

ORIGINAL ARTICLE

Airway Diseases

Sputum cytology during late-phase responses to inhalation challenge with different allergens

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Abstract

Background: In mouse models of allergic asthma, exposure to different allergens can trigger distinct inflammatory subtypes in the airways. We investigated whether this observation extends to humans.

Methods: We compared the frequency of sputum inflammatory subtypes between mild allergic asthma subjects (n = 129) exposed to different allergens in inhalation challenge tests. These tests were performed using a standardized protocol as part of clinical trials of experimental treatments for asthma, prior to drug randomization. Five allergen types were represented: the house dust mites *Dermatophagoides pteronyssinus* and *Dermatophagoides farinae*, ragweed, grass, and cat.

Results: Of 118 individuals with a sputum sample collected before allergen challenge (baseline), 45 (38%) had paucigranulocytic, 51 (43%) eosinophilic, 11 (9%) neutrophilic, and 11 (9%) mixed granulocytic sputum. Of note, most individuals with baseline paucigranulocytic sputum developed eosinophilic (48%) or mixed granulocytic (43%) sputum 7 hours after allergen challenge, highlighting the dynamic nature of sputum inflammatory subtype in asthma. Overall, there was no difference in the frequency of sputum inflammatory subtypes following challenge with different allergen types. Similar results were observed at 24 hours after allergen challenge.

Conclusions: Unlike reported in mice, in humans the sputum inflammatory subtype observed after an allergen-induced asthma exacerbation is unlikely to be influenced by the type of allergen used.

KEYWORDS

airway inflammation, allergen inhalation, allergens, allergic asthma, sputum inflammatory subtype

1 | INTRODUCTION

Allergic asthma is an inflammatory disease of the airways triggered by the inhalation of aero-allergens in previously sensitized individuals. Within hours of allergen exposure, various immune cell types infiltrate the airways, where they release pro-inflammatory mediators that result in narrowing of the airways and the characteristic symptoms of wheezing and shortness of breath.¹ Among the most

abundant cells in the airway immune infiltrate are eosinophils and neutrophils. The relative abundance of these cell types in induced sputum varies widely between asthmatics and is often used to categorize the underlying inflammatory response.^{2–6} Currently, 4 major sputum subtypes have been described:^{6,7} (i) eosinophilic, with elevated eosinophils but not neutrophils; (ii) neutrophilic, with elevated neutrophils but not eosinophils; (iii) mixed granulocytic, with both eosinophils and neutrophils elevated; and (iv) paucigranulocytic, with

both eosinophils and neutrophils within the normal range. Recognizing this heterogeneity in inflammatory subtype is important because it can help identify the subsets of patients that can benefit most from specific targeted therapies.⁸ For example, patients with eosinophilic asthma typically respond well to corticosteroids,^{9–11} which is not the case for those with noneosinophilic asthma.^{11–14} However, despite its importance, the factors that contribute to the heterogeneity in sputum inflammatory subtype in humans remain poorly understood.

Mouse models of asthma have provided some clues into the factors that might contribute to the type of sputum inflammatory subtype that develops upon allergen challenge. As in humans, airway inflammation can be induced in mice by challenging previously sensitized animals with a specific allergen. The allergen most commonly used to trigger airway inflammation in mice is ovalbumin (OVA), which typically induces eosinophilic inflammation. However, higher concentrations of endotoxin in the OVA solution can shift the inflammation developed in the airways to a mixed granulocytic subtype.^{15,16} Furthermore, the adjuvant that is co-administered with OVA to stimulate the immune response also influences the inflammation that develops in the airways. For example, co-administering alum promotes stronger eosinophilia than if no adjuvant is used.¹⁷ Similarly, house dust mite (HDM), a common household allergen, is frequently used in mouse models of asthma and triggers an airway inflammation rich in eosinophils.^{18,19} In contrast, cockroach and *Alternaria* allergens, to which humans are also commonly sensitized, promote an inflammation rich in both eosinophils and neutrophils in mice.^{20–22} Less common are allergens that induce an increase in neutrophils but not eosinophils in mice: 1,3- β -glucan, the major cell wall component of fungi, is one example.^{23,24} Collectively, these observations suggest that different allergen types are capable of promoting distinct inflammatory subtypes in mice.

To our knowledge, no study has previously tested the hypothesis that different allergens can induce distinct inflammatory subtypes in humans. As in mice, the inflammatory response to allergen exposure can be studied in humans in a controlled setting using an allergen inhalation challenge test. In this test, subjects with mild allergic asthma inhale increasing concentrations of an allergen solution until an early asthmatic response (EAR) is triggered, typically consisting of a 20% drop in lung function from baseline.²⁵ The allergen used in the inhalation challenge test is usually that which triggers the largest allergic response in a skin prick test.²⁵ The primary outcome measured is often the late asthmatic response (LAR), which follows the natural recovery from the EAR and corresponds to the drop in lung function from baseline recorded between 3 and 8 hours after allergen challenge. The magnitude of the LAR reflects the severity of airway inflammation induced by allergen exposure²⁶ and can vary considerably between individuals. Importantly, Boulet et al found that some of this variation in the magnitude of the LAR can be explained by the allergen type used in the challenge.²⁷ On the other hand, the allergen used in the inhalation challenge did not determine the absolute number of eosinophils or neutrophils measured in

sputum, but these cell types were analyzed individually and sputum inflammatory subtypes were not considered. In this study, we extend the analysis of Boulet et al to test the association between sputum inflammatory subtypes and the allergen type inhaled, based on information from 129 individuals with a documented EAR and LAR.

2 | METHODS

2.1 | Study design and participants

We performed a retrospective analysis of data from 21 clinical trials of experimental asthma treatments that included an allergen inhalation challenge test. The trials (summarized in Table S1) were conducted between 2003 and 2013 in 2 centers (Laval University and McMaster University); protocols were standardized between sites. Briefly, in all studies, each participant completed an allergen inhalation challenge test during the screening phase of the trial, that is, prior to receiving any experimental treatment. The test was carried out on 3 consecutive days and consisted of the following: (i) skin prick titration test and methacholine challenge test on day 1, to determine the dose of allergen to be administered in the inhalation test; (ii) the allergen inhalation challenge test on day 2; and (iii) a follow-up methacholine challenge on day 3. All measurements included in our analysis refer to data collected only in these 3 days. A total of 153 participants (57% female) were recruited, aged 18–61 years. Subjects had mild asthma, defined by (i) a forced expiratory volume in 1 seconds (FEV_1) > 70% of the predicted normal value (ie, sufficient lung function such that a 50% fall in FEV_1 would be safe and tolerated), (ii) a provocative concentration of methacholine that caused a 20% drop in FEV_1 (PC_{20}) that was ≤ 16 mg/mL (see below), and (iii) no requirement for anti-inflammatory treatments that could interfere with allergen-induced responses, with the exception of short-acting beta-agonists (SABA) to relieve symptoms less than twice weekly.

2.2 | Skin prick tests

Each participant completed 2 skin prick tests—an allergen skin test and an allergen skin titration test.

The aim of the allergen skin test, typically performed during the first clinical visit, was to determine the allergen type to be used subsequently in the inhalation challenge test. Briefly, skin reactivity was measured 15 minutes after exposure (scratching or pricking) to (i) an allergen; (ii) a positive control (1 mg/mL histamine); and (iii) a negative control (diluent). Allergens tested included alder, white birch, horse, *Alternaria*, *Dermatophagoides farinae* (HDMDf), ragweed, grass, cat, and *Dermatophagoides pteronyssinus* (HMDP); and were purchased from Omega Laboratories, Greer, and ALK. Positive skin reactions were defined as a wheal (raised itchy bump) greater than 2×2 mm in size. The allergen that resulted in the largest positive skin reaction after exposure was selected for the inhalation challenge (the test allergen).

