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Sputum basophils are increased in eosinophilic asthma compared with non-eosinophilic asthma phenotypes

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Abstract

Sputum basophil numbers are increased in allergic asthmatics, but it is unclear what role airway basophils play in “TH2-low” asthma phenotypes. Using flow cytometry we found that basophils were significantly increased in all asthmatics (n=26) compared with healthy controls (n=8) (p=0.007) with highest levels observed in eosinophilic asthma (EA; median 0.22%, IQR 0.11-0.47%; n=14) compared with non-EA (0.06%, 0.00-0.20%; n=12; p<0.05). In asthmatics, basophils were positively correlated with sputum eosinophils (r=0.54; p<0.005), and inversely with sputum neutrophils (r=-0.46; p<0.05), but not with FEV₁ (% predicted), FEV₁/FVC, or bronchodilator reversibility. In a subgroup initially identified as inadequately controlled asthma (n=7), there was a trend (p=0.08) towards a reduction in sputum basophils following increased ICS treatment. Our findings suggest that basophils may be particularly important in eosinophilic asthma, and that sputum basophil assessment could be a useful additional indicator of ‘TH2-high’ asthma.

Key words:

Asthma, basophil, flow cytometry, eosinophil, biomarker

Author contributions

All authors were involved in the conception and design of the study. CRB, CV and JLS recruited participants, collected and processed samples, and conducted experiments. CRB and CVD conducted data analyses in consultation with JD, IFH, JLS and PGG. CRB wrote the

initial manuscript. All authors were involved in critical appraisal and revision of the manuscript, and all approved the final version.

Conflict of interest

Dr Gibson reports grants from GSK and Novartis, and personal fees from GSK, AstraZeneca, and Novartis, outside the submitted work. The remaining authors declare no conflict of interest.

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Basophils are important effector cells in TH2-mediated allergic immune responses (1). Previous studies of sputum and bronchial biopsies show that basophil numbers are increased in the airways of asthmatics, and further increase during exacerbations (2) or following allergen inhalation challenge (3). However, these studies primarily focussed on allergic asthmatics. Asthma is highly heterogeneous, and not exclusively associated with allergy hallmarks such as an increased TH2-gene signature or sputum eosinophil numbers (4). It is currently unclear what role basophils play in “TH2-low” asthma phenotypes such as non-eosinophilic, paucigranulocytic or neutrophilic asthma (5).

Whilst earlier studies were limited by the use of light microscopy and the small number of basophils in the airways, recent flow cytometric methods have considerably improved the ability to investigate rare cell populations in sputum (6-8). The aim of this study was to compare sputum basophil numbers between adult asthmatics and healthy controls and among

asthma inflammatory subtypes. We also examined the association between sputum basophil and eosinophil counts, and between sputum basophils and asthma characteristics. Finally, in a subgroup of asthmatics we determined whether basophil numbers changed following an increase in inhaled corticosteroid (ICS) dose.

Thirty four subjects were recruited from Wellington, New Zealand (16 asthmatics/7 non-asthmatics) and Newcastle, Australia (10 asthmatics/1 non-asthmatic). Asthma (n=26) was identified on the basis of a doctor's diagnosis and current wheeze, and in Newcastle, was confirmed by demonstrated airway hyperreactivity to hypertonic saline. All participants provided written informed consent and successfully underwent spirometry, skin-prick testing and sputum induction using hypertonic saline as described previously (5,7). Seven participants with inadequately controlled asthma at initial assessment (who were also amenable to asthma medication changes) had their treatment changed according to National Asthma Education and Prevention Program (NAEPP) Expert Panel Report 3 (EPR 3) guidelines (9) and were re-assessed 4-6 weeks after their first visit (10) to determine whether an increase in the prescribed ICS dose affected sputum basophil frequencies.

