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Lack of an exaggerated inflammatory response upon virus infection in cystic fibrosis

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ABSTRACT

Respiratory virus infections play an important role in cystic fibrosis (CF) exacerbations, but underlying pathophysiological mechanisms are poorly understood. We aimed at assessing whether an exaggerated inflammatory response of the airway epithelium upon virus infection could explain the increased susceptibility of CF patients towards respiratory viruses.

We used primary bronchial and nasal epithelial cells obtained from healthy control subjects (n=24) and CF patients (n=18). IL-6, IL-8/CXCL8, IP-10/CXCL10, MCP-1/CCL2, RANTES/CCL5 and Gro-α/CXCL1 levels in supernatants and mRNA expression in cell lysates were measured before and after infection with rhinoviruses (RV-16; RV-1B) and RSV. Cytotoxicity was assessed by LDH assay and flow cytometry.

All viruses induced strong cytokine release in both control and CF cells. The inflammatory response upon virus infection was heterogeneous and depended on cell type and virus used, but was not increased in CF compared to control cells. On the contrary, there was a marked trend towards lower cytokine production associated with increased cell death in CF cells.

An exaggerated inflammatory response to virus infection in bronchial epithelial cells does not explain the increased respiratory morbidity after virus infection in CF patients.

INTRODUCTION

Respiratory virus infections have been associated with both short- and long-term pulmonary morbidity in cystic fibrosis (1-7). Although they occur at similar frequency and seasonal distribution in CF as in the general population, their clinical impact is greater than in healthy subjects, with a longer duration and more severe course of the disease (1, 6). Respiratory viruses have been increasingly recognized as important agents of pulmonary exacerbations and hospitalizations in both adults and children with CF (2, 3, 5, 7). Amongst viruses implicated in CF pulmonary morbidity, rhinoviruses (RVs) and respiratory syncytial virus (RSV) play a major role. RVs are the most common agents associated with exacerbations (4, 7), and RSV is an important cause of early acute respiratory tract morbidity in young infants with CF (2, 5).

The pathophysiology of virus induced CF exacerbations is unclear. Exaggerated production of inflammatory mediators and the consequent increased influx of inflammatory cells are proposed mechanisms leading to the increased respiratory morbidity during respiratory virus infections in CF (8-11). It is well established that inflammation is a key contributor to the pathophysiology of CF lung disease, associated with progressive destructive changes (12).

In addition to being the main site of virus replication, the airway epithelium has been shown to actively participate in the inflammatory response during respiratory virus infections (13). Epithelial-derived cytokines and chemokines including IL-6, IL-8/CXCL8, IP-10/CXCL10, MCP-1/CCL2, RANTES/CCL5 and Gro-α/CXCL1, all of which have been shown to be induced upon virus infection (13) and to be associated with magnitude of inflammation and respiratory morbidity in CF (14), are important mediators involved in the initiation of

inflammatory processes as they recruit and activate effector cells of innate and adaptive immunity, such as eosinophils, neutrophils, dendritic cells, macrophages and T cells.

We hypothesized that there is an exaggerated inflammatory response of the CF airway epithelium upon virus infection. To test this hypothesis, we assessed epithelial cytokine and chemokine production upon infection with different respiratory viruses using various models of CF and control airway epithelial cells.

MATERIAL AND METHODS

Cell culture. Primary human airway epithelial cells were obtained from healthy control subjects and from CF patients (15). Nasal epithelial cells (HNECs) were obtained by brushing the inferior surface of the middle turbinate of both nostrils with a cytology brush (Dent-o-Care, UK). Bronchial epithelial cells (HBECs) were grown from brushings of the bronchial tree performed at the time of a clinically indicated bronchoscopy or through the endotracheal tube in subjects undergoing elective surgery under general anaesthesia (16). This study was conducted with the approval by the University Children`s Hospital Ethics Committee, Bern, Switzerland. Informed consent was obtained from study participants and/or caregivers. Primary cultures were established by seeding freshly brushed cells into Bronchial Epithelial Growth Medium supplemented with Single Quots (Lonza, Switzerland), Primocin (InvigoGen, US) and 10% fetal calf serum (FCS; Invitrogen, US). 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.

