Thorax Online First, published on February 12, 2009 as 10.1136/thx.2008.108290

Granulocyte macrophage colony stimulating factor expression in induced sputum and bronchial mucosa in asthma and COPD

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KEY WORDS: Asthma, COPD, GM-CSF, GM-CSFR, sputum, bronchus, airway smooth muscle
WORD COUNT: 2,359

ABSTRACT

Background: Granulocyte macrophage colony stimulating factor (GMCSF) has been implicated as an important mediator in the pathogenesis of asthma and chronic obstructive pulmonary disease (COPD). However, the expression of GMCSF and its receptor in airway samples in asthma and COPD across disease severity needs to be further defined.

Methods: We measured sputum GM-CSF in 18 control subjects, 45 subjects with asthma, and 47 subjects with COPD. Enumeration of GM-CSF+ cells in the bronchial submucosa and airway smooth muscle bundle was performed in 29 control subjects, 36 subjects with asthma, and 10 subjects with COPD.

Results: The proportion of subjects with measurable GM-CSF in the sputum was raised in the moderate (7/14) and severe (11/18) asthmatics, and in those with COPD GOLD stage II (7/16), III (8/17) and IV (7/14) compared to controls (1/18) and mild asthmatics (0/13); p=0.001). The sputum GM-CSF concentration was correlated with the sputum eosinophilia in subjects with moderate-severe asthma (r_s=0.41; p=0.018). The median (interquartile range) GM-CSF and GM-CSFR+ cells/mm^2 submucosa was increased in severe asthma (1.4 [3.0]) and (2.1 [8.4]) compared to mild-moderate asthmatics (0 [2.5]) and (1.1 [5]), and healthy controls (0[0.5]) and (0 [1.6]), (p=0.004, and p=0.02 respectively).

Conclusions: Our findings support a potential role for GM-CSF in asthma and COPD and suggest that over-expression of GM-CSF in sputum and the bronchial mucosa is a particular feature of severe asthma.

INTRODUCTION

The airway diseases asthma and chronic obstructive pulmonary disease (COPD) are common and cause significant morbidity and mortality worldwide. Asthma affects 10% of children and 5% of adults, and its prevalence continues to rise (1). Severe asthma accounts for about 10% of asthma, but is particularly important as it leads to debilitating chronic symptoms despite optimal standard asthma treatment and contributes to over half of the health care costs attributed to asthma (1-3). COPD is a major public health problem and will rank as the 3rd cause of death in 2030 (4). Both conditions are characterised by airflow obstruction with airway inflammation, and remodelling. Although the inflammatory profiles of asthma and COPD have been described as overlapping (5), asthma is more commonly associated with Th2 mediated eosinophilic inflammation (6) whereas in COPD neutrophilic inflammation is more predominant (5). Several cytokines and chemokines have been implicated in driving the airway inflammatory response in asthma and COPD.

Granulocyte macrophage-colony stimulating factor (GM-CSF) is a major regulator of inflammatory cells of the myeloid lineage and has been implicated in asthma and COPD (7). It is released by a range of structural and inflammatory cells, including airway epithelium, airway smooth muscle, fibroblasts, T-lymphocytes, mast cells, eosinophils, and macrophages. GM-CSF has recently been shown to signal via a ternary receptor complex (GMCSFR) composed a 2:2:2 hexamer consisting of two βc chains, two GMRα chains and two GM-CSF molecules (8). GM-CSF is a pleiotrophic and proinflammatory cytokine that stimulates myelopoiesis, promotes leukocyte survival and activation and regulates mucosal immunity and inflammation in part via modulation of Toll-like receptor-4 (9) and neutrophil function (10). Its importance in airways disease is supported by evidence from mouse models of COPD (7) and asthma (11), whereby administration of anti-GM-CSF antibody attenuates the neutrophilic and eosinophilic inflammatory response respectively. Importantly, in human disease GM-CSF expression is increased in sputum, BAL and bronchial biopsies in asthma (12-17). In contrast, in COPD there is a lack of direct evidence of increased GM-CSF expression in airway secretions or biopsy tissue. However, in culture GM-CSF secretion by ex vivo sputum cells is increased in COPD (18). Similarly, whether GM-CSFR expression is increased in airways disease is contentious with one study suggesting that GMCSFR is increased in non-atopic, but not atopic asthma (19). Therefore, GM-CSF and GM-CSFR expression in airways disease needs to be further defined.

