



TLR7/8 regulates type I and type III interferon signalling in rhinovirus 1b-induced allergic asthma

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TLR7/8 agonist reduced IFN- α R1 but induced IFN- λ R1 in PBMCs of pre-school age children. This induction of IFN- λ R1 offers a new mechanism for therapy of children with RV1b-exacerbated asthma. <https://bit.ly/2QWnoTp>

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ABSTRACT

Introduction: Interferon (IFN) responses have been reported to be defective in rhinovirus (RV)-induced asthma. The heterodimeric receptor of type I IFN (IFN- α/β) is composed of IFN- α R1 and IFN- α R2. Ligand binding to the IFN- α/β receptor complex activates signal transducer and activator of transcription (STAT) proteins STAT1 and STAT2 intracellularly. Although type III IFN (IFN- λ) binds to a different receptor containing IFN- λ R1 and interleukin-10R2, its triggering leads to activation of the same downstream transcription factors. Here, we analysed the effects of RV on IFN type I and III receptors, and asked about possible Toll-like receptor 7/8 (TLR7/8) agonist R848-mediated IFN- α R1 and IFN- λ R1 regulation.

Methods: We measured IFN- α , IFN- β and IFN- λ and their receptor levels in peripheral blood mononuclear cell (PBMC) supernatants and cell pellets stimulated with RV1b and R848 in two cohorts of children with and without asthma recruited at pre-school age (PreDicta) and at primary school age (AGENDAS) as well as in cell supernatants from total lung cells isolated from mice.

Results: We observed that R848 induced IFN- λ R mRNA expression in PBMCs of healthy and asthmatic children, but suppressed IFN- α R mRNA levels. In murine lung cells, RV1b alone and together with R848 suppressed IFN- α R protein in T-cells compared with controls and in total lung IFN- λ R mRNA compared with RV1b infection alone.

Conclusions: In PBMCs from pre-school age children, IFN- α R mRNA was reduced and IFN- λ R1 mRNA was induced upon treatment with the TLR7/8 agonist R848, thus suggesting new avenues for induction of antiviral immune responses in paediatric asthma.

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Introduction

Asthma is a chronic inflammatory disease of the respiratory tract that has variable clinical occurrences. More than 300 million people worldwide are affected and the cumulative incidence is still rising [1]. It has been indicated that T-lymphocytes play a prominent role in the pathogenesis of asthma. In particular, T-helper type 2 (Th2) cells and their cytokines, *e.g.* interleukin (IL)-5 and IL-13, are overexpressed in allergic asthma [2] starting from childhood [3, 4]. Key problems in asthma are chronic inflammation of the airways and hyperresponsiveness that lead to episodes of coughing, wheezing and shortness of breath [5]. Repeated viral infections may accompany the immune shift towards a Th2 phenotype and are also considered as crucial factors for asthma exacerbation in children and airway remodelling [4].

Rhinovirus (RV) infections are common [6], but there is evidence that affected patients with asthma may show marked variability in symptoms upon infection [7]. RV is a member of the Picornaviridae family [6]. Major group RV (parts of serotypes of RV-A and all serotypes of RV-B) enters the body through the upper airways [6] and cells *via* intercellular adhesion molecule 1, while minor group RV (parts of serotypes of RV-A, *i.e.* RV1b) [6, 8] enters *via* members of the low-density lipoprotein receptor family [9, 10]. RV-C enters the cells *via* cadherin-related family member 3 (CDHR3) [11]. Toll-like receptor (TLR) 7, TLR8, TLR9 and TLR3 detect virus nucleic acids [12], and lead to the activation of a myeloid differentiation primary response protein (MyD88)-dependent pathway [12], resulting in phosphorylation of interferon (IFN) regulatory factor (IRF) 7 and IRF3 that leads to the transcription of type I (IFN- α and IFN- β) and type III (IFN- λ) IFNs [9, 12].

After IFN- α binds to the heterodimeric receptor complex containing the transmembrane glycoproteins IFN- α receptor chain 1 (IFN- α R1) and IFN- α R2, tyrosine kinase 2 (TYK2) and Janus kinase 1 (JAK1) become activated and phosphorylate the transcription factors signal transducer and activator of transcription protein (STAT) 1 and STAT2 [13]. Phosphorylated STAT1 and STAT2 form a complex with IRF9, the so-called IFN-stimulated gene factor 3 (ISGF3) complex that translocates to the nucleus and promotes the transcription of IFN-stimulated genes [13]. The signalling pathway of IFN- λ (also known as IL-28A, IL-28B and IL-29) is similar to IFN- α , but IFN- λ R has a different structure compared with IFN- α R and consists of IFN- λ R1 and IL-10R2 [14, 15]. The latter receptor chain is also used for some other cytokine signalling pathways, *e.g.* IL-10 and IL-22 signalling pathways [14, 15]. However, the IFN type III-induced immune response is more restricted, because IFN- λ R1 is expressed on fewer cells compared with IFN- α R1 [14, 16, 17].

Asthmatic subjects seem to have a defect in their type I IFN levels upon viral infection [18–20], but we recently described that they can produce IFN- α in the case of acute exacerbation [9]. Several reports suggested an increased secretion of IFN- λ in asthmatic subjects [14, 21, 22]. Our group also studied IFN- λ secretion after RV1b infection and found an increased production in peripheral blood mononuclear cells (PBMCs) of pre-school age asthmatic children that had a positive RV detection *in vivo* [9, 14].

