types is in play. Our study is the first to examine the effect of asymptomatic picornavirus presence on the airway mucosal immune signature in healthy neonates.

In this study, respiratory viruses were detected with a PCR technique, and specific virus cultivation and antibody detection was not performed. All PCR assays were conducted with positive and negative controls, and our finding that 12% of the samples were virus positive, with the majority being picornavirus, is comparable to findings of other studies investigating the presence of viruses in asymptomatic children [15, 16].

It is a limitation of our study that we cannot quantify the load of virus based on the applied methodology. Moreover, we tested only for the presence of the most common pathogenic respiratory viruses and not for adenovirus. However, this biases the results toward the null hypotheses, because such false-negatives would have increased the immune mediator levels in the control group. Rhinoviruses were not differentiated into substrains, and use of the most recent primers may have found more positive samples.

Another limitation of our study is that we restricted our mucosal lining fluid analyses to 20 cytokines and chemokines, none of which belonged to the group of type I or III interferons. These particular mediators were carefully chosen a priori to represent both innate and adaptive mediators involving activation of type 1, type 2, type 17, and regulatory type responses, providing a representative view of mediators produced by the different airway immune cells. We use these classification terms to underscore that a variety of leukocytes can be producers of, for example, interferon γ, including natural killer cells, ILC1 cells, Th1 cells, and CD8+ T cells [17, 18]. Along the same lines, type 2 cytokines, such as IL-4, IL-5, and IL-13, can be produced by a variety of cells, including Th2 cells, ILC2 cells (IL-5, IL-13), and eosinophils (IL-4) [19, 20].

The high number of immune mediators raises the concern of multiple testing. To circumvent this problem, we also included a data-driven multivariate approach involving a PLS model. Concordance between results obtained from the conventional statistics and the data-driven approach enhances confidence in the findings.

The combination of pathogenic airway bacteria and picornavirus was associated with an additive effect. Importantly, we found no interaction between the effect of pathogenic airway bacteria and picornavirus and no evidence of a synergistic effect.

**Interpretation**

We found an overall immune-stimulatory effect of picornavirus in the airway mucosa of asymptomatic neonates, with a predominant enhancement of type 1–based inflammatory mediators. This profile was driven primarily by CXCL10 but also by CCL4, and TNF-α.

CXCL10 is a key type 1–related chemokine responsible for the early immune response to viral infection produced in response to both type I and type II interferon stimulation [21]. CXCL10 attracts various CXCR3+ cells, including plasmacytoid dendritic cells, Th1 cells, and CD8+ T cells, important for elimination of viruses. In general, the type 1 immune response is known to be important for intracellular clearance of pathogens such as viruses [22], and the observed type 1–based cytokine signature associated with picornavirus presence is thus biologically meaningful.

The Picornaviridae family includes, among others, the genera rhinovirus and enterovirus, with rhinovirus being the most prevalent [23, 24]. Picornaviruses in general and rhinoviruses in particular are well-known triggers of asthma and asthma exacerbations as well as the causal agents for upper and lower respiratory tract infections [25, 26]. We found a general immune-stimulatory effect of picornavirus on the airway immune profile in asymptomatic neonates, primarily driven by rhinovirus.

Furthermore, picornavirus infections, predominantly rhinovirus in the first year of life, have been proposed to be the main external trigger for wheezing and later asthma development [27, 28]. The effect of early asymptomatic presence of picornavirus has not been studied in this context, and the observed effect of picornavirus in the airways of healthy newborns may play an important role in programming the specific type of memory response to picornavirus during first encounter in early life.

We found an overall elevated level of immune mediators, with predominance of type 1–related mediators, which could simply reflect a normal immune response against virus or represent one early event involved in skewing the Th1-Th2 balance in favor of Th1 in healthy children. Moreover, the type 17–related mediators interleukin 17, IL-1β, and CXCL8 were enhanced by picornavirus, as were regulatory factors such as transforming growth factor β1 and interleukin 10, suggesting that various immune paths are activated by the viral trigger, including both proinflammatory trails as well as those involved in immune cessation.

We also observed an exaggerated type 2 response, which could be explained by the viruses evading type 1–based immunity by inducing a Th2 response [29]. It is well known that early life skewing toward Th2, or type 2 responses has long-term consequences for childhood health, because enhanced production of type 2–related mediators, such as IL-4, IL-5, and IL-13, predispose to development of asthma and allergy [30, 31]. Accordingly, we have shown elsewhere that elevated levels of type 2–associated chemokines within cord blood are associated with increased total immunoglobulin E production in preschool-age children [32].
Presumably, the main external trigger of early life Th1/Th2/Th17 immune development is the composition of the human microbiome [33–35]. Factors influencing the human microbiota and the immunological consequences of such influence is demonstrated in studies of farming communities, where it is evident that being subjected to animals early in life modulates the microbiome and the immunological fingerprint in a type 1– or regulatory-oriented direction, resulting in protection against asthma and allergy development [36,37]. Similarly, the presence of older siblings has been found elsewhere to inversely relate to the incidence of hay fever [38], and both the presence of older siblings in the home and early daycare attendance have been reported to protect against asthma and wheezy symptoms in later childhood [39]. Because other children in the home or in daycare are the main reservoirs for common respiratory microbes [40], such findings indicate that being subjected to microbes early in life is important for an optimal immune maturation.

In conclusion, the asymptomatic presence of picornavirus in the neonatal airway is a potent activator of the airway mucosal immune system, with predominant enhancement of key proinflammatory mediators of type 1 origin.

Supplementary Data

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Author contributions. The guarantor of the study is H. B., who has been responsible for the integrity of the work as a whole, from conception and design to conduct of the study and acquisition of data, analysis and interpretation of data, and writing of the manuscript. H. M. W., N. V. F., S. Birch, S. Brix, T. T. H., S. L. J., T. K., B. L. C., and K. B. were responsible for data analysis, interpretation, and writing of the manuscript. S. Brix was responsible for all the intellectual input and approval of the final version of the manuscript.

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