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Article type : Original Article-Asthma and Rhinitis

Title: Neutrophilic asthma features increased airway classical monocytes

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Running title: Monocyte dysregulation in asthma

Word count: Text: 4297; 2 tables; 5 figures

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This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/CEA.13811
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Accepted Article

Key words: Monocyte, macrophage, asthma, neutrophil, severe asthma, mannose receptor, sputum

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Abstract

Background: Monocytes and macrophages are critical innate immune cells of the airways. Despite their differing functions, few clinical studies discriminate between them and little is known about their regulation in asthma.

Objective: We aimed to distinguish and quantify macrophages, monocytes and monocyte subsets in induced sputum and blood, and examine their relationship with inflammatory and clinical features of asthma.

Methods: We applied flow cytometry to distinguish macrophages, monocytes and subsets in sputum and blood (n=53; 45 asthma, 8 non-asthma) and a second asthma sputum cohort (n=26). Monocyte subsets were identified by surface CD14/CD16 ($CD14^{++}CD16^{-}$ classical, $CD14^{+}CD16^{+}$ intermediate and $CD14^{+}CD16^{++}$ non-classical monocytes). Surface CD206, a marker of monocyte tissue differentiation, was measured in sputum. Relationship to airway inflammatory phenotype (neutrophilic n=9, eosinophilic n=14, paucigranulocytic n=22) and asthma severity (severe n=12, non-severe n=33) was assessed.

Results: Flow cytometry- and microscope-quantified sputum differential cell proportions were significantly correlated. Sputum macrophage number was reduced (p=0.036), while classical monocyte proportion was increased in asthma vs non-asthma (p=0.032). Sputum classical monocyte number was significantly higher in neutrophilic vs paucigranulocytic asthma (p=0.013). $CD206^{-}$ monocyte proportion and number were increased in neutrophilic vs eosinophilic asthma (p<0.001, p=0.013). Increased sputum classical and $CD206^{-}$ monocyte numbers in neutrophilic asthma were confirmed in the second cohort. Blood monocytes did not vary with airway inflammatory phenotype, but blood classical monocyte proportion and number were increased in severe vs non-severe asthma (p=0.022, p=0.011).

Conclusion & Clinical relevance: Flow cytometry allowed distinction of sputum macrophages, monocytes and subsets, revealing compartment-specific dysregulation of monocytes in asthma. We observed an increase in classical and $CD206^{-}$ monocytes in sputum in neutrophilic asthma, suggesting co-recruitment of monocytes and neutrophils to the airways in asthma. Our data suggest further investigation of how airway monocyte dysregulation impacts on asthma-related disease activity is merited.

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Introduction:

Asthma is characterized by chronic inflammation of the airways, driven by the complex interplay of immune and stromal cells (1). The roles of eosinophils and neutrophils in the context of asthma have been widely investigated, and four airway inflammatory phenotypes have been defined based on their varying proportions in sputum cell counts (2). Airway inflammatory phenotyping can be useful, for example sputum eosinophilia predicts clinical responsiveness to corticosteroid treatment in mild-moderate asthma or anti-IL-5 biologic treatment in severe asthma (3, 4). No treatments are available targeting neutrophilic asthma, a subtype of asthma associated with corticosteroid resistance (5), more severe asthma (6) and other clinical characteristics such as asthma exacerbation (7), airflow limitation (8) and increased airway bacterial burden (9, 10). Pharmacological blockade of neutrophil recruitment to the airways did not provide clinical benefit in uncontrolled asthma, although this trial did not select for neutrophilic participants using sputum count (11). Alternatively, this trial finding may suggest that the presence of airway neutrophils does not necessarily indicate a pathological role for neutrophils and that alternative cellular and molecular factors may be relevant in neutrophilic asthma.

Dysregulation of mononuclear phagocytes (which include monocytes, monocyte-derived cells, dendritic cells (DC) and macrophages (12, 13)) is less well understood in the context of asthma, although several lines of evidence suggest a crucial role in initiation, progression and resolution of airway inflammation. In the airway lumen, macrophages are considered to exert important functions in tissue homeostasis and repair. Macrophages and monocytes are known to play diverse and distinct roles in promotion and resolution of inflammatory responses (14). Thus, impaired monocyte/macrophage homeostasis could have a detrimental pathological outcome in asthma, as occurs in other autoimmune diseases such as lupus, rheumatoid arthritis, and inflammatory bowel disease (15). Monocytes are subdivided by surface expression of CD14 and CD16 into classical (CM: $CD14^{++}CD16^{-}$), intermediate (IM: $CD14^{+}CD16^{+}$) and non-classical (NCM: $CD14^{+}CD16^{++}$) subsets, linked to specific functions (16). For example, CM and IM respond to LPS by secreting pro- and anti-inflammatory cytokines. 'Patrolling' NCM crawl along the endothelial blood vessels, exhibit varying responses to LPS (17, 18), and secrete TNFα and IL-1β upon TLR7/8 induction, suggesting a role in viral defense (19). Little is known about monocyte subset dysregulation in asthma. Circulating IM were found to be increased in severe asthma compared to control subjects and mild to moderate asthma (20), and studies of monocyte subset composition in the asthma airways are lacking. CD206 (mannose receptor) has been used as a marker for airway macrophages (21), but is also expressed on several discrete populations of differentiating monocytes, but not DC, in the extravascular lung (22). In contrast, pulmonary arterial monocytes do not express CD206, suggesting that its expression relates to monocyte differentiation driven by local environmental cues following entry into the lung tissue. Expression of this receptor is linked to pathogen recognition and phagocytosis (21), and

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reduced receptor expression on alveolar macrophages has been linked to impaired phagocytosis in COPD (23). Despite important functions in homeostasis, CD206 expression on sputum monocytes has not yet been investigated in the context of asthma. With increased understanding of the airway mononuclear lineage further asthma inflammatory phenotypes might be defined and novel therapeutic targets identified.

Here we apply flow cytometry to induced sputum and blood samples from participants with and without asthma to discriminate macrophages and monocytes and quantify monocyte subsets. We hypothesized that the macrophage and monocyte proportion, number and subsets would be altered in asthma and that dysregulation would be linked to asthma inflammatory phenotype, disease severity and clinical traits of asthma.

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Methods:

Participants:

Participants aged 18 years or over provided written informed consent. Sputum samples for flow cytometry analysis were acquired in an opportunistic manner from ongoing studies of inflammatory mechanisms in asthma. Clinical trials were approved by the Hunter New England Human Research Ethics Committee and the University of Newcastle Human Research Ethics Committee (approval numbers: 16/04/20/3.0; 17/02/15/3.04; 15/03/18/3.04; 17/04/12/4.03). Participants with asthma (n=45) had a doctor diagnosis of asthma with variable airflow obstruction defined as outlined in the online supplement. Questionnaires were administered to participants to collect demographic information, asthma history, medication use and adherence, asthma symptoms based on Juniper asthma control questionnaire 6 (24), exacerbation- and smoking history. Asthma severity was defined based on inhaled corticosteroid (ICS) dose, the use of a second controller, and meeting one of the criteria for uncontrolled asthma as outlined by ERS/ATS guidelines (25). Non-asthma control participants (n=8) were eligible if they were not pregnant or breastfeeding, had not smoked within the last six months and did not have a current respiratory disease. Research data are not shared. When appropriate, studies were registered with the Australian New Zealand Clinical Trials Registry (ACTRN12614001003662, ACTRN12617000395336, ACTRN12617000991314). Key flow cytometry findings were validated in a second independent cohort consisting of 26 individuals with asthma described previously who were not also part of the cohort described above (26).

Spirometry, sample collection and processing

Spirometry, sputum induction and sample processing were performed as previously described (27). Pre-bronchodilator spirometry (FEV_1, FVC, FEV_1/FVC) was performed (Medgraphics CPFS/D USB, Ascencia). Sputum induction and processing was performed as outlined in the online supplement. Asthma inflammatory phenotypes were determined based on sputum differential cell counts as previously described (28): Eosinophilic asthma (EA) ≥3% eosinophils and <61% neutrophils, neutrophilic asthma (NA) <3% eosinophils and ≥61% neutrophils, paucigranulocytic asthma (PGA) <3% eosinophils and <61% neutrophils, mixed granulocytic asthma (MGA) ≥3% eosinophils and ≥61% neutrophils. Venous blood samples were collected in ethylenediaminetetraacetic acid anticoagulant collection tubes and an automated full blood count was performed (CELL-DYN Ruby, Abbott).

Flow cytometry of whole blood and sputum

Staining was performed as outlined in the online supplement. 100 µL of whole blood and 1×10^5 – 1.5×10^6 sputum cells were labelled with antibodies directed against CD45 (BD Biosciences, clone HI30), CD14 (BD Biosciences, clone M5E2), CD16 (BD Biosciences, clone 3G8), HLA-DR (BD Biosciences, clone G46-6). Sputum cells were further stained with antibody against CD206 (Biolegend, clone MMR)

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and a viability dye (7-AAD, BD Biosciences). Samples were run on a flow cytometer (LSRFortessa X-20, BD Biosciences) and subsequent analyses performed with the FlowJo software (Version 10). Sputum monocytes, macrophages, neutrophils and eosinophils were identified based on the gating strategy illustrated in supplemental Figure S1. Fluorescence minus one controls were performed in set-up experiments to establish gating positions (Figure S2), a sputum gating template produced, and gates manually adjusted by a single blinded investigator on a sample to sample basis to account for sample heterogeneity in sputum. The gating strategy for blood is given in Figure S3. A detailed justification of the gating strategy is provided in the online supplement. Data were expressed as a proportion of $CD45^+$ cells (number of events after selecting $CD45^+$, single, whole cells) and absolute numbers as cells per milliliter. Absolute numbers of flow cytometry-quantified cell types in sputum samples were calculated as outlined in the online supplement. Monocyte subsets were additionally expressed as a proportion of total monocytes.

Statistical analysis

Statistical analyses were performed using the software Stata 15 and GraphPad Prism 8. Normality and lognormal distribution were assessed using the Shapiro-Wilk test. Student's *t* test for two group- and ANOVA with Holm-Sidak post-hoc correction for multiple comparison were applied for comparison of normally distributed data. Mann-Whitney *U* rank sum test and Kruskal-Wallis with Dunn's test for multiple comparison were performed when data were non-parametric. Two-sample test of proportions and Fisher's exact test were used for comparison of proportions. Correlation analyses were performed using Spearman's rank for non-parametric data.

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Results:

Patient characteristics

Clinical characteristics of the flow cytometry study cohort are summarized in Table 1. Blood and sputum differential cell counts are given in Table S1. The asthma group had significantly lower lung function (FEV_1, p=0.005; FEV_1/FVC, p=0.005), higher BMI (p=0.008), a higher proportion of sputum eosinophils (p<0.001) and peripheral blood eosinophils (p=0.005), elevated numbers of sputum and blood eosinophils (p<0.001, p=0.010) and decreased numbers of macrophages (p=0.046) than the non-asthma control group. Other baseline parameters did not differ significantly between the groups.

Flow cytometry enables direct airway sampling

Monocytes and monocyte subsets ($CD45^+$, SSC^{med}, HLA-DR^+, $CD14^{+/++}$, $CD206^{-/medium}$, $CD16^{-/+}$, 7-AAD^-), macrophages ($CD45^+$, $SSC^{medium/high}$, HLA-DR^+, $CD14^+$, $CD206^{high}$, $CD16^+$, 7-AAD^{medium}), neutrophils ($CD45^+$, SSC^{medium}, HLA-$DR^{-/low}$, $CD14^-$, $CD206^-$, $CD16^{++}$, 7-AAD^-) and eosinophils ($CD45^+$, SSC^{medium}, HLA-$DR^{-/low}$, $CD14^-$, $CD206^-$, $CD16^-$, 7-AAD^-) were identified by flow cytometry as outlined in Figure S1. In contrast to differential sputum cell counts, which summarizes all mononuclear phagocytes as macrophages, our gating strategy distinguished between monocytes and macrophages, and allowed us to identify monocyte subsets. We compared the proportions of eosinophils, neutrophils and macrophages distinguished by microscope-based differential sputum cell analysis with eosinophils, neutrophils and HLA-DR^+ cells (including monocytes, macrophages and DCs) as proportions of $CD45^+$ cells determined by flow cytometry. We observed a high correlation between the immune cell proportions distinguished with the two methods, with all associated p values being lower than 0.0001 (Figure S4A-C). We isolated sputum macrophages, monocytes, neutrophils and eosinophils by fluorescence-activated cell sorting and prepared cytospins of these populations (Figure S4D-G) that confirmed the validity of our sputum gating strategy (Figure S1).

Reduced sputum macrophages in asthma are associated with airway neutrophilia

Using flow cytometry data, we explored sputum monocyte/macrophage dysregulation in asthma by comparing the proportion (expressed as % of $CD45^+$ cells) and absolute number (expressed as cells/mL) of sputum macrophages and monocytes between participants with asthma and non-asthma controls (Figure 1). The proportion of macrophages did not differ significantly between asthma and non-asthma controls (Figure 1A), while absolute numbers were significantly reduced in asthma (p=0.036, Figure 1B). Neither the proportion of monocytes (Figure 1C), nor their absolute number (Figure 1D), differed between the two groups.

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We investigated if absolute numbers (cells/mL) or proportions (percentage of $CD45^+$ cells) of sputum monocytes and macrophages were associated with other immune cells quantified by flow cytometry (Table 2). Monocyte number correlated significantly with the number of neutrophils ($r=0.60$, $p<0.001$) and eosinophils ($r=0.47$, $p<0.001$), while macrophages number did not. The proportion of monocytes weakly correlated with the proportion of eosinophils ($r=0.28$, $p=0.045$), whereas the proportion of macrophages significantly correlated negatively with the proportion of neutrophils ($r=-0.60$, $p<0.001$).

Our data revealed significant dysregulation in the monocyte/macrophage lineage in asthmatic airways. We observed a significant reduction of macrophage number in sputum of participants with asthma compared to non-asthma controls, while their proportion was negatively associated with airway neutrophilia. Monocytes were not significantly altered in asthma but showed a significant association with airway neutrophils and eosinophils.

Macrophages but not monocytes are decreased in neutrophilic asthma

Since both monocyte numbers and macrophage proportions were significantly correlated with eosinophils and/or neutrophils, we investigated the differences between the three airway inflammatory phenotypes; neutrophilic asthma (NA, n=9), eosinophilic asthma (EA, n=14) and paucigranulocytic asthma (PGA, n=22), categorized by differential sputum cell count (29, 30). None of the participants displayed mixed granulocytic asthma. Demographics and clinical traits of inflammatory phenotype groups are summarized in Table S2, and blood and sputum cell counts are given in Table S3. Asthma duration was significantly higher in NA than in EA ($p=0.003$). We observed a reduction in macrophage proportion measured by flow cytometry in NA, which was significantly lower than in EA ($p=0.013$) and PGA ($p=0.001$; Figure 2A). Absolute numbers of macrophages were significantly decreased in NA vs PGA ($p=0.035$; Figure 2B), although we did not replicate this finding in the second sputum cohort (Figure S7). Proportions and absolute numbers of total monocytes did not differ significantly between inflammatory phenotypes (Figure 2C, D). NA featured a significantly higher proportion and number of flow cytometry-quantified neutrophils in comparison to EA and PGA (Figure 2E, F). Representative dot plots from NA (Figure 2G) and PGA asthma participants (Figure 2H) illustrate the relative decrease of macrophages in NA.

