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Impaired macrophage phagocytosis in non-eosinophilic asthma

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Summary

Background Many patients with non-eosinophilic asthma have increased numbers of neutrophils in the airways. The explanation for this chronic inflammation remains unclear, but may result from an impaired ability of alveolar macrophages to phagocytose apoptotic cells (a process termed 'efferocytosis'), as we have shown in chronic obstructive pulmonary disease (COPD).

Objectives To examine induced sputum as a non-invasive technique to characterize efferocytosis in chronic lung diseases and to compare efferocytosis in patients with non-eosinophilic asthma, eosinophilic asthma and COPD.

Methods Participants with stable asthma (20 with eosinophilic and 30 with non-eosinophilic) and COPD ($n = 11$) underwent clinical assessment including allergy skin tests, saline challenge and sputum induction. Sputum cells were dispersed using dithiothreitol and resuspended in culture medium. Efferocytosis of apoptotic bronchial epithelial cells by sputum-derived macrophages was determined using flow cytometry.

Results There were no significant differences in efferocytosis between paired sputum and bronchoalveolar lavage macrophages from three subjects. Efferocytosis was significantly impaired in patients with non-eosinophilic asthma [mean (SD) 0.95 (0.24)] compared with eosinophilic asthma [1.17 (0.19)] and to a similar degree as patients with COPD [1.04 (0.16)]. Sputum neutrophils were significantly higher in patients with COPD and non-eosinophilic asthma compared with eosinophilic asthma.

Conclusion and Clinical Relevance Induced sputum provides a reliable and non-invasive method for studying macrophage efferocytosis in chronic lung disease. Macrophage efferocytosis is impaired in non-eosinophilic asthma to a similar degree as that in COPD and may explain the persistent airway neutrophilia and chronic inflammation that characterizes this asthma subtype.

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Introduction

We, and others, have shown that asthma is composed of several subtypes with up to 50% of all asthma cases showing no evidence of eosinophilic inflammation and a persistence of airway neutrophilia [non-eosinophilic asthma (NEA)] [1–5]. NEA was initially described in uncontrolled asthmatics with normal sputum eosinophil counts [6], and since this time, it has been identified in stable [1] and acute asthma [7, 8], severe corticosteroid-dependent asthma [9] and persistent asthma [10].

NEA is associated with a poor response to inhaled corticosteroids [6]. NEA also occurs in steroid-free individuals [11], and the absence of eosinophils in NEA has also been confirmed in bronchial tissue by both endo-bronchial biopsy [4, 9] and post-mortem examination [12].

The presence of non-eosinophilic exacerbations has also been well documented in studies of acute asthma where viral infection induces airway neutrophilia [13]. Importantly, patients with persistent asthma experience more non-eosinophilic exacerbations than eosinophilic

exacerbations; these exacerbations are not prevented by corticosteroid treatment. There is therefore a need to characterize more fully the reasons for the chronic inflammation and neutrophilic accumulation and to define effective therapeutic options for NEA.

Our previous studies have focused on the role of apoptosis and macrophage dysfunction in chronic obstructive pulmonary disease (COPD) which is also characterized by defective airway repair, chronic inflammation and an accumulation of neutrophils in the airway. We have shown that alveolar macrophages from subjects with COPD have significantly reduced ability to phagocytose apoptotic bronchial epithelial cells (a process termed 'efferocytosis') [14–17]. We have shown that the impaired clearance of accumulated apoptotic cells has the potential to lead to secondary necrosis of the uncleared material and perpetuation of inflammation [18], and that macrophage-directed therapies have the potential to improve efferocytosis and reduce airway inflammation in COPD and smoking mice [17, 19–21].

Efferocytosis is also likely to be important in the airways of patients with NEA, where many of the disease characteristics appear to mirror those found in COPD (e.g. the neutrophilic influx, chronic inflammation and relative insensitivity to corticosteroids); however, despite numerous studies of phagocytosis of bacteria in asthma, there have been limited studies of efferocytosis in asthma and no studies specifically assessing macrophage function in NEA. One study investigated the ability of alveolar macrophages to phagocytose apoptotic T cells in severe, oral steroid-dependent patients with asthma [22] and found that alveolar macrophages had reduced ability to phagocytose the apoptotic cells. The patients, however, were not grouped on the basis of eosinophilic or NEA. Previous methods for assessing pulmonary macrophage phagocytic or efferocytic function have relied largely on obtaining alveolar macrophages from flexible bronchoscopy. This method has proved to be reliable and has produced several key findings with regard to the pathogenesis of COPD [14–17]; however, it is relatively invasive and not suited to large-scale studies of pathophysiology and treatments.

