ORIGINAL ARTICLE

Impaired type I and type III interferon induction and rhinovirus control in human cystic fibrosis airway epithelial cells

Marjolaine Vareille,1,2,3,4 Elisabeth Kieninger,1,4 Marco P Alves,1,4 Brigitte S Kopf,1,4 Alexander Möller,5 Thomas Geiser,6 Sebastian L Johnston,7 Michael R Edwards,7 Nicolas Regamey1,4

ABSTRACT

Background Rhinoviruses are important triggers of pulmonary exacerbations and possible contributors to long-term respiratory morbidity in cystic fibrosis (CF), but mechanisms leading to rhinovirus-induced CF exacerbations are poorly understood. It is hypothesised that there is a deficient innate immune response of the airway epithelium towards rhinovirus infection in CF.

Methods Early innate immune responses towards rhinoviruses (RV-16, major-type and RV-1B, minor-type) in CF and non-CF bronchial epithelial cell lines and primary nasal and bronchial epithelial cells from patients with CF (n=13) and healthy controls (n=24) were studied.

Results Rhinovirus RNA expression and virus release into supernatants was increased more than tenfold in CF cells compared with controls. CF cells expressed up to 1000 times less interferon (IFN) type I (IFN-α and IFN-β) and type III (λ) mRNA and produced less than half of IFN-β and IFN-λ protein compared with controls. In contrast, interleukin 8 production was increased, indicating a selective deficiency in the innate antiviral defence system. Deficient IFN production was paralleled by lower expression of IFN-stimulated genes including myxovirus resistance A, 2′,5′-oligoadenylate synthetase, vimentin and nitric oxide synthase 2. Additional deficiency of type I and III IFNs, particularly IFN-λ, restored antiviral pathways and virus control in CF cells, underlining the crucial role of these molecules.

Conclusions This study describes a novel mechanism to explain the increased susceptibility of patients with CF to rhinovirus infections. A profound impairment of the antiviral early innate response in CF airway epithelial cells was identified, suggesting a potential use of IFNs in the treatment of rhinovirus-induced CF exacerbations.

Introduction

Respiratory virus infections have a significant impact on patients with cystic fibrosis (CF).1–3 They have been linked to increased respiratory symptoms,4 antibiotic use,4 prolonged hospitalisations4 and pulmonary function deterioration in both children and adults with CF.5–7 Up to 40% of pulmonary exacerbations in CF and half of the hospital admissions of infants with CF are associated with respiratory viruses.1,3,5,6 Rhinoviruses (RVs), the agents of the common cold, have been shown to precipitate the majority of asthma exacerbations5 and nearly half the exacerbations of chronic obstructive pulmonary disease (COPD).3 RVs have consistently been identified as the predominant agents during virus-associated pulmonary exacerbations in CF.9,10 The airway epithelium is central to innate immune responses in the lung, which are crucial in the defence against respiratory viruses.11 Interaction between respiratory viruses and airway epithelial cells (AECs) results in production of antiviral substances including type I (α and β) and III (λ) interferons (IFNs) which contribute to virus clearance.12,13 IFN-β and IFN-λ (IFN-λ1/IL-29, IFN-λ2/IL-28A and IFN-λ3/IL-28B) are the major IFNs produced by bronchial epithelial cells upon virus infection.14 After binding to their specific receptors, they send a signal to the nucleus through the Jak-STAT pathway to induce IFN-stimulated genes (ISGs) leading to the production of several antiviral proteins. These include 2′,5′-oligoadenylate synthetase (2′,5′-OAS), myxovirus resistance (Mx) proteins, viperin and nitric oxide synthase (NOS) 2, which mediate the antiviral actions of IFNs by inhibiting virus replication.11 Recently, deficient induction of type I and III IFNs in bronchial epithelial cells from patients with asthma upon RV infection has been proposed as a mechanism to explain the susceptibility of

Key messages

What is the key question?

What are the mechanisms leading to rhinovirus-induced exacerbations in cystic fibrosis (CF)?

What is the bottom line?

Deficient virus control by the CF airway epithelium may explain the increased susceptibility of patients with CF towards rhinoviruses.

Why read on?

This study identifies defective interferon type I and III production as a key pathophysiological mechanism leading to impaired virus control by the CF airway epithelium.

Correspondence to
Professor Nicolas Regamey, Division of Respiratory Medicine, Department of Paediatrics, University Children’s Hospital, Bern, Inselspital, Bern, Switzerland; nicolas.regamey@insel.ch

MV and EK contributed equally.

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these patients towards RVs.\textsuperscript{15,16} We hypothesised that similar deficiencies may be found in CF, and studied the early innate antiviral immune response of CF AECs towards RVs. In this paper we report increased RV replication due to deficient epithelial type I and III IFN production as a novel mechanism to explain the increased susceptibility of patients with CF to RV infections, and thus identify type I and III IFNs as potential treatments for virus-induced exacerbations in CF.

