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Research article

THE EXPRESSION OF THE EOTAXINS IL-6 AND CXCL8 IN HUMAN EPITHELIAL CELLS FROM VARIOUS LEVELS OF THE RESPIRATORY TRACT

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Abstract: Airway epithelium acts as multifunctional site of response in the respiratory tract. Epithelial activity plays an important part in the pathophysiology of obstructive lung disease. In this study, we compare normal human epithelial cells from various levels of the respiratory tract in terms of their reactivity to pro-allergic and pro-inflammatory stimulation. Normal human nasal, bronchial and small airway epithelial cells were stimulated with IL-4 and IL-13. The expressions of the eotaxins IL-6 and CXCL8 were evaluated at the mRNA and protein levels. The effects of pre-treatment with IFN-γ on the cell reactivity were measured, and the responses to TNF-α, LPS and IFN-γ were evaluated. All of the studied primary cells expressed CCL26, IL-6 and IL- 8 after IL-4 or IL-13 stimulation. IFN-γ pre-treatment resulted in decreased CCL26 and increased IL-6 expression in the nasal and small airway cells, but this effect was not observed in the bronchial cells. IL-6 and CXCL8 were produced in varying degrees by all of the epithelial primary cells in cultures stimulated with TNF-α, LPS or IFN-γ. We showed that epithelial cells from the various levels of the respiratory tract act in a united way, responding in a similar manner to stimulation with IL-4 and IL-13, showing similar reactivity to TNF-α and LPS, and giving an almost unified response to IFN-γ pre-stimulation.

Key words: CXCL8, Eotaxin-3, Inflammation, Interferon gamma, Interleukin 6, Respiratory epithelium, United airways

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Abbreviations used: ATCC - American Type Culture Collection; BEGM – bronchial epithelial cell growth medium; COPD – chronic obstructive pulmonary disease; IFN-γ – interferon γ; LPS – lipopolysaccharide; TGF-β – transforming growth factor beta; TNF-α – tumor necrosis factor alpha

INTRODUCTION

The epithelium of the respiratory tract is more than a mechanical barrier between the outside environment and the internal parenchyma. It responds to inhaled microbes and noxious stimuli that overcome the mucociliary barrier, and is therefore vital for host defense [1]. The airway epithelium is involved in the immunological response to infections and participates in pathological processes in non-infectious lung diseases [2]. It is a source of numerous pro-inflammatory mediators, which cause a flow of inflammatory cells into the airway lumen [3-5]. The local influx of eosinophils, basophils, Th2 lymphocytes and macrophages to the bronchoalveolar space during respiratory diseases, such as asthma, pulmonary infections, sarcoidosis and COPD, is a secondary source of diverse cytokines, cytotoxic proteins and other mediators of inflammation [6-9].

The respiratory epithelium plays a complex and pivotal role in obstructive respiratory diseases. The epithelium is a major producer of eotaxins (eotaxin-1/CCL11, eotaxin-2/CCL24, eotaxin-3/CCL26), which attract eosinophils, the important cytological markers of asthma. Eosinophil inflammation in the sputum is present in 50% of cases of asthma [10]. IL-4 and IL-13 are Th2 cytokines important in allergies and asthma. They stimulate eotaxin release from the respiratory epithelium [11]. Although these cytokines share the same receptor, which is expressed on airway epithelial cells, among others, their effector profiles are not identical [12]. IL-6 and CXCL8 are important cytokines isolated from the airways during obstructive respiratory diseases. IL-6, known as a classic marker of systemic inflammation, is produced by airway epithelial cells. The respiratory epithelium constitutively expresses the IL-6 receptor [13]. IL-6 production is increased after mast cell interaction and stimulation with IL-1β, TGF-β, LPS or TNF-α [5, 14-16]. IL-6 is also known as an immunomodulator that can change the properties of other cells [17]. CXCL8 is a potent neutrophil chemotactic factor. It is a crucial mediator in neutrophil-dependent inflammation, especially in infections and COPD. CXCL8 expression in respiratory epithelial cells is increased by pro-inflammatory cytokines (TNF-α, IL-1β) and bacterial infections (LPS) [18-20].

The "united airway disease" concept implies that upper and lower airway diseases are different manifestations of the same inflammatory process [21]. Thus, asthma and allergic rhinitis would be different symptoms of the same allergic airway inflammation. According to some authors, the combined results of numerous studies suggest that allergic rhinitis, asthma and chronic rhinosinusitis are all connected by united allergic airways, a concept that implies similarities in the pathophysiology, epidemiology and treatment [22, 23].

Although human nasal and bronchial airway epithelial cells are similar in size, shape and growth characteristics, their biochemical features are not identical [24]. Only a few studies have compared the pro-inflammatory abilities of the nasal and bronchial epithelial cells. Experiments with cultures of paired undifferentiated nasal and bronchial epithelial cells obtained from patients

suffering from various respiratory disorders showed differences in the production of IL-6, CXCL8 and numerous mediators of inflammation in response to TNF-α and IL-1β [25]. Comer *et al.* showed that nasal epithelial cells obtained from COPD subjects could not be used as a substitute for bronchial epithelial cells for *in vitro* studies due to the substantially different IL-6 release [26]. Also, nasal and bronchial epithelial cells from children with or without asthma behaved differently under basal conditions and after IL-13 stimulation [27]. It seems that the question of the reactivity of respiratory epithelial cells in the context of the united airways concept is not unequivocally resolved.

We focused on how normal human airway epithelial cells from various levels of the respiratory tract react to selected pro-allergic or pro-inflammatory stimulation. The aim of the study was to compare some fundamental activities of nasal, bronchial and small airway epithelial primary cells, in particular:

• The expression of the eotaxins IL-6 and CXCL8 after stimulation with the allergic reaction mediators IL-4 or IL-13, and the effect of pre-stimulation with IFN-γ on this activity

• The expression of IL-6 and CXCL8 upon stimulation with selected mediators of inflammation: TNF-α, LPS or IFN-γ.

