

Eosinophil protein in airway macrophages: A novel biomarker of eosinophilic inflammation in patients with asthma

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Background: Noneosinophilic asthma is common across asthma severities. However, in patients with moderate-to-severe disease, the absence of sputum eosinophilia cannot distinguish between asthmatic subjects with eosinophilic inflammation controlled by corticosteroids versus those in whom eosinophilic inflammation is not a component of the disease.

Objectives: We sought to develop a method to quantify eosinophil proteins in airway macrophages as a novel biomarker of eosinophilic airway inflammation.

Methods: Eosinophil proteins in airway macrophages were assessed by means of flow cytometry, immunofluorescence, and cytoplasmic hue change after ingestion of apoptotic eosinophils. Airway macrophage median percentage of red-hued area in stained sputum cytospin preparations was assessed by means of image analysis from (1) subjects with mild-to-severe asthma, subjects with nonasthmatic eosinophilic bronchitis, and healthy control subjects; (2) subjects with eosinophilic severe asthma after treatment with prednisolone; and (3) subject with noneosinophilic asthma before corticosteroid withdrawal.

Results: Eosinophil proteins were detected in airway macrophages, and cytoplasmic red hue increased after ingestion of apoptotic eosinophils. Airway macrophage percentage red-hued area was increased in subjects with moderate-to-severe asthma compared with that seen in subjects with mild asthma and healthy control subjects, was similar in those with or without a sputum eosinophilia, and was increased after corticosteroid therapy. In asthmatic subjects without sputum eosinophilia, the airway macrophage percentage red-hued area was increased in subjects who did versus those who did not have sputum eosinophilia after corticosteroid withdrawal.

Conclusions: Eosinophil proteins can be reliably measured in airway macrophages. In combination with sputum eosinophilia, the macrophage eosinophil protein content might further define

the asthma phenotype and provide an additional tool to direct therapy. (*J Allergy Clin Immunol* 2010;■■■■:■■■-■■■.)

Key words: Asthma, macrophage, eosinophil, computer-assisted image analysis, induced sputum

There is increasing recognition that asthma is a heterogeneous condition, particularly in those with severe disease.¹ The currently available biomarkers are able to detect recent but not ongoing inflammation, especially in treated subjects. Sputum induction has allowed the analysis of large numbers of asthmatic subjects and has led to the identification of different inflammatory phenotypes defined by their granulocytic composition, namely eosinophilic, neutrophilic, mixed granulocytic, and paucigranulocytic.²

Eosinophilic inflammation is considered a hallmark of asthma, but only about 50% of asthmatic subjects have sputum evidence of eosinophilic inflammation.³ The eosinophilic phenotype is associated with a good response to corticosteroids⁴ and anti-IL-5 mAb therapy.^{5,6} Directing corticosteroid therapy to normalize the sputum eosinophil count in subjects with severe asthma has also led to a reduction in exacerbations without an increase in overall corticosteroid therapy.^{7,8} The success of this approach was due to the appropriate up-titration and down-titration of therapy. However, after corticosteroid withdrawal in subjects with severe asthma, there is often an emergence of eosinophilic inflammation.⁹ The natural variation in disease, corticosteroid treatment, and resolution can alter the sputum eosinophil differential count.¹⁰ Therefore a normal eosinophil count might reflect controlled eosinophilic inflammation as a consequence of corticosteroid treatment or might identify a group of asthmatic subjects in which the eosinophil is not an important factor in the disease's pathogenesis. Therefore there is a need for noninvasive biomarkers to estimate the eosinophilic load within the airway.

The burden of eosinophilic airway inflammation within a subject is a constant balance between eosinophil influx into the airway wall and clearance by airway macrophages.¹¹ Although eosinophil products are demonstrated in macrophages,^{11,12} to date, the eosinophil load within the macrophage is not quantified or used to assess past eosinophilic inflammation. This might provide additional insight into the kinetics of eosinophilic inflammation and identify patients with high macrophage eosinophil content that hitherto have been labeled as noneosinophilic.

We hypothesized that airway macrophage eosinophil protein content is increased in subjects with severe asthma independent of sputum eosinophil count and that the combination of a normal

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Abbreviations used

APC: Allophycocyanin
 ECP: Eosinophil cationic protein
 EPO: Eosinophil peroxidase
 FITC: Fluorescein isothiocyanate
 GINA: Global Initiative for Asthma
 NAEB: Nonasthmatic eosinophilic bronchitis

sputum eosinophil count and low airway macrophage eosinophil protein content identifies a true noneosinophilic phenotype that predicts success of weaning from corticosteroids. To test our hypothesis, we aimed to (1) develop a method to quantify eosinophil proteins in airway macrophages as a novel biomarker of eosinophilic airway inflammation and (2) compare airway macrophage eosinophil content across asthma severities, in subjects with nonasthmatic eosinophilic bronchitis (NAEB),

and in healthy control subject subjects, as well as its response to corticosteroid therapy and withdrawal.

METHODS**Subjects**

Subjects with asthma ($n = 72$) and NAEB ($n = 15$) and healthy control subjects ($n = 10$) were recruited from patients attending Glenfield Hospital, Leicester, United Kingdom; hospital staff; and respondents to local advertising. Asthma was defined and classified according to the Global Initiative for Asthma (GINA) guidelines,¹³ and NAEB was defined as per the American College of Chest Physicians' guidelines.¹⁴ Subjects underwent standardized clinical assessment, including spirometry, skin prick testing to common aeroallergens, methacholine challenge testing,¹⁵ and sputum induction.¹⁶ All of the subjects had participated in previous studies.^{5,7,17,18} The study was approved by the Leicestershire Research Committee, and subjects provided written informed consent.

Romanowsky-stained sputum cytospin preparations were obtained from (1) subjects with mild (GINA class I), moderate (GINA class II/III), and severe