This study addressed the hypothesis that analysis of induced sputum would provide a non-invasive technique to characterize macrophage efferocytic function in chronic lung diseases including asthma and COPD. We further hypothesized that efferocytosis would be impaired in NEA to a similar extent as that found in COPD and that this defect may contribute to the persistent airway neutrophilia and chronic inflammation that characterizes this asthma subtype. We investigated efferocytosis using macrophages from induced sputum from participants with stable asthma (both eosinophilic and NEA) and COPD.

Materials and methods

Subject population

Efferocytosis was investigated in participants with stable asthma (20 with eosinophilic and 30 with NEA) or COPD ($n = 11$). No patients were receiving oral corticosteroids. Subjects underwent a clinical assessment which included history of smoking, respiratory symptoms and allergy, and sputum induction. Ethical approval was granted by both the Royal Adelaide Hospital Ethics Committee and the Hunter New England Human Research Ethics Committee. Written informed consent was obtained for each patient or control recruited for the study. The diagnosis of COPD was established using the GOLD criteria ($FEV_1/FVC < 70\%$) with clinical correlation [23]. The diagnosis of asthma was based on a history of variable symptoms and the presence of symptoms with airways hyperresponsiveness to hypertonic saline or a clinically significant bronchodilator response ($> 12\%$ improvement in FEV_1).

Sputum induction

Spirometry (KoKo PD Instrumentation, Louisville, CO, USA) and sputum induction with hypertonic saline (4.5%) were performed as previously described [24]. A fixed sputum induction time of 15 min was used for all subjects.

Processing of induced sputum

Induced sputum was processed as we have previously reported [24]. Briefly, sputum cells were dispersed using dithiothreitol, prepared for differential cell counts and cells resuspended in RPMI1640 (1% FCS, 0.5% HEPES, 2% Pen/strep, 1% Amphotericin Fungizone) [24]. Samples were collected from two Australian sites and all testing performed at 24 h following collection.

Flexible bronchoscopy

For comparison with macrophages obtained from induced sputum, alveolar macrophages were obtained from bronchoalveolar lavage (BAL) from three healthy subjects within 5 days of sputum induction. Bronchoscopy was performed according to American Thoracic Society recommendations as previously reported [14–18]. Cells from BAL were washed in RPMI 1640 (Gibco, BRL, Germany) and re-suspended in RPMI supplemented with 10% fetal calf serum and 1% weight per volume penicillin/streptomycin (Gibco) (culture medium). Macrophages were purified by adhesion to plastic for 1 h as previously described [14–18].

Efferocytosis ability of sputum-derived macrophages

Efferocytosis was investigated as we have previously described [14–17]. Briefly, our *in vitro* flow-cytometric assay quantifies phagocytosis of target cells (apoptotic bronchial epithelial cells) by macrophages (re-suspended at a concentration of 4×10^5 macrophages/mL and purified by adhesion to plastic). Apoptosis is induced in the target cell by exposure to UV [these cells are stained with mitotracker red (Molecular Probes, Eugene, OR, USA)] and ingested cells identified using flow cytometry and co-staining with a macrophage marker {CD33 [phycoerythrin cyanide-5 (PC-5)] (Immunotech/Coulter, Marseille, France)} and mitotracker red.

Optimization of techniques

Influence of time post-collection. We optimized specimen delivery between the centres by investigating efferocytosis in sputum collected from three healthy and one participant with asthma at 0- and 24-h post-collection. Efferocytosis was assessed as described above.

BAL vs. sputum-derived macrophages. A comparison between the 'gold standard technique' of measuring efferocytosis in BAL-derived alveolar macrophages and sputum-derived macrophages was undertaken in three healthy subjects from whom both sputum and BAL were collected and tested within a 5-day period.

Intra-subject variability in sputum parameters and efferocytosis. To assess intra-subject variability in sputum parameters and efferocytosis in samples collected at different time points, repeat sputum was collected from 12 subjects 2 weeks following the first collection. Efferocytosis and patient classification (eosinophilic or NEA) were compared.

