

Macrophage characteristics do not correlate with clinical outcomes in exacerbations of neutrophilic asthma

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Abstract

Airway inflammation in acute exacerbations of asthma is frequently neutrophilic. Macrophages have the potential to promote inflammation or to accelerate resolution of inflammation, however, little is known about their contribution to the pathogenesis of asthma. The aim of the study was to explore the relationship between sputum macrophages in exacerbations of neutrophilic and paucigranulocytic asthma with clinical outcomes. Paucigranulocytic (PA; n = 18) and neutrophilic (NA; n = 21) asthmatic subjects were recruited upon an exacerbation of asthma. Clinical data [lung function, exhaled nitric oxide (FeNO), and the Asthma Control Questionnaire (ACQ-6)] and sputum were collected at presentation and on 14- and 28-days. Sputum macrophage subtypes were characterized by flow cytometry. Sputum cells were further assessed by bulk RNA sequencing. Both PA and NA groups displayed impaired lung function upon exacerbation that improved by day 28. Sputum monocytes and alveolar macrophages were significantly elevated upon exacerbation among NA participants compared to PA participants. However, neither macrophage number nor immunophenotype correlated with any clinical outcome assessed. These findings were further supported by bulk RNA sequencing as macrophage enrichment did not correlate with clinical outcomes at the time of exacerbation. Macrophage characteristics do not correlate with clinical outcomes during exacerbations or during recovery, regardless of asthma phenotype.

Introduction

Asthma is the most common chronic respiratory disease, with an estimated 300 million cases globally (1–3). It is characterized by airway inflammation, variable airflow obstruction, and airway hyperresponsiveness to endogenous or exogenous bronchoconstrictive stimuli (4). Acute asthmatic exacerbations contribute significantly to morbidity, healthcare costs, and progressive lung function decline (5, 6). Viral illness is the most frequent trigger of exacerbations, in particular rhinovirus (7). It is highly relevant to understand the cellular and molecular mechanisms that govern these events.

Asthma is broadly classified as either type 2 (T2)-high or T2-low (8). T2-high asthma is the most prevalent form among stable asthmatics and is characterized by elevated immunoglobulin E, blood and sputum eosinophilia, and type-2 cytokines including interleukin (IL)-4, IL-5, and IL-13. Consequently, it is responsive to inhaled corticosteroid treatment and with biologics blocking type 2 cytokines that reduce severe asthma exacerbations (9). In contrast, T2-low asthma is driven by innate immune cells and by T-helper 1 (Th1) and Th17 immune responses, and is less responsive to conventional anti-inflammatory treatments (10). T2-low asthma can be sub-categorized based on the abundance of sputum neutrophils as neutrophilic asthma (NA) or paucigranulocytic asthma (PA). Neutrophil-predominant asthma is more frequent than other forms of asthma during acute exacerbations (11). The role of other innate immune effector cells is less well understood.

Macrophages are the most abundant immune cell in the lung and are classified as alveolar macrophages (AMs), interstitial macrophages (IM), or monocyte-derived macrophages, depending on their origin and tissue niche (12). Certain cellular functions of macrophages, including phagocytosis, efferocytosis, and inflammation, are impaired in asthma (13, 14). AMs in NA have increased TNF- α and IL-1 β expression, which are both implicated in the disease pathology (15–18). Specifically in NA, impaired efferocytosis of neutrophils may exacerbate the inflammatory phenotype (14). Macrophages have also been implicated in the removal of eosinophils (19) and therefore potentially masking a contribution of eosinophils to the inflammatory response. However, the role of macrophages in acute asthma remains poorly understood.

Macrophages in stable NA by RNASeq have a specific macrophage transcriptomic program (20). Notably, certain macrophage clusters are correlated with clinical outcomes. However, whether an association between macrophages and lung function is present during asthmatic exacerbations requires further exploration. Therefore, we hypothesized that macrophages are elevated in the sputum during asthma exacerbation and correlate with lung function impairment in NA, but not PA. To address this, we recruited individuals at the time of acute exacerbations of asthma. At presentation, 14-, and 28-days, we collected lung function data and sputum macrophages for flow cytometry and RNASeq. Ultimately, while macrophage populations were elevated in NA compared to PA at exacerbation, we found no correlation between lung function and macrophage abundance or phenotypes.

Results

Cohort characteristics

A total of 39 participants were included and retrospectively classified as having paucigranulocytic asthma (PA; n=18) or neutrophilic asthma (NA; n=21) based on sputum collected at the time of exacerbation (visit 1; table 1). All participants with eosinophilic inflammation were excluded from analysis. Sex distribution was similar between groups, with females comprising 61.1% of the PA group and 61.9% of the NA group. The NA group was significantly older than the PA group (65.0 ± 14.3 vs. 51.3 ± 13.9 years). BMI did not differ significantly between groups (30.0 ± 8.0 vs. 32.7 ± 8.1 kg/m²).

Smoking status, severe asthma prevalence, and maintenance oral corticosteroid use were not significantly different between groups. Sputum total cell count was higher in the NA group than the PA group, but this difference did not reach statistical significance (1.2×10^6 vs. 0.6×10^6 ; $p=0.055$). Sputum inflammatory cell profiles differed significantly between groups. The PA group had a higher proportion of sputum macrophages than the NA group ($42.8 \pm 15.6\%$ vs. $16.6 \pm 10.9\%$), while the NA group had a higher proportion of sputum neutrophils ($79.4 \pm 10.9\%$ vs. $37.5 \pm 16.5\%$). Sputum eosinophils were also significantly higher in the NA group, though levels remained low overall ($0.6 \pm 1.2\%$ vs. $0.0 \pm 0.0\%$). Finally, both groups displayed comparable rates of documented viral infections (PA- 33.3% vs NA- 28.6%).

Lung function recovery over the follow-up period

The best FEV₁ (percent predicted) recorded over the preceding two years did not differ significantly between PA and NA groups (figure 1a). Similarly, the change in FEV₁ (percent predicted) from the best value prior to the time of exacerbation was not significantly different between groups (figure 1b).