In addition, three well-characterized human bronchial epithelial cell lines, each with a non-CF (control) and CF phenotype, were used as comparisons to primary cells: UNCN2T/UNCCF2T (F508del/F508del CFTR mutation) (17), 16HBE14o-/CFBE41o- (F508del/F508del CFTR mutation) (18) and IB3-S9/IB3-1 (F508del/W1282X CFTR mutation) (19).

Virus Culture. RV-16 (major group RV) and RV-1B (minor group RV) stocks were generated and titrated from infected culture of Ohio-HeLa cells (European Collection of Cell Cultures) and stocks prepared as HeLa-lysates at $1x10^7$ $TCID_{50}$/ml (20). The A2 strain of RSV was grown on Hep-2 cells. These stocks had a titer of $1.8x10^7$ plaque forming units (PFU)/ml (21).

Virus infection. Virus titration experiments were conducted to assess optimal multiplicity of infection (MOI) (Supplemental Figure 1 in OLS). Cells were infected apically with RVs at MOI of 2 at room temperature and with RSV at MOI of 1.5 at 37ºC for 1h with shaking. Virus preparations were removed and plates incubated at 37ºC. Cell lysates and supernatants were harvested at 8h, 24h and 48h after infection. Medium-treated cells and filtrated-viruses (20) served as negative controls. Efficacy of virus infection was confirmed by intracellular staining of viral antigen.

Cytotoxicity. Cytotoxicity was assessed by measuring LDH actitivy in culture supernatants (Cytotoxicity Detection Kit; Roche, Switzerland). Biotinylated-annexinV with streptavidin-conjugated allophycocyanin as detection antibody (all eBioscience, US) and propidium iodide (PI) (Invitrogen, US) stainings were performed to quantify apoptosis and necrosis. Analysis was done with flow cytometry.

Multiplex suspension arrays. Levels of IL-6, IL-8/CXCL8, IP-10/CXCL10, MCP-1/CCL2, RANTES/CCL5 and Gro-α/CXCL1 were measured in supernatants overlying uninfected or infected cell cultures using the Bioplex suspension array technique (Biorad, Switzerland) and the Milliplex®-Map Kit for human cytokines/chemokines (Millipore, US).

Taq-Man® real-time PCR. Quantitative PCR (RNeasy Kit, Omniscript RT kit, Qiagen, Switzerland) was carried out using specific primers and probes for IL-6, IL-8 and RANTES (Supplemental Table 1 in online supplement [OLS]). Reactions were performed on iCycler® (Biorad, US). Gene expression was normalized to 18S rRNA, compared to standard curves and expressed as copies/µgRNA.

Statistical analysis. Data are presented as the mean ± standard error of the mean. Mann-Whitney *U,* non-parametric ANOVA with Kruskal-Wallis tests and Bonferroni corrections for multiple testing were used to determine differences between two or more groups. Associations were tested by multivariable regression analysis taking gender, age and atopy into account; $p<0.05$ was considered statistically significant.

Additional information is provided in the OLS.

RESULTS

Primary airway epithelial cell cultures from control subjects and CF patients

Primary cell cultures were obtained from 24 control subjects (mean age 17.5 ± 2.2 years) and 18 CF patients (mean age 17.7 ± 2.5 years) (Table 1).

Our success rate in establishing primary cell cultures was almost 95% with the number of cells (840 000 ± 160 000 cells/mL) and the proportion of viable cells (89 ± 2.2 %) obtained from nasal and bronchial brushings being similar between control and CF subjects.

Lack of exaggerated inflammatory response after virus challenge in primary airway epithelial cells from CF patients