We hypothesised that GM-CSF and GM-CSFR expression is increased in asthma and COPD and is related to disease severity. To test our hypothesis we have measured the sputum GM-CSF concentration and enumerated in bronchial mucosa the number of GM-CSF+ and GM-CSFR+ cells in asthma and COPD.

Methods

Subjects

Subjects were recruited from hospital staff, general respiratory and the 'Difficult Asthma' clinics at Glenfield Hospital, Leicester, local primary health care, and by local advertising. Asthma was defined according to the current global initiative for asthma (GINA) guidelines (20). Subjects with asthma had typical symptoms and the presence of one or more of the following objective criteria: significant bronchodilator reversibility of FEV_1 >200mls, a provocation concentration of methacholine causing a 20% fall in FEV_1 (PC_{20}) of less than 8mg/ml or a peak flow amplitude % mean over 2 weeks of more than 20%. Asthma severity was classified using the GINA treatment steps (20). COPD was diagnosed and severity categorised by using Global initiative for chronic Obstructive Lung Disease (GOLD) criteria (21). COPD subjects that demonstrated partial bronchodilator reversibility were not excluded. Subjects were recruited as three independent cross-sectional groups, to assess sputum GM-CSF concentration in asthma and COPD (*Group 1*); and GM-CSF and GM-CSFR expression in proximal airways in asthma (*Group 2*) and COPD (*Group 3*). Subjects were free from exacerbations for at least 6 weeks. Healthy controls had normal spirometry and some smokers with >10 pack year history were included to enable comparisons between healthy smokers and COPD subjects. All subjects gave written informed consent with study approval from the Leicestershire ethics committee.

Protocol

For all subjects demographics and spirometry was recorded. Subjects with asthma and healthy controls in group 1 and 2 also underwent methacholine inhalation test using the tidal breathing method (22) and allergen skin prick tests for *Dermatophagoides pteronyssinus*, dog, cat and grass pollen. Sputum induction using incremental concentrations of nebulised hypertonic saline 3, 4 and 5% each for 5 minutes was also performed in all subjects in groups 1 and 2 (23).

In group 2 subjects underwent bronchoscopy conducted according to the British Thoracic Society guidelines (24), and biopsies were taken from the right middle and lower lobe carinae. In group 3 proximal airway samples were collected from surgical specimens. All bronchial mucosal specimens were fixed in acetone and embedded in glycomethacrylate as described previously (25).

Sputum GM-CSF measurement

Sputum was selected, dispersed using the mucolytic dithiothreitol (DTT) and processed to generate a sputum differential cell count and cell free supernatants were stored at -80^0C for later analysis as described previously (26).

Sputum GM-CSF was measured by ELISA (Caltag-Medsystems, Buckinghamshire, UK). The lower limit of detection was 10pg/g sputum. The GM-CSF assay was validated in line with European Respiratory Society recommendations to assess the effect of DTT and the recovery of exogenous spiking with recombinant cytokine (27). GM-CSF recovery was not affected by DTT and recovery of exogenous spiked GM-CSF was 103 (15)% (n=4).

GM-CSF and GM-CSFR expression in endobronchial biopsies

2µm sections were cut and stained using monoclonal antibodies against GM-CSF (clone: BVD2-21C11, Cambridge BioScience Ltd.**)**, GM-CSFR (clone: 2B7, a monoclonal antibody raised to the extracellular domain of GM-CSFR, gift from Dr Sleeman. MedImmune, Grant Park Cambridge), or appropriate isotype controls (Rat IgG2a [RαD systems Europe Ltd, Abingdon, UK] and mouse IgG1: clone DAK-Go1 [Dako UK Ltd, Cambridge] respectively). The number of positive nucleated cells was enumerated per mm^2 of bronchial submucosa or ASM-bundle by a blinded observer as described previously (28, 29).

Statistical Analysis

Statistical analysis was performed using PRISM Version 4. Parametric data were expressed as mean (SEM), data that had a normal log distribution were log transformed and described as geometric mean (95% confidence interval) and non-parametric data were described as median (interquartile range [IQR]). One-way analysis of variance (Kruskal-Wallis for non-parametric data) was used for across group comparisons with Tukey's and Dunn's post-hoc tests for between group comparisons respectively. Chi squared tests were used to compare categorical data. Correlations were assessed by Spearman rank correlation coefficients.