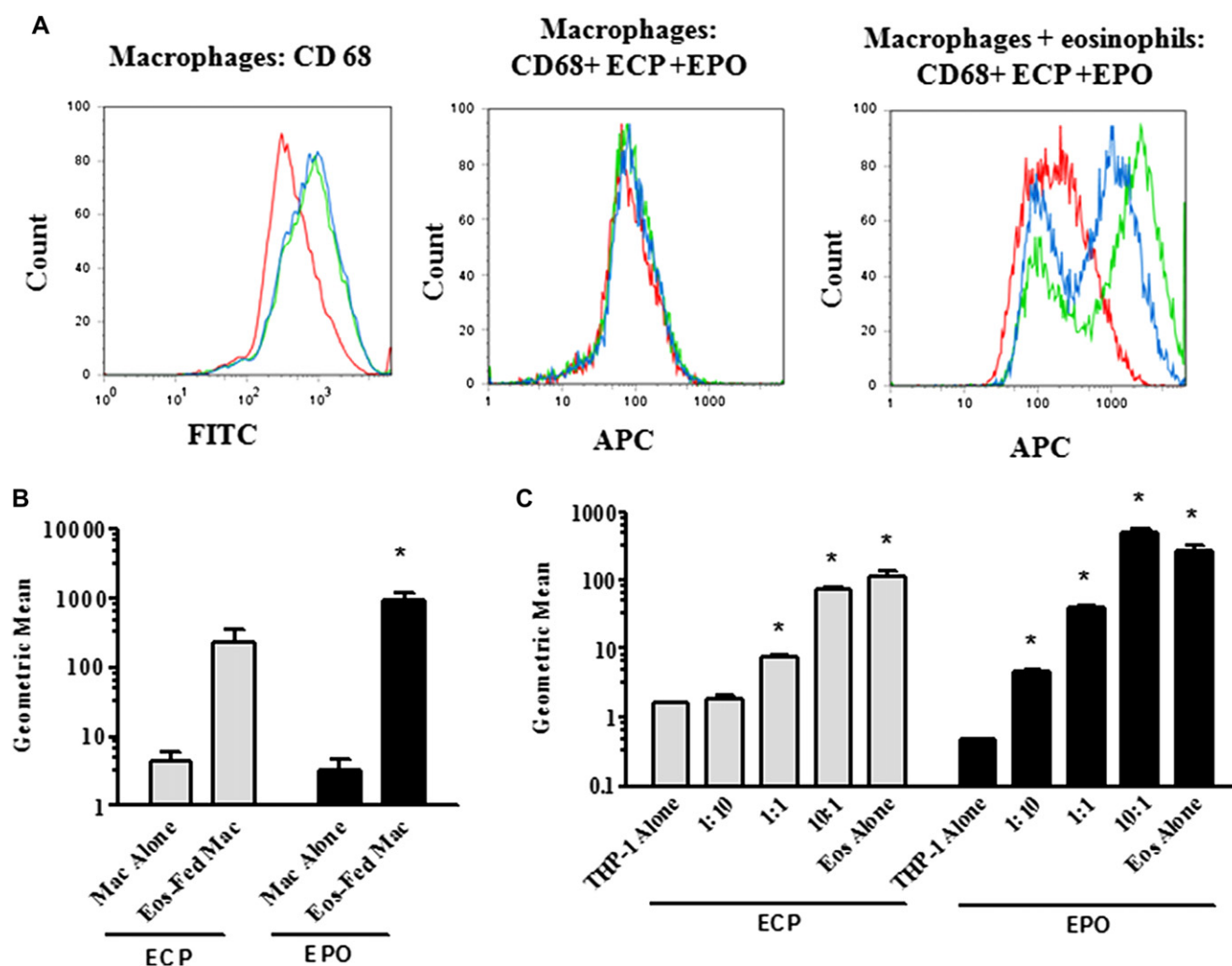


FIG 1. ECP and EPO expression within macrophages and THP-1 cells: **A** macrophages gated by size, granularity, and CD68 expression (left panel) and protein expression (middle and right panels). Representative histograms of macrophages cultured in the absence (middle panel) or presence (right panel) of apoptotic eosinophils are shown. ECP (blue), EPO (green), and isotype (red) values are shown. **B** and **C**, Geometric mean fluorescence above control value of macrophages (Fig 1, B) or THP-1 cells (Fig 1, C; $n = 3$). * $P < .05$ compared with control.

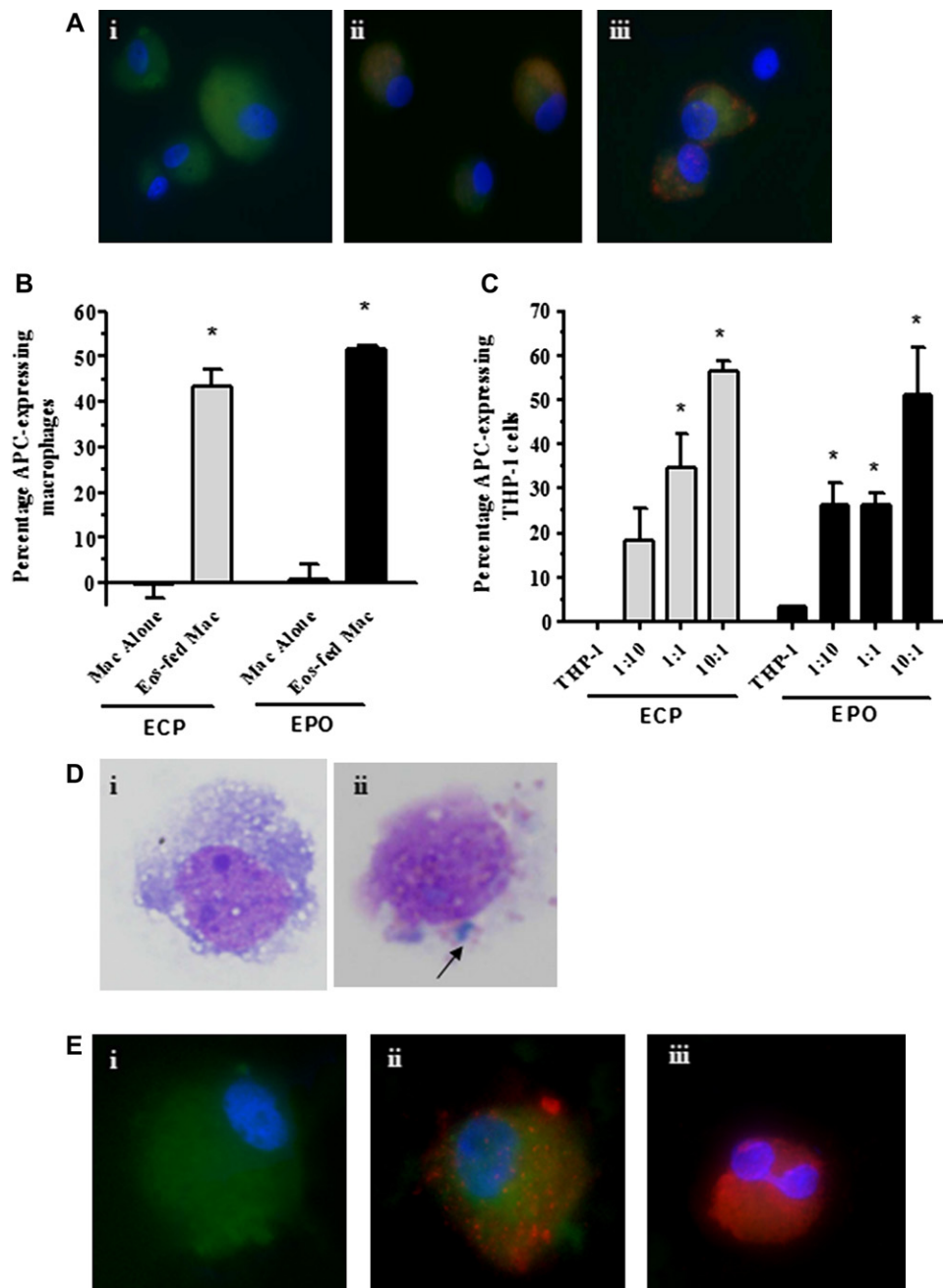


FIG 2. **A**, Representative immunofluorescence staining of macrophages cultured alone (*i*) or with apoptotic eosinophils (*ii* and *iii*). Macrophages were stained with CD68-FITC (green) and ECP-APC (red; *ii*) or EPO-APC (red; *iii*) and 4',6-diamidino-2-phenylindole (blue). **B** and **C**, Percentage of APC-expressing cells above control values for macrophages (Fig 2, **B**) and THP-1 cells (Fig 2, **C**). * $P < .05$ compared with control. **D**, THP cells cultured alone (*i*) show blue cytoplasm, and THP cells cultured with apoptotic eosinophils (*ii*) show color change in Romanowsky stained cytopsin. **E**, Representative immunofluorescence staining: *i*, isotype control macrophage; *ii*, macrophage; *iii*, eosinophil. EPO-APC (red), CD68-FITC (green), and 4',6-diamidino-2-phenylindole (blue) staining in sputum cytopsin preparation is shown.