In this study, we further investigated the production and expression of type I and type III IFNs in asthmatic children. Accordingly, we examined the pathways of those IFNs and their receptors with particular reference to IFN- α R1 and IFN- λ R1. Moreover, we used a TLR7/8 agonist (R848) that is known to upregulate IFN type I expression [23] in order to study IFN production in asthma patients upon RV1b infection.

Material and methods

Additional details are provided in the supplementary material.

Human studies PreDicta and AGENDAS

In the European study PreDicta (Post-infectious immune reprogramming and its association with persistence and chronicity of respiratory allergic diseases) Work Package 1 (WP1), healthy and asthmatic pre-school children aged 4–6 years were recruited and examined at recruitment as well as 2 years later (in collaboration with the Dept of Allergy and Pneumology, Children's Hospital, Friedrich-Alexander-Universität (FAU) University Hospital, Erlangen, Germany). The study in Erlangen was approved by the Ethics Committee of the FAU Erlangen-Nürnberg (4435) and was registered retrospectively in the German Clinical Trials Register with identifier DRKS00004914.

The study AGENDAS (Gender, age and environmental factors that modify immune-response and the development of allergic asthma during the school age in childhood) was performed in Erlangen with the same team that performed PreDicta. In this longitudinal study, primary school children aged 6–11.9 years were recruited and analysed in collaboration with the Dept of Allergy and Pneumology, Children's Hospital, FAU University Hospital. The study was approved by the local Ethics Committee of the FAU Erlangen-Nürnberg (212_12 B) and is registered in the German Clinical Trials Register with identifier DRKS00004927.

The recruitment of the subjects, inclusion and exclusion criteria as well as the time scale for clinical visits and data collection have been described previously [9, 24, 25].

In vitro cell culture of human PBMCs with RV1b or R848

PBMCs were adjusted to a concentration of 1×10^6 viable cells·mL⁻¹ in complete culture medium. PBMCs were cultured for 48 h in complete culture medium at 37°C/5% CO₂, whereby parts of them were challenged *in vitro* with RV1b or 200 µL·mL⁻¹ R848 (0.2 mg·mL⁻¹) (resiquimod; InvivoGen, Toulouse, France).

Details on the growth of RV1b and the description of the RV1b infection itself have been published elsewhere [9, 26, 27]. Following challenge, supernatants were carefully removed and stored at -80°C. For RNA extraction, PBMCs were diluted in QIAzol Lysis Reagent (Qiagen Sciences, Germantown, MD, USA) and stored at -80°C.

Mice

All mice were maintained under specific pathogen-free conditions with free access to food and water. The government of Mittelfranken (Bavaria, Germany) approved the experiments (55.2-2532-2-633).

Total lung cell isolation and in vitro cell culture (RV1b and/or R848)

Whole lungs from sacrificed 9-week-old mice were removed and used to isolate total lung cells: lungs were sectioned and mixed with 10 mL PBS, 300 U·mL⁻¹ collagenase type Ia, 0.015% DNase I and digested for 45 min at 37°C. Digested lungs were passed through a cell strainer (40 µm) prior to centrifugation. Pellets were then mixed with ammonium-chloride-potassium lysing buffer and incubated for 2 min at room temperature before they were centrifuged again. Pellets were washed with PBS. Isolated lung cells were centrifuged and counted. Isolated lung cells were cultured in complete culture medium (RPMI 1640 medium supplemented with 10% fetal calf serum, 1% L-glutamine and 1% penicillin/streptomycin) for 24 h at 37°C/5% CO₂. For RV infection, total lung cells were incubated with RV1b for 1 h at 33°C on a horizontal shaker, washed with medium and cultured in complete culture medium for 24 h at 37°C/5% CO₂. For R848 stimulation, cells were cultured in complete culture medium and 200 µL·mL⁻¹ R848 for 24 h at 37°C/5% CO₂.

Flow cytometric analysis and cellular staining

After 24 h, cultured total lung cells were harvested, washed and stained with fluorochrome-conjugated anti-CD4, anti-CD8, anti-CD25, anti-FOXP3 and anti-IFN-αR1 antibodies (supplementary table S2) in staining buffer (PBS/0.1% sodium azide/1.0% fetal bovine serum) at 4°C for 30 min and then washed in PBS. Fluorescence-labelled cells were acquired by fluorescence-activated cell sorting (FACS) (FACSCanto II; BD Bioscience, Heidelberg, Germany) and analysed with FlowJo (Tree Star, Ashland, OR, USA).

Statistical analysis

For statistical analysis, we used Prism version 7 for Windows (GraphPad, San Diego, CA, USA). Differences were evaluated for significance by using the Kruskal–Wallis test to generate p-values and corrected for multiple comparisons using Dunn's statistical hypothesis test, except for figures 2b–e where we used the Mann–Whitney U-test.

Results

RV1b infection suppressed average IFN-αR mRNA expression in PBMCs from asthmatic children

All children analysed in the longitudinal study PreDicta were aged between 4 and 6 years at the time of recruitment (baseline: B0) and were followed up for 2 years until the final visit (visit F4) (figure 1a) [9]. Within the PreDicta cohort, asthmatic children with RV in the airways had a defect in IFN type I levels in their PBMCs and in serum at recruitment, whereas during exacerbation of disease, close to infection, this defect in IFN type I in serum was not present in asthmatic children [9]. As these findings indicated that IFN-α expression is usually upregulated within hours upon infection, we next looked at later time-points by using PBMC supernatants 48 h after an infection with RV1b *in vitro* (figure 1a). Accordingly, cell culture supernatants from PBMCs were collected and analysed for cytokine content. Comparing IFN-α production in the supernatants of unstimulated *versus* RV1b-stimulated PBMCs, we did not find any RV1b-dependent IFN-α regulation (figure 1b).