RESULTS

Sputum GM-CSF concentration in asthma and COPD

Clinical and sputum characteristics for subjects in group 1 are shown in table 1. The proportion of subjects with measurable GM-CSF in the sputum was raised in the moderate (7/14) and severe (11/18) asthmatics, and in those with COPD GOLD stage II (7/16), III (8/17) and IV (7/14) compared to controls (1/18) and mild asthmatics (0/13) (p=0.001). The sputum GM-CSF concentration was increased in subjects with COPD across severity compared to controls (p=0.02 Kruskal-Wallis; p<0.05 for COPD all severities compared to controls; Figure 1). Similarly, the sputum GM-CSF concentration was increased in severe asthma compared to mild asthma and controls and in moderate asthma compared to mild disease (p<0.001 Kruskal-Wallis; p<0.05 for between group comparisons; Figure 1). The sputum GM-CSF concentration was increased in the subjects with moderate-severe asthma combined compared to those with COPD GOLD II-IV combined (p=0.004). The sputum GM-CSF concentration was correlated with the sputum eosinophilia in subjects with disease as a whole group (r_s=0.28; p=0.007), all asthmatics (r_s=0.3; p=0.04) and moderate-severe disease (r_s=0.41; p=0.018), but not COPD. There was no association in subjects with asthma or COPD with sputum GM-CSF concentration and % predicted FEV_1 (r=-0.26, p=0.09; r=-0.07, p=0.7) or FEV_1/FVC ratio (r=0.06, p=0.7; r=0.1, p=0.5) respectively.

GM-CSF/R expression in large airway tissue specimens

Examples of GM-CSF and GM-CSFR+ cells in the bronchial submucosa in asthma are as shown in figure 2. Clinical characteristics of group 2 are as shown in table 2. The median (IQR) GM-CSF cells/mm^2 submucosa was increased in severe asthma (1.4 [3.0]) compared to mild-moderate asthmatics (0 [2.5]), and healthy controls (0[0.5]), (p=0.004, Kruskal-Wallis; between group comparisons are as shown Figure 3a). The number of GM-CSFR+ cells/mm^2 submucosa and ASM was increased in severe asthma (2.1 [8.4]) and 2.4 (5.5) compared to healthy controls (0 [1.6]) and 0 (0.8), but not mild-moderate asthma (1.1 [5]) and 1.2 (2.2) (p=0.02 and p=0.049 respectively Kruskal-Wallis; p<0.05 severe asthma versus control, Figure 3b, c). The number of GM-CSF+ cells in the ASM-bundle was very low in subjects with asthma and healthy controls.

There were no differences in the number of GM-CSF or GM-CSFR+ cells within the submucosa, or ASM-bundle in lung resection tissue from subjects with COPD and controls with and without a significant smoking history (Table 3).

Table 1 Clinical and sputum characteristics of Asthmatic and COPD subjects in the sputum groups

	Normal	Mild Asthma (GINA 1)	Moderate Asthma (GINA 2-4)	Severe Asthma (GINA 5)	GOLD 2	GOLD 3	GOLD 4
Number	18	13	14	18	16	17	14
Age [#]	54 (3)	53 (4)	51 (4)	49 (5)	71 (2)	68 (2)	72 (11)
Male/ Female	4/14	8/5	4/10	7/11	9/7	12/5	13/1
Never/current/ex-smokers	9/0/9	11/0/11	10/0/4	16/0/2	2/9/5	0/6/11	0/4/10
Pack years[#]	17 (5)	2 (1)	6 (3)	4 (3)	43 (8)	52 (4)	56 (9)
Atopy n (%)	6 (33)	7 (54)	11 (79)	13 (72)	7 (44)	7 (41)	5 (36)
$PC_{20}FEV_1$ (mg/ml)[^]	>16	1 (0.3-4.2)	0.4 (0.1-1.5)	0.1 (0-1.6)	X	X	X
FEV_1% predicted[#]	98.7 (3.0)	80.4 (5.1)	66.4 (4.4)	56.4 (6.8)	60.1 (1.5)	39.9 (1.3)	24.1 (1.3)
Pre-BD FEV_1/FVC %[#]	77.6 (1.7)	72.1 (3.6)	67.5 (3.0)	69.7 (2.8)	59.4 (2.2)	50.9 (2.1)	40.3 (2.0)
BD response (%)[#]	1.5 (0.7)	6.0 (3.6)	5.0 (4.6)	9.6 (2.0)	4.3 (2.2)	6.0 (1.8)	4.1 (3.2)
Sputum Cell Counts							
TCC[#]	3.8 (0.9)	2.34 (0.82)	3.31 (1.8)	6.57 (3.3)	3.3 (0.6)	4.3 (0.9)	11.2 (3.2)
Eosinophil %^	0.5 (0.3-0.8)	2.3 (0.6-7.9)	2.7 (1.0-7.3)	3.8 (1.8-8.0)	2.3 (1.7-6.3)	2.6 (1.4-4.7)	1.0 (0.4-2.3)
Neutrophil %[#]	55.8 (6.0)	67.3 (6.9)	57.8 (5.9)	64.3 (6.0)	72.2 (5.0)	71.0 (4.3)	85.6 (2.9)
Macrophage %[#]	38.5 (5.3)	20.6 (4.6)[∞]	21.4 (4.7)[∞]	19.2 (4.9)[∞]	29.2 (4.6)	21.3 (3.3)	8.7 (1.7)
Lymphocyte %[#]	0.4 (1.4)	0.5 (0.2)	0.4 (0.1)	1.9 (1.5)	1.6 (1.1)	0.6 (0.2)	1.0 (0.3)
Epithelial cells %[#]	3.7 (1.4)	3.7 (1.8)	3.6 (1.7)	3.3 (1.6)	3.3 (0.6)	4.3 (0.9)	11.2 (3.2)

