

Impaired virus replication and decreased innate immune responses to viral infections in nasal epithelial cells from patients with allergic rhinitis

Condensed title: **Innate immune responses to viral infections in nasal epithelium**

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Summary

Purpose: The aim of the study was to assess the immune response to parainfluenza virus type 3 (PIV3), rhinovirus 1B (RV1B) and intracellular Toll-like receptors (TLR) agonists in nasal epithelial cells (NECs) from patients with allergic rhinitis and healthy controls.

Methods: NECs were obtained from 8 patients with allergic rhinitis (AR) and 11 non-atopic healthy controls (HC) by nasal scraping, grown to confluence and exposed to PIV3, RV1B infection or TLR3 and TLR7/8 agonists. IFN- λ 1, IFN- α , IFN- β and RANTES release into the cell culture supernatants was assessed at 8, 24 and 48h

upon infection or 8 and 24h after stimulation with Poly(I:C) and R848. mRNA levels of IFNs, RANTES, IRF3, IRF7 and viral gene copy number were determined using Real-Time PCR.

Results: PIV3 but not RV1B replication 48h after infection was significantly lower ($p<0.01$) in NECs from AR patients as compared to HC. PIV3 infection induced significantly less IFN- λ 1 (both protein and mRNA) in NECs from AR compared to HC. IFN- β mRNA expression and RANTES protein release and mRNA expression tended to be smaller in AR as compared HC cells in response to both viruses. Stimulation with TLR3 agonist (Poly (I:C)) induced similar IFN- λ 1 and RANTES generation in AR and HC subjects. Viral infections in NECs induced IRF7 expression, which correlated with interferon and RANTES expression.

Conclusions: These data suggest that virus proliferation rates and the immune response profile are different in nasal epithelial cells from patients with allergic rhinitis as compared to healthy individuals.

INTRODUCTION

Respiratory viruses which cause common cold and provoke acute illness of lower respiratory tract (bronchitis, pneumonia) in healthy individuals (1) may also trigger asthma or COPD exacerbation. (2) The role of viral infections in pathogenesis of chronic upper airway diseases, including allergic rhinitis, is less well documented (3). Respiratory virus infections may exacerbate existing allergic symptoms in the nasal mucosa and increase inflammatory response in the airway epithelium. (4) (5) Patients with atopic sensitization have significantly higher risk of upper respiratory viral infections as compared to non-atopic subjects, although the difference is not so striking as for the lower respiratory tract infections. (6) Little is known about antiviral response to viruses in the upper airways of patients with allergic rhinitis. (3) (7) Recently, an impaired expression of TLR3 receptors and increased rhinovirus replication in nasal epithelial cells from patients with allergic rhinitis have been documented. (8)

As opposed to the upper airway epithelium, respiratory infections in bronchial epithelial cells from asthmatics (including allergic asthma (9) (10)) have been extensively studied during the last ten years. (11) (12) (13) Several studies documented significantly abnormal immune responses to viral infections in primary bronchial epithelial cells from allergic patients with asthma as compared to healthy individuals. (14) The effect of Th2 cytokines on increased susceptibility to rhinovirus infection in bronchial epithelial cells (8) as well as on virus-induced asthma exacerbation have been shown. (15) However, other studies have failed to confirm defective immune response in asthma upon virus infection (13) and similar

transcriptional response to rhinovirus in bronchial epithelial cells from healthy and asthmatic has been reported. (16)

Rhinoviruses and parainfluenza viruses are the most common type of respiratory viruses detected in patients with perennial allergic rhinitis. (17)

Human parainfluenza viruses, including type 3 (PIV3) are single-stranded RNA viruses belonging to the *Paramyxoviridae* family and causing both upper and lower respiratory tract illness. Paramyxovirus infection in infants, children and immunocompromised individuals may cause severe lower airway infections often associated with hospitalization. (18) (19) It has been demonstrated that parainfluenza virus infections contribute to 3-13 % of asthma exacerbations (20) (21) (22) and seem to be a major cause of post-infectious olfactory dysfunction potentially associated with chronic rhinosinusitis exacerbations. (23)

Rhinoviruses (RV), members of the *Picornaviridae* family are known to cause common cold but they are also involved in virus-induced asthma exacerbations. Different RV serotypes have been divided into groups according to the type of their cellular receptor. RV1B that belongs to the minor group binds to the low-density lipoprotein receptors (LDL-R). (24)

The role of PIV3 and RV1B infections in allergic rhinitis exacerbation is unknown, and the immune response to these viruses in the upper airway epithelial cells of allergic patients has not been studied.