The allergen skin titration test was performed the day before the inhalation challenge test. The aim of this test was to determine the weakest dilution of the test allergen that resulted in a positive (>2 mm wheal) skin prick response. Briefly, skin reactivity was measured 10 minutes after exposure to serial dilutions of the test allergen. The lowest titration that caused a positive reaction corresponded to the "skin test endpoint," which is required to calculate the PC of the test allergen that is expected to cause a 20% drop in FEV₁, that is, the allergen PC₂₀.²⁸

2.3 | Methacholine inhalation challenge

A methacholine challenge test was performed the day before the allergen inhalation test to quantify airway sensitivity, as this is required to calculate the expected allergen PC₂₀, in addition to the skin test endpoint.²⁸ Briefly, methacholine chloride stock was prepared at a concentration of 128 mg/mL in physiological saline and further diluted in physiological saline to achieve working concentrations from 0.031 to 128 mg/mL. A Wright nebulizer with an output of 0.13 mL/min was used to produce methacholine aerosols. Participants were instructed to wear nose clips and to breathe normally from a Hans Rudolf valve connected to the nebulizer, during a 2-minutes period. First, subjects inhaled normal saline for 2 minutes. This was followed by inhalation of doubling concentrations of methacholine (starting with 0.031 mg/mL) for periods of 2 minutes each. FEV₁ was measured at 30, 90, 180, and 300 seconds after each inhalation, until a fall of 20% in FEV₁ was observed. The concentration at which a 20% decline in FEV₁ was observed corresponds to the methacholine PC₂₀.

2.4 | Allergen inhalation challenge

The allergen PC₂₀ was determined based on the skin test endpoint and methacholine PC₂₀ using an equation developed elsewhere.²⁸ In turn, the allergen PC₂₀ determined the first concentration of the test allergen that was administered in the inhalation test, specifically corresponding to 3-4 doubling dilutions below the PC₂₀. Starting with that concentration, allergen was inhaled at tidal breathing for 2 minutes, and FEV₁ measured 10 minutes after challenge. Increasing doubling concentrations of allergen (up to 1:4) were administered until a concentration was reached that caused a 20% decline in FEV₁, which corresponds to the observed allergen PC₂₀. FEV₁ was then recorded at regular intervals until 7 hours after inhalation of the last allergen dose. At 7 hours, all participants were treated with a SABA to reverse bronchoconstriction from allergen challenge. Participants received no anti-inflammatory medications, including inhaled corticosteroids, before or after allergen challenge, with the exception of SABA to relieve symptoms less than twice weekly.

A positive EAR was defined by a decline of $\geq 20\%$ in FEV₁ from baseline within 3 hours of the last allergen inhalation. A positive LAR was defined by a decline of $\geq 15\%$ in FEV₁ 3-7 hours post-allergen inhalation. Only individuals who experienced both an EAR and an LAR were selected for analysis.

2.5 | Sputum collection and classification of inflammatory subtype

Sputum was collected at 3 time points: after the methacholine challenge on day 1, after the allergen challenge on day 2 (7 hours postinhalation), and after the methacholine challenge on day 3. Relative to the start of the allergen inhalation test, we refer to these time points as -24, 7, and 24 hours postchallenge. The sputum samples collected therefore reflect 3 different inflammatory states: baseline (ie, prechallenge), LAR, and persistence of LAR.

Sputum was induced and processed as described previously,²⁹ which included obtaining total and differential cell counts. To account for differences in sputum yield, analyses of absolute cell numbers were performed relative to sputum weight. The proportion of eosinophils and neutrophils in sputum was calculated relative to the total cell count and used to categorize sputum inflammatory subtype into 4 categories as described elsewhere⁶: (i) eosinophilic, >1.01% eosinophils and <61% neutrophils; (ii) neutrophilic, <1.01% eosinophils and >61% neutrophils; (iii) mixed granulocytic, >61% neutrophils and >1.01% eosinophils; or (iv) paucigranulocytic, <61% neutrophils and <1.01% eosinophils. These thresholds were determined based on the 95th percentiles observed in a cohort of healthy individuals.⁶

2.6 | Statistical analysis

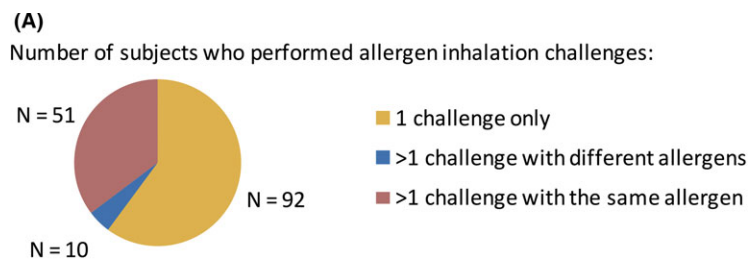
Outcome variables measured on a continuous scale (cell counts, EAR, and LAR) were assessed for normality using the Shapiro-Wilkson test. Neutrophil counts, eosinophil counts, and LAR were not normally distributed and so were transformed with a rank-based inverse-normal transformation. A paired *t* test was used to compare cell counts between prechallenge (-7 hours) and postchallenge (7 and 24 hours) time points. A one-way ANOVA was used to test the effect of allergen type on variation in outcome variables, with post hoc comparisons between allergen types performed with the Tukey-Kramer test. Associations between sputum subtype, which is a categorical variable, and predictors of interest (eg, allergen type) were tested with a multinomial log-linear model, using the multinom function from the nnet R package.³⁰ Sputum subtype proportions were compared between groups using the McNemar test. All outcomes were assessed for association with covariates (age, sex, height, weight, and body mass index) before analysis. Analyses were performed using R version 3.2.3.

3 | RESULTS

3.1 | Dataset and subject characteristics at baseline

The dataset available for analysis included 305 allergen challenges performed by 153 unique individuals and including 9 allergen types (Figure 1). Four allergen extracts (alder, white birch, horse,

FIGURE 1 Summary of steps used to obtain the dataset analyzed. A, The original dataset consisted of 305 allergen challenges performed by 153 unique individuals, including 9 different types of allergen. Most individuals ($n = 92$) completed a single allergen challenge. Of the 61 individuals who completed 2 or more allergen challenges, 51 were challenged with the same allergen and 10 were challenged with different allergens. B, Four data management steps were applied to select individual allergen challenges for analysis. First, challenges performed with an allergen extract (alder, white birch, horse, *Alternaria*) tested in <10 individuals were excluded. Second, results from challenges performed by the same individual with the same allergen type in different studies were averaged. Third, for individuals challenged with different allergen types in different studies, results for the least common allergen(s) were excluded. Fourth, results from individuals who did not experience both an early asthmatic response and an late asthmatic response were excluded



Number of subjects challenged: 153
Total number of allergen challenges: 305

Number of allergen inhalation challenges performed with each allergen type:

	Alder	White birch	Horse	Alternaria	HDMDP	Ragweed	Grass	Cat	HDMDP
Original dataset	1	1	6	9	20	27	39	84	118
From unique individuals retained for analysis	0	0	0	0	12	16	22	39	40

(B)

N subjects	153	(1)	145	(2)	145	(3)	145	(4)	129
N allergens	9		5		5		5		5
N challenges	305		288		158		145		129