Across study follow-up, lung function showed broadly similar trajectories between PA and NA groups. FVC (percent predicted) increased from visit 1 to visit 2 and remained relatively stable at visit 3 in both groups (figure 1c). FEV₁ (percent predicted) followed a similar pattern, with improvement from visit 1 to visit 2 and minimal change thereafter (figure 1d).

Fractional exhaled nitric oxide (FeNO), a marker of airway inflammation, was significantly elevated in the NA group at visit 1 but decreased by visit 2 and remained stable at visit 3 (figure 1d). FeNO in the PA group remained stable across visits. Finally, ACQ-6 scores declined over time in both groups, indicating improvement in symptom control during follow-up (figure 1f).

Overall, PA and NA exacerbation phenotypes showed no significant differences in severity of exacerbation or recovery during follow-up. Only FeNO at the time of recruitment into the study was significantly different between the groups, which is consistent with increased inflammation associated with the NA phenotype.

Macrophage abundance during exacerbation and recovering asthma

We next assessed sputum-isolated macrophage abundance by flow cytometry from exacerbation at initial visit to recovering asthma at visit 3. Absolute monocyte numbers significantly decreased from visit 1 to 3 in both groups but were not significantly different between PA and NA groups at any visit (figure 2a).

Absolute alveolar macrophage numbers also declined significantly from visit 1 to 3 among all participants (figure 2b). For interstitial macrophages, there was no significant difference between visit 1 to 3. However, NA participants had significantly elevated numbers at the initial visit compared to PA (figure 2c).

Since differences in absolute cell counts are potentially caused by variations in sputum production, we also assessed relative percentages of macrophage types as a fraction of all CD45⁺ immune cells. When expressed as a percentage of CD45⁺ cells, monocytes, the most abundant population, showed a trend to increase over the period of follow-up but comparably in both groups ($p=0.08$; figure 2d). In contrast, alveolar macrophages and interstitial macrophage percentages did not significantly change across follow-up, nor were there differences between PA and NA groups (figure 2e, f).

Finally, to investigate the functional capacity of macrophages, we performed immunocytochemistry to assess efferocytosis of eosinophils. Macrophages positive for the eosinophil-specific marker major basic protein (MBP), are indicative of internalized eosinophils (figure 2g). At visit 1, both PA and NA groups have comparable levels of MBP⁺ macrophages. This percentage significantly increases in both groups at visit two. By visit 3, the proportion of MBP⁺ macrophages differs significantly between groups as it declines in the NA group but remains stable in the PA group. The percentage of MBP⁺ macrophage did not correspond to the number or abundance of sputum eosinophils (data not shown).

Macrophage phenotype during exacerbation and recovering asthma

We further investigated the immunophenotypes of the macrophages. Classical “M1” macrophage markers CD80 and CD86 and non-classical “M2” macrophage markers CD163 and HLA-DR were assessed at the time of exacerbation and in the recovery phase. At visit 1, CD163⁺ monocytes were significantly elevated in NA compared to PA (figure 3a). Meanwhile, alveolar macrophages showed no significant differences between PA and NA groups for expression of any markers (figure 3b). Further, HLA-DR⁺ interstitial macrophages were significantly elevated in NA compared to PA (figure 3c).

The relative frequency of monocytes expressing each marker was comparable between NA and PA groups, with CD163 being the most frequently expressed marker, detected in approximately 50% of monocytes (figure 3d). Similarly, marker expression in both alveolar and interstitial macrophages was comparable between groups during exacerbation, with nearly all cells expressing HLA-DR in both populations (figure 3e, f).

During recovery at visit 3, CD163⁺ monocytes remained elevated in the NA group relative to PA (figure 3g). Further, HLA-DR⁺ alveolar macrophages were significantly elevated in NA (figure 3h). Interstitial macrophages were comparable in both groups (figure 3i). Finally, percentages of monocytes, alveolar macrophages, and interstitial macrophages expressing each marker were comparable between each group at visit 3 (figure 3j-l). MBP-positivity did not correlate with any clinical outcomes at visit 1 (data not shown).

Macrophage abundance and phenotypes do not correlate to clinical outcomes during exacerbation or recovering asthma

To determine whether macrophage abundance or phenotypes correlated with clinical outcomes during acute exacerbations or during recovery from the exacerbation, we created several correlation matrices, stratified by NA and PA status. Clinical outcomes assessed were FeNO, change in FEV₁ (percent predicted) from historic best, FEV₁ (percent predicted), FEV₁/FVC, and ACQ-6.

During exacerbation, neither total monocyte number nor number of CD80⁺, CD86⁺ CD163⁺, and HLA-DR⁺ monocytes correlated to any clinical outcome measured (figure 4a). This was also true for alveolar and interstitial macrophages (figure 4b, c).

In the NA group however, monocyte number was significantly positively correlated with FeNO (figure 4d). Alveolar macrophage number was negatively correlated with ACQ-6 (figure 4e); however, significance was lost when a multiple linear regression was performed and adjusted for age, sex, height, and baseline oral corticosteroid use. Further, there were no associations found between interstitial macrophages and clinical outcomes (figure 4f). Similarly at visit 3, monocyte, alveolar macrophage and interstitial macrophage numbers did not correlate to any clinical outcomes in the PA or NA groups (supplemental figure 1).

Finally, MBP-positivity did not correlate with any clinical outcomes at visit 3 (data not shown).

Based on previous work demonstrating a relationship between macrophage gene signatures and lung function in stable NA patients (20), we performed bulk RNASeq on sputum cells isolated at the time of exacerbation. Overall, there were 5 PA and 5 NA participants included in the analysis. Both groups were of comparable sex, race, BMI, smoking status, and viral infection status; however, the NA group was significantly older than the PA group (supplemental table 1). No individuals included in the RNASeq analysis were receiving maintenance OCS at the time of exacerbation.