[#]mean (SE), ^ geometric mean (95% confidence interval [CI]), BD-bronchodilator

Table 2. Clinical and sputum characteristics of biopsy group asthma

	Normal	**Mild-moderate Asthma (GINA 1-3)**	**Severe Asthma (GINA 4-5)**
Number	10	15	12
Age [#]	38 (4)	48 (4)	50 (4)
Male/ Female	4/6	8/7	6/6
Never/current/ex-smokers	9/0/1	9/0/6	10/0/2
Pack years[#]	0 (0)	3 (1)	3 (2)
Atopy n (%)	5 (50)	10 (66)	9 (75)
$PC_{20}FEV_1$ (mg/ml)[^]	>16	0.3 (0.1-0.9)	0.4 (0.1-1.4)
FEV_1% predicted[#]	98.4 (4.5)	89.7 (4.9)	80.2 (6.6)
Pre-BD FEV_1/FVC %[#]	77.9 (3.3)	74.4 (2.9)	74.5 (3.4)
BD response (%)[#]	1.1 (1.2)	8.8 (4.1)	12.5 (5.7)
Sputum Cell Counts			
TCC[#]	2.1 (0.6)	2.9 (0.7)	2.7 (0.6)
Eosinophil %^	0.4 (0.1-0.8)	0.9 (0.4-2.8)	2.9 (0.8-10.6)
Neutrophil %[#]	48.8 (17.7)	55.2 (7.0)	59 (9.7)
Macrophage %[#]	47.4 (11.7)	37.1 (6.3)	25.1 (5.8)
Lymphocyte %[#]	1.9 (1.2)	1.1 (0.2)	1.5 (0.7)
Epithelial cells %[#]	1.4 (1.2)	4.0 (1.6)	6.9 (3.3)
GM-CSF			
Submucosa[~]	0 (0.5)	0 (2.5)	1.4 (3.0)
ASM[~]	0 (0)	0 (0)	0 (0)
GM-CSFR			
Submucosa[~]	0 (1.6)	1.1 (5)	2.1 (8.4)
ASM[~]	0 (0.8)	1.2 (2.2)	2.4 (5.5)

[#]mean (SE), ^ geometric mean (95% CI), ~median (IQR), BD-bronchodilator

Table 3. Clinical characteristics and GM-CSF/R expression in proximal airway from lung resection

	Normal	**Smoker**	**COPD**
Number	8	11	10
Age[#]	58 (3)	60 (3)	66 (3)
Male/ Female	7/1	8/3	7/3
Never/current/ex-smokers	6/0/2	0/0/11	0/0/10
Pack years[#]	0 (1)	30 (7)	39 (6)
FEV_1[#]	2.8 (0.2)	2.6 (0.2)	1.8 (0.2)
FEV_1 % pred[#]	87 (3)	87 (4)	64 (4)
Pre-BD FEV_1/FVC[#]	79 (3)	81 (4)	55 (3)
GM-CSF			
Submucosa~	0.5 (1)	0.8 (2.4)	0.2 (1.1)
ASM~	0 (0)	0 (0.1)	0 (0)
GM-CSFR			
Submucosa~	2.3 (6.1)	0.3 (1.3)	0.5 (4.4)
ASM~	0 (0.1)	0 (0)	0 (0)