**METHODS**

**Cell and virus culture**

Three human bronchial epithelial cell lines with CF and non-CF phenotypes were used: UNCCF2T (F508del/F508del cystic fibrosis transmembrane conductance regulator (CFTR) mutation)/UNCN2T\textsuperscript{17}, CFBE41o- (F508del/F508del CFTR mutation)/16HBE14o-\textsuperscript{18,19} (both kindly donated by Drs S Randell and D C Gruenert) and IB3-1 (F508del/W1282X CFTR mutation)/IB3-S9.\textsuperscript{20} Cells were grown as previously described.\textsuperscript{21} Prior to infection, cells were seeded in 12-well tissue culture plates (Nunc, Rochester, USA) and placed into culture medium without supplements for 24 h. RV16 and RV1B stocks were grown in Ohio HeLa cells (European Collection of Cell Cultures) and stocks prepared as HeLa lysates at 1 \( \times 10^7 \) TCID\textsubscript{50}/ml.\textsuperscript{22}

**Primary human airway epithelial cells**

Primary human AECs obtained from patients with CF and healthy subjects were grown in Bronchial Epithelial Growth Medium (Lonza, Switzerland) according to the manufacturer’s recommendations, as described previously.\textsuperscript{21} At passage 2, cells were seeded onto 12-well plates until 80–90% confluency and grown without supplements for 24 h prior to infection.

**Virus infection**

Cells were infected with RV16 or RV1B at a multiplicity of infection (MOI) of 2 for 1 h at room temperature with shaking. Virus preparations were removed, cells washed and 1 ml of fresh medium added. As negative controls, cells were treated with medium alone or filtered virus.\textsuperscript{22} Plates were incubated at 37\(^\circ\)C in 5% CO\(_2\). Cell lysates and supernatants were harvested at various time points and stored at \(-80\)^\circ\)C.

**RT-qPCR**

Quantitative RT-qPCR was carried out using specific primers and probes for RV, IFN-\(\lambda\), IFN-\(\beta\), interleukin (IL)-8, MxA, 2',5'-OAS, viperin, NOS2, Toll-like receptor 3 (TLR3), melanoma differentiation-associated gene 5 (MDA5), retinoic acid inducible gene 1 (RIG-I) and 18S (see table E1 in online supplement). Reactions were performed on iCycler (Biorad, USA). Gene expression was normalised to 18S rRNA\textsuperscript{23} and expressed as copies per \(\mu\)g of total RNA.

**Measurement of virus particle release**

Supernatants were serially diluted in Dulbecco’s Modified Eagle Medium containing 4% FCS (Invitrogen, USA) and titrated on HeLa cells to determine the TCID\textsubscript{50}/ml of RV in the supernatants as described previously.\textsuperscript{24}

**ELISA**

Protein levels of IL-8, IFN-\(\beta\) and IFN-\(\lambda\), (IFN-\(\lambda\)/IL-29, IFN-\(\lambda\)/IL-28B) were quantified in supernatants from untreated and infected cell cultures using ELISA kits for human IL-8 (Amersham Biosciences, USA), IFN-\(\beta\) (Biosource International, USA) and IFN-\(\lambda\) (R\&D Systems, USA).

**Biological effect of IFN-\(\beta\) and IFN-\(\lambda\)**

Cells were seeded and treated with/without different doses of IFN-\(\beta\) and/or IFN-\(\lambda\) before and after infection (Peprotech, USA).

**Transfection and RNA interference**

Healthy primary nasal AECs were transfected with 100 pmol of scrambled control or three different CFTR siRNAs by using lipofectamin 2000 (Invitrogen, USA) according to the manufacturer’s instructions. siRNA sequences\textsuperscript{25} and CFTR sequences of primers/probes are given in the online supplement and in supplemental table E1.

**Figure 1** Time course of rhinovirus (RV) replication and release in cystic fibrosis (CF) bronchial epithelial cells. (A) RV16 expression was measured by RT-qPCR after 8 h, 24 h and 48 h of infection (UNCCF2T/UNCN2T cells). Virus replication increased over time and was significantly higher in CF cells (closed circles) than in non-CF cells (open circles) at both 24 h and 48 h. (B) RV16 release into the supernatants of infected cells was determined by calculating the TCID\textsubscript{50}/ml by titration assay on Ohio HeLa cells. Virus load was significantly increased in CF compared with non-CF cells (open circles) at both 24 h and 48 h. TCID\textsubscript{50}, 50% tissue culture infective dose. Data are presented as median (IQR) of 3–6 independent experiments.
Rhinovirus (RV) replication and release in cystic fibrosis (CF) and non-CF bronchial epithelial cells

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<th>CF cells</th>
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<td>88.2 (39.0−6735.0)×10^5</td>
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<td>RV16 titre (TCID50/ml)</td>
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<td>RV1B RNA expression (copies/μg RNA)</td>
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<td>RV1B titre (TCID50/ml)</td>
<td>0.01 (0.004−0.09)×10^5</td>
<td>0.2 (0.1−0.20)×10^5</td>
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Data are presented as median (IQR). The Mann–Whitney U test was used to determine differences between groups. Associations were tested by univariable and multivariable regression analyses.

Statistics

Data are presented as median (IQR). The Mann–Whitney U test was used to determine differences between groups. Associations were tested by univariable and multivariable regression analyses.