Principal component analysis of normalized bulk RNASeq data showed that PC1 and PC2 explained 44.0% and 17.8% of the total variance, respectively (figure 5a). PA and NA samples showed substantial overlap in the PCA space, with no complete separation between groups.

Macrophage-associated expression was evaluated using a multi-gene signature including canonical markers CD68, CD163, CSF1R, C1QA, C1QB, C1QC, LST1, AIF1, TYROBP, FCER1G, MRC1, MSR1, VSIG4, and APOE. The gene-wise standardized heatmap demonstrated marked heterogeneity in macrophage-marker expression across individual samples (figure 5b). Overall, macrophage-associated transcriptional enrichment score varied substantially within both PA and NA groups and did not appear to be associated with group classification (figure 5c).

Further, we investigated whether macrophage enrichment scores correlated with clinical outcomes at the time of exacerbation. We found no association between macrophage enrichment and FeNO, FEV₁ (percent of predicted), FVC (percent of predicted), FEV₁/FVC, or ACQ-6 (figure 5d-h). Further, when stratified by asthma phenotype, neither PA nor NA groups displayed any significant correlations (supplemental figure 2). Similarly, macrophage enrichment during exacerbation did not correspond to any clinical outcome at visit 3 (supplemental figure 3). These findings persisted for M1 and M2 macrophage enrichment as well (data not shown).

Discussion

Macrophage dysfunction is increasingly recognized as a feature of asthma, although its contribution to disease pathogenesis and clinical heterogeneity remains incompletely understood (14). The present study is novel in that we assessed the macrophage subsets at the time of exacerbation of asthma and changes in phenotype from the acute to the recovery phase. We observed a significant reduction in macrophage subsets from the assessment on enrolment (visit 1) to visit 3 on day 28. Moreover, one aspect of macrophage function, efferocytosis of eosinophils, differed significantly between PA and NA participants during recovery. By visit 3, PA participants had an elevated percentage of MBP⁺ macrophages compared to NA participants. While this process is critical for resolving inflammation in the context of acute asthma exacerbations (21), we did not observe a relationship between efferocytosis and clinical outcomes. This dissociation between macrophage efferocytosis and short-term clinical recovery suggests that macrophage-mediated clearance of inflammatory cells may represent one component of a broader resolution program rather than a primary determinant of symptom or lung-function improvement. The delayed emergence of a difference in MBP⁺ macrophages between phenotypes may also indicate that macrophage function becomes more relevant during resolution than during the initial inflammatory response.

Both alveolar and interstitial macrophages were numerically significantly different between NA and PA at visit 1. CD163 expression on monocytes was significantly greater in NA compared to PA. Similarly, HLA-DR expression was greater in interstitial macrophages from NA participants than PA participants. In contrast, alveolar macrophages showed no significant differences between PA and NA groups for expression of any markers. Despite these findings, there was no difference in the severity of the attack as assessed by ACQ-6 or spirometry. Similarly, the improvement over 28 days was not different. With the exception of the association between monocyte abundance and FeNO, macrophage abundance and phenotype were not meaningfully associated with clinical outcomes during either exacerbation or recovery. Consistent with these findings, RNA sequencing did not identify associations between macrophage-related transcriptional signatures and clinical outcomes at the time of exacerbation. Collectively, these results suggest that macrophage abundance and activation state may differ between inflammatory phenotypes during acute exacerbations but may not directly reflect short-term physiological outcomes. One interpretation is that sputum macrophage characteristics more closely reflect the local inflammatory milieu than the overall clinical severity of an exacerbation. Asthma symptoms and airflow obstruction arise from multiple interacting processes, including airway smooth-muscle contraction, epithelial dysfunction, mucus production, and granulocyte-mediated inflammation, consequently, modest differences in macrophage phenotype may not translate into measurable differences in ACQ-6 or spirometry. The absence of strong clinical associations therefore does not necessarily imply that macrophages are biologically unimportant, but rather that their contribution may be phenotype-specific, temporally dependent, or insufficiently captured by conventional clinical endpoints.

In stable asthma, the neutrophilic asthma phenotype has been associated with older age (22), which is consistent with the findings of the current study. In some but not all studies, NA has been associated with more severe asthma, although severity is more generally associated with neutrophilia when there is concurrent eosinophilic inflammation (23). Exclusion of participants with concurrent eosinophilia may therefore partly explain the absence of greater clinical severity in the NA group.

Macrophages express a large number of molecules relevant to asthma (14). Macrophages in asthmatics when activated *ex vivo* release larger quantities of granulocyte/macrophage colony stimulating factor, tumour necrosis factor and interleukin-1 β (24). Impaired phagocytosis by macrophages harvested from asthmatics also has been shown (25). Whether impaired efferocytosis contributes to the inflammatory process has not been confirmed, although is a theoretical possibility. Together, these observations support a model in which macrophages may influence asthma through several functions, including inflammatory signalling, antigen presentation, and clearance of apoptotic cells, rather than through changes in cell number alone. Functional heterogeneity may therefore be particularly important when interpreting macrophage involvement in different asthma phenotypes. CD163 is a hemoglobin-haptoglobin scavenger receptor commonly associated with monocyte/macrophage differentiation and inflammation-resolution programs, including efferocytosis. Altered CD163 expression has previously been reported in airway macrophages from patients with asthma (26). Greater monocyte CD163 expression in NA could therefore indicate altered monocyte recruitment or differentiation during neutrophilic inflammation. The parallel increase in HLA-DR, a marker of antigen-

presentation capacity, supports broader monocyte activation in NA. However, because neither marker was associated with exacerbation severity or recovery, their clinical significance remains uncertain. The simultaneous expression of markers associated with both inflammatory activation and resolution also emphasize that macrophage phenotypes are unlikely to conform to a simple pro-inflammatory versus anti-inflammatory classification. Instead, airway macrophages during exacerbation may occupy mixed or transitional activation states shaped by the surrounding cytokine and cellular environment.