Neutrophilic asthma features increased sputum classical monocytes

We identified CD14/CD16 monocyte subsets in sputum (Figure 3, Figure S5) and peripheral blood (Figure S6) in order to investigate potential dysregulations within the monocyte subset composition in asthma and asthma airway inflammatory phenotypes. The proportion of CM was significantly increased in sputum of participants with asthma compared to non-asthma controls ($p=0.032$, Figure 3A), while their absolute numbers did not differ (Figure 3B). We observed similar proportions of CM between asthma inflammatory

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phenotypes (Figure 3C) and detected a significant increase in CM absolute number in NA compared to PGA (p=0.013, Figure 3D). Representative dot plots of total monocytes in NA (Figure 3E) vs PGA (Figure 3F) show the relative increase of CM in NA. We found no differences between airway inflammatory phenotypes in proportion and absolute numbers of intermediate and non-classical sputum monocytes (Figure S5) and observed similar subset compositions between disease groups and airway inflammatory phenotypes in blood (Figure S6).

Neutrophilic asthma is associated with increased proportion and number of sputum CD206$^-$ monocytes

We identified both CD206$^-$ and CD206$^+$ monocytes in our sputum samples. CD206$^+$ monocytes showed low side scatter similar to CD206$^-$ monocytes and were distinct from the macrophage population (Figure S1). CD206$^-$ and CD206$^+$ monocytes expressed moderate to high CD14 and/or CD16, which are lacking on conventional DC subsets. We expressed CD206$^-$ and CD206$^+$ monocytes as a proportion of total monocytes as well as absolute number and performed comparisons between asthma vs non-asthma controls and between airway inflammatory phenotypes (Figure 4). CD206$^-$ monocytes as a proportion of total monocytes were significantly reduced in asthma vs non-asthma (p=0.041, Figure 4A), while absolute numbers of CD206$^-$ or CD206$^+$ monocytes did not differ (Figure 4B-C). We observed a significantly higher proportion of total monocytes that were CD206$^-$ in NA compared to EA (p<0.001) and PGA (p=0.038, Figure 4D). Proportions were further significantly higher in PGA vs EA (p=0.020). The absolute number of CD206$^-$ monocytes was significantly elevated in NA compared to EA (p=0.013, Figure 4E). Absolute numbers of CD206$^+$ monocytes did not differ significantly between inflammatory phenotypes (Figure 4F). Representative dot plots from NA (Figure 4G) and PGA (Figure 4H) illustrate the relative increase of CD206$^-$ monocytes in NA.

To verify our key findings, we investigated macrophage abundance and sputum monocyte subsets relative to asthma inflammatory phenotypes in a second, independent cohort (Table S4, Figure S7). We observed a significant decrease in the proportion of macrophages in NA vs PGA (p=0.005), however, the finding of decreased numbers was not replicated in the validation cohort. Absolute numbers of sputum CM numbers were increased in NA (n=9) vs EA (n=5, p=0.015) and PGA (n=12, p=0.015), together with increased numbers of CD206$^-$ monocytes in NA vs PGA (p=0.007), confirming our prior results of dysregulated monocyte subsets in NA.

CD206$^-$ monocytes feature increased CM phenotype vs CD206$^+$ monocytes

Acquisition of CD206 surface staining and IM and NCM status have both been linked to discrete immune functions and also to differentiation of extravasated monocytes in lung tissue (22). We therefore hypothesized that CD206$^+$ status would be associated with increased IM and/or NCM status in the airways

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and investigated whether monocyte subset proportions differed between $CD206^-$ vs $CD206^+$ sputum monocytes (Figure S8). Indeed, the $CD206^-$ monocyte population consisted of a significantly higher proportion of CM ($p<0.001$, Figure S8A) and a significantly decreased proportion of IM ($p<0.001$, Figure S8B) compared to the $CD206^+$ monocyte population. The proportion of NCM was comparable within the $CD206^-$ and $CD206^+$ monocyte populations (Figure S8C).

Circulating but not airway monocyte subsets are altered in severe asthma

In order to investigate potential alterations in blood and sputum monocyte subset compositions between asthma severities, we performed comparisons between data from participants with severe (n=12) vs non-severe asthma (n=33, Figure 5, Figure S9, Figure S10). Demographics and cell counts are summarized in Table S5 and Table S6. In blood we observed a significant increase in CM as a proportion of $CD45^+$ cells as well as their absolute number in severe compared to non-severe asthma ($p=0.022$, Figure 5A; $p=0.011$, Figure 5B). In contrast to blood, sputum CM did not differ between severe and non-severe asthma (Figure 5C, D). The comparison of total blood monocytes in mild (Figure 5E) vs severe paucigranulocytic asthma (Figure 5F) shows the higher relative abundance of CM in blood of participants with severe asthma. Intermediate and non-classical monocytes did not differ between asthma severities, neither in blood (Figure S9) nor in sputum (Figure S10). We further investigated relative proportions of $CD206^-$ monocytes and numbers of $CD206^-$ and $CD206^+$ monocytes in severe vs non-severe asthma and observed no differences (Figure S11).

Sputum macrophage number is negatively associated with age and asthma duration

To examine if our findings were linked to clinical traits, we investigated the correlation between absolute numbers of sputum monocytes (total monocytes and $CD206^-$ monocytes) and macrophages with clinical characteristics (age, pre-bronchodilator FEV_1, FVC, FEV_1/FVC, BMI, ACQ6, asthma duration, ICS dose and smoking history as pack-years). We used absolute numbers since they are not influenced by the abundance of other cell types (primarily neutrophils). We chose $CD206^-$ monocytes as a marker for 'newly arrived' monocytes to investigate if ongoing monocyte recruitment to the airways is linked to asthma clinical traits. Macrophage numbers negatively correlated with age ($r=-0.36$, $p=0.015$) and asthma duration ($r=-0.34$, $p=0.025$; Table S7). None of the other parameters were significantly associated.

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Discussion:

Monocytes and macrophages have important functions linked to initiation, progression and resolution of inflammation (14). However, their specific roles and dysregulation in asthma are poorly understood as they are not commonly distinguished in clinical studies of airway-derived material. Flow cytometry enabled us to distinguish between monocytes, macrophages, and monocyte subsets. Correlation analyses with conventional cell count and cell sorting of sputum immune cell populations confirmed the validity of the flow cytometry method to assess sputum leukocyte populations. While airway neutrophils were negatively associated with macrophage proportion, they were positively correlated with the absolute numbers of monocytes. Further, NA was associated with significantly increased numbers of sputum CM compared to PGA and $CD206^-$ monocytes in comparison to EA, findings which were confirmed in a separate set of asthma sputum samples. In severe asthma we observed a significant compartment-specific increase of CM in blood compared to non-severe asthma. Together these results imply a significant dysregulation of the monocyte/macrophage lineage in asthma and demonstrate increased presence of airway CM and $CD206^-$ monocytes in neutrophilic asthma consistent with recruitment of neutrophils and monocytes to the airways in concert.

We report for the first time that NA is associated with increased presence of CM and $CD206^-$ monocytes in the airway. CM are the naïve precursor of IM and NCM, whereas NCM are considered the most mature subset (31). While all monocyte subsets have the potential to differentiate into macrophages *in vitro*, only CM can give rise to DCs *in vitro* (32), although recent *in vivo* studies in mice cast doubt on the relevance of these findings (33). The mannose receptor CD206 is important for pathogen recognition and phagocytosis (21). Its expression is a feature of differentiating monocytes and tissue macrophages, suggesting that $CD206^-$ monocytes have been recently recruited to the airways and not exposed to airway specific environmental factors for long enough to induce the expression of CD206 (22). The observation that particularly CM and $CD206^-$ monocytes are elevated in NA implies an increased influx of monocytes to the neutrophil enriched airways. We noted some variation in sputum CM numbers across the NA phenotype, indicating this was not a uniform feature of NA. However, we confirmed this finding in a separate cohort (Figure S7). Increased proportion of neutrophils in NA was associated with a decrease of macrophage proportion as expected. The observation that the proportion of CM was not reduced in NA but consistent across inflammatory phenotypes is further indicative of increased CM in NA. Also consistent with this finding, we observed increased CM phenotype in $CD206^-$ vs $CD206^+$ monocytes. Monocyte subsets express differential patterns and levels of chemoreceptors enabling subset specific recruitment (19). CCR2 is highly abundant on CM and IM. Its ligand CCL2 was found to be increased in primary airway smooth muscle cells in asthma vs healthy controls, upregulated in blood of patients with severe asthma vs healthy controls, and elevated in sputum of patients with cell wall thickening compared to those without cell wall thickening

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(34). Thus, CCL2 could potentially contribute to the increased recruitment of CM, however, it's implication in NA remains to be investigated. CCR5 (highly abundant on CM and IM) has been linked to asthma pathogenesis, as a naturally occurring mutation in this receptor was associated with a reduced risk of developing asthma (35). In addition to chemokines alone, cross-talk between immune cells appears to play a role in immune cell trafficking. In COPD, enhanced monocyte migration to CX3CR1 (highly expressed on IM and NCM) and CCR5 chemokines compared to non-smoking and smoking non-COPD controls was associated with simultaneous migration of lymphocytes (36). Co-dysregulation of neutrophils and monocytes has been implicated in other diseases affecting other extra-pulmonary systems such as atherosclerosis, inflammatory bowel disease and glomerulonephritis. Neutrophils contribute to monocyte recruitment by the secretion of chemoattractants, adhesion molecules and by altering the cytoskeletal structure and thus promoting monocyte transmigration (37). Inversely, patrolling monocytes have been shown to promote neutrophil recruitment and activation in a murine model of glomerular injury (38). CM can express pro-inflammatory cytokines including chemoattractants for neutrophils (IL-8) and monocytes (CCL2, CCL3, CCL5), potentially contributing to NA. Our data are the first demonstration of an association between neutrophil and monocyte migration to the airways in the context of asthma, supporting the concept of cross-talk between these cell types. Further mechanistic studies investigating the interplay between these two cells and the role of airway monocytes in modifying asthma disease activity are therefore merited.

Through distinction of monocytes and macrophages in sputum, we were able to observe that airway macrophages are decreased in asthma vs non-asthma controls. We observed reduced numbers of macrophages in NA vs PGA in our main cohort but were unable to replicate this result in a separate cohort. Several functions of macrophages have been reported to be impaired in asthma including inflammatory mediator production, inflammatory resolution and efferocytosis (39). Whether or not macrophage functions are impaired on a per cell basis in asthma as outlined above, a reduced number of macrophages could produce similar effects in terms of reducing efferocytosis, phagocytosis and impairing inflammatory resolution. Investigation of macrophage abundance and function in relation to neutrophilic airway inflammation in a larger cohort is merited.

In our study severe asthma was associated with alterations in the circulating monocyte subset composition and number. Reports about monocyte subset compositions in peripheral blood in asthma are inconsistent in the literature. Moniuszko *et al* observed increased proportions of IM in severe vs mild-moderate asthma and healthy volunteers, however Al-Radoushi *et al* did not find any differences, which could be explained by different methodological approaches or differences in the clinical cohorts and their classification. Of note, aging has been linked to altered monocyte subset composition (40) and the average age in our severe

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asthma group was numerically higher than the non-severe group (68 yr vs 54 yr, p=0.064), suggesting that age could be a confounder for our observations. Modulatory effects of corticosteroids on monocytes could help to explain dysregulation of circulatory monocyte subsets in severe asthma, however, the literature is contradictory (41, 42), highlighting the need for further studies in this area. Nevertheless, our observations demonstrate differences between monocyte dysregulation detected in blood and airways, suggesting that blood is not an appropriate surrogate for sputum in this context.

Our study has several limitations. Firstly, samples were obtained through a cross-sectional study. It would be of interest to test whether blood and sputum monocyte subset compositions are stable over time within individuals. Secondly, CM, IM and NCM gates in sputum samples were conservatively gated in order to exclude DC and other $CD14^{-}CD16^{-}$ non-monocytes. However, co-expression of CD14 and CD16 on inflammatory monocyte-derived DC has been reported (43) and therefore we cannot exclude the possibility that this subset may have been included in monocyte analysis. Due to limited material for sputum flow cytometry, we could not run fluorescence minus one (FMO) controls for every sample. Gating was based off initial FMO control experiments, manually adjusted on a per sample basis, and to prevent any possible bias, monocyte subset gating was performed by a blinded observer and confirmed by a second blinded observer. Including a positive marker for DC could help in future studies to distinguish them from the monocyte population. Some populations could not be definitively identified using the present marker set and were excluded from analysis, for example cells with intermediate HLA-DR expression falling between the macrophage, monocyte and DC gate and the granulocyte gate. Additional markers have recently been assessed for improved monocyte subset identification to increase reproducibility and robustness of flow cytometry and should be applied in future studies where possible (44). The background fluorescence intensity in the 7-AAD viability channel varied across different sputum cell types, requiring an adjusted gating strategy where non-viable cells were excluded from immune cell populations after the identification of cell types, consistent with a prior sputum flow cytometry study (45). Our $CD45^{+}$ cell population therefore consisted of viable and non-viable cells. In future studies use of alternative viability dyes may allow exclusion of non-viable cells in the whole $CD45^{+}$ population. The criteria for sputum samples might have introduced certain bias as only samples with sufficient cells for conventional differential cell count and flow cytometry were used for analysis. The non-asthma control sample size was relatively low, and had a significantly lower BMI, although BMI was not related to key study outcomes such as CM number or $CD206^{-}$ monocyte number. Age and sex did not significantly differ between the asthma cohort and the non-asthma control group. Further studies are required to investigate any impact of female gender and/or obesity on monocyte/macrophage mechanisms in NA.

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Our study shows that flow cytometry is a valid method to distinguish between monocytes, their subsets and macrophages in sputum samples. By applying this technique, we were able to gain novel insights in immune cell dysregulation in asthma. We observed significant differences in the monocyte/macrophage profile in neutrophilic and severe asthma, subtypes of asthma which are yet poorly understood and lack effective medication. Our findings support the concept of cross-talk between monocytes and neutrophils in inflammatory diseases and provide novel evidence for this paradigm being applicable to the airways and NA. This was an observational study, however our data prompt new hypotheses that may inform future clinical and mechanistic studies. We conclude that neutrophilic airway inflammation in asthma is not exclusively "neutrophilic" but also involves dysregulation of monocytes and macrophages. Dysfunctional macrophages have been proposed as a crucial factor in promoting this discrete inflammatory phenotype (39), and here we hypothesize that dysregulation of monocytes and their subsets may promote inflammatory and clinical features of NA. Thus, future studies should aim to understand the disease modifying activity of airway monocytes in asthma.

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Acknowledgements:

This study was supported by the National Health and Medical Research Council (NHMRC) Centre of Research Excellence in Severe Asthma (project identifier 1078579), NHMRC project grants, Asthma Australia, and the John Hunter Hospital Charitable Trust. M.F. was supported by a Thoracic Society of Australia and New Zealand-AstraZeneca Respiratory Research Fellowship. P.G.G. was supported by an NHMRC Practitioner Fellowship. The authors would like to acknowledge the support of the clinical and laboratory team members at HMRI who conducted participant recruitment, spirometry, specimen collection, processing and analysis: Catherine Delahunty, Netsanet Negewo, Penelope Chan, Naomi Fibbens, Lakshitha Gunawardhana, Bridgette Donati, Heather Macdonald, Amber Smith and Allison Kautto. We would like to thank Nicole Cole (University of Newcastle) for assistance with flow cytometry and Dr Collin Brooks (Massey University, NZ) for technical advice.