- 1) Remove allergens used in <10 challenges
- 2) Average outcomes of subjects with >1 challenge with the same allergen
- 3) Restrict data to challenges with least common allergens for subjects with >1 challenge with different allergens
- 4) Restrict to subjects who experienced an EAR and an LAR

and *Alternaria*) were tested in a small number of individuals ($n < 10$), so these data were excluded from further analysis. Sixty-one individuals participated in 2 or more clinical trials and so completed multiple allergen challenge tests. For each of these individuals, outcomes were averaged across allergen challenge tests performed with the same allergen type in different studies. If an individual was challenged with different allergens in different studies, data were included for the challenge performed with the allergen type that was least common in the dataset, to maximize the number of challenges with each allergen included in the analyses. Lastly, data were excluded from 16 allergen challenges with a negative EAR and/or negative LAR, as it was not clear whether allergen challenge triggered a typical acute asthmatic response. After these exclusions, data were available for 129 allergen challenges performed by 129 individuals and including 5 allergen types: HDMDP ($n = 12$), ragweed ($n = 16$), grass ($n = 22$), cat ($n = 39$), and HDMDP ($n = 40$). Sputum samples were available for 118 subjects before allergen challenge (-24 hours), and for 123 (7 hours) and 124 (24 hours) subjects after allergen inhalation challenge. For 111 subjects, sputum samples were available across all 3 time points.

Subjects subsequently tested with different allergen types had similar characteristics at baseline, including, for example, comparable sputum subtype frequencies (Table S2). Age, height, weight,

and BMI had no significant effect on variation in sputum subtype frequencies (not shown).

3.2 | Overall effect of allergen challenge on sputum granulocyte counts and inflammatory subtype

At baseline (ie, before allergen challenge), the sputum inflammatory subtype of most individuals was either paucigranulocytic (38%) or eosinophilic (43%) (Table 1).

At 7 hours postchallenge, both eosinophils and neutrophils in sputum increased significantly (Figure S1; Table S3), causing the inflammatory subtype to change for most subjects (62%; Tables S4 and S5). For example, of the 44 individuals with paucigranulocytic sputum at baseline, 48% and 43% had eosinophilic and mixed granulocytic subtype at 7 hours postchallenge, respectively. Overall, relative to baseline, there was an increase in the proportion of individuals with the eosinophilic (from 43% to 55%) and mixed granulocytic (9% to 38%) subtypes, and a decrease in the proportion of individuals with the paucigranulocytic (38% to 6%) and neutrophilic subtypes (9% to 1%) (Figure 2; Table 1).

At 24 hours postchallenge, eosinophils and neutrophils in sputum remained elevated compared to baseline and largely unchanged from 7 hours postchallenge (Figure S1; Table S3). However, when cell counts were assessed as a fraction of the total cell count, there was

TABLE 1 Proportion of sputum inflammatory subtypes before (–24 h) and after (7 and 24 h) an allergen inhalation challenge

Subtype		–24 h	7 h	<i>P</i> ^a	24 h	<i>P</i> ^a	<i>P</i> ^b
		Proportion	Proportion		Proportion		
		n = 118	n = 123		n = 124		
All allergens	Paucigranulocytic	.38	.06	2.5×10^{-10}	.04	4.5×10^{-9}	.4795
	Eosinophilic	.43	.55	.0253	.76	3.6×10^{-7}	1.0×10^{-4}
	Neutrophilic	.09	.01	.0016	.02	.0114	.3173
	Mixed granulocytic	.09	.38	3.3×10^{-7}	.19	.0184	1.0×10^{-4}
		n = 37	n = 38		n = 38		
HDMDP	Paucigranulocytic	.41	.05	3.0×10^{-4}	.05	3.0×10^{-4}	1
	Eosinophilic	.43	.53	.4669	.76	.0047	.0126
	Neutrophilic	.08	.00	.0833	.00	.0833	NA
	Mixed granulocytic	.08	.42	.0029	.18	.1573	.0126
		n = 36	n = 38		n = 38		
Cat	Paucigranulocytic	.31	.05	.0016	.00	.0016	.1573
	Eosinophilic	.56	.66	.285	.84	.0067	.0348
	Neutrophilic	.03	.00	.3173	.00	.3173	NA
	Mixed granulocytic	.11	.29	.0522	.16	.3173	.0956
		n = 20	n = 19		n = 22		
Grass	Paucigranulocytic	.45	.00	.0027	.00	.0027	NA
	Eosinophilic	.35	.42	.3173	.68	.0196	.1573
	Neutrophilic	.10	.05	.3173	.05	.3173	NA
	Mixed granulocytic	.10	.53	.0047	.27	.1797	.1573
		n = 15	n = 16		n = 14		
Ragweed	Paucigranulocytic	.60	.19	.0082	.07	.0082	.1573
	Eosinophilic	.13	.50	.0143	.71	.0082	.0833
	Neutrophilic	.13	.00	.1573	.07	1	.3173
	Mixed granulocytic	.13	.31	.0833	.14	NA	.1573
		n = 10	n = 12		n = 12		
HDMDF	Paucigranulocytic	.10	.00	.3173	.17	.5637	.1573
	Eosinophilic	.60	.58	1	.67	.6547	.6547
	Neutrophilic	.30	.00	.0833	.00	.0833	NA
	Mixed granulocytic	.00	.42	.0455	.17	.3173	.0833

^aVersus –24 h.^bVersus 7 h.

a significant decrease in the proportion of neutrophils from 7 to 24 hours postchallenge (Figure S2). This resulted in a significant decrease in the proportion of individuals with the mixed granulocytic subtype (38%-19%) at 24 hours, and a significant increase in the proportion of individuals with the eosinophilic subtype (55%-76%) (Figure 2; Table 1).

When considering the difference in sputum cell counts between the baseline and postchallenge time points, which reflects how many cells entered the airways after the challenge, there was no association between the severity of the LAR and the number of eosinophils or neutrophils (Table S6). However, individuals with higher eosinophil ($P = .014$) and neutrophil ($P = .018$) sputum counts at baseline developed a more severe LAR upon allergen challenge (Table S6).

3.3 | Effect of allergen type on sputum granulocyte counts and inflammatory subtype

Compared to baseline, all allergens promoted a significant (Figure 3) and comparable (Figure S3 and Table S3) increase in sputum eosinophil counts, both at 7 and 24 hours postchallenge. On the other hand, neutrophil counts only increased significantly at 7 hours postchallenge for HDMDP, cat, and grass allergens (Figure 3), although a similar trend was observed with HDMDF and ragweed (Figure S3 and Table S3). At 24 hours postchallenge, all allergens promoted a significant increase in neutrophils, suggesting a delayed infiltration of neutrophils with some allergens.

