Sputum interleukin-5 concentration is associated with a sputum eosinophilia and attenuated by corticosteroid therapy in COPD

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Short title: Sputum IL-5 in COPD with eosinophilic airway inflammation

ABSTRACT

Background: Airway inflammation in chronic obstructive pulmonary disease (COPD) is predominately neutrophilic, but some subjects demonstrate eosinophilic airway inflammation. Whether these inflammatory phenotypes have differential cytokine and chemokine expression is unknown.

Aims: To assess the sputum concentrations of cytokines and chemokines and their response to oral corticosteroid therapy in COPD subjects with or without a sputum eosinophilia.

Methods: Cytokine and chemokine concentrations were measured using the meso-scale device platform. To assess validity, recovery of exogenous spikes was examined. The concentrations of the validated mediators were measured in COPD sputum from subjects with or without a sputum eosinophilia. In a subgroup with a sputum eosinophilia the response to oral prednisolone 10mg for 1 month was examined.

Results: The recovery in sputum of exogenous spiked mediators was >80% in 11/26 cytokines and chemokines. In supernatants from eosinophilic (n=39) versus non-eosinophilic (n=59) sputa the geometric mean [95%CI] concentration was increased for IL-5 (9.0 [4.5-18] pg/ml versus 3.6 [2.7-6.3] pg/ml; p=0.03). IL-5 alone was correlated with sputum eosinophil counts (R^2=0.11, p=0.001), and was attenuated following treatment with prednisolone (n=9; mean difference 2.3 pg/mL [0.2 to 4.3, 95%CI]; p=0.03).

Conclusion: We have validated the use of the meso-scale device platform for cytokine and chemokine measurements in the sputum supernatants in COPD. Sputum IL-5 was associated with a sputum eosinophilia and was attenuated following oral corticosteroid therapy. Whether this cytokine is important in the pathogenesis of COPD in a subgroup of patients warrants further investigation.

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is a major cause of morbidity and mortality worldwide (1;2). It is characterised by progressive airflow limitation, with pulmonary and systemic inflammation, partly mediated by locally secreted cytokines and chemokines (3). The identification of key inflammatory mediators may yield further novel therapies to improve symptoms and reduce exacerbations.

Airway neutrophilia and CD8+ T-lymphocyte infiltration have been the hallmark of COPD inflammation (4) with increased numbers of CD8+ T-lymphocytes associated with worsening severity of disease (5). However sputum and airway wall studies in COPD have also shown the presence of eosinophilic inflammation (6-8). This evidence supports that eosinophilic inflammation (≥3% sputum eosinophil count) is present in up to 40% of COPD patients in stable disease (9) and randomised control trials have shown that this type of inflammation is associated with a favourable response to corticosteroid therapy (10) with reduction in severe exacerbation rates (11).

Whether the cytokine and chemokine concentrations are differentially expressed in the sputum supernatants from COPD subjects with or without eosinophilic inflammation is unknown. Several studies have examined the concentration of mediators in sputum supernatants, but to date the measurement of only a few of these mediators has been validated. The development of high throughput platforms such as meso-scale device has presented the opportunity to measure a large panel of mediators in small volumes of sputum supernatants. Therefore, in this study our aim was to assess the validity, repeatability and responsiveness of measuring cytokines and chemokines using meso-scale device in sputum supernatants from COPD subjects.

METHODS

Subjects

Ninety-seven sputum supernatant samples were available from 34 COPD subjects with stable disease, who had participated in a 12 month longitudinal randomised control study looking at eosinophilic airway inflammation and exacerbation rates (11). This included baseline samples from 13 subjects. The clinical characteristics of the 34 subjects included in this study are shown in table 1. Subjects were recruited from the respiratory clinics at the Glenfield Hospital (Leicester, UK) between February 2003 and March 2004. All subjects had a medical diagnosis of COPD, as per GOLD criteria (2) The study was approved by the Leicestershire, Rutland and Northamptonshire ethics committee, and all subjects gave signed written consent.