Statistical analyses

Data were analysed using Stata 11 (Stata Corporation, College Station, TX, USA). Results are reported as mean

(SD) or median (interquartile range) unless otherwise indicated. Analysis was performed using the two-sample Wilcoxon rank sum test, and the Kruskal–Wallis test was used for more than two groups. Fisher's exact test was used for categorical data. Phagocytosis data were log transformed and analysed using ANOVA with Bonferroni correction. Associations between data were determined using the Spearman rank correlation. All results were reported as significant when $P < 0.05$. Predictor variables were included in the multiple linear regressions if $P < 0.1$ in simple linear regression and known confounders (age and gender) were included in all models. Predictor variables were tested for collinearity using STATA's variance inflation factors post-estimation. Bland–Altman plots of Difference vs. Average were used to assess within-subject variation.

Results

Subject demographics

Subjects with eosinophilic asthma were slightly younger than the NEA and COPD groups (Table 1). Patients with COPD had smoked more in the past and had a reduced FEV₁/FVC compared with NEA. Significantly more patients with asthma were taking ICS compared with those with COPD; however, there was no difference in the reported dose (Table 1). Using simple linear regression, there was an association between ICS dose and phagocytosis but not between smoking pack years and phagocytosis ($P > 0.100$). For ICS dose $n = 57$, $P = 0.060$, coefficient -0.0000723 (-0.0001479 to -0.0000326).

Sputum neutrophils were significantly higher in patients with COPD and NEA compared with eosinophilic asthma (Table 2).

Efferocytosis ability of sputum macrophages

Efferocytosis was significantly impaired in patients with non-eosinophilic asthma [$\log_{10}$ mean (SD) 0.95 (0.24)]

Table 1. Clinical characteristics of subjects used for macrophage phenotype analyses

	EA	NEA	COPD	P
N	20	30	11	
Age, years mean (SD)	57 (15)*	62 (14)	73 (10)	0.010
Sex, male (%)	9 (45)	13 (43)	9 (82)	0.089
Ex-smokers n (%)	6 (30)*	12 (40)*	10 (91)	0.003
Pack years, median (q1, q3)	8.0 (0.5–28.6)*	10.4 (1.8–48.0)*	86.5 (42–105)	0.007
Taking ICS n (%)	19 (95)	28 (93)	6 (55)	0.007
ICS dose bdp equivalents median (q1, q3)	1000 (800–2000)	1800 (1000–2000)	1500 (1000–2000)	0.258
FEV ₁ % predicted mean (SD)	70 (21)	70 (18)	63 (20)	0.570
FEV ₁ /FVC% mean (SD)	64 (11)	69 (10)*	58 (8)	0.017

* $P < 0.05$ vs. COPD.

Table 2. Inflammatory cell counts

	EA	NEA	COPD	P
Total cells $\times 10^6/\text{mL}$	6.5 (2.6–8.1)	7.1 (3.2–14.0)	5.0 (3.1–7.8)	0.309
Viability, %	71 (57–79)	81 (63–90)	80 (63–89)	0.082
Neutrophils, %	35.1 (15.4–54.0)* [†]	51.3 (28.3–70.5)	63.3 (48.3–71.5)	0.014
Neutrophil $\times 10^4/\text{mL}$	88.20 (48.26–336.96)	248.06 (94.19–1224)	355.01 (202.30–579.92)	0.071
Eosinophils, %	8.8 (4.5–21.9)* [†]	0.8 (0.5–1.3)	1.1 (0.0–2.0)	<0.001
Macrophages, %	44.1 (23.6–59.1)	42.3 (23.0–67.3)	29.5 (21.5–43.3)	0.485
Lymphocytes, %	0.6 (0.0–1.3)	0.5 (0.0–1.0)	0.8 (0.3–1.8)	0.658
Columnar epithelial cells, %	1.1 (0.1–5.5)	0.8 (0.3–3.3)	2.0 (0.3–7.3)	0.803
Squamous, %	2.9 (1.2–7.5)	2.5 (0.7–5.0)	4.6 (2.9–14.4)	0.275

* $P < 0.05$ vs. COPD.† $P < 0.05$ vs. NEA.

compared with eosinophilic asthma [1.17 (0.19)] and to a similar degree as patients with COPD [1.04 (0.16)] (Fig. 1).

Multiple regression showed that age was associated with sputum macrophage efferocytosis, independently of gender and ICS dose (co-efficient -0.006 , $P = 0.008$, Model adjusted R^2 15.98%).

Optimization of techniques

Influence of time post-collection. Efferocytosis was reduced in induced sputum at 24-h vs. 0-h post-collection although not significantly (mean 0 h 17.4% $\pm$ SEM 3.0% vs. 24 h 13.0% $\pm$ SEM 1.4%). Therefore, all testing of samples from both centres was performed at exactly 24-h post-collection.

