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ORIGINAL ARTICLE

Asthma and Lower Airway Disease

An altered sputum macrophage transcriptome contributes to the neutrophilic asthma endotype

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Abstract

Background: Neutrophilic asthma (NA) is a clinically important asthma phenotype, the cellular and molecular basis of which is not completely understood. Airway macrophages are long-lived immune cells that exert important homeostatic and inflammatory functions which are dysregulated in asthma. Unique transcriptomic programmes reflect varied macrophage phenotypes in vitro. We aimed to determine whether airway macrophages are transcriptomically altered in NA.

Methods: We performed RNASeq analysis on flow cytometry-isolated sputum macrophages comparing NA (n = 7) and non-neutrophilic asthma (NNA, n = 13). qPCR validation of RNASeq results was performed (NA n = 13, NNA n = 23). Pathway analysis (*PANTHER*, *STRING*) of differentially expressed genes (DEGs) was performed. Gene set variation analysis (GSVA) was used to test for enrichment of NA macrophage transcriptomic signatures in whole sputum microarray (cohort 1 - controls n = 16, NA n = 29, NNA n = 37; cohort 2 U-BIOPRED - controls n = 16, NA n = 47, NNA n = 57).

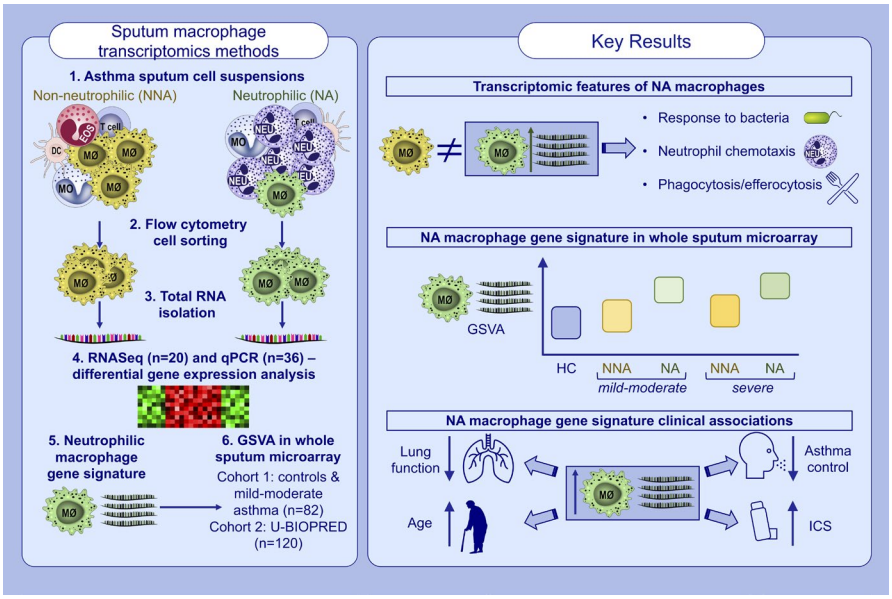
Abbreviations: AHR, Airways hyperresponsiveness; DC, dendritic cell; EOS, eosinophil; ES, Enrichment score; FEV_1, Forced expiratory volume in 1 second; GCSF, Granulocyte colony stimulating factor; GO, Gene ontology; GSVA, gene set variation analysis; HC, healthy control; ICS, inhaled corticosteroid; IFN, Interferon; IL, Interleukin; LPS, Lipopolysaccharide; MØ, macrophage; MO, monocyte; NA, neutrophilic asthma; NNA, non-neutrophilic asthma; NEU, neutrophil; QC, Quality control; qPCR, Quantitative polymerase chain reaction; RNASeq, RNA sequencing; TNF, Tumour necrosis factor.

Results: Flow cytometry-sorting significantly enriched sputum macrophages (99.4% post-sort, 44.9% pre-sort, $p < .05$). RNASeq analysis confirmed macrophage purity and identified DEGs in NA macrophages. Selected DEGs (*SLAMF7*, *DYSF*, *GPR183*, *CSF3*, *PI3*, *CCR7*, all $p < .05$ NA vs. NNA) were confirmed by qPCR. Pathway analysis of NA macrophage DEGs was consistent with responses to bacteria, contribution to neutrophil recruitment and increased expression of phagocytosis and efferocytosis factors. GSVA demonstrated neutrophilic macrophage gene signatures were significantly enriched in whole sputum microarray in NA vs. NNA and controls in both cohorts.

Conclusions: We demonstrate a pathophysiologically relevant sputum macrophage transcriptomic programme in NA. The finding that there is transcriptional activation of inflammatory programmes in cell types other than neutrophils supports the concept of NA as a specific endotype.

KEYWORDS

asthma, endotype, macrophage, neutrophil, transcriptome



GRAPHICAL ABSTRACT

RNASeq of isolated sputum macrophages reveals transcriptomic alterations in neutrophilic asthma (NA) consistent with responses to bacteria, promotion of neutrophil recruitment, and regulation of phagocytosis/efferocytosis. NA macrophage gene signature is enriched in NA whole sputum microarray datasets across asthma severities. NA macrophage gene signature correlates with increasing age, worse lung function, poorer asthma control, and increased ICS dose.

Abbreviations: U-BIOPRED, Unbiased Biomarkers for the Prediction of Respiratory Disease Outcomes; DC, dendritic cell; EOS, eosinophil; GSVA; gene set variation analysis; HC, healthy control; ICS, inhaled corticosteroid; MØ, macrophage; MO, monocyte; NA, neutrophilic asthma; NNA, non-neutrophilic asthma; NEU, neutrophil; qPCR, quantitative PCR; RNA, ribonucleic acid; RNASeq, RNA sequencing

1 | INTRODUCTION

Airway inflammation causes asthma pathophysiology and symptoms through promotion of airway remodelling, airways hyperresponsiveness and asthma flare-ups. Airway inflammation in asthma can be categorized into eosinophilic/non-eosinophilic or type 2 high/type-2 low based on the presence or absence of airway and systemic biomarkers.[1,2] Eosinophilic airway inflammation is more responsive to inhaled corticosteroid treatment in mild-moderate asthma and can be targeted with biologics blocking type 2 cytokine signalling in severe asthma to reduce flare-ups.[1,3] In contrast, effective targeted anti-inflammatory treatment for non-eosinophilic asthma is lacking.[2] Greater understanding of the cellular and molecular inflammatory pathways present in non-eosinophilic asthma are required to identify new therapeutic targets.