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Conflict of interest:

N.N. has received PhD scholarship funding from NHMRC and declares no conflict of interest. K.J.B. has received research funding from the NHMRC and fellowship funding from Lung Foundation Australia and declares no conflict of interest in relation to this paper. J.L.S. declares no conflict of interest in relation to this paper. H.S. has received research and fellowship funding from Asthma Australia, National Asthma Council, AstraZeneca and Thoracic Society of Australia and New Zealand, and declares no conflict of interest. LQ reports grants from NHMRC and funding from national clinical key specialist scheme at Xiangya Hospital and declares no conflict of interest. P.G.G. has received research and fellowship funding from the NHMRC, research funding from AstraZeneca, GlaxoSmithKline, and Novartis, and speaker fees from AstraZeneca, GlaxoSmithKline, Novartis, and Sanofi, unrelated to the current manuscript. M.F. has received research and fellowship funding from the NHMRC, Thoracic Society of Australia and New Zealand and AstraZeneca, and declares no conflict of interest in relation to this paper.

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Tables

Table 1. Demographics and clinical characteristics of the study population.

	Non-asthma (n=8)	Asthma (n=45)	p value
Age (years)[a]	57 (35, 66)	60 (46, 70)	0.296
Sex (female)[b]	7 (87.5 %)	26 (57.8 %)	0.110
BMI (kg/m^2)[c]	24.6 (3.7)	30.6 (6.0)	**0.008**
Ex-smoker[b]	1 (12.5 %)	5 (11.1 %)	0.909
Pack-years[a]	4.4	1.0 (0.45, 1.45)	-
Pre-bronchodilator FEV_1 (% predicted)[a]	104.7 (89.7, 108.5)	79.0 (65.9, 93.6)	**0.005**
Pre-bronchodilator FVC (% predicted)[a]	101.7 (90.5, 106.8)	92.1 (79.6, 100.7)	0.109
Pre-bronchodilator FEV_1/FVC (%)[a]	81.0 (73.9, 83.3)	71.1 (62.9, 78.3)	**0.005**
Taking ICS[b]	-	40 (88.9%)	-
ICS dose (BDP equivalent [µg])[a]	-	1000 (500, 1800)	-
ACQ6 score[a]	-	1.25 (0.5, 1.9) [n=40]	-
Duration asthma (yr)[a]	-	28 (12, 53) [n=43]	-

Data are presented as median (1st and 3rd quartile)[a], number (percentage)[b] or mean (SD)[c]. Mann-Whitney U for non-parametric data, two-sample test of proportions for proportions, Student's *t* test for normally distributed data. BMI, body mass index; FEV_1, forced expiratory volume in one second; FVC, forced vital capacity; ICS, inhaled corticosteroids; BDP, beclomethasone dipropionate; ACQ6, asthma control questionnaire 6. Statistically significant results are highlighted in bold.

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Table 2. Interrelation between flow cytometry quantified sputum immune cells.

Cells/mL	**Macrophages**	**Neutrophils**	**Eosinophils**
Monocytes	r = 0.14	r = 0.60	r = 0.47
	p = 0.337	**p < 0.001**	**p < 0.001**
Macrophages	-	r = -0.14	r = -0.06
		p = 0.303	p = 0.654
% of CD45$^+$ cells	**Macrophages**	**Neutrophils**	**Eosinophils**
Monocytes	r = -0.07	r = 0.09	r = 0.28
	p = 0.637	p = 0.536	p = 0.045
Macrophages	-	r = -0.60	r = -0.23
		p < 0.001	p = 0.097

Spearman correlation was performed and the corresponding correlation coefficient (r) and p value are reported. Significant correlations are highlighted in bold.

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Figure legends:

Figure 1. Comparison of proportions and absolute numbers of sputum macrophages and monocytes quantified by flow cytometry in subjects with asthma vs non-asthma controls. Non-asthma (n=8), asthma (n=45). Macrophages and monocytes expressed as proportion of $CD45^+$ cells (A, C) and as absolute cell number per mL sputum (B, D). Mann-Whitney U for two-sample comparison unless Student's *t* test specified. Bars and whiskers indicate mean with SD for parametric data (D) and median with interquartile range for non-parametric data. P values are shown in the graph, significant differences are highlighted in bold.

Figure 2. Comparison of proportions and absolute numbers of sputum macrophages, monocytes and neutrophils quantified by flow cytometry between asthma inflammatory phenotypes. Eosinophilic asthma (EA, n=14), neutrophilic asthma (NA, n=9) and paucigranulocytic asthma (PGA, n=22). Macrophages, monocytes, and neutrophils expressed as a proportion of $CD45^+$ cells (A, C, E) and as absolute cell number per mL sputum (B, D, F). Dot plots of the monocyte vs macrophage population in NA (G) vs PGA (H). Kruskal Wallis for multiple comparisons unless ANOVA specified. Bars and whiskers indicate mean with SD for parametric data (D) and median with interquartile range for non-parametric data. P values for overall comparison are shown in the graph, significant differences are highlighted in bold. * $p<0.05$, ** $p<0.005$.

Figure 3. Comparison of classical monocytes in subjects with asthma vs non-asthma controls and between asthma inflammatory phenotypes in sputum. (A, B**)** Non-asthma (n=8), asthma (n=45); (C, D) eosinophilic asthma (EA, n=14), neutrophilic asthma (NA, n=9), paucigranulocytic asthma (PGA, n=22). CM, classical monocytes; expressed as % of $CD45^+$ cells (A, C) and absolute numbers/mL sputum (B, D). Dot plots of sputum monocyte subsets in NA (E) vs PGA (F). Mann-Whitney U and Kruskal Wallis. Bars and whiskers indicate median with interquartile range. P values for overall comparison are shown in the graph, significant differences are highlighted in bold. * $p<0.05$.

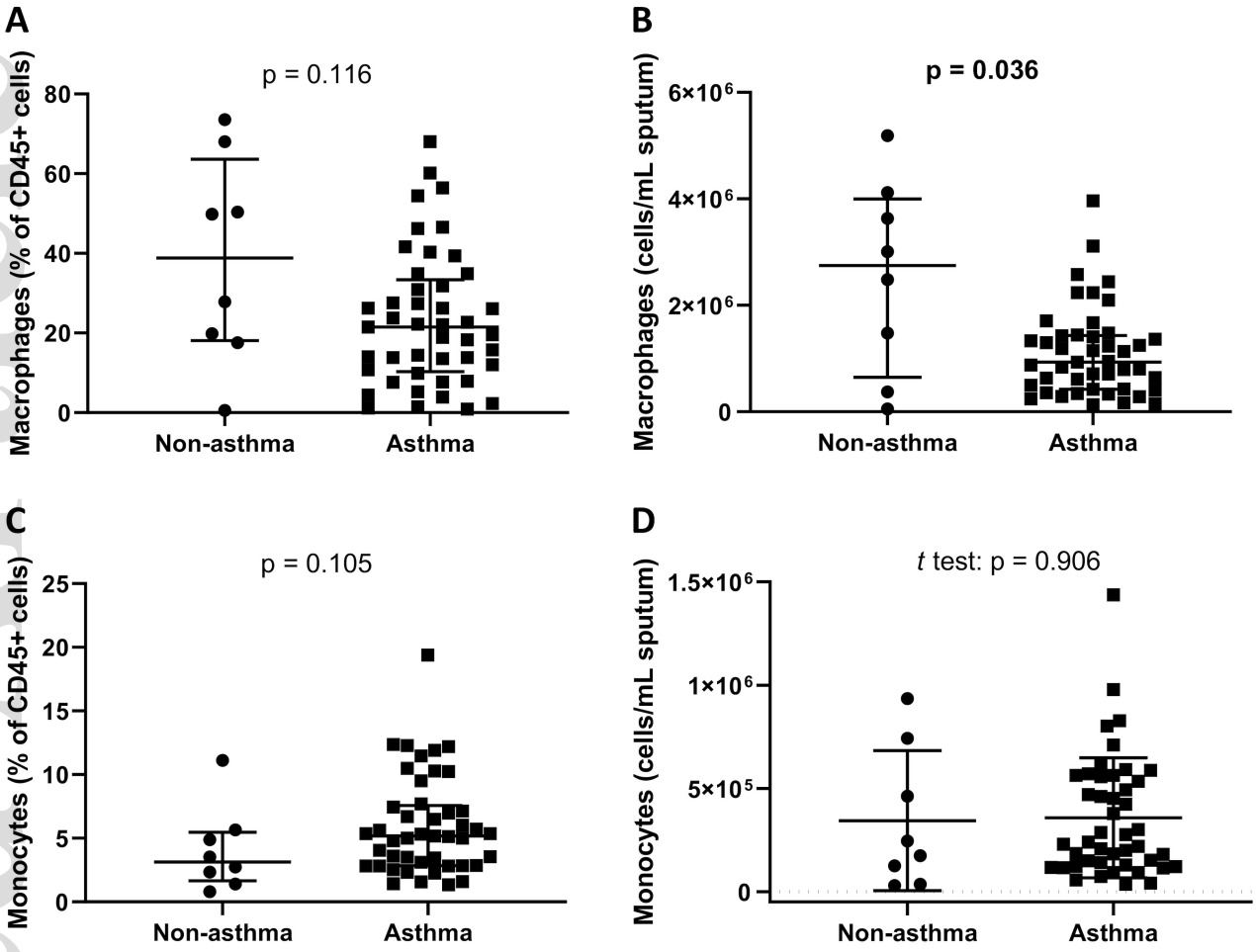
Figure 4. $CD206^{-/+}$ monocyte subsets in sputum. (A-C) Non-asthma (n=8), asthma (n=45); (D-F) eosinophilic asthma (EA, n=14), neutrophilic asthma (NA, n=9), paucigranulocytic asthma (PGA, n=22). Dot plots of $CD206^-$ and $CD206^+$ monocytes in NA (G) vs PGA (H). Mann-Whitney U for two group comparison, Kruskal Wallis for multiple comparison unless ANOVA specified. Bars and whiskers indicate mean with SD for parametric data (D) and median with interquartile range for non-parametric data. P values for overall comparison are shown in the graph, significant differences are highlighted in bold. * $p<0.05$, *** $p<0.001$.

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Figure 5. CM in severe vs non-severe asthma. Severe asthma (n=12), non-severe asthma ($n_{\%}$=33; $n_{cells/mL\ blood}$=32); CM, classical monocytes expressed as % of $CD45^+$ cells and absolute numbers/mL blood (A, B) and sputum (C, D). Dot plots of blood monocyte subsets in mild (E) vs severe PGA (F). Mann Whitney U unless Student's *t* test specified. Bars and whiskers indicate mean with SD for parametric data (C) and median with interquartile range for non-parametric data. P values are shown in the graph, significant differences are highlighted in bold.

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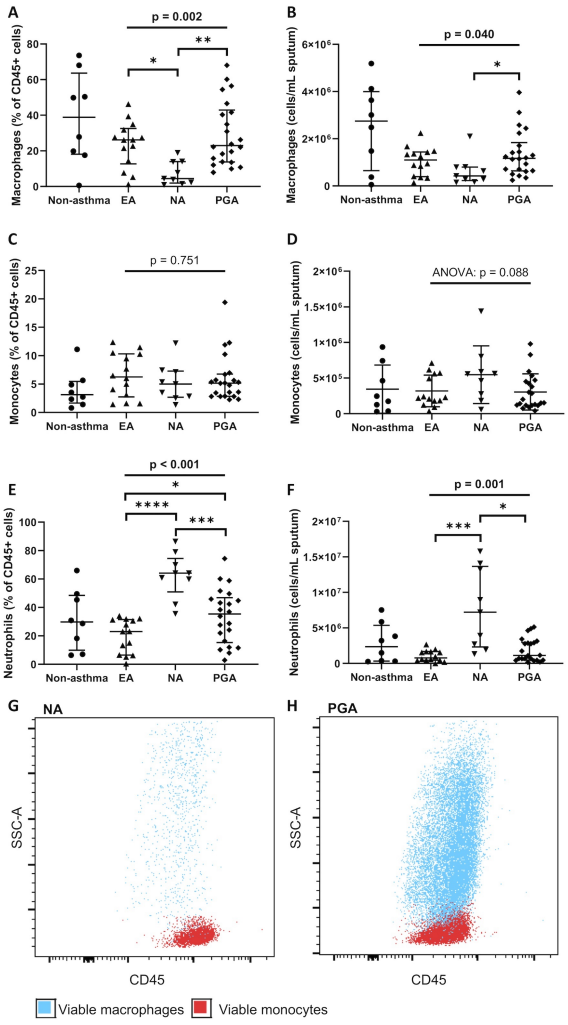