MATERIALS AND METHODS

Cell cultures

All the epithelial cell cultures used were primary cells obtained from healthy donors. Human nasal epithelial cells (PromoCell; C-12620; donor: Caucasian woman, aged 30) were cultured in Airway Epithelial Cell Basal Medium (PromoCell; C-21260) supplemented with Airway Epithelial Cell Growth Medium Kit (PromoCell; C-21160). Human small airway epithelial cells (PromoCell; C12642; donor: Caucasian woman, aged 81) were cultured in Small Airway Epithelial Cell Basal Medium (PromoCell; C-21270) supplemented with Small Airway Epithelial Cell Growth Medium Kit (PromoCell; C-21170). Human primary bronchial/tracheal epithelial cells (ATCC; ATCC-PCS-300-010; donor: Caucasian man, aged 37) were cultured in Airway Epithelial Cell Basal Medium (ATCC cat. No. PCS-300-030) with Bronchial Epithelial Cell Growth Kit (ATCC cat. No. PCS-300-040). All of the primary cells were cultured on plastic dishes (Nunc) with 100 U/ml penicillin and 100 μg/ml streptomycin (PAA) at 37ºC in 5% CO_2. The nasal and small airway epithelial cells were in their 3rd passage and the bronchial epithelial cells in their 4th passage.

Cell stimulation

When the cells reached 80% confluence, they were stimulated with IL-4, IL-13 (30 ng/ml), TNF-α (30 ng/ml), IFN-γ (50 ng/ml; R&D Systems) or LPS (25 μg/ml; Sigma-Aldrich) for 24 h, or pre-incubated with IFN-γ for 1 h and stimulated with IL-4 or IL-13 for 24 h. The cytokines were dissolved in water and a minimal amount of bovine serum albumin. LPS was dissolved in epithelial

basal medium. Four experiments were performed, each in triplicate. The experiments were performed in the basal medium without a supplement.

Protein measurement

Culture supernatants were used to detect CCL24, CCL26, IL-6 and CXCL8 using ELISA kits (R&D Systems) according to the manufacturer's instructions. The optical density was read at 450 nm with correction at 570 nm.

RNA isolation and determination of eotaxin mRNA levels

Total RNA was isolated from cells using Trizol (Invitrogen). The purity and concentration of the isolated RNA was measured in a DU650 spectrophotometer (Beckman) using the 260/280 nm absorbance ratio. One microgram of RNA was added for reverse transcription using a RevertAid Premium First Strand cDNA Synthesis Kit (Fermentas, Thermo Fisher Scientific). Quantitative real-time PCR evaluation was performed with an ABI-Prism 7500 Sequence Detector System (Applied Biosystems). Table 1 shows the sequence of primers used in PCR. For real-time PCR, 1 µl of cDNA was amplified in a 20-µl PCR volume containing Power SYBR Green PCR master mix (Applied Biosystems) with 150 nmoles of specific primers. Each sample was measured in duplicate. The PCR conditions were: 40 cycles of 15 s at 95ºC and 1 min at 60ºC. 18S rRNA was used to normalize the expression levels of the examined amplicon in all epithelial cells. The Endogenous Control was chosen based on results from the TaqMan Array Human Endogenous Controls (Applied Biosystems, Life Technology).

Table 1. Sequence of primers used in real-time PCR.

	Forward primer	Reverse primer	Product length
CCL11	CTCGCTGGGCCAGCTTCTGTC	GGCTTTGGAGTTGGAGATTTTTGG	227 bp
CCL24	CACATCATCCCTACGGGCTCT	GGTTGCCAGGATATCTCTGGACAGGG	288 bp
CCL26	GGAACTGCCACACGTGGGAGTGAC	CTCTGGGAGGAAACACCCTCTCC	354 bp
IL-6	CCGGGAACGAAAGAGAAGCT	GCGCTTGTGGAGAAGGAGTT	68 bp
CXCL8	GAGCACTCCATAAGGCACAAACT	ATCAGGAAGGCTGCCAAGAG	150 bp
18s rRNA	GGATGAGGTGGAACGTGTGAT	AGGTCTTCACGGAGCTTGTTG	150 bp

The relative abundance values were calculated using the $2^{-\Delta\Delta CT}$ method [28]. The cycle thresholds (CT) were determined for the target amplicon and the endogenous control (18s rRNA) for each sample. Differences were calculated between these two CTs (ΔCT) to account for the differences in the amount of total nucleic acid taken for each reaction. The value of ΔCT for unstimulated cells (calibrator) was subtracted from the ΔCT of each experimental sample from stimulated cells to give the value termed ΔΔCT. The value of the target normalized to the endogenous control for the experimental samples relative to that in the calibrator was then calculated using the formula $2^{-\Delta\Delta CT}$. The results are expressed as relative quantification units (fold change).

Statistical analysis

Differences between cell types were evaluated with the nonparametric Wilcoxon rank-sum test. P values < 0.05 were considered statistically significant. All numerical results are presented as mean values and SEM.

RESULTS

The expression of eotaxins in airway epithelial cells after IL-4 or IL-13 stimulation

A comparison of eotaxin mRNA and protein expression in nasal, bronchial and small airway epithelial cell cultures upon IL-4 or IL-13 simulation is shown in Figs 1 and. 2 and Suppl. Tables 1 and 2 in supplementary material at http://dx.doi.org/10.2478/s11658-013-0107-y. According to the PCR results, CCL26 was the highest-expressed eotaxin in all of the epithelial cells 24 h after IL-4 or IL-13 stimulation. Small airway epithelial cells expressed significantly higher levels of CCL26 mRNA after IL-4 or IL-13 stimulation compared to the two other epithelial cell types. Protein measurements were performed for CCL26 only. Unstimulated cells did not produce measurable amounts of CCL26.