Non-eosinophilic asthma can be sub-categorized by presence or absence of elevated sputum neutrophils. Neutrophilic asthma (NA) is associated with corticosteroid resistance, asthma flare-ups, airflow

limitation and airway dysbiosis.[4–6] The cellular and molecular pathways that promote airway neutrophilia and clinical features of NA are incompletely understood. Transcriptomic profiling of whole sputum samples demonstrated alterations in NA[7] or a transcriptomic cluster with elevated neutrophils,[8] including enrichment of gene expression relating to IL-1β, TNF and IFNγ signalling. It is unclear if sputum transcriptomic alterations in NA reflect contribution of altered proportions of distinct immune cell types to the total RNA pool, and/or reflect a genuine transcriptional activation of inflammatory programmes in specific cell types. Better understanding of cell-specific dysregulation of inflammatory pathways and their contribution to airway inflammation in asthma may inform development of new therapeutics.

Macrophages are a key immune cell of the airway lumen that mount functionally diverse responses and exert important homeostatic and regulatory functions.[9] Macrophage functions are dysregulated in asthma and may relate to NA.[10] In vitro studies have demonstrated that transcriptomic alterations underpin functional differences in macrophages exposed to varying type 1 (yielding M1 macrophages) and type 2 (yielding M2 macrophages) inflammatory stimuli.[10,11] Sputum macrophages constitute a major lumenal immune cell population across asthma airway inflammatory phenotypes. Their longevity as well as phenotypic and functional plasticity prompt the concept that they may play context-specific roles in promoting/regulating asthma airway inflammatory phenotypes. We hypothesized that sputum macrophages would display transcriptomic alterations in NA and that these alterations would also be reflected in previously described transcriptomic data derived from whole sputum samples. To address this, we developed a flow cytometry-based method to isolate highly pure preparations of sputum macrophages and performed an exploratory transcriptomic analysis of sputum macrophages using a bulk RNAseq approach to compare macrophage transcriptomes in NA vs non-neutrophilic asthma (NNA).

2 | METHODS

2.1 | Study population

Clinical studies were approved by the Hunter New England Health Human Research Ethics Committee (16/04/20/3.0; 17/02/15/3.04; 15/03/18/3.04; 17/04/12/4.03). All participants provided written informed consent. Participants were aged 18 or over and had a doctor diagnosis of asthma with documented evidence of variable airflow limitation in the past 10 years in the form of: AHR (PD15 <15 ml to hypertonic saline OR mannitol); bronchodilator response (change post-bronchodilator FEV1 > 12% OR 200 ml) or; FEV1 variability >12% (two values measured within 2 months of each other) or; Peak Flow variability >12% over at least 1 week of monitoring. At time of visit, participants had stable asthma (no respiratory infection, asthma exacerbation or change in asthma maintenance therapy in the past month). Participants were never smokers or had no recent history of smoking (prior 6 months), were not pregnant or breastfeeding, and had no current lung cancer or other blood, lymphatic or solid organ malignancy. For U-BIOPRED cohort-based microarray analysis, severe asthma was defined as previously described.[12] For all other analyses, severe asthma was defined according to ERS/ATS guidelines.[13]

2.2 | Spirometry and sputum induction

Spirometry and sputum induction with hypertonic saline (4.5%) was performed and sputum cell suspensions and cytospins prepared as previously described (see online supplement).[14] Samples were categorized into inflammatory subtypes based on sputum differential cell count using a threshold ≥ 61% neutrophils for NA vs. <61% NNA.[14] Additional cell suspension was immediately processed for flow cytometry-mediated isolation of sputum macrophages.

2.3 | Flow cytometry-mediated sputum macrophage isolation

General sputum flow cytometry methods including fluorescence minus one controls have been described,[15] for details see online supplement. Sputum macrophages were identified as $CD45^+$, $HLA\text{-}DR^+$, $CD14^{intermediate}$, CD16 high, $CD206^+$, side scatter (SSC) high events (Figure 1), consistent with prior reports.[16,17] Sputum cell suspensions were run on a BD FACSAria III cell sorter, using a 70 micron filter and macrophages sorted directly into PBS or TRIzol LS (*ThermoFisher*) chilled at 4°C for preparation of cytospins or RNA preparation respectively. Cytospins of sorted sputum macrophages were stained with May-Grünwald and Giemsa and conventional differential cell count performed on 400 cells.

2.4 | RNA isolation, cDNA synthesis, qPCR

RNA was isolated from macrophages lysed in TRIzol LS (see online supplement). 200–500 pg of sputum macrophage RNA was reverse transcribed to produce cDNA using a SuperScript™ IV VILO™ Master Mix (*ThermoFisher*) following manufacturer's instructions. qPCR was run using standard TaqMan reagents and probesets (*ThermoFisher*) as previously described.[7] Probesets used were: *SLAMF7* – Hs00904275_m1; *DYSF* – Hs01002513_m1; *GPR183* – Hs00953886_m1; *MERTK* – Hs01031979_m1; *CSF3* – Hs00738432_g1; *CCR7* - Hs01013469_m1; *PI3* - Hs00160066_m1. Relative mRNA abundance of target to the housekeeper *ACTB* was calculated ($2^{-\Delta Ct}$).

2.5 | RNASeq library preparation and sequencing

Library preparation, library QC and sequencing service was provided by Ramaciotti Centre for Genomics (University of New South Wales, Sydney, Australia). cDNA library synthesis was conducted using a