[#]mean (SE), ~median (IQR), BD-bronchodilator

DISCUSSION

We report here for the first time that the sputum GM-CSF concentration was increased in COPD, independent of disease severity, and confirm that in asthma the sputum GM-CSF concentration is associated with more severe disease. In asthma our sputum findings were supported by increased GM-CSF and GM-CSFR expression in bronchial biopsies in severe disease. Our study therefore supports our hypothesis that GM-CSF and GM-CSFR expression is increased in asthma and COPD and in asthma is related to disease severity.

Several lines of evidence support a role for GM-CSF in COPD. GM-CSF is induced by the presence of airway pathogens (30, 31) and is known to be an important regulator of the activation and survival of key effector cells in COPD namely the neutrophil and macrophage (32, 33). Critically, neutralisation of GM-CSF in animal models attenuates airway inflammation in response to cigarette smoking (7). However, to date there has been a paucity of direct evidence of increased GM-CSF expression in airway secretions. Indeed sputum GM-CSF concentration was not increased in subjects at exacerbations compared to their stable state at recovery (34), although in contrast GM-CSF release by sputum cells in culture was increased (18). *In vivo* and *in vitro* GM-CSF is rapidly internalised following receptor binding and therefore it is likely that the measurement of sputum GM-CSF is under-estimated by ELISA (7, 35). Importantly, the concentration of free GM-CSF is under tight control with measurable GM-CSF autoantibodies in healthy controls as well as disease (36). In spite of this we have here validated the measurement of GM-CSF in sputum and found that it was increased in COPD subjects across all disease severities compared to smoking and non-smoking controls,although there was no relationship between sputum GM-CSF concentration and disease severity. In the resection samples we were unable to confirm that expression of GM-CSF or its receptor was increased. However, we only studied COPD subjects with milder disease and the control subjects often had underlying lung cancer which may have masked differences between COPD and controls. Therefore the role of GM-CSF in COPD needs to be further defined and future studies need to include analysis of bronchial tissue in moderate to severe COPD.

In asthma there is a wealth of data supporting a role for GM-CSF. In particular GM-CSF is pivotal in eosinophil maturation and survival (37), a key effector cell in asthma. In animal models GM-CSF neutralisation attenuates airway inflammation and GM-CSF knockout mice (38) do not develop a bronchial eosinophilia in response to allergen challenge. In contrast to COPD, in asthma there are several reports of increased GM-CSF expression in airway secretions and tissue (12-17). In particular increased sputum GM-CSF expression is associated with more severe disease (12, 13). We have confirmed these earlier reports and found that sputum GM-CSF concentration was increased in moderate-severe asthma, but not in mild disease. In addition the intensity of the sputum GM-CSF expression was related to the sputum eosinophilia. We report here for the first time that GM-CSF and GM-CSFR expression was also increased in the bronchial submucosa in more severe asthma. Therefore, in severe asthma there is a generalised upregulation in the GM-CSF/GM-CSFR axis suggesting that this mediator may play a prominent role in severe asthma.

Our study design allowed for direct comparison of the sputum GM-CSF concentration in asthma and COPD, but not expression in tissue as samples were obtained using different methods. Comparisons in sputum GM-CSF concentrations were undermined by the relative insensitivity of

our assay with a large number of subjects that had concentrations below the level of detection of our assay. This is likely to reflect the rapid internalisation of GM-CSF. In spite of this limitation we found that sputum GM-CSF was increased in both COPD and moderate-severe asthma, and importantly it was greater in moderate-severe asthma than COPD. Therefore whether GM-CSF plays a more important role in severe asthma than COPD warrants further investigation.