At 7 hours postchallenge, the eosinophilic subtype was found at a similar frequency across allergens, ranging from 42% of individuals

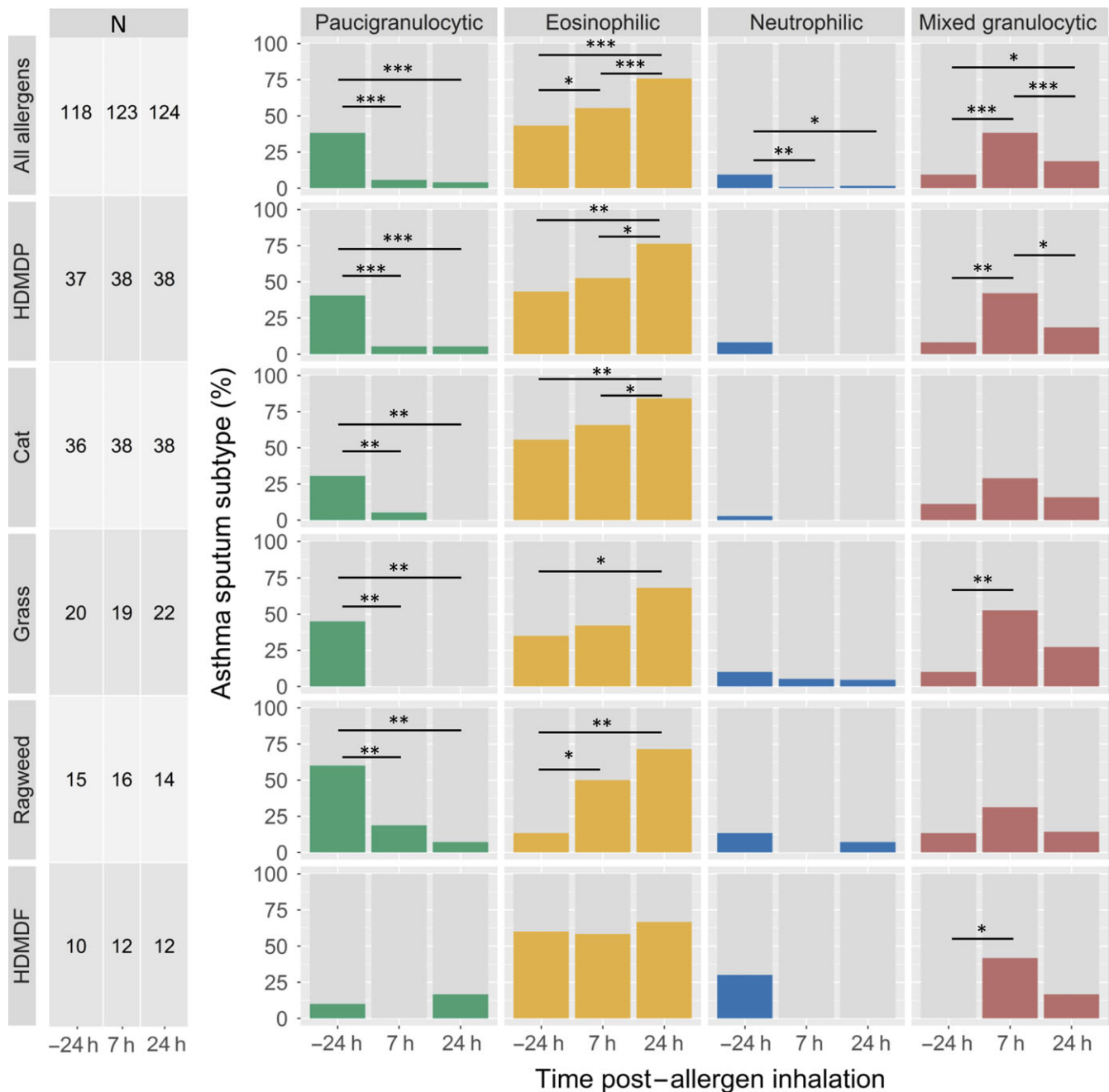


FIGURE 2 Proportion of sputum inflammatory subtypes before (–24 h) and after (7 and 24 h) an allergen inhalation challenge. Sample sizes for each time point are shown on the left. * $P < .05$, ** $P < .01$, *** $P < .001$

tested with grass to 66% of those tested with cat (Figure 2 and Table 1). The second most common subtype at this time point, mixed granulocytic sputum, also occurred with similar frequency across allergen types, ranging from 29% for cat to 53% for grass allergen. Overall, there were no significant differences in the frequency of sputum inflammatory subtypes between the 5 allergen types tested ($P = .298$).

At 24 hours postchallenge, the eosinophilic subtype was found in 66% (HDMDF) to 84% (cat) of individuals, and the mixed granulocytic subtype was found in 14% (ragweed) to 27% (grass) of

individuals, again with no overall statistically significant differences in subtype frequencies between allergen types ($P = .255$).

4 | DISCUSSION

In this study, we analyzed data from allergen inhalation challenges to test the hypothesis that different allergen types can induce distinct inflammatory profiles in the airways. The study population included exclusively individuals with mild allergic asthma.

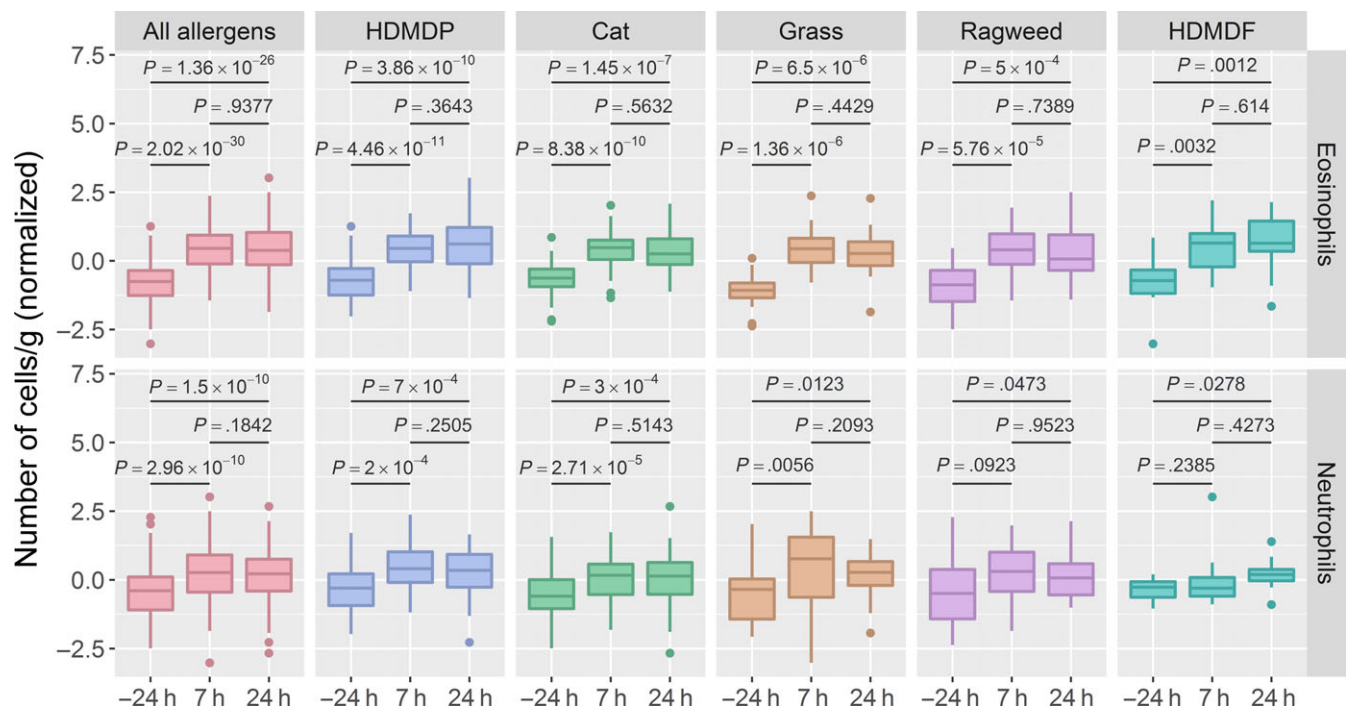


FIGURE 3 Eosinophil and neutrophil cell counts in sputum collected before (–24 h) and after (7 and 24 h) allergen inhalation challenge, stratified by allergen

Unexpectedly, we found no association between the number of eosinophils and neutrophils that infiltrate into the airways after allergen challenge and the severity of the LAR. On the other hand, individuals with increased eosinophil and neutrophil sputum counts at baseline had a more severe LAR, consistent with results from Boulet et al²⁷ These results suggest that the presence of larger numbers of granulocytes at baseline, perhaps reflecting ongoing or chronic inflammatory processes, is a risk factor for a more severe asthma exacerbation upon acute allergen exposure.

