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Differential gene expression and cytokine production from neutrophils in asthma phenotypes

K.J. Baines*,#, J.L. Simpson*,#, N.A. Bowden¶, R.J. Scott¶ and P.G. Gibson*,#

ABSTRACT: Asthma is characterised into eosinophilic and non-eosinophilic phenotypes based on inflammatory cell patterns in airway secretions. Neutrophils are important in innate immunity, and are increased in the airways in non-eosinophilic asthma. The present study investigated the activity of neutrophils in asthma phenotypes.

Participants with eosinophilic (n=8) and non-eosinophilic asthma (n=9) and healthy controls (n=11) underwent sputum induction and blood collection. Neutrophils were isolated and cultured with or without lipopolysaccharide. Cytokines were measured by ELISA, and gene expression was analysed using a gene expression microarray and quantitative PCR.

In non-eosinophilic asthma, blood neutrophils released significantly higher levels of interleukin-8 at rest. Cytokine gene expression and sputum neutrophil protein production did not differ between asthma subtypes. Microarrays demonstrated closely related expression profiles from participants with non-eosinophilic asthma that were significantly distinct from those in eosinophilic asthma. A total of 317 genes were significantly altered in resting neutrophils from participants with non-eosinophilic asthma *versus* eosinophilic asthma, including genes related to cell motility and regulation of apoptosis.

Non-eosinophilic and eosinophilic asthma are associated with specific gene expression profiles, providing further evidence that these phenotypes of asthma involve different molecular mechanisms of disease pathogenesis at the systemic level. The mechanisms of non-eosinophilic asthma may involve enhancement of blood neutrophil chemotaxis and survival.

KEYWORDS: Airway inflammation, asthma phenotypes, gene expression, innate immunity, interleukin-8, neutrophils

The inflammatory response in asthma is heterogeneous, involving a well-characterised eosinophilic pathway that is triggered by the inhalation of allergens, and which involves activation of type-2 T-helper lymphocytes and interleukin (IL)-5 production. Non-eosinophilic asthma represents an alternative asthma phenotype in which patients exhibit asthma symptoms and heightened airway responsiveness in the absence of significant eosinophilia [1–4]. The mechanisms underlying non-eosinophilic inflammation in asthma are unclear; however, neutrophils may be important since studies of non-eosinophilic asthma find increased numbers of neutrophils and elevated levels of the neutrophil chemoattractant IL-8 in the airways [5]. Furthermore, neutrophilic asthma is associated with innate immune activation, specifically increases in the expression of the Toll-like receptors (TLRs) TLR2, TLR4 and CD14, as well as the pro-inflammatory cytokines IL-8 and IL-1β, in airway samples [6]. The levels of these innate immune mediators measured in the sputum correlate with the number of neutrophils in the airways, implicating a role for neutrophils in the local production of these mediators.

Neutrophils have long been considered phagocytes whose main purpose is to engulf and degrade microorganisms. However, recent microarray studies have provided substantial evidence that neutrophils are capable of extensive gene expression changes that are important in the regulation of many neutrophil functions, as well as modulation of the immune response. A wide range of genes are expressed in unstimulated neutrophils, and this gene profile is dramatically changed in response to bacterial exposure [7], transmigration to the

AFFILIATIONS
*Priority Research Centre for Asthma and Respiratory Diseases, The University of Newcastle,
¶Priority Research Center for Information Based Medicine, Hunter Medical Research Institute, The University of Newcastle, Callaghan, and
#Dept of Respiratory and Sleep Medicine, Hunter Medical Research Institute, John Hunter Hospital, New Lambton, Australia.

CORRESPONDENCE
K.J. Baines
Level 3, Hunter Medical Research Institute
John Hunter Hospital
Locked Bag 1
Hunter Region Mail Centre
Newcastle
NSW 2310
Australia
E-mail: katherine.baines@newcastle.edu.au

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airways [8] and neutrophil-mediated diseases [9]. Marked changes in neutrophil gene expression occur following experimental exposure to soluble lipopolysaccharide (LPS) [8, 10] and whole bacteria [11]. LPS, a potent stimulus of innate immune responses, leads to alterations in gene expression that include genes that encode cytokines and receptors, genes involved in host defence, apoptosis-related genes, genes encoding transcription factors and chromatin-remodelling genes [11].

Although neutrophils are present in increased numbers in non-eosinophilic asthma, the precise mechanisms of their recruitment and accumulation remain largely unknown. Whole-genome gene expression analysis has not been widely used to investigate the molecular mechanisms underlying asthma, but could provide useful information relating to the heterogeneity of disease. The present study investigated the activation of circulating and sputum neutrophils in non-eosinophilic asthma, including the production of innate immune mediators, specifically the pro-inflammatory cytokines IL-8, IL-1β, tumour necrosis factor (TNF)-α and oncostatin M (OSM), the expression of TLR2 and TLR4, and whole-genome gene expression using microarrays. We hypothesised that neutrophils would show increased activation in non-eosinophilic asthma compared to eosinophilic asthma, demonstrated by increased protein release and expression of important innate immune mediator genes.