RV-16, RV-1B and RSV infection of human nasal (HNECs) and bronchial (HBECs) epithelial cells from healthy controls and CF patients induced a significant and robust time-dependent production of IL-6, IL-8, IP-10, MCP-1, RANTES and Gro-α, with values exceeding several thousand pg/ml for some cytokines (Figures 1 and 2). Spontaneous production of cytokines was similar in primary control and CF cells. After virus infection there was an overall trend towards lower cytokine production in CF cells especially in HNECs (Figure 1). However, for some cytokines at specific time points, higher values in CF HBECs were observed compared to control HBECs (e.g. IL-8 after infection with RV-16 at 48h: 4789 ± 416 vs. 2717 ± 451, p=0.01) (Figure 2). Cytokine levels were consistently lower in both control and CF HBECs compared to HNECs (e.g. IL-6 production after RV-1B infection at 48h: CF HBECs 382 ± 103 vs. CF HNECs 2686 ± 1749, p=0.03). When comparing the inflammatory responses between viruses, we observed that RV-1B led to a higher response than RV-16 and RSV in both HBECs and HNECs and for both control and CF cells (Figures 1 and 2). There was no difference in the cytokine response when looking at different subgroups (e.g. atopic vs. non-atopic subjects), neither an association between cytokine levels before or after virus infection and clinical parameters in CF patients (age, atopy, FEV_1 and Pseudomonas aeruginosa colonization).

Increased cytotoxicity in primary airway epithelial cells from CF patients after virus infection

We observed a higher cytotoxicity as a measure of cell damage after virus infection in CF primary airway epithelial cells. In CF HNECs the increase in cytotoxcity compared to control cells after virus infection at 48h was 109.4% for RV-16, 121% for RV-1B and 183% for RSV. In HBECs it was 120% for RV-16, 108.6% for RV-1B and 137% for RSV. Higher cytotoxicity was found in Pseudomonas aeruginosa (PA)-positive compared to PA-negative CF patients (60% vs. 10%, p=0.01). Taking into account cell damage occurring after virus infection, data were also adjusted for increased cell death (9). However, this did not change overall results and the trend towards lower cytokine levels in primary airway epithelial cells from CF remained unchanged (data not shown).

Comparison of primary airway epithelial cells and bronchial epithelial cell lines

We performed the same experiments on bronchial epithelial cell lines and found that with the exception of the recently described UNCN2T/UNCCF2T cell line (17), which showed increased basal production of several cytokines in CF cells, the spontaneous production of most cytokines was similar in CF and control cells (Supplemental Tables 2-4 in the OLS). As for the findings in primary cells, all viruses induced a vigorous inflammatory response in both control and CF cells, and the inflammatory response upon virus infection was not exaggerated in CF cells. The expression of *IL-6, IL-8* and *RANTES* genes at the mRNA level was also analysed. In accordance with the observations made at the protein production level, gene expression values after virus infection were similar between control and CF cells (Supplemental Figure 2 in the OLS). In line with the primary cell data, increased cytotoxicity upon virus infection was found in CF cells (e.g. 48h after infection with RV-16: 199 ± 14% vs. 96 ± 15%, p=0.01; Supplemental Figure 3 in the OLS). We further determined in the

UNCN2T/UNCCF2T cell line whether the observed increase in cell lysis upon virus was due to apoptosis or necrosis (22). Apoptosis levels were similar between control and CF cells. However, the number of necrotic cells was significantly higher in CF cells after infection with RV-16 ($p=0.04$) and RSV ($p=0.03$), and there was a trend for increased necrosis in CF cells after infection with RV-1B (Figure 3). An inverse relationship was found between cell death and cytokine production in control and CF cells. Intact cells (i.e. control cells) responded to virus infection with large cytokine production, whereas damaged cells (i.e. CF cells) produced less cytokines (Supplemental Figure 4 in the OLS).

DISCUSSION

In this study we performed a comprehensive investigation of the inflammatory response of CF airway epithelial cells upon virus infection. Strong cytokine production was found in all cells studied, with the magnitude and type of inflammation differing depending on cell type and virus used. There was no exaggerated inflammatory response in CF, neither at the cytokine production nor at the transcriptional level, which is contrary to what we expected. We rather observed a trend towards lower cytokine production in CF airway epithelial cells after virus infection, which was associated with increased cell death.

There has been a long-lasting debate as to whether the CF airway epithelium is pro-inflammatory and dysregulated *per se* (23). While this was not the main purpose of this study, our findings do not support an intrinsically pro-inflammatory phenotype in CF. In both primary nasal and primary bronchial cells and in two out of three cell lines, spontaneous production of cytokines did not differ between control and CF cells.