RESULTS

Increased virus replication and release in CF bronchial epithelial cells

To investigate whether CF cells were more susceptible to RV infection than non-CF cells, we analysed vRNA expression at 8, 24 and 48 h after infection. We found more than 10-fold increase of vRNA expression in CF cells (UNCCF2T) compared with non-CF cells (UNCN2T) (figure 1A). These results were confirmed by measuring virus particle release into supernatants (figure 1B). Experiments performed with the minor-group RV1B gave similar results (table 1). RV replication (2^a), RV RNA expression (3^b) and IFN-α levels (4^c) were lower in CF cells than in non-CF cells (figure 2A–C). A similar positive relationship was found between ISG expression and IFN-λ (not shown). Associations remained significant after adjusting for disease group in multivariable regression analysis.

Impaired IFN induction in CF bronchial epithelial cells

Type I (α,β) and III (λ1, λ2/5) IFNs have been described as having strong antiviral properties against RVs. Type I IFNs (IFN-α, IFN-β) were upregulated in infected CF and non-CF cells. IFN-λ1 and IFN-λ2/5 gene expression was upregulated after infection in both CF and non-CF cells. IFN-λ1 was significantly higher in CF than in non-CF cells (figure 2A–C). IFN-α mRNA was poorly expressed and not upregulated. In accordance with mRNA data, IFN-β and IFN-λ protein production was increased in CF and control cells upon RV infection but impaired in CF, both at 24 h and 48 h (figure 2D,E).

Specific IFN deficiency in CF bronchial epithelial cells

We assessed whether IFN production deficiency in CF cells was specific and not due to a global downregulation of gene expression and protein production by analysing induction of the proinflammatory cytokine IL-8. IL-8 gene expression was not upregulated after infection in CF or control cells (figure 2F), but similar large amounts of IL-8 protein were secreted at both 24 h and 48 h after infection in CF and non-CF cells (figure 2G).

Deficient induction of IFN-stimulated genes in CF bronchial epithelial cells

To study the downstream effects of impaired IFN induction in CF, we investigated the expression of ISGs (i.e., 2',5'-OAS, viperin and NOS2). Expression of all ISGs was induced in CF and non-CF cells upon infection, but CF cells expressed up to 1000 times less ISG mRNA (figure 3A). ISG expression was inversely related to RV replication (2',5'-OAS: R^2=0.52, p=0.005; viperin: R^2=0.12, p=0.142; NOS2: R^2=0.29, p=0.042) and positively to IFN-β production (2',5'-OAS: R^2=0.61, p=0.002; viperin: R^2=0.16, p=0.106; NOS2: R^2=0.39, p=0.021) (data for MxA shown in figure 3B,C). A similar positive relationship was found between ISG expression and IFN-λ production (not shown). Associations remained significant after adjusting for disease group in multivariable regression analysis.

Exogenous IFN restores virus control and decreases virus-induced cytotoxicity in bronchial epithelial CF cells

Having demonstrated increased virus replication and a selective IFN production deficiency in CF after RV infection, we tested the ability of exogenous IFN-β/IFN-λ to restore antiviral pathways. Both exogenous IFN-β and IFN-λ (100 pg/ml) increased levels of ISGs (figure 4A) and decreased RV16 RNA expression and release into supernatants of CF cells (figure 4B,C) to the level of control cells. IFN-β had a more pronounced effect than IFN-λ. No additional effect was observed when CF cells were treated with IFN-β and IFN-λ together. There was no effect of IFN treatment in control cells. Additionally, previously reported increased cytotoxicity upon RV infection in CF cells could be decreased to the level of control cells after exogenous IFN treatment (see figure E1 in online supplement).

Impaired virus control and IFN induction in primary CF airway epithelial cells

To assess whether our findings in cell lines could be replicated in primary AECs, we investigated virus replication in nasal and...
We found impaired control of RV replication in CF, with the virus load being 100-fold higher in nasal CF AECs and tenfold higher in bronchial CF AECs than in non-CF cells (Figure 5A,B). IFN-β mRNA expression was 100 times lower in nasal CF AECs and 10 times lower in bronchial CF AECs compared with controls (Figure 5C,D). Similar findings were obtained for IFN-β protein both at 24 h and 48 h (Figure 5E,F). There was no association between virus load or IFN production and clinical markers in patients with CF (age, forced expiratory volume in one second, Pseudomonas aeruginosa colonisation). However, there was an inverse relationship between IFN-β levels and RV replication in nasal AECs ($R^2=0.59$, $p<0.001$) and bronchial AECs ($R^2=0.29$, $p=0.03$; Figure 6) which remained significant after adjusting for disease group in a multivariable regression analysis.

As pattern recognition receptors (PRRs) including TLR3, MDAs5 and RIG-1 play a central role in RV recognition and virus-induced IFN responses, we investigated whether expression of these receptors differed in CF compared with non-CF. Basal expression of all receptors was similar in CF and non-CF (Figure E2 in online supplement).