MATERIALS AND METHODS

Participants

Nonsmoking adults with stable asthma (n=17) were defined using the American Thoracic Society criteria, had a doctor's diagnosis of symptomatic asthma and demonstrated evidence of airways hyperresponsiveness to hypertonic saline. Healthy controls (n=11) exhibited no respiratory symptoms, with normal spirometric results and airways hyperresponsiveness. Participants were excluded if they had had a course of oral corticosteroids, antibiotics or a respiratory infection within 4 weeks prior to the visit. Participants were recruited through the Respiratory Ambulatory Care Service at the John Hunter Hospital (New Lambton, Australia) or by advertisement (healthy controls) and underwent clinical assessment, an allergy skin-prick test, spirometry, sputum induction and blood collection. All participants gave informed consent prior to their inclusion in the study and the Hunter Area Health Service and The University of Newcastle (Callaghan, Australia) Research Ethics Committees approved this study.

Sputum induction and analysis

Spirometry (KoKo; PD Instrumentation, Louisville, CO, USA) and sputum induction with hypertonic saline (4.5%) were performed as previously described [12]. A fixed sputum induction time of 15 min was used for all participants. Selected sputum was dispersed using dithiothreitol. The suspension was filtered, and total leukocyte count and cell viability were determined. Cytospins were prepared and stained (May–Grünwald Giemsa), and a differential cell count obtained from 400 nonsquamous cells.

Asthma subtype classification

Based on previous studies [2], participants with a sputum eosinophil count of $\geqslant$1% alone were classified as having eosinophilic asthma, and participants with sputum eosinophil counts of <1% were classified as having non-eosinophilic asthma. Those participants with increased neutrophils (>63%) and eosinophils (>1%) were classified as having non-eosinophilic asthma [2].

Neutrophil isolation and culture

Peripheral blood neutrophils were isolated from 50 mL whole blood using a Percoll density gradient and magnetic cell separation using CD16 microbeads (Miltenyi Biotec, Gladbach, Germany). CD16-positive cells were isolated from the remainder of the sputum sample using magnetic cell separation. Highly pure blood neutrophils (median 100% (interquartile range 96–100%)) and the neutrophil-enriched cell fraction (59% (30–78%) neutrophils; 35% (22–57%) macrophages) were cultured with or without LPS (100 ng·mL^{-1}) for 24 h. Further details are provided in the supplementary material.

Detection of mediators

Cytokine production was assessed from isolated airway and peripheral blood neutrophils after 24 h of culture. The concentrations of IL-8, IL-1β, TNF-α and OSM were determined by ELISA (R&D Systems, Minneapolis, MN, USA). The standard curves for these assays ranged 31.3–2,000 pg·mL^{-1} for IL-8, TNF-α and OSM, and 7.8–250 pg·mL^{-1} for IL-1β. Target gene expression was analysed using real-time PCR. RNA was prepared and reverse transcribed to cDNA as described previously [13]. PCR probes were purchased in kit form (Applied Biosystems, Foster City, CA, USA). PCR primers and probes were combined with the reference gene eukaryotic 18S ribosomal RNA in duplex real-time PCRs as previously described (7500 Real-Time PCR System; Applied Biosystems) [13]. The amount of target present was calculated relative to the 18S housekeeping gene and an internal calibrator ($2^{-\Delta\Delta Ct}$, where Δ is change and Ct is the cycle threshold).

Gene expression profiling

Selected blood neutrophil samples were processed for gene expression analysis, including those from four participants with non-eosinophilic asthma who had sputum neutrophil counts of >63% and five with eosinophilic asthma who had sputum eosinophil counts of >2.5%. RNA was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany) and quantified using the Quant-iT RiboGreen RNA Quantitation Assay Kit (Molecular Probes, Inc, Invitrogen, Eugene, OR, USA). Fluorescence was measured at wavelengths of 485 nm for excitation and 520 nm for emission (FLUOstar Optima; BMG LABTECH, Mornington, Australia). A total of 500 ng RNA was reverse transcribed into cRNA and labelled with biotin-uridine triphosphate using the Illumina TotalPrep RNA Amplification Kit (Ambion, Austin, TX, USA). A total of 850 ng cRNA was hybridised to Illumina Sentrix HumanRef-8 v1.1 Expression BeadChips (Illumina, San Diego, CA, USA) using standard protocols. Each BeadChip measured the expression of 24,354 genes and was scanned using the Illumina Bead Station and captured using BeadScan 3.5.11 (Illumina).

Statistical analysis

Data were analysed using Stata 9 (Stata Corporation, College Station, TX, USA). All data, unless otherwise stated, are nonparametric and reported as median (interquartile range). In the case of age, percentage predicted forced expiratory volume in

1 s (FEV_1) and FEV_1/forced vital capacity, data are reported as mean±SD, and significant differences were determined using either the two-sample unpaired t-test or multiple-sample ANOVA. For all other data, significant differences ($p<0.05$) were detected using the two-sample Wilcoxon rank-sum test or the multiple-sample Kruskal–Wallis test. For categorical data (sex and atopy) Fisher's exact test was applied. Associations between data were determined using Spearman's rank correlation.