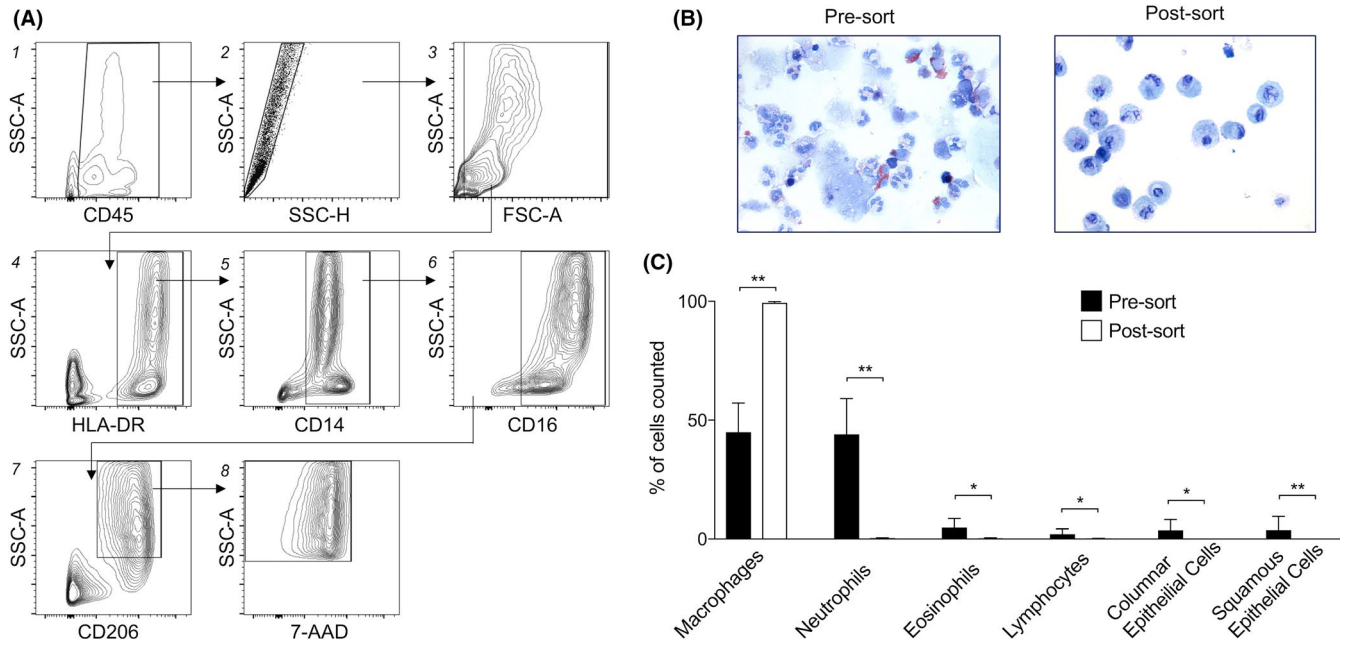


FIGURE 1 Flow cytometry-mediated sorting and isolation of highly pure sputum macrophage preparations. (A) Flow cytometry-sorting strategy for isolation of sputum macrophages. Macrophages were gated as CD45+, HLA-DR+, CD14+, CD16+, CD206+ with a high side scatter (SSC) profile. (B) Representative May Grünwald Giemsa-stained cytospin images of sputum cell suspensions pre flow cytometry sort (*left hand panel*), and post flow cytometry sort for macrophages (*right hand panel*). (C) Differential cell count of cytospins of pre- and post- flow cytometry sort sputum samples (n = 5). Mann-Whitney test. * = $p < .05$, ** = $p < .01$

SMARTer® Stranded Total RNA-Seq kit v2 (Takara Bio), following the manufacturer's instructions. This library synthesis kit was selected due to its compatibility with picogram inputs of total RNA of variable quality. Between 500–1000 pg total RNA was used as input for cDNA library preparation. All samples processed successfully yielded libraries based on confirmation of expected DNA concentration and lack of dimer peaks. Libraries were sequenced on the NovaSeq 6000 platform (single NovaSeq S1 2 × 100bp lane), generating an average of 45.5 M paired end reads per sample (lowest was 36.3 M).

2.6 | Bioinformatics processing of RNASeq data and differential gene expression analysis

Paired read RNASeq data analysis consisted of paired adapter and quality trimming (TrimGalore 0.6.3, cutadapt 1.18), followed by sequence alignment (HISAT2 2.0.2[18]) and count (subread 1.6.4[19]) against the Homo sapiens GRCH38 v97 transcriptomic reference plus decoys. Quality control was performed at every step with FastQC and Multiqc,[20] mapping efficiency was above 90% and 72% of paired end reads had unique mappings on average. An average of 25% of unique reads were assigned to transcripts. Differential expression analysis was performed in R with the DESeq2 package[21] and included logarithmic fold change and significance p value estimation (s value) with the approximate posterior estimation for generalized linear model (apeglm) package,[22] which outperforms the normal shrinkage estimators. A detailed report on bioinformatics analysis pipelines is provided in the supplemental data (Figure S1).

2.7 | Gene ontological analysis

Gene ontological (GO) analysis was performed using a PANTHER Overrepresentation Test (www.pantherdb.org) using the GO Biological Process annotation data set and fisher's exact test with correction for false discovery rate. STRING functional protein association network tool (https://string-db.org/, version 11) was used to investigate protein-protein interactions of DEGs. A minimum required interaction score of high confidence (>0.7) was applied and only the 1st shell query proteins were included in the analysis for the maximum number of interactions. Network edges were marked as confidence where line thickness indicates strength of data support. Disconnected nodes and interactions between only two proteins were removed from the analysis.

2.8 | Gene set variation analysis

Gene set variation analysis (GSVA) was used to identify differences in expression of predefined NA macrophage gene sets in two previously published whole sputum microarray datasets comparing non-asthma controls, NA and NNA: the first dataset primarily included participants with mild-moderate asthma,[7,23,24] the second being the U-BIOPRED sputum microarray sub-cohort which primarily included participants with severe asthma.[8,25] An enrichment score (ES) ranging from −1 to +1 was calculated for the expression of each gene set for each sample across the whole sample population.

2.9 | Statistics

Mann-Whitney test was used for two group comparisons and Kruskal-Wallis with Dunn's multiple comparisons for multiple group comparisons of non-parametric data. p values <.05 were considered statistically significant.

3 | RESULTS

3.1 | Flow cytometry isolation of sputum macrophages

Induced sputum cell suspensions were rapidly processed for flow cytometry-mediated sorting of sputum macrophages. Sputum macrophages were identified as a distinct population on the basis of CD45+, HLA-DR+, CD14+, CD16+, CD206+ with a high side scatter profile (Figure 1A). Other immune cells, including monocytes, visible in subpanel 7 of Figure 1A as a population with low SSC and low CD206 signal, were excluded through gating. Differential cell count of unsorted sputum cell suspensions vs flow cytometry-sorted macrophages (Figure 1B) confirmed the enrichment to a high level of purity of macrophages (mean 44.9% macrophages pre-sort, 99.4% macrophages post-sort, $p = .008$ Figure 1C).

3.2 | Bulk transcriptomic analysis of isolated sputum macrophages by RNASeq

RNA sequencing was performed on 20 macrophage samples from participants with asthma (Table 1). Of the 20 samples, seven were NA and 13 were of NNA airway inflammatory phenotype. Clinical features did not differ between NA and NNA participants, and sputum neutrophil and macrophage proportions differed as expected. Across all 20 samples high expression of macrophage signature genes and low to undetectable expression of signature genes representing lung neutrophils, monocytes, dendritic cells, mast cells, T- and NK cells and B cells[26] was observed (Figure S2), consistent with flow cytometry isolation of sputum macrophages to a high degree of purity. PANTHER gene ontology (GO) analysis of the 200 most highly expressed RNA species across all macrophage samples revealed statistical overrepresentation of transcripts relating to cellular iron homeostasis, T cell mediated immunity, positive regulation of the adaptive immune response, wound healing, granulocyte chemotaxis, leukocyte chemotaxis, response to LPS, protein folding and actin filament organization, consistent with known macrophage roles in maintaining airway homeostasis and orchestration of immune responses (Table S1).