Among numerous antiviral factors released by infected cells, type III interferon, IFN- λ 1, is considered to be a critical component of the immune response in the airway epithelial cells and was shown to be secreted by epithelial cells in response to single stranded RNA viruses. (11, 25-27) Recently, we have documented that type II IFN (IFN- γ) may be also generated by human nasal epithelial cells in response to

PIV3 infection. (28) Similarly, intracellular Toll-like receptors (TLRs) in the airway epithelial cells, which sense viral RNA, seem to be involved in IFNs production during infection with respiratory viruses such as rhinovirus (29), influenza A virus (30) as well as respiratory syncytial virus (RSV) that belongs to paramyxoviruses. (31) Study of Sabbah et al. (32) demonstrated that PIV3 infection in A549 (human respiratory epithelial cells) caused induction of IFN- β through the activation of IRF3 (interferon regulatory factor 3). In parallel to IFNs, proinflammatory cytokines, including RANTES, are generated by the airway epithelial cells in response to virus infection. (33) (34)

Given that little is known about the role of respiratory virus infections in allergic rhinitis, we sought to explore the effect of PIV3, RV1B and TLR3 and TLR 7/8 agonists on the induction of the immune response in primary nasal epithelial cells from allergic rhinitis patients. We compared virus replication rates, generation of type I and type III IFNs and RANTES as well as the expression level of interferon regulatory factors in cultured nasal epithelial cells from patients with allergic rhinitis and healthy non-allergic controls in response to PIV3 and RV1B infection and also to stimulation with TLRs agonists.

MATERIALS AND METHODS

Subjects and sample collection

Eight patients with persistent allergic rhinitis, diagnosed according to ARIA criteria, aged 20 to 59 years (3 males/5 females) were recruited from our clinic. All 8 patients had perennial nasal symptoms and 5 patients additionally reported seasonal

exacerbations during tree and/or grass pollen season. Allergic sensitization was confirmed by skin prick testing (SPT) with a battery of inhalant allergens. Patient characteristics are presented in Table 1. Patients received intranasal corticosteroids and oral antihistamines, which were withdrawn 2 weeks before the sample collection, thus during cell sampling patients were symptomatic (suffered from mild rhinitis symptoms). One patient had also mild bronchial asthma, and received low doses of inhaled corticosteroids, which were stopped 12 hours before the sampling. In patients with seasonal exacerbations nasal sample collection was done out of the relevant pollen season. Eleven non-atopic, healthy volunteers with negative SPTs to a panel of 14 locally relevant inhaled allergens were recruited from the university students and department personnel. None of the study subjects was smoking or had had respiratory tract infection in the preceding 4 weeks. Study was approved by the MUL ethics committee and all subjects gave informed consent.

Culture of nasal epithelial cells

Nasal scrapings were performed using sterile cytology brushes (DAGA MED, Poland), after topical anesthesia with 10% lidocaine (EGIS, Poland) administered on the nasal mucosa. Before the procedure, participants blew their nose to remove mucus lining from the nasal cavity and surface of the mid-part of the inferior turbinate was scraped. Epithelial cells obtained were transferred to Bronchial Epithelial Cell Growth Medium (BEGM; Lonza, Germany) containing 1% Penicillin/Streptomycin and cells were seeded onto the flask of 25 cm² coated with collagen and fibronectin, and cultured with hormonally supplemented BEGM. Fresh medium was replaced every 48h until a monolayer formed. For the first three medium replacements BEGM containing 1% of serum substitute - Ultrosor G (Cytogen, Poland) - was used. At the

passage 2 cells were split onto 24 well plates (Corning Costar, USA) and incubated at 37°C with 5% CO₂ until 80-90% confluence was achieved, which usually occurred after 7 to 10 days. The epithelial nature of cultured cells was confirmed by intracellular staining for anti-cytokeratin 19 assessed by cytofluorometry using BD LSR Fortessa Analyzer.