Conflicting results regarding the inflammatory response of the CF airway epithelium upon virus infection have been reported. Black *et al*. used primary nasal epithelial cells taken from patients with or without CF, as well as control and CF cell lines, and found no difference in the magnitude or in the duration of the IL-8 response upon RSV infection (24). In contrast, others reported that primary bronchial CF cells obtained from lung transplant recipients and autopsies reacted with a greater inflammatory response compared to control cells upon infection with human parainfluenza virus 3 (11) and influenza A (10), and recently, Sutanto *et al.* found an increased inflammatory response upon RV infection in bronchial epithelial cells obtained from young CF children (9). Animal models have also yielded inconsistent evidence. Whereas an aberrant pro-inflammatory response upon RSV infection was described in CF mice (8), this was not confirmed in another study (25). In the present study, we investigated this issue comprehensively. Primary airway epithelial cells from the upper and lower airways were obtained from a relatively large and homogenous group of CF patients and control subjects with similar age distribution. Additionally, potential confounders such as atopy were adjusted for. We studied a wide range of cytokines and chemokines after infection of these cells with three different respiratory viruses. Despite some differences between CF and control cells for certain cytokines (some of them up- and some of them downregulated), our results indicate that overall the inflammatory response towards virus infection in CF airway epithelial cells is not exaggerated. However, our findings do not rule out that under certain circumstances, some cytokines may be upregulated in CF airway epithelial cells after virus infection. For instance, Sutanto *et al*. found increased levels of IL-6 and IL-8 after RV infection of primary bronchial CF cells compared to control cells, but only when using the minor type RV-1B (no difference found upon infection with the major type RV-14) and only at a high MOI of 25 (no difference found upon infection at a MOI of 3.1) (9). It is also possible that technical issues such as type of virus used (e.g. human parainfluenza virus 3 and

influenza A in the studies by Zheng *et al.* and Xu *et al.,* respectively) and cell culture conditions (e.g. different plate coating or use of supplements during virus infection in the study of Sutanto *et al.*) account for these apparently dissimilar results between previous studies and ours.

A possible explanation for the lack of an exaggerated inflammatory response in the CF airway epithelium upon virus infection could be the occurrence of increased cell death. We indeed observed increased virus induced cytotoxicity due to necrosis in CF cells. Our findings are consistent with the concept of increased epithelial cell death in CF, also described by Sutanto et *al.* (9, 26, 27), suggesting increased susceptibility of the CF airway epithelium towards toxic effects of respiratory viruses. Chronic inflammatory stimulation as seen in the context of bacterial colonization could lead to such increased susceptibility to cytotoxic stimuli. The observed increased virus induced cytotoxicity in Pseudomonas-positive patients compared to Pseudomonas-negative ones supports this concept. Alternatively, impaired ability to clear viruses due to failure of increasing airway surface liquid upon virus infection (28), or generating anti-viral mediators such as nitric oxide (8) or type I and III interferons (10, 11, 29), could lead to increased viral replication and further cell death (29). However, as a lower inflammatory response in CF was still observed after data adjustment for cell death, additional pathophysiological mechanisms such as interactions between antiviral and pro-inflammatory pathways are likely to be involved (30). It could be speculated that because of chronic activation of pro-inflammatory pathways, CF airway epithelial cells are not able to respond adequately to further stimuli such as virus infections. This might in turn lead to a deficient recruitment of effector immune cells resulting in longer duration and more severe respiratory symptoms.

The airway epithelial inflammatory response towards respiratory viruses is of considerable variability and depends amongst other factors on virus type, strain and load, cell culture conditions and infected cell type (31-33). Thus data obtained from only a single *in vitro* model system should be interpreted with caution. Based on these considerations our study has several strengths. Different respiratory viruses and various culture conditions and models were used to account for this variability. Primary airway epithelial cultures were included to overcome the inherent drawbacks of complementation, derivation from single individuals and changes of the phenotypic characteristics over time of immortalized cell lines (31). Long-term (passage 2) cell cultures were used to overcome the initial pro-inflammatory phenotype seen in epithelial cells freshly isolated from CF airways (34, 35), and cells were treated without any additional supplements prior to infection to overcome potential overlapping effects of culture media components. However our study also has limitations and following points have to be considered when interpreting our findings. The effects of genetic and environmental factors on the inflammatory response, such as recent exposure to viral, bacterial or fungal infection, are unknown. Full differentiation of cell cultures including cell polarization of epithelial cells or factors of the naturally occurring *in vivo* micro-environment, such as mucus, surfactant and cytokines (36) might be needed to mimic specific effects of CFTR dysfunction on epithelial processes. Synergisms between viruses and bacteria were also not examined though they may be a further mechanism responsible for increased respiratory morbidity towards virus infection in CF patients (37, 38).