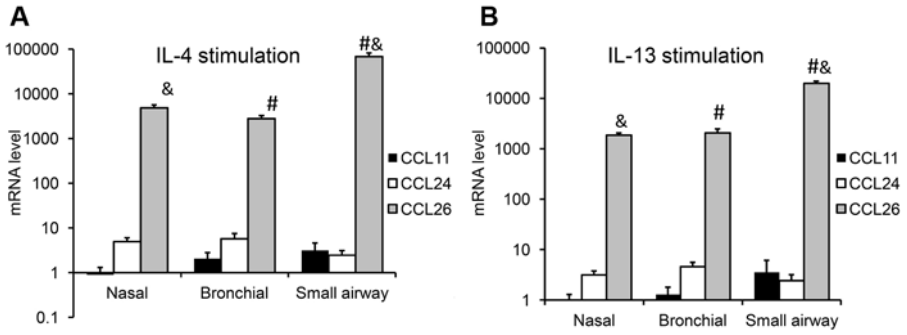

Fig. 1. CCL11, CCL24 and CCL26 mRNA expression in nasal, bronchial and small airway epithelial cells after IL-4 or IL-13 stimulation. Cells were stimulated with 30 ng/ml IL-4 (A) or IL-13 (B) for 24 h. The results are shown as the fold change in the relative mRNA level compared to unstimulated cells. The expression was normalized vs. 18s rRNA ($2^{-\Delta\Delta CT}$ method). The mean values and SEM are shown. $^{\#}P < 0.05$ bronchial vs. small airway and $^{\&}P < 0.05$ small airway vs. nasal epithelial cells.

In contrast to the PCR results, ELISA data showed that the bronchial epithelial cells produced the largest amount of CCL26. This was observed in cultures stimulated with IL-4 or IL-13. The mean and SEM values of CCL26 concentrations after IL-4 stimulation were: 78.29 ± 18.30, 773.42 ± 208.60 and 52.5 ± 12.06 pg/ml in bronchial, nasal and small airways epithelial cell cultures, respectively. After IL-13 stimulation the corresponding values were 21.94 ± 5.75, 522.05 ± 133.75 and 32.58 ± 5.98 pg/ml (Fig. 2, Suppl. Table 2).

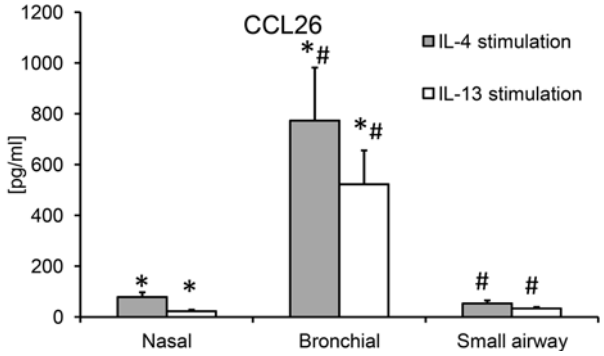

Fig. 2. CCL26 protein production by nasal, bronchial and small airway epithelial cells after IL-4 or IL-13 stimulation. Cells were stimulated with 30 ng/ml IL-4 or IL-13 for 24 h. Mean values and SEM are shown. *P < 0.05 nasal vs. bronchial, #P < 0.05 bronchial vs. small airway epithelial cells.

The expression of IL-6 and CXCL8 in airway epithelial cells after IL-4 or IL-13 stimulation

A comparison of IL-6 and CXCL8 mRNA and protein expression in nasal, bronchial and small airway epithelial cell cultures upon IL-4 or IL-13 simulation is shown in Figs 3 through 5 and Suppl. Tables 3 through 7. Unstimulated cells produced different levels of IL-6 and CXCL8 proteins. The highest concentrations of these cytokines were noted in the small airway epithelial cell culture while the bronchial cells produced the lowest amounts of IL-6. The absolute values of IL-6 protein were significantly different in all of the evaluated primary cells. Small airway epithelial cells produced significantly higher amounts of CXCL8 protein than nasal and bronchial epithelial cells (Fig. 3, Suppl. Table 3).

Since the qRT-PCR results are shown as the fold change compared to unstimulated cells ($2^{-\Delta\Delta CT}$ method), we decided to present the ELISA results as the percentage of the control values for unstimulated cells as well.

The expression of both IL-6 and CXCL8 mRNA was relatively low in the stimulated epithelial cells. The bronchial cells were the most reactive in terms of IL-6 mRNA expression after IL-4 or IL-13 stimulation (4.5 ± 2.28 fold change and 12.52 ± 8.32 fold change, respectively; Fig. 3). These cells were also the most potent IL-6 protein producer (449.75 ± 181.14% of the control values after IL-4 stimulation, and 469.39 ± 121.47% of the control values after IL-13 stimulation; Fig. 4).

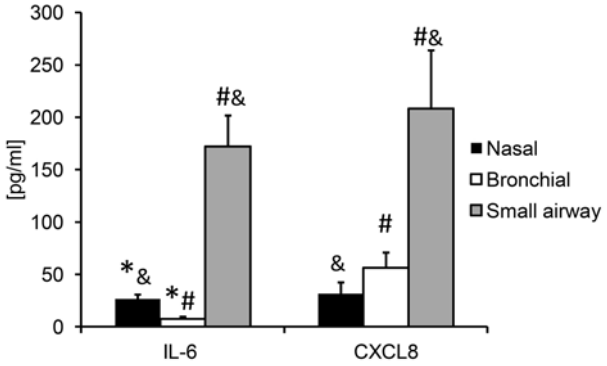

Fig. 3. IL-6 and CXCL8 protein production by unstimulated nasal, bronchial and small airway epithelial cells. Mean values and SEM are shown. *P < 0.05 nasal vs. bronchial, #P < 0.05 bronchial vs. small airway and &P < 0.05 small airway vs. nasal epithelial cells.