The increased abundance of all macrophage subsets at enrolment, followed by a reduction during recovery, may reflect acute recruitment into the airway during exacerbation. However, all subjects received oral corticosteroids following initial presentation, which suppress airway inflammation and can modify immune-cell abundance and phenotype (27). Consequently, the effects of spontaneous inflammatory resolution cannot be separated from those of treatment.

The present study has several limitations. Small sample sizes may have limited the statistical power to reveal associations of macrophage subsets to asthma phenotype, especially with regards to the RNASeq results for which there was a samples size of 5 per group. Additionally, the use of sputum as a method of isolating monocyte-derived, alveolar, and interstitial macrophages may introduce a bias, favoring isolation of macrophages within the luminal space. Sputum-derived cells may therefore provide an incomplete representation of macrophage populations located within the distal airways and lung interstitium. Future studies may benefit from more robust collection methods such as bronchoalveolar lavage or lung tissue biopsy.

In conclusion, acute asthma exacerbations were associated with increases in sputum macrophage abundance, while NA and PA differed modestly in macrophage abundance and phenotype. These differences were not accompanied by differences in exacerbation severity, recovery, or macrophage-associated transcriptional signatures. The findings therefore support a model in which macrophage responses accompany acute airway inflammation but are not straightforward biomarkers of short-term clinical severity. Instead, their potential importance may lie in regulating the inflammatory environment and subsequent resolution in ways that vary across asthma phenotypes. Larger studies with alternative macrophage-isolating protocols may be required to conclusively determine whether macrophages are relevant to the pathogenesis of acute exacerbations of asthma.

Methods

Study Design and Ethics Approval

Subjects were recruited during a prospective observational cohort study conducted at the Montreal Chest Institute (MCI) Day Hospital, a quaternary-level respiratory referral center affiliated with the McGill University Health Centre (MUHC), Montréal, Canada. Recruitment took place between January 2022 and December 2023. The study was approved by the MUHC Research Ethics Board (protocol BIA V3, 13 July 2022) and all participants provided written informed consent. All methods were performed in accordance with the relevant guidelines and regulations and with the Declaration of Helsinki.

Participants

Adults aged 18 years or older with a physician-confirmed diagnosis of asthma for at least 12 months, who presented to the MCI Day Hospital with an acute exacerbation requiring systemic corticosteroids were eligible for recruitment. An exacerbation was defined as an unscheduled visit for worsening asthma requiring the administration of intravenous or high-dose oral corticosteroids. Participants were on stable maintenance therapy for at least three months prior to enrolment and were excluded if they had clinically significant pulmonary disease other than asthma, major active comorbidities likely to affect asthma outcomes or active COVID-19 infection. Patients on maintenance oral corticosteroids and those receiving biologic therapy were eligible and included.

Clinical Procedures and Assessments

Study visits were conducted at day 0 (day of presentation, baseline), day 14, and day 28. At each visit, spirometry, fractional exhaled nitric oxide (FeNO) was assessed. FeNO was measured using an expiratory flow of 50 mL/s using a NIOX VERO® device (Circassia, Oxford, UK) according to ATS guidelines (28). Spirometry was performed according to ATS/ERS 2019 standards (29) using a calibrated spirometer. Pre-bronchodilator FEV₁, FVC, and the FEV₁/FVC ratio were recorded. Patient-reported outcomes included the six-item Asthma Control Questionnaire (ACQ-6), which captures asthma symptom control on a 0–6 scale (0 = well controlled, 6 = uncontrolled), the minimal important difference (MID) is 0.5 points (30, 31).

Sputum Collection and Cell Isolation

When an FEV₁ of less than 1.0L was recorded, expectorated sputum only was collected. For sputum induction, an ultrasonic nebulizer (Drive DeVilbiss Healthcare, New York, USA) was used. Hypertonic saline administration was as follows: 10 ml of 3% concentration for 7 minutes, 10 ml of 4% concentration for 7 minutes, 10 ml of 5% concentration for 7 minutes. Spirometry was performed following each dose of hypertonic saline and induction was stopped if FEV₁ fell by more than 20% of baseline. Samples were collected in plastic sterile containers and kept on ice before processing

Sputum liquefaction was performed by mixing freshly isolated sputum with Sputalysin and sterile water. Following incubation, the suspension was filtered using a 70µm cell strainer, and pelleted and cells were counted.

Asthma Phenotype Classification

Approximately 30,000 cells were deposited on a glass slide by cytocentrifugation at 1000rpm for 5 minutes using the EpreDia™ Cytospin™ 4 Cytocentrifuge. Cells were air fixed and stained with Diff-Quik (Fisher Scientific, Kalamazoo, MI) according to manufacturer's instructions. Differential cell

counts were determined by calculating the percent of macrophages, lymphocytes, neutrophils, and epithelial cells from a count of three hundred cells. Patients with less than 2% total eosinophil abundance were classified as non-eosinophilic. Further, NA was defined by at least 65% neutrophil abundance while PA was defined by less than 65% neutrophil abundance.

Immunocytochemistry

For immunocytochemistry, sputum cells were deposited on a glass slide as described above. Cells were then fixed with 10% formalin, permeabilized with tween 20, blocked, and incubated overnight with a primary antibody for major basic protein (Bio Rad Laboratories, USA, catalogue number: MCA5751). The slides were then rinsed and incubated with a secondary antibody (ThermoFisher Scientific, USA, catalogue number: A-21247) and 4',6-diamidino-2-phenylindole (DAPI). Cells were then visualized using the Zeiss Confocal LSM700 microscope and analysed with ImageJ software.

Flow cytometry

For flow cytometry, up to 10 million cells were collected for staining. Lung monocyte/macrophage panel and gating strategy is adapted from Yu et al. (32). Flow cytometry staining method was adapted from Chen et al. (33) with the following modification: viability staining with eFluor780 viability dye was performed prior to surface staining.

RNA isolation

For RNA isolation, 2.5×10^5 cells were isolated from sputum and suspended in TRIzol (Thermo Fisher, USA). For long term storage, samples were maintained at -80°C .