TLR7/8 agonist R848 induced IFN-α but suppressed IFN-αR mRNA in PBMCs from pre-school age children with asthma

We next investigated if TLR7/8, a single-stranded viral RNA receptor that triggers a rapid innate immune response [28], could restore IFN responses in PBMCs. In these experiments, we incubated PBMCs from asthmatic and control subjects with R848 followed by analysis of IFN-α levels. R848 treatment induced

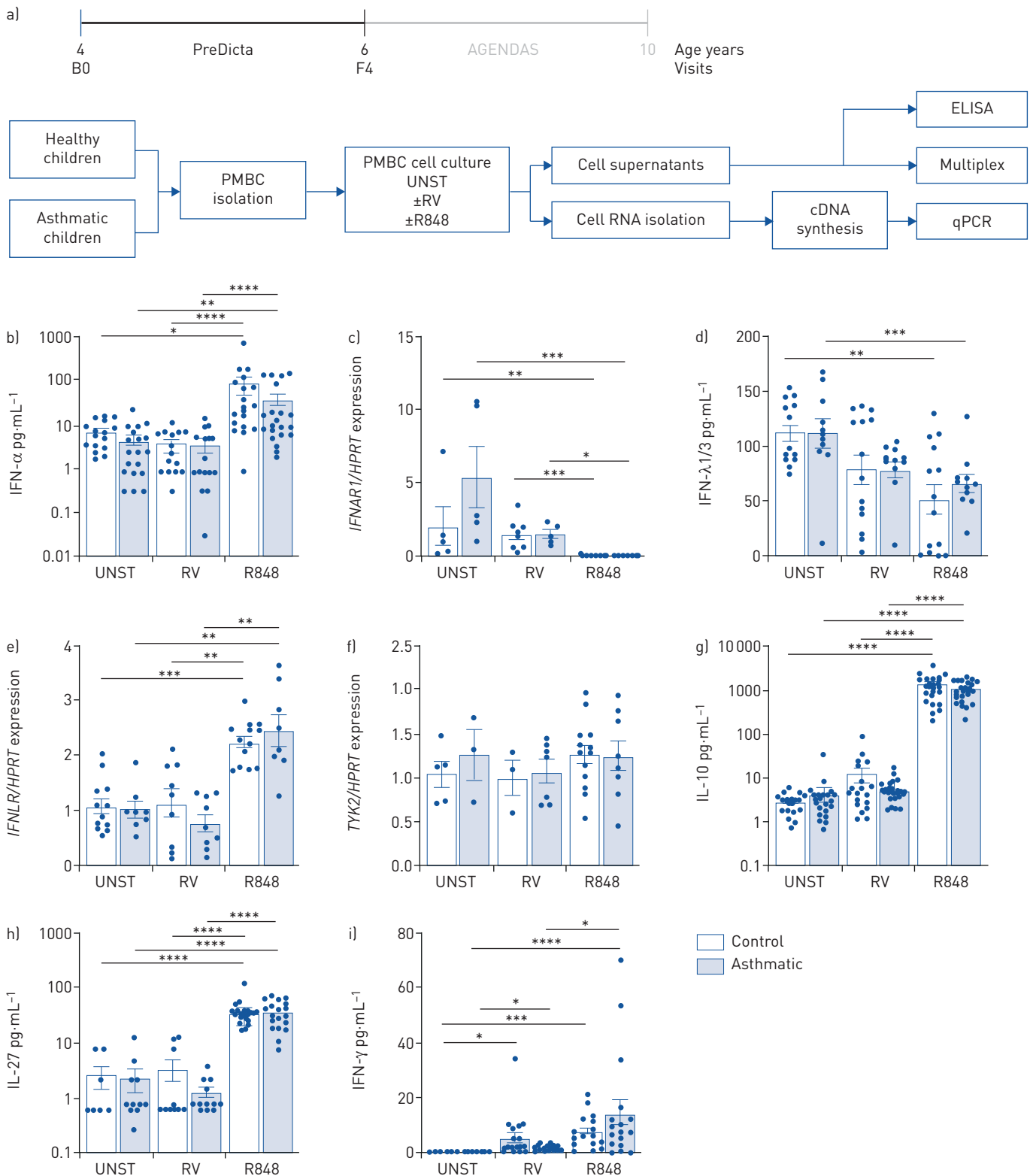


FIGURE 1 Immunoregulatory and immunosuppressive function of Toll-like receptor 7/8 agonist R848 in peripheral blood mononuclear cells (PBMCs) of healthy [control] and asthmatic pre-school age children (PreDicta). a) Design of PreDicta. B0: baseline visit; F4: follow-up visit after 2 years; UNST: unstimulated; RV: rhinovirus; qPCR: quantitative PCR. b) Interferon (IFN)- α levels in PBMC supernatants after *in vitro* cell culture with RV1b or R848: B0 [control: UNST n=13, RV n=16, R848 n=19; asthmatic: UNST n=20, RV n=19, R848 n=21]. c) IFN- α R1 mRNA expression in PBMCs after *in vitro* cell culture with RV1b or R848: B0 [control: UNST n=5, RV n=8, R848 n=17; asthmatic: UNST n=5, RV n=5, R848 n=16]. d) IFN- λ levels in PBMC supernatants after *in vitro* culture with RV1b or R848: F4 [control: UNST n=14, RV n=14, R848 n=14; asthmatic: UNST n=11, RV n=11, R848 n=11]. e) IFN- λ R mRNA expression in PBMCs after *in vitro* culture with RV1b or R848: F4 [control: UNST n=12, RV n=9, R848 n=13, asthmatic: UNST n=8, RV n=9, R848 n=8]. f) TYK2 mRNA expression in PBMCs after *in vitro* culture with RV1b or R848: F4 [control: UNST