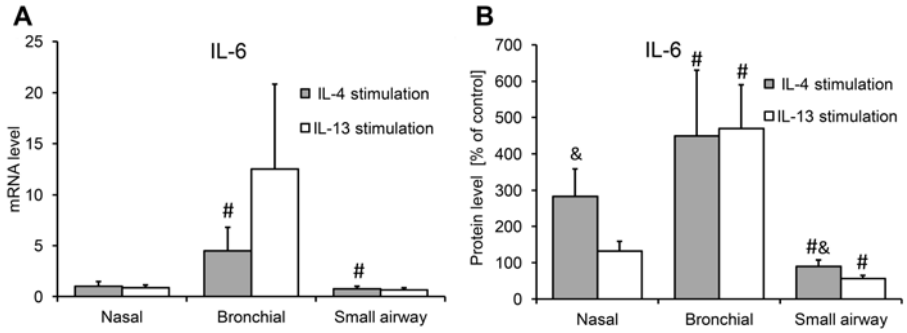

Fig. 4. IL-6 mRNA (A) and protein (B) expression by nasal, bronchial and small airway epithelial cells after IL-4 or IL-13 stimulation. Cells were stimulated with 30 ng/ml IL-4 or IL-13 for 24 h. PCR results are shown as the fold change of the relative mRNA level compared to unstimulated cells. The mRNA expression was normalized vs. 18s rRNA ($2^{-\Delta\Delta CT}$ method). The protein concentration in the cell supernatants was measured in pg/ml and is presented as a percentage of the control values. The mean values and SEM are shown. #P < 0.05 bronchial vs. small airway, &P < 0.05 small airway vs. nasal epithelial cells.

The mRNA and protein results are compatible in this section. We observed a more significant difference between the nasal, bronchial and small airway cells at the protein level. As with the PCR results, the highest amounts of IL-6 protein were produced by the bronchial cells. The lowest IL-6 protein production was observed in the culture of small airway cells independently of stimulation. The concentration of IL-6 in these cells was significantly lower (89.5 ± 17.95% of the control value) than in the nasal (283.81 ± 75.08% of the control value) or bronchial (449.75 ± 181.14% of the control value) cells upon stimulation with IL-4, and significantly lower than in bronchial cells in cultures stimulated with IL-13: 56.17 ± 8.85% of the control value vs. 469.39 ± 121.47% of the control value (Fig. 4, Suppl. Table 4). We did not notice any significant differences in

CXCL8 expression at the mRNA and protein level between the nasal, bronchial and small airway epithelial cells after IL-4 or IL-13 stimulation (Fig. 5, Suppl. Table 5).

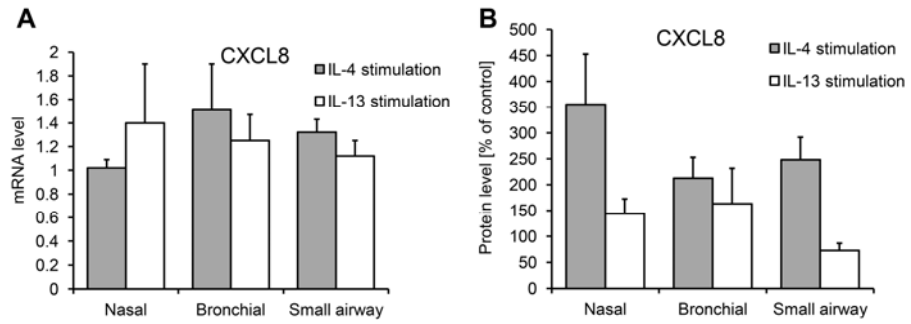

Fig. 5. CXCL8 mRNA (A) and protein (B) expression by nasal, bronchial and small airway epithelial cells after IL-4 or IL-13 stimulation. Cells were stimulated with 30 ng/ml IL-4 or IL-13 for 24 h. PCR results are shown as the fold change of the relative mRNA level compared to unstimulated cells. The mRNA expression was normalized vs. 18s rRNA ($2^{-\Delta\Delta CT}$ method). The protein concentration in the cell supernatants was measured in pg/ml and is presented as a percentage of the control values. Mean values and SEM are shown.

The effect of IFN-γ pre-stimulation on CCL26, IL-6 and CXCL8 expression in airway epithelial cells stimulated with IL-4 or IL-13

The effects of IFN-γ pre-stimulation on CCL26 mRNA and protein expression in nasal, bronchial and small airway cell cultures stimulated with IL-4 or IL-13 are summarized in Fig. 6 and Suppl. Table 8, and those on IL-6 and CXCL8 mRNA and protein expression in Figs 7 and 8 and Suppl. Tables 9 and 10. The results are presented relative to the mRNA expression or protein concentration after IL-4 or IL-13 stimulation alone.

Pre-incubation with INF-γ changed the effects of IL-4 or IL-13 stimulation on CCL26, IL-6 and CXCL8 expression in almost the same manner in the nasal, bronchial and small airway cells. It inhibited CCL26 expression, and stimulated IL-6 and CXCL8 expression (except for CXCL8 expression in the bronchial cells). The mRNA and protein results are consistent with the slight differences between the PCR and ELISA results. We observed significant differences between the nasal, bronchial and small airway epithelial cells in terms of CCL26 mRNA expression after INF-γ pre-incubation, but these differences were not confirmed statistically at the protein level (Fig. 6, Suppl. Table 8).