Our study has a number of possible criticisms. This is a cross-sectional observational study. We were unable to demonstrate an association between GM-CSF expression and lung function. Whether GM-CSF expression is related to longitudinal clinical outcomes such as disease progression, lung function decline and exacerbations requires further examination. Similarly, we are unable to determine whether differences observed between mild and severe asthma reflect disease severity or are a consequence of differences in treatment. Therefore the effects of corticosteroids on GM-CSF need to be fully elucidated, although previous work suggests that GM-CSF expression in tissue is attenuated by corticosteroids (39). The rapid turnover of GM-CSF in vivo limits the interpretation of protein expression by ELISA and immunohistochemistry. We have not defined the cellular source of GM-CSF or determined whether the increased expression of GM-CSF is associated with an increase in the total number of infiltrating cells within the bronchial submucosa. In addition protein expression in tissue often reflects granular stores and may underestimate GM-CSF expression in cells that release rather than store GM-CSF such as T-cells. Further studies are therefore required to confirm our findings and to determine the relative expression of GM-CSF in bronchial tissue by different cell types.

In conclusion, we found that sputum GM-CSF concentration was increased in COPD, independent of disease severity, and in moderate to severe asthma. Increased bronchial submucosal expression of both GM-CSF and its receptor was a particular feature of severe asthma. Our findings therefore do support a potential role for GM-CSF in asthma and possibly COPD. Efficacy studies of therapeutic strategies targeted at GM-CSF are eagerly awaited and will further define the functional importance of GM-CSF in airways disease.

ACKNOWLEDGEMENTS
Mrs Sue Mckenna and Mrs Beverley Hargadon for clinical characterisation of the subjects and Mr William Monteiro and Ms Natalie Neale for technical support.

FUNDING
Asthma UK, MedImmune Ltd, DOH Clinical Scientist award (CB) and Wellcome Senior Clinical Fellowship (CB)

COMPETING INTERESTS
CB has received research funding from AstraZeneca, MedImmune and GlaxoSmithKline and consultancy fees from MedImmune and GlaxoSmithKline. MS and ESC are employees of MedImmune.

Figure Legends

Figure 1: Sputum GM-CSF concentration
Sputum GM-CSF concentration in the control group, subjects with COPD (GOLD 2-4), mild asthma (GINA 1), moderate asthma (GINA 2-4), or severe asthma (GINA 5). Across group comparisons by Kruskal-Wallis were $p<0.05$, p values for Dunn's post-hoc test for between group comparisons are as shown.

Figure 2: Examples of GM-CSF+ and GM-CSFR+ cells in the submucosa and ASM-bundle in asthma
Representative photomicrographs of bronchial biopsy sections from severe asthmatics illustrating isotype controls a) Rat IgG2a b) Mo IgG1, c) GM-CSF+ cells present in the bronchial submucosa and d) GM-CSFR+ cells in the submucosa (x400). GM-CSF/R+ cells highlighted by arrows.

Figure 3: The number of GM-CSF+ and GM-CSFR cells in the submucosa in asthma
The number of a) GM-CSF+ and b) GM-CSFR+ cells in the bronchial submucosa and c) GM-CSFR+ cells in the airway smooth muscle bundle (ASM) in healthy controls, mild-moderate asthma (GINA 1-3) and severe asthma (GINA 4-5). Across group comparisons by Kruskal-Wallis were all $p<0.05$, p values for Dunn's post-hoc test for between group comparisons are as shown.