n=5, RV n=3, R848 n=13; asthmatic: UNST n=3, RV n=7, R848 n=8). g) Interleukin (IL)-10 levels in PBMC supernatants after *in vitro* culture with RV1b or R848 (control: UNST n=17, RV n=20, R848 n=20; asthmatic: UNST n=22, RV n=23, R848 n=21). h) IL-27 levels in PBMC supernatants after *in vitro* culture with RV1b or R848: PreDicta [B0] (control: UNST n=8, RV n=11, R848 n=20; asthmatic: UNST n=12, RV n=12, R848 n=21). i) IFN- γ levels in PBMC supernatants after *in vitro* culture with RV1b or R848: B0 (control: UNST n=6, RV n=18, R848 n=17; asthmatic: UNST n=12, RV n=15, R848 n=18). Expression data were normalised to the hypoxanthine-guanine phosphoribosyltransferase (HPRT) housekeeping gene. Data are presented as individual points and mean $\pm$ SEM. Differences between the cultures were evaluated for significance using the Kruskal-Wallis test and corrected for multiple comparisons using Dunn's statistical hypothesis test. *: $p \leq 0.05$; **: $p \leq 0.01$; ***: $p \leq 0.001$; ****: $p \leq 0.0001$.

IFN- α levels in healthy control children as well as in asthmatic children (figure 1b). Furthermore, R848 significantly suppressed the expression of IFN- α R mRNA in PBMCs from both cohorts of children (figure 1c).

TLR7/8 agonist R848 reduced IFN- λ and induced IFN- λ R mRNA in PBMCs from pre-school age children

The IFN type III pathway is thought to be alternatively activated in the absence of type I IFNs (supplementary figure S1a) [15]. Accordingly, we next analysed IFN- λ in PBMC supernatants and found that TLR7/8 agonist R848 treatment reduced IFN- λ levels in the supernatants of asthma and control subjects compared with untreated cells (figure 1d). In contrast, R848 induced IFN- λ R mRNA expression in asthma and control children (figure 1e). RV1b infection did not lead to any significant regulation of IFN- λ R mRNA in PBMCs both in control and asthmatic children (figure 1e). In contrast, TYK2 mRNA was not regulated after TLR7/8 treatment or RV1b infection *in vitro* (figure 1f).

Immunosuppressive and immunostimulatory role of TLR7/8 agonist R848 in PBMCs from pre-school age children

As RV-induced lower airway inflammation and impaired lung function in patients with asthma are strongly related to deficient IFN- γ and IL-10 responses [29], we next studied expression of these cytokines as well as the expression of IL-27, a potent IFN- γ inducer [30]. R848 treatment significantly induced the levels of the cytokine IL-10 in the supernatants of PBMCs from control and asthmatic children compared with untreated control samples (figure 1g). Beside this immunosuppressive effect, R848 treatment induced the immunostimulatory cytokine IL-27 (figure 1h). However, in the presence of elevated IL-10 and IL-27 levels, the resulting induction of the Th1 cytokine IFN- γ upon R848 treatment was relatively modest (figure 1i). Nevertheless, a significant induction of IFN- γ levels was detected in R848-treated samples from asthma and control subjects compared with untreated samples (figure 1i).

Age-dependent decreased IFN- α and IFN- α R1 mRNA levels in asthma

We then analysed a new cohort of older control and asthmatic children at primary school age (AGENDAS) (figure 2a and table 1), and asked whether the inhibition of IFN- α and IFN- α R1 signalling was also present in this cohort. IFN- α was significantly induced in serum (figure 2b) and tendentially induced at the mRNA level in PBMCs from asthmatic children (supplementary figure S1c). Furthermore, we could not detect any difference in IFN- α R1 mRNA in these PBMCs (supplementary figure S1d).

Improved IFN- λ signal transduction in the serum of primary school asthmatic children resulted in a reduced number of RV infections in the upper airways

We next found that IFN- λ levels were elevated in the serum of asthmatic children compared with control children (figure 2c). Furthermore, IFN- λ R mRNA was upregulated in PBMCs of primary school age children with asthma compared with control children (figure 2d). Consistent with an improved IFN type I and type III immune response, the expression of transcription factor STAT2 mRNA (figure 2e) was tendentially upregulated. Moreover, STAT1A mRNA (figure 2f), the antiviral component of STAT1 [31], but not STAT1B mRNA (figure 2g) was tendentially upregulated in PBMCs from asthmatic children after RV1b infection *in vitro*. In addition, STAT1B mRNA was elevated in PBMCs from control children after RV1b infection *in vitro* (figure 2g). It could be possible that this is the reason why this cohort of children had fewer RV infections in the airways compared with the younger PreDicta cohort of children (table 1) [9]. Further research is needed.