When we compared the frequency of sputum inflammatory subtypes between individuals challenged with different allergen types, we found no overall significant differences. This is in contrast with findings from studies of experimental acute allergic asthma conducted in isogenic mice. Such differences could arise, for example, if there is a significant genetic contribution to variation in sputum subtype in humans. Blood cell counts are highly heritable in humans, with about 70% of the variation in eosinophil and neutrophil numbers explained by genetic factors.³¹ There is also a significant positive correlation in granulocyte counts between blood and sputum, most strongly for eosinophils.^{4,32,33} Therefore, it is possible (if not likely) that sputum cell counts are also heritable. On the other hand, environmental factors might also contribute to variation in sputum subtype after acute allergen challenge in humans but not (or less so) in mouse studies. For example, recent studies have shown that depending on the microbiota composition of the lungs, the airway inflammatory phenotype may adopt a neutrophilic- or eosinophilic-like profile.^{34–37} The intestinal microbiome can also modulate the allergic responses in the airways through the production of by-products that regulate cell function and differentiation.³⁸ Thus, unlike

described in mice, allergen type is likely to have no (or only a small) contribution to variation in sputum subtype following acute allergen challenge.

The changes in sputum inflammatory cells seen in the paucigranulocytic subtype warrant some discussion, especially as the pathogenesis of this asthma subtype is not well understood. Our findings indicate that individuals with paucigranulocytic asthma can readily develop an eosinophilic or mixed granulocytic sputum subtype after allergen exposure. This suggests that it is not correct to regard paucigranulocytic asthma as a static phenotype but one in which airway inflammation can develop dynamically in response to sensitizing allergens.

There are a number of limitations that need to be considered when interpreting results from our study. First, the study design used does not explicitly control for many factors that could potentially contribute to interindividual variation in sputum subtype. This could decrease power to detect a true effect of allergen type on variation in sputum subtype frequency. For example, in mouse studies, animals are isogenic, kept in a comparable environment, fed the same diet, and challenged within days of each other. As such, the effect of genetic and (many) environmental factors on sputum subtype variability is minimized, which was not the case in our study. Therefore, in mice, allergen type might explain a larger fraction of sputum subtype variability, and so such effect might be detectable with relatively small sample sizes. A comparable and more desirable study design in humans would be to test whether different allergens trigger different sputum subtypes in the same individual, with different allergens tested in a relatively narrow time frame. Such data were available for only a

small number of individuals in our study and so were not considered in our analyses. Second, to facilitate the analysis and interpretation of results, we averaged observations from individuals who completed multiple allergen challenges using the same allergen. Although this is unlikely to have affected the robustness of our findings, a more powerful approach would have been to use mixed models and include random effects to account for the presence of nonindependent observations. We explored this approach but often had convergence problems, thus questioning the validity of the model estimates. Nonetheless, with this caveat in mind, results obtained with mixed models were consistent with our conclusions (not shown). Third, not all allergens tested in these analyses were equally represented. HDMDP, the most common allergen in the dataset, was used in 40 allergen inhalation tests, whereas HDMD, the rarest allergen in the dataset, was only used in 12 allergen inhalation tests. Small sample sizes could affect the power to detect differences between allergens. Another caveat relates to the allergen groupings used in our analysis. We opted to analyze the 2 HDM species separately (instead of combining them into a broader HDM group) because known differences between these allergens^{39,40} could potentially trigger distinct immunological responses. The same holds for the 2 types of pollen that were analyzed separately in our study (ragweed and grass). When we repeated our analyses with only 3 major allergen groups (HDM, pollen, and cat), results remained largely unchanged (not shown). Lastly, the outcome of interest in these analyses was the sputum subtype, which is defined by the proportion of eosinophils and neutrophils in sputum. The thresholds we used to classify sputum subtype were proposed by Simpson et al⁶ corresponding to the 95th percentiles observed in healthy individuals. However, other studies have proposed slightly different thresholds,^{4,41,42} which might result in a different sputum subtype classification for some individuals in our study. Which thresholds are more appropriate to define the different sputum subtypes is still an open question. However, when we categorized sputum subtype according to other published thresholds, results remained unchanged, with no significant effect of allergen type on the frequency of sputum subtypes (not shown).

In conclusion, we found no association between the type of allergen used in inhalation challenge tests and the sputum inflammatory subtype developed. This observation is important because it suggests that there are no specific allergen types that can be used reliably to enrich allergen-induced responses for an inflammatory subtype of interest, as might be important in clinical trials of biologic therapies for asthma.

ACKNOWLEDGMENTS

M.A.R.F. was supported by a fellowship from the National Health and Medical Research Council of Australia (APP1124501). J.A.R. is supported by a doctoral grant (SFRH/BD/92907/2013) from Fundação para a Ciência e Tecnologia, Portugal.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

AUTHOR CONTRIBUTIONS

JAR, GMG, and MARF conducted the analyses. KJK, PMO, LPB, and GMG were involved in data collection. All authors wrote the manuscript.

