Effect of TH2 cytokines and interferon gamma on beat frequency of human respiratory cilia

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BACKGROUND: In asthmatic airways secondary ciliary dyskinesia contributes to impaired mucociliary clearance. To investigate underlying mechanisms, we studied the effects of cytokines associated with asthma phenotype on the ciliary beat frequency (CBF) in a cell culture model of ciliated human respiratory epithelial cells.

METHODS: Nasal respiratory epithelial cells of 21 patients were used to prepare multicellular cells (spheroids) in the presence of the T helper (TH) 2 cytokines interleukin (IL)-4, IL-5, IL-9 and IL-13, and the TH1 cytokine interferon gamma (IFN-γ). CBF was determined by high-speed video microscopy.

RESULTS: Addition of IL-4 and IL-13 and IL-4 + IL-13 decreased the mean CBF by 17, 21, and 22%, respectively, compared with untreated controls. Addition of IL-5 and IL-9 lead to an increase in mean CBF (20 and 10%, respectively). Lower concentrations of IFN-γ (0.1 and 1 ng/ml) decreased mean CBF and higher concentrations (10 ng/ml) increased CBF by 6%. Addition of IFN-γ to IL-13 reversed the effect of IL-13 on the CBF of spheroids.

CONCLUSION: Cytokines directly influence the ciliary function of respiratory epithelium and contribute to the impaired mucociliary clearance in asthmatic disease. Our study encourages further research to investigate IFN-γ as a treatment option in diseases with impaired mucociliary clearance like asthma.

Asthma is a major cause of morbidity. It affects up to 300 million individuals worldwide with a global prevalence ranging from 1 to 16% (1,2). Clinical asthma manifestations include recurrent episodes of at least partially reversible wheezing, dyspnoea, cough, and increased airway mucus production. The asthmatic airway is characterized by epithelial damage, mucus hypersecretion, submucosal glandular hypertrophy, and goblet cell hyperplasia finally impairing mucociliary clearance (3–7). While efficient mucociliary clearance plays a fundamental role in the innate immune response system of the conducting airways, the impaired mucociliary clearance in asthma leads to prolonged exposure of inhaled pollutants and aeroallergens to the respiratory epithelium and an increased susceptibility to infections (4). This aggravates clinical symptoms and contributes to the disease burden of asthma. Ciliary function is an important factor for the mucociliary clearance (8,9). However, studies on ciliary function in asthma are scarce. Since the T helper (TH) 2 cytokines interleukin (IL)-4, IL-5, IL-9, and IL-13 are known to play a key role in the pathophysiology of TH2 high asthmatic airways (8,10), we evaluated whether they alter the ciliary beat frequency (CBF) of respiratory epithelium cell cultures. Additionally, we investigated the effect of the cytokine interferon gamma (IFN-γ). IFN-γ is fundamental for many aspects of the innate and adaptive immunity and it inhibits TH2-mediated response (11).

RESULTS

Addition of IL-4 (**Figure 1**) and IL-13 (**Figure 2**) to the spheroids resulted in CBF decline of 17% (IL-4) and 21% (IL-13), respectively (**Table 1**). In ciliary cultures containing both TH2 cytokines IL-4 and IL-13 (10 ng/ml each), we observed a CBF reduction of 22% (**Figure 3**; **Table 1**), hence no additive effect as compared to IL-4 alone. IL-4 was added in different concentrations (0.1, 1 and 10 ng/ml) to the spheroids and showed a dose-dependent effect on the CBF decrease (**Figures 1** and **3**).

IL-5 showed a dose-dependent increase in CBF with a maximum increase in CBF by 20% (**Figure 4**; **Table 1**) and IL-9 showed a statistically significant increase in CBF by 10% with a concentration of 0.1 ng/ml. There was a trend for increasing CBF with concentrations 1 and 10ng/ml, both did not reach statistically significance (**Figure 5**; **Table 1**).