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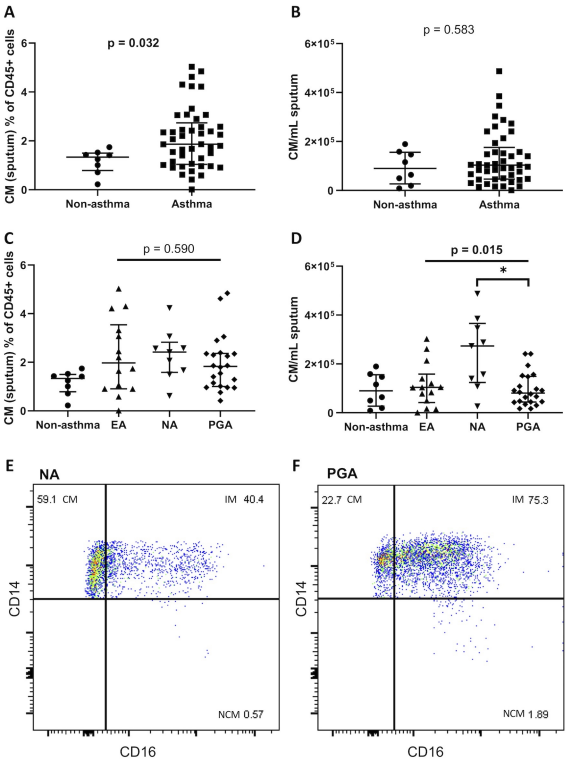
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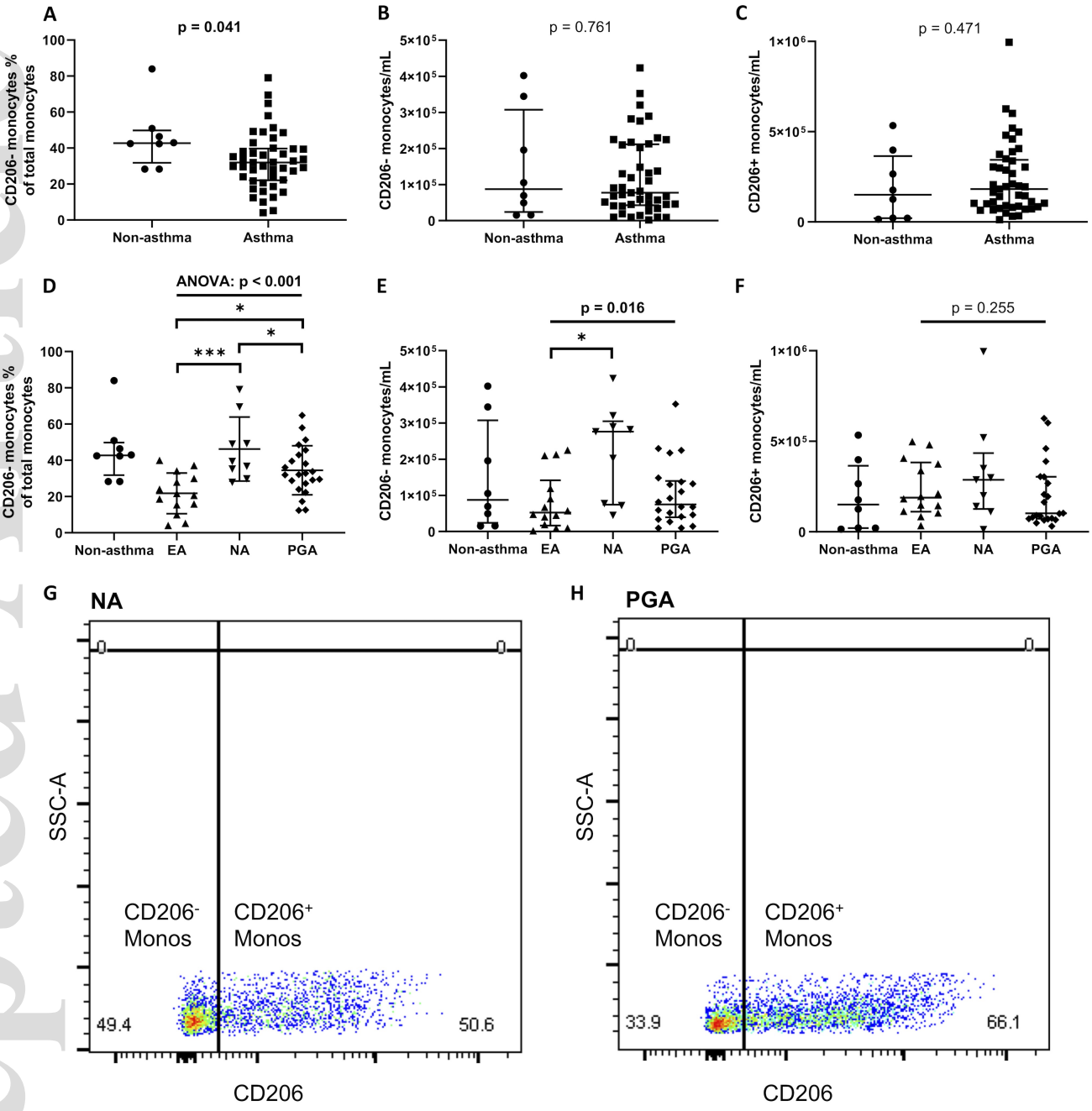
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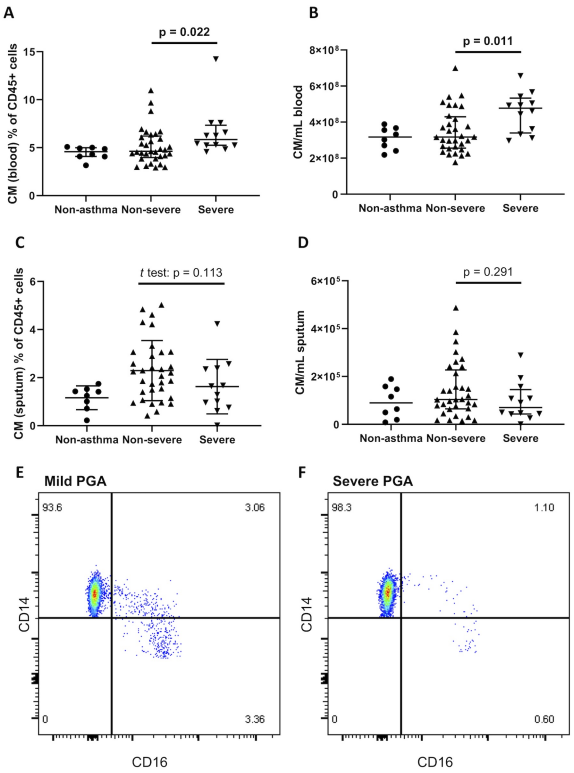
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Title: Neutrophilic asthma features increased airway classical monocytes

Authors: Natalie M. Niessen, MSc, Katherine J. Baines, PhD, Jodie L. Simpson, PhD, Hayley A. Scott, PhD, Ling Qin, MD, Peter G. Gibson, MBBS FRACP DMed, Michael Fricker, PhD

SUPPLEMENTAL ONLINE INFORMATION

Supplemental methods:

Study inclusion and exclusion criteria

Variable airflow obstruction was defined as airway hyperresponsiveness (PD_{15}<15mL to hypertonic saline or other standard challenge agent), bronchodilator responsiveness (change in post-bronchodilator FEV_1>12% or >200 mL), peak flow variability >12% over at least one week of monitoring, or FEV_1 variability >12% (two values measured in two months of each other). Exclusion criteria were respiratory infection or asthma exacerbation requiring oral corticosteroids, antibiotics or change in maintenance therapy in the last month, current lung cancer or other malignancy, smoking within the last six months, pregnancy or breastfeeding, and respiratory diseases other than asthma.

Sputum induction

Sputum induction (after administration of salbutamol) or sputum challenge and induction (withholding the reliever) were performed by hypertonic saline challenge. Nebulized saline (4.5%) was inhaled for increasing time periods (30 seconds, 1 minute, 2 minutes, and 3 x 4 minutes) from an Ultra-NEB™ ultrasonic nebulizer (DeVilbiss, Model 2000 or similar) up to a maximum of 15.5 minutes or until FEV_1 had fallen by more than 20%. One minute after each saline dose, FEV_1 was measured and participants were asked to expectorate into a sterile container. Sputum was processed by separating mucus from saliva and dispersing the cells with dithiothreitol and phosphate buffered saline (PBS). A total cell count was performed under the light microscope. Cell viability was assessed by trypan blue exclusion. Differential cell counts were performed on cytospins stained with May-Grünwald-Giemsa, counting 400 non-squamous cells.

Flow cytometry of whole blood and sputum

Whole blood (100 µL) was added to an antibody cocktail consisting of 50 µL Brilliant Stain Buffer (BD Biosciences) and optimized amounts of antibodies against CD45 (BD Biosciences, clone HI30), CD14 (BD Biosciences, clone M5E2), CD16 (BD Biosciences, clone 3G8) and HLA-DR (BD Biosciences, clone G46-6). Antibody concentrations were established through antibody titration. Samples were incubated in the dark at room temperature for 20 minutes. Erythrocyte lysis buffer (154 mM NH_4Cl, 10 mM Tris, pH 7.5) was added and incubated for 15 minutes in the dark at room temperature. Cells were pelleted by centrifugation (500 x g, 5 minutes, 4°C) and washed with flow cytometry buffer (PBS, 2 mM ethylenediaminetetraacetic acid, 1 % fetal calf serum, 0.01 % sodium azide), followed by repeat centrifugation. The cell pellet was resuspended in flow cytometry buffer and analyzed on a flow cytometer (LSRFortessa X-20, BD Biosciences).

Sputum cells were pelleted by centrifugation (400 x g, 10 minutes) and resuspended in flow cytometry buffer. A minimum of 1 x 10^5 and, when possible, a maximum of 1.5 x 10^6 cells were used for staining. Human Fc block (BD Biosciences) was added and samples were incubated for 10 minutes on ice, following manufacturer's instructions. Cells were washed with flow cytometry buffer and centrifuged (400 x g, 7 minutes, 4°C). The cell pellet was resuspended in the antibody cocktail consisting of the same antibodies used for blood and an additional antibody directed against CD206 (Biolegend, clone MMR). The volumes of antibodies were adjusted according to the numbers of sputum cells to be stained. Cells were incubated for 20 minutes on ice, pelleted (400 x g, 7 minutes, 4°C) and resuspended in flow cytometry buffer. A viability dye (7-AAD, BD Biosciences) was added. After 10 minutes incubation on ice, samples were analyzed on the flow cytometer.

Blood cell flow cytometry gating

Gating was performed blinded to participant ID and characteristics. Events were collected during consistent flow rate (*upper left hand* panel, Figure S3). Doublets were excluded and $CD45^+$ leukocytes were selected. Based on side scatter and CD45 expression, granulocytes, monocytes and lymphocytes were identified. HLA-DR and CD14 negative eosinophils and neutrophils within the granulocyte population were distinguished by CD16 staining. Monocytes were selected based on CD14, CD16 and

HLA-DR expression as suggested by Abeles *et al.*, (1). 'Inclusion gating' and selection of 'true monocytes' was performed to exclude $CD16^+$ neutrophils and natural killer cells from the monocyte gate. Quadrant gates were applied to distinguish between $CD14^{++}CD16^-$, $CD14^+CD16^+$ and $CD14^+CD16^{++}$ monocyte subsets based on the gating strategy published by Jardine *et al* (2). CD14 negative events were excluded from the monocyte gate as all monocytes are considered CD14 positive to varying degrees.

Sputum cell flow cytometry gating

Gating was performed blinded to participant ID and characteristics. Sputum samples were analyzed based on gating strategies published by Lay *et al.* and Brooks *et al.* (3, 4). Sputum sample immune cell populations were less defined than blood samples and more steps were necessary to ensure that selected cell populations did not include other cell types with overlapping marker expression patterns. Fluorescence minus one controls were performed in initial set-up studies to determine gating strategy (see figure S2) but due to variable and limited amounts of sputum cell suspension, it was not possible to run FMO as standard. An overall gating template was created in FlowJo software, applied to each sample and gates were manually adjusted on a sample by sample basis by a single blinded investigator. As previously described (4), it was not possible to exclude dead cells as an initial step in sputum due to varying fluorescence of individual cell populations in the 7-AAD channel therefore dead cells were gated once individual immune cell populations had been gated. The first gates excluded events collected during inconsistent flow rate, doublets, $CD45^-$ cells and cell debris. Lymphocytes were removed from further analysis by excluding $SSC^{low}CD14^-$ events. Mononuclear phagocytes were distinguished from granulocytes based on HLA-DR expression. We observed events with intermediate HLA-DR expression. Since eosinophils and neutrophils rarely express HLA-DR (5, 6) and mononuclear phagocytes can show decreased HLA-DR expression under certain conditions (6, 7), we were unable to definitively identify HLA-DR intermediate events and they were excluded from further analyses. Non-monocytes were excluded on the basis of gating out $HLA\text{-}DR^+CD14^-CD16^-$ events (3) before selecting monocytes ($SSC^{low}CD206^{-/+}$) and macrophages ($SSC^{high}CD206^+$) in the $HLA\text{-}DR^+$ population. Events

with negative or very high CD14 expression were excluded from the monocyte population and $CD206^{-/+}$ subpopulations determined. Classical, intermediate and non-classical monocytes were identified in the $CD206^{-}$, $CD206^{+}$ and total monocyte populations based on CD14 and CD16 expression in a manner consistent with prior literature on blood monocyte subset studies (2, 8). Quadrant gates determined in the blood sample were applied in the corresponding matched sputum monocyte plot and minor manual adjustment by a blinded observer was performed to account for the higher heterogeneity in sputum samples. Undefined events within the HLA-DR negative sputum granulocyte population were removed by excluding $CD14^{+}$ and $CD206^{+}$ events and re-selecting defined $CD45^{+}$ populations and evaluating CD16 positive or negative status. Non-viable 7-$AAD^{medium/high}$ cells gated in individual immune cell populations were excluded from analyses.

Calculation of absolute cell numbers as cells/mL sputum

Absolute numbers of cell types in sputum samples quantified by flow cytometry were calculated with the number of total cells counted (TCC) on a haemocytometer during sputum processing and the proportion of leukocytes (neutrophils, eosinophils, macrophages and lymphocytes) determined by the microscopy-based differential cell count and flow cytometry analysis. TCC (which also includes columnar epithelial cells not quantified by flow cytometry) was adjusted to represent only the TCC of immune cells (akin to quantification of only CD45+ immune cells by flow cytometry). An explanatory equation is given below.

$$\%leukocytes = 43.25\%\, neutrophils + 0.25\%\, eosinophils + 54.5\%\, macrophages + 1.75\ \%\, lymphocytes = 99.75\ \%$$

$$Total\ cell\ counted(TCC) = 2.97\ x\ 10^{6}\ {}^{cells}/_{mL} = 100\%$$

$$\#\ leukocytes = \frac{TCC * \%leukocytes}{100} = \frac{2.97 * 10^{6}\ {}^{cells}/_{mL} * 99.75\%}{100\%} = 2.96 * 10^{6}\ {}^{cells}/_{mL}$$

Absolute numbers of the cell type of interest were then calculated with the following equation:

$$\frac{\#cell\ type\ of\ interest\ quantified\ by\ flow\ cytometry * \#leukocytes}{\#\ CD45{+}(single,\ whole)\ cells\ quantified\ by\ flow\ cytometry}$$

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Supplemental Tables

Table S1. Sputum and blood cell counts of the study population.

	Non-asthma (n=8)	Asthma (n=45)	p value
Total sputum cell count (10^6 cells/mL)	8.33 (4.32, 9.68)	5.67 (4.05, 7.65)	0.326
Sputum neutrophils (%)	32.00 (9.00, 52.75)	43.25 (28.00, 58.25)	0.297
Sputum eosinophils (%)	0 (0, 0.13)	1.50 (0.75, 6.25)	< **0.001**
Sputum macrophages (%)	48.75 (41.38, 87.63)	41.25 (27.20, 60.25)	0.188
Sputum lymphocytes (%)	3.25 (1.50, 4.38)	2.00 (0.75, 3.00)	0.205
Columnar epithelial cells (%)	0.25 (0.25, 0.75)	2.50 (0.50, 7.50)	0.105
Sputum neutrophils (10^6 cells/mL)	2.42 (0.42, 6.08)	2.21 (0.90, 4.27)	0.823
Sputum eosinophils (10^6 cells/mL)	0 (0, 0.01)	0.15 (0.03, 0.28)	< **0.001**
Sputum macrophages (10^6 cells/mL)	3.79 (2.24, 6.22)	2.14 (1.44, 3.09)	**0.046**
Sputum lymphocytes (10^6 cells/mL)	0.21 (0.05, 0.51)	0.10 (0.03, 0.18)	0.192
Columnar epithelial cells (10^6 cells/mL)	0.03 (0.02, 0.04)	0.13 (0.02, 0.32)	0.149
Total blood cell count (10^9 cells/mL)	7.01 (6.07, 7.88)	6.50 (5.81, 7.92) [n=44]	0.648
Blood neutrophils (%)	57.25 (55.60, 67.65)	58.25 (53.00, 60.60) [n=44]	0.439
Blood eosinophils (%)	1.62 (1.28, 2.11)	3.23 (2.46, 4.59) [n=44]	**0.005**
Blood monocytes (%)	6.49 (6.11, 7.07)	7.48 (6.05, 9.02) [n=44]	0.259
Blood lymphocytes (%)	32.25 (23.40, 33.90)	30.00 (25.30, 33.90) [n=44]	0.949
Blood neutrophils (10^9 cells/mL)	4.48 (3.70, 4.74)	3.79 (3.18, 4.63) [n=44]	0.281
Blood eosinophils (10^9 cells/mL)	0.10 (0.09, 0.16)	0.23 (0.16, 0.28) [n=44]	**0.010**
Blood monocytes (10^9 cells/mL)	0.46 (0.37, 0.53)	0.49 (0.38, 0.67) [n=44]	0.432
Blood lymphocytes (10^9 cells/mL)	2.00 (1.53, 2.56)	1.98 (1.64, 2.25) [n=44]	0.970

Data are presented as median (1^{st} and 3^{rd} quartile). Mann-Whitney U. Statistically significant results are highlighted in bold.