3.3 | Differential gene expression analysis reveals transcriptomic alteration of sputum macrophages in neutrophilic asthma

We conducted an exploratory analysis to address our hypothesis that sputum macrophages would display inflammatory specific transcriptomic changes in NA. Analysis of NA ($n = 7$) vs. NNA ($n = 13$) samples returned 44 RNA species with s values (equivalent of p value) below 0.05, the majority of which were increased in NA vs. NNA (Table S2). Thus, NA was associated with a specific transcriptomic alteration of sputum macrophages.

3.4 | qPCR validation of NA macrophage DEGs

We ranked genes in terms of level of differential expression between NA and NNA using the ratio of s value to (absolute) log fold change, with smaller values representing greater differential expression, generating a list of the 200 most differentially expressed genes, which included the 44 RNA species with s values below 0.05 (Table S2). To verify this list as truly differentially expressed between NA and NNA, we performed qPCR in a larger sample set of 36 flow cytometry-isolated sputum macrophage samples, 14 of which were included in the RNASeq cohort (NA $n = 13$, NNA $n = 23$, Table S3). For validation, we selected genes from the top 200 DEG list with known immune functions and with a range of average expression in the RNASeq dataset (Table S4: *MERTK*, *SLAMF7*, *DYSF*, *GPR183*, *CSF3*, *PI3*, *CCR7*). Expression was normalized to the housekeeper gene *ACTB* (β-actin), which was highly expressed across all samples and was not differentially expressed between NA vs NNA in the RNASeq dataset (log2 fold change −0.008, s value 0.98). All genes tested by qPCR were significantly increased in NA vs NNA in accordance with the RNASeq data, except *MERTK* which showed a similar trend (Figure 2A-G). Thus, qPCR confirmed increased expression of DEGs identified by RNASeq as increased in NA vs NNA sputum macrophages.

3.5 | Pathway analysis of NA macrophage DEGs

We next performed GO and protein network analyses to characterize the altered transcriptomic landscape of macrophages in NA vs. NNA. GO analysis using PANTHER of the top 200 DEGS from NA vs. NNA macrophages revealed overrepresentation of genes involved in positive regulation of Janus kinase activity, regulation of granulocyte chemotaxis, regulation of neutrophil migration, positive regulation of leukocyte chemotaxis, regulation of the inflammatory response, response to bacterium, lymphocyte activation and defence response (Table 2). The STRING protein interaction network tool identified 163 protein-coding transcripts amongst the top 200 DEGs, and three networks containing three or more members, including a major node containing 38 genes (Figure 3A-C), with a significant PPI enrichment value ($p < 1 \times 10^{13}$), indicating that the proteins identified were at least partially biologically connected as a group. STRING also identified statistical functional enrichment in numerous GO biological process categories, many of which were common with PANTHER GO analysis (Table S5).

3.6 | Enrichment of the macrophage-derived NA signature in whole sputum samples

We performed GSVA analysis to test for enrichment of NA macrophage gene signatures in a whole sputum microarray dataset, which primarily featured

TABLE 1 Clinical characteristics and sputum differential cell count of sputum macrophage RNASeq cohort

	All ($n = 20$)	NNA ($n = 13$)	NA ($n = 7$)
Female[†]	14 (70)	9 (69)	5 (71)
Age[‡]	59.1 (13.1)	58.3 (13.1)	60.5 (13.9)
BMI[#]	28.8 (24.7, 39.2)	32.6 (26.7, 39.5)	26 (23.8, 28.9)
Pre-β2 FEV1 (% pred)[‡]	69.5 (14.7)	69.1 (14.5)	70.2 (16.1)
Pre-β2 FEV1/FVC[‡]	64.2 (11.2)	65.6 (9.6)	61.5 (14.1)
Ex-smoker[†]	5 (25)	4 (30.8)	1 (14.3)
ACQ6[§]	0.8 (0.5, 1.7)	0.7 (0.5, 2.1)	1 (0.2, 1.2)
ICS treated[†]	18 (90)	12 (92)	6 (86)
LABA treated[†]	17 (85)	11 (85)	6 (86)
ICS dose (fluticasone equivalent)[§]	500 (500, 937.5)	500 (375, 1000)	500 (500, 500)
OCS treated[†]	0 (0)	-	-
LAMA treated[†]	7 (35)	4 (31)	3 (43)
Severe asthma[†]	4 (20)	2 (15)	2 (29)
Sputum TCC ($\times 10^6$/ml)[§]	6.4 (4.1, 13.2)	4.7 (2.8, 8,5)	15 (7.1, 23.9)*
Sputum macrophage (%)[§]	44.1 (26.1, 63)	58.1 (44.1, 70.6)	18.3 (10.8, 28)***
Sputum neutrophil (%)[§]	45.9 (29.6, 67.8)	37.5 (20.4, 45.9)	74.7 (64, 83.8)***
Sputum eosinophil (%)[§]	1.4 (0.6, 3.6)	2 (0.7, 4.0)	1 (0.3, 2)
Sputum lymphocyte (%)[§]	1.5 (0.8, 2.4)	2 (1.3, 2.5)	0.5 (0, 1.8)*
Sputum macrophage ($\times 10^4$/ml)[§]	229 (172, 425)	279 (186, 411)	228 (76, 456)
Sputum neutrophil ($\times 10^4$/ml)[§]	267 (137, 564)	148 (95, 358)	1323 (559, 1526)***
Sputum eosinophil ($\times 10^4$/ml)	9 (3, 23)	9 (4, 18)	17 (2, 31)
Sputum lymphocyte ($\times 10^4$/ml)	9 (2, 17)	10 (5, 18)	2 (0, 17)

Abbreviations: BMI, body mass index; FEV1, forced expiratory volume in 1 second; FVC, forced vital capacity; ACQ, asthma control questionnaire; ICS, inhaled corticosteroid; TCC, total cell count.
[†]Number (%),[‡]Mean (SD), [§]Median (IQR). $^*p < 0.05$ NA vs NNA. $^{***}p < 0.001$ NA vs NNA.

participants with mild-moderate asthma (non-asthma controls $n = 16$, NA $n = 29$, NNA $n = 37$, Table S6).[7,23] Of the 200 most differentially expressed RNA species between NA and NNA macrophages, 150 were also present in the microarray dataset (gene set 1, Table S7). Gene set 1 was significantly enriched in NA vs NNA and non-asthma controls (Figure 4A). A significantly increased enrichment score was also observed in NA vs NNA when gene set 1 was restricted to genes which had s values below 0.05 (Figure 4B, gene set 2, Table S7). These results were then validated through analysis of whole sputum microarray data in a second independent cohort, the U-BIOPRED cohort, primarily composed of participants with severe asthma.[8,25] Both gene set 1 and 2 produced increased enrichment scores in NA vs NNA and non-asthma controls (Figure 4C-D). When the U-BIOPRED microarray cohort was further categorized into those with mild-moderate and those with severe asthma, we observed increased enrichment scores in NA irrespective of asthma severity, and no difference between mild-moderate and severe asthma enrichment scores in NA or NNA (Figure 4E-F).