Taken together, our findings suggest that the increased morbidity in CF patients after virus infection is not due to an exaggerated inflammatory response of the airway epithelium but rather linked to increased cell death. They thus provide a rationale for implementing therapies aimed at controlling viruses and their replication rather than primarily targeting inflammation.

In this respect, a promising candidate is the macrolide-antibiotic azithromycin, which is increasingly used in CF patients as a beneficial immunmodulatory agent (39) and has recently been shown to possess anti-viral properties (40).

In this context, our results provide the basis for a better understanding of the possible action of those drugs and shed further light upon the increased respiratory morbidity after virus infection in CF.

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Ethics approval: This study was conducted with the approval by the University Children`s Hospital Ethics Committee, Bern, Switzerland.

Author contributions: Conceived and designed the study: EK, ME, NR. Acquisition of data: EK, MV, BK, FB, MA, FG, TG, CC, NR. Analysis and interpretation: EK, MA, PL, CC, TG, ME, SJ, NR. Drafting the manuscript for important intellectual content: EK, MA, PL, CC, TG, ME, SJ, NR. Final approval of the manuscript: all authors.

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Table 1. Characteristics of healthy control subjects and CF patients from whom airway epithelial cell cultures were established

Nasal Cells

	gender	age (years)	atopy[1]	steroid use[2]	FEV_1(%)	Pseudomonas aeruginosa colonization[3]	genotype
Controls							
1	m	26.5	no	no			
2	f	29.9	no	no			
3	f	26.9	no	no			
4	f	25.1	no	no			
5	f	26.8	yes	no			
6	m	25.6	yes	no			
7	f	23.7	yes	no			
8	m	48.9	no	no			
9	f	27.2	no	no			
10	f	25.4	no	no			
11	m	6.3	no	no			
12	f	16.2	yes	no			
13	m	10.9	no	no			
14	m	5.9	no	no			
CF patients							
1	m	13.8	yes	no	88	no	F508del/R553X
2	m	16.4	yes	no	81	no	N1303K/2347delG
3	f	13.5	yes	no	86	yes	F508del/F508del
4	m	45.7	yes	no	68	yes	F508del/F508del
5	f	11.8	no	no	85	yes	F508del/F508del
6	m	17.7	yes	no	55	yes	F508del/F508del
7	m	24.9	yes	no	64	yes	F508del/F508del
8	m	22.1	yes	no	48	no	F508del/F508del
9	m	22.6	no	no	84	yes	F508del/F508del
10	m	23.9	no	no	21	yes	F508del/F508del

Bronchial Cells

	gender	age (years)	atopy[1]	steroid use[2]	FEV_1(%)	Pseudomonas aeruginosa colonization[3]	genotype
Controls							
1	f	7.1	no	no			
2	m	6.3	no	no			
3	m	14.4	no	no			
4	f	8.8	no	no			
5	m	9.9	yes	no			
6	m	4.5	no	no			
7	f	16.2	no	no			
8	m	10.9	no	no			
9	m	10.6	no	no			
10	m	5.9	no	no			
CF patients							
1	m	0.2	no	no	n.d.	no	F508del/F508del
2	f	14.2	no	no	85	yes	F508del/3905insT
3	m	27.8	no	no	88	yes	F508del/G542X
4	f	15.9	no	no	73	no	F508del/F508del
5	f	29.1	no	no	66	no	F508del/F508del
6	m	0.4	no	no	n.d.	no	F508del/R553X
7	m	10.4	no	no	82	yes	Q525X/ Q525X
8	f	8.7	no	no	92	yes	F508del/3659Cdel

[1]atopy defined as positive history of hay fever, eczema or asthma
[2]steroid use defined as any treatment with either systemic, inhaled or nasal steroids within the past three months
[3]Pseudomonas aeruginosa (PA) colonization defined as one or more PA-positive oropharyngeal cultures during the preceding 12 months (41)
FEV_1: forced expiratory volume in one second as percent predicted; n.d.: not done