Table S2. Demographics and clinical characteristics of asthma airway inflammatory phenotype subgroups.

	Eosinophilic asthma (n=14)	**Neutrophilic asthma (n=9)**	**Paucigranulocytic asthma (n=22)**	**p value**
Age (years)[a]	62 (41, 68)	67 (56, 72)	55 (43, 70)	0.535
Sex (female)[b]	6 (42.86 %)	6 (66.68 %)	14 (63.64%)	0.454
BMI (kg/m^2)[c]	31.46 (5.09)	28.99 (6.18)	30.75 (6.56)	0.634
Ex-smoker[b]	1 (7.14 %)	0 (0 %)	4 (18.18 %)	0.476
Pack-years[a]	1.45	-	0.73 (0.35, 4.97)	-
Pre-bronchodilator FEV_1 (% predicted)[a]	81.34 (74.00, 88.37)	66.51 (60.22, 76.99)	83.35 (65.91, 97.48)	0.284
Pre-bronchodilator FVC (% predicted)[a]	89.86 (81.30, 100.00)	92.55 (84.91, 94.80)	93.15 (75.38, 103.69)	0.870
Pre-bronchodilator FEV_1/FVC (%)[a]	70.75 (65.68, 76.96)	71.10 (54.36, 75.98)	71.57 (62.93, 80.59)	0.446
Taking ICS[b]	13 (92.86 %)	8 (88.89 %)	19 (86.36 %)	> 0.999
ICS dose (BDP equivalent [µg])[a]	1000 (800, 1000)	1000 (500, 2000)	1000 (500, 1600)	0.972
ACQ6 score[a]	1.67 (0.67, 1.92) [n=12]	1.33 (1.00, 2.17) [n=7]	1.00 (0.50, 1.83) [n=21]	0.647
Duration asthma (yr)[a]	18 (7, 40) [n=14]	53.5 (37, 61.5)* [n=8]	26 (18, 44) [n=21]	**0.023**

Data are presented as median (1st and 3rd quartile)[a], number (percentages)[b] or mean (SD)[c]. Kruskal-Wallis for non-parametric data, Fisher's exact test for proportions, one-way ANOVA for normally distributed data. *: $p<0.05$ vs EA. FEV_1, forced expiratory volume in one second; FVC, forced vital capacity; ICS, inhaled corticosteroids; BDP, beclomethasone dipropionate; BMI, body mass index; ACQ6, asthma control questionnaire 6. Statistically significant results are highlighted in bold.

Table S3. Sputum and blood cell counts of asthma airway inflammatory phenotype subgroups.

	Eosinophilic asthma (n=14)	**Neutrophilic asthma (n=9)**	**Paucigranulocytic asthma (n=22)**	**p value**
Total sputum cell count (10^6 cells/mL)	5.85 (3.06, 6.57)	11.25 (5.13, 16.83)	5.00 (3.60, 7.20)	0.064
Sputum neutrophils (%)	30.13 (17.75, 37.25)	73.50 (68.00, 82.00)**,††	41.38 (20.50, 56.75)	< **0.001**
Sputum eosinophils (%)	10.38 (6.75, 13.00)††,‡‡	1.00 (0.40, 1.50)	0.88 (0.25, 2.00)	< **0.001**
Sputum macrophages (%)[b]	49.25 (41.25, 61.00)	16.25 (13.75, 26.00)**,††	51.00 (37.00, 63.25)	< **0.001**
Sputum lymphocytes (%)	2.00 (0.50, 5.50)	0.75 (0.20, 2.25)	2.13 (1.25, 2.75)	0.243
Columnar epithelial cells (%)	2.50 (0.50, 7.50)	1.80 (0.50, 8.25)	2.63 (0.25, 6.25)	0.890
Sputum neutrophils (10^6 cells/mL)	1.94 (0.60, 2.82)	9.23 (3.77, 13.40)**,††	1.32 (0.86, 3.62)	< **0.001**
Sputum eosinophils (10^6 cells/mL)	0.38 (0.28, 1.23)††,‡	0.06 (0.03, 0.17)	0.04 (0.01, 0.09)	< **0.001**
Sputum macrophages (10^6 cells/mL)	2.04 (1.38, 2.88)	1.83 (0.76, 2.31)	2.61 (1.59, 3.13)	0.438
Sputum lymphocytes (10^6 cells/mL)	0.10 (0.03, 0.25)	0.03 (0.01, 0.17)	0.10 (0.05, 0.16)	0.845
Columnar epithelial cells (10^6 cells/mL)	0.10 (0.02, 0.35)	0.10 (0.08, 0.33)	0.13 (0.02, 0.28)	0.996
Total blood cell count (10^9 cells/mL)	6.38 (5.96, 7.69) [n=13]	6.49 (6.07, 6.57)	6.53 (5.62, 8.06)	0.854
Blood neutrophils (%)	57.30 (55.10, 59.50) [n=13]	60.40 (53.30, 60.80)	58.30 (50.10, 61.90)	0.807
Blood eosinophils (%)	4.32 (2.91, 7.37) [n=13]	2.55 (2.29, 4.10)	3.12 (2.08, 3.91)	0.078
Blood monocytes (%)	6.27 (4.93, 7.44) [n=13]	8.32 (6.81, 9.92)	8.00 (5.96, 9.33)	0.085
Blood lymphocytes (%)	30.1 (25.3, 33.8) [n=13]	29.2 (25.3, 33.1)	30.00 (26.00, 34.80)	0.776
Blood neutrophils (10^9 cells/mL)	3.80 (3.23, 4.54) [n=13]	3.77 (3.59, 3.99)	3.84 (2.96, 4.85)	0.985
Blood eosinophils (10^9 cells/mL)	0.27 (0.20, 0.51) [n=13]	0.19 (0.15, 0.24)	0.24 (0.12, 0.26)	0.107
Blood monocytes (10^9 cells/mL)	0.38 (0.34, 0.50) [n=13]	0.55 (0.43, 0.70)	0.54 (0.42, 0.67)	0.099
Blood lymphocytes (10^9 cells/mL)	2.10 (1.54, 2.34) [n=13]	1.74 (1.53, 2.17)	1.95 (1.77, 2.17)	0.551

Data are presented as median (1st and 3rd quartile). Kruskal-Wallis. **: p<0.005 vs EA; ††: p<0.005 vs PGA; ‡: p<0.05 vs NA; ‡‡: p<0.005 vs NA. Statistically significant results are highlighted in bold.

Table S4. Demographics and clinical characteristics of the second independent sputum flow cytometry study population.

	Asthma (n=26)
Age (years)[a]	54.8 (39.5, 68.2)
Sex (female)[b]	16 (61.5 %)
BMI (kg/m^2)[c]	30.9 (6.6)
Ex-smoker[b]	11 (42.3 %)
Pack-years[a]	9 (2.2, 23)
Pre-bronchodilator FEV_1 (% predicted)[a]	82.6 (71.3, 95.8)
Pre-bronchodilator FVC (% predicted)[a]	90.4 (80.4, 99.3)
Pre-bronchodilator FEV_1/FVC (%)[a]	72.4 (62.9, 80.8)
Taking ICS[b]	22 (84.6%)
ICS dose (BDP equivalent [µg])[a]	1000 (500, 1125)
ACQ6 score[a]	0.59 (0.27, 1.4)
Duration asthma (yr)[a]	32 (11, 52)

Data are presented as median (1st and 3rd quartile)[a], number (percentage)[b] or mean (SD)[c]. FEV_1, forced expiratory volume in one second; FVC, forced vital capacity; ICS, inhaled corticosteroids; BDP, beclomethasone dipropionate; BMI, body mass index; ACQ6, asthma control questionnaire 6.

Table S5. Demographics and clinical characteristics of asthma severity subgroups.

	Non-severe (n=33)	**Severe (n=12)**	**p value**
Age (years)[a]	54 (40, 70)	68 (60, 71)	0.064
Sex (female)[b]	21 (63.64)	5 (41.67)	0.187
BMI (kg/m^2)[c]	30.63 (6.21)	30.58 (5.62)	0.978
Ex-smoker[b]	3 (9.09)	2 (16.67)	0.475
Pack-years[a]	1.45 (0.45, 8.93)	0.63 (0.25, 1)	0.248
Pre-bronchodilator FEV_1 (% predicted)[a]	82.3 (73.4, 96.7)	67.8 (60.5, 85.9)	0.051
Pre-bronchodilator FVC (% predicted)[a]	92.6 (81.2, 103.7)	90.5 (74.2, 95.0)	0.190
Pre-bronchodilator FEV_1/FVC (%)[a]	75.9 (63.6, 80.4)	67.9 (55.2, 72.0)	0.051
Taking ICS[b]	28 (84.85)	12 (100)	0.153
ICS dose (BDP equivalent [µg])[a]	800 (500, 1000)	2000 (900, 2000)	**0.009**
ACQ6 score[a]	1 (0.5, 1.83) [n=29]	1.67 (1, 3.17) [n=11]	0.097
Duration asthma (yr)[c]	27 (19.6)	47 (19.2)	**0.005**

Data are presented as median (1^{st} and 3^{rd} quartile)[a], number (percentage)[b] or mean (SD)[c]. Mann-Whitney U for non-parametric data, two-sample test of proportions for proportions, Student's *t* test for normally distributed data. FEV_1, forced expiratory volume in one second; FVC, forced vital capacity; ICS, inhaled corticosteroids; BDP, beclomethasone dipropionate; BMI, body mass index; ACQ6, asthma control questionnaire 6. Statistically significant results are highlighted in bold.

Table S6. Sputum and blood cell counts of asthma severity subgroups.

	Non-severe (n=33)	Severe (n=12)	p value
Total sputum cell count (10^6 cells/mL)	5.22 (3.60, 7.65)	6.66 (4.19, 7.92)	0.653
Sputum neutrophils (%)	37.25 (28.50, 56.75)	55.25 (14.13, 64.75)	0.564
Sputum eosinophils (%)	2.25 (0.75, 9.00)	1.38 (0.50, 2.25)	0.335
Sputum macrophages (%)	43.50 (27.20, 59.75)	39.25 (26.75, 73.25)	0.888
Sputum lymphocytes (%)	2.00 (1.00, 3.25)	1.63 (0.63, 2.75)	0.520
Columnar epithelial cells (%)	2.25 (0.25, 8.50)	2.63 (1.00, 4.88)	0.918
Sputum neutrophils (10^6 cells/mL)	2.16 (0.90, 3.88)	2.71 (0.79, 5.11)	0.878
Sputum eosinophils (10^6 cells/mL)	0.17 (0.03, 0.30)	0.07 (0.02, 0.20)	0.426
Sputum macrophages (10^6 cells/mL)	1.95 (1.44, 3.04)	2.45 (1.15, 4.49)	0.644
Sputum lymphocytes (10^6 cells/mL)	0.10 (0.03, 0.20)	0.08 (0.04, 0.15)	0.572
Columnar epithelial cells (10^6 cells/mL)	0.10 (0.02, 0.32)	0.16 (0.05, 0.28)	0.939
Total blood cell count (10^9 cells/mL)	6.53 (5.81, 7.75) [n=32]	6.31 (5.81, 8.90)	0.843
Blood neutrophils (%)	58.25 (53.00, 60.4) [n=32]	57.35 (50.70, 63.95)	0.874
Blood eosinophils (%)	3.12 (3.67, 4.22) [n=32]	4.01 (2.61, 5.84)	0.292
Blood monocytes (%)	7.16 (5.90, 8.45) [n=32]	8.63 (6.45, 9.73)	0.155
Blood lymphocytes (%)	30.10 (25.65, 34.15) [n=32]	29.50 (22.90, 32.10)	0.493
Blood neutrophils (10^9 cells/mL)	3.79 (3.24, 4.48) [n=32]	3.76 (2.92, 5.51)	0.948
Blood eosinophils (10^9 cells/mL)	0.21 (0.15, 0.27) [n=32]	0.26 (0.17, 0.42)	0.231
Blood monocytes (10^9 cells/mL)	0.43 (0.37, 0.68) [n=32]	0.60 (0.51, 0.66)	0.133
Blood lymphocytes (10^9 cells/mL)	1.95 (1.64, 2.24) [n=32]	2.01 (1.64, 2.25)	0.916

Data are presented as median (1^{st} and 3^{rd} quartile). Mann-Whitney U.

Table S7. Correlation between monocytes/macrophages and clinical characteristics.

	Total sputum monocytes/mL	**CD206$^-$ sputum monocytes/mL**	**Macrophages/mL**
Age	r = -0.01 p = 0.928	r = -0.07 p = 0.636	**r = -0.36** **p = 0.016**
Pre-BD FEV_1 (% pred)	r = -0.10 p = 0.523	r = -0.10 p = 0.525	r = 0.10 p = 0.509
Pre-BD FVC (% pred)	r = -0.09 p = 0.545	r = -0.03 p = 0.855	r = -0.07 p = 0.655
Pre-BD FEV_1/FVC (%)	r = -0.01 p = 0.949	r = -0.09 p = 0.542	r = 0.20 p = 0.194
BMI	r = -0.03 p = 0.847	r = -0.09 p = 0.578	r = 0.22 p = 0.155
ACQ6 score	r = 0.06 p = 0.726	r = 0.04 p = 0.828	r = -0.10 p = 0.535
Asthma duration	r = 0.08 p = 0.593	r = 0.20 p = 0.200	**r = -0.34** **p = 0.024**
ICS dose (BDP equivalent)	r = -0.15 p = 0.344	r = -0.21 p = 0.190	r = -0.08 p = 0.608
Packyears	r = -0.20 p = 0.783	r = 0.10 p = 0.950	r = -0.40 p = 0.517

Spearman correlation was performed and the corresponding correlation coefficient (r) and p value are reported. Significant correlations are highlighted in bold.

Supplemental figure legends:

Figure S1. Gating strategy for sputum cells and overlay of identified cell populations in a final dot plot. Identification and quantification of viable monocytes ($CD45^{+}$, SSC^{med}, HLA-DR^{+}, $CD14^{+/++}$, $CD206^{-/medium}$, $CD16^{-/+}$, 7-AAD^{-}, orange), viable macrophages ($CD45^{+}$, $SSC^{medium/high}$, HLA-DR^{+}, $CD14^{+}$, $CD206^{high}$, $CD16^{+}$, 7-AAD^{medium}, blue), viable neutrophils ($CD45^{+}$, SSC^{medium}, HLA-$DR^{-/low}$, $CD14^{-}$, $CD206^{-}$, $CD16^{++}$, 7-AAD^{-}, purple) and viable eosinophils ($CD45^{+}$, SSC^{low}, HLA-$DR^{-/low}$, $CD14^{-}$, $CD206^{-}$, $CD16^{-}$, 7-AAD^{-}, red). SSC, side scatter; FSC, forward scatter; Macs, macrophages; Monos, monocytes; Eos, eosinophils; CM, classical monocytes; IM, intermediate monocytes; NCM, non-classical monocytes.

Figure S2. Fluorescence minus one (FMO) controls and fully stained sputum samples. FMO's were performed with initial samples to establish the gating strategy. FMO's are shown on the left and the corresponding stained sample on the right.