We observed a variable effect of IFN-γ at different concentrations (0.1, 1, and 10 ng/ml) on the CBF. Whereas lower concentrations resulted in a CBF decrease (**Figure 6**; **Table 1**), a CBF increase of 6% was observed at the highest IFN-γ concentration (10 ng/ml). Addition of increasing concentrations of IFN-γ (0.1, 1, and 10 ng/ml) to the spheroid cultures containing IL-13 (10 ng/ml) resulted in an increase in CBF, which was statistically significant for 0.1 ng/ml and with a trend to a dose-dependent increase in CBF for 1 and 10 ng/ml (**Figure 7**; **Table 1**). Thus, IFN-γ reversed the negative effect of IL-13 on CBF.

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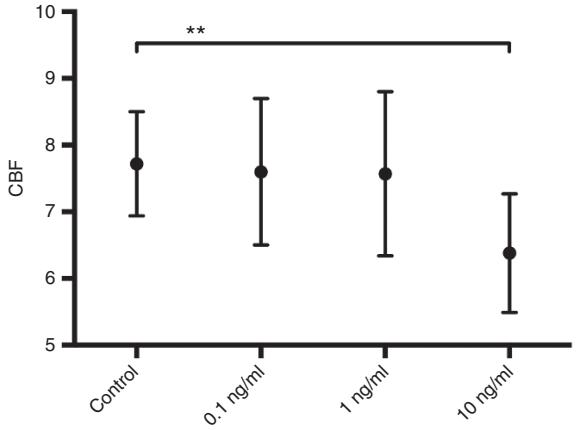

Figure 1. Influence of IL-4 on the ciliary beat frequency. Presentation of the mean ciliary beat frequency (CBF) in IL-4-treated cells. The CBF is significantly decreased after IL-4 treatment. The plots indicate mean values and SD. **Statistically significant with $P < 0.001$.

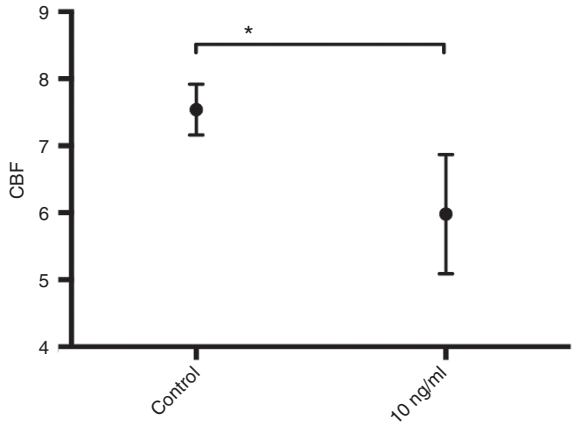

Figure 2. Influence of IL-13 on the ciliary beat frequency. Presentation of the mean ciliary beat frequency (CBF) in IL-13-treated cells. The CBF is significantly decreased after IL-13 treatment. The plots indicate mean values and SD. *Statistically significant with $P < 0.05$.

DISCUSSION

We show in a human respiratory cell culture model that the cytokines IL-5 and IL-9 increase the CBF in ciliated spheroid cultures, whereas the TH2 cytokines IL-4 and IL-13 impair the ciliary function. The TH1 cytokine IFN-γ improves the ciliary function and dominates the effect of IL-13 on the CBF.

IL-13 plays a key role in the pathophysiology of TH2 high asthma (12–14). IL-13 is produced by innate lymphoid cells and TH2 cells and induces several characteristic changes in the asthmatic airway epithelium resulting in subepithelial fibrosis, mucinous metaplasia, altered ciliated cell differentiation and function and an increase in goblet cells (15–17). Impairment of ciliary function has previously been shown for IL-13 in human cell culture models (15,18). Our data confirm these results.

IL-4 also impairs mucociliary clearance by inducing mucin hypersecretion (19,20). IL-4 stimulates TH2 cell development and suppresses TH1 cell development, and it induces immunoglobulin E switching in B cells and mediates tissue adhesion and inflammation in asthma (21–23). We here show that IL-4 directly impairs ciliary function. Incubation of spheroids with both IL-4 and IL-13 results in a CBF decrease, too, but we did not find an additive effect. A reason for that may be both cytokines competitively bind to the type II IL-4R receptor that was completely saturated at the concentrations used. Overall, IL-4 had a bigger effect on the CBF than IL-13, possibly because IL-4 additionally binds to the type I IL-4R receptor whereas IL-13 does not (20,24,25).