Measurements

Baseline characteristics were recorded including age, gender, smoking history and exacerbation frequency in the previous year. At each visit spirometry (Vitalograph, Maids Moreton, UK), pre and post bronchodilation following 400μg inhaled salbutamol, and health status using the chronic respiratory health questionnaire were recorded (12). Sputum was collected from subjects, and processed as previously described to produce cytospins for differential cell counts and supernatant for fluid-phase measurements using dithiothreitol (DTT)(13). Cytokine and chemokine levels were measured using the meso-scale device platform.

Sub-groups

The sputum supernatant chemokine and cytokine concentrations were measured to address the following aims: i) to define the pattern of chemokine and cytokine concentrations in

supernatant from samples with or without sputum eosinophilia (>3% non-squamous cells) at stable state, including baseline ii) to assess the repeatability of cytokines and chemokines identified from (i) as differentially expressed between eosinophilic and non-eosinophilic and the relationship between these mediators and the intensity of eosinophilic inflammation, and iii) to investigate the response of the mediators identified in (ii) from COPD subjects with a sputum eosinophilia following treatment with prednisolone 10mg daily for 1 month.

Meso-Scale Measurements

Meso-scale multi-array platforms were used to measure multiple chemokines and cytokines. The meso-scale platform [Meso Scale Discovery, Maryland, U.S.A.] uses electroluminescence technology, where antibodies have been lined in a patterned array to measure multiple mediators simultaneously. We assessed 26 mediators in duplicate. This required a minimum of 150μL of sputum DTT cell free supernatant for all analysis. The use of mucolytic dithiothrietol (DTT) in the processing of the sputum samples using the meso-scale multi-array platform was validated using sputum samples from COPD subjects with stable disease. The limit of detection of cytokine and chemokine recovery from the meso-scale platform is presented in table 2.

To assess recovery standard chemokine or cytokine spike was added to the sputum samples and to buffer controls (n=5), and then validated for its concentration by comparing spike recovery from sputum and buffer controls. A recovery of >65% from sputum and buffer spike identified initial analytes with a satisfactory recovery. Further analysis was then performed on these analytes that demonstrated a percentage recovery from sputum and buffer of at least 80% (14).

Analysis

Statistical analysis was performed using PRISM Version 4. Parametric data were expressed as mean (SEM), data that had a log normal distribution was log transformed and described as geometric mean (95% confidence interval) and non-parametric data were described as median (range). One-way analysis of variance and Student's t-tests were used for across and between group comparisons respectively for parametric data and Wilcoxon matched paired test for non-parametric data. Correlations were assessed by Pearson and Spearman correlations for parametric and non-parametric data and repeatability assessed by intra-class correlation coefficients. A p value of <0.05 was taken as the threshold for statistical significance.

RESULTS

Recovery of all cytokines and chemokines measured by the meso-scale multi-array platform are illustrated in figure 1. Recovery was >80% in 11/26 mediators and the chemokines CXCL8 and CCL22 were found to be the most abundant.

Baseline sputum samples were available in 13 COPD subjects, of which 6 had a sputum eosinophilia. We found differential expression in the eosinophilic vs. non eosinophilic group at baseline for IL-5 only with a mean (SEM) of 4.46 (1.62) vs. 0.47 (0.12) pg/ml, p=0.02 respectively (see figure 2).

For all sputum supernatant samples (n=97) the pattern of expression of the 11 validated cytokines and chemokines was different in the COPD samples with or without evidence of eosinophilic inflammation (table 3). Compared to the samples with non-eosinophilic inflammation those with eosinophilic inflammation had increased concentrations of IL-5 (mean difference [95% CI] 0.4 [0.2-0.9] pg/ml; p=0.03). There was no difference in the dosage of inhaled corticosteroid in the eosinophilic group versus the non-eosinophilic group (1200 [130] vs. 1346 [152], p=0.94).

The repeatability of IL-5 was assessed in paired sputum samples from 9 stable COPD subjects separated by 4 weeks whilst the subject was stable without evidence of an exacerbation 6 weeks before the baseline sample and without changes in the subjects' treatment between visits. The Pearson correlation coefficient (r) and intraclass correlation (ICC) for IL-5, was r=0.78, and ICC=0.34 respectively.

IL-5 was the only cytokine which significantly correlated with the sputum eosinophil count (R=0.33, p=0.001) (figure 3). There was no correlation of IL-5 with FEV_1 percent predicted or health status as measured by the CRQ_{total} (R=0.03, p=0.73; R=0.07, p=0.51 respectively).