Figure S3. Gating strategy for blood cells and overlay of identified cell populations in a final dot plot. Identification and quantification of monocytes ($CD45^{+}$, $SSC^{low/medium}$, HLA-DR^{+}, $CD14^{+/++}$, $CD16^{-/+}$, orange), neutrophils ($CD45^{+}$, SSC^{high}, HLA-DR^{-}, $CD14^{-}$, $CD16^{++}$, purple) and eosinophils ($CD45^{+}$, SSC^{high}, HLA-DR^{-}, $CD14^{-}$, $CD16^{-}$, red). FSC, forward scatter; SSC, side scatter; Eos, eosinophils; CM, classical monocytes; IM, intermediate monocytes; NCM, non-classical monocytes.

Figure S4. Correlation between the proportion of cells determined by flow cytometry vs differential sputum cell count and images of isolated immune cells via fluorescence-activated cell sorting. Spearman correlation coefficient (r) and the corresponding p value are indicated in the graph. Monos, monocytes; Macs, macrophages; DC, dendritic cells. Correlation between neutrophils (A), eosinophils (B), monocytes, macrophages and dendritic cells (C) and microscope images of neutrophils (E), monocytes (F) and macrophages (G).

Figure S5. Comparison of CD14/CD16-based monocyte subsets in asthma vs non-asthma controls and between asthma airway inflammatory phenotypes in sputum. (A-D) Non-asthma (n=8), asthma (n=45); (E-H) eosinophilic asthma (EA, n=14), neutrophilic asthma (NA, n=9), paucigranulocytic asthma (PGA, n=22). IM, intermediate monocytes; NCM, non-classical monocytes; expressed as % of $CD45^+$ cells (A, C, E, G) and absolute numbers/mL (B, D, F, H). Mann-Whitney U and Kruskal Wallis. Bars and whiskers indicate median with interquartile range. P values for overall comparison are shown in the graph.

Figure S6. Comparison of CD14/CD16-based monocyte subsets in asthma vs non-asthma controls and between asthma inflammatory phenotypes in blood. **(**A-F) Non-asthma (n=8), asthma ($n_{\%}$=45, $n_{cells/mL}$=44); (G-L) eosinophilic asthma (EA, $n_{\%}$=14; $n_{cells/mL}$=13), neutrophilic asthma (NA, n=9), paucigranulocytic asthma (PGA, n=22). CM, classical monocytes; IM, intermediate monocytes; NCM, non-classical monocytes; expressed as % of $CD45^+$ cells (A-C**,** G-I) and absolute numbers/mL (D-F, J-L). Mann-Whitney U for two group comparison unless Student's *t* test specified, Kruskal Wallis for multiple comparison unless ANOVA specified. Bars and whiskers indicate mean with SD for parametric data (D, L) and median with interquartile range for non-parametric data. P values for overall comparison are shown in the graph.

Figure S7. Validation of key results in an independent cohort. Macrophages expressed as % of $CD45^+$ cells (A) and absolute numbers/mL sputum (B). CM, classical monocytes; expressed as % of $CD45^+$ cells (C) and absolute numbers/mL sputum (D). $CD206^-$ monocytes expressed as % of total monocytes (E) and absolute cells/mL sputum (F). EA, eosinophilic asthma (n=5); NA, neutrophilic asthma (n=9); PGA, paucigranulocytic asthma (n=12). Kruskal Wallis for multiple comparison unless ANOVA specified. Bars and whiskers indicate mean with SD for parametric data (A, D, E) and median with interquartile range for non-parametric data. P values for overall comparison are shown in the graph, significant differences are highlighted in bold. * $p<0.05$, ** $p<0.005$. If no asterisk is indicated despite a significant p value for multiple comparison, the post-hoc test revealed no significant differences between groups.

Figure S8. Comparison of CD14/CD16 monocyte subset occurrence between $CD206^-$ vs $CD206^+$ monocyte populations in sputum of participants with asthma. Subsets are expressed as a proportion of either $CD206^-$ monocytes or $CD206^+$ monocytes. (A) CM, classical monocytes; (B) IM, intermediate monocytes; (C) NCM, non-classical monocytes. Mann-Whitney U to compare $CD206^-$ vs $CD206^+$ subsets (n=45). Bars and whiskers indicate mean with SD for parametric data (A) and median with interquartile range for non-parametric data. P values are shown in the graph, significant differences are highlighted in bold.

Figure S9: Blood monocyte subset composition in severe asthma vs non-severe asthma. Non-severe asthma ($n_{\%}$=33; $n_{cells/mL}$=32), severe asthma (n=12). IM, intermediate monocytes; NCM, non-classical monocytes; expressed as a proportion of $CD45^+$ cells (A, C) and absolute cells/mL blood (B, D). Mann Whitney U. Bars and whiskers indicate median with interquartile range. P values are shown in the graph.

Figure S10. Sputum monocyte subset composition in severe asthma vs non-severe asthma. Non-severe asthma (n=33), severe asthma (n=12). IM, intermediate monocytes; NCM, non-classical monocytes; expressed as a proportion of $CD45^+$ cells (A, C) and absolute cells/mL sputum (B, D). Mann Whitney U. Bars and whiskers indicate median with interquartile range. P values for the two groups comparison are shown in the graph.

Figure S11. Comparison of $CD206^-$ vs $CD206^+$ monocyte subsets in severe vs non-severe asthma. Non-severe asthma (n=33), severe asthma (n=12). Monocyte subsets are expressed as a proportion of $CD45^+$ cells (A), and absolute cells/mL sputum (B, C). Mann Whitney U unless Student's *t* test specified. Bars and whiskers indicate mean with SD for parametric data (A) and median with interquartile range for non-parametric data. P values are shown in the graph.

Supplemental figures:

Figure S1

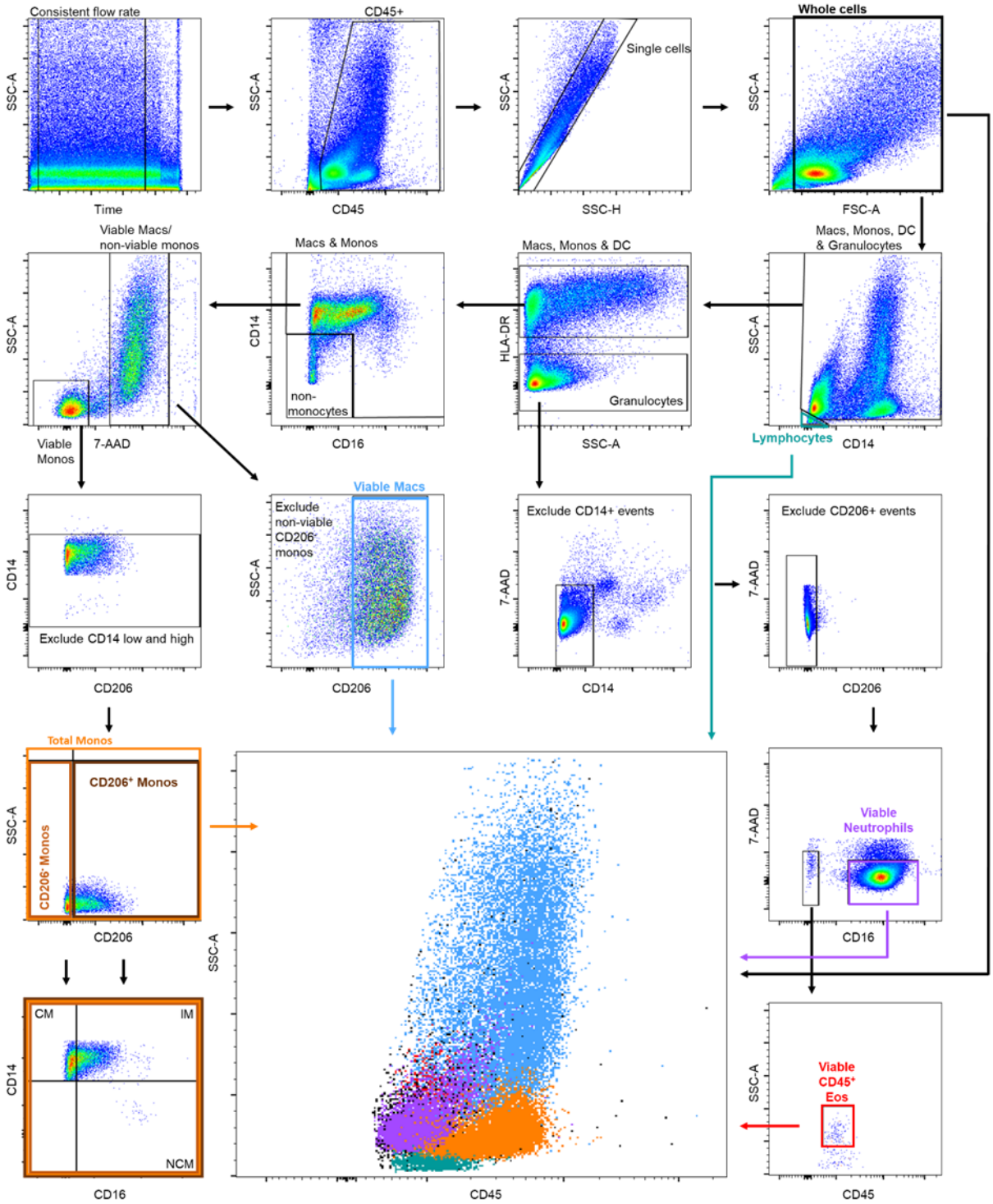


Figure S2

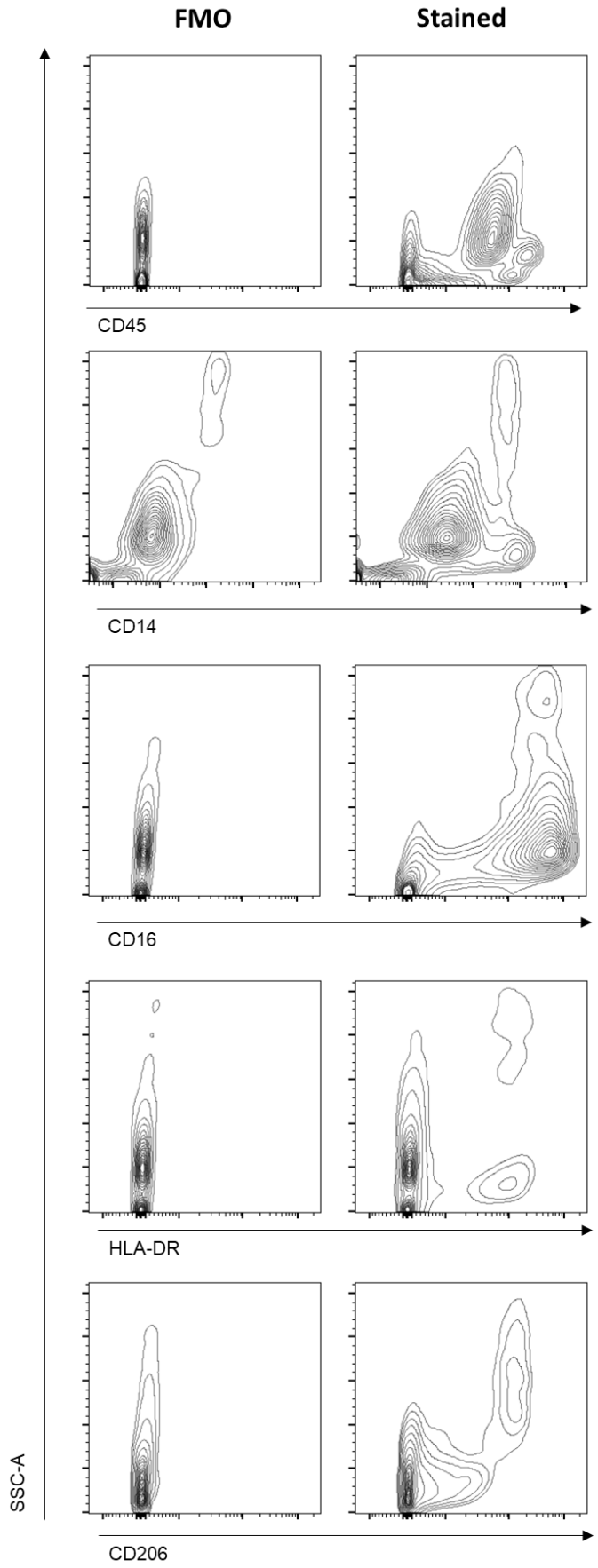


Figure S3

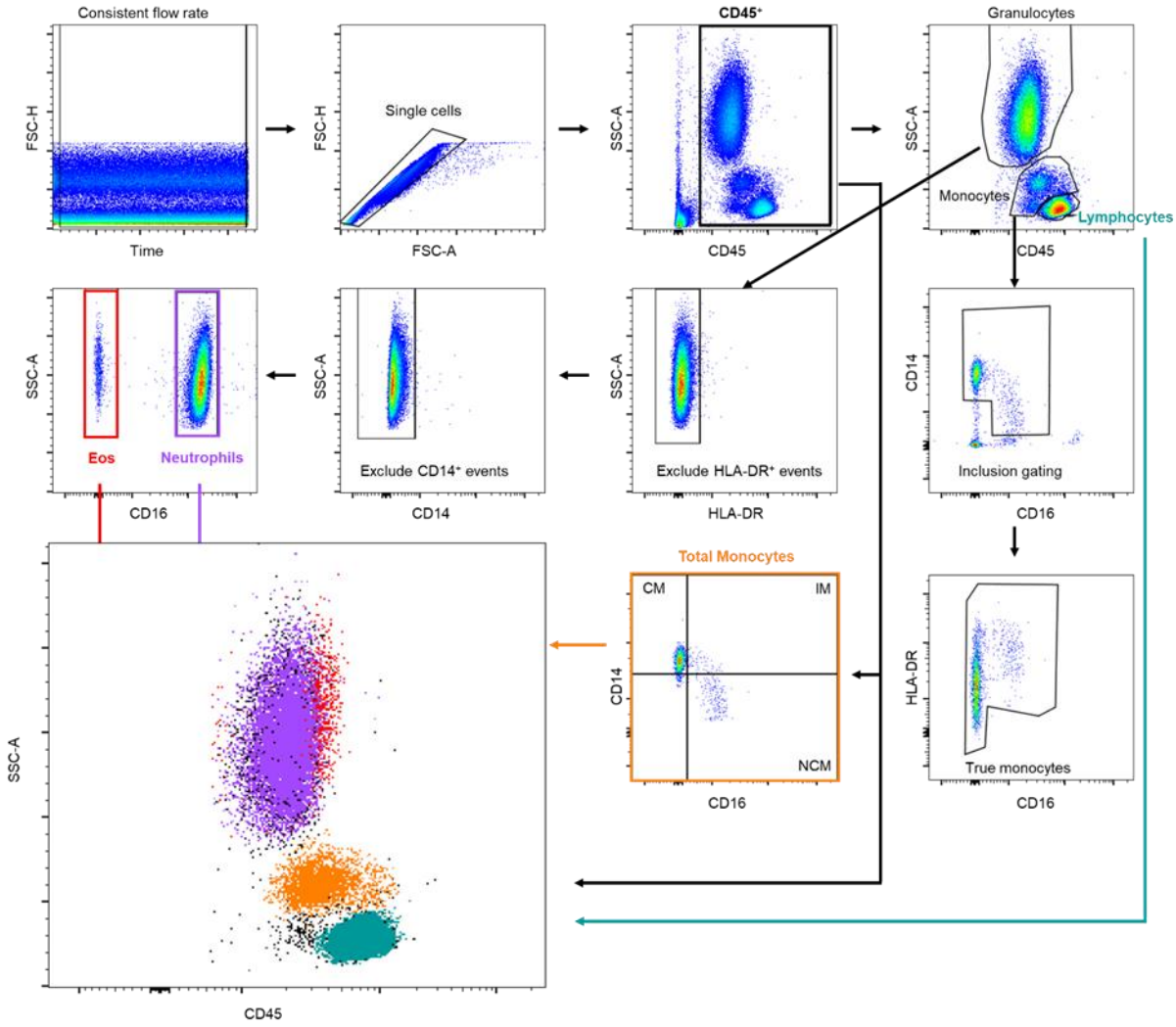

Consistent flow rate
FSC-H
Time
Single cells
FSC-H
FSC-A
CD45+
SSC-A
CD45
Granulocytes
SSC-A
Monocytes
Lymphocytes
CD45
CD14
Inclusion gating
CD16
HLA-DR
True monocytes
CD16
Exclude HLA-DR+ events
SSC-A
HLA-DR
Exclude CD14+ events
SSC-A
CD14
Eos
Neutrophils
SSC-A
CD16
Total Monocytes
CM
IM
NCM
CD14
CD16
SSC-A
CD45