The most pronounced stimulatory effect of IFN-γ was observed for IL-6 expression in the nasal cell culture independently of the stimulator used. The increase in IL-6 mRNA and protein levels after IFN-γ pre-stimulation was the highest in nasal cells (significantly higher compared to small airway cells after IL-4 stimulation and compared to bronchial cells after IL-13 stimulation; significantly higher compared to both other cell types at the protein level). Pre-stimulation with IFN-γ increased IL-6 protein production under all the

experimental combinations, but the magnitude of this stimulation was significant between nasal, bronchial and small airway epithelial cells (Fig. 7, Suppl. Table 9). The effects of IFN-γ pre-treatment on IL-4- or IL-13-stimulated CXCL8 expression were highly cell type specific. In bronchial cells, IFN-γ decreased CXCL8 expression following stimulation with IL-4 or IL-13. At the mRNA level, the decrease was 101.41 ± 34.08% after IL-4 stimulation and 64.54 ± 11.38% after IL-13 stimulation, and at the protein level, 58.86 ± 16.36% after IL-4 stimulation and 118.45 ± 59.91% after IL-13 stimulation. By contrast, in the other two epithelial types, IFN-γ pre-treatment enhanced the CXCL8 expression, but this effect was less pronounced than that on IL-6 expression. The strongest effect of IFN-γ on CXCL8 protein production was found in nasal cultures stimulated with IL-13. The differences between the effects of IFN-γ pre-treatment on CXCL8 production in bronchial cells and the nasal and small airway cells were statistically significant at the protein level. However, at the mRNA level, the effects were much less pronounced between the three types of primary cell studied. The only statistically significant differences were between nasal and bronchial cells and between bronchial and small airway cells stimulated with IL-13.

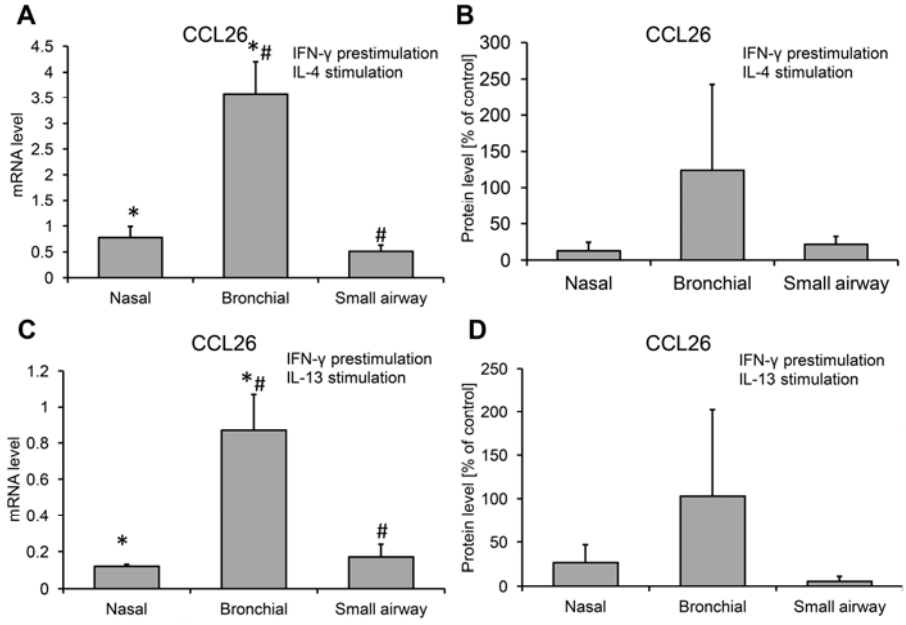

Fig. 6. The effect of IFN-γ pre-treatment on CCL26 expression after IL-4 (A, B) or IL-13 (C, D) stimulation in nasal, bronchial and small airway epithelial cells. Cells were pre-incubated with or without 50 ng/ml IFN-γ and then stimulated with 30 ng/ml IL-4 or IL-13 for 24 h. The results are shown as percentages of the values for CCL26 expression after IL-4 or IL-13 stimulation alone. mRNA expression was measured via PCR using the $2^{-\Delta\Delta CT}$ method and protein levels were measured using ELISA. The mean values and SEM are shown. *$P < 0.05$ nasal vs. bronchial, $^{\#}P < 0.05$ bronchial vs. small airway epithelial cells.