**Increase of virus replication and impaired IFN responses are not directly linked to CFTR dysfunction**

We examined RV replication and IFN responses after CFTR inhibition in healthy primary AECs. Upon transfection of siRNA targeting CFTR, levels of CFTR mRNA were decreased up to 80% in control and RV16-infected cells (Figure 7A). However, there were no differences in virus replication or in IFN mRNA levels between control and CFTR siRNA-transfected cells (Figure 7B, C).

### DISCUSSION

Strategies aimed at controlling respiratory virus infections may limit exacerbations and deterioration of pulmonary function in patients with CF. Approaches targeting respiratory viruses in CF have included yearly immunisations, the use of neuraminidase inhibitors or adamantanes against influenza and prophyllactic administration of immunoglobulins against respiratory syncytial virus. However, no treatment against rhinoviruses is currently available. Impaired ISG production linked to impaired innate antiviral immunity has been identified in primary AECs from patients with CF and healthy controls. This is the first time impaired antiviral immunity to RV through limited production of IFN-β/IFN-λ and associated ISG induction in CF AECs. This impaired antiviral status was restored by topical administration of IFN-β/IFN-λ, which are critical mediators of the host innate immune response against respiratory viruses. These novel studies provide a mechanism to explain the previous observations with STAT1/NOS2 inhibition in cystic fibrosis.

We first identified differences in innate antiviral responses between CF and non-CF cells using three different well-characterised cell lines. Our data show that all cell lines have a similar phenotype with regard to impaired antiviral responses. We conclude that this is not an artefact of cell line immortalisation or transformation or specific to a particular cell line. These results were confirmed in primary AECs from patients with CF and healthy controls. This is the first time impaired innate antiviral immunity has been identified in both nasal and bronchial AECs obtained from patients with CF and healthy subjects at a median (IQR) age of 15.1 (9.6–26.7) years (Table 2).
This is important, as many respiratory virus infections may start as upper airway infections and proceed to the lower airway. Experimental RV infections are often given in the nose and proceed to the lung causing lower airway inflammation, symptoms and reduction in lung function.36–38 Our data support a hypothesis that impaired antiviral immunity in the nose may lead to greater virus dissemination and hence greater risk of lower airway infection.

To understand mechanisms behind the increased susceptibility of the CF airway epithelium towards RVs, we investigated epithelial IFN responses. We observed significant IFN-β, IFN-λ-1 and IFN-α2/β production after infection in both CF and non-CF, which is consistent with data from the literature reporting that IFN-β and IFN-λ but not IFN-γ or IFN-α are produced by the airway epithelium in response to viruses.14 IFN induction was profoundly impaired in CF cells at the mRNA expression and protein level. Impaired IFN production was a selective defect, as the proinflammatory cytokine response in CF ACEs was similar in CF and non-CF. The relevance of this defective IFN production was demonstrated by the inverse relationship between IFN production and virus replication: whereas primary AECs obtained from patients with CF produced low levels of IFN and were unable to control virus replication, healthy AECs produced high levels of IFN associated with low virus replication. The addition of exogenous IFN to CF cells restored control of virus replication, proving that IFN deficiency is the cause of impaired virus control in CF. Zheng et al.32 previously reported increased parainfluenza replication in CF AECs from lung explants. They identified defective IFN signalling with impaired induction of NOS2 and 2',5'-OAS as possible mechanisms, but did not study IFN-β, and the IFN-λ family was only very recently described at the time. Our findings of impaired IFN induction associated with deficient induction of ISGs including NOS2 and 2',5'-OAS suggest that their results32 33 were a consequence of defective IFN induction, although we cannot rule out the possibility that other mechanisms independently contribute to impaired antiviral control of the CF airway epithelium. Others have reported similar IFN responses upon RV infection in well-differentiated AECs from patients with CF and healthy controls.39 Technical issues such as virus strains used, infection intensity and cell culture conditions might account for these apparently dissimilar results. Although we did not formally rule out increased virus binding or endocytosis in CF cells, our findings of similar viral loads at early time points in CF and non-CF cells and of defective IFN induction argue against this mechanism as a cause of increased susceptibility of the CF airway epithelium towards RVs. In addition, similar expression of RV receptors...
Our findings may have direct therapeutic relevance as we corrected impaired virus control and defective ISG induction by adding exogenous IFN. IFN-β had the most pronounced effect, suggesting it as the preferred candidate. Addition of IFN-β restored levels of ISGs to control levels, which was not achieved by the addition of IFN-λ in our study nor by the addition of IFN-γ or a cytokine cocktail in the study by Zheng et al. So far, there has been only one therapeutic trial using IFN.

### Figure 4
Exogenous interferon (IFN) restores the antiviral response to rhinovirus (RV) in cystic fibrosis (CF) bronchial epithelial cells.