Decreased percentage of lung CD8⁺IFN- α R⁺ and CD4⁺IFN- α R⁺ T-cells after RV1b infection and R848 treatment in vitro

We next wanted to directly study the immunomodulatory mechanisms mediated by RV1b and TLR7/8 agonist R848 on IFN- α R and IFN- λ R expression. To this aim, we isolated total lung cells from C57BL/6JRj mice and cultured them with RV1b or R848, or both (figure 3a). Isolated total lung cells stained with Annexin V and propidium iodide confirmed that 96% of the isolated cells were viable (supplementary figure S2a). Moreover, FACS analysis (supplementary figure S2b) of these lung cells showed that IFN- α R⁺ lymphocytes (figure 3c) as well as CD8⁺IFN- α R⁺ (figure 3d) and CD4⁺IFN- α R⁺ (figure 3e) cells were

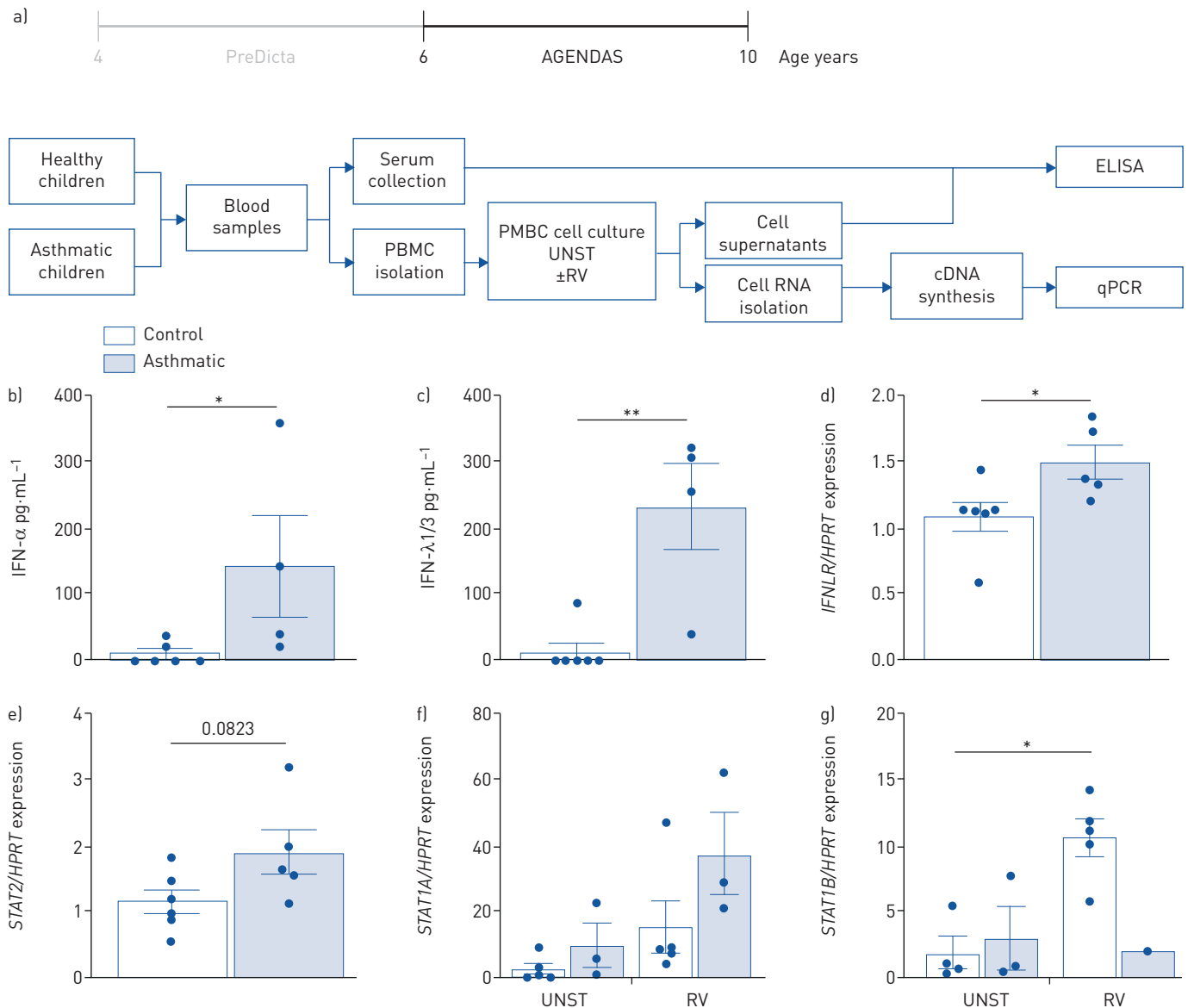


FIGURE 2 Asthmatic primary school age children (AGENDAS) have more interferon (IFN)- λ and signal transducer and activator of transcription (STAT) 2 compared with healthy (control) children and improved *in vivo* rhinovirus (RV) clearance. a) Design of AGENDAS. PBMC: peripheral blood mononuclear cell; UNST: unstimulated. b) IFN- α levels in serum (control: n=6; asthmatic: n=4). c) IFN- λ levels in serum (control: n=6; asthmatic: n=4). d) IFN- λ R mRNA expression in PBMCs (control: n=6; asthmatic: n=5). e) STAT2 mRNA expression in PBMCs (control: n=6; asthmatic: n=5). f) STAT1A mRNA expression in PBMCs without and with RV1b infection *in vitro* (control: UNST n=5, RV n=5; asthmatic: UNST n=3, RV n=3). g) STAT1B mRNA expression in PBMCs without and with RV1b infection *in vitro* (control: UNST n=4, RV n=5; asthmatic: UNST n=3, RV n=1). Expression data were normalised to the hypoxanthine-guanine phosphoribosyltransferase (HPRT) housekeeping gene. Data are presented as individual points and mean $\pm$ SEM. b–e) Differences between healthy and asthmatic children were evaluated for significance using the Mann–Whitney U-test; f, g) differences between the cultures were evaluated for significance by using the Kruskal–Wallis test and corrected for multiple comparisons using Dunn’s statistical hypothesis test. *: $p \leq 0.05$; **: $p \leq 0.01$.

significantly decreased after RV1b infection together with R848 treatment *in vitro*. However, IFN- λ R1 mRNA expression was downregulated after R848 treatment together with RV1b infection *in vitro* compared with RV1b infection alone (figure 3f). Successful RV1b infection in the absence and presence of R848 was confirmed by RV1b PCR (figure 3g).

TLR7/8 agonist R848 suppresses TYK2 mRNA expression in murine lung cells

TYK2 mRNA, in contrast to the results in PBMCs of pre-school age children, was significantly downregulated by R848 treatment but not by RV1b infection alone in lung cells (figure 3h). These results are consistent with the different expression of IFN- λ R mRNA in PBMCs and murine lung cells after R848 treatment *in vitro*. R848 in combination with RV1b also resulted in decreased expression of TYK2 mRNA (figure 3h). Moreover, RV1b and R848 in combination inhibited IFN- α production by lung cells

TABLE 1 Characteristics of the children recruited in AGENDAS

	Healthy control children	Asthmatic children
Subjects	6	6
Sex		
Male	33.3 (2/6)	50 (3/6)
Female	66.7 (4/6)	50 (3/6)
Mean age years	6.68	7.73
FEV₁ <90% predicted	0 (0/6)	0 (0/6)
FEV₁/FVC <70%	0 (0/6)	0 (0/6)
Treatment		