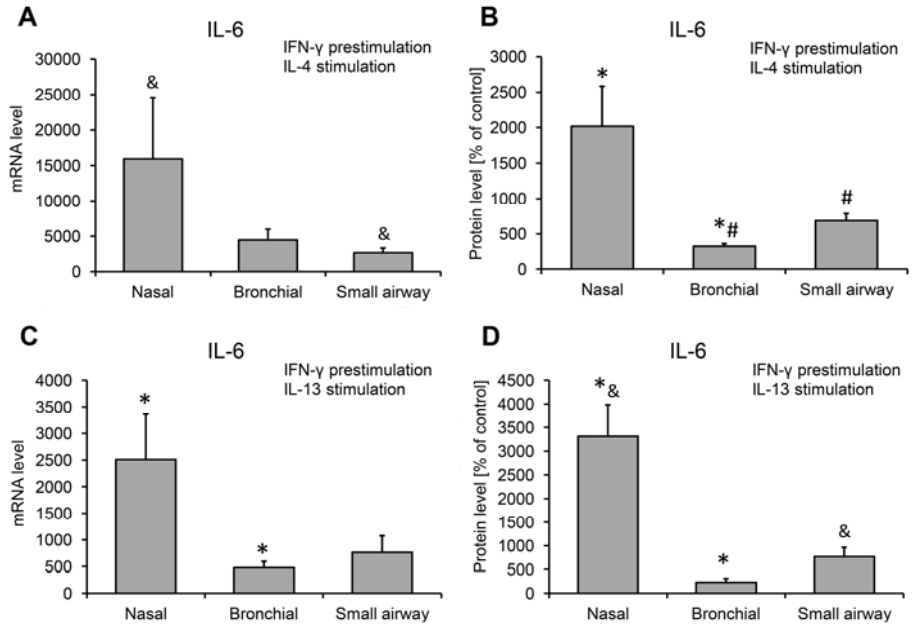

Fig. 7. The effect of IFN-γ pre-treatment on IL-6 expression after IL-4 (A, B) or IL-13 (C, D) stimulation in nasal, bronchial and small airway epithelial cells. Cells were pre-incubated with or without 50 ng/ml IFN-γ and then stimulated with 30 ng/ml IL-4 or IL-13 for 24 h. Results are shown as percentages of the IL-6 expression values after IL-4 or IL-13 stimulation alone. mRNA expression was measured via PCR using the $2^{-\Delta\Delta CT}$ method and protein levels were measured using ELISA. Mean values and SEM are shown. *P < 0.05 nasal vs. bronchial, $^{\#}$P < 0.05 bronchial vs. small airway, $^{\&}$P < 0.05 small airway vs. nasal epithelial cells.

The expression of IL-6 and CXCL8 in airway epithelial cells after stimulation with TNF-α, LPS or IFN-γ

The IL-6 and CXCL8 mRNA and protein expression in nasal, bronchial and small airway epithelial cell cultures after TNF-α, LPS or IFN-γ stimulation is depicted in Figs 9 and 10 and Suppl. Tables 11 and 12.

The reactivity to TNF-α, LPS and IFN-γ stimulation depends on the type of primary cell and the stimulator used. The highest IL-6 mRNA expression was noted in bronchial cells. It was significantly lower in the nasal and small airway cells. TNF-α was the most effective stimulator. The observed differences reached statistical significance for IL-6 mRNA expression in TNF-α- or LPS-stimulated bronchial cells vs. nasal and small airway cells. Similar results were obtained for the IL-6 protein level although the only statistically significant difference was between bronchial and small airway epithelial cells after TNF-α stimulation: 24545.54 ± 4211.97 vs. 849.58 ± 151.02 pg/ml, respectively. A statistically significant difference was found between the IL-6 protein level in nasal and small airway epithelial cells after IFN-γ stimulation (658.08 ± 214.74 vs. 198.49 ± 49.94 pg/ml, respectively; Fig. 9). The corresponding mRNA levels were not significantly different.

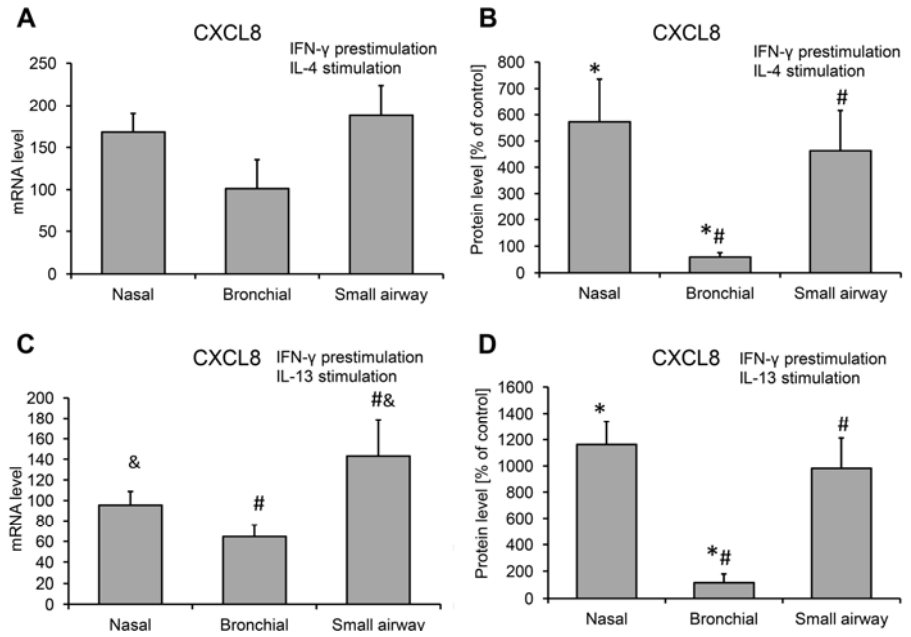

Fig. 8. The effect of IFN-γ pre-treatment on CXCL8 expression after IL-4 (A, B) or IL-13 (C, D) stimulation in nasal, bronchial and small airway epithelial cells. Cells were pre-incubated with or without 50 ng/ml IFN-γ and then stimulated with 30 ng/ml IL-4 or IL-13 for 24 h. Results are shown as percentages of CXCL8 expression after IL-4 or IL-13 stimulation alone. mRNA expression was measured via PCR using the $2^{-\Delta\Delta CT}$ method and protein levels were measured using ELISA. Mean values and SEM are shown. *P < 0.05 nasal vs. bronchial, $^{\#}$P < 0.05 bronchial vs. small airway, $^{\&}$P < 0.05 small airway vs. nasal epithelial cells.