RNA was extracted from TRIzol-lysed cells according to the manufacturer's protocol. Briefly, following chloroform-induced phase separation, the aqueous phase was collected, and RNA was precipitated with isopropanol, washed with 75% ethanol, and resuspended in TE buffer (Thermo Fisher, USA). RNA concentration and purity were assessed by spectrophotometry.

Bulk RNA Sequencing

RNA sequencing was performed by Plasmidsaurus using Oxford Nanopore Technology with custom analysis and annotation. Quality of the fastq files was assessed using FastQC v0.12.1. Reads were then quality filtered using fastp v0.24.0 with poly-X tail trimming, 3' quality-based tail trimming, a minimum Phred quality score of 15, and a minimum length requirement of 50 bp. Quality-filtered reads were aligned to the reference genome using STAR aligner v2.7.11 with non-canonical splice junction removal and output of unmapped reads, followed by coordinate sorting using samtools v1.22.1. PCR and optical duplicates were removed using UMI-based deduplication with UMICollapse v1.1.0. Alignment quality metrics, strand specificity, and read distribution across genomic features were assessed using RSeQC v5.0.4 and Qualimap v2.3, with results aggregated into a comprehensive quality control report using MultiQC v1.32. Gene-level expression quantification was performed using featureCounts (subread package v2.1.1) with strand-specific counting, multi-mapping readfractional assignment, exons and three prime UTR as the feature identifiers, and grouped by gene_id. Final gene counts were annotated with gene biotype and other metadata extracted from the reference GTF file. Sample-sample correlations for sample-sample heatmap and PCA were calculated on normalized counts (TMM, trimmed mean of M-values) using Pearson correlation. Differential expression was done with edgeR v4.0.16 using standard practice including filtering for low-expressed genes with edgeR::filterByExpr with default values. Functional enrichment, when available, is performed using gene set enrichment analysis with gseapy v0.12 using the MSigDB Hallmark gene set.

Statistical Analysis

GraphPad Prism 11 software (GraphPad Software Inc., San Diego, CA, USA) or RStudio version 2026.07.0 (Posit Software, PBC, Boston, MA) was used for statistical analysis. The non-parametric Mann-Whitney U test was used to compare two groups. Two-way ANOVA was used for repeated measures. Correlations were performed using Pearson's correlations. All analyses were two-tailed tests with a 95% confidence interval. A p-value less than 0.05 was considered statistically significant.

Abbreviations

PA: paucigranulocytic asthma; NA: neutrophilic asthma; BMI: body mass index; OCS: oral corticosteroid; TCC: total cell count

Declarations

Author Contributions

Conceptualization: RS, SJ, JGM

Methodology: RS, SJ, TM, NBA, TN, AM, SW

Investigation: SJ, RS

Funding acquisition: JGM

Supervision: JGM

Writing – original draft: SJ

Writing – review & editing: SJ, JGM, ND

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Data availability

RNASeq data were deposited into the Gene Expression Omnibus database under accession number GSE339251.

For reviewer access, the data set can be retrieved with the token **odovqymexxgvjwz** using the link

[https://can01.safelinks.protection.outlook.com/?](https://can01.safelinks.protection.outlook.com/?url=https%3A%2F%2Fwww.ncbi.nlm.nih.gov%2Fgeo%2Fquery%2Facc.cgi%3Facc%3DGSE339251&data=05%7C02%7Csydney.joy%40mail.mcgill.ca%7Ca9296)

<url=https%3A%2F%2Fwww.ncbi.nlm.nih.gov%2Fgeo%2Fquery%2Facc.cgi%3Facc%3DGSE339251&data=05%7C02%7Csydney.joy%40mail.mcgill.ca%7Ca9296>

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Table

Table 1. Demographic information at time of exacerbation

	PA (n=18)	NA (n= 21)	Significance
Female[†]	11 (61.1)	13 (61.9)	0.96 ^x
Age [‡]	51.3 (13.9)	65.0 (14.3)	0.0045 [4.50 to 22.90] ^z
Caucasian[†]	16 (88.9)	19 (90.0)	0.87 ^x
BMI [‡]	32.7 (8.1)	30.0 (8.0)	0.31 [-2.60 to 7.80] ^z
Smoker[†]	2 (11.1)	3 (14.3)	0.77 ^x
Severe asthma[†]	16 (88.8)	18 (85.7)	0.77 ^x
Maintenance OCS[†]	1 (5.5)	3 (14.3)	0.37 ^x
Sputum TCC[‡]	0.6x10 ⁶ (0.6x10 ³)	1.2x10 ⁶ (1.1x10 ⁶)	0.055 [-0.01 to 1.21] ^z
Sputum macrophage (%)^{‡§}	42.8 (15.6)	16.6 (10.9)	<0.0001 [17.33 to 35.18] ^z
Sputum neutrophil (%)^{‡§}	37.5 (16.5)	79.4 (10.9)	<0.0001 [32.56 to 51.24] ^z
Sputum eosinophil (%)^{‡§}	0.0 (0.0)	0.6 (1.2)	0.035 [0.047 to 1.13]
Viral infection[†]	6 (33.3)	6 (28.6)	0.677 ^x

[†]Number (%), [‡] Mean (SD), ^zt-test [95% confidence interval], ^xChi-squared test