3.7 | Clinical correlations of macrophage-derived NA signature

Enrichment scores for gene set 1 (Figure 4A) were tested for correlation with clinical characteristics in participants with asthma ($n = 66$). Increased expression of gene set 1 was significantly associated with increased age, worse lung function, poorer asthma control and increased ICS dose (Table 3).

4 | DISCUSSION

Macrophages are important, functionally diverse, innate immune cells of the airways whose dysregulation has been implicated in asthma. Altered transcriptomes are considered to underpin macrophage functional plasticity in response to varying extracellular queues; however, it is unclear from transcriptomic studies of airway samples including sputum whether macrophage transcriptomic identity is altered in asthma. Here we performed bulk RNASeq analysis of flow cytometry-isolated sputum macrophages and demonstrate an altered macrophage transcriptome in NA. Transcriptomic alterations in NA macrophages identified by RNASeq were confirmed by qPCR in a larger sample set. Bioinformatic analysis suggested NA macrophage transcriptomic alteration may reflect response to an altered extracellular environment including bacteria, potential contribution to neutrophil recruitment and clearance, and an immunomodulatory role. Finally, we demonstrate that NA macrophage gene signatures are enriched in whole sputum microarray from NA

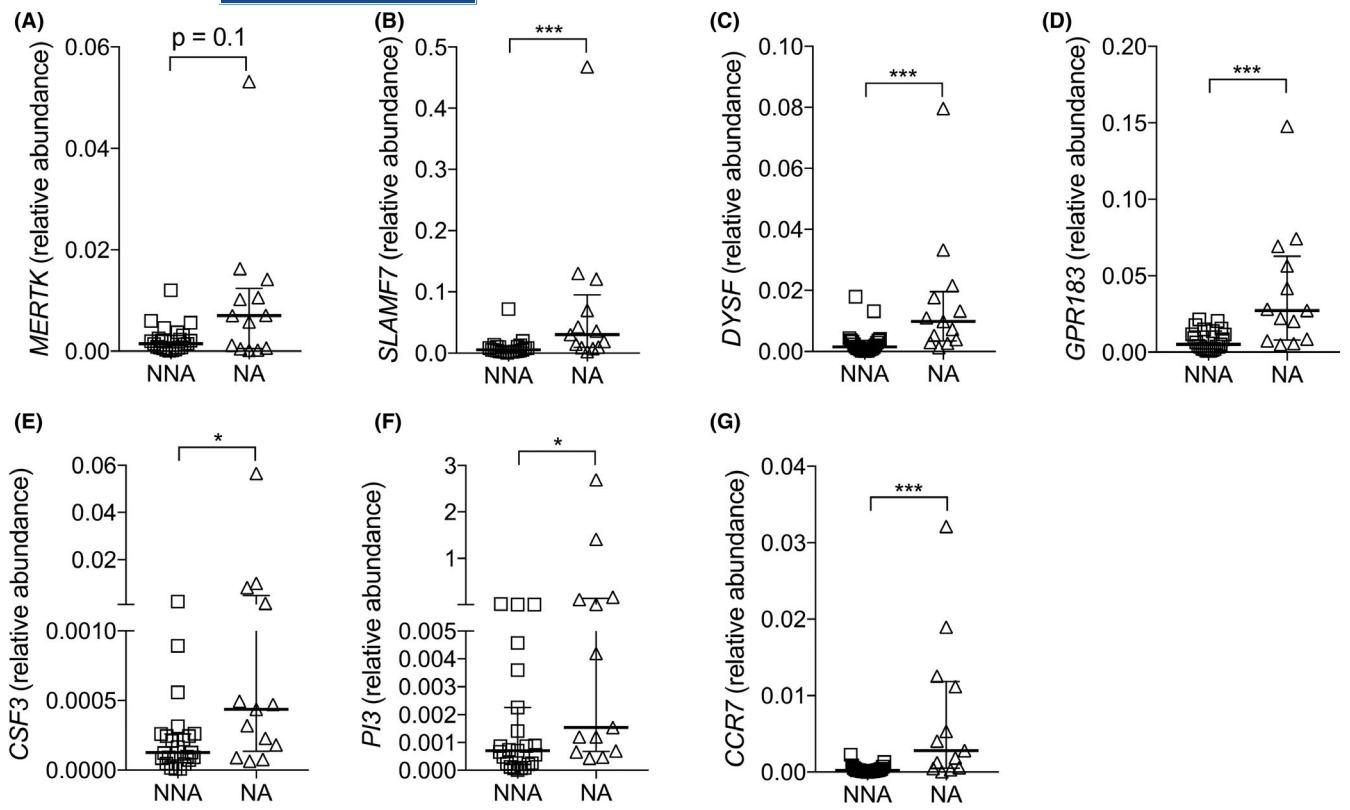


FIGURE 2 qPCR validation of neutrophilic asthma sputum macrophage DEGs. RNA was isolated from flow cytometry-sorted sputum macrophages and cDNA prepared (n = 36). qPCR was run measuring relative abundance of *MERTK* (A), *SLAMF7* (B), *DYSF* (C), *GPR183* (D), *CSF3* (E), *PI3* (F) and *CCR7* (G) relative to the housekeeper gene *ACTB*. Relative mRNA abundance of target mRNA to the housekeeper *ACTB* was calculated ($2^{-\Delta Ct}$) and compared between NA ($n = 13$) and NNA ($n = 23$), Mann-Whitney test. * = $p < .05$, *** = $p < .001$. Data is plotted as median ± interquartile range

in both mild-moderate and severe asthma and correlate with important clinical characteristics including worse lung function and poorer asthma control, demonstrating a clinically-relevant macrophage-specific element of the previously described whole sputum NA transcriptome.