Table 1. Influence of cytokines on ciliary beat frequency

	CBF	ΔCBF	*P* value	*n*
IL-4				
Control	7.72 (0.78)			156
0.1 ng/ml	7.60 (1.11)	−0.12 (0.52)	0.8169	166
1 ng/ml	7.57 (1.23)	−0.15 (0.46)	0.7463	110
10 ng/ml	6.38 (0.89)	−1.34 (0.17)	<0.0001**	142
IL-5				
Control	7.28 (0.40)			116
0.1 ng/ml	8.20 (0.38)	0.92 (0.77)	0.2359	96
1 ng/ml	8.63 (0.02)	1.35 (0.38)	0.0004**	124
10 ng/ml	8.76 (0.11)	1.48 (0.29)	<0.0001**	128
IL-9				
Control	6.60 (0.06)			109
0.1 ng/ml	7.26 (0.35)	0.66 (0.29)	0.0235*	91
1 ng/ml	7.15 (0.48)	0.55 (0.42)	0.1970	99
10 ng/ml	7.31 (0.56)	0.71 (0.50)	0.1592	126
IL-13				
Control	7.54 (0.38)			70
10 ng/ml	5.98 (0.89)	−1.56 (0.51)	0.0028*	75
IL-4 (…ng/ml) + IL-13 (10 ng/ml)				
Control	8.69 (0.62)			139
0.1 ng/ml	8.51 (0.91)	−0.18 (0.34)	0.6232	111
1 ng/ml	7.87 (1.39)	−0.82 (0.79)	0.3077	138
10 ng/ml	6.79 (0.92)	−1.90 (0.32)	<0.0001**	119
IFN-γ				
Control	11.11 (1.38)			158
0.1 ng/ml	9.42 (1.36)	−1.69 (0.44)	0.0001**	120
1 ng/ml	10.33 (1.01)	−0.78 (0.52)	0.1362	131
10 ng/ml	11.80 (1.18)	0.69 (0.34)	0.0411*	139
IFN-γ (…ng/ml) + IL-13 (10 ng/ml)				
Control	7.54 (0.42)			178
0.1 ng/ml	7.85 (0.32)	0.31 (0.15)	0.0354*	155
1 ng/ml	7.97 (0.72)	0.43 (0.45)	0.3436	158
10 ng/ml	8.32 (0.65)	0.78 (0.46)	0.0892	195

The table shows the mean CBF with SD within the different experiments. Estimates for mean and SD are based on the underlying two-level random intercept mixed model. *P* values refer to associated pairwise comparisons with control.
CBF, ciliary beat frequency; ΔCBF, intervention minus control CBF; IFN-γ, interferon gamma; IL, interleukin; *n*, number of spheroids examined.
*Statistically significant with $P < 0.05$; **Statistically significant with $P < 0.001$.

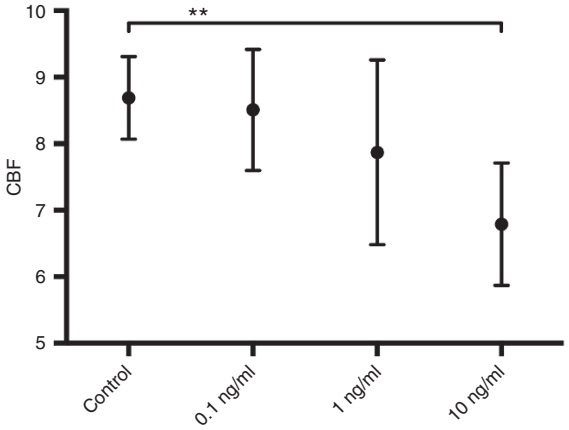

Figure 3. Influence of IL-4 plus IL-13 on the ciliary beat frequency. Presentation of the mean ciliary beat frequency (CBF) in IL-4 (different concentrations given on the *x*-axis) + IL-13 (10 ng/ml)-treated cells. The CBF is significantly decreased after IL-4 + IL13 treatment. The plots indicate mean values and SD. **Statistically significant with $P < 0.001$.