Figures

FIGURE 1

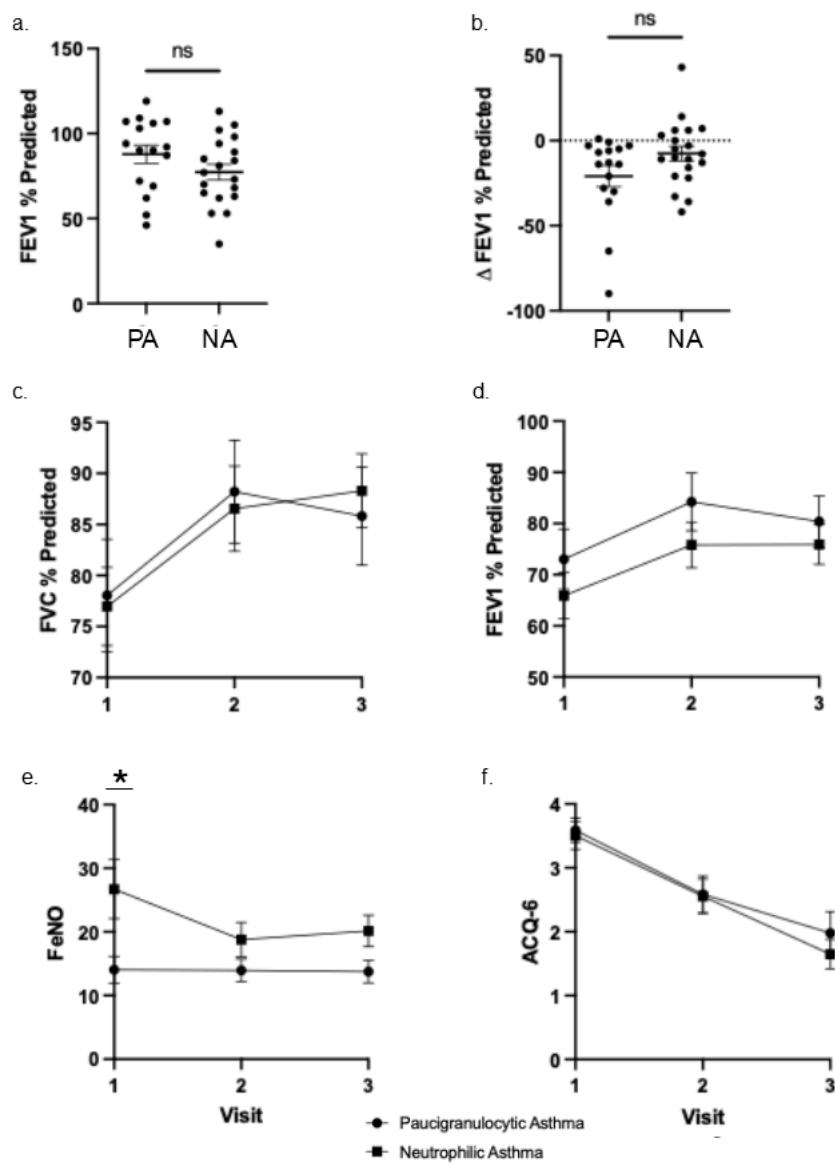


Figure 1

Clinical outcomes are comparable between paucigranulocytic and neutrophilic asthmatic participants following exacerbation. **a.** Best forced expiratory volume in 1 second (FEV₁; percent of predicted) recorded over the previous 2 years. **b.** Change in FEV₁ (percent of predicted) at time of exacerbation compared to historic best. **c.** Forced vital capacity (FVC; percent of predicted) over visits 1-3. **d.** FEV₁ (percent of predicted) over visits 1-3. **e.** FeNO over visits 1-3. **f.** Asthma control questionnaire (ACQ)-6 over visits 1-3. Data shown as mean $\pm$ SEM.

FIGURE 2

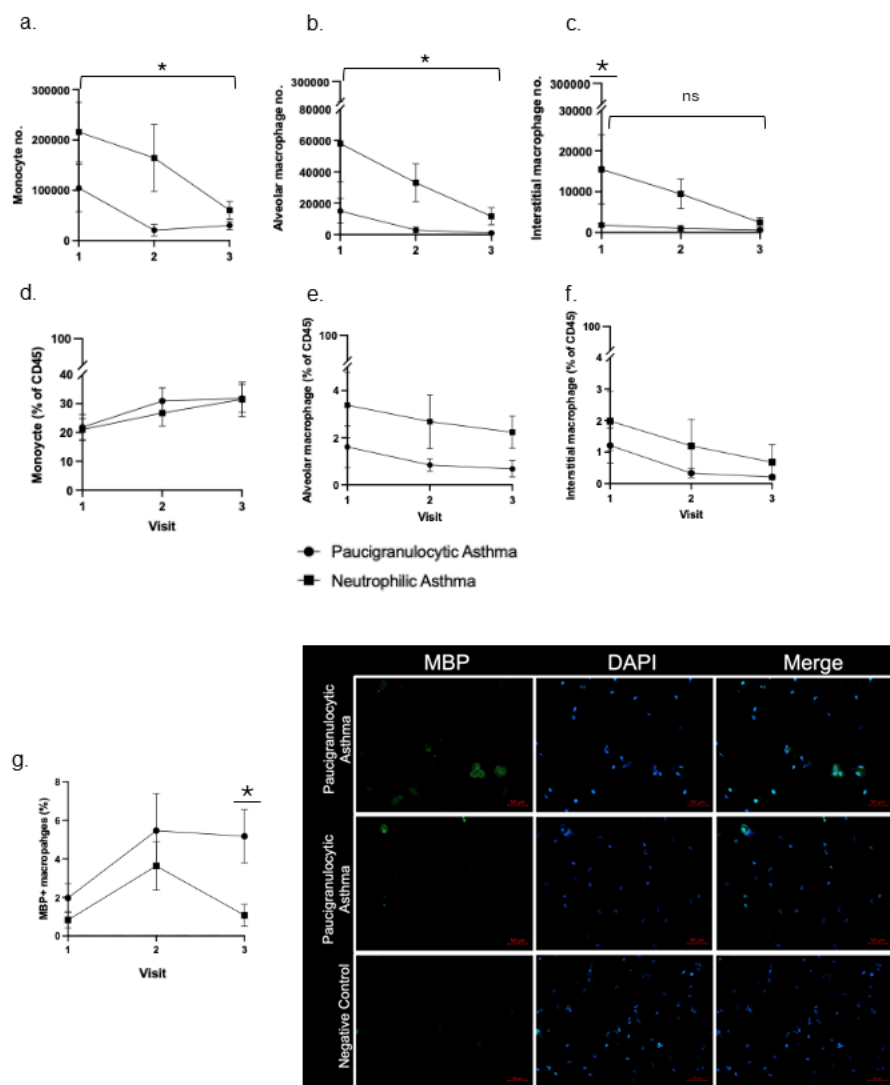
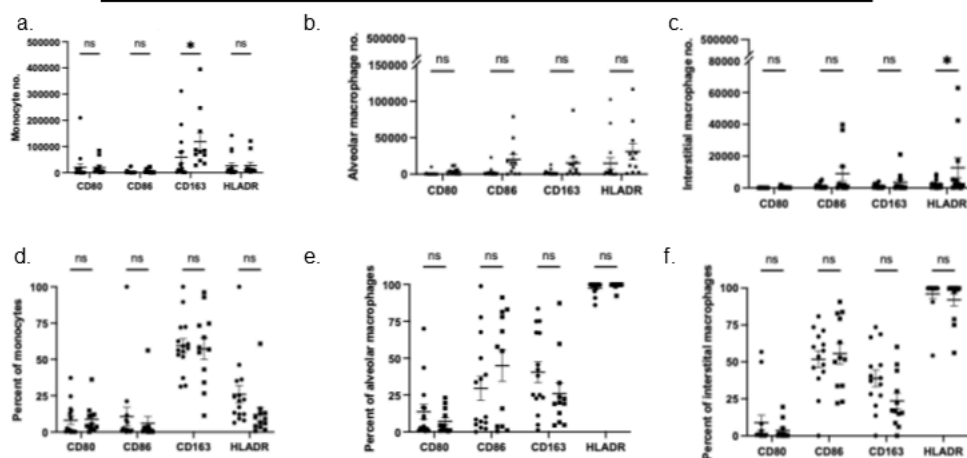