Infection with PIV3 and RV1B

Parainfluenza virus type 3 a was kind gift of Dr E. Paradowska (Institute for Medical Biology, Polish Academy of Sciences, Lodz, Poland). Viral stocks were used at 3.02×10^7 TCID₅₀/ml (tissue culture infectious dose 50/ml). Rhinovirus serotype 1B was provided courtesy of Professor S. Johnston from the Department of Respiratory Medicine, Imperial College London, UK. Viral stocks were used at 7.96×10^7 TCID₅₀/ml.

Nasal epithelial cells (NECs) monolayers seeded in 24 well plates at 1×10^5 were treated with PIV3 or RV1B at a multiplicity of infection (MOI) of 0.5 at room temperature or exposed to medium alone (negative control). After 1 hour of incubation virus was removed, cells were washed three times with warm PBS, which was replaced by fresh BEGM medium at the end of washing step. Cell culture supernatants and lysates were harvested at 8, 24 and 48h after infection.

In parallel experiments cells were incubated with TLR agonists - Polyinosinic-polycytidylic acid sodium salt, Poly(I:C) (Sigma-Aldrich, Germany) - 10 µg/ml (TLR3); imidazoquinoline compound R848 (InvivoGen, France) - 1 µM (TLR7/8) and cell culture supernatants and lysates were collected after 8 and 24h.

Cytotoxicity assay

Lactate dehydrogenase (LDH) activity was determined using Pierce LDH Cytotoxicity Assay (ThermoFisher Scientific, Poland) according to the manufacturer's instructions.

RNA isolation and quantitative PCR

Total RNA was extracted from nasal epithelial cells using RNeasy Mini Kit (Qiagen, Germany) according to manufacturer's instruction and used for cDNA synthesis by RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Scientific, Austria). Primers specific for IFN- α , IFN- β , IFN- λ 1, IRF3, IRF7 and RANTES were purchased from Life Technologies. TaqMan analysis of mRNA expression was performed using Step One Plus™ Real-Time PCR System (Applied Biosystems, USA). Results were normalized to 18S rRNA. The mRNA expression was assessed using the relative quantification method ($2^{-\Delta\Delta C_t}$ method).

Real Time-PCR (RT-PCR) method with the Genesig Kit (PrimerDesign, UK) was used for detection and quantification of PIV3 and RVB.

Protein measurement

IFN- α and RANTES protein levels in cell culture supernatants were assessed using Cytometric Bead Array (CBA; Becton Dickinson, Poland). IFN- β (PBL Assay Science, USA) and IFN- λ 1 (eBioscience, Germany) were measured by ELISA. The detection limits for described assays are: 50 pg/ml (IFN- β), 15 pg/ml (IFN- λ 1), 1.5 pg/ml (IFN- α), and 10 pg/ml (RANTES).

Statistical analysis

Statistical analysis was performed using Statistica 10 PL (StatSoft; INC, USA) and GraphPad Prism Version 5.01 (GraphPad Software; San Diego, California, USA). Gene expression induction and protein levels between allergic rhinitis patients and healthy individuals were compared depending on normality of data using U Mann-Whitney test or t test. Changes in protein release and mRNA expression were analyzed using Wilcoxon's ranked-pairs analysis for paired data. Normality of data were assessed using Shapiro-Wilk test. Kruskal Wallis followed by Dunn's multiple comparisons test was used to compare differences between time points. Correlations were evaluated with the Spearman rank correlation test or Pearson test. Data were considered as significantly different when $p < 0.05$ unless otherwise noted.

RESULTS

PIV3 and RV1B replication and NECs viability

The PIV3 gene copy number significantly increased at 48h post infection as compared to 8h ($p < 0.01$) only in healthy controls (HC), while it was not changed in the cells from allergic rhinitis patients (AR) (Figure 1A). Although at 8h p.i. PIV3 gene copy number was 4.8-fold higher ($p < 0.05$) in AR group than in HC, at later time points after infection PIV3 proliferation was significantly higher in HC (10.3-fold higher ($p < 0.05$) and 129.6-fold higher ($p < 0.01$) at 24h and 48h, respectively). In contrast, the RV1B copy number in NECs from HC did not change markedly during the time course, whereas in AR there was a significant increase in virus copy number between 8 and 48h. However, there was no significant difference between AR and HC at any time point (Figure 1B).