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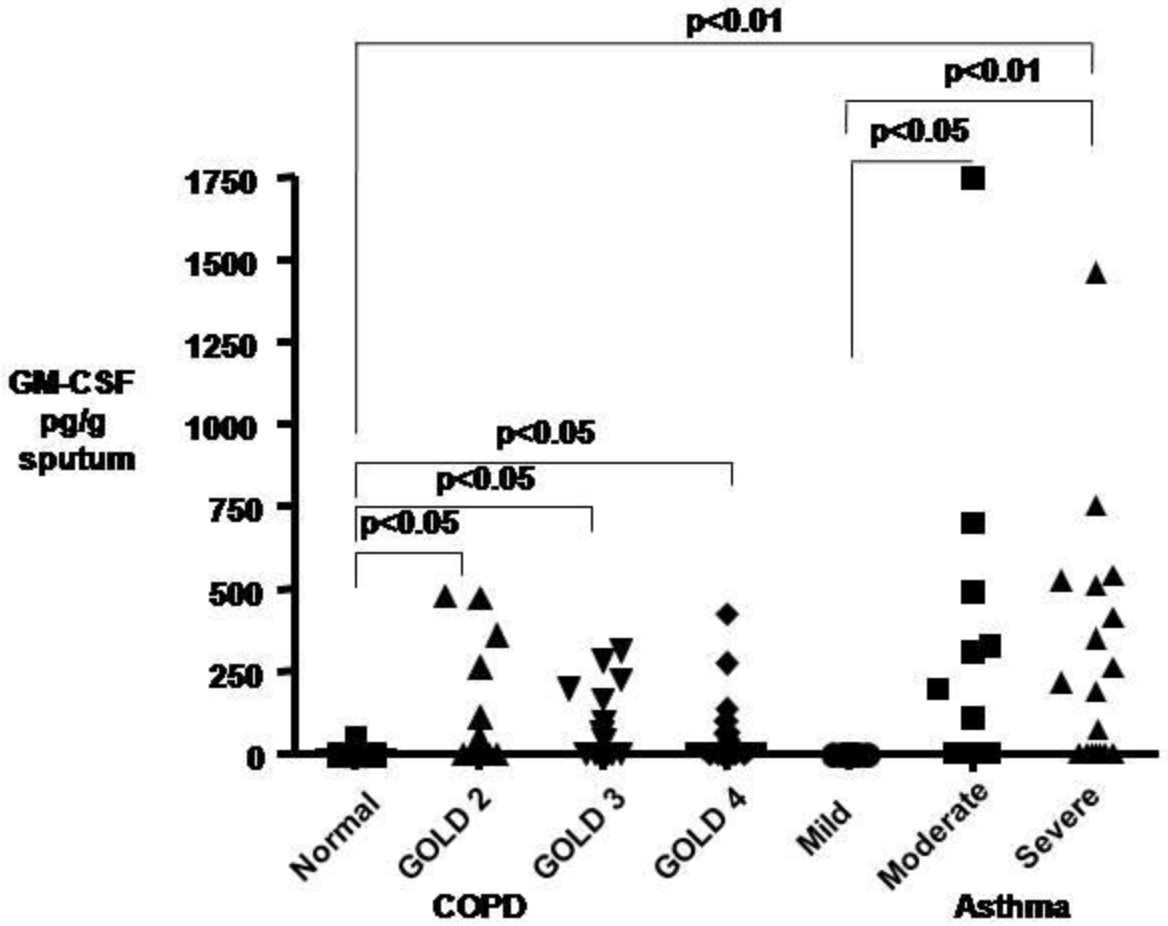
Figure 1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p<0.01
p<0.05
p<0.05
p<0.05
p<0.05
GM-CSF pg/g sputum
1750
1500
1250
1000
750
500
250
0
Normal
GOLD 2
GOLD 3
GOLD 4
Mild
Moderate
Severe
COPD
Asthma