(GINA class IV/V) asthma, as well as subjects with NAEB and healthy control subjects; (2) subjects with sputum eosinophilia⁴ and severe asthma before and after prednisolone, 30 mg daily, for 2 weeks; and (3) subjects without baseline sputum eosinophilia before corticosteroid withdrawal in the sputum arm of a randomized, controlled 1-year study of the use of sputum analysis to direct corticosteroid therapy.⁷ These subjects were dichotomized into those for whom corticosteroids were weaned to maintenance of 0 to 400 μ g of beclomethasone equivalent per day without the emergence of sputum eosinophilia

(ie, successful weaning) versus those with sputum eosinophilia on corticosteroid withdrawal (ie, unsuccessful weaning).

Eosinophil proteins in macrophages after ingestion of apoptotic eosinophils

Macrophages from lung resection and peripheral blood eosinophils from healthy volunteers were obtained as previously described.^{19,20} Apoptosis in

TABLE I. Demographic characteristics, induced sputum differential count, median percentage area of red hue/airway macrophage for the healthy, eosinophilic bronchitis, and asthma groups

	Healthy (n = 10)	NAEB (n = 15)	GINA class I (n = 8)	GINA class II-III (n = 18)	GINA class IV-V (n = 46)
Age (y), mean (SE)	32 (5)	48 (3)	46 (7)	55 (3)	50 (2)
Male sex, no. (%)	5 (50)	8 (53)	4 (50)	10 (56)	24 (52)
FEV ₁ (% predicted), mean (SE)	106 (6)	100 (3)	94 (4)	87 (5)	78 (4)
Induced sputum (%), median (range)					
Eosinophils	0.4 (0-2.5)	8.3 (3.3-68)	0.9 (0-33.5)	1.1 (0.1-45.3)	1.5 (0-90.5)
Neutrophils	40.7 (8.3-66.8)	47.3 (8.75-82.7)	20 (0.4-77.2)	60.8 (5.0-95.0)	54.4 (2-93.5)
Macrophages	51.4 (28.8-90)	27 (06-84)	76 (12.4-90.6)	26.5 (3.5-78.5)	25.9 (5.2-78.50)
Lymphocytes	1.2 (0-9.8)	0.5 (0-2.2)	0.2 (0-0.5)	0.8 (0-2.0)	0.8 (0-4.5)
Percentage area of red hue/airway macrophage, median (IQR)	2.1 (1.9-3.8)	36.3 (14.3-40.7)	2.0 (0.9-9.1)	10.7 (3.0-38.7)	28.0 (11.8-68.0)

IQR, Interquartile range.

eosinophils was induced by aging in culture.²¹ For additional information, see the Methods section in this article's Online Repository at www.jacionline.org. Lung macrophages or THP-1 cells (a macrophage cell line) were cocultured with apoptotic eosinophils at a ratio of 10:1 to 1:10 for 4 hours.²² Eosinophil cationic protein (ECP) and eosinophil peroxidase (EPO) expression in macrophages and THP-1 cells was analyzed by means of immunofluorescence and intracellular flow cytometry with mAbs for ECP or EPO (Diagnostics Development, Uppsala, Sweden) indirectly labeled with allophycocyanin (APC) and CD68–fluorescein isothiocyanate (FITC) and appropriate isotype controls. Cytospin preparations were obtained and stained by using the Romanowsky method.

Eosinophil proteins in sputum macrophages

Sputum macrophages from subjects with asthma were analyzed for the presence of eosinophil proteins by means of immunolabeling with mAbs for ECP and EPO and indirectly labeling with APC and CD68-FITC and appropriate isotype controls. For additional information, see the Methods section in this article's Online Repository.

Imaging and color-image analysis of cytospin preparations

From the cytospin preparations, 100 macrophages were imaged by using $\times 40$ objective at ultrahigh resolution (4080×3072 ; Olympus DP70 digital camera; AnalySIS, Soft Imaging System GmbH, Munster, Germany). Image J software (ImageJ 1.40g/java 1.6.0_05, National Institutes of Health Image) was used to analyze the percentage area of cytoplasm with a red hue by means of thresholding (see Fig E1 in this article's Online Repository at www.jacionline.org). Median percentage area of cytoplasm with a red hue (primary measure) in 100 macrophages per subject was used as a measure of eosinophil protein content. Imaging and image analysis was performed by a person blinded to clinical characteristics. Intraobserver and interobserver variability was assessed in 50 airway macrophages. Inclusions of apoptotic eosinophils were frequently observed in the macrophages (see Fig E2 in this article's Online Repository at www.jacionline.org).

Statistics

Statistical analysis was performed with GraphPad Prism 5 software (GraphPad Software, Inc, San Diego, Calif). Subjects' characteristics are presented as means (SEs) or medians (ranges or interquartile ranges). Interobserver and intraobserver variation was assessed by using intraclass correlation. Comparisons across groups were analyzed with the Kruskal-Wallis test for nonparametric data, with between-group comparisons using the Mann-Whitney test for unpaired data and the Wilcoxon matched pairs test for paired data as appropriate and proportions using the Fisher exact test. Differences were considered significant at a *P* value of less than .05.

RESULTS

Eosinophil proteins in macrophages after ingestion of apoptotic eosinophils

Example flow cytometric histograms showing that CD68⁺ macrophages expressed ECP and EPO after apoptotic eosinophil ingestion are as shown in Fig 1, A. The geometric mean fluorescence intensity of macrophages after ingesting apoptotic eosinophils and stained with EPO antibodies was significantly higher than that for macrophages alone (887 [95% CI, 356-2,209] vs 2 [95% CI, 0-27] *P* = .016; Fig 1, B). This was confirmed in THP-1 cells fed with eosinophils. The geometric mean fluorescence intensity increased with the increasing proportion of eosinophils to macrophages stained with ECP and EPO (*P* < .001; Fig 1, C).

Similarly, the expression of ECP and EPO by macrophages after apoptotic eosinophil ingestion was confirmed by means of immunofluorescence. Example immunofluorescent photomicrographs illustrating macrophages expressing ECP and EPO after ingestion of eosinophils are as shown in Fig 2, A. The percentage of positively stained lung macrophages or THP-1 cells was significantly higher after phagocytosis of eosinophils and related to the ratio of eosinophils to macrophages (Fig 2, B and C).

THP-1 cells had red granules and apoptotic eosinophils in them after phagocytizing apoptotic eosinophils, changing the macrophage cytoplasm color to red-purple (Fig 2, D).

Airway macrophage eosinophil protein staining in sputum cytospin preparations

The presence of eosinophil proteins (ECP and EPO) in sputum macrophages was confirmed by means of immunofluorescence. Example immunofluorescent photomicrographs are shown in Fig 2, E.

The intraclass correlation coefficient of intraobserver and interobserver variation for the image analysis method to calculate the median percentage of red-purple hue per airway macrophage was 0.99 (95% CI, 0.99-0.99) and 0.97 (95% CI, 0.96-0.98), respectively.