Infection with PIV3 did not affect epithelial cell viability measured with LDH release in either AR or HC (Figure 2A). Upon RV1B infection a slight increase in cell cytotoxicity was observed at 48h compared to the earlier time points, but without difference between AR and HC (Figure 2B).

Immune response to virus infection

PIV3 infection induced IFN- $\lambda 1$ protein release from NECs at 48h p.i. (but not earlier) and the protein level was detectable in cell culture supernatants from 7 out of 9 HC subjects and 2 of 7 AR donors; the mean concentration of IFN- $\lambda 1$ was significantly lower in patients with AR ($p < 0.05$) (Figure 3A). When NECs were infected with RV1B, IFN- $\lambda 1$ protein was detectable at 48h in 5 of 7 HC and 6 of 7 AR

patients; the mean IFN- λ protein concentration was 5.5-fold lower in NECs supernatants from AR compared to HC, although this difference did not reach statistical significance (Figure 3B).

In parallel to protein release, IFN- λ 1 mRNA expression was strongly induced by PIV3 at 48h in HC cells and was significantly ($p < 0.05$) higher as compared to AR (Figure 3C). In RV1B-infected cells the mean IFN- λ 1 mRNA expression levels tended to be lower in AR as compared to HC cells. However, only in AR group IFN- λ 1 mRNA expression increased significantly ($p < 0.01$) at 48h compared to 8h (Figure 3D).

Neither IFN- α nor IFN- β protein were detectable in cell culture supernatants at any time point after PIV3 or RV1B infection. Upon PIV3 infection IFN- β gene expression increased markedly only in HC at 48h as compared to 8h ($p < 0.05$) and at 48h tended to be lower in AR as compared to HC cells ($p = 0.053$) (Figure 4A). Following RV1B infection IFN- β mRNA expression level continued to increase over the time course in both groups and there was significant difference in gene expression between 8h and 48h after infection in AR ($p < 0.01$) and HC ($p < 0.05$), although no difference between these two groups was observed (Figure 4B).

RANTES protein which was not measurable 8 or 24h after either PIV3 or RV1B infection (data not shown), at 48h after PIV3 infection was detected in 5 of 8 individuals from HC group and only in 1 of 7 AR patients (Figure 5A), while following RV1B infection in 4 of 8 HC and 5 of 7 patients in AR group (Figure 5B). Mean level of RANTES protein at 48h after either PIV3 or RV1B infection tended to be lower in AR as compared to HC, however the difference was not statistically significant. At 48h RANTES gene expression was 31.9-times and 12.0-times lower in AR cells as compared to HC in PIV3-infected (Figure 5C) and RV1B-infected (Figure 5D) NECs,

respectively, although the difference was statistically significant only in PIV3-infected cells.

Virus-induced IRF7 and IRF3 gene expression

Following PIV3 infection IRF7 gene expression tended to increase over the time course in NECs from HC but not in AR (Figure 6A). RV1B infection induced significant IRF7 mRNA expression at 48h as compared to 8h in both groups (Figure 6B). However, no differences between AR and HC at any time point were observed. IRF3 mRNA expression has not been changed in any group upon either PIV3 or RV1B infection (Figure 6C-D).

IRF7 expression induced by PIV3 infection correlated strongly with IFN- λ 1 gene expression both in AR (at all time points) and in HC (at 24h) (Table 2), while following RV1B infection IRF7 mRNA level correlated with IFN- λ 1 gene expression only in HC at 24h and 48h (data not shown).

PIV-3 induced IRF7 gene expression correlated also with RANTES mRNA expression at 8h, 24h and 48h in AR and at 24h in HC. After RV1B infection IRF7 expression correlated with RANTES mRNA expression only in HC individuals (at 24h and 48h), whereas these two genes did not correlate in AR subjects (data not shown).

