IFN-α/IFN-λ responses to respiratory viruses in paediatric asthma

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ABSTRACT We analysed the influence of rhinovirus (RV) in nasopharyngeal fluid (NPF) on type I and III interferon (IFN) responses (e.g. IFN-α and IFN-λ) and their signal transduction, at baseline and during disease exacerbation, in cohorts of pre-school children with and without asthma.

At the time of recruitment into the Europe-wide study PreDicta, and during symptoms, NPF was collected and the local RV colonisation was analysed. Peripheral blood mononuclear cells (PBMCs) were challenged in vitro with RV or not. RNA was analysed by quantitative real-time PCR and gene arrays. Serum was analysed with ELISA for IFNs and C-reactive protein.

We found that PBMCs from asthmatic children infected in vitro with the RV1b serotype upregulated MYD88, IRF1, STAT1 and STAT2 mRNA, whereas MYD88, IRF1, STAT1 and IRF9 were predominantly induced in control children. Moreover, during symptomatic visits because of disease exacerbation associated with RV detection in NPF, IFN-α production was found increased, while IFN-λ secretion was already induced by RV in asthmatic children at baseline.

During asthma exacerbations associated with RV, asthmatic children can induce IFN-α secretion, indicating a hyperactive immune response to repeated respiratory virus infection.

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Introduction

Viral infections, especially with rhinovirus (RV), a member of the family Picornaviridae, play an important role in asthma development, asthma exacerbations in children and airway remodelling [1–4]. RV enters cells via members of the low-density lipoprotein receptor family or intercellular adhesion molecule 1 (online supplementary figure S1) [2, 5]. Within the cell, single-stranded virus RNA is detected by Toll-like receptors TLR7, TLR8 and especially TLR3 [5]. A myeloid differentiation primary response protein (MyD88)-dependent pathway finally leads to phosphorylation and activation of the interferon (IFN) regulatory factor IRF7, as well as IRF3 and NF-κB. Activated IRF7, IRF3 and NF-κB translocate to the nucleus and trigger antiviral immune responses [1, 6].

One large group of agents with antiviral activity is the family of IFNs. Based on structural characteristics, receptor usage and biological activities, IFNs are classified in three different types [7]. Type I and III IFNs are primarily responsible for antiviral activities in humans [1, 7]. While type I IFN combines 13 species of IFN-α, there is a single species of each IFN-β, IFN-ε, IFN-κ and IFN-ω. In asthma, type I IFNs are described to be downregulated [6] and it has been shown that treatment with a pegylated form of IFN-α effectively eliminates persistent RV in patients with immunosuppression [8]. Furthermore, type III IFN responses have also been shown to be impaired during asthma exacerbations [9].

The receptor for type I IFNs is composed of two chains of transmembrane glycoproteins, called IFNAR1 and IFNAR2. Several IFN-regulated signalling elements and cascades have been discovered [10, 11]. While most cell types express type I and II IFN receptors, type III IFN receptors are primarily found on cells of epithelial derivation, including myeloid lineage cells such as dendritic cells and macrophages [10, 12].

The intracytoplasmic domains of the receptors donate binding sites for Janus kinase 1 (Jak1) and tyrosine kinase 2 (Tyk2). Binding of the ligand activates auto- and trans-tyrosine phosphorylation of Jak1 and Tyk2, and their principle targets for phosphorylation, i.e. signal transducer and activator of transcription (STAT) proteins STAT1 and STAT2 [10, 11, 13]. Together with IRF9, phosphorylated STAT1 and STAT2 form the so-called IFN-stimulated gene factor 3 (ISGF3) complex. Subsequently, this heterotrimeric complex translocates to the nucleus, detects a specific DNA motif, termed the IFN-stimulated response element, and advances the transcription of IFN-stimulated genes [10, 11, 13].

Negative regulatory factors, like suppressor of cytokine signalling 1 (SOCS1), limit the scale and duration of the antiviral reaction by directly inhibiting kinase activity via binding to the IFNAR1–Tyk2 complex [10, 14].