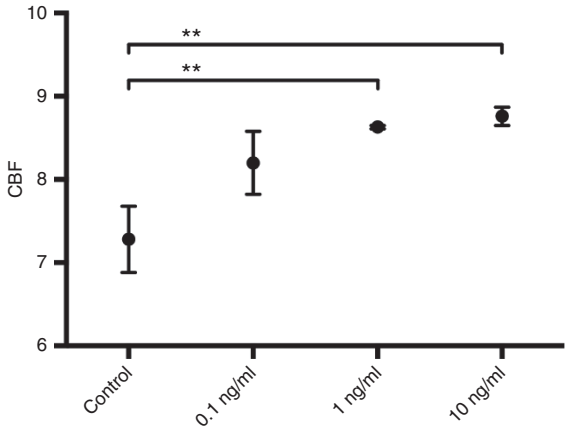

Figure 4. Influence of IL-5 on the ciliary beat frequency. Presentation of the mean ciliary beat frequency (CBF) in IL-5-treated cells. The CBF is significantly increased after IL-5 treatment. The plots indicate mean values and SD. **Statistically significant with $P < 0.001$.

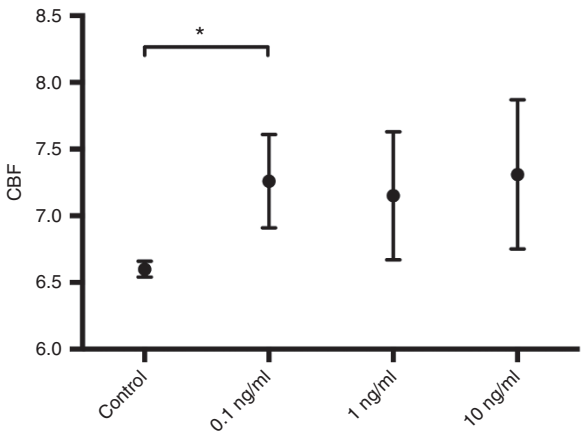

Figure 5. Influence of IL-9 on the ciliary beat frequency. Presentation of the mean ciliary beat frequency (CBF) in IL-9-treated cells. The CBF is significantly increased after IL-9 treatment. The plots indicate mean values and SD. *Statistically significant with $P < 0.05$.

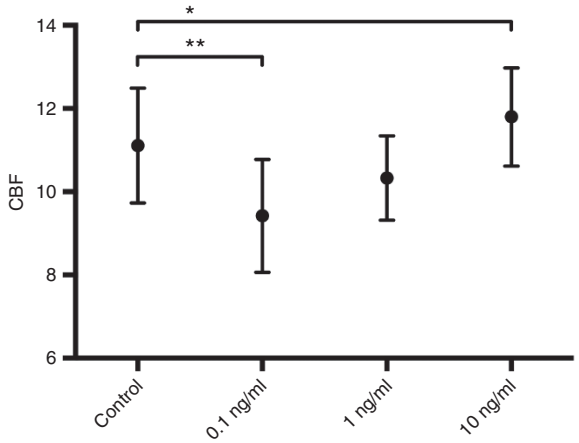

Figure 6. Influence of IFN-γ on the ciliary beat frequency. Presentation of the mean ciliary beat frequency (CBF) in IFN-γ-treated cells. The CBF is decreased in lower concentrations of IFN-γ and it is increased after treatment with IFN-γ in 10 ng/ml solution. The plots indicate mean values and SD. *Statistically significant with $P < 0.05$; **Statistically significant with $P < 0.001$.