Response to TLR agonists

IFN- λ 1 protein release from NECs was induced after Poly(I:C) (TLR3 agonist) (Figure 7A) in both groups, but significant increase between 8h and 24h was observed only in the cells from HC. There was no difference in the mean IFN- λ 1 release between AR and HC. Changes in IFN- λ 1 mRNA expression did not parallel the protein release

after TLR3 stimulation (Figure 7B). We were not able to detect type I IFNs (IFN- α and IFN- β) protein release following TLR3 agonist stimulation. IFN- β gene expression (but not IFN- α mRNA) was induced after Poly(I:C) treatment in both groups at 8h and then decreased significantly at 24h both in AR ($p<0.01$) and HC subjects ($p<0.05$) (Figure 7C).

Poly(I:C) stimulation induced measurable RANTES protein release at 8h and 24h (Figure 7D) and there was a tendency towards lower mean RANTES release in AR compared to HC. However, such differences were not observed at mRNA level (Figure 7E).

IRF7 induction after Poly(I:C) stimulation of NECs from AR and HC was similar at both time points. Similarly to PIV3 infection, stimulation with TLR3 agonist did not affect IRF3 expression in any group (data not shown).

Stimulation with TLR7/8 agonist (resiquimod – R848), an imidazoquinoline-based compound, did not cause any significant type I and III IFNs induction or significant changes in IRF7 and IRF3 mRNA expression (data not shown).

DISCUSSION

This is the first report comparing the innate immune response to PIV3 and RV1B infection in primary nasal epithelial cells (NECs) from allergic rhinitis patients (AR) and healthy non-atopic subjects (HC). PIV3 and RV1B infections are common causes of common cold symptoms and may induce exacerbations of asthma and COPD. (12) (22) (35) Infection of NECs from AR patients with PIV3 resulted in significant replication of the virus at 8h, but the replication rate subsided after 24 and 48h of infection. In contrast, virus copy number in NECs from HC subjects, which was

negligible at 8h, dramatically increased at later time points and at 48h was on average 129.6-fold higher as compared to AR patients.

When NECs were infected with rhinovirus RV1B, virus proliferation tended to be less vigorous as compared to PIV3 infection and the rhinovirus load in NECs was not significantly different between AR and HC subjects.

These data suggest that PIV3 (but not RV1B) virus entry, proliferation rate and/or virus clearance in NECs may differ between AR and HC subjects. Rapid increase in PIV3 load at 8h after infection indicates that virus entry to the epithelial cells was not impaired in NECs from AR donors, but might have been even increased as compared to the controls. PIV3 entry into the cells could be facilitated in NECs from AR donors due to an augmented paracellular permeability associated with the epithelial barrier disruption caused by proteolytic activity of the inhaled allergens and ongoing allergic inflammation. (36) (37) However, at the later time of infection significant PIV3 replication was observed in NECs from HC subjects and the total virus load at 48h was significantly higher as compared to AR patients. Since virus can replicate only in the living cells, cell death could limit viral replication, therefore causing subsequent decrease in virus copy number. However, LDH cytotoxicity assay did not show any significant differences in NECs viability between AR and HC upon viral infection, therefore this explanation is unlikely.

Another hypothetical explanation for decreased replication rate of PIV3 in NECs from AR patients may refer to the potential differences in the intracellular mechanisms regulating virus proliferation, survival and clearance, which might be associated with ongoing allergic inflammation. It should be emphasized that AR patients recruited to our study were sensitized to perennial allergens and were kept off intranasal corticosteroids for 2 weeks before the cell sampling. Thus, patients

were symptomatic at the time of the cell collecting and ongoing airway inflammation might have affected virus proliferation/clearance in the epithelial cells. In line with our findings regarding PIV3, decreased load of RSV, another *Paramyxoviridae* family member, has been demonstrated in virus-infected human bronchial epithelial cells from patients with moderate/severe asthma (but not mild disease) as compared to healthy controls. (12) In contrast to PIV3, rhinovirus proliferation in our study tended to be higher in NECs from AR patients at 48h and significant increase of RV1B replication rate between 8 and 48h postinfection was observed in AR patients, but not in HC. Similarly, previous studies on rhinovirus infection documented increased viral proliferation in bronchial epithelium and concomitant downregulation of rhinovirus-induced epithelial inflammatory response under the influence of atopic environment. (38) Recently, it has been demonstrated that Th2-driven attenuation of the antiviral response was accompanied by increased rhinovirus replication. (8)

These data suggest, that proliferation rate of respiratory viruses in nasal epithelial cells from AR patients may be either decreased or increased as compared to HC subjects, depending on the type of virus.