BAL vs. sputum-derived macrophages. A comparison between efferocytosis using BAL-derived alveolar macrophages and sputum-derived macrophages from three

of healthy subjects from whom both sputum and BAL were collected within a 5-day period was undertaken. Efferocytic function of macrophages was not significantly different between the two methods of collection (mean BAL 15.2% $\pm$ SEM 4.4% vs. Sputum 14.7% $\pm$ SEM 4.9%).

Intra-subject variability in sputum parameters and efferocytosis. The phagocytosis results were highly correlated over two visits $r = 0.73$, $P = 0.007$ and the intraclass correlation co-efficient was 0.8514 (95% CI 0.5639–0.9548). Inflammatory phenotype classification showed good agreement over two visits with a kappa statistic of 0.792 (0.413–1.00). Bland–Altman plots of Difference vs. Average showed that the variability was even and small, with a bias at -1.082 suggesting that the second sample gave a lower result of only 1% (Fig. 2).

Discussion

In this study, we confirmed our previous studies that have shown differences in mechanistic pathways

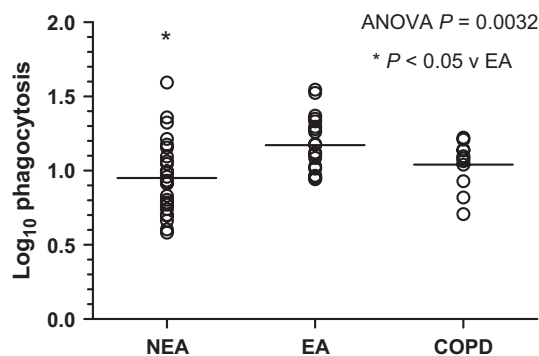


Fig. 1. Efferocytosis of apoptotic bronchial epithelial cells by sputum-derived macrophages from asthmatics with non-eosinophilic (NEA) or eosinophilic (EA) disease and patients with COPD. Results are individual data points expressed as the $\log_{10}$ percentage of macrophages ingesting apoptotic cells. The horizontal line represents the mean value for each group * $P < 0.05$ compared to patients with eosinophilic asthma.

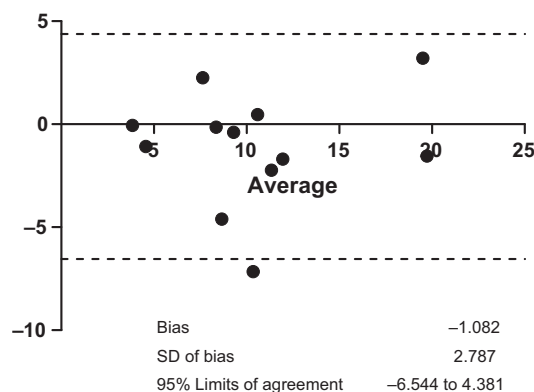


Fig. 2. Bland–Altman plots of Difference vs. Average. Note even and small variability, with a bias at -1.082 suggesting that the second sample gave a lower result of only 1%.

between NEA and eosinophilic asthma. We found that the efferocytosis ability of airway macrophages obtained from induced sputum was comparable with that in macrophages obtained from the more invasive technique of bronchoscopy and BAL. Using induced sputum we showed that compared with patients with eosinophilic asthma, efferocytosis is significantly reduced in the airway of patients with NEA, to a similar extent as found in COPD. Our previous studies have focused on the role of apoptosis and macrophage dysfunction in COPD which is also characterized by defective airway repair, chronic inflammation and the accumulation of neutrophils in the airway. We were the first to identify the accumulation of apoptotic material and impaired clearance of this material by macrophages in the airways of smokers and patients with COPD [13–17] and have shown that uncleared apoptotic material may undergo secondary necrosis with pro-inflammatory effects [17]. Our current findings suggest that NEA may follow this pattern as the significant reduction in efferocytic function observed in NEA was similar to that found in our COPD subjects. It is probable that these defects may have diverse effects in the lung and may perpetuate a chronic inflammatory response, tissue damage and persistent neutrophilia in NEA. It is noteworthy that in asthma the epithelium is fragile and shedding of airway surface epithelium have been reported in histologic studies, although comparisons between NEA and eosinophilic asthma in this regard has not been undertaken [25]. Although the present study focused on phagocytosis of apoptotic airway epithelial cells, it is also likely that phagocytosis of neutrophils is an important mechanism for regulation of their numbers in asthma, as we have previously shown that the phagocytic defect in COPD subjects was comparable whether airway epithelial cells or neutrophils were used as phagocytic targets [14]. Our further study showed that treating COPD patients with low-dose azithromycin improved phagocytosis of both cell types (neutrophil data unpublished) and this was associated with significant decrease in the total WCC, a non-significant decrease in neutrophil numbers and reduced inflammation (hsCRP) [16].