Bioinformatic analyses of DEGs in sputum macrophages isolated from NA identified enrichment of genes related to processes with pathophysiological relevance in NA. Among these were GO categories relating to regulation of inflammatory responses and responses to bacteria. Airway dysbiosis is evident in NA,[6] and our data suggest macrophage transcriptomes and consequently function are altered in response to bacterial queues. Many of the DEGs identified in our analysis are inducible by the bacterial ligand LPS in macrophages or monocytes. These included genes that promote LPS inflammatory responses (eg signalling adaptor *TRAF1*,[27] transcription factor *POU2F2*/Oct2[28]) but also genes that suppress LPS responses through mechanisms including LPS binding (*CRISPLD2*[29]), suppression of LPS-induced pro-inflammatory gene transcription (*IL10*[30]) and modulation of intracellular signalling (*MARCKS*[31]). This suggests sputum macrophages initiate and regulate inflammatory responses to the altered airway bacterial environment in NA. Also consistent with an immune modulatory/homeostatic role in NA was upregulation of several genes involved in efferocytosis (*MERTK*, *STAB2*, *SIGLEC10*, *SLAMF* members)[32,33] and phagocytosis (*FAIM3*, *HRH2*, *STAB2*, *MARCKS*),[34-36] increasing cellular components that facilitate macrophage uptake of host cells and microbes. This is consistent with an M2-like phenotype where increased uptake of dead/dying cells and bacteria promotes inflammatory resolution. Defective efferocytosis by airway and monocyte-derived macrophages has been demonstrated in non-eosinophilic asthma, suggesting any potential defect in efferocytosis by NA macrophages may be post-transcriptional.[37,38] *CSF3* and *CSF3R*, encoding granulocyte-colony stimulating factor (GCSF) and its receptor, were increased in NA macrophages. CSF3R is expressed on mature neutrophils but also monocytes, and GCSF, a *POU2F2*/Oct2-inducible gene,[28] can induce altered differentiation of monocytes towards an immunosuppressive M2-like macrophage phenotype.[39] Aberrant GCSF signalling has recently been linked to NA[40] and severe asthma.[41] *CSF3* and *CSF3R* clustered with several other notable DEGs using the STRING tool. These included receptors for IL1β (*IL1R*) and IL6 (*IL6R*), two cytokines postulated to play important roles in neutrophilic and severe asthma,[42,43] as well as the IL23 subunit *IL23A*, which has links to airway obstruction in IL17-mediated neutrophilic airways disease.[44] Thus, macrophages display numerous transcriptomic alterations consistent with both inflammatory promotion and resolution in NA, which may reflect functional plasticity and diversity amongst the population studied.

TABLE 2 PANTHER gene ontology analysis of top 200 DEGs from NA vs NNA macrophages

PANTHER GO biological process	Fold Enrichment	raw *p* value	FDR
Positive regulation of activation of Janus kinase activity	62.48	5.55E−05	2.00E−02
Positive regulation of receptor signalling pathway via JAK-STAT	8.06	1.52E−04	4.13E−02
Regulation of response to stimulus	1.59	5.39E−05	2.00E−02
Positive regulation of receptor signalling pathway via STAT	7.81	1.79E−04	4.46E−02
Positive regulation of protein phosphorylation	2.40	1.52E−04	4.20E−02
Positive regulation of phosphorylation	2.42	6.59E−05	2.22E−02
Positive regulation of phosphate metabolic process	2.28	1.29E−04	3.84E−02
Positive regulation of phosphorus metabolic process	2.28	1.29E−04	3.91E−02
Regulation of activation of Janus kinase activity	41.65	1.31E−04	3.75E−02
Regulation of granulocyte chemotaxis	13.15	1.24E−05	9.44E−03
Regulation of leukocyte chemotaxis	7.09	2.85E−05	1.49E−02
Regulation of response to external stimulus	2.38	5.26E−05	1.99E−02
Regulation of locomotion	2.79	2.89E−06	5.49E−03
Regulation of leukocyte migration	5.11	4.12E−05	1.84E−02
Regulation of immune system process	2.11	4.75E−05	1.95E−02
Regulation of cell migration	2.74	1.52E−05	1.05E−02
Regulation of cell motility	2.80	4.37E−06	4.73E−03
Regulation of cellular component movement	2.66	6.53E−06	5.83E−03
Regulation of localization	1.91	4.33E−06	5.05E−03
Regulation of neutrophil migration	12.62	8.12E−05	2.62E−02
Positive regulation of leukocyte chemotaxis	8.33	3.51E−05	1.67E−02
Positive regulation of leukocyte migration	6.17	7.16E−05	2.36E−02
Leukocyte chemotaxis	6.82	1.20E−05	9.60E−03
Cell chemotaxis	4.78	1.63E−04	4.27E−02
Chemotaxis	3.31	4.00E−05	1.84E−02
Response to stimulus	1.38	3.50E−05	1.72E−02
Taxis	3.29	4.23E−05	1.84E−02
Locomotion	2.56	3.92E−06	4.96E−03
Response to external stimulus	2.19	1.39E−07	7.03E−04
Cell migration	2.85	8.40E−06	7.09E−03
Cell motility	2.61	2.17E−05	1.22E−02
Movement of cell or subcellular component	2.38	3.78E−06	5.74E−03
Localization of cell	2.61	2.17E−05	1.27E−02
Localization	1.48	4.33E−05	1.83E−02
Leukocyte migration	4.61	3.68E−06	6.20E−03
Immune system process	1.79	1.18E−04	3.64E−02
Regulation of inflammatory response	4.04	8.86E−07	3.36E−03
Regulation of defence response	2.58	1.30E−04	3.79E−02
Response to bacterium	3.46	3.88E−06	5.36E−03
Response to other organism	2.50	6.05E−06	6.13E−03
Response to external biotic stimulus	2.50	6.05E−06	5.74E−03
Response to biotic stimulus	2.43	1.33E−05	9.65E−03
Lymphocyte activation	3.34	1.95E−04	4.78E−02
Cell activation	2.37	5.66E−05	2.00E−02
Regulation of system process	3.06	1.59E−04	4.24E−02

(Continues)

TABLE 2 (Continued)

PANTHER GO biological process	Fold Enrichment	raw *p* value	FDR
Regulation of multicellular organismal process	1.68	1.72E−04	4.35E−02
Cation transport	2.72	6.23E−05	2.15E−02
Cell adhesion	2.69	4.84E−05	1.93E−02
Biological adhesion	2.67	5.16E−05	2.01E−02
Defence response	2.57	2.37E−06	5.15E−03
Immune response	2.18	1.59E−05	1.05E−02
Regulation of biological quality	1.58	1.64E−04	4.22E−02
Multicellular organismal process	1.43	1.47E−04	4.12E−02
RNA processing	< 0.01	8.28E−05	2.62E−02
RNA metabolic process	.17	2.28E−05	1.23E−02
Nucleic acid metabolic process	.20	1.44E−06	3.64E−03
Nucleobase-containing compound metabolic process	.20	4.27E−08	6.48E−04
Cellular nitrogen compound metabolic process	.28	7.95E−08	6.04E−04
Organic cyclic compound metabolic process	.38	1.84E−05	1.17E−02
Heterocycle metabolic process	.29	1.28E−06	3.88E−03
Cellular aromatic compound metabolic process	.38	2.87E−05	1.45E−02
Gene expression	.23	2.11E−05	1.28E−02

Abbreviations: FDR, False Discovery Rate (Benjamini-Hochberg); GO, gene ontology.