Our study demonstrated that PIV3 and RV1B induce IFN- λ 1 generation both at the protein and mRNA levels in human nasal epithelial cells. This observation is in line with earlier studies documenting that PIV3 induces type III interferon response in the epithelial cells, although may interfere with downstream antiviral signaling pathways. (39) Type I interferon – IFN- β was detected only at mRNA level after infection with both RV1B and PIV3. Therefore, our study confirmed previous observations (25) that antiviral response to respiratory viruses in nasal epithelial cells involves primarily type III, but not type I interferons.

Viral infections are associated with induction of proinflammatory cytokines e.g. RANTES in the airway epithelial cells, but in our study both viruses tended to induce smaller RANTES production in AR patients as compared to HC. Upon PIV3 infection measurable RANTES protein levels were detected at 48h in NECs from most of HC, but only in one AR subject and mean RANTES gene expression was significantly lower in AR patients. Similar trend was observed upon RV1B infection, although the differences in RANTES generation between both groups did not reach statistical significance.

Observation of impaired immune and inflammatory response in nasal epithelial cells from AR patients is intriguing and has not been reported previously. However, it may be referred to the previous studies showing an impaired antiviral IFN response in both lower airway epithelial cells and peripheral blood leukocytes from atopic patients with asthma and suggesting defective intrinsic immune mechanism in allergic airway epithelium. (40) (41) (42) Studies in bronchial epithelium documented downregulation of rhinovirus-induced epithelial inflammatory response and attenuation of antiviral response under the influence of atopic environment. (8) (38) Since our patients were sensitized to perennial allergen (HDM) and were studied during symptomatic period, it is likely, that observed downregulation of the innate immune response in NECs, similarly to that observed in the lower airways, resulted from the Th2 microenvironment in the nasal mucosa.

Alternatively, impaired immune response to PIV3 in NECs from AR patients documented in our study could result from impaired virus replication and therefore diminished stimulation of antiviral and proinflammatory gene expression and protein release. Nevertheless, this explanation is less likely since diminished immune

response in AR epithelial cells was also observed upon RV1B infection despite higher viral replication rate.

Following virus entry to the epithelial cells viral RNA is detected by endosomal receptors such as TLR3, TLR7 and TLR8, which may result in the activation of intracellular antiviral responses and proinflammatory pathways. (43). Poly(I:C) (a TLR3 agonist) treatment caused similar IFN- λ 1 and RANTES protein production in NECs from both groups, although there was a trend toward weaker response to stimulation with TLR3 agonist in AR as compared to HC. Furthermore, significant increase in IFN- λ 1 protein release between 8 and 24h was observed only in the cells from HC. Since Poly(I:C) induced much stronger interferon response as compared to virus infection, we cannot exclude that stimulation was supramaximal, and that stimulation of NECs with lower Poly(I:C) concentration could reveal significant differences between the groups. Earlier study by Wagener et al. reported similar induction of interferon-related genes (CCL22, FOSL1, IFN- β 1) in NECs in response to Poly(I:C) in healthy controls and rhinitis, but not in asthmatic patients. (44) This observation can be also referred to the recent study, which has shown similar induction of IFN- λ 1 and IFN- β after stimulation with Poly(I:C) in the lower airway epithelial cells of asthmatic as compared to non-asthmatic individuals. (45)