Figure 2

Macrophage abundance does not differ significantly between paucigranulocytic and neutrophilic asthmatic during exacerbation. **a.** Number of monocytes isolated from sputum over visits 1-3. **b.** Number of alveolar macrophages isolated from sputum over visits 1-3. **c.** Number of interstitial macrophages isolated from sputum over visits 1-3. **d.** Monocytes as a percentage of CD45+ cells over visits 1-3. **e.** Alveolar macrophages as a percentage of CD45+ cells over visits 1-3. **f.** Interstitial macrophages as a percentage of CD45+ cells over visits 1-3. **g.** Summary data and representative images of major basic protein (MBP)-positive sputum-isolated macrophages.

FIGURE 3

Visit 1



Visit 3

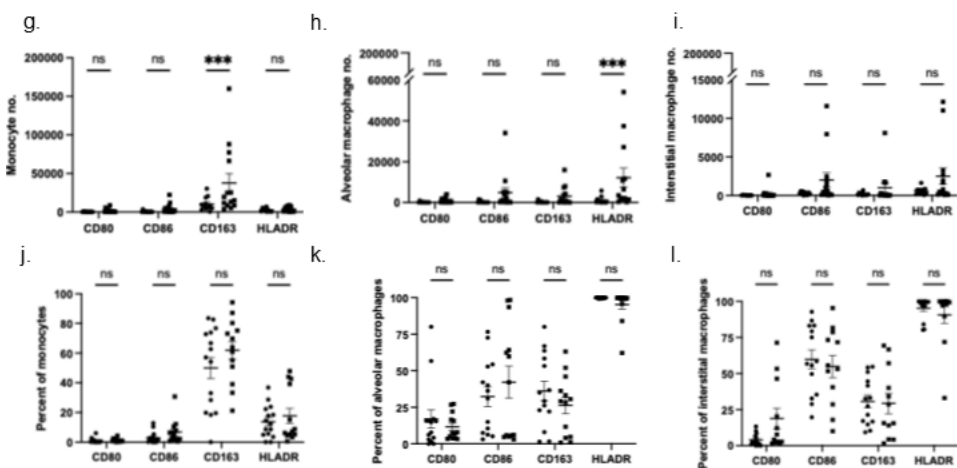


Figure 3

Macrophage phenotypes do not differ significantly between paucigranulocytic and neutrophilic asthmatic during exacerbation or stable asthma. a-c. Number of monocytes, alveolar macrophages, and interstitial macrophages expressing CD80, CD86, CD163, or HLA-DR at visit 1. Percentage of monocytes (d), alveolar macrophages (e), or interstitial macrophages (f) expressing CD80, CD86, CD163, and HLA-DR at visit 1. Number of monocytes (g), alveolar macrophages (h), and interstitial macrophages (i) expressing CD80, CD86, CD163, and HLA-DR at visit 3. Percentage of monocytes (j), alveolar macrophages (k), and interstitial macrophages (l) expressing CD80, CD86, CD163, and HLA-DR at visit 1. Data shown as mean $\pm$ SEM.