Figure legends

Figure 1. Cytokine production in primary nasal epithelial cells from healthy control subjects and CF patients before and after virus infection. IL-6 (A), IL-8 (B), IP-10 (C), MCP-1 (D), RANTES (E) and Gro-α (F) levels were measured in the supernatants of primary human nasal (HNECs) epithelial cells by multiplex suspension array technique before (medium; open bars) and after infection with RV-16 (solid bars), RV-1B (dark shaded bars) and RSV (bright shaded bars) at 24h and 48h. Data from 14 healthy controls and 10 CF patients are presented as mean ± SEM; * = different to control cells (*p<0.05).

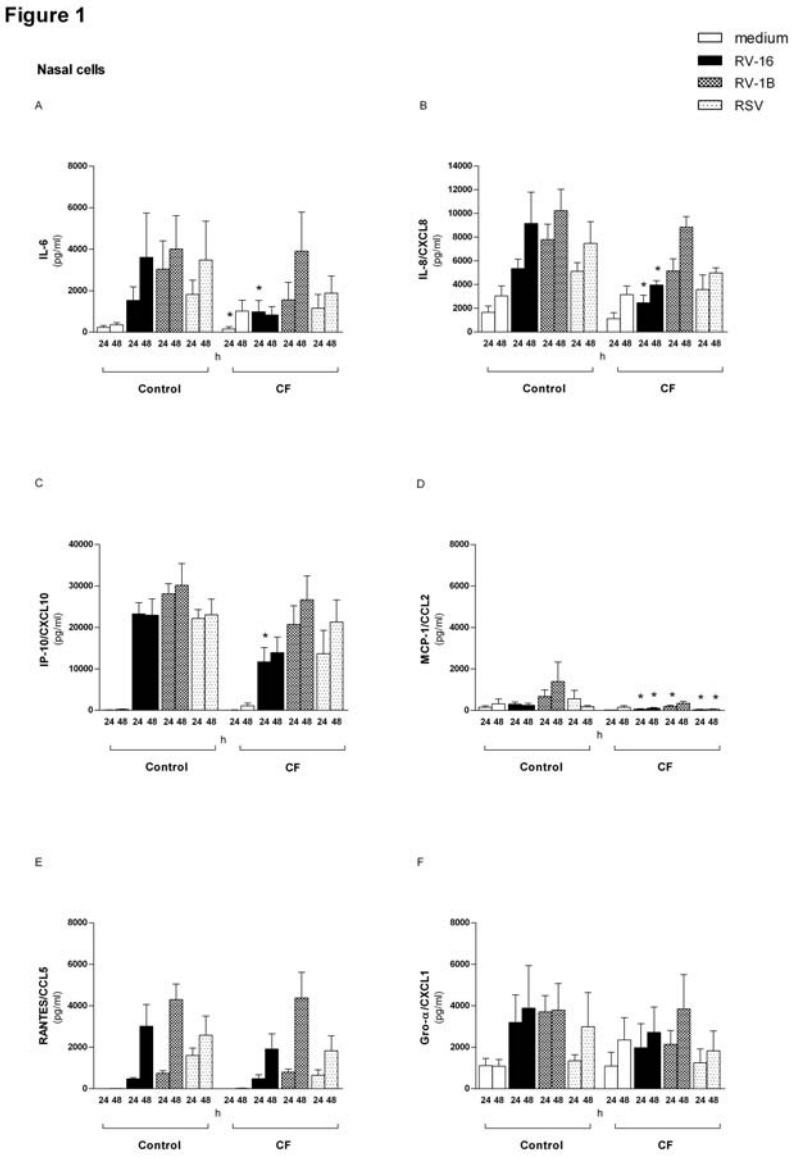

Figure 2. Cytokine production in primary bronchial epithelial cells from healthy control subjects and CF patients before and after virus infection. IL-6 (A), IL-8 (B), IP-10 (C), MCP-1 (D), RANTES (E) and Gro-α (F) levels were measured in the supernatants of primary human bronchial (HBECs) epithelial cells by multiplex suspension array technique before (medium; open bars) and after infection with RV-16 (solid bars), RV-1B (dark shaded bars) and RSV (bright shaded bars) at 24h and 48h. Data from 10 healthy controls and 8 CF patients are presented as mean ± SEM; * = different to control cells (*p<0.05, ** p<0.01).