Even though type III IFNs, also called IFN-λ1 (or interleukin (IL)-29), IFN-λ2 (or IL-28A) and IFN-λ3 (or IL-28B), operate a different receptor complex from type I IFNs, they activate a similar antiviral signalling, resulting in phosphorylation of different STAT proteins [7].

As there is a need to better understand and manage asthma especially in small children [15] and since asthma often begins in early childhood, we focused on pre-school children aged 4–6 years. In children the only virus types significantly associated with asthma exacerbations are RVs [5]; therefore, in this study, we asked how RV infection would regulate type I and III IFNs in paediatric asthma at baseline and during disease exacerbations associated with RV.

Material and methods

Human study PreDicta

Our investigations are part of the Europe-wide study PreDicta (Post-infectious immune reprogramming and its association with persistence and chronicity of respiratory allergic diseases). This multicentre prospective cohort study is carried out in five different centres in Europe across major cultural and climatic regions. The Dept of Molecular Pneumology in collaboration with the Dept of Paediatrics and Adolescent Medicine of the Children’s Hospital of the Friedrich-Alexander Universität Erlangen-Nürnberg (Erlangen, Germany) is one of these study centres.

Subject recruitment and the timescale of the study are described in detail in the online supplementary material and elsewhere [16]. Some of the clinical aspects of the children are reported in table 1 and were published recently [16–19].

The study was approved by the Ethics Committee of the Universitätsklinikum Friedrich-Alexander Universität Erlangen-Nürnberg (Re-No 4435). Informed consent was obtained from the parents/guardians of all participants. The study is registered in the German Clinical Trial Register (www.germanctr.de: DRKS00004914).

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Detailed information about sample recovery and subsequent analysis is given in the online supplementary material.

**Statistical analysis**

Differences were evaluated for statistical significance using the nonparametric two-tailed Mann–Whitney test (Prism version 6 for Windows; GraphPad, La Jolla, CA, USA).

**Results**

**Clinical data of the cohorts of children analysed in this study**

All subjects analysed in this study were pre-school children aged between 4 and 6 years. Whereas all healthy children received no treatment at all, asthmatic children were treated with either steroids, nonsteroid drugs (e.g. bronchodilators or antihistamines) or a combination of both. The clinical and virology data of the PreDicta cohort in Erlangen were previously described, and will be collectively reported in a manuscript in preparation [16–18]. Tables 1 and 2 give an overview of important clinical data of the Erlangen cohorts at the time of recruitment into the PreDicta study.

**RV induced STAT1/STAT2 in PBMCs of asthmatic children and IRF9 in control children, whereas IRF1 was upregulated in both groups**

It is known that after cellular RV infection both IFN-α and IFN-λ are secreted from various cells, e.g. plasmacytoid dendritic cells, and that these cytokines induce STAT1 signalling in T-cells (online supplementary figure S1) [4, 7, 11].

We thus wanted to investigate the genes upregulated in PBMCs after *in vitro* exposure to RV and subsequent cell culture for 48 h, by using RNA gene arrays (figure 1a). As a demonstration that RV challenged the cells in both control and asthmatic PBMCs cell culture, it was noted that incubation with RV *in vitro* led to an upregulation of *MYD88* expression (figure 1b and c). Moreover, we identified *STAT1*

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**TABLE 1** Demographic data of the children recruited in Erlangen

<table>
<thead>
<tr>
<th></th>
<th>Healthy control children</th>
<th>Children with asthma</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Subjects</strong></td>
<td>22</td>
<td>24</td>
</tr>
<tr>
<td><strong>Sex</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>59.1 [13/22]</td>
<td>62.5 [15/24]</td>
</tr>
<tr>
<td>Female</td>
<td>40.9 [9/22]</td>
<td>37.5 [9/24]</td>
</tr>
<tr>
<td><strong>Age years</strong></td>
<td>4.7±0.84</td>
<td>4.8±0.64</td>
</tr>
<tr>
<td><strong>FEV1 &lt;100% predicted</strong></td>
<td>27 [6/22]</td>
<td>54 [13/24]</td>
</tr>
<tr>
<td><strong>Treatment</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No treatment</td>
<td>100 [22/22]</td>
<td>21 [5/24]</td>
</tr>
<tr>
<td>Nonsteroid</td>
<td></td>
<td>17 [4/24]</td>
</tr>
<tr>
<td>Steroid</td>
<td></td>
<td>63 [15/24]</td>
</tr>
<tr>
<td>Steroid+nonsteroid</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>NPF: RV</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>45 [10/22]</td>
<td>54 [13/24]</td>
</tr>
</tbody>
</table>

Data are presented as n, % (n/N) or mean±SD. FEV1: forced expiratory volume in 1 s; NPF: nasopharyngeal fluid; RV: rhinovirus.