Cross-sectional assessment of subjects with asthma, subjects with NAEB, and healthy control subjects

The clinical characteristics of subjects are as shown in Table I. Examples of sputum cytospin preparations showing the

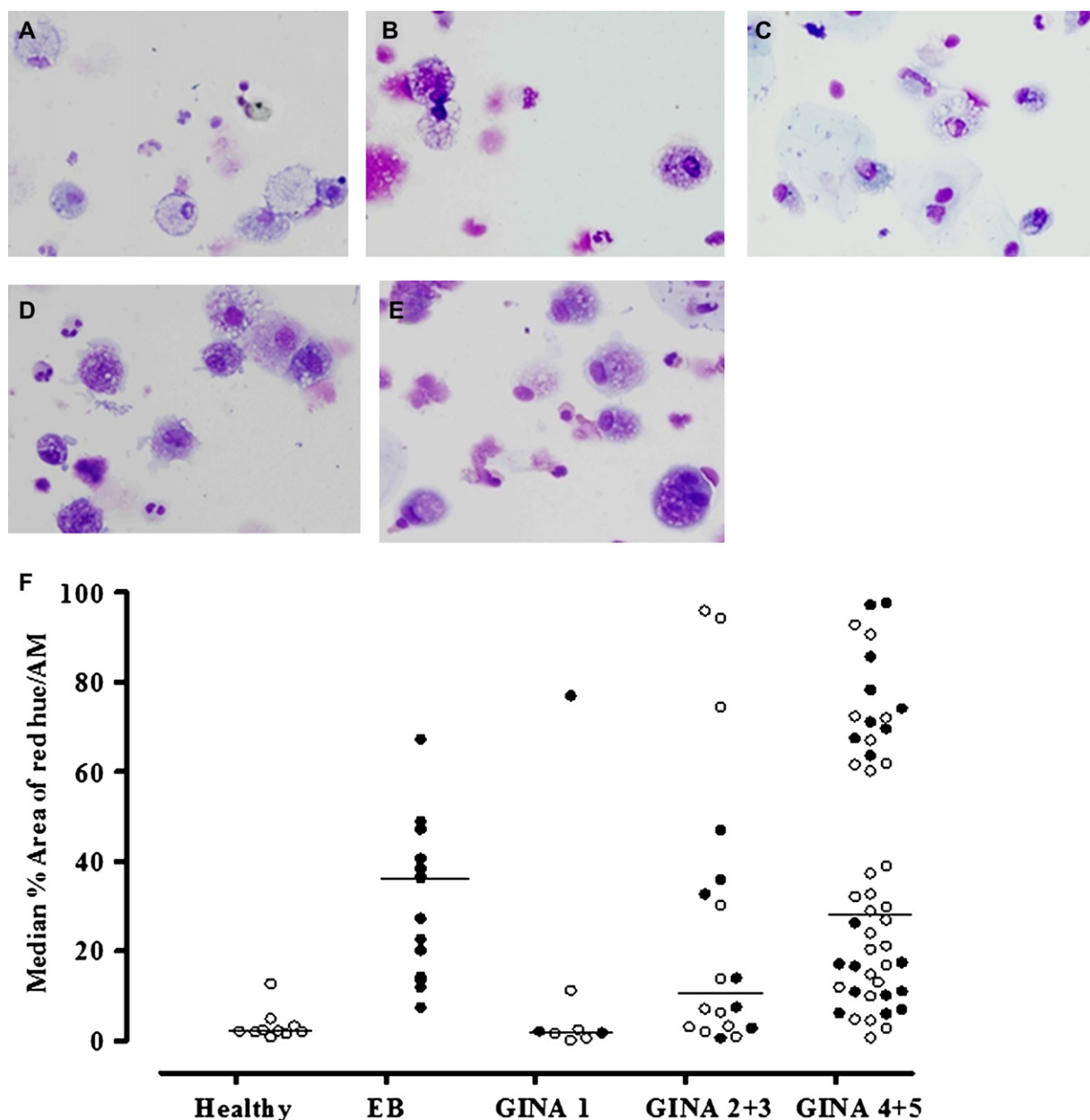


FIG 3. Representative images of sputum macrophages: **A**, healthy control subjects; **B**, subjects with NAEB; **C**, subjects with GINA class I asthma; **D**, subjects with severe noneosinophilic asthma; **E**, subjects with severe eosinophilic asthma. **F**, Scatter plot showing comparison of median percentage area of red-purple hue in sputum macrophages in the healthy, NAEB (EB), and GINA class I, GINA class II and III, and GINA class IV and V asthma groups. *Solid circles* represent a sputum eosinophil count of greater than 1.9, and *open circles* represent a sputum eosinophil count of less than 1.9.

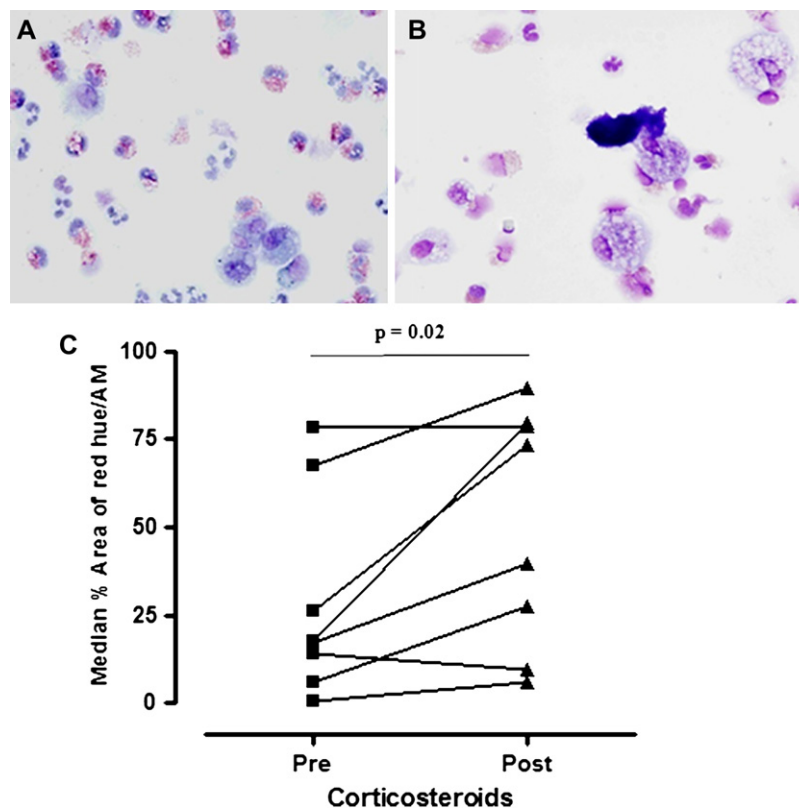
differences in the macrophage color from healthy control subjects, subjects with mild asthma, subjects with NAEB, and subjects with severe asthma with and without sputum eosinophilia are shown (Fig 3, A-E). The median airway macrophage percentage red-hued area was significantly higher in subjects with severe asthma compared with that seen in healthy control subjects ($P < .0001$) and subjects with mild asthma ($P = .002$), trends toward significance when compared with that seen in subjects with moderate asthma ($P = .065$), but was similar to that seen in subjects with NAEB ($P = .9$; Kruskal-Wallis $P < .0001$; Fig 3, F, and Table I). The subjects in GINA class IV and V appear to be divided

into 2 groups (Fig 3, F): one with median airway macrophage red-hued percentage area of less than 50 ($n = 29$) and one with median airway macrophage red-hued percentage area of greater than 50 ($n = 17$). A further comparison of these 2 groups did not show any significant differences in age, sex, FEV₁, and eosinophil differential count. There was no significant difference in the median airway macrophage percentage red-hued area in those asthmatic subjects with ($n = 29$; median, 17.5 [interquartile range, 7.3-70.3]) versus those without ($n = 43$; median, 20.4 [interquartile range, 4.6-60.2]) sputum eosinophilia ($P = .39$, examples are shown in Fig 3, D and E). The subjects with eosinophilic and