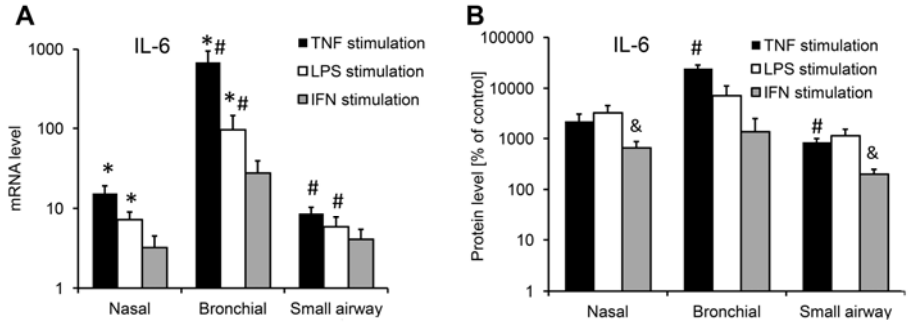

Fig. 9. IL-6 mRNA (A) and protein (B) expression by nasal, bronchial and small airway epithelial cells after TNF-α, LPS or IFN-γ stimulation. Cells were stimulated with 30 ng/ml TNF-α, 25 μg/ml LPS or 50 ng/ml IFN-γ for 24 h. PCR results are shown as the fold change in the relative mRNA level compared to the values for unstimulated cells. The mRNA expression was normalized vs. 18s rRNA ($2^{-\Delta\Delta CT}$ method). The protein concentration in the cell supernatants was measured in pg/ml and is presented as a percentage of the values for the control. Mean values and SEM are shown. $^{\#}$P < 0.05 bronchial vs. small airway, $^{\&}$P < 0.05 small airway vs. nasal epithelial cells.

CXCL8 mRNA expression remained practically unchanged regardless of the stimulation used. The ELISA results were not exactly the same. We observed one significant difference: bronchial epithelial cells produced significantly more CXCL8 after TNF-α stimulation than the small airway epithelial cells did: respectively 18369.76 ± 4562.64% vs. 1125.94 ± 276.68% of the control value (Fig. 10).

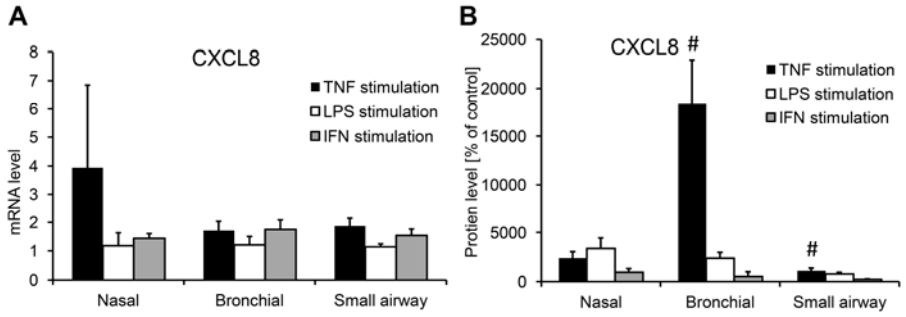

Fig. 10. CXCL8 mRNA (A) and protein (B) expression by nasal, bronchial and small airway epithelial cells after TNF-α, LPS or IFN-γ stimulation. Cells were stimulated with 30 ng/ml TNF-α, 25 μg/ml LPS or 50 ng/ml IFN-γ for 24 h. PCR results are shown as the fold change in the relative mRNA level compared to the values for unstimulated cells. The mRNA expression was normalized vs. 18s rRNA ($2^{-\Delta\Delta CT}$ method). Protein concentration in cell supernatants was measured in pg/ml and is presented as a percentage of the values for the control. Mean values and SEM are shown. $^{\#}P < 0.05$ bronchial vs. small airway, $^{\&}P < 0.05$ small airway vs. nasal epithelial cells.

DISCUSSION

This study was designed to compare normal human epithelial cells from various levels of the respiratory tract in terms of their reactivity to diverse immune stimuli. We assessed the production of mediators of immunological and inflammatory reactions resulting from these stimuli. In general, the reactivity of the nasal, bronchial and small airway epithelial cells was qualitatively similar, although in some cases substantial differences were observed. Similar differences in the reactivity of paired cultures of human nasal and bronchial epithelial cells were also observed by other authors [26, 29].

We confirmed the earlier observations of many authors that CCL26 was the main eotaxin detected in cultures of human bronchial epithelial cells stimulated with IL-4 or IL-13 [30, 31]. Some of the PCR and ELISA results for CCL26 expression are inconsistent. The expression of CCL26 mRNA was the highest in small airway epithelial cells, while bronchial epithelial cells produced the greatest amounts of CCL26 protein. We presume that the nasal, bronchial and small airway epithelial cells may differ in terms of CCL26 metabolism.

There are two possible direct reasons for the discrepancy between the PCR and ELISA results. First, the kinetics of CCL26 expression after IL-4 or IL-13

stimulation may be different in the cells studied: the bronchial epithelial cells reach the CCL26 mRNA maximum earlier and produce the CCL26 protein most efficiently 24 h after IL-4 or IL-13 stimulation, while in small airway cells, the accumulation of CCL26 mRNA increased steadily at least until 24 h post-stimulation, with CCL26 protein production lagging behind. Second, it cannot be excluded that CCL26 protein may be directed by some post-transcriptional mechanism in small airway epithelial cells. These results may indicate that the quickest, the most effective response to the Th2 cytokines takes place at the bronchial level of the respiratory tract. In consequence, we can imagine that the biggest influx of eosinophils after the allergic response should be placed at the bronchial level. Some studies suggest that infiltration of the bronchial epithelium by eosinophil leukocytes is a feature of both large and small airways, with a greater intensity in the proximal airways [32, 33].

There is little experimental data regarding IL-6 and CXCL8 release in airway epithelial cells upon IL-4 or IL-13 stimulation. We found that this stimulation caused a several-fold increase in the IL-6 and CXCL8 protein levels relative to unstimulated cells. This reaction could mean that normal respiratory epithelial cells have the potency to be active in the allergic cascade as a response to IL-4 and IL-13.