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**TABLE 2** Nasopharyngeal rhinovirus (RV) colonisation and treatment in the asthmatic group

<table>
<thead>
<tr>
<th></th>
<th>−RV</th>
<th>+RV</th>
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<tbody>
<tr>
<td><strong>NS</strong></td>
<td>40 [2/5]</td>
<td>60 [3/5]</td>
</tr>
<tr>
<td><strong>S+NS</strong></td>
<td>58 [11/19]</td>
<td>42 [8/19]</td>
</tr>
</tbody>
</table>


Data are presented as % [n/N]. NS: nonsteroid treatment; S: steroid treatment. a: in the top two rows the children are subdivided first in accordance with their treatment and then by RV colonisation; in the bottom two rows the children are subdivided first in accordance with RV colonisation and then by treatment.
FIGURE 1 Peripheral blood mononuclear cells (PBMCs) from asthmatic children exposed to rhinovirus (RV) in vitro showed upregulated MYD88 and STAT1A/STAT2 after 48 h. a) Experimental design for RNA arrays of PBMCs incubated for 1 h in the presence or absence of the RV1b serotype and then cultured for an additional 48 h. b, c) Heatmaps for mRNA isolated from PBMCs of b) asthmatic and c) control children exposed (+RV) or not exposed (–RV) to RV. Differential expression analysis of the regulated genes is shown (log2 average +RV/average –RV). nasthma=7, ncontrol=5. d, e) Quantitative real-time PCR for d) STAT1A and e) STAT2 mRNA in PBMCs from control and asthmatic children as used in arrays. ncontrol,RV-negative=5 (–RV), ncontrol,RV-positive=5 (+RV), nasthma,RV-negative=3 (–RV), nasthma,RV-positive=5 (+RV). Data are presented as mean±SEM. *: p≤0.05.
and STAT2 as two candidate genes upregulated in RV-challenged PBMCs from asthmatic children (figure 1b and online supplementary figure S2). Quantitative real-time PCR (qPCR) confirmed these data as the STAT1A isoform [20] and STAT2 were found 7- and 2-fold, respectively, upregulated by RV in children with asthma (figure 1d and e). Medications, specifically steroids, do not seem to be responsible for the upregulation of STAT1/STAT2 in these steroid-treated children with asthma, as steroids have an opposite effect on STAT1 isoforms A and B (online supplementary figure S2). In addition, control children showed upregulated IRF9 (figure 1c), while both groups analysed showed upregulated IRF1, an antiviral transcription factor (figure 1b and c), after RV exposure, as assessed by gene arrays. Taken together, these data support the presence of downstream genes upregulated by IFN in PBMCs challenged with RV both from controls and children with asthma.

Regulation of IFNA mRNA expression in PBMCs of pre-school asthmatic children with respiratory virus infection and IFNA gene analysis

Next, upstream of STAT1/STAT2, IFNA mRNA levels were analysed by qPCR in the above-described cohorts of pre-school children with and without asthma in isolated and untouched PBMCs (figure 2a).

Here, we found a statistically significant decrease of IFNA mRNA expression in asthmatic patients as compared with control children (figure 2b). A mutational screening analysis in these cohorts did not show any genetic alterations in the IFNA gene (online supplementary table S2).

We next hypothesised that environmental factors such as RV might regulate IFN-α expression differently in blood cells in asthmatic children.

To address this point, we searched for the presence of RV in the nasopharynx of these children, which might have caused airway infections, a feature recognised especially in pre-school age. On average, RV RNA detection at baseline showed no notable difference between healthy and asthmatic children (table 1).