TABLE II. Lung function, sputum differential count, and median percentage area of red hue before and after prednisolone treatment in subjects with severe asthma (n = 8)

	Before prednisolone	After prednisolone	P value
FEV ₁ (L), mean (SD)	2.47 (0.98)	2.77 (0.82)	.09
FVC (L), mean (SD)	3.48 (1.25)	3.70 (0.88)	.51
FeNO (ppb), mean (SD)	53 (52)	34 (16)	.26
Induced sputum (%), median (range)			
Eosinophils	31.63 (3.5-90.5)	0.87 (0.0-16.25)	.03
Neutrophils	29.88 (2-74.25)	66.65 (4.18-87.0)	.05
Macrophages	35.39 (6.5-56.75)	28.50 (9.75-80.23)	.90
Percentage area of red hue/AM, median (IQR)	17.4 (8.0-57.2)	56.6 (13.9-79.3)	.02

AM, Airway macrophage; FeNO, fraction of exhaled nitric oxide; FVC, forced vital capacity; IQR, interquartile range.

**FIG 4.** Representative images of sputum macrophages after treatment with prednisolone, 30 mg/d, for 2 weeks. **A** and **B**, High number of eosinophils and macrophages with blue cytoplasm before treatment (Fig 4, **A**) and reduced eosinophil counts with increase in eosinophilic staining of macrophages in the same patient after treatment (Fig 4, **B**). **C**, Graph showing the median percentage of red hue/airway macrophage before and after oral corticosteroids.

noneosinophilic asthma are shown in Fig 3, *F*, as solid and open symbols, respectively.

Response to corticosteroids in subjects with severe eosinophilic asthma

Clinical characteristics before and after prednisolone are as shown in Table II. Examples of sputum cytopsin preparations showing differences in macrophage color before and after prednisolone are shown in Fig 4, **A** and **B**. The median airway macrophage percentage red-hued area significantly increased after treatment with prednisolone ($P = .02$; Fig 4, **C**, and Table II).

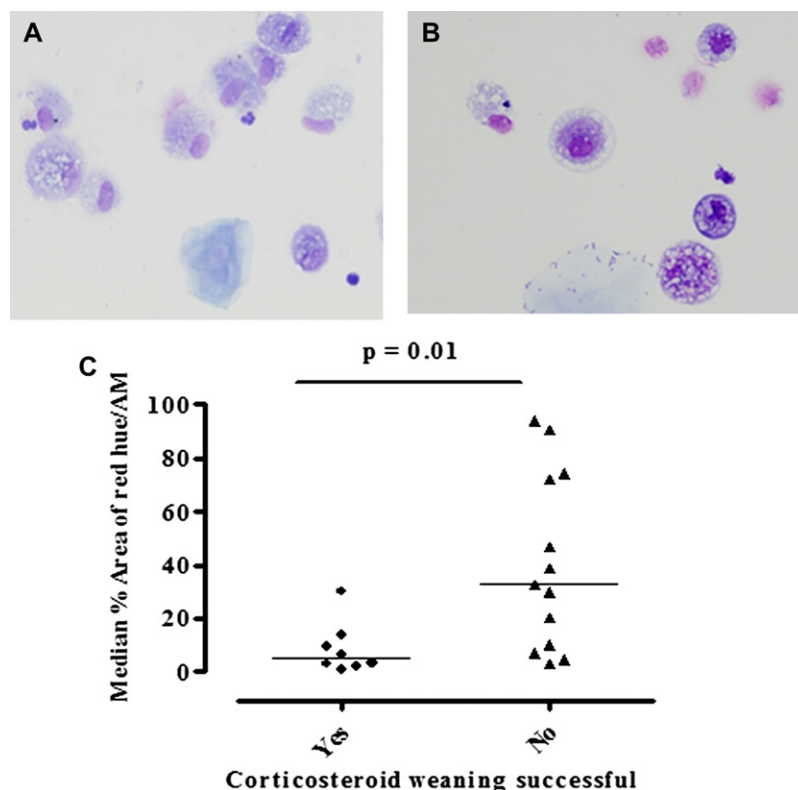
Airway macrophage hue and corticosteroid-weaning success

Clinical characteristics of the subjects with a normal sputum eosinophil count who had successful or unsuccessful corticosteroid weaning are as shown in Table III. At baseline, there was no significant difference in age, lung function, and sputum eosinophil differential between those who were successfully weaned and those who were not. Representative sputum cytopsin preparations from the 2 groups are as shown in Fig 5, **A** and **B**. The median airway macrophage percentage red-hued area at baseline was significantly lower in those who were successfully weaned versus those who were not ($P = .01$; Fig 5, **C**, and Table III).

TABLE III. Demographic, lung function, and sputum differential count in subjects with successful and unsuccessful weaning

	Successful weaning (n = 8)	Unsuccessful weaning (n = 13)	P value
Age (y), mean (SE)	56 (6)	52 (4)	.55
Male sex, no. (%)	5 (63)	6 (46)	.65
FEV ₁ (% predicted), mean (SE)	84 (24)	80 (8)	.78
Induced sputum (%), median (range)			
Neutrophils	61 (5-93)	48 (12-95)	.79
Macrophages	35 (6-79)	36 (4-72)	.97
Eosinophils	0.37 (0.1-1.0)	0.25 (0.1-1.5)	.63
Percentage area of red hue/AM, median (IQR)	4.9 (2.3-12.8)	32.8 (8.6-73.4)	.01

AM, Airway macrophage; IQR, interquartile range.

**FIG 5.** A and B, Representative images of induced sputum macrophages in subjects with low eosinophil counts (<1.9%) in whom weaning of corticosteroid treatment was successful (Fig 5, A) and unsuccessful (high eosinophilic staining; Fig 5, B). C, Scatter plots comparing median macrophage percentage of red hue in these 2 groups.

DISCUSSION

We describe here that the eosinophil protein content of airway macrophages is a reliable biomarker of ongoing eosinophilic airway inflammation. The quantity of eosinophil protein content in macrophages can be determined by the measurement of the percentage of red hue of macrophages on routinely stained sputum cytopsin preparations. Importantly, airway macrophage percentage of red hue was increased in subjects with moderate-to-severe asthma independent of current sputum eosinophil counts, suggesting that the absence of sputum eosinophilia in these subjects was a consequence of corticosteroid therapy rather than a reflection of a true noneosinophilic phenotype. In addition to its potential role in phenotyping asthma, the combination of a normal sputum eosinophil count and macrophage percentage

of red hue determined from the same cytopsin preparation was a good predictor of successful weaning from corticosteroid treatment.