In addition to IL-4 and IL-13, we used TNF-α, LPS and IFN-γ as stimulators and observed significant differences in IL-6 production after LPS or TNF-α stimulation between the nasal, bronchial and small airway epithelial cells. The highest changes in IL-6 expression were noted in bronchial cells, but only after mediator stimulation. This is contrary to the results of Comer *et al.*, who showed a higher IL-6 protein production in a nasal epithelial cell culture and even a lack of IL-6 production by bronchial epithelial cells after LPS stimulation [26]. This apparent discrepancy between the studies could be due to the different models of data presentation. Comer *et al.* showed absolute values of the interleukin concentration while we presented the results as a percentage of the control values. Looking at the absolute values in the controls in our study, the bronchial epithelial cells produced the smallest amounts of IL-6 protein. This is consistent with previously published results [26, 34].

Stimulation with TNF-α resulted in a greater increase in the IL-6 level in bronchial cells. Our results differ from those of other researchers [25]. McDougall *et al.* showed comparable levels of IL-6 in cultures of bronchial and nasal epithelial cells after TNF-α and IL-1β stimulation (median 163 vs. 179 pg/ml, respectively). Pringle *et al.* showed significantly higher IL-6 expression in a culture of nasal cells compared to bronchial epithelial cells (42.2 vs. 7.4 pg/ml, respectively). It is difficult to unequivocally assess the reasons for these discrepancies. The most important were probably differences in the origins of the primary cells. In both cited studies, the epithelial cells were from patients with COPD, cancer or other illnesses. Differences in methodological conditions could also affect the results. For example, in the studies of McDougall *et al.* and Comer *et al.*, epithelial cells were cultured on collagen-coated plastic plates,

while we cultured cells on uncoated dishes. In the studies of McDougall *et al.*, Comer *et al.* and Pringle *et al.*, cells were growth in BEGM, which is similar to the medium used here for nasal cells. The other media differ in composition, especially that for bronchial cells (ATCC). For example, it contains human serum albumin, L-glutamine, linoleic acid and extract P, which are not present in BEGM. The most important methodological difference between our study and the above-mentioned studies is that we performed the experiments using the basal medium, while the cited authors used a medium with supplements which include, e.g. hydrocortisone or retinoic acid. Finally, differences related to the age of the donor cannot be excluded. We presented the results calculated as percentages of the values for the control, to minimize age-dependent differences in the constitutive expression of some mediators.

Our study revealed some interesting data about CXCL8 expression in the airway epithelium. It was relatively high in all three cell types. Moreover, stimulation with Th2 cytokines or typical inflammatory mediators did not change the mRNA expression compared to unstimulated cells. Also, earlier studies showed constitutive CXCL8 expression in primary nasal and bronchial epithelial cells [35, 36]. We showed only one significant difference in protein production after TNF-α stimulation between the bronchial and small airway epithelial cells. This contrast between the unchanged CXCL8 mRNA level and the changing CXCL8 protein amount indicates an engagement of a post-transcriptional or post-translational regulation of CXCL8 production, at least following TNF-α action. According to Villarete and Remick, CXCL8 mRNA abundance is regulated at both the transcriptional and post-transcriptional levels [37]. It is known that nitric oxide, prostaglandin E2 and Toll-like receptor 5 regulate CXCL8 expression post-transcriptionally [38-40]. It is possible that these mechanisms are ERK1/2- and p38 MAPK-dependent [41].

Pre-stimulation with IFN-γ significantly decreased CCL26 expression and generally increased IL-6 and CXCL8 expression (except for CXCL8 in bronchial cells) after IL-4 or IL-13 stimulation in the nasal, bronchial and small airway epithelial cells. Similar effects have been shown before [42, 43], but to the best of our knowledge, no one had previously studied the effect of IFN-γ on CCL26, IL-6 and CXCL8 production in the airway epithelium. The differences in CXCL8 expression after IFN-γ pre-stimulation found here between the bronchial and the other primary cells could be an argument against the united airway concept. However, it is possible that this difference was due to the particular features of the bronchial cell donor. This issue requires further investigation using more than a single line of each type of cells, and preferably matched sets from several donors. That being said, the overall similarity of the response of the various types of airway epithelial cell studied here to IFN-γ pre-incubation speaks in favor of a functional integration of these cells.

The concept of “united airway disease” is often used to describe pathological developments in asthma and allergic rhinitis. It is a clinical expression and clearly does not relate to any specific structure or cells of the respiratory system.

In this manuscript, we approach the concept of the "united airways" using one of the structural components of the respiratory tract: the airway epithelium. Especially at the submucosal level, the epithelium shows some morphological differences at various levels of the respiratory tract. Those include: pseudofiltrated columnar cells in the nasal region; ciliated, undifferentiated columnar, secretory and basal cells in the large airways of the lower respiratory tract, and no cilia and the advantage of the Clara cells in the small airways. Our results indicate that the immunological response of the airway epithelium is similar at various levels of the respiratory tract, but that these reactions differ in their severity. Because the bronchial epithelium showed some disproportions in its response to the studied mediators when the nasal and small airways were compared, we assume that the bronchial level is an important place where the inflammatory process is influenced during airway disease. This conclusion is consistent with the observation that the inflammation during asthma is largely restricted to the conducting airways, but as the disease becomes more chronic, the inflammatory infiltrates spread both proximally to involve the trachea and larynx and distally to the small airways and occasionally adjacent alveoli [44].

CONCLUSION

The normal human nasal, bronchial and small airway epithelial cells evaluated here are similar, but not identical in their immunological reactivity. Although the absolute amounts of the produced cytokines differ between the epithelial cells from various levels of the respiratory tract, we can conclude that nasal, bronchial and small airway epithelial cells act in similar scheme. Despite these conclusions, it is noteworthy that the bronchial epithelium is distinct from other epithelium cell types in its CCL26 protein production and changes in CXCL8 expression after IFN-γ incubation.

Conflict of interest. The authors declare that there is no conflict of interest.

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