Paired samples from another 9 COPD subjects with eosinophilic inflammation were available before and after treatment with prednisolone 10mg/day for 1 month. Following corticosteroid therapy there was a significant reduction in IL-5 (mean difference [95%CI] 2.3 [0.2 to 4.3] pg/mL; p=0.032) and sputum eosinophil count (mean difference [95% CI] 6.0 [3.2 to 8.7] %; p=0.001) (figure 4), whilst there was a non-significant increase in lung function and health status following corticosteroid therapy (table 4).

DISCUSSION

We have validated the measurement of 11 cytokines and chemokines in sputum supernatants from subjects with COPD using the meso-scale device platform. We report here for the first time that in COPD sputum IL-5 concentration was increased in those sputum samples with, compared to those without, evidence of eosinophilic inflammation. The sputum IL-5 concentration was repeatable; related to the degree of sputum eosinophilia and decreased in response to systemic corticosteroids. Therefore sputum IL-5 is a valid, repeatable and responsive measure in COPD.

The meso-scale multi-array platform employs a sandwich immunoassay format, where antibodies are lined in a patterned array. We have been able to demonstrate the use of this format in the analysis of sputum supernatant from COPD subjects demonstrating good recovery of several cytokines and chemokines. Detection of many cytokines including that of sputum IL-5 using enzyme-linked immunosorbent assay (ELISA) has proven to be difficult in asthmatics previously (15), improved only upon the addition of protease inhibitors (16). The use of DTT is known to reduce detectable levels of mediators measured by ELISA or substrate assay (17), however using the meso-scale platform and DTT for our samples, we were able to get recovery of >80% for 11/26 analytes including IL-5, without the need of protease inhibitors.

In this study we found differential expression of IL-5 in subjects with or without sputum eosinophilia, demonstrating increase in IL-5 concentrations in those with sputum eosinophils. Interleukin-5 (IL-5) is a T-helper 2 (TH_2) cytokine, and is integral to the development, differentiation, recruitment, activation and survival of eosinophils (18). The evidence for IL-5 is established in asthma, with new therapies aimed at blocking IL-5 production (20). Despite

the overlap between asthma and COPD, current dogma suggests that IL-5 does not play a role in stable COPD, irrespective of disease severity (21). In bronchial biopsies IL-5 is consistently not elevated in COPD (22;23). Whereas in contrast Betz et al found that sputum IL-5 was increased in asymptomatic subjects with airway hyper-responsiveness and subjects with COPD compared to healthy controls, although correlation with sputum eosinophils was not examined (24). Our study extends this earlier observation to show that the sputum IL-5 concentration was increased in COPD sputum samples with eosinophilic inflammation.

In addition to an increased sputum IL-5 concentration, in those COPD samples with an eosinophilia, we identified a positive albeit weak correlation between the sputum IL-5 and eosinophil count. This correlation has previously observed in asthmatic subjects only. Interestingly, in response to systemic corticosteroid therapy there was a reduction in the sputum eosinophil count and sputum IL-5 concentration. This is similar to studies conducted in asthmatic patients (25), further demonstrating similarities to COPD and identifying the role of IL-5 in COPD subjects with an eosinophilic phenotype. This relationship infers that blocking IL-5 may be a novel therapeutic strategy in COPD (20). In addition to increase of IL-5 in our non-eosinophilic group, we have noted that there was a trend to reduction of TNF-α and CD120b in the non-eosinophilic group. Pulmonary and systemic inflammation in COPD has shown elevated pro-inflammatory mediators, but whether this inflammation is differential according to the corresponding airway inflammation warrants further analysis.

One potential limitation of our study is that we reserved our study to sputum samples obtained whilst patients were stable and not at exacerbations. Sputum eosinophilia is known to be increased in exacerbations of COPD (26), and more so in those with a viral aetiology (27). Further studies measuring IL-5 and infective parameters during an exacerbation are

required. The numbers of subjects studied before and after corticosteroid therapy was small and not placebo controlled, but the magnitude of the response of the sputum eosinophil count and IL-5 suggests this observation is robust. Other studies of cytokine and chemokine concentration in sputum have been limited to small panels of mediators and have confronted several technical difficulties.