Macrophage ingestion of apoptotic eosinophils is a well-documented phenomenon resulting in resolution of inflammation.¹¹ Woolley et al¹¹ have reported the presence of eosinophil products in sputum macrophages after treatment with corticosteroids. Similar findings have been confirmed *in vitro*²² and in electron microscopic images of macrophages.¹² However, to date, the quantity of eosinophil proteins in airway macrophages has not been used as a marker of eosinophilic inflammation. We have confirmed with flow cytometry and immunofluorescence that eosinophil proteins (ECP and EPO) are detectable in airway macrophages after ingestion of apoptotic eosinophils. An increase in

macrophage eosinophil protein content with a higher eosinophil/macrophage ratio confirmed a positive dose response. Apoptotic eosinophils were observed within macrophages but in some macrophages intact eosinophils were no longer visible. We therefore chose to examine the potential application of the red color on Romanowsky-stained cytospin preparations, which reflects material staining for eosin as a measure of ingested eosinophils and their products. Macrophage color changed from blue to red-purple after ingestion of apoptotic eosinophils, a method that was applied to analyze cytospin preparations. This color change was analyzed by using hue spectrum in image analysis because it corresponds to the color perceived by the human eye and does not depend on light intensity or saturation. Importantly, the described image analysis method had excellent interobserver and intraobserver variation. We are therefore confident that macrophage color reflects eosinophil protein content and has potential as a biomarker of ongoing eosinophilic airway inflammation.

There is increasing recognition that sputum eosinophilia is only present in about 50% of asthmatic subjects across all severities.^{3,23} This observation has undermined previous dogma that asthma is predominately characterized by T_H2-mediated eosinophilic inflammation and has introduced the possibility that other inflammatory pathways are dominant in different asthma subphenotypes.²⁴ This view has enormous implications for asthma treatment and pathogenesis. In corticosteroid-naïve asthmatic subjects the absence of sputum eosinophilia (<1.9% non-squamous cells) on 2 or more occasions has been used to define noneosinophilic asthma.^{4,23} However, in asthmatic subjects treated with corticosteroid therapy, it is not possible to determine whether the lack of sputum eosinophilia is a consequence of corticosteroid therapy or represents a true noneosinophilic phenotype. Longitudinal sputum assessments and corticosteroid withdrawal studies suggest that the presence of a sputum eosinophil count in asthmatic subjects treated with corticosteroids is variable and changes in response to disease stability and current therapy.^{8,9,25,26} One of our most important findings in this report is that in patients with moderate-to-severe asthma, the macrophage percentage of red hue was markedly increased independent of the sputum eosinophil count, with no difference between the noneosinophilic and eosinophilic groups (see the Results section and Fig 3, D and E). This suggests that in asthmatic subjects treated with corticosteroids, the absence of sputum eosinophilia reflects response to therapy (ie, adequate clearance by macrophages). This view was further supported by our findings that the macrophage percentage of red hue increased in response to 2 weeks of prednisolone therapy in subjects with severe asthma. Therefore true noneosinophilic asthma is probably less common in subjects with moderate-to-severe asthma than previously considered.

This might provide an explanation for the observation that children with noneosinophilic severe asthma responded to corticosteroids²⁷ and suggests that eosinophil-directed therapy might be applicable to a large group of subjects with severe asthma. This is supported by our recent study of mepolizumab, which demonstrated that this anti-IL-5 mAb therapy led to a marked reduction in asthma exacerbations in subjects with severe asthma, even though one third of these subjects did not have sputum eosinophilia at study entry.⁵ These subjects did have at least 1 episode of sputum eosinophilia in the last 2 years, which underscores the current need in some patients for analysis of repeated sputum samples to define the asthma phenotype. This therefore highlights

the potential added value of using the eosinophil protein content in macrophages together with the differential cell count. Indeed, we found that subjects with severe asthma followed up for 1 year in a study designed to test the application of sputum analysis to direct corticosteroid therapy who had a normal sputum eosinophil count also had a low macrophage percentage of red hue among those who were successfully weaned off or onto maintenance low-dose inhaled corticosteroids, whereas those subjects with normal sputum eosinophil counts but moderate-to-high eosinophil protein content in their macrophages (at the start of weaning) were unsuccessful at corticosteroid withdrawal. The high eosinophil protein content in macrophages suggests ongoing eosinophilia. Therefore the combination of the sputum eosinophil count and macrophage color on a single sputum cytospin preparation might have clinical utility in directing therapy and warrants further testing in prospective studies.

Some subjects had low airway macrophage red hue in combination with high eosinophil counts, which suggests (1) an inadequate amount of corticosteroid reaching the airways or (2) or an inherent defect of phagocytosis. However, an increase in airway macrophage hue after treatment with systemic steroids differentiates between the 2 possible causes. When the airway macrophage red hue and eosinophil count are both high, it suggests a possibility of ongoing eosinophilia beyond the capacity of macrophage clearance mechanisms.

Interestingly, subjects with corticosteroid-naïve NAEB had similar content of eosinophilic staining as those with severe asthma. One possible explanation is that there is more spontaneous resolution of eosinophilic airway inflammation in subjects with NAEB compared with that seen in subjects with asthma. This might be due to increased eosinophil apoptosis in subjects with NAEB, which could be due to the decreased IL-13 concentration in the airway in subjects with NAEB, which is known to promote eosinophil survival.²⁸ This important distinction between mild asthma and NAEB requires further study.

One potential limitation of our study is that the macrophage color does not quantify the exact amount of eosinophil proteins, which might reduce its accuracy. However, we have demonstrated that the test is repeatable, responds to corticosteroid therapy, and has the potential to direct therapy. It provides additional and complementary information to the differential cell count. Importantly, it does not require further processing of sputum; moreover, ImageJ is freely downloadable software. The macro speeds up the process significantly by combining the majority of the analysis steps in to a single step (see Fig E1). Critically, data in this study are derived from the analysis of retrospective sputum cytospin preparations collected as part of previous studies and need to be replicated in prospective studies. Whether this new biomarker can be more widely applied to other airway diseases, such as chronic obstructive pulmonary disease or chronic cough, requires further study.

In conclusion, macrophage eosinophil protein content can be reliably assessed from sputum cytospin preparations by measuring macrophage color and reflects ongoing airway eosinophilia. The absence of sputum eosinophilia and normal macrophage eosinophil protein content is unusual in subjects with moderate-to-severe asthma. The combination of macrophage eosinophil protein content and eosinophil count is useful in predicting response to treatment withdrawal. Prospective studies of the clinical utility of sputum macrophage color as a new biomarker of eosinophilic inflammation are warranted.

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Clinical implications: Sputum macrophage eosinophil content (1) is useful in identifying ongoing eosinophilia, even with normal eosinophil counts, and (2) might be useful, along with eosinophil counts, in predicting response to treatment withdrawal.

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METHODS

Eosinophil protein in macrophages after ingestion of apoptotic eosinophils

Lung filtrate. Lung resection tissue was obtained from patients undergoing surgery for carcinoma at the Glenfield Hospital in Leicester, United Kingdom. Macroscopically normal tissue was excised at least 2 cm from the tumor. The specimen usually weighed between 10 and 40 g.