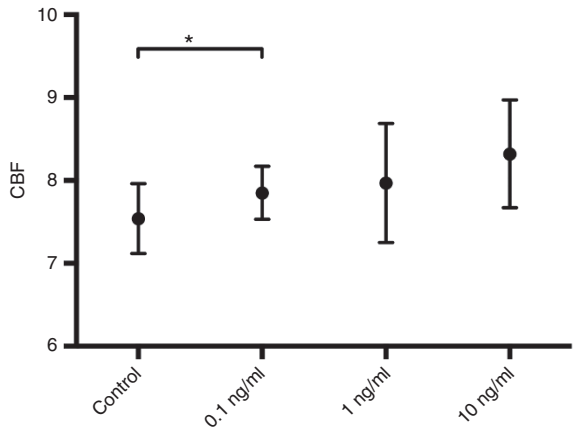

Figure 7. Influence of IL-13 plus IFN-γ on the ciliary beat frequency. Presentation of the mean ciliary beat frequency (CBF) in IL-13 + IFN-γ-treated cells. The CBF is significantly increased after IL-13 + IFN-γ treatment. 10 ng/ml IL-13 is added to different concentrations of IFN-γ solutions (0.1, 1, and 10 ng/ml). The plots indicate mean values and SD. *Statistically significant with $P < 0.05$.

It has been shown that a decreased CBF impairs mucociliary clearance (26). Our results suggest that the impaired mucociliary clearance in asthma is not only caused by epithelial damage, mucus metaplasia, and changes in mucus rheology, but also by direct effects of TH2 cytokines on the ciliary function. Impaired mucociliary clearance increases the time aeroallergens and pathogens spend on airways. This likely increases the risk of exacerbations and infections in asthma and contributes consequently to the burden of asthma.

IFN-γ has several effects in asthmatic bronchial epithelium: in an asthma cell culture model in mice it has been shown that IFN-γ reduces the mucous cell metaplasia (16). Nebulized IFN-γ inhibits eosinophilic airway inflammation (27,28). In subjects who benefited from allergen immunotherapy, an increased IFN-γ production of T-lymphocytes has been

demonstrated suggesting a protective role for IFN-γ in allergic diseases (29). IFN-γ is suggested to be involved in downregulating TH2 asthma (30,31). We have shown that IFN-γ is a potentiator of ciliary function by increasing the CBF. In addition, IFN-γ reverses the negative effect of IL-13 on the CBF. Our study adds evidence that IFN-γ enhances the mucociliary clearance by improving ciliary function. This may encourage clinical trials with topical IFN-γ or drugs that increase the IFN-γ airway concentration as a possible treatment option in well-defined asthma cases.

Other cytokines in asthma pathophysiology are IL-5 and IL-9. IL-5 is a promoter for activation, differentiation, and adhesion of eosinophils. IL-5 levels in bronchoalveolar lavage fluids correlate with asthma severity (32,33). Treatment with substances targeting IL-5 reduces exacerbation rate and sputum eosinophilia and increases the quality of life in clinical trials with patients having refractory eosinophilic asthma (34). IL-9 has effects on ciliated cell differentiation and mucus hypersecretion (16,35,36). Although IL-5 and IL-9 are relevant cytokines in asthma pathophysiology their influence on CBF is not yet known. We show that both IL-5 and IL-9 improve ciliary function and therefore deserve further research as potential agents that may enhance mucociliary clearance.

We used respiratory epithelium from nonasthmatic subjects. This approach is beneficial, as we were able to study the direct influence of different TH2 cytokines and IFN-γ on the ciliary function of respiratory epithelium naive to typical secondary changes seen in asthmatic subjects. Our primary research focus was to show whether ciliary function in human respiratory cells is dependent on TH2 cytokines or IFN-γ. We did not try to study other mechanisms affecting mucociliary clearance beyond ciliary function such as mucus composition, structural tissue damage or presence of inflammatory cells: a cell culture model would not be sufficient to address these questions. The majority of the observed effects showed dose dependency and were statistically significant. We did not investigate the interactions between cytokines and the function of ciliary motor protein complexes. It is known that the CBF is regulated by cAMP, intracellular calcium concentration, cGMP, changes in pH, and possibly intracellular bicarbonate (37) or NO signaling pathways (38). It is not clear, whether or which of these mechanisms are involved in the regulation of the CBF by cytokines. Our study was not designed to investigate the underlying mechanisms leading to the observed effects of cytokines on CBF. Future research will be needed to elucidate these questions. A better understanding of CBF regulation may help developing drugs that improve mucociliary clearance in diseases like asthma, cystic fibrosis or primary ciliary dyskinesia, in which impaired mucociliary clearance is a major pathophysiological mechanism.