We were able to validate the meso-scale discovery platform using DTT processed samples and without the need for protease inhibitors. This multi-array high throughput device removes the length of the assay time, the small dilutional range known to occur with ELISA, whilst requiring small quantities of analyte.

In conclusion, we have demonstrated differential expression of the cytokine and chemokine pattern in COPD sputum samples with and without an eosinophilia using the meso-scale discovery platform. Importantly, sputum IL-5 was increased in samples with an eosinophilia, which was correlated with the intensity of eosinophilic inflammation and responsive to systemic corticosteroid therapy. Whether in a subgroup of COPD subjects IL-5 is an important cytokine in the pathogenesis of disease, a potential biomarker to guide steroid responsiveness or a novel therapeutic target warrants further investigation.

Conflicts of Interest: MB, SS, RS, WM, do not have any financial relationship with a commercial entity that has an interest in the subject of this manuscript. AstraZeneca R & D, Charnwood, employs MM, PR, PD, and PN. IDP received $2000 for speaking at conferences organised by GlaxoSmithKline and $5000 for speaking at conferences organised by AstraZeneca; and has received a grant of $500,000 from GlaxoSmithKline for a study in severe asthma. CEB has received a total of $2.2 million in research funds from AstraZeneca, Cambridge Antibody Technology, and GlaxoSmithKline; he has received less than $10,000 in consultancy fees from Cambridge Antibody Technology, AstraZeneca, GlaxoSmithKline, Roche and Pfizer; and has participated in scientific meetings organised and financed for AstraZeneca, GlaxoSmithKline, Boehringer Ingelheim, MSD and Pfizer.

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Table 1

Baseline clinical characteristics for COPD subjects.

Data presented as mean (SEM). ^beclomethasone dipropionate equivalent

	COPD Subjects (n=34)
Age	68 [52-80]
Gender (female/male)	6/28
Current Smoker (%)	27
Ex-Smoker (%)	74
Smoking Pack Years	45 (4.5)
Baseline inhaled steroid dose/day^	1290 (174)
Number exacerbations in previous year	2.8 (0.4)
FEV_1 (L)	1.0 (0.1)
FEV_1 percentage predicted (%)	36.0 (2.3)
FEV_1 post-bronchodilator (L)	1.1 (0.1)
FEV_1/FVC (%)	47.6 (2.0)
CRQ_{TOTAL}	15.4 (0.7)

Table 2

Limit of detection of cytokines and chemokines from meso-scale discovery platform (pg/ml)

Cytokine	LDD (mean)		Chemokine	LDD (mean)	
IL-1β	0.7		CCL2	5.9	
IL-4	0.3		CCL4	9.8	
IL-5	0.1		CCL3	119.0	
IL-6	2.6		CCL5	0.3	
IL-10	1.6		CCL11	4.9	
IL-13	18.4		CCL13	20.0	
IL-17	4.5		CCL17	8.0	
IFN-γ	1.0		CCL22	256.7	
TNF-α	1.4		CCL26	3.0	
CD120a	10.0		CXCL8	0.2	
CD120b	0.8		CXCL10	32.9	
CD126	0.2		CXCL11	6.2	
GM-CSF	2.3		VEGF	9.7	

Table 3

Cytokine and chemokine concentrations (g/ml) in sputum supernatant samples categorised by sputum eosinophil count. Data presented as geometric mean [95% CI]

	Eosinophil < 3% **(n=58)**	**Eosinophil > 3%** **(n=39)**	p-value
IL-1β	509 [298 to 871]	248 [158 to 392]	0.06
IL-6	4126 [2867 to 5938]	3335 [2419 to 4600]	0.41
CXCL8	31830 [19626 to 51623]	30602 [22415 to 41768]	0.90
CXCL10	8471 [6324 to 11346]	7038 [5700to 8692]	0.35
CCL22	13510 [11615 to 15712]	11291 [9418 to 13541]	0.13
CCL4	4718 [3207 to 6940]	7332 [4924to 10405]	0.11
CD120a	6228 [4576 to 8476]	4314 [3440to 5411]	0.08
CD120b	2158 [1457 to 3198]	1255 [899 to 1752]	0.05
TNF-α	123 [82 to 186]	69 [47 to 103]	0.05
IL-5	**4 [3 to 6]**	**9 [5 to 18]**	***0.03***
VGEF	8319 [6542 to 10579]	7595 [6293 to 9167]	0.58

Table 4

Sputum cytokine and chemokine concentrations, lung function and health status before and after 1 month of prednisolone 10mg (n=9 COPD subjects). Data presented as mean (SEM).