Investigating IFNA mRNA expression in PBMCs and RV RNA detection in vivo in nasopharyngeal fluid (NPF) reveals another difference between healthy and asthmatic subjects. In healthy children, RV detection in vivo in NPF was found associated with an increase in IFNA mRNA expression in their whole blood cells.
PBMCs as compared with asthmatic children with positive RV detection in their NPF (figure 2c). This was also detected at the protein level in serum, although no statistical significance was observed (figure 2d). In summary, asthmatic patients with in vivo RV detection did not show upregulated IFN-α levels as RV-positive control patients did. Furthermore, in PBMCs treated in vitro with steroids, we could not find any significant influence of steroids on IFN-α mRNA expression (online supplementary figure S3).

Taken together, these findings suggest an impairment in the antiviral immune response in the blood of children with asthma when RV is present in their airways. In addition, we did not see any relationship between IFN-α regulation and the medications nor the allergic status in these patients (tables 3 and 4).

C-reactive protein levels in control and asthmatic children at the time of recruitment
Comparing asthmatic and control children with and without nasopharyngeal RV RNA detection in NPF, we found that the levels of C-reactive protein (CRP), a protein with a half-life of 19 h which is produced during inflammation and infection [21], in serum of control and asthmatic children did not differ at the time of recruitment (figure 2e), even though asthmatic children were recruited preferentially during the winter as compared with the control children (online supplementary figure S4).

Role of RV or other respiratory viruses in NPF on STAT1/SOCS1 in asthmatic patients at baseline
To better analyse IFN-α regulation in asthmatic patients, we then carried out analysis of the methylation status of five CpG islands within the IFNAR1 and SOCS1 genes, which also did not reveal any difference in methylation in healthy and asthmatic children (online supplementary figure S5 and table S3). Consistently, we could not find any regulation of SOCS1 at the mRNA level after qPCR (supplementary figure S6).

When we looked at STAT1 mRNA expression, downstream of the IFN-α receptor, to see if we could find impaired IFN signalling, STAT1 expression in total blood was not found increased in asthmatic children with positive RV detection in vivo (online supplementary figure S7). We next analysed if steroids would have influenced STAT1 and found that this was not the case; rather, steroids decrease STAT1 mRNA expression (online supplementary figure S7).

RV RNA detection in NPF was found associated with IFN-λ induction in the blood of children with asthma at baseline
As STAT1/STAT2 map downstream of different IFN family members, including IFN-λ (a strong antiviral factor), we next examined type III IFN in serum. We found a significant increase of IFN-λ protein in serum

| TABLE 3 Virus detection, medication and relative IFN-α mRNA expression in peripheral blood mononuclear cells (baseline) |
|-----------------|----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|

obtained from children with asthma and with RV detected in their nasopharyngeal tract as compared with RV-negative asthmatic children at baseline (figure 3). The same tendency was observed in control children.

Acute asthma exacerbation with RV and other respiratory virus infection in NPF in asthmatic children is associated with induced IFN-α secretion

The above results show that children with asthma and RV detection in the NPF have reduced IFN-α levels in the serum as compared with RV-colonised healthy children at baseline. Interestingly, after an asthma

| Asthma patient ID | Baseline | Symptomatic | Medication | Skin test
group |
<table>
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<tbody>
<tr>
<td></td>
<td>RV#</td>
<td>IFN-α pg·mL⁻¹</td>
<td>IL-29/IL-28B pg·mL⁻¹</td>
<td>RV#</td>
</tr>
<tr>
<td>203</td>
<td>–</td>
<td>ND</td>
<td>452.52</td>
<td>NSA</td>
</tr>
<tr>
<td>204</td>
<td>–</td>
<td>ND</td>
<td>713.86</td>
<td>NSA</td>
</tr>
<tr>
<td>205</td>
<td>–</td>
<td>1103.05</td>
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<td>NSA</td>
</tr>
<tr>
<td>207</td>
<td>–</td>
<td>ND</td>
<td>245.98</td>
<td>++</td>
</tr>
<tr>
<td>216</td>
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<td>210.70</td>
<td>NSA</td>
</tr>
<tr>
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<td>ND</td>
<td>323.47</td>
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<td>213</td>
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<td>ND</td>
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<tr>
<td>223</td>
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<td>21.94</td>
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<td>224</td>
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<td>960.89</td>
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<td>ND</td>
<td>NSA</td>
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</tr>
<tr>
<td>229</td>
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<td>ND</td>
<td>115.22</td>
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</tr>
<tr>
<td>230</td>
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<td>ND</td>
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<td>++</td>
</tr>
<tr>
<td>238</td>
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<td>51.00</td>
<td>769.14</td>
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<tr>
<td>239</td>
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<td>NSA</td>
</tr>
<tr>
<td>242</td>
<td>++</td>
<td>NSA</td>
<td>2505.94</td>
<td>++</td>
</tr>
<tr>
<td>243</td>
<td>++</td>
<td>7.31</td>
<td>773.50</td>
<td>+++</td>
</tr>
</tbody>
</table>