No treatment	100 (6/6)	16.7 (1/6)
Steroid	0 (0/0)	33.3 (2/6)
Steroid+nonsteroid	0 (0/0)	50 (3/6)
NPF: RV		
Negative	100 (5/5)	66.7 (4/6)
Positive	0 (0/5)	33.3 (2/6)

Data are presented as n or % (n/N). FEV₁: forced expiratory volume in 1 s; FVC: forced vital capacity; NPF: nasopharyngeal fluid; RV: rhinovirus.

(figure 3i), while TLR7/8 agonist treatment alone had no significant effect. However, IFN- α production induced by RV1b and R848 together is not different from RV1b alone, meaning that R848 does not modify the response to RV1b infection for this parameter.

Discussion

Previous studies suggested that asthmatic children have a defect in IFN type I production [18–20]; however, this defect has been shown to be reversible [9]. In this study, we investigated whether this defect in IFN responses could be ameliorated by treatment with the TLR7/8 agonist R848. As a result, treatment of PBMCs with R848 *in vitro* led to an increase of IFN- α in the supernatants of PBMCs, consistent with a previous report [23]. However, we discovered that R848 also has a limiting effect on type I IFN signalling, because it additionally decreased the expression of IFN- α R1 mRNA. The increase of IFN- α in the supernatants of PBMCs after R848 treatment *in vitro* could be the result of decreased ligand–receptor binding as a result of IFN- α R mRNA expression. Due to some limitations of the remaining biological material of the study, we could not use one donor for one stimulation for this evaluation. Nevertheless, similar effects were noted on total murine lung cells upon *in vitro* R848 treatment and such treatment in combination with RV1b infection *in vitro* reduced IFN- α R1 expression on both CD4⁺ and CD8⁺ lung T-cells. However, R848 treatment augmented IFN- λ R mRNA expression in PBMCs of pre-school age children, suggesting that TLR7/8 activation may favour antiviral immune responses *via* type III IFN signalling.

RV1b infection *in vitro* led to a decrease of IFN- α R1 expression in murine lung cells as well as tendentially in PBMCs of asthmatic pre-school age children, suggesting suppression rather than activation of type I IFN signalling upon RV1b infection. It is possible that we could not detect a significant difference when analysing murine CD4⁺ and CD8⁺ lymphocytes because we used RV1b instead of RV-C (whose receptor is CDHR3) [11] or a major group RV serotype [6]. Alternatively, other as-yet unidentified types of lymphocytes may have been affected. Because of this limitation of our study, further analysis with RV-C or major group rhinovirus serotypes is needed [32]. RV-C-related wheezing illnesses were found associated with an increased risk of respiratory hospital admission [33], particularly in those children with asthma [34]. Although separate investigations are needed to reveal the underlying mechanism of these results, it is possible that this is a mechanism by which the RV escapes the immune system of the host either by inhibiting IFN production or by decreasing its stability and, more interestingly, by downregulating IFN- α R. Consistent with this type of virus immune escape mechanism, it has been shown that hepatitis C virus and vesicular stomatitis virus infection resulted in IFN- α R degradation leading to weakened IFN type I immune responses [35, 36]. Further research is needed.