Macrophage purification protocol

A modified method similar to one described before^{E1} was used. Briefly, the lung specimen was placed in media and finely chopped with scissors. The cell suspension was then filtered twice through 100- μ m gauze. The resulting filtrate was centrifuged for 10 minutes at 1,000 rpm, after which erythrocytes were lysed with distilled water for 30 seconds. An equal volume of 2 $\times$ PBS was immediately added and washed once. The cell suspension was thereafter incubated in T75 flasks for 2 hours at 37°C in a 5% CO₂ atmosphere to allow adherence of the macrophages to the plastic. Nonadherent cells were removed, and the resulting macrophages were cultured in macrophage serum-free media (Invitrogen, Paisley, UK) supplemented with penicillin (100 U/mL), streptomycin (100 μ g/mL), Amphotericin B (0.25 μ g/mL), nonessential amino acids (100 μ mol/L), and macrophage colony-stimulating factor (10 ng/mL; R&D Systems, Abingdon, UK). Purity was assessed with Kimura stain. Trypan Blue (Sigma, Gillingham, UK) and propidium iodide (BD Biosciences, Oxford, United Kingdom) were used to assess viability.

THP-1 cells

The human monocytic cell line THP-1 (kind gift from AstraZeneca, Charnwood Loughborough, United Kingdom) was maintained in Dulbecco modified Eagle medium with high glucose, no phenol red supplemented with penicillin (100 U/mL), streptomycin (100 μ g/mL), Amphotericin B (0.25 μ g/mL), nonessential amino acids (100 μ mol/L, Invitrogen), sodium pyruvate (1 mmol/L), and FBS (10%, Sigma). THP-1 cells were differentiated into an adherent macrophage-like phenotype through stimulation with 10 nmol/L phorbol 12-myristate 13-acetate for 48 hours.

Eosinophil purification protocol and apoptosis induction

Eosinophils were isolated from heparinized peripheral venous blood from healthy control subjects or mild atopic volunteers by using established techniques, as described previously.^{E2} Briefly, 100 mL of peripheral blood was taken to which 1,000 U/mL heparin was added. Six percent volume dextran was added and gently mixed before allowing 45 minutes for the erythrocytes to settle. The plasma layer was removed to a fresh tube and centrifuged at 1,000 rpm for 10 minutes without a break. The cell pellet was resuspended before layering onto Histopaque 1083 (Sigma-Aldrich) and centrifuged at 1,400 rpm for 25 minutes without a break. The cell pellet was lysed with ice-cold distilled water for 30 seconds before adding an equal volume of 2 $\times$ buffer (20% 10 $\times$ Hanks balanced salt solution, 3% 1 mol/L Hepes, 2% 0.5 mol/L EDTA, and 75% dH₂O) and centrifuged at 1,200 rpm for 8 minutes at 4°C. Eosinophils were negatively selected by using a combination of anti-CD14-, anti-CD16-, and anti-CD3-conjugated microbeads and LS columns (Miltenyi Biotech, Surrey, United Kingdom). Finally, purity was assessed with Kimura stain. Trypan blue (Sigma), Annexin V, and propidium iodide (both from BD Biosciences) were used to assess viability.

Apoptosis in eosinophils was induced by aging in culture.^{E3} Briefly, after isolation, eosinophils were washed and incubated in Dulbecco modified Eagle medium supplemented with penicillin (100 U/mL), streptomycin (100 μ g/mL), Amphotericin B (0.25 μ g/mL), nonessential amino acids (100 μ mol/L), and heat-inactivated FCS (10%). Cells were cultured at 1 $\times$ 10⁶/mL for 48 hours at 37°C and 5% CO₂. After cytokine-deprived aging, eosinophils were assessed for apoptosis by means of morphologic assessment and with Annexin V binding. Cultures used were at least 70% apoptotic.

Eosinophil uptake assay

Freshly isolated adhered macrophages or stimulated THP-1 cells were cocultured with apoptotic eosinophils at known concentrations (eosinophil/macrophage = 1:10, 1:1, and 10:1) and incubated at 37°C in a 5% CO₂ atmosphere for 4 hours. Nonphagocytosed eosinophils were gently washed off before the macrophages/THP-1 cells were removed with a cell lifter (Fisher Scientific, Loughborough, UK). These cells were subsequently used to prepare cytospin preparations (at 3.75 $\times$ 10⁴/cytospin preparation) for Romanowsky or immunofluorescence staining or for flow cytometric analysis.

Flow cytometry

Cells were fixed and permeabilized with 4% paraformaldehyde and 0.1% saponin and dual stained with either murine anti-human ECP or EPO (Diagnostics Development) at 1 μ g/mL followed with APC secondary antibody at 1.25 μ g/mL and CD68 directly conjugated with FITC at 10 mg/mL or respective control antibodies. Analysis was performed with 2-color flow cytometry on the BD FACSCanto flow cytometer.

Immunofluorescence

Cells (from the *in vitro* experiments and sputum cytospin preparations) were fixed and permeabilized with 4% paraformaldehyde and 0.1% saponin and blocked with 3% BSA. Cells were dual labeled with either murine anti-human ECP or EPO at 2.0 μ g/mL (for 90 minutes) followed with APC secondary (R&D Systems) at 2.5 μ g/mL and CD68 directly conjugated with FITC (DAKO, Glostrup, Denmark) at 10 mg/mL or respective control antibodies. Cells were counterstained with 4',6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich) and mounted with photo bleach retardant mounting medium.

Imaging and color-image analysis

One hundred macrophages were imaged at ultrahigh resolution (4080 $\times$ 3072) with a $\times$ 40 objective (Olympus BX50 microscope and AnalySIS). White balance was performed before imaging the macrophages, and images were stored in TIFF format. Image J software (ImageJ 1.40g/java 1.6.0_05) was used to analyze the area of cytoplasm with red hue (Fig E1). The cytoplasmic area of each macrophage was selected and added as a region of interest. The red/green/blue image was converted to a hue/saturation/brightness stack. Hue image was thresholded to include pixels with red hue at the same threshold (190 on a scale of 0-260) for all images. The hue was used to identify eosinophilic staining as it corresponds to the color perceived by the human eye and was not dependent on light intensity or saturation. The threshold of 190 was selected because it matched the observer-identified purple-red color in multiple images. Median percentage area of cytoplasm with a red hue (primary measure) in 100 macrophages per subject was used as a measure of eosinophil protein content. Multiple airway macrophages in one image were analyzed by using a macro built in-house for this analysis. Imaging and image analysis were performed by a person blinded to clinical characteristics. Intra-observer and interobserver variability were assessed in 50 airway macrophages. The inclusion of apoptotic eosinophils was frequently observed in the macrophages (Fig E2).