	Pre corticosteroid therapy	Post corticosteroid therapy	Mean difference (95% CI)	p-value
Eosinophils (%)	**8.1 (1.2)**	**2.2 (0.6)**	**6.0 [3.2 to 8.7]**	***0.001***
FEV1 (L)	0.95 (0.1)	0.97 (0.1)	-0.01 [-0.17 to 0.13]	0.78
FEV1 % predicted	37.7 (6.5)	39.0 (7.4)	-1.3 [-7.0 to 4.5]	0.62
CRQ_{TOTAL}	15.8 (1.3)	16.2 (1.4)	-0.4 [-2.5 to 1.6]	0.64
IL5 (pg/mL)	**3.0 (1.2)**	**0.7 (0.4)**	**2.3 [0.2 to 4.3]**	***0.032***

Figure Legends

Figure 1

Percentage recovery of exogenous spike of cytokine and chemokines to COPD sputum and buffer samples (n=5). Horizontal bar set at 65% recovery to highlight analytes with sufficient recovery from sputum and buffer. Asterisked analytes considered to be valid assays, with recovery of >80% from sputum compared to buffer. Data presented as mean (SEM).

Figure 2

Mean IL-5 concentrations in subjects according to sputum eosinophil (< 3% or ≥ 3) counts at baseline visit.

Figure 3

Correlation between sputum IL-5 concentration and sputum eosinophil differential cell count.

Figure 4

a) IL-5 concentration (pg/mL) and b) sputum eosinophil count (%) before and after oral prednisolone for 1 month (n=9).