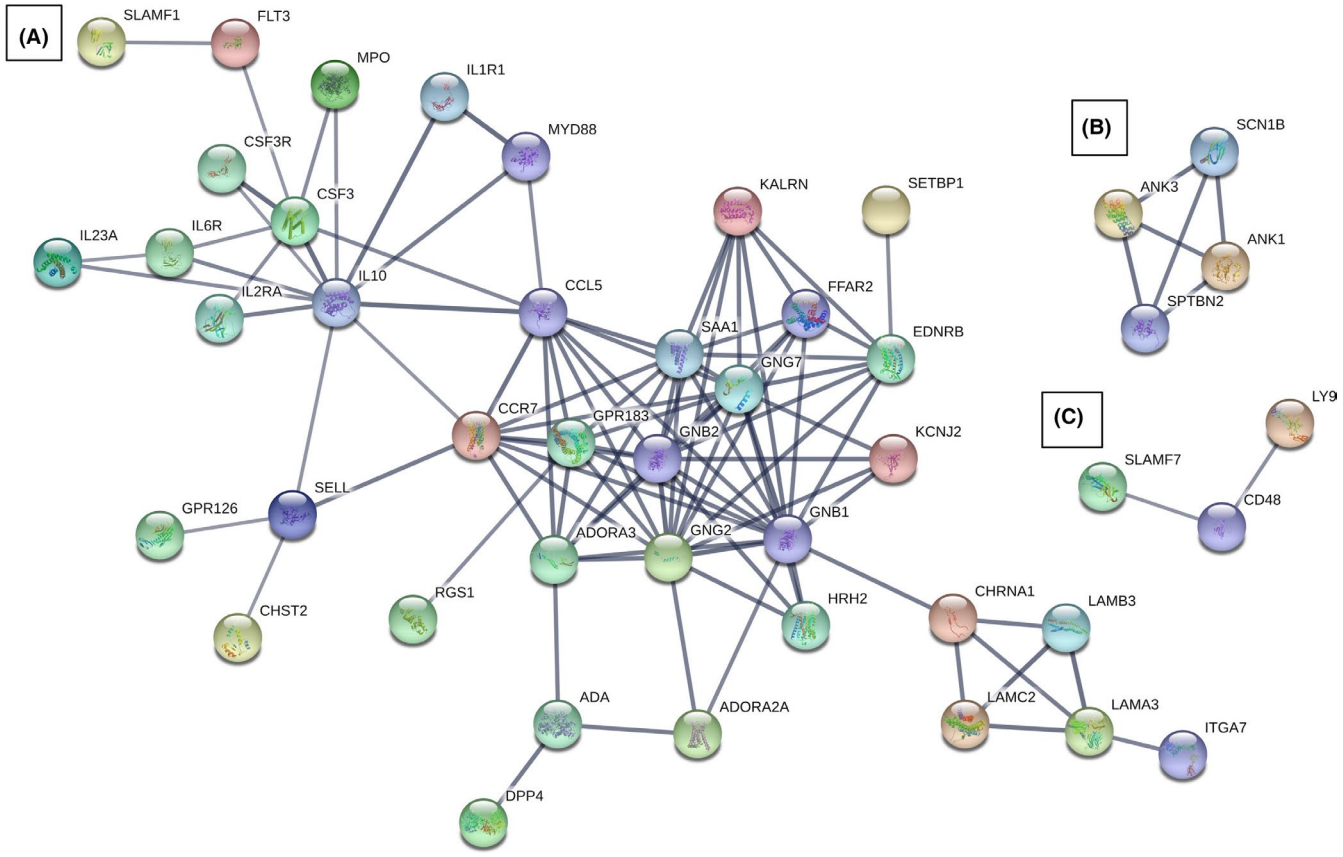


FIGURE 3 STRING database protein interaction network of top 200 DEGs identified in NA vs NNA sputum macrophages. Of the top 200 DEGs, STRING identified 167 encoded protein-coding genes. Modules with 2 or more interacting genes displayed (labelled A-C)