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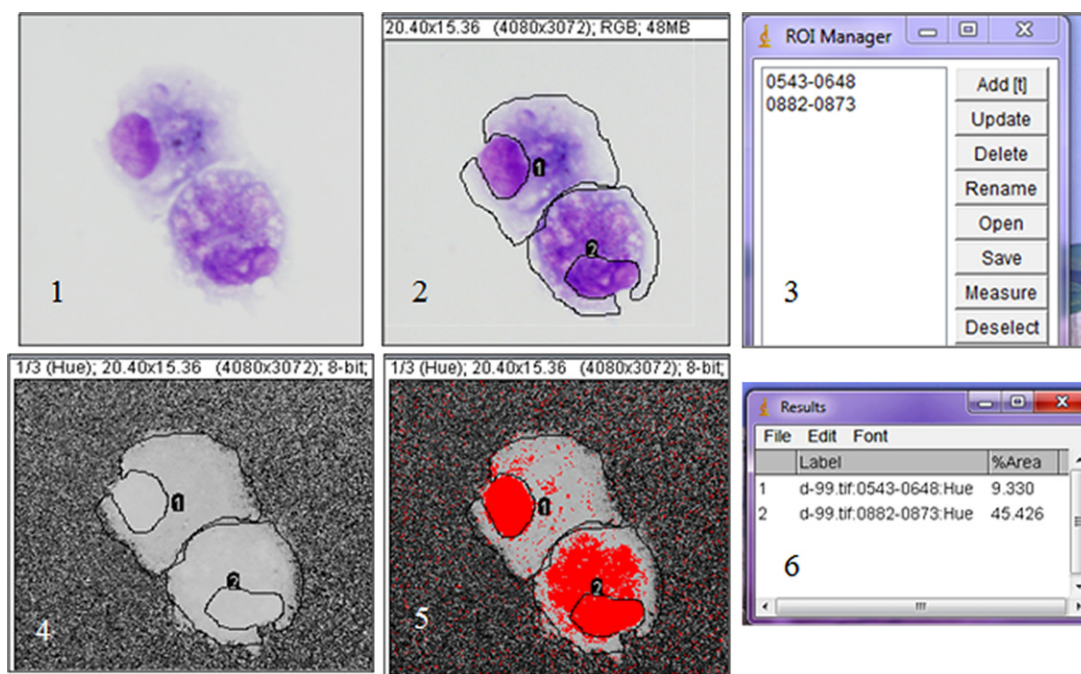


FIG E1. Method to calculate the percentage of eosinophilic staining by thresholding the hue image. 1, The saved image of airway macrophage (TIFF file) is opened in the ImageJ program. 2, The free hand-drawing tool is used to define the cytoplasmic area (which is selected as single area excluding the nucleus). 3, The selection is then added to the region of interest manager. All the macrophages identified in one image are added for batch analysis (example shown). 4, The red/green/blue image is converted to a hue/saturation/brightness stack. The hue image (example shown, which is seen as a grayscale image) is used to threshold for red-purple hue. 5, All the pixels with red-purple hue (those between 190-256) are identified by thresholding (shown as red-colored areas in the example, with the nucleus excluded from measurement). 6, The percentage of this area to total macrophage cytoplasmic area is measured. One hundred macrophages per subject are analyzed, and results are copied into an Excel sheet. The median percentage area of red-purple hue per airway macrophage per subject was calculated. The macro used for analysis of percentage area of red hue in macrophages (steps 4-6) was as follows:

```
run("HSB Stack");
n = roiManager("count");
for (i=0; i<n; i++) {
    roiManager("select", i);
    setThreshold(190, 255);
    run("Set Measurements...", "area_fraction limit display redirect=None decimal=3");
    run("Measure");
    updateResults();
    selectWindow("Results");
    runMacro("copyResultsToClipboard.txt");
}
roiManager("deselect");
roiManager("Delete");
run("Open Next");
```

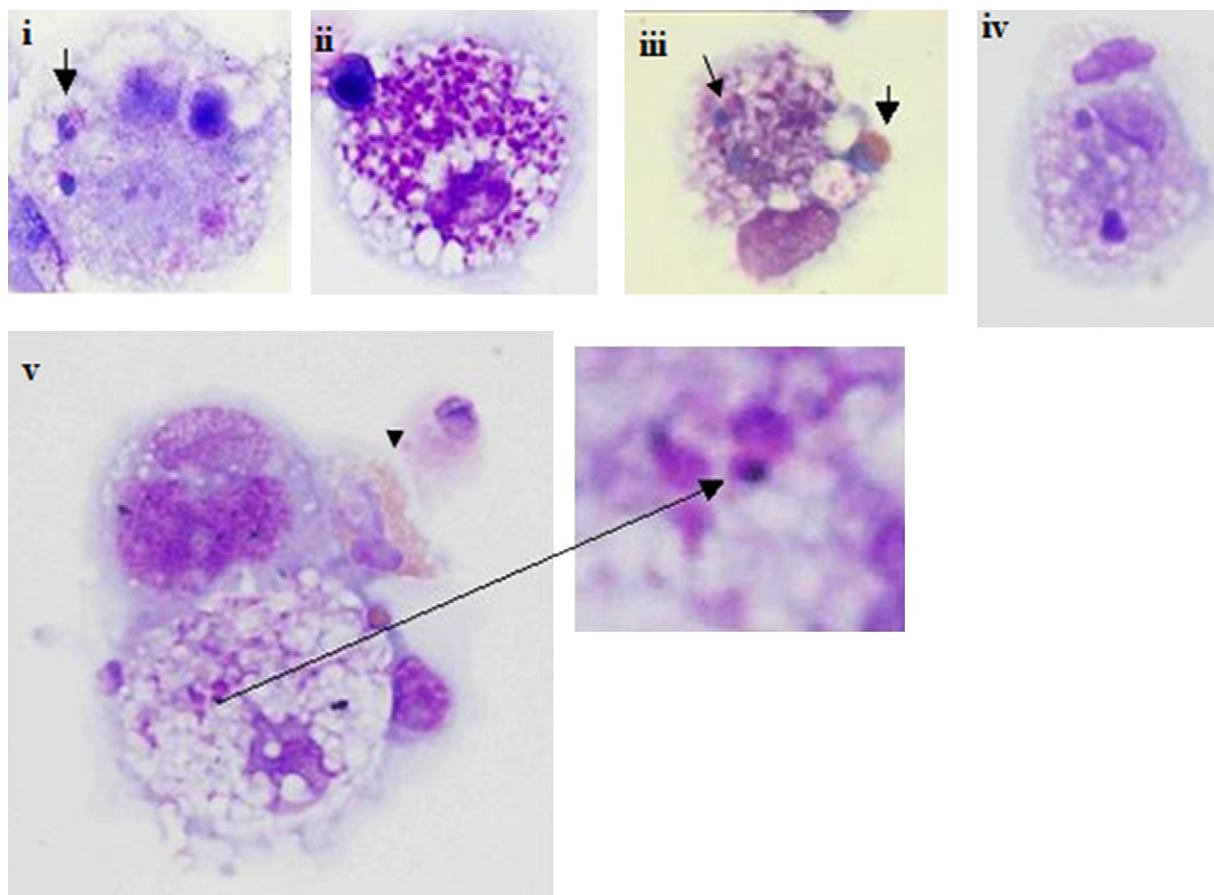


FIG E2. Examples of induced sputum macrophages with apoptotic eosinophils and red hue of cytoplasm. Apoptotic eosinophils (*arrows*) can be identified inside macrophages in *panels i and iii*. In other panels (*ii, iv, and v*) the ingested eosinophils appear as dense red areas with blue dots (nuclei of eosinophils). In addition, an intact uningested eosinophil (*arrowhead*) is seen in panel *v*. All slides were stained with Romanowsky stain and imaged at $\times 40$ by using ultrahigh resolution on a digital camera.