(A) Effect of exogenous IFN-β and IFN-λ (100 pg/ml) on myxovirus resistance A (MxA), 2',5'-oligoadenylate synthetase (OAS), viperin and nitric oxide synthase (NOS)-2 gene expression was tested at 8 h after RV16 infection by RT-qPCR in UNCCF2T cells. Both IFN-β and IFN-λ increased all IFN-stimulated genes (*p < 0.05, **p < 0.01, ***p < 0.001), but the effect was most pronounced for IFN-β. (B) Virus replication in UNCCF2T and UNCN2T cells was measured by RT-qPCR at 8 h, 24 h and 48 h after RV16 infection in the presence or absence of exogenous IFN-β and IFN-λ (100 pg/ml). There was a reduction in virus RNA expression in CF cells treated with IFN-β and/or IFN-λ to the level of control cells (p < 0.001 for both IFN-β and IFN-λ). (C) RV16 release into the supernatants of infected cells was determined by calculating the 50% tissue culture infective dose (TCID50)/ml by titration assay on Ohio HeLa cells at 8 h, 24 h and 48 h after infection in the presence or absence of exogenous IFN-β and IFN-λ. There was a reduction in virus load in supernatants of CF cells treated with IFN-β and/or IFN-λ to the level of control cells (p < 0.05 for both IFN-β and IFN-λ). Data are presented as median (IQR) of 3–4 independent experiments.

### Table 2
Characteristics of patients with cystic fibrosis (CF) and healthy control subjects

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*Atopy defined as a positive history of hay fever, eczema or asthma.
†Steroid use defined as any treatment with systemic, inhaled or nasal steroids within the past 3 months.
‡Pseudomonas aeruginosa (PA) colonisation defined as one or more PA-positive oropharyngeal cultures during the preceding 12 months; negative: no growth of PA in previous 12 months.

FEV1, forced expiratory volume in 1 s as percentage predicted; ND, not done.

ICAM-1, sICAM-1 and VLDL) and viral binding/endocytosis in CF and control cells has recently been reported.39–41

Our findings may have direct therapeutic relevance as we corrected impaired virus control and defective ISG induction by adding exogenous IFN. IFN-β had the most pronounced effect, suggesting it as the preferred candidate. Addition of IFN-β restored levels of ISGs to control levels, which was not achieved by the addition of IFN-λ in our study nor by the addition of IFN-γ or a cytokine cocktail in the study by Zheng et al. So far, there has been only one therapeutic trial using IFN-
application in patients with CF. Moss et al. tested the effect of aerosolised IFN-γ1b in patients with CF with mild to moderate lung disease but did not find any beneficial effect. The lack of efficacy of this approach may be due to the fact that IFN-γ1b is a type II IFN which is mainly produced by natural killer cells and T lymphocytes and not by the bronchial epithelium. Topical type I IFNs have successfully been used in the treatment of severe asthma, leading to improved lung function and reduced need for oral corticosteroids. A similar approach may benefit patients with CF.

We were able to gain an insight into the mechanisms leading to deficient IFN induction in the CF airway epithelium. We and others have recently demonstrated a lack of exaggerated inflammatory responses and increased AEC necrosis upon RV infection in CF. Here we show that impaired IFN induction is not a consequence of virus-induced increased cell death as exogenous IFN decreased cytotoxicity in CF cells to the level of control cells, but rather that impaired IFN induction results in enhanced cell death. Further, we found that deficient IFN production was not due to altered virus recognition by CF ACEs as expression of dsRNA PRRs was similar in CF and control cells. Our data suggest that aberrant PRR signalling leads to deficient IFN production in CF. Indeed, Chattoraj et al. recently demonstrated inhibited RV-stimulated Akt phosphorylation and decreased interferon regulatory factor-3 phosphorylation in CF but not in normal AECs after Pseudomonas aeruginosa infection. Susceptibility to RV infection associated with deficient IFN production is not unique to CF and has been shown in asthma and COPD. Because CF, asthma and COPD are all chronic inflammatory airway diseases, we speculate that
deficient antiviral responses are the consequence of a chronic proinflammatory state rather than directly linked to disease-specific characteristics such as CFTR dysfunction. Indeed, in our study, CFTR inhibition in healthy AECs did not lead to impaired IFN responses and higher virus replication. Recent evidence suggests that a chronic inflammatory milieu, such as that seen in the context of bacterial infection or atopy, suppresses IFN responses towards virus infections in the airways. However, it might be that, in CF, both inflammatory milieu and loss of CFTR function lead to defective epithelial IFN production upon virus infection through, for example, increased and persistent oxidative stress.

Our study has some limitations. We have used primary and immortalised ACEs grown as monolayers, and full differentiation of cells with addition of factors of the naturally occurring microenvironment may have been needed to better mimic virus effects on ACEs. Furthermore, the number of study subjects was relatively small, which did not allow us to examine the effects of genetic and environmental factors. Finally, we did not explore synergisms between viruses and bacteria which might be of relevance to antiviral responses of the CF airway epithelium.

In conclusion, our findings demonstrate a deficient antiviral response to RV infection in CF AECs. Impaired induction of IFN-β, IFN-λ and ISGs results in increased virus replication. This defect can be restored in vitro by the addition of exogenous IFN which limits virus replication to levels observed in normal cells. We therefore suggest that IFNs, particularly IFN-β, might provide novel therapeutic avenues for the treatment of virus-induced CF exacerbations for which very few therapeutic options currently exist.