Sputum samples were processed according to a well-characterised protocol, with staining and assessment of sputum leucocyte proportions by light microscopy to identify asthma inflammatory phenotypes (EA was identified on the basis of >2% eosinophils (11), and NEA (<2% eosinophils) was stratified into NA and PGA on the basis of 61% neutrophils (5)). A fraction of sputum cells were prepared for flow cytometry as described previously (7). Briefly, samples were washed in Dulbecco's phosphate buffered saline (DPBS), incubated with live/dead fixable blue dye (Molecular Probes, OR, USA) on ice for 15 minutes, washed again (in FACS buffer; DPBS with 10mM ethylenediaminetetraacetic acid (EDTA) (Sigma-

Aldrich, MO, USA), 0.01% sodium azide (Sigma-Aldrich) and 2% foetal calf serum (FCS; SAFC Biosciences, KS, USA), and stained for 30 minutes on ice with antibody panels containing anti-CD45-APC-Cy7, anti-CD14-APC, anti-CD16-Alexa 700, anti-CD123-PE, anti-FcER1-FITC, and anti-HLA-DR-PerCP (all BD Biosciences, CA, USA; titrated to optimal concentrations). Aliquots were washed and re-suspended in FACS buffer/ neutral buffered formalin (Sigma-Aldrich, MO, USA). Flow cytometric analyses were conducted using a LSR II or FACSCanto flow cytometer, data collected using FACSDIVA™ (all BD Biosciences), and analysed using FlowJo software (Treestar Inc, OR, USA). Basophils were identified as a CD45 intermediate, non-debris/SSC low, live/dead blue low, HLA-DR low, CD16 low, CD14 low, CD123+ cell population also expressing FcεR1+, consistent with previous reports (6,8,12). Statistical associations (Prism 5; Graphpad Software, La Jolla, CA) were assessed using Spearman's rank correlation; differences between groups were assessed using the Mann-Whitney u-test, or Wilcoxon matched-pairs test for paired data, and a multi-group comparison was conducted using the Kruskal-Wallis test with Dunn's post-test.

Baseline characteristics are presented in Table 1. Basophil frequencies (expressed as a percentage of viable CD45+ leukocytes) were significantly higher in asthmatics (median 0.15%, IQR 0.06-0.28%) compared with healthy controls (0.02%, 0.01-0.03%; $p=0.007$; Figure 1A). When asthma was stratified into EA and NEA phenotypes (on the basis of $>2\%$ eosinophils (11)), basophil frequencies were significantly higher in EA (0.22%, 0.11-0.47%) than NEA (0.06%, 0.00-0.20%; $p<0.05$); NEA and healthy controls did not significantly differ (Figure 1B). Similar findings were observed using 1% or 3% eosinophil cut-offs to identify phenotypes (data not shown). Further stratification of NEA into PGA and NA (5) suggested that neither phenotype was specifically associated with increased sputum basophil

percentages (Figure 1C), although 4/7 of PGA and 1/5 NA had basophil frequencies substantially higher (at least three times) than any of the healthy controls tested.

There was a significant positive correlation between basophils and sputum eosinophils in participants with asthma ($r=0.54$; $p=0.005$; Figure 1D), and an inverse association with sputum neutrophils ($r=-0.46$; $p=0.02$; Figure 1E). No significant associations with demographic (i.e. age, gender) or lung function parameters (i.e. FEV1 (% predicted), FEV1/FVC, or bronchodilator reversibility) were observed (data not shown).

Of the group undergoing treatment changes, 5 were initially identified as EA and 2 as PGA. A non-significant trend towards a basophil reduction was observed following an increase in ICS dose (0.47%, 0.1-1.29% versus 0.21%, 0.09-0.53%; $p=0.08$; Figure 1F) with basophil frequency reduced in 5/7 asthmatics (4 of whom were initially identified as EA, 1 as PGA).

These results support previous findings showing that airway basophil numbers are significantly increased in asthma and (although only borderline statistically significant in our study) may also be further increased in inadequately controlled asthma (2,3,6,12). In addition, we found that increased sputum basophils are associated with the “TH2-high” phenotype and are positively correlated with eosinophils and inversely with neutrophils. Whilst an earlier study (3) observed that basophil frequencies may be associated with alterations in physiological parameters such as airway hyperreactivity or bronchodilator response, we observed no association with clinical characteristics or lung function. However, as the aforementioned study assessed only atopic asthmatics with pre-identified allergen reactivity, and we investigated a heterogeneous group (without challenge testing), direct comparison is difficult.