ND: not determined; NSA: no sample available; LTRA: leukotriene receptor antagonist. #: genome copy number in sample: +++: high; ++: intermediate; +: low; -: negative. ¶: more “+” symbols refer to a higher atopic status; –: negative.

FIGURE 3 Rhinovirus (RV) detected in vivo associated with upregulation of interferon (IFN)-λ (interleukin (IL)-29/IL-28B) levels in serum of asthmatic patients: amount of IFN-λ in serum measured by ELISA and RV detected in vivo in nasopharyngeal fluid. n_control_RV-negative=4 (–RV), n_control_RV-positive=14 (+RV), n_asthma_RV-negative=7 (–RV), n_asthma_RV-positive=12 (+RV). Data are presented as mean±SEM. *: p≤0.05.
exacerbation or infection, when asthmatic children were asked to come to the hospital for an examination within the next 48 h after the onset of symptoms (symptomatic visit "S"; figure 2a), the concentration of IFN-α in the serum was elevated in the presence of RV in NPF, in contrast to RV-positive children at the recruitment visit (figure 4a). The serum concentration of IFN-λ was not found to be altered by acute asthma exacerbations (figure 4b) and remained upregulated as at baseline. A differential analysis of the CRP level in the serum of asthmatic children revealed that there is a tendency towards elevated CRP values at the time of a symptomatic visit and further investigation provided evidence that even higher values were present after RV detection and/or infection (figure 4c and d). These results indicate that asthmatic children, with RV detection in NPF, seem to have impaired IFN-α production and/or release under steady-state conditions. However, this observation seems to be time dependent; asthmatic children are able to significantly upregulate IFN-α production during an acute infection, while IFN-λ is not further regulated during this process. We also extended this analysis to three additional cohorts of PreDicta and observed an overall induction of IFN-α in asthmatic children, whose serum was analysed during symptomatic visits at their children’s hospital, when their serum CRP was significantly elevated in association with a respiratory virus infection in their airways (online supplementary figure S9). In accordance with the cohort from Erlangen, no significant difference was observed in IL-29/IL-28B serum levels (online supplementary figure S9).

Discussion

Previous studies showed defective RV IFN responses in bronchoalveolar lavage fluid and bronchial epithelial cells from asthmatic patients [6, 22]. In this study, we support and expand the notion of a previously described defect in type I IFN production in asthmatic adult patients [6] to pre-school children. We found decreased IFNA levels in blood cells from asthmatic children compared with healthy children at the time of recruitment. Moreover, we found that RV RNA detection in the upper airways was associated with induced IFNA mRNA expression in blood cells of control children as compared with asthmatic pre-school children.
exacerbations associated with RV, had significantly upregulated IFN-α. We discovered that asthmatic children, during a visit to the hospital within 48 h after onset of disease, show a decrease of this cytokine as soon as 10 h after virus infection [24]. Moreover, in the past, several approaches to improve the treatment of RV infection have been developed, including administration of IFN-α to adults with persistent RV infection. This trial showed promising results [8]. However, there are also some drawbacks with the treatment with IFN-α. Although it has been shown to clear RV RNA rapidly [8], it seems to reduce symptoms only modestly and patients suffer from strong side-effects, including flu-like illness, nausea and headaches [1, 5, 25].