Acknowledgements The authors thank all study participants and their families for participating in the study; Kathrin Mühlmann and Susanne Aebi from the Department of Infectious Diseases, University of Bern, Amiq Gazzah, Patrizia Castiglioni and Fabian Blank from the Division of Respiratory Medicine, University of

Figure 6 Relationship between interferon (IFN) production and rhinovirus (RV) replication in cystic fibrosis (CF) primary airway epithelial cells. Levels of IFN-β protein released 48 h after infection with RV16 by (A) primary nasal epithelial cells and (B) bronchial epithelial cells were inversely related to virus replication. Linear regression R² was 0.59, p<0.001 for nasal cells and R² was 0.29, p=0.03 for bronchial cells. Associations remained significant when adjusting for disease group in multivariable regression analysis. Data are presented as median (IQR) of 6–7 CF subjects and 7–11 healthy controls for bronchial cells. Open circles represent control cells and closed circles represent CF cells.

Figure 7 Virus replication after CFTR inhibition in healthy primary airway epithelial cells. (A) CFTR levels in untreated (medium (M)) and RV16-infected primary nasal epithelial cells. (B) RV16 and (C) interferon (IFN)-λ2/3 mRNA expression were measured by RT-qPCR 24 h after infection in control and CFTR siRNA cells. Neither RV16 expression nor levels of IFN-λ2/3 mRNA differed between control and CFTR siRNA cells. Data are presented as median (IQR) of five healthy controls. CFTR, cystic fibrosis transmembrane conductance regulator; RV, rhinovirus; siRNA, small interfering RNA.
REFERENCES


Retraction

Marjolaine Vareille,1,2,3,4* Elisabeth Kieninger,1,4* Marco P. Alves,1,4 Brigitte S. Kopf,1,4 Alexander Möller,5 Thomas Geiser,6 Sebastian L. Johnston,7 Michael R. Edwards,7 and Nicolas Regamey1,4

1Department of Clinical Research, University of Bern, 3010 Bern, Switzerland
2Institute for Infectious Diseases, University of Bern, 3010 Bern, Switzerland
3Laboratoire d’Immunologie, EA 4233, Facultés de Médecine et Pharmacie, 63001 Clermont-Ferrand, France
4Division of Respiratory Medicine, Department of Paediatrics, University Children’s Hospital of Bern, Inselspital, 3010 Bern, Switzerland
5Department of Respiratory Medicine, University Children’s Hospital, 8091 Zürich, Switzerland
6Division of Respiratory Medicine, University Hospital of Bern, Inselspital, 3010 Bern, Switzerland
7Department of Respiratory Medicine, National Heart and Lung Institute, Wright Fleming Institute of Infection and Immunity & MRC and Asthma UK Centre in Allergic Mechanisms of Asthma, Imperial College London, Norfolk Place, London W2 1PG, UK


In our article recently published in *Thorax*, we described a novel mechanism explaining the increased susceptibility of patients with cystic fibrosis (CF) to rhinovirus infections, namely defective interferon type I and III production by CF airway epithelial cells. In experiments performed after publication of the article we were unable to consistently replicate our findings of deficient interferon type I and III production by CF airway epithelial cells upon rhinovirus infection. In the light of these results, we carried out detailed investigations of the data reported in the above manuscript and regrettably found evidence of deliberate manipulation by the first author Dr M. Vareille. This manipulation was accompanied in some instances by absence of original data files. The manipulation/original data absence involved data presented in most, if not all of the figures, thus we wish to fully retract the paper and apologise to the readers of *Thorax* and to the scientific community for the inconvenience this has caused.

We also checked data published by our group in manuscripts on which Dr Vareille was a co-author and found that data published in these manuscripts had not been manipulated. These two manuscripts, whose data and conclusions we stand by are:


and


Dr. Vareille has received a letter from the Secretary General of the University of Bern condemning her scientific misconduct as a severe offence against the rules of scientific integrity. Her current employers have also been informed.

All co-authors of the publication including Dr. Vareille concur with the retraction statement.

Online Supplement

Impaired type I and III interferon induction and rhinovirus control in human cystic fibrosis airway epithelial cells

Marjolaine Vareille, Elisabeth Kieninger, Marco P. Alves, Brigitte S. Kopf, Alexander Möller, Thomas Geiser, Sebastian L. Johnston, Michael R. Edwards and Nicolas Regamey
SUPPLEMENTAL METHODS

**Cell culture.** UNCCF2T/UNCN2T cells were grown in CnT-17 medium (CELLnTEC, Switzerland) supplemented with 1% Penicillin/Streptomycin and 10% fetal calf serum (FCS, Invitrogen US) in a humidified incubator (37°C, 5% CO₂). CFBE41o-/16HBE14o- cells were grown in Minimum Essential Medium and IB3-1/IB3-S9 cells in Dulbecco’s Modified Eagle Medium plus Glutamax, both additionally supplemented as above (InvivoGen, US).

**Virus culture.** The identities of each RV were confirmed by neutralisation using serotype specific antibodies. Virus stocks were negative for *Mycoplasma* infection.