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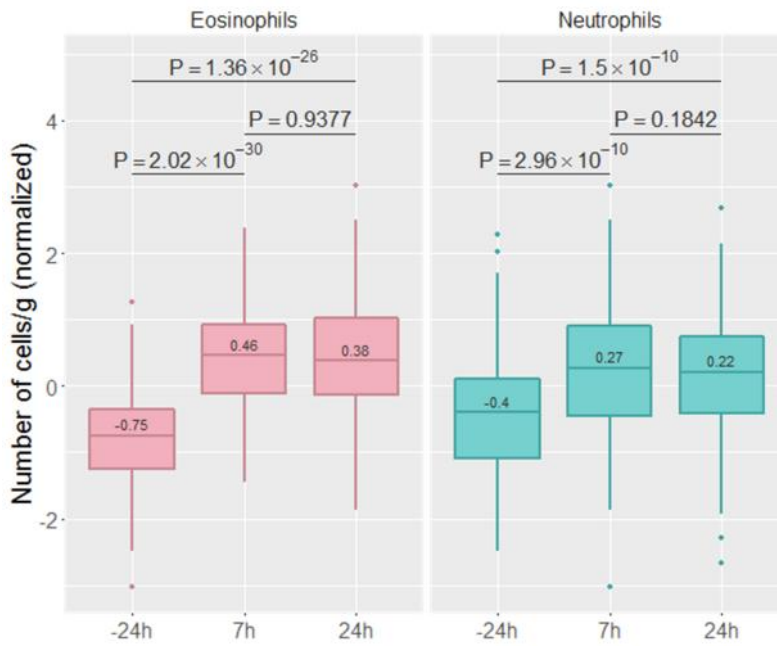
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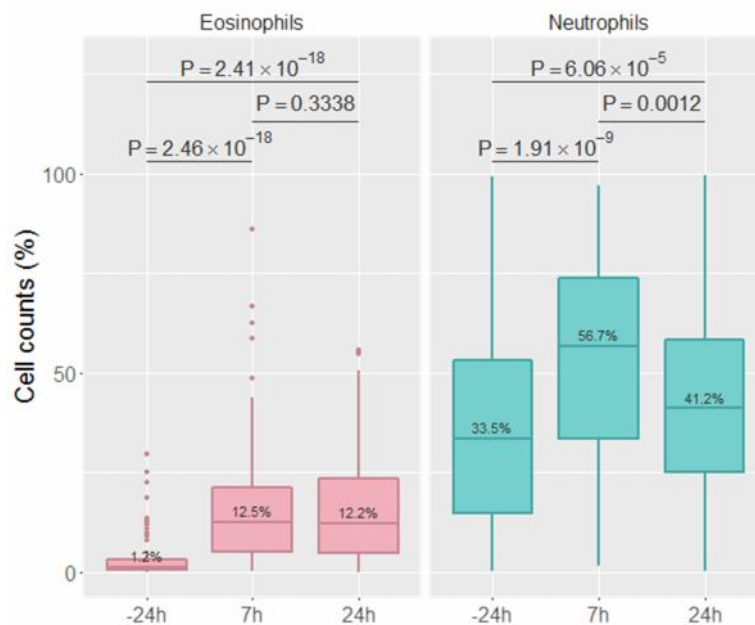
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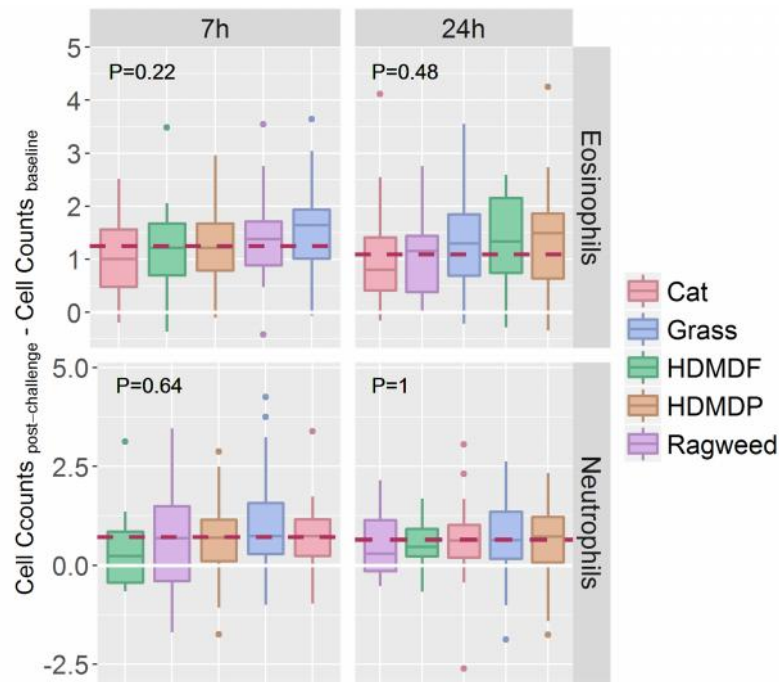
How to cite this article: Revez JA, Killian KJ, O'Byrne PM, et al. Sputum cytology during late-phase responses to inhalation challenge with different allergens. *Allergy.* 2018;73:1470-1478. <https://doi.org/10.1111/all.13415>



Supplementary Figure 1. Eosinophil and neutrophil cell counts in sputum collected before (-24 h) and after (7 h and 24 h) allergen inhalation challenge



Supplementary Figure 2. Eosinophils and neutrophil cell proportions in sputum collected before (-24h) and after (7 h and 24 h) allergen inhalation challenge



Supplementary Figure 3. Change in sputum eosinophil and neutrophil cell counts from baseline (-24 h) to 7 and 24 h post allergen inhalation challenge, stratified by allergen.

Differences from baseline were calculated based on normalized cells counts per gram of sputum. The dashed line represents the overall median change from baseline.

14 **Supplementary Table 1. Demographics of participants from 21 clinical trials of experimental asthma.**

Study	N participants	Sex (% female)	Age (years)	BMI (kg/m ²)	Allergens tested (allergen type, N)
1	22	66.7	32 ± 11.2	25 ± 4	Alternaria, 1; Cat, 5; Grass, 3; HDMDf, 1; HDMDP, 6; Horse, 4; Ragweed, 2
2	20	50	33 ± 13.2	26 ± 4.6	Alder, 1; Cat, 8; Grass, 3; HDMDP, 6; Ragweed, 1; White birch, 1
3	11	54.5	26 ± 8.3	24 ± 2.1	Cat, 4; Grass, 1; HDMDf, 1; HDMDP, 2; Horse, 1; Ragweed, 2
4	24	45.8	26 ± 8.7	NA	Alternaria, 1; Cat, 6; Grass, 5; HDMDP, 9; Ragweed, 3
5	9	55.6	25 ± 9.8	NA	Alternaria, 1; Cat, 2; HDMDP, 5; Ragweed, 1
6	10	60	31 ± 12.6	NA	Cat, 4; Grass, 1; HDMDf, 1; HDMDP, 4
7	18	52.4	31 ± 13.7	25 ± 5.9	Alternaria, 2; Cat, 6; Grass, 3; HDMDf, 2; HDMDP, 7; Ragweed, 1
8	11	54.5	38 ± 13.2	28 ± 8.7	Alternaria, 1; Cat, 5; Grass, 1; HDMDP, 4
9	21	57.1	27 ± 10.6	NA	Cat, 4; Grass, 2; HDMDf, 3; HDMDP, 10; Ragweed, 2
10	10	30	33 ± 15.3	24 ± 2.7	Alternaria, 1; Cat, 2; HDMDf, 1; HDMDP, 4; Ragweed, 2
11	7	28.6	29 ± 11	NA	Alternaria, 1; Cat, 1; Grass, 1; HDMDP, 3; Ragweed, 1
12	21	42.9	29 ± 10.3	NA	Cat, 11; Grass, 2; HDMDf, 1; HDMDP, 7
13	5	80	32 ± 15.4	25 ± 4.8	Alternaria, 1; Cat, 1; Grass, 1; HDMDP, 2
14	20	52.4	36 ± 13.7	27 ± 6.3	Cat, 9; Grass, 2; HDMDP, 8; Ragweed, 2
15	15	46.7	29 ± 10.6	NA	Cat, 3; Grass, 5; HDMDf, 2; HDMDP, 5
16	11	72.7	29 ± 10.8	NA	Cat, 2; Grass, 2; HDMDf, 2; HDMDP, 5
17	10	30	33 ± 13.3	NA	Cat, 1; HDMDf, 1; HDMDP, 6; Ragweed, 2
18	19	73.7	27 ± 11	NA	Cat, 3; HDMDf, 2; HDMDP, 9; Ragweed, 5
19	13	64.3	31 ± 13.1	27 ± 5.8	Cat, 5; Grass, 2; HDMDP, 5; Horse, 1; Ragweed, 1
20	7	57.1	33 ± 14.9	NA	Cat, 2; Grass, 3; HDMDP, 2
21	16	75	26 ± 9.1	NA	Grass, 2; HDMDf, 3; HDMDP, 9; Ragweed, 2

15 BMI, body mass index. Results are presented as means ± SD.

16 **Supplementary Table 2. Demographics and clinical characteristics of the study participants, stratified by allergen type inhaled.**