In asthmatic pre-school age children with RV infection *in vivo*, higher IFN- λ levels were noted in serum [9]. We observed that school age asthmatic children have significantly higher IFN- λ levels in serum and higher IFN- λ R mRNA expression in PBMCs compared with healthy children, indicating an activation of the IFN- λ pathway.

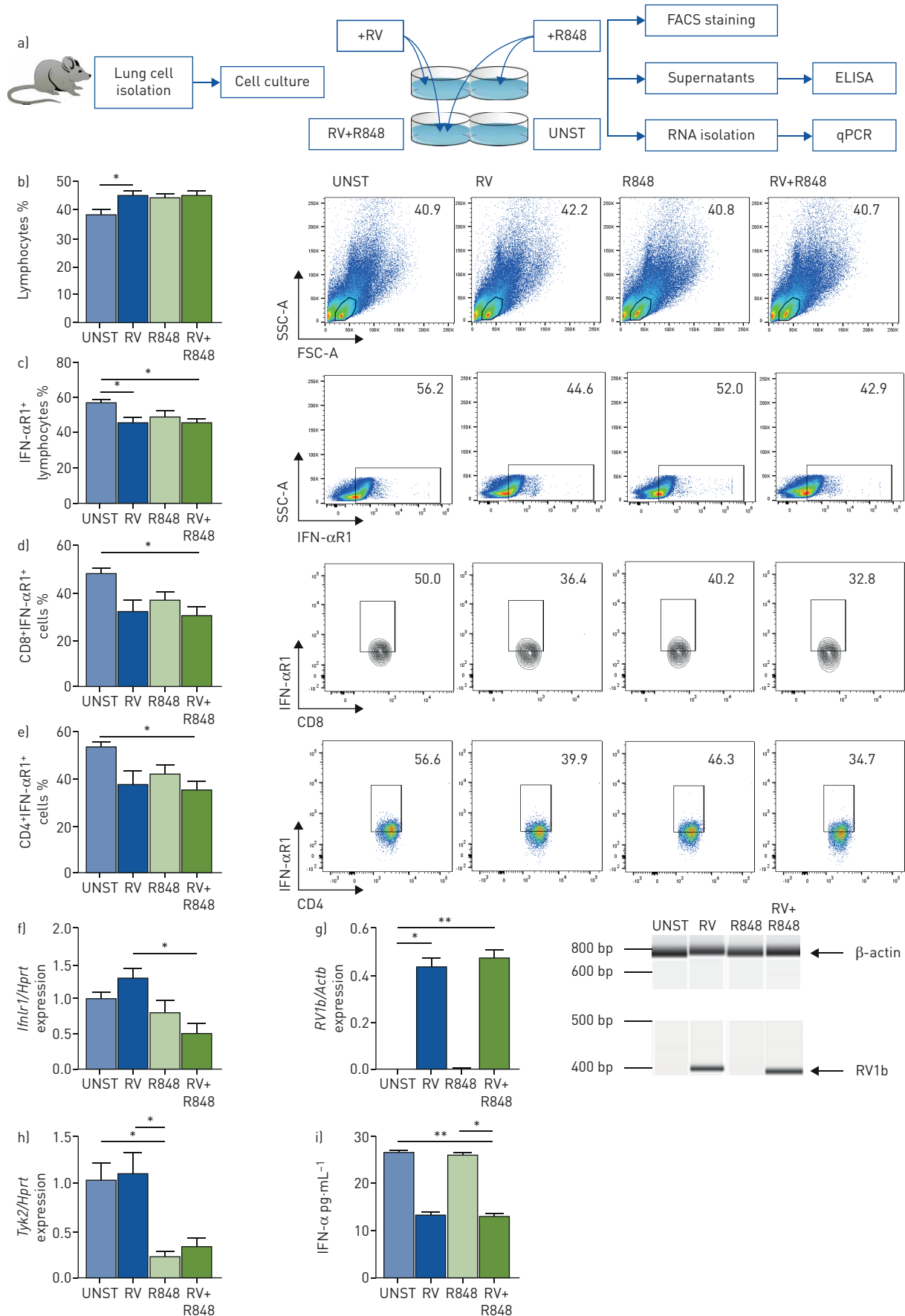


FIGURE 3 Downregulation of interferon (IFN)- α receptor (IFN- α R) in lung cells after culture with rhinovirus 1b (RV) and Toll-like receptor 7/8 agonist R848. a) Experimental design of the mouse experiment. This figure is based on an original created using Servier Medical Art templates (smart.servier.com), which are licensed under a Creative Commons Attribution 3.0 Unported License. UNST: unstimulated; FACS: fluorescence-activated cell sorting; qPCR: quantitative PCR. b) Flow cytometry analysis of lymphocytes gated on all cells after *in vitro* culture of total lung cells with RV1b, R848 or both (UNST n=5, RV n=5, R848 n=5, RV+R848 n=5). SSC: side scatter; FSC: forward scatter. c) Flow cytometry analysis of IFN- α R1⁺ lymphocytes gated on lymphocytes after *in vitro* culture of total lung cells with RV1b, R848 or both (UNST n=5, RV n=5, R848 n=5, RV+R848 n=5). d) Flow cytometry analysis of CD8⁺IFN- α R1⁺ cells gated on CD8⁺ lymphocytes after *in vitro* culture of total lung cells with RV1b, R848 or both (UNST n=5, RV n=5, R848 n=5, RV+R848 n=5). e) Flow cytometry analysis of CD4⁺IFN- α R1⁺ cells gated on CD4⁺ lymphocytes after *in vitro* culture of total lung cells with RV1b, R848 or both (UNST n=5, RV n=5, R848 n=5, RV+R848 n=5). f) IFN- λ R1 mRNA expression in total lung cells after *in vitro* culture of total lung cells with RV1b, R848 or both (UNST n=4, RV n=5, R848 n=5, RV+R848 n=5). g) RV1b mRNA expression in total lung cells after *in vitro* culture of total lung cells with RV1b, R848 or both (UNST n=4, RV n=5, R848 n=5, RV+R848 n=5). h) TYK2 mRNA expression in total lung cells after *in vitro* culture of total lung cells with RV1b, R848 or both (UNST n=4, RV n=5, R848 n=5, RV+R848 n=5). i) IFN- α levels per 10⁶ cells in the supernatant of total lung cells after *in vitro* culture of total lung cells with RV1b, R848 or both (UNST n=5, RV n=5, R848 n=5, RV+R848 n=5). Expression data were normalised to hypoxanthine-guanine phosphoribosyltransferase or β -actin housekeeping genes. Data are presented as mean $\pm$ SEM. Differences were evaluated for significance using the Kruskal–Wallis test and corrected for multiple comparisons using Dunn’s statistical hypothesis test. *: p $\leq$ 0.05; **: p $\leq$ 0.01.

IFN- λ has already been suggested as an alternative antiviral pathway in the presence of a suboptimal type I IFN response [17]. In an additional series of studies, we investigated whether R848 was able to induce the IFN- λ signalling pathway. We observed an induction of IFN- λ R mRNA expression in PBMCs of pre-school age children after R848 treatment *in vitro*, but a decrease of IFN- λ in the supernatants of PBMCs. The lower levels of IFN- λ could be at least partially due to compensatory reduction of cytokine production after induction of receptor expression or high IFN- λ binding to the induced IFN- λ R.

It is already known that asthma symptoms may change with age [37]. Consistently, in agreement with the proposed functional relevance of IFN- λ signalling, we found an upregulation of IFN- λ R expression in the AGENDAS cohort that was associated with a lower percentage of RV infection in the airways compared with the younger PreDicta cohort [9]. This effect was not due to differential use of corticosteroids, as similar percentages of children (pre-school age children 79.2% [9] and school age children 83.3%) were treated with corticosteroids.