**Primary human airway epithelial cells.** Nasal AECs were obtained by brushing the inferior surface of the middle turbinate of both nostrils with a 3-mm-cytology brush (Dent-o-Care, UK). Bronchial AECs were grown from brushings of the bronchial tree performed during clinically-indicated bronchoscopies or through the endotracheal tube in anaesthetised subjects undergoing elective surgery [E1]. Primary cultures were established by seeding freshly brushed cells into Bronchial Epithelial Growth Medium supplemented with Single Quots (Lonza, Switzerland), Primocin (100µg/ml; InvigoGen, US) and 10% FCS (Invitrogen, US). CF AECs were additionally treated with ceftazidime (100µg/ml; GlaxoSmithKline, Switzerland) and amphotericin B (2.5mg/l; Sigma, US) during first days of culture. Immunocytochemical staining for cytokeratin-13 and -19 (Abcam, UK) and occludin (Invitrogen, US) confirmed epithelial origin >95% of the cells obtained. At passage two, cells were seeded onto 12-well plates (Nunc, Rochester, US) until 80-90% confluency and placed into Bronchial Epithelial Basal Medium (Lonza, Switzerland) without any supplements for 24h prior to infection.
**RT-qPCR.** Cell lysates were obtained by adding 350µl of RLT-buffer (Qiagen, Switzerland). Total RNA was extracted (RNase Kit, Qiagen, Switzerland) and 2µg used for cDNA synthesis (Omniscript-RT-Kit, Qiagen, Switzerland). qPCR was carried out using specific primers (sense 300nM; antisense 900nM) and probes (175nM) for RV, IFN-λ1, IFN-λ2/3, IFN-β, IL-8, MxA, 2’,5’-OAS, viperin, NOS2, Toll-like receptor 3 (TLR3), melanoma differentiation-associated gene 5 (MDA5), retinoic acid inducible gene I (RIG-I) and 18S (Supplemental Table E1). Reactions consisted of 2µl of cDNA (cDNA for 18S was diluted 1:100) and 12.5µl QuantiTect-Probe PCR-MasterMix (Qiagen, Switzerland) and were performed on iCycler® (Biorad, US). Gene expression was normalized to 18S rRNA, which has been shown to be a stable housekeeping gene for studies involving human airway epithelial cells [E2], and expressed as copies per µg of total RNA using a standard curve generated by amplification of plasmid DNA bearing the cDNA of the gene of interest.

Table E1. Sequences of primers and probes

<table>
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<th>Gene</th>
<th>Sequence of primers and probes</th>
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| RV        | Forward-5’-GTGAAGAGCCSCRTGTGCT-3’  
Reverse-5’-GCTSCAGGGTTAAGTTAGCC-3’  
Probe-5’-FAM-TGAGTCTCCTGGCCCCTGAATG-TAMRA-3’ |
| IFN-β     | Forward-5’-CGCCGCTTGGCAGCCATCTA-3’  
Reverse-5’-GACATTAGCCAGCTTTAGCTCA-3’  
Probe-5’-FAMTCAGACAAGATTACATCTAGCTGCTGATAMRA-3’ |
| IFN-λ1    | Forward-5’-GAAAGCCTCGAGGATCCATTTA-3’  
Reverse-5’-AGAAGCCTAGCAGGCTTTAGCTCA-3’  
Probe-5’-FAMAGTTGCAAGGCTTCTCTTCATGCTGATAMRA-3’ |
| IFN-λ2/3  | Forward-5’-CTGCCCATAGCCCATCTA-3’  
Reverse-5’-AGAAGCCTGACTTTAAATAGGTCTTAGCA-3’  
Probe-5’-FAMTCCTCCACAGGAGCAGCTGGCCTTATAMRA-3’ |
| IL-8      | Forward-5’-TGCCGCTTGGCAGCCATCTA-3’  
Reverse-5’-AGAAGGGGCTTCTCTTACATGCTGCTA-3’  
Probe-5’-FAMTCCTCAGCAGGCTGGGCTTATAMRA-3’ |
| MxA       | Forward-5’-AGACCTTGAGGCTTATCATAC-3’  
Reverse-5’-AGAAGCCTGACTTTAAATAGGTCTTAGCA-3’  
Probe-5’-FAMAGTCAGGCAAGGCTGCTGATAMRA-3’ |
| Viperin   | Forward-5’-AGAAGGCATATATATTTCTTATGAGGCAATGAGG-3’  
Reverse-5’-AACGAAGGTGCTGCTGATAMRA-3’ |
**ELISA.** IFN-β and IL-8 proteins were quantified in supernatants from untreated and infected cell cultures using ELISA-kits for human IFN-β (Biosource International, US) and IL-8 (Amersham Biosciences, US) according to the manufacturer’s instructions. The detection limits were 2.5U/ml (IFN-β) and 3.5pg/ml (IL-8). To quantify IFN-λ1 in supernatants, we used a monoclonal anti-human IFN-λ1 as capture-antibody, a polyclonal anti-IFN-λ1 as secondary-antibody and biotin-conjugated donkey-anti-goat-IgG as third-antibody (R&D Systems, US), followed by streptavidin-conjugated horseradish-peroxidase (Biosource, US). Recombinant human IFN-λ1 (Peprotech, US) was used as standard. The sensitivity of the assay was 25pg/ml. This assay also detects IFN-λ2/3 due to 25% cross-reactivity [E3].