Production of type I and type III IFNs after virus infection may be regulated by several transcription factors including IRF3 and IRF7 operating downstream to TLR3 receptors. (46) (47) IRF3 is involved in the regulation of IFN- α and IFN- β as well as RANTES gene expression upon activation by virus infection, whereas IRF7 is a key transcriptional factor regulating type I IFNs, which is expressed in epithelial and plasmacytoid cells. Reduced IRF7 response to rhinovirus infection in PBMCs from asthmatics has been reported. (48) (49) Both IFN- λ 1 and IFN- β undergo regulation

by IRF3 and IRF7, (50) however the organization of the regulatory elements in IFN- λ 1 gene seems to be different in comparison to IFN- β . (50) (51) (52) In order to get some insight into the mechanism of innate response to virus infection IRF3 and IRF7 expression was studied in parallel to interferons. Previous studies documented that paramyxoviral proteins tend to interfere with IRF3 and IRF7 activation to prevent interferon production. (53) (54) In our study IRF3 expression was affected neither by PIV3 and RV1B infection nor TLR agonists stimulation, but changes in IRF7 expression in relation to viral infections and correlations with expression of IFN- λ 1 and RANTES were noticed. In HC there was a trend for increased expression of IRF7, while a tendency toward decreased IRF7 mRNA level over the course of PIV3 infection was observed in AR patients. In RV1B-infected cells IRF7 expression level increased significantly between 8 and 48h postinfection in both groups, however there was no difference in IRF7 gene expression between atopic and non-atopic subjects.

IRF7 strongly correlated with IFN- λ 1 and RANTES gene expression upon PIV3 infection in both atopic and non-atopic donors, whereas in RV1B-infected cells IRF7 mRNA level correlated with IFN- λ 1 and RANTES mRNA only in HC group. These data suggest that IRF7 expression is associated with the generation of innate immune response to PIV3 infection in the upper airway epithelial cells. However, IRF7 expression seems to be differentially regulated in allergic and non-allergic subjects, which is in line with the observed differences in IFN and RANTES expression.

In conclusion, this study documented significant differences in PIV3 virus proliferation and innate response to PIV3 (and to a lesser extent to RV1B) infection in primary upper airway epithelial cells from allergic rhinitis patients as compared to

healthy controls. Impaired response to respiratory virus infection in NECs from AR subjects may have consequences for acute symptom exacerbations but also for clinical persistence and chronicity of symptoms.

Further studies are needed to confirm these findings and to elucidate the mechanism underlying impaired PIV3 proliferation and attenuated innate responses to viral infections in nasal epithelial cells from patients with allergic rhinitis.

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CONFLICT OF INTEREST STATEMENT

The authors declare that there are no conflicts of interest.

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Table 1.

Patient characteristics.

		AR	HC
Number		8	11
Mean age (range)		34 (20-59)	30 (23-50)
Male/Female		3/5	3/8
Allergic rhinitis (positive SPT)	Perennial and seasonal	5	0
	Perennial only	3	0

Table 2.

Correlations between IRF7 mRNA and RANTES mRNA, IFN- λ 1 mRNA and IFN- λ 1 protein in PIV3-infected NECs from healthy control (HC) and allergic rhinitis (AR) subjects. Correlations were analyzed using Spearman's rank correlation test or Pearson test. Data were considered as significantly different when $p < 0.05$ unless otherwise noted. n/a=not applicable

HC					AR			
	n	time point	correlation coefficient	p	n	time point	correlation coefficient	p
IRF7 mRNA vs RANTES mRNA	7	8h	0.64	0.14	7	8h	0.86	<0.05
		24h	0.86	<0.05		24h	0.79	<0.05
		48h	0.61	0.17		48h	0.86	<0.05
IRF7 mRNA vs IFN-λ1 mRNA	7	8h	0.54	0.24	7	8h	0.96	<0.01
		24h	0.79	<0.05		24h	0.89	<0.05
		48h	0.71	0.09		48h	0.86	<0.05
IRF7 mRNA vs IFN-λ1 protein	7	48h	0.53	0.24			n/a	n/a

Figure legend

Figure 1.

Virus copy number in NECs from patients with allergic rhinitis (AR) and healthy controls (HC).