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One previous study (13) showed that non-atopic asthmatics had intermediate levels of basophils compared to atopic asthmatics (who had the highest levels) and non-asthmatics, but no comparisons on the basis of airway inflammation was conducted. Thus, to our knowledge, this is the first study to assess sputum basophils in different asthma phenotypes/endotypes, the results of which suggest that basophils may be particularly important in EA (associated with TH2-inflammation). However, in some individuals (5/12) classified as NEA (of which 4 PGA, 1 NA), basophil levels were at least three times higher than the maximum found in non-asthmatics. As sputum basophils levels were negligible (less than 0.05% of viable sputum leukocytes) in healthy controls, regardless of atopic status, we speculate that the presence of even a small discrete population of basophils, as observed in those classified as NEA, may be indicative of underlying TH2-mediated airway pathology. If true, using a 2% eosinophil cut-off to differentiate between the TH2-high EA and TH2-low NEA phenotypes might result in misclassification. The assessment of basophil numbers in addition to eosinophils may therefore be a useful additional indicator of TH2-high asthma phenotypes, and/or in further elucidating the involvement of different effector cells involved in airway inflammation in asthma. This is important when considering that, despite the correlation between sputum eosinophils and basophils observed in our study, numbers of both cell types (and neutrophils) may differentially fluctuate over time in affected airways. In particular, although basophils and eosinophils have similar recruitment pathways, there are examples of selective, temporally variable recruitment of one or other cell population into affected tissues during allergic inflammation (14), which may partly explain the relatively moderate associations found.

We observed little evidence of increased basophils in NA, with a significant negative correlation observed between sputum basophils and neutrophils and only 1/5 NA having more than 0.01% sputum basophils. Our data therefore supports previous reports suggesting that there is little or no involvement of TH2-mediated processes in NA, which is more likely to involve innate cytokine driven responses (involving IL-1 α and IL-8) to epithelial cell-derived alarmins or subclinical infection (15).

This study has some limitations. Firstly, only small numbers of asthmatics were represented in each sub-group. Secondly, with the exception of the seven asthmatics undergoing change in treatment, this was a cross-sectional study. We were unable to determine temporal variance in basophil frequencies and what, if any effect current ICS use had upon basophil frequencies in the remaining asthmatics. It therefore remains unclear to what extent basophil numbers are reduced in optimally controlled asthma and whether this is dependent on asthma phenotype. Although we saw reduced basophils in 5/7 asthmatics with increased doses of ICS, median basophil numbers remained significantly higher than in non-asthmatics, but these findings were based on a small group consisting of largely EA (i.e. 5/7). Larger studies are thus required.

In conclusion, this study, using flow cytometry, confirmed that sputum basophils are increased in asthmatics compared to non-asthmatics, and that basophil frequency correlates with eosinophil numbers. As a consequence there were also significant differences in basophil frequencies between EA and NEA. ICS treatment may reduce basophil levels, but results were inconclusive. Measurement of sputum basophils may be a useful additional biomarker of TH₂-mediated asthmatic inflammation, however, further studies are required to assess the stability of basophil numbers over time and in response to ICS treatment.

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Figure 1. Sputum basophil analysis.

(A) Sputum basophil percentages (of viable leucocytes) in asthma/no asthma. (B) Sputum basophil percentages in EA, NEA and no asthma. (C) Sputum basophil percentages in asthma inflammatory phenotypes as described in (5). (D) Correlation of sputum basophils and eosinophils. (E) Correlation of sputum basophils and neutrophils. (F) Change in sputum basophil percentage following an increase in ICS dose. For inclusion of data on figures 1D and E, when eosinophils or basophils were undetected (0%), data is expressed as 0.1-0.001% i.e. at least one log below detectable. *P<0.05, **P<0.005, ***P<0.001

Table 1. Clinical characteristics of participants at baseline visit (expressed as number, percentage or median/IQR). P values calculated using the Mann-Whitney U test or Fisher's exact test.

	Asthma	Non-asthma	P value
Number (female)	26 (17)	8 (5)	
Age	39.5 (34.8-56.3)	54.0 (34.5-63.0)	0.310
Atopy % (number)	80% (20)	25% (2)	0.008
ICS use % (number)	85% (22)	-	
FEV ₁ (% predicted)	89.3 (76.7-94.8)	94.8 (87.4-102.8)	0.142
FEV ₁ /FVC	0.69 (0.65-0.74)	0.78 (0.74-0.83)	0.010
Bronchodilator reversibility (% change)	9.9 (5.8-16.0)	3.2 (1.8-4.7)	0.006
Sputum eosinophils %	2.1 (1.2-5.3)	0.0 (0.0-0.8)	0.0005
Sputum neutrophils %	35.4 (22.4-44.8)	41.9 (18.2-55.3)	0.53