	HDMDf	Ragweed	Grass	Cat	HDMDP
<i>n</i>	12	16	22	39	40
Sex (% female)	41.7	50	22.7	68.4	65
Age (years)	27 ± 10.5 (19-52)	23 ± 5.9 (18-40)	29 ± 10.4 (18-48)	31 ± 13.8 (19-60)	29 ± 10.5 (19-54)
Height (cm)	174 ± 12 (155-192)	170 ± 9.9 (150-186)	176 ± 10.9 (155-195)	170 ± 8.7 (155-187)	169 ± 8.4 (153-188)
Weight (kg)	71 ± 6.1 (63-77)	73 ± 10.1 (59-83)	86 ± 21.3 (53-115)	75 ± 17.9 (52-113)	69 ± 13.5 (50-101)
BMI (kg/m ²)	23 ± 1.5 (21-24)	26 ± 2.1 (24-29)	27 ± 3.5 (22-32)	27 ± 6 (19-41)	24 ± 3.6 (19-32)
LAR (% fall in FEV ₁)	28 ± 7.7 (15-41)	24 ± 9.2 (15-49)	24 ± 6.7 (17-42)	22 ± 6 (16-44)	26 ± 7.2 (15-44)
<i>Baseline sputum characteristics</i>					
TCC (x 10 ⁶ /g mucus)	2 ± 1.3 (0-4)	5 ± 5.9 (0-24)	3 ± 4.1 (0-19)	3 ± 2.2 (1-12)	4 ± 2.8 (1-13)
Eosinophils (x 10 ⁶ /g mucus)	16 ± 33.6 (0-110)	10 ± 16.8 (0-61)	4 ± 6.6 (0-26)	12 ± 20.8 (0-111)	16 ± 34.6 (0-172)
Neutrophils (x 10 ⁶ /g mucus)	111 ± 52.2 (32-191)	286 ± 573.9 (2-2255)	185 ± 337.6 (4-1518)	140 ± 195.5 (1-915)	179 ± 212.7 (4-1022)
Subtype (% Pauci, % Eos, % Neut, % MG)	10, 60, 30, 0	60, 13, 13, 13	45, 35, 10, 10	31, 56, 3, 11	41, 43, 8, 8

17 BMI, body mass index; Eos, eosinophilic; FEV₁, forced expiratory volume in 1 s; LAR, late airway response; MG, mixed granulocytic; Neut,
18 neutrophilic; Pauci. paucigranulocytic; TCC, total cell counts. Results are presented as frequencies (in %) or means ± SD (range).

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Supplementary Table 3. Change in eosinophil and neutrophil cell counts from baseline (-24 h) to 7 and 24 h post allergen inhalation challenge. Differences from baseline were calculated based on normalized cells counts per gram of sputum. Δ , mean difference from baseline.

	Allergen	7 h post challenge			24 h post challenge		
		N	Δ	<i>P</i> -value	N	Δ	<i>P</i> -value
Eosinophils	All allergens	112	1.2	2.0×10^{-30}	113	1.1	1.4×10^{-26}
	Grass	18	1.6	1.4×10^{-6}	20	1.3	6.5×10^{-6}
	Ragweed	15	1.4	5.8×10^{-5}	13	1.2	5.0×10^{-4}
	HDMDP	10	1.2	0.0032	10	1.3	0.0012
	HDMDP	35	1.2	4.5×10^{-11}	36	1.5	3.9×10^{-10}
	Cat	34	1.0	8.4×10^{-10}	34	0.8	1.5×10^{-7}
Neutrophils	All allergens	112	0.7	3.0×10^{-10}	113	0.6	1.5×10^{-10}
	Ragweed	15	0.7	0.0923	13	0.3	0.0473
	Grass	18	0.7	0.0056	20	0.6	0.0123
	Cat	34	0.7	2.7×10^{-5}	34	0.6	3.0×10^{-4}
	HDMDP	35	0.7	2.0×10^{-4}	36	0.7	7.0×10^{-4}
	HDMDP	10	0.2	0.2385	10	0.5	0.0278

Supplementary Table 4. Proportion of sputum inflammatory subtypes observed 24 h before allergen inhalation challenge, stratified by the sputum subtype developed 7 h post challenge.

		Baseline				Total at 7h
		Paucigranulocytic	Eosinophilic	Neutrophilic	Mixed granulocytic	
7 h post challenge	Paucigranulocytic	9%	0%	0%	0%	4
	Eosinophilic	48%	69%	27%	60%	64
	Neutrophilic	0%	0%	9%	0%	1
	Mixed granulocytic	43%	31%	64%	40%	45
	Baseline total	44	49	11	10	114

32 **Supplementary Table 5. Sputum inflammatory subtypes observed before (-24 h) and**
33 **after (7 h and 24 h) allergen inhalation challenge.** N/A, no sputum sample available.

Participant	-24 h	7 h	24 h	Allergen
1	Eosinophilic	Eosinophilic	Eosinophilic	Cat
2	Eosinophilic	Eosinophilic	Eosinophilic	Cat
3	Eosinophilic	Eosinophilic	Eosinophilic	Cat
4	Eosinophilic	Eosinophilic	Eosinophilic	Cat
5	Eosinophilic	Eosinophilic	Eosinophilic	Cat
6	Eosinophilic	Eosinophilic	Eosinophilic	Cat
7	Eosinophilic	Eosinophilic	Eosinophilic	Cat
8	Eosinophilic	Eosinophilic	Eosinophilic	Cat
9	Eosinophilic	Eosinophilic	Eosinophilic	Cat
10	Eosinophilic	Eosinophilic	Eosinophilic	Cat
11	Eosinophilic	Eosinophilic	Eosinophilic	Cat
12	Eosinophilic	Eosinophilic	Eosinophilic	Cat
13	Eosinophilic	Eosinophilic	Eosinophilic	Cat
14	Eosinophilic	Eosinophilic	Eosinophilic	Cat
15	Eosinophilic	Eosinophilic	Eosinophilic	Cat
16	Eosinophilic	Eosinophilic	Eosinophilic	Grass
17	Eosinophilic	Eosinophilic	Eosinophilic	Grass
18	Eosinophilic	Eosinophilic	Eosinophilic	Grass
19	Eosinophilic	Eosinophilic	Eosinophilic	Grass
20	Eosinophilic	Eosinophilic	Eosinophilic	HDMDP
21	Eosinophilic	Eosinophilic	Eosinophilic	HDMDP
22	Eosinophilic	Eosinophilic	Eosinophilic	HDMDP
23	Eosinophilic	Eosinophilic	Eosinophilic	HDMDP
24	Eosinophilic	Eosinophilic	Eosinophilic	HDMDP
25	Eosinophilic	Eosinophilic	Eosinophilic	HDMDP
26	Eosinophilic	Eosinophilic	Eosinophilic	HDMDP
27	Eosinophilic	Eosinophilic	Eosinophilic	HDMDP
28	Eosinophilic	Eosinophilic	Eosinophilic	HDMDP
29	Eosinophilic	Eosinophilic	Eosinophilic	HDMDP
30	Eosinophilic	Eosinophilic	Eosinophilic	Ragweed
31	Eosinophilic	Eosinophilic	Eosinophilic	Ragweed
32	Eosinophilic	Eosinophilic	Mixed granulocytic	Grass
33	Eosinophilic	Eosinophilic	Mixed granulocytic	HDMDP
34	Eosinophilic	Eosinophilic	Paucigranulocytic	HDMDP
35	Eosinophilic	Mixed granulocytic	Eosinophilic	Cat
36	Eosinophilic	Mixed granulocytic	Eosinophilic	Cat
37	Eosinophilic	Mixed granulocytic	Eosinophilic	Cat
38	Eosinophilic	Mixed granulocytic	Eosinophilic	Cat
39	Eosinophilic	Mixed granulocytic	Eosinophilic	Grass
40	Eosinophilic	Mixed granulocytic	Eosinophilic	HDMDP
41	Eosinophilic	Mixed granulocytic	Eosinophilic	HDMDP