A. PIV3 gene copy number relative to known standards (AR: n=6; HC: n=5). B. RV1B gene copy number relative to known standards (AR: n=5; HC: n=7 unless otherwise noted). Data presented as mean ($\pm$ SD). Statistical differences between AR and HC are denoted as * when $p < 0.05$ or ** when $p < 0.01$. ## represents statistical differences between 8 and 48h postinfection in HC ($p < 0.01$), whereas & denotes statistical difference between 8 and 48h postinfection in AR ($p < 0.05$).

Figure 2.

Cell cytotoxicity upon viral infections in NECs from patients with allergic rhinitis (AR) and healthy controls (HC).

A. PIV3-induced cytotoxicity in nasal epithelial cells (AR: n=5; HC: n=8 unless otherwise noted). B. RV1B-induced cytotoxicity in nasal epithelial cells (AR: n=7; HC: n=9 unless otherwise noted). Data presented as mean ($\pm$ SD).

Figure 3.

Virus-induced type III interferon (IFN- λ 1) generation in NECs from allergic rhinitis (AR) and healthy controls (HC).

A. PIV3-induced IFN- λ 1 protein release at 48h (AR: n=7; HC: n=9). B. RV1B-induced IFN- λ 1 protein release at 48h (AR: n=7; HC: n=7). C. PIV3-induced IFN- λ 1 gene expression (AR: n=7; HC: n=7). D. RV1B-induced IFN- λ 1 gene expression (AR: n=7; HC: n=8 unless otherwise noted). Data presented as mean ($\pm$ SD). Statistical difference between AR and HC is denoted as * ($p < 0.05$). && represents significant difference between 8 and 48h in AR ($p < 0.01$).

Figure 4.

Virus-induced type I interferon (IFN- β) generation in NECs from allergic rhinitis (AR) and healthy controls (HC).

A. PIV3-induced IFN- β gene expression (AR: n=7; HC: n=7). B. RV1B-induced IFN- β gene expression (AR: n=7; HC: n=8 unless otherwise noted). Data presented as mean ($\pm$ SD). # represents significant difference between 8 and 48h in HC ($p < 0.05$). && represents significant difference between 8 and 48h in AR ($p < 0.01$).

Figure 5

Virus-induced RANTES generation in NECs from allergic rhinitis (AR) and healthy controls (HC).

A. PIV3-induced RANTES protein release at 48h (AR: n=7; HC: n=8). B. RV1B-induced RANTES protein release at 48h (AR: n=7; HC: n=8). C. PIV3-induced RANTES gene expression (AR: n=7; HC: n=7). D. RV1B-induced RANTES gene expression (AR: n=7; HC: n=8 unless otherwise noted). Data presented as mean ($\pm$ SD). Statistical difference between AR and HC is denoted as * ($p < 0.05$). Significant difference between 8 and 48h in HC are denoted as # when $p < 0.05$ or ### when $p < 0.001$.

Figure 6.

Virus-induced IRF7 and IRF3 gene expression in NECs from allergic rhinitis (AR) and healthy controls (HC). A. PIV3-induced IRF7 gene expression (AR: n=7;

HC: n=7). B. RV1B-induced IRF7 gene expression (AR: n=7; HC: n=8 unless otherwise noted). C. PIV3-induced IRF3 gene expression (AR: n=7; HC: n=7).

D. RV1B-induced IRF3 gene expression (AR: n=7; HC: n=8 unless otherwise noted).

Data presented as mean ($\pm$ SD). &&& denotes statistical difference between 8 and 48h postinfection in AR ($p < 0.001$). # represents significant differences between 8 and 48h in HC ($p < 0.05$).

Figure 7.

Poly (I:C)-induced IFN- λ 1, IFN- β and RANTES generation in NECs from allergic rhinitis (AR) and healthy controls (HC).

A. IFN- λ 1 protein release (AR: n=8; HC: n=8). B. IFN- λ 1 gene expression (AR: n=7; HC: n=7). C. IFN- β gene expression (AR: n=7; HC: n=7). D. RANTES protein release (AR: n=8; HC: n=8). E. RANTES gene expression (AR: n=7; HC: n=7). Data presented as mean ($\pm$ SD). # represents significant difference between 8 and 24h in HC when $p < 0.05$; ## when $p < 0.01$ or ### when $p < 0.001$. && represents significant difference between 8 and 24h in AR ($p < 0.01$).