Figure 1

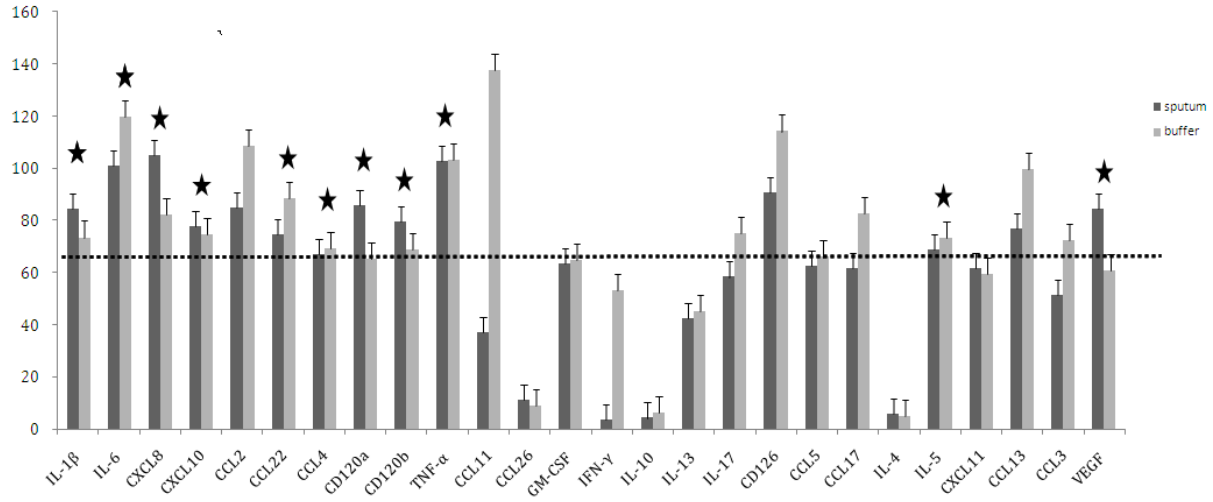


Figure 2

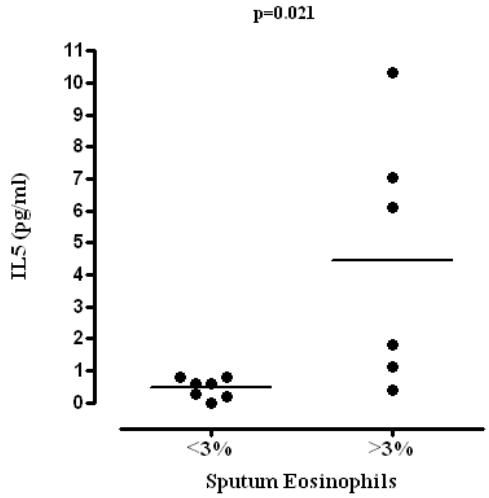


Figure 3

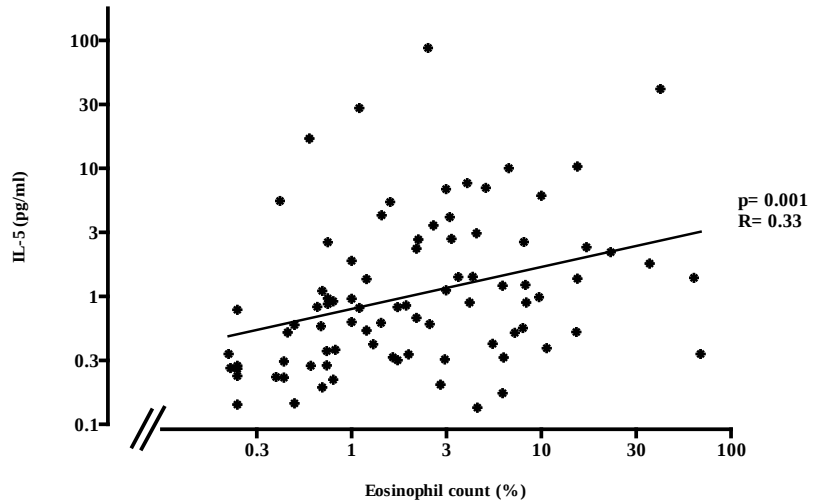
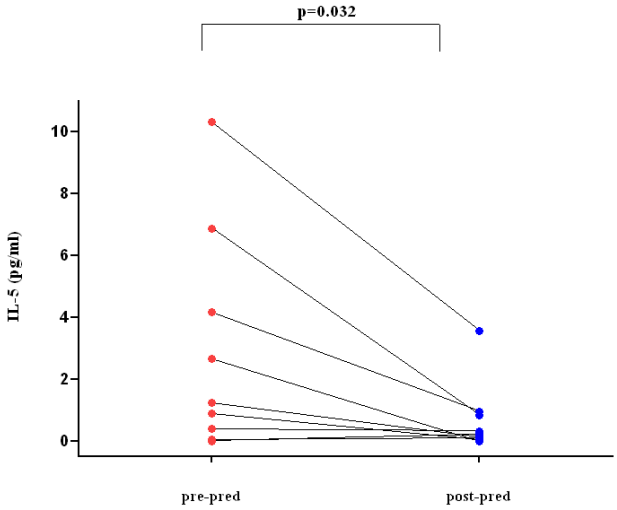
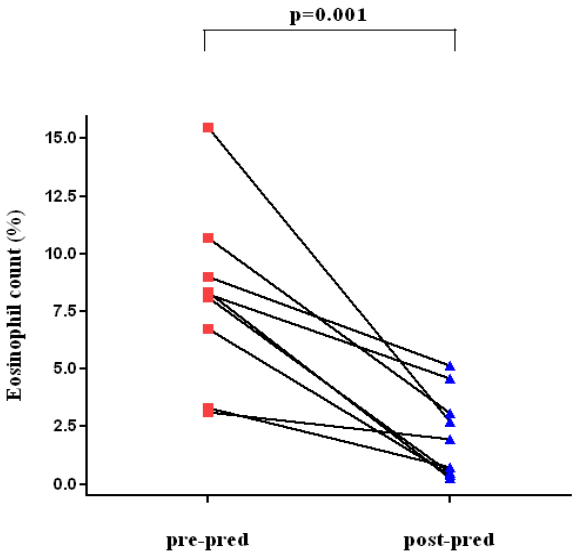


Figure 4

a)



b)


p=0.001
Eosinophil count (%)
15.0
12.5
10.0
7.5
5.0
2.5
0.0
pre-pred
post-pred