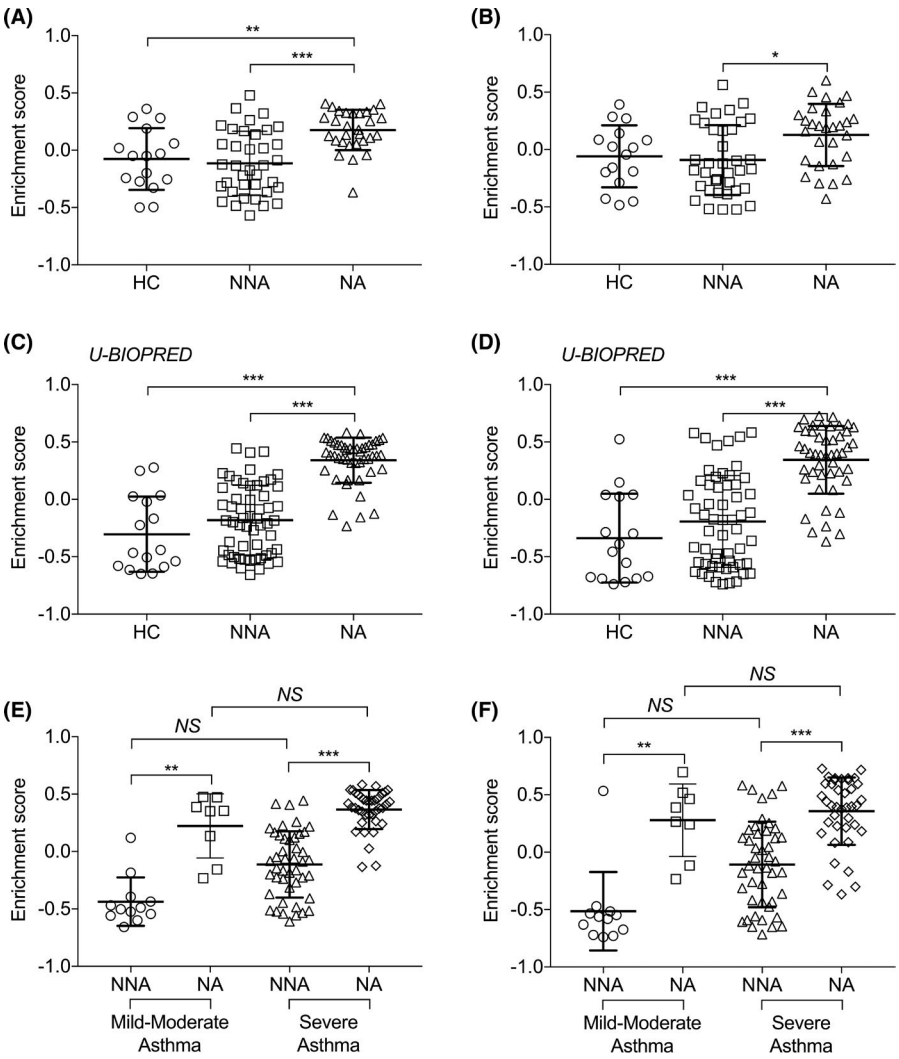


FIGURE 4 Gene set variation analysis demonstrates enrichment of NA sputum macrophage gene signatures in whole sputum microarray. Gene sets tested using GSVA were the 150 genes present in both the top 200 DEGs of NA vs NNA macrophages and the whole sputum microarray dataset (gene set 1, A), and those genes within gene set 1 that had an s value below 0.05 in NA vs NNA RNASeq differential expression comparison (gene set 2, B). Gene set lists are provided in the online supplement. Gene set enrichment scores were calculated across 82 whole sputum microarray samples (non-asthma controls n = 16, NA *n* = 29, NNA *n* = 37; A, B). Gene set 1 and 2 were also tested by GSVA in the U-BIOPRED sputum microarray dataset (non-asthma controls *n* = 16, NA *n* = 47, NNA *n* = 57; C, D), and comparing mild-moderate vs severe asthma by neutrophilic phenotype (mild-moderate NNA *n* = 12, mild-moderate NA *n* = 8, severe NNA *n* = 45, severe NA *n* = 39; E, F). Kruskal-Wallis test with Dunn's multiple comparisons test. * = $p < .05$, ** = $p < .01$, *** = $p < .001$

TABLE 3 Correlation of NA sputum macrophage gene signature (gene set 1) GSVA enrichment scores with clinical characteristics

	Spearman *r* value
Age	.364**
BMI	.003
ACQ6	.284*
Pre-β2 FEV1 (% pred)‡	−.539***
Pre-β2 FEV1/FVC‡	−.471***
ICS dose (fluticasone equivalent)	.254*

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

Whole sputum microarray studies show alteration of the transcriptome in NA[7] or severe asthma with elevated sputum neutrophils.[8] Use of immune cell-specific gene signatures implicates altered contribution of specific immune cell gene pools to altered sputum transcriptomes of type 2 high vs type 2 low asthma.[45] A recent study employing GSVA of *in vitro* derived macrophage gene signatures[11] in whole sputum microarray was suggestive of a contribution of altered macrophage transcriptomes to altered sputum transcriptomes in severe and neutrophilic asthma.[46] Here we demonstrate enrichment of a NA macrophage gene signature in whole sputum microarray data from participants with NA. This is the first direct evidence that altered macrophage transcriptomes contribute to the overall gene expression differences observed in whole sputum microarray in NA, and imply altered macrophage function is a significant facet of the airway inflammatory milieu in NA. Analysis of the U-BIOPRED sputum microarray dataset demonstrated similar alterations in mild-moderate vs severe NA, implying the altered macrophage transcriptome is a feature of NA irrespective of asthma severity. Importantly, increased expression of the NA macrophage gene signature correlated with clinical features including worse lung function and poorer asthma control. NA is a clinically important phenotype with limited therapeutic options for those with corticosteroid resistant disease symptoms, and neutrophilia appears unaffected by corticosteroid treatment. However, trials of CXCR2 antagonists that reduce neutrophil recruitment to the airways have failed to show clinical benefit in severe asthma.[2] Our present data and those above suggest therapeutic modulation of immune cells such as macrophages could be considered for further investigation in the context of neutrophilic airway inflammation. These data also suggest the term 'neutrophilic' could be considered something of a misnomer,[47] and perhaps a more precise description

encompassing cellular and molecular alterations beyond the neutrophil may be enabled by this and other studies.

This study has limitations. Our RNASeq analysis should be considered exploratory due to the relatively low sample numbers used, although we confirmed DEGS using qPCR in a larger cohort. Low sample number in the RNASeq cohort prevented meaningful analysis of relationship of identified gene signatures to other clinical characteristics. Our analysis is limited to transcriptomic alterations and further studies should seek to confirm these observations using protein and functional assays, however, altered macrophage transcriptomes underpin macrophage functional plasticity in response to differing extracellular queues.[11] Future studies should aim to test whether smoking or prior smoking alters macrophage phenotype in asthma.[48] We performed bulk RNASeq on isolated sputum macrophage populations, and thus, our dataset lacks the resolution to identify specific transcriptomic and functional subsets of macrophages that may be present. We cannot exclude that contaminating cells such as neutrophils contributed to our macrophage RNA pool, although all analyses indicated that we achieved highly pure macrophage preparations, DEGs identified did not include lung neutrophil signature genes,[26] and the majority of DEGs appeared plausibly derived from macrophages based on literature searches. We were also careful to exclude monocytes from our macrophage preparations in order to be able to specifically evaluate the macrophage niche independently of monocyte influx.[49]

In sum, this work reveals inflammatory context-specific modulation of airway macrophage phenotype in NA. Bioinformatic analyses indicated that these transcriptomic alterations relate to both pathophysiologically-relevant inflammation promoting and resolving responses to an altered extracellular milieu, including response to bacteria, promotion of neutrophil recruitment and enhanced clearance of bacteria and host cells. This work demonstrates transcriptomic activation of inflammatory programmes in a cell type other than the neutrophil in NA, supporting the concept of neutrophilic asthma as a discrete asthma endotype.

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CONFLICT OF INTEREST

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AUTHOR CONTRIBUTIONS

M.F. designed, performed and supervised laboratory studies, designed and performed data analysis, interpreted data and wrote the manuscript. L.Q. performed laboratory studies and edited the manuscript. S.S-O designed and performed data analysis and edited the manuscript. J.L.S. supervised clinical studies and edited the manuscript. K.J.B. supervised clinical studies, designed analysis and edited the manuscript. C.R. designed and performed data analysis, interpreted data and edited the manuscript. H.A.S. supervised clinical studies and edited the manuscript. L.G.W. supervised clinical studies and edited the manuscript. P.A.B.W., N.Z.K. and K.F.C. supervised data analysis and edited the manuscript. P.G.G. designed laboratory studies, supervised clinical studies, designed data analysis, interpreted data and edited the manuscript.

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SUPPORTING INFORMATION

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