FIGURE 4

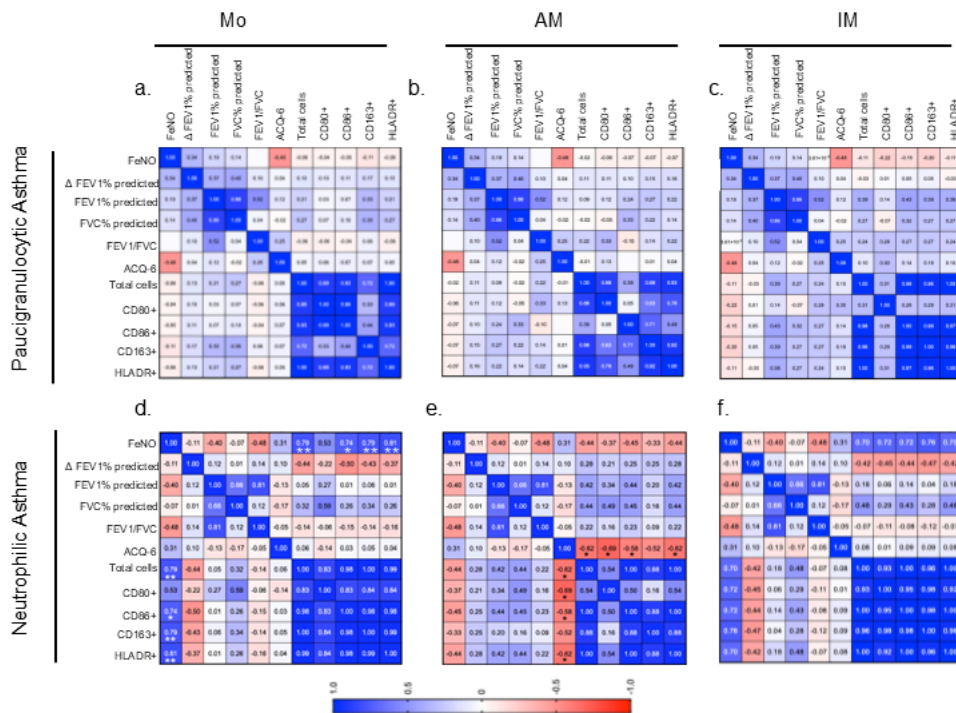


Figure 4

Macrophages do not correlate with clinical outcomes at exacerbation of asthma. Heatmaps show Pearson's correlations between FeNO, spirometric measures, ACQ-6, and cell populations across monocyte (a, d), alveolar macrophage (b, e), and interstitial macrophage (c, f). Results are stratified by granulocytic phenotype, with paucigranulocytic participants shown in the top row and neutrophilic participants in the bottom row. Blue indicates positive correlation and red indicates negative correlation, with color intensity proportional to Pearson's correlation magnitude.

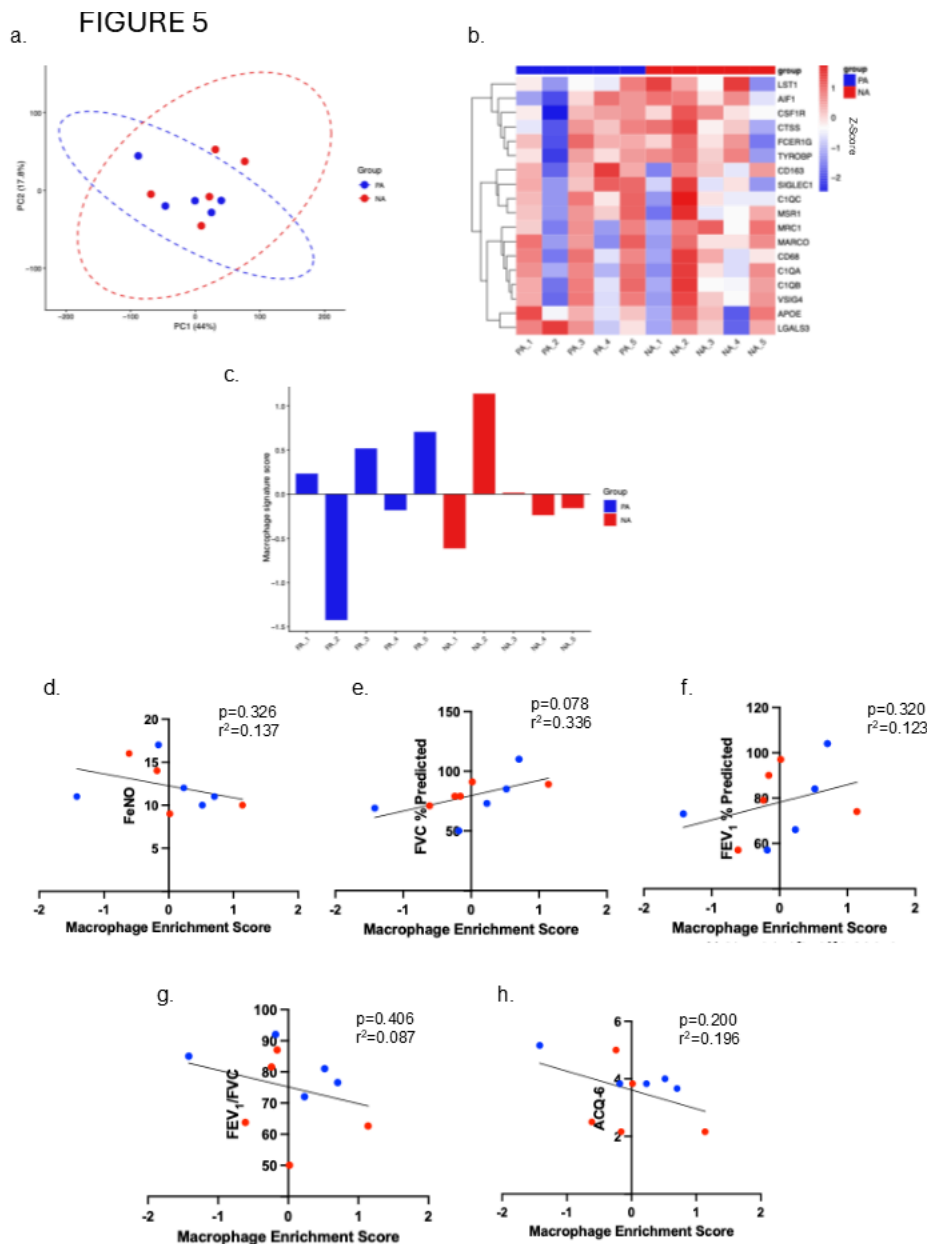


Figure 5

Macrophage enrichment does not correlate with clinical outcomes during exacerbations of asthma. **a.** PCA of paucigranulocytic asthma and neutrophilic asthma sputum samples generated from bulk RNA sequencing. **b.** Heatmap of macrophage-associated genes by sample. **c.** Macrophage enrichment score by sample. Pearson's correlation between macrophage enrichment score and **(d)** FeNO, **(e)** Percent of predicted FEV₁, **(f)** Percent of predicted FVC, **(g)** FEV₁/FVC, **(h)** ACQ-6. Blue denotes paucigranulocytic asthma subjects and red denotes neutrophilic asthma subjects. n=5/group.

Supplementary Files

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