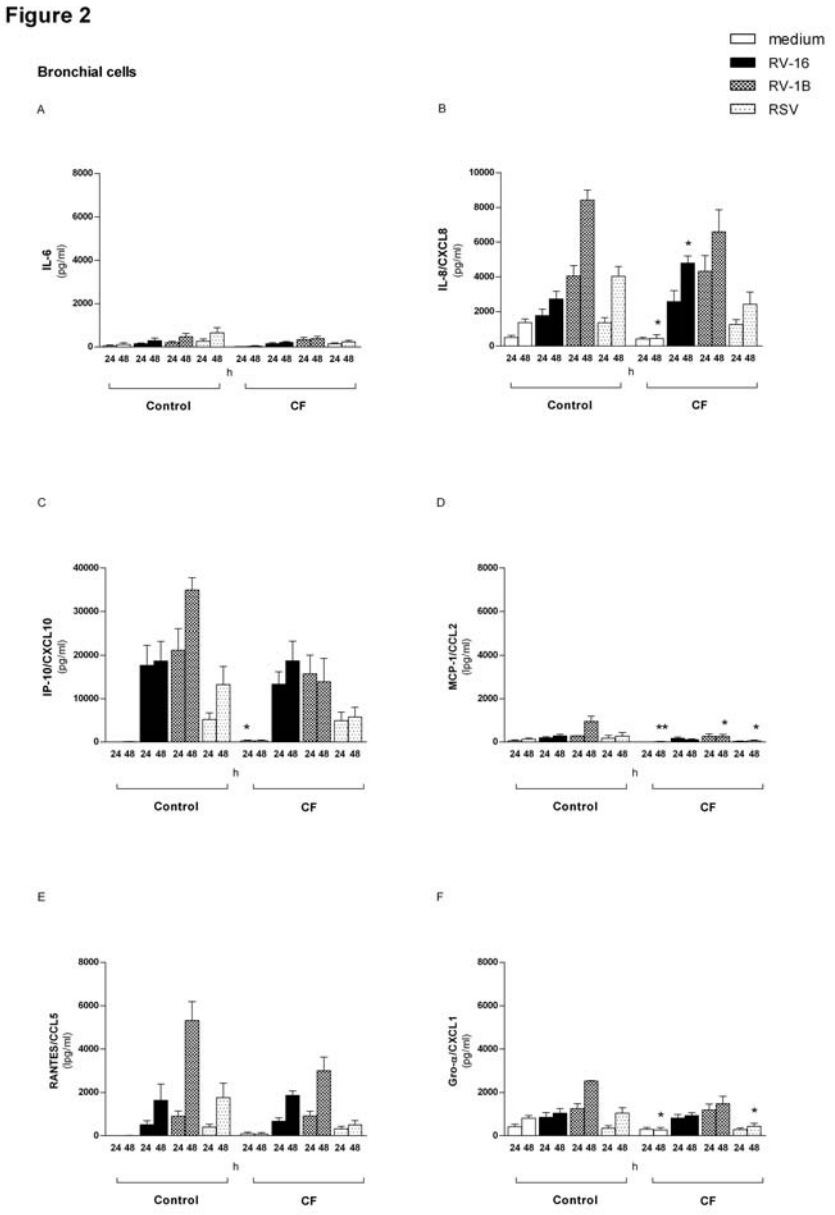

Figure 3. Virus induced cell death in bronchial control and CF cells (UNCN2T/UNCCF2T cell line). Cell death was determined before (medium) and 24h after exposure to RV-16, RV-1B and RSV by flow cytometry using Annexin V (apoptosis; A) and propodium iodide (PI) staining (necrosis; B). Data from four independent experiments are presented as fold increase from baseline as mean ± SEM; * = different to control cells (*p<0.05). (C) Total number of apoptotic (Annexin V-positive) and necrotic (PI-positive) cells in CF compared to control cells. Data are presented as flow cytometry dot plots representative of five independent experiments.

Figure 3