Figure S4

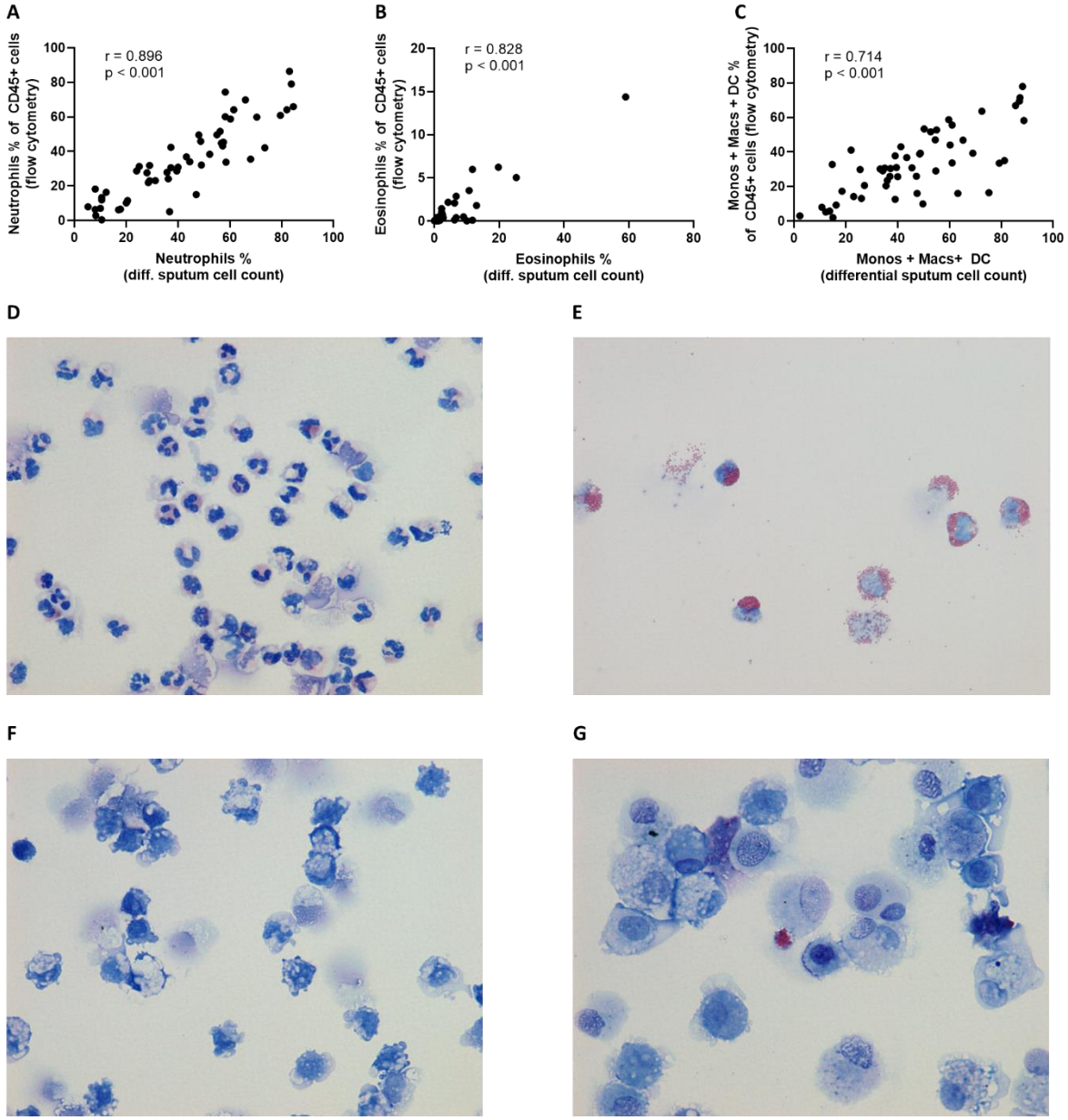

A
Neutrophils % of CD45+ cells (flow cytometry)
r = 0.896
p < 0.001
Neutrophils %
(diff. sputum cell count)
B
Eosinophils % of CD45+ cells (flow cytometry)
r = 0.828
p < 0.001
Eosinophils %
(diff. sputum cell count)
C
Monos + Macs + DC % of CD45+ cells (flow cytometry)
r = 0.714
p < 0.001
Monos + Macs+ DC
(differential sputum cell count)
D
E
F
G

Figure S5

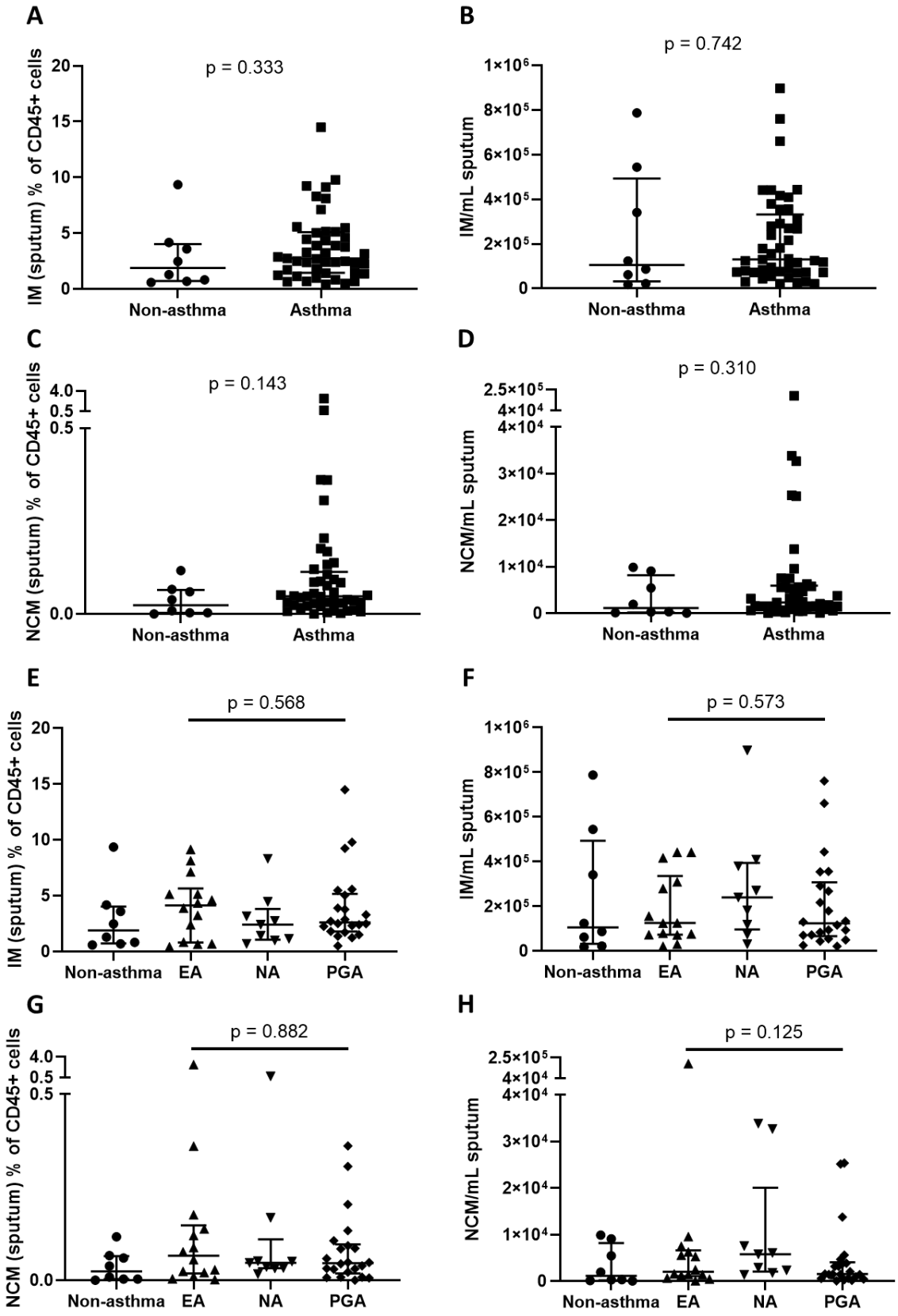

A
p = 0.333
IM (sputum) % of CD45+ cells
20
15
10
5
0
Non-asthma
Asthma
B
p = 0.742
IM/mL sputum
1×10⁶
8×10⁵
6×10⁵
4×10⁵
2×10⁵
0
Non-asthma
Asthma
C
p = 0.143
NCM (sputum) % of CD45+ cells
4.0
0.5
0.5
0.0
Non-asthma
Asthma
D
p = 0.310
NCM/mL sputum
2.5×10⁵
4×10⁴
4×10⁴
3×10⁴
2×10⁴
1×10⁴
0
Non-asthma
Asthma
E
p = 0.568
IM (sputum) % of CD45+ cells
20
15
10
5
0
Non-asthma
EA
NA
PGA
F
p = 0.573
IM/mL sputum
1×10⁶
8×10⁵
6×10⁵
4×10⁵
2×10⁵
0
Non-asthma
EA
NA
PGA
G
p = 0.882
NCM (sputum) % of CD45+ cells
4.0
0.5
0.5
0.0
Non-asthma
EA
NA
PGA
H
p = 0.125
NCM/mL sputum
2.5×10⁵
4×10⁴
4×10⁴
3×10⁴
2×10⁴
1×10⁴
0
Non-asthma
EA
NA
PGA

Figure S6

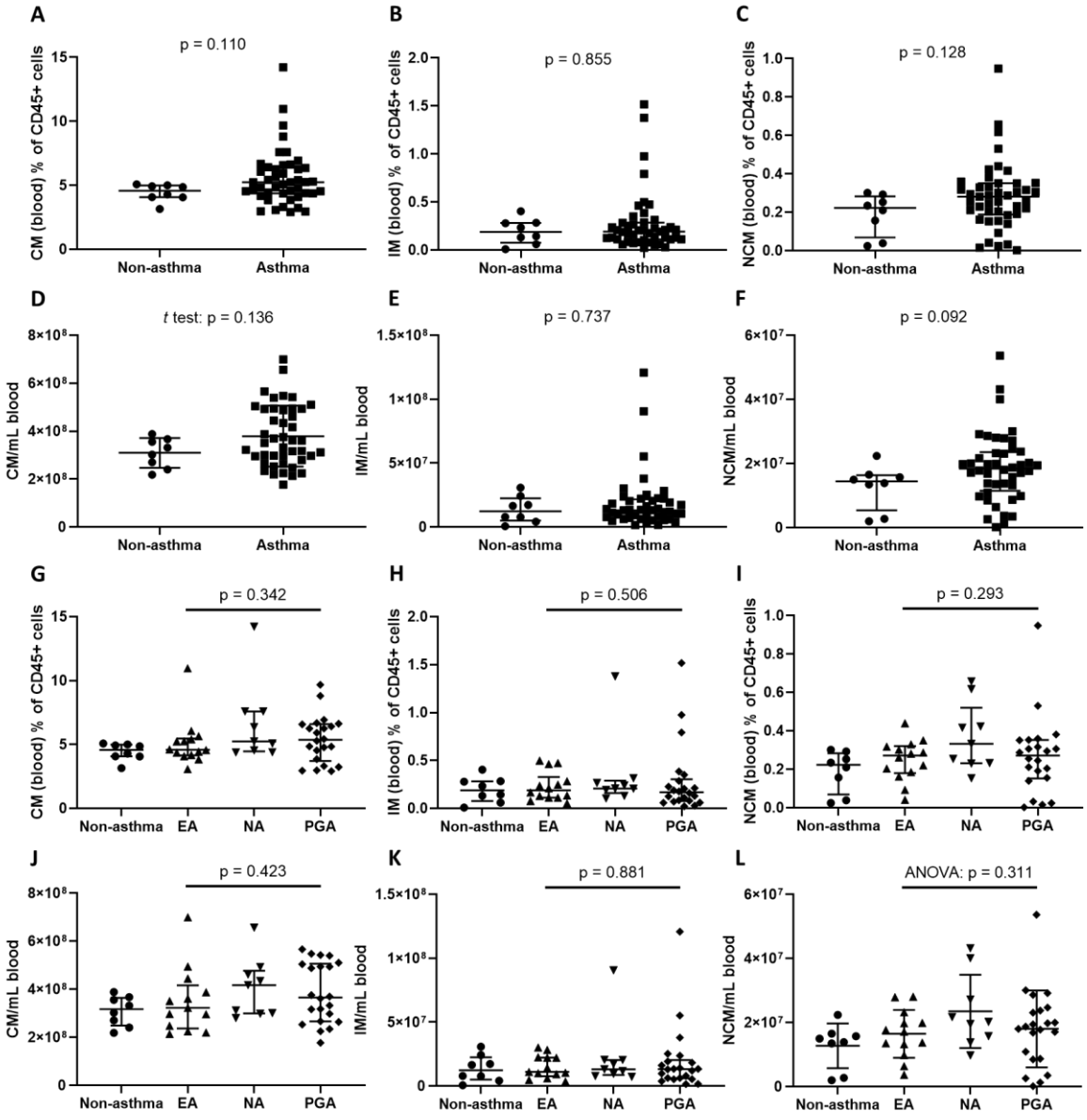

A
p = 0.110
CM (blood) % of CD45+ cells
Non-asthma
Asthma
B
p = 0.855
IM (blood) % of CD45+ cells
C
p = 0.128
NCM (blood) % of CD45+ cells
D
t test: p = 0.136
CM/mL blood
E
p = 0.737
IM/mL blood
F
p = 0.092
NCM/mL blood
G
p = 0.342
EA
NA
PGA
H
p = 0.506
I
p = 0.293
J
p = 0.423
K
p = 0.881
L
ANOVA: p = 0.311