In addition to IFN type I and III signalling, we found that R848 also regulated IFN- γ and IFN- γ -inducing cytokines such as IL-27 [38]. R848 caused an induction of IFN- γ and IL-27 cytokine responses that may favour antiviral immune responses. Moreover, R848 augmented IL-10 production. As RV-induced lower airway inflammation and impaired lung function in patients with asthma are strongly related to deficient IFN- γ and IL-10 responses [29], these findings suggest that R848 may induce several important cytokines that control mucosal immune responses and thus may potentially affect the clinical outcome of RV infection.

In conclusion, we show for the first time that R848 reduces IFN- α R mRNA expression. Additionally, our data indicate an important role of the IFN- λ pathway for the clearance of RV1b infection, especially in asthmatic children. Furthermore, we demonstrate that this pathway can be induced *via* the TLR7/8 agonist R848 that causes upregulation of IFN- λ R mRNA and regulates both IFN- γ and IL-10 responses. Thus, triggering of TLR7/8 responses may offer a new therapeutic approach for asthmatic children. It is currently unclear whether this TLR7/8-dependent IFN- λ R response is limited to RV1b infection or whether it might be considered for treatment of other severe respiratory tract infections, such as respiratory syncytial virus (RSV) or severe acute respiratory syndrome coronavirus (SARS-CoV), because MORDSTEIN *et al.* [17] have already shown that IFN- λ R has a role in restricting RSV and SARS-CoV infections. Collectively, our results indicate an induction of IFN- λ signalling upon treatment with the TLR7/8 agonist R848, thus suggesting new avenues for induction of antiviral immune responses in paediatric asthma.

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Data sharing: All data reported in the figures and as supplementary will be shared upon request to collaborate with the corresponding author.

Author contributions: J. Krug generated the laboratory results, correlated, analysed and interpreted all of the data, and generated all of the figures, tables and supplementary material. She wrote the introduction, methods, results and the discussion sections. She was involved in drafting and editing the manuscript, and contributed to the revision of the manuscript. A. Kiefer recruited, analysed and diagnosed the children from the PreDicta WP1 cohort in Erlangen and the AGENDAS cohort. J. Koelle helped with the murine data recruitment. T. Vuorinen provided the respiratory virus analysis for PreDicta and AGENDAS. M. Akdis and B. Stanic provided the multiplex cytokine data for the PreDicta

WP1 cohort in Erlangen that are reported and correlated in figures 1c and 2b–d. N.G. Papadopoulos was the coordinator of the PreDicta studies and P. Xepapadaki helped N.G. Papadopoulos to establish the different European cohorts in PreDicta. M.T. Chiriac helped generating the laboratory results regarding the IFN- λ R qPCR. T. Zimmermann was involved in the recruitment and analysis of the PreDicta studies. S. Finotto contributed to the design of the study, writing the first draft and editing the manuscript, analysed and mentored all of the studies for the PreDicta and AGENDAS part in Erlangen, and contributed to the revision of the manuscript.

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