Our hypothesis of a time-dependent regulation of IFN-α was further supported by the extension of our observation to different cohorts of children from the PreDicta study. It is also in accordance with our analysis of IFNA1 and IFNA13, where we did not find any pathogenic mutation, and the methylation analysis, which showed no difference in epigenetics of IFNAR1 and SOCS1 between asthmatic and control children. However, the exact mechanism behind deficient IFN-α in asthmatic subjects and the ability to overcome this lack in acute infections remains unknown. One possibility relates to the time point of respiratory virus detection. It is possible that during the symptomatic visit, the asthmatic children are closer to the RV infection as compared with the baseline visit. This is also supported by the higher CRP levels during the symptomatic visit. IFN-α kinetics show a decrease of this cytokine as soon as 10 h after virus infection [24]. Moreover, in the past, several approaches to improve the treatment of RV infection have been developed, including administration of IFN-α to adults with persistent RV infection. This trial showed promising results [8]. However, there are also some drawbacks with the treatment with IFN-α. Although it has been shown to clear RV RNA rapidly [8], it seems to reduce symptoms only modestly and patients suffer from strong side-effects, including flu-like illness, nausea and headaches [1, 5, 25].

Our hypothesis of a time-dependent regulation of IFN-α was further supported by the extension of our observation to other cohorts of the PreDicta study (i.e., Athens, Lodz and Turku). In this analysis, we observed a variability which might be attributed to different confounders such as atopy, sampling during pollen season, antibiotics for bacterial complication or other oral medications, or even disease activity [6, 26]. The use of steroid treatment, however, was not observed to influence IFNA mRNA expression. This is in line with the finding that corticosteroids have been shown to have a clinical benefit in children with RV-associated first-time wheezing [27, 28]. Further studies in this direction are underway.

Moreover, during acute exacerbation in asthma associated with RV, looking at type III IFNs, such as IFN-λ, we found it induced in serum, although an increase in CRP and IFN-α was observed. This could be consistent with a delayed kinetic of IFN-λ.

Consistent with their antiviral activity, virus-infected cells usually induce both type I and type III IFNs. One reason might be common upstream regulatory elements in type I and type III IFN genes, such as those for NF-κB, IRF3 and IRF7 [7]. However, transcriptional regulation of type I and type III IFNs is not completely identical. Moreover, studies using neutralising antibodies showed that activation of type I and III receptor systems in response to viral infections is independent [7], but the different groups of IFNs do interact. For example, IFN-α is able to upregulate IFN-λ, probably via increasing expression of the genes for Toll-like receptors and IRF7 [7]. As IFN-λ production and severity of symptoms showed an inverse correlation [9], type III IFN is thought to have a protective role in asthma [5]. Further research is required to better understand the interactions of IFN pathways after RV infection in asthmatic as well as healthy children. Our in vitro gene array data suggest that both control and asthmatic children upregulated significantly more IRF1, and whereas asthmatic children upregulated the STAT1A and STAT2 components, control children preferentially activated STAT1A and IRF9 downstream of IFN in PBMCs infected with RV. Thus, since STAT1A/STAT2 are downstream of IFN-α/IFN-λ it seems reasonable that these two types of IFNs are not defective during RV exacerbation seen in paediatric asthma. The exact mechanism of this differential regulation needs to be addressed in further studies.

Regarding the clinical conditions, it is already known that the presence of viruses, as well as bacteria, influences the immune system and increases the risk of exacerbation in chronic pulmonary disease [1–4, 29]. Moreover, RV infections play an important role not only in asthma development [1, 2, 25], but are the only virus types that are significantly associated with asthma exacerbations in children [1, 3, 5, 23]. Additionally, it is known that RV can reach, penetrate and replicate in the lower airways after nasal inoculation, and thus lead to lower airway inflammation and, in the long term, airway remodelling [1, 30]. In this study, we focused especially on RV detection in NPF and its effects on the immune responses on pre-school children. These results are also consistent with the fact that individuals with atopic asthma are not at greater risk of RV infections, but suffer from more frequent infections and more severe symptoms [31].
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