**Biological effect of IFN-β and IFN-λ.** Cells were seeded and treated for 24h before and for up to 48 hours after RV infection with/without different doses of IFN-β and/or IFN-λ1

| Probe-5’-FAMCCCTGAATCTAACAGAAAGATGAAAGACTCTAMRA-3’ |
| 2',5'-OAS Forward-5’-CTGACGFCTGACCTGTTGCTT-3’ |
| Reverse-5’-CCCCGGCGATTTAATGATGTA-3’ |
| Probe-5’-FAMCCCTAGCTCCTCCACCACTCTTCTAMRA-3’ |
| NOS2 Forward-5’-GGTGGGACGGTGAAAAGG-3’ |
| Reverse-5’-TGCTGGGACGGGAGATGGA-3’ |
| Probe-5’-FAMAGAAGAACAAAGGACGCTACCATACGACCCGAMRA-3’ |
| TLR3 Forward-5’-ATAATTTCAAGGTTTCTCCAGGCTT-3’ |
| Reverse-5’-ATTTCTCGAAATGATGCTTGTCG-3’ |
| Probe-5’-FAM-CTGAGGCTCCCTCTATGAMRA-3’ |
| RIG-I Forward-5’-CCACAGTCATTCTATCCAT-3’ |
| Reverse-5’-TACCAGCTGAGTCTCTTCT-3’ |
| Probe-5’-FAM-CCACACGAAATGTGCCAATGCAAGTCCTTAMRA-3’ |
| MDA5 Forward-5’-GCCACCCACATGCGAATG-3’ |
| Reverse-5’-GGCCACCCCTGAGTCTCTTCT-3’ |
| Probe-5’-FAM-GGATGCTCTGCGGATTCTCTAMRA-3’ |
| CFTR Forward-5’-AGCTTGCAACCCGTGTCTCAGATA-3’ |
| Reverse-5’-TGAGGATGCTGCACGCAATTC-3’ |
| Probe-5’-FAM-CACACGAAATGTGCCAAGTCTCTTAMRA-3’ |
| 18S Forward-5’-CGCCGCTAGGTGAAATCTT-3’ |
| Reverse-5’-CATTCTGGCAAAATGCTTCC-3’ |
| Probe-5’-FAMCCCGCGCAAGGACCAGATAMRA-3’ |
Cytotoxicity. Cytotoxicity was assessed by measuring LDH activity in culture supernatants (Cytotoxicity Detection Kit; Roche, Switzerland).

Transfection and RNA interference. Healthy primary nasal AECs were cultured until 80-90% confluency and transfected with 100pmol of scrambled control or three different CFTR siRNAs by using lipofectamin 2000 (Invitrogen, US) according to manufacturer’s instructions. Following sequences of functional-siRNAs were used: siCFTR.1 (5’-CGUGUGUCUGUAAACUGAUGGCUAA-3’), siCFTR.2 (5’-CCCUUCUGUUGAUCUGCUGACAAU-3’), siCFTR.3 (5’-GGCAUAGGCUUAUGCC UUCUCUUAUA-3’). Control-siRNA for the exclusion of non-target effects was 5’-UACCGUCUCACUUGAUCGdTdT-3’ (E4).

Statistics. Descriptive statistics and analyses were performed using Stata™ (STATA Corporation, College Station, US). As most of the data were not normally distributed, we present them as median (interquartile range [IQR]). P-values <0.05 were considered statistically significant.
SUPPLEMENTAL REFERENCES


SUPPLEMENTAL FIGURE LEGENDS

**Figure E1. Exogenous IFN decreases RV-induced cytotoxicity in CF bronchial epithelial cells** (UNCN2T/UNCCF2T cell line). Cytotoxicity (%), assessed by measuring LDH activity in supernatants, was determined after exposure to RV16 in the presence or absence of exogenous IFN-β (100 pg/ml) and IFN-λ (100 pg/ml) at different timepoints (8h, 24h, 48h) and compared to untreated cells. Cytotoxicity was significantly increased in CF cells compared to non-CF cells (p=0.02). There was a reduction in cytotoxicity in CF cells treated with IFN-β and/or IFN-λ to the level of control cells (p=0.004 for both IFN-β and IFN-λ). Data are presented as median (IQR) of 3-4 independent experiments.

**Figure E2. Similar expression of double-stranded RNA recognition receptors in CF and non-CF primary airway epithelial cells.** Basal expression of MDA5 (A), TLR3 (B) and RIG-I (C) were measured by RT-qPCR in primary bronchial epithelial cells obtained from CF patients or healthy control donors. Expression of all receptors was similar in CF and non-CF cells. Data are presented as median (IQR) of 4 CF subjects and 4 healthy controls.
Figure E1

Graph showing the cytotoxicity (%) of control cells, CF cells, CF cells + IFN-β, and CF cells + IFN-γ over time (hours). Time points are at 0 (medium), 8, 24, and 48 hours.

- Control cells
- CF cells
- CF cells + IFN-β
- CF cells + IFN-γ

Statistical significance:
- p = 0.02
- all p = 0.004