Figure S7

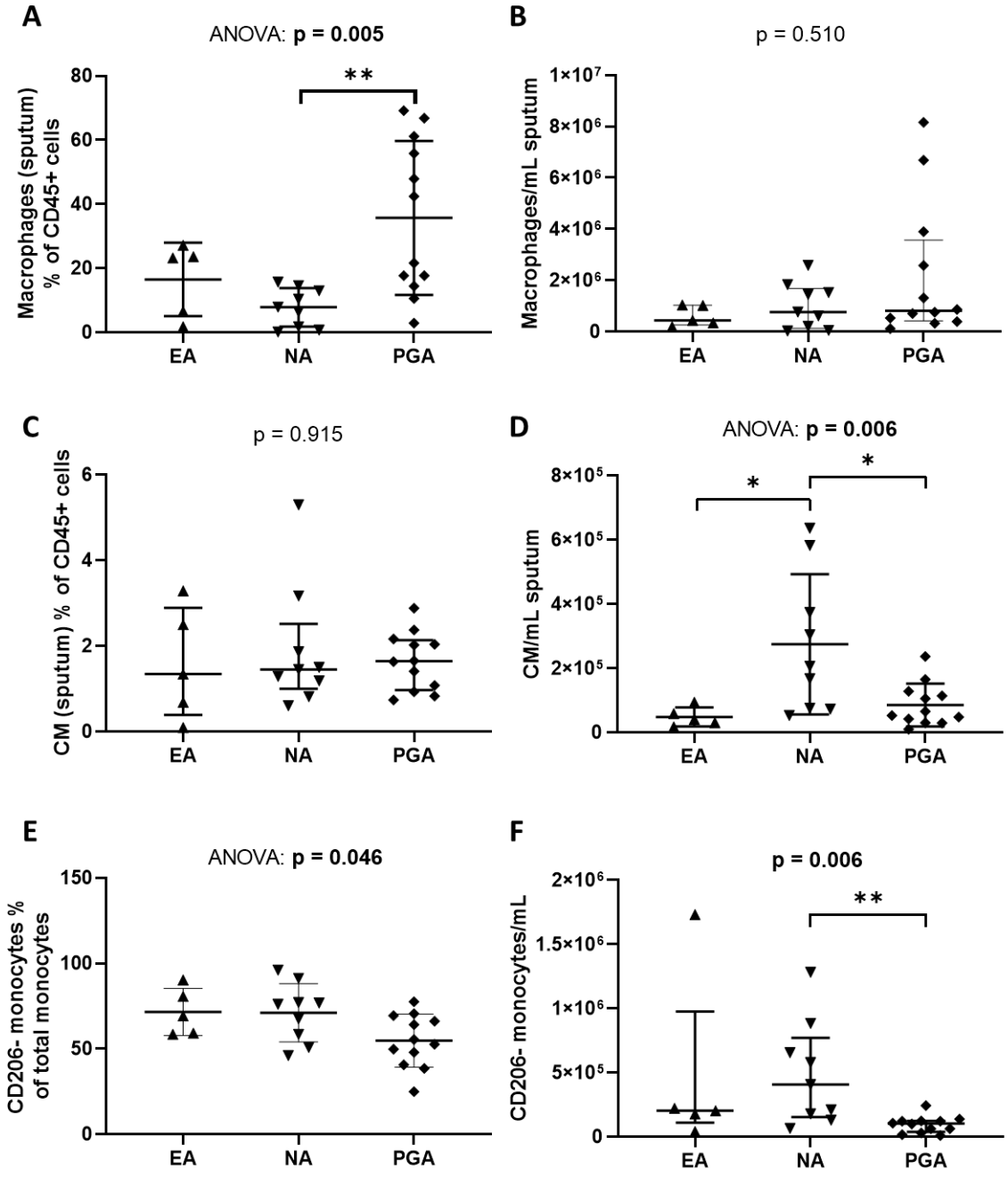

A
ANOVA: p = 0.005
**
Macrophages (sputum) % of CD45+ cells
80
60
40
20
0
EA
NA
PGA
B
p = 0.510
Macrophages/mL sputum
1×10⁷
8×10⁶
6×10⁶
4×10⁶
2×10⁶
0
EA
NA
PGA
C
p = 0.915
CM (sputum) % of CD45+ cells
6
4
2
0
EA
NA
PGA
D
ANOVA: p = 0.006
*
*
CM/mL sputum
8×10⁵
6×10⁵
4×10⁵
2×10⁵
0
EA
NA
PGA
E
ANOVA: p = 0.046
CD206- monocytes % of total monocytes
150
100
50
0
EA
NA
PGA
F
p = 0.006
**
CD206- monocytes/mL
2×10⁶
1.5×10⁶
1×10⁶
5×10⁵
0
EA
NA
PGA

Figure S8

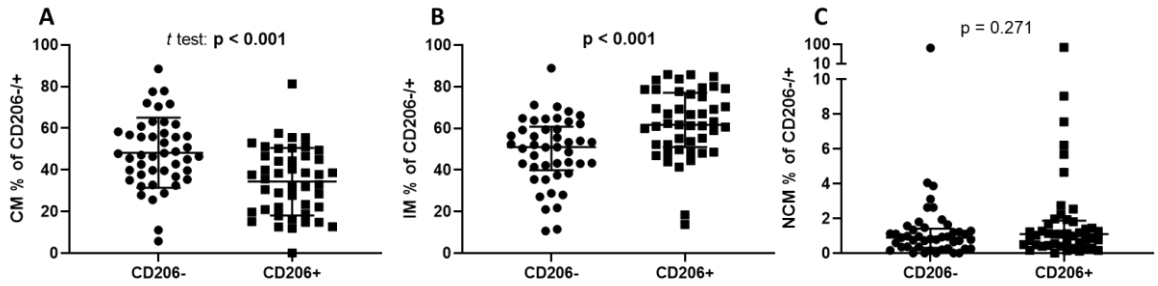

A
t test: p < 0.001
CM % of CD206-/+
100
80
60
40
20
0
CD206-
CD206+
B
p < 0.001
IM % of CD206-/+
100
80
60
40
20
0
CD206-
CD206+
C
p = 0.271
NCM % of CD206-/+
100
10
10
8
6
4
2
0
CD206-
CD206+

Figure S9

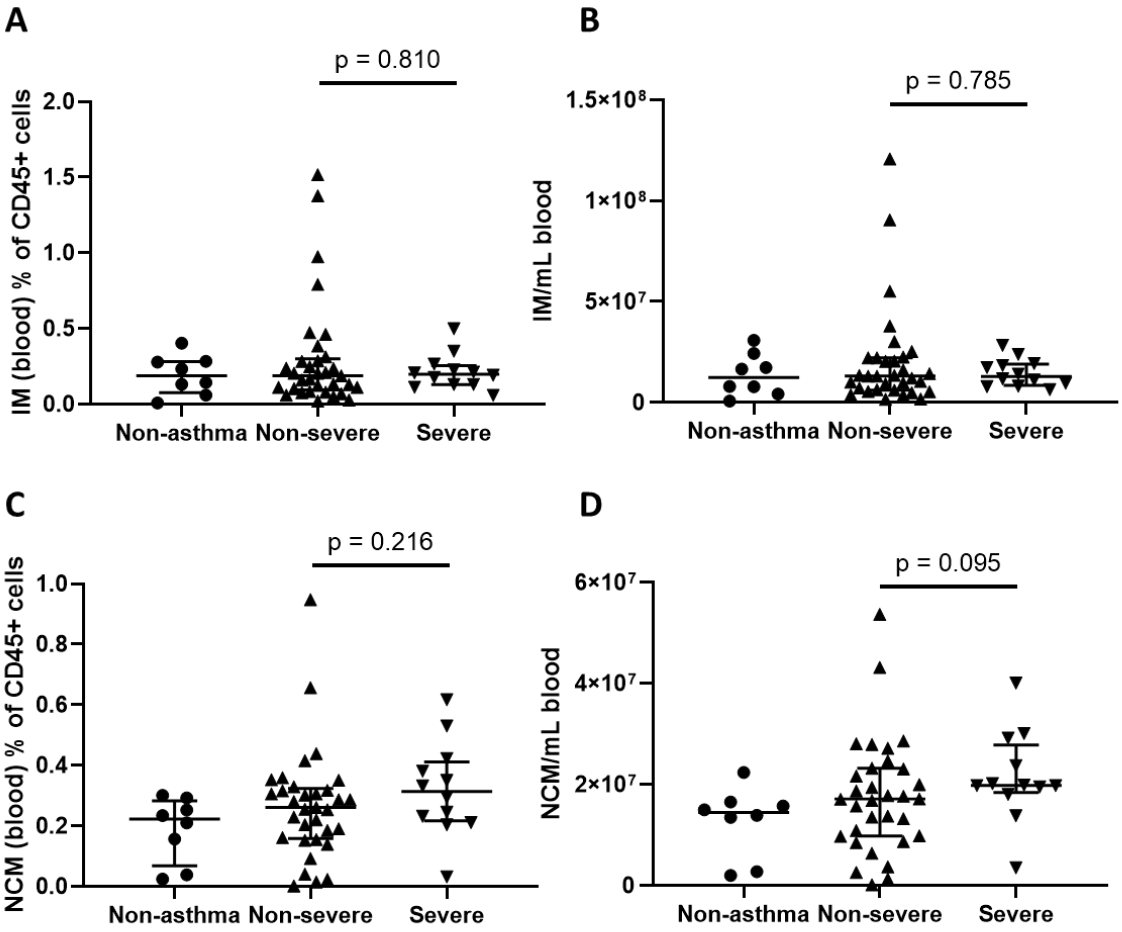

A
p = 0.810
IM (blood) % of CD45+ cells
2.0
1.5
1.0
0.5
0.0
Non-asthma
Non-severe
Severe
B
p = 0.785
IM/mL blood
1.5×10⁸
1×10⁸
5×10⁷
0
Non-asthma
Non-severe
Severe
C
p = 0.216
NCM (blood) % of CD45+ cells
1.0
0.8
0.6
0.4
0.2
0.0
Non-asthma
Non-severe
Severe
D
p = 0.095
NCM/mL blood
6×10⁷
4×10⁷
2×10⁷
0
Non-asthma
Non-severe
Severe

Figure S10

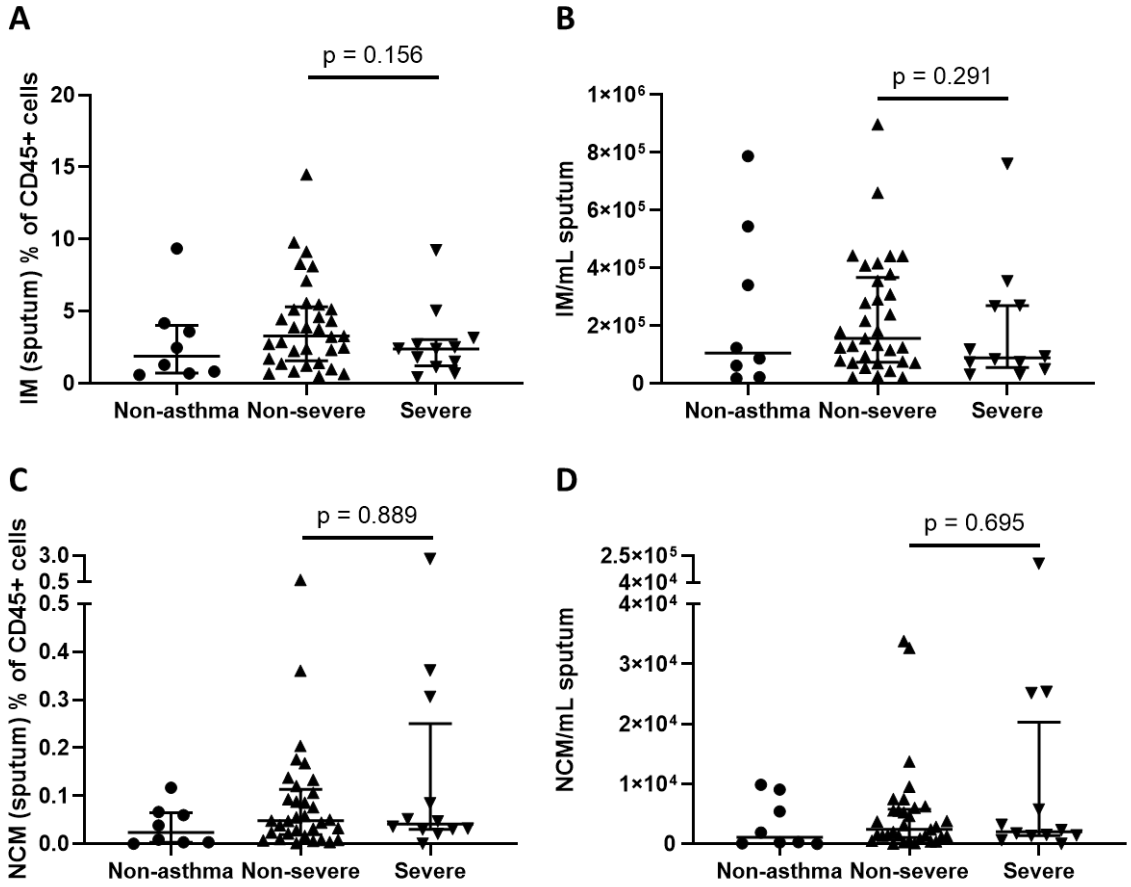
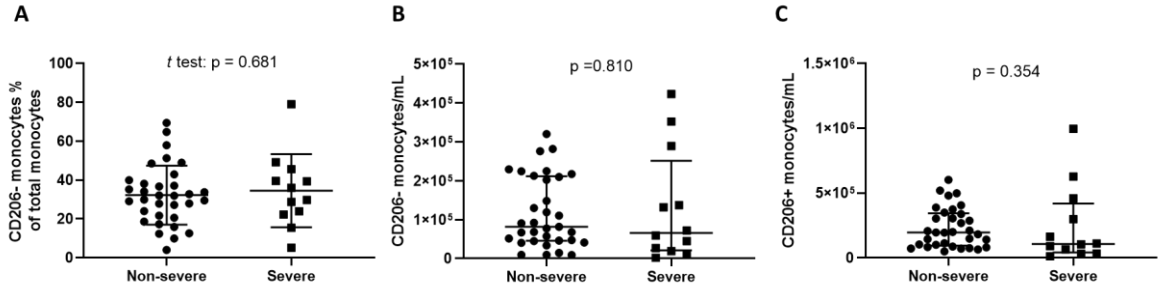

A
p = 0.156
IM (sputum) % of CD45+ cells
20
15
10
5
0
Non-asthma
Non-severe
Severe
B
p = 0.291
IM/mL sputum
1×10⁶
8×10⁵
6×10⁵
4×10⁵
2×10⁵
0
Non-asthma
Non-severe
Severe
C
p = 0.889
NCM (sputum) % of CD45+ cells
3.0
0.5
0.5
0.4
0.3
0.2
0.1
0.0
Non-asthma
Non-severe
Severe
D
p = 0.695
NCM/mL sputum
2.5×10⁵
4×10⁴
4×10⁴
3×10⁴
2×10⁴
1×10⁴
0
Non-asthma
Non-severe
Severe

Figure S11


A
t test: p = 0.681
CD206- monocytes % of total monocytes
100
80
60
40
20
0
Non-severe
Severe
B
p =0.810
CD206- monocytes/mL
5×10⁵
4×10⁵
3×10⁵
2×10⁵
1×10⁵
0
Non-severe
Severe
C
p = 0.354
CD206+ monocytes/mL
1.5×10⁶
1×10⁶
5×10⁵
0
Non-severe
Severe