Figure 2

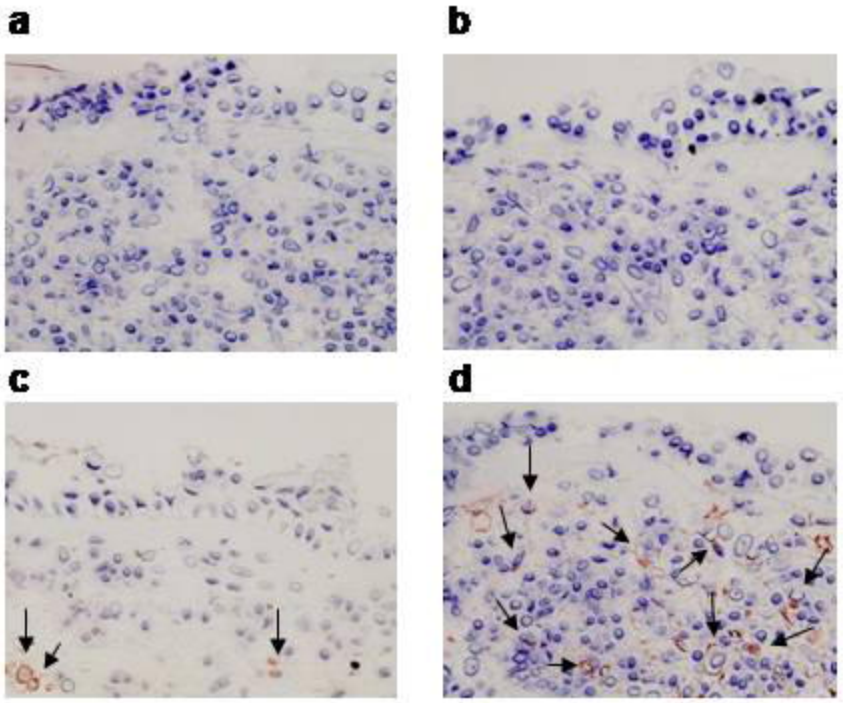
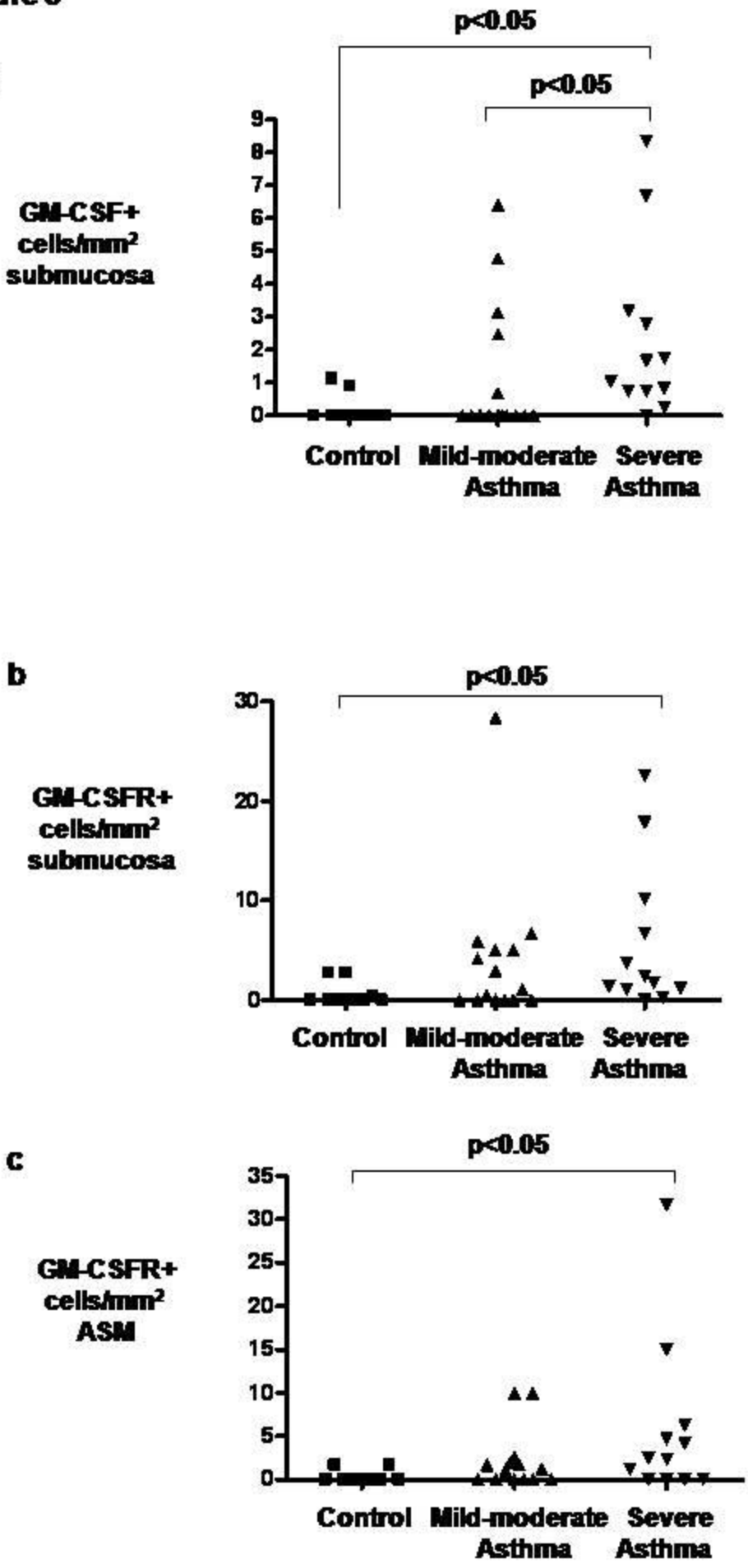


Figure 3


a
p<0.05
p<0.05
GM-CSF+ cells/mm² submucosa
9
8
7
6
5
4
3
2
1
0
Control
Mild-moderate Asthma
Severe Asthma
b
p<0.05
GM-CSFR+ cells/mm² submucosa
30
20
10
0
Control
Mild-moderate Asthma
Severe Asthma
c
p<0.05
GM-CSFR+ cells/mm² ASM
35
30
25
20
15
10
5
0
Control
Mild-moderate Asthma
Severe Asthma