We have previously established that in NEA there is dysfunction of the innate immune response with increased gene expression for toll-like receptors 2 and 4, increased IL-8 and IL-1 β [26] and increases in the proteolytic enzymes neutrophil elastase and total matrix metalloproteinase-9 (MMP-9) [27]. Our current findings indicate that these changes may at least partially result from the presence of an increased inflammatory burden in the NEA airway as a result of uncleared apoptotic material.

Defective macrophage phagocytic ability has been previously reported in asthma, although most of the

previous studies focused on phagocytosis of bacteria or IgG opsonized yeast [28, 29]. Fitzpatrick and colleagues [29] compared normal volunteers with non-asthmatic children with chronic cough and those with moderate and severe asthma. A decreased ability of alveolar macrophages to ingest *S. aureus* was noted in the children with poorly controlled asthma. Increased rates of infection by agents that include rhinovirus cause significant exacerbations of asthma. Oliver et al. recently reported that rhinovirus exposure caused a reduced macrophage phagocytic response to labelled bacterial particles but not to latex beads, suggesting a specific defect in macrophage phagocytic ability in response to rhinovirus infection [30]. Despite these numerous studies of phagocytosis of bacteria, there have been few studies of efferocytosis in asthma. Huynh and colleagues compared the ability of alveolar macrophages to phagocytose apoptotic T-cell line Jurkats in normal volunteers, mild to moderate asthmatics, and severe, oral steroid dependant asthmatics [22]. They initially noted a reduced number of phagocytic bodies in the severe asthmatics but not in the mild-moderate group compared with normal subjects. Further *ex-vivo* studies confirmed that alveolar macrophages from severe asthmatics had reduced ability to phagocytose the apoptotic cells. Interestingly, macrophages from the severe group were resistant to phagocytosis-stimulating effect of LPS, but were responsive to dexamethasone, whereas macrophages from the mild to moderate asthmatics responded in a similar fashion to the normal subjects. The severe asthmatics were not categorized on the basis of eosinophilic or non-eosinophilic disease, although there was a trend for an increased percentage of neutrophils in the severe asthma group.

Tobacco smoking is common in asthma; up to 30% of asthmatic subjects are current or ex-smokers. Cigarette smoking induces an additional neutrophilic burden [31]. In this study, we excluded asthmatics who were currently smoking, although a larger study is warranted to fully assess the potential effect of smoking history on macrophage function in NEA and eosinophilic asthma. It is noteworthy that in our studies of COPD subjects, a comparable defect in macrophage function was found for both current- and ex-smokers with the disease. A potential drawback of the study was that subjects with eosinophilic asthma were younger than the COPD group; however, we have previously shown that patients with eosinophilic asthma are younger than those with asthma without eosinophils and high proportions of neutrophils [2]. Although we standardized our investigations by assessing phagocytosis for all subjects at 24-h post-collection, the slight decrease in phagocytosis observed over the 24-h time frame in our optimization experiments was interesting. It is conceivable

that macrophages from patients with NEA have a different time course but a similar peak effect, and this requires further study. In addition, the use of image analysis to assess cytoplasmic hue change after ingestion of apoptotic material may provide a potentially more sensitive biomarker of eosinophilic airway inflammation [32].

The optimal treatment of NEA is not known; however, corticosteroids have little efficacy in this subtype of asthma [4, 6]. This is consistent with the dominant action of corticosteroids to reduce eosinophilic inflammation, and their ability to potentiate neutrophilia by inhibition of neutrophil apoptosis. In addition to their established anti-bacterial role, there is both *in vitro* and *in vivo* evidence for an anti-inflammatory activity of macrolides, and some evidence that they may be efficacious in neutrophil-mediated airway diseases. We have shown that in patients with COPD, azithromycin improved the phagocytosis of apoptotic epithelial cells, apoptotic neutrophils and bacteria [16,19]. While the exact anti-inflammatory mechanisms of macrolide antibiotics are unknown, our data suggest that part of the inflammatory action of macrolides may be through restoration of phagocytosis and removal of apoptotic cells prior to secondary necrosis.

The findings of this study suggest that investigating efferocytosis using sputum macrophages provides a relatively non-invasive technique for large-scale studies of asthma pathophysiology and treatments, and we are currently investigating efferocytosis as a component of a large multi-centre trial of azithromycin treatment in asthma.

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