42	Eosinophilic	Mixed granulocytic	Eosinophilic	HDMDP
43	Eosinophilic	Mixed granulocytic	Eosinophilic	HDMDP
44	Eosinophilic	Mixed granulocytic	Eosinophilic	HDMDP
45	Eosinophilic	Mixed granulocytic	Eosinophilic	HDMDP
46	Eosinophilic	Mixed granulocytic	Mixed granulocytic	Cat
47	Eosinophilic	Mixed granulocytic	Mixed granulocytic	HDMDP
48	Eosinophilic	Mixed granulocytic	Mixed granulocytic	HDMDP
49	Eosinophilic	Mixed granulocytic	Mixed granulocytic	HDMDP
50	Eosinophilic	N/A	Eosinophilic	Grass
51	Eosinophilic	N/A	Eosinophilic	HDMDP
52	Mixed granulocytic	Eosinophilic	Eosinophilic	Cat
53	Mixed granulocytic	Eosinophilic	Eosinophilic	HDMDP
54	Mixed granulocytic	Eosinophilic	Eosinophilic	HDMDP
55	Mixed granulocytic	Eosinophilic	Mixed granulocytic	Cat
56	Mixed granulocytic	Eosinophilic	Mixed granulocytic	Cat
57	Mixed granulocytic	Eosinophilic	Mixed granulocytic	HDMDP
58	Mixed granulocytic	Mixed granulocytic	Mixed granulocytic	Cat
59	Mixed granulocytic	Mixed granulocytic	Mixed granulocytic	Grass
60	Mixed granulocytic	Mixed granulocytic	Mixed granulocytic	Ragweed
61	Mixed granulocytic	Mixed granulocytic	Mixed granulocytic	Ragweed
62	Mixed granulocytic	N/A	Eosinophilic	Grass
63	Neutrophilic	Eosinophilic	Eosinophilic	HDMDP
64	Neutrophilic	Eosinophilic	Eosinophilic	Ragweed
65	Neutrophilic	Eosinophilic	Paucigranulocytic	HDMDP
66	Neutrophilic	Mixed granulocytic	Eosinophilic	HDMDP
67	Neutrophilic	Mixed granulocytic	Eosinophilic	HDMDP
68	Neutrophilic	Mixed granulocytic	Mixed granulocytic	Cat
69	Neutrophilic	Mixed granulocytic	Mixed granulocytic	Grass
70	Neutrophilic	Mixed granulocytic	Mixed granulocytic	HDMDP
71	Neutrophilic	Mixed granulocytic	Mixed granulocytic	HDMDP
72	Neutrophilic	Mixed granulocytic	N/A	Ragweed
73	Neutrophilic	Neutrophilic	Neutrophilic	Grass
74	Paucigranulocytic	Eosinophilic	Eosinophilic	Cat
75	Paucigranulocytic	Eosinophilic	Eosinophilic	Cat
76	Paucigranulocytic	Eosinophilic	Eosinophilic	Cat
77	Paucigranulocytic	Eosinophilic	Eosinophilic	Cat
78	Paucigranulocytic	Eosinophilic	Eosinophilic	Cat
79	Paucigranulocytic	Eosinophilic	Eosinophilic	Cat
80	Paucigranulocytic	Eosinophilic	Eosinophilic	Grass
81	Paucigranulocytic	Eosinophilic	Eosinophilic	Grass
82	Paucigranulocytic	Eosinophilic	Eosinophilic	HDMDP
83	Paucigranulocytic	Eosinophilic	Eosinophilic	HDMDP
84	Paucigranulocytic	Eosinophilic	Eosinophilic	HDMDP
85	Paucigranulocytic	Eosinophilic	Eosinophilic	HDMDP
86	Paucigranulocytic	Eosinophilic	Eosinophilic	HDMDP
87	Paucigranulocytic	Eosinophilic	Eosinophilic	HDMDP

88	Paucigranulocytic	Eosinophilic	Eosinophilic	HDMDP
89	Paucigranulocytic	Eosinophilic	Eosinophilic	Ragweed
90	Paucigranulocytic	Eosinophilic	Eosinophilic	Ragweed
91	Paucigranulocytic	Eosinophilic	Eosinophilic	Ragweed
92	Paucigranulocytic	Eosinophilic	Eosinophilic	Ragweed
93	Paucigranulocytic	Eosinophilic	Mixed granulocytic	Grass
94	Paucigranulocytic	Eosinophilic	N/A	Ragweed
95	Paucigranulocytic	Mixed granulocytic	Eosinophilic	Cat
96	Paucigranulocytic	Mixed granulocytic	Eosinophilic	Cat
97	Paucigranulocytic	Mixed granulocytic	Eosinophilic	Cat
98	Paucigranulocytic	Mixed granulocytic	Eosinophilic	Grass
99	Paucigranulocytic	Mixed granulocytic	Eosinophilic	Grass
100	Paucigranulocytic	Mixed granulocytic	Eosinophilic	Grass
101	Paucigranulocytic	Mixed granulocytic	Eosinophilic	Grass
102	Paucigranulocytic	Mixed granulocytic	Eosinophilic	Grass
103	Paucigranulocytic	Mixed granulocytic	Eosinophilic	HDMDP
104	Paucigranulocytic	Mixed granulocytic	Eosinophilic	HDMDP
105	Paucigranulocytic	Mixed granulocytic	Eosinophilic	HDMDP
106	Paucigranulocytic	Mixed granulocytic	Eosinophilic	HDMDP
107	Paucigranulocytic	Mixed granulocytic	Eosinophilic	HDMDP
108	Paucigranulocytic	Mixed granulocytic	Eosinophilic	Ragweed
109	Paucigranulocytic	Mixed granulocytic	Mixed granulocytic	Grass
110	Paucigranulocytic	Mixed granulocytic	Mixed granulocytic	HDMDP
111	Paucigranulocytic	Mixed granulocytic	Neutrophilic	Ragweed
112	Paucigranulocytic	Mixed granulocytic	Paucigranulocytic	HDMDP
113	Paucigranulocytic	Mixed granulocytic	N/A	Cat
114	Paucigranulocytic	Paucigranulocytic	Eosinophilic	HDMDP
115	Paucigranulocytic	Paucigranulocytic	Eosinophilic	Ragweed
116	Paucigranulocytic	Paucigranulocytic	Paucigranulocytic	HDMDP
117	Paucigranulocytic	Paucigranulocytic	Paucigranulocytic	Ragweed
118	Paucigranulocytic	N/A	Mixed granulocytic	Cat
119	N/A	Eosinophilic	Eosinophilic	Cat
120	N/A	Eosinophilic	Eosinophilic	HDMDP
121	N/A	Eosinophilic	Eosinophilic	HDMDP
122	N/A	Eosinophilic	N/A	HDMDP
123	N/A	Mixed granulocytic	Mixed granulocytic	Grass
124	N/A	Mixed granulocytic	Mixed granulocytic	HDMDP
125	N/A	Paucigranulocytic	Eosinophilic	Cat
126	N/A	Paucigranulocytic	Eosinophilic	Cat
127	N/A	Paucigranulocytic	Eosinophilic	Ragweed
128	N/A	N/A	Eosinophilic	Grass
129	N/A	N/A	N/A	HDMDP

Supplementary Table 6. Association between the severity of the LAR and sputum granulocyte counts and inflammatory subtype. Sputum subtype and cell counts were assessed before (-24 h; baseline) and after (7 h) allergen challenge.

	N	beta	P-value
Sputum subtype at baseline	118	NA	0.32388
Neutrophils at baseline	117	0.19	0.01824
Eosinophils at baseline	117	0.26	0.01371
Sputum subtype at 7 h post challenge	123	NA	0.51214
Neutrophils at 7 h post challenge	121	0.03	0.69377
Eosinophils at 7 h post challenge	121	0.16	0.09762
Difference in neutrophils between 7 h and baseline	112	-0.07	0.2989
Difference in eosinophils between 7 h and baseline	112	0.03	0.76726