In conclusion, we show that cytokines directly influence ciliary function in human respiratory epithelial cells. Thus, targeting these effects seems a promising approach in improving mucociliary clearance disorders such as asthma where these cytokines are altered.

METHODS

Primary Respiratory Cell Cultures and Ciliogenesis

Respiratory epithelial cells were obtained from nasal conchae or polyps from 21 patients who underwent ear, nose, and throat surgery. The removed tissue was washed with saline and epithelial cells were dissociated by incubation with 0.1% filtered pronase (protease XIV, Sigma-Aldrich, Taufkirchen, Germany) overnight at 4 °C in 10 ml Ham's F12-DMEM 1/1 (Invitrogen, Karlsruhe, Germany). The cell suspension was incubated in 5 ml medium for 1 h in an uncoated T25 culture flask at 37 °C, 5% CO_2 to remove fibroblasts. The remaining cells were suspended in 15 ml medium containing 2% Ultroser G (Cytogen GmbH, Sinn, Germany) and plated on collagen-coated T75 tissue flasks. Medium was replaced three times per week. After ~3 wk cells reached confluency. The collagen gel was resolved using collagenase type IV (200 U/ml, Worthington Biochemical Corporation, St. Katharinen, Germany) and the cell sheet was slightly disintegrated in smaller pieces with a cell scraper. 10 ml medium (containing Ultroser G) was added to the cell assemblies and placed in uncoated T25 culture flasks. To establish suspension cultures, the flask was placed on a rotary shaker (80 rpm) and incubated at 37 °C. After 24 h, the composition of the medium was changed to Ham's F12-DMEM 1/1 supplemented with 10% NU-Serum (Schubert and Weiss GmbH, München, Germany) which was replaced every other day. Stable cell aggregates (spheroids) started to differentiate into ciliated respiratory epithelium.

At 1 wk of suspension culture, the cytokines (all cytokines were obtained from Sigma-Aldrich, Germany) were added to the medium in different concentrations. The cells were exposed to a given cytokine (IL-4, IL-5, IL-9, IL-13, IL-4 + IL-13, IFN-γ and IFN-γ + IL-13) for 14 d. After 3 wk of suspension culture, spheroids exhibited multiple motile cilia and cells were processed for high-speed video microscopy.

Assessment of Ciliary Function by High-Speed Video Microscopy

CBF was determined with Sisson–Ammons Video Analysis (39). Respiratory epithelial cells were analyzed with an Olympus IMT-2 microscope (40× phase contrast objective) equipped with a Redlake ES-310 Turbo monochrome high-speed video camera (Redlake, San Diego) set at 125 frames per second.

Statistics

The statistical endpoints for this report are the CBF and cytokine concentration (control, 0.1, 1 and 10 ng/ml). The cytokines considered in this report are: IL-4, IL-5, IL-9, IL-13, IL-4 + IL-13, IFN-γ, and IFN-γ + IL-13. Depending on the cytokine, cellular material was generated from 2 to 4 patients and was randomly assigned to the different concentration levels.

Univariable distribution of metric variables is described by mean and SD. Normal distribution of metric outcomes was assessed graphically and by statistical measures (skewness, kurtosis). To analyze the impact of cytokine concentration on CBF, a two-level random intercept mixed model (40) was fitted for each cytokine. The mixed models were built with CBF as metric outcome, cytokine concentration as fixed effect and a patient-specific random intercept while allowing different variances within the distinct concentration levels. The random intercept is included to account for dependence between repeated, unordered measurements in cellular material from the same individual.

Analyses are regarded as explorative with *P* values displayed for descriptive reasons to detect and study meaningful effects. In particular, no adjustment for multiple testing was performed and "significance" refers to local statistical significance defined as a local, unadjusted *P* value below 0.05.

Statistical analyses and graphs were performed using SPSS version 19 (SPSS Inc., Chicago, IL), the SAS 9.4 software package (SAS Institute, Cary NC) and GraphPad Prism (version 6.0, GraphPad Software, La Jolla, California).

The study was approved by the Institutional Ethics Review Board at the University of Freiburg. Written informed consent to participate in this study was obtained from each individual.

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