For whole-genome gene expression, data were normalised using a cubic spline in the BeadStudio 2.0 software (Illumina), exported to GeneSpring 7.3.1 software (Agilent Technologies, Santa Clara, CA, USA) and further normalised to the median. Using the Wilcoxon–Mann–Whitney test, three comparisons were carried out between: 1) resting and LPS-stimulated neutrophils, 2) resting neutrophils in non-eosinophilic asthma *versus* eosinophilic asthma, and 3) LPS-stimulated neutrophils in non-eosinophilic asthma *versus* eosinophilic asthma. Using standard correlation and distance in GeneSpring 7.3.1, a dendrogram was created to show relationships between samples (Experiment Tree), and a second dendrogram to show relationships between gene expression levels across the samples (Gene Tree). Genes were judged to be differentially regulated only when: 1) the gene was present in all samples studied, 2) the difference in expression was >1.5-fold, and 3) the extent of difference in expression was significant ($p<0.05$ in Wilcoxon–Mann–Whitney test).

RESULTS

Clinical features and inflammatory cells

Clinical details and total and differential inflammatory cell counts from the induced sputum samples collected are shown in table 1. Healthy controls (n=11) without respiratory disease or symptoms exhibited an FEV_1 of >80% pred. All participants with asthma were receiving inhaled corticosteroid therapy, and 88% (15) of participants were taking combination therapy with a long-acting β_2-agonist. Eight (47%) of the 17 participants had eosinophilic asthma, and the remaining nine (53%) non-eosinophilic asthma. The asthma pattern was classified as intermittent (n=1; 6%), mild (n=5; 29%), moderate (n=6; 35%) or severe persistent (n=5; 29%). There was no significant difference between eosinophilic and non-eosinophilic asthma regarding the clinical parameters measured; however, sputum eosinophil numbers were increased in eosinophilic asthma and sputum neutrophil numbers in non-eosinophilic asthma. Whole-genome gene expression microarray experiments were performed on selected participants with eosinophilic and non-eosinophilic asthma, and their clinical details were comparable (table E1 in the supplementary material).

Innate immune responses of peripheral blood neutrophils

Resting peripheral blood neutrophils from participants with non-eosinophilic asthma released significantly more IL-8 than did those from participants with eosinophilic asthma ($p=0.03$) (fig. 1). Resting neutrophils did not release detectable levels of TNF-α, and 93% (26) of resting neutrophil samples had undetectable levels of IL-1β and OSM. There was a trend towards upregulation of IL-8 gene expression (fig. 1b) in non-eosinophilic asthma compared to eosinophilic asthma; however, OSM, IL-1β, TNF-α, TLR2 and TLR4 gene expression did not differ significantly between asthma phenotypes (table E2 in the supplementary material).

LPS stimulation induced the release of IL-8, IL-1β, TNF-α and OSM, and increased IL-8, IL-1β, TNF-α, OSM, TLR2 and TLR4 gene expression. LPS-stimulated neutrophils isolated from

TABLE 1 Clinical characteristics and induced sputum inflammatory cell counts of healthy controls and participants with eosinophilic and non-eosinophilic asthma

	Controls	Asthma		p-value
		Eosinophilic	Non-eosinophilic	
Subjects n	11	8	9	
Age yrs	56±18	53±20	65±9	0.35
Males/females n	5/6	4/4	4/5	1.0
Atopy	6 (55)	7 (88)	7 (78)	0.29
FEV_1 % pred	98±17	77±19	66±18[¶]	<0.01
FEV_1/FVC %	77±7	70±9	65±10[¶]	0.01
ICS dose[#] μg		750 (400–1500)	1000 (500–2000)	0.24
Asthma control score		0.7 (0.3–1.4)	1.0 (0.9–1.1)	0.74
TCC 10^6 cells·mL^{-1}	3.7 (2.4–5.6)	5.3 (2.8–8.3)	10.1 (4.4–17.6)	0.13
Neutrophils %	30.5 (14.5–37.4)	31.2 (16.0–41.1)	58.5 (24.5–72.0)	0.08
Neutrophils 10^4 cells·mL^{-1}	90.9 (49.1–150.7)	104.7 (64.4–218.7)	589.7 (81.6–1043.3)	0.06
Eosinophils %	0 (0–0.3)	5.1 (2.1–8.6)[¶,+]	0.2 (0–0.8)	<0.01
Eosinophils 10^4 cells·mL^{-1}	0 (0–1.4)	16.1 (8.8–47.7)[¶]	0.8 (0–13.2)	<0.01
Macrophages %	66.1 (58.4–82.4)	52.6 (49.8–62.8)	40.5 (22.6–48.3)[¶]	<0.01
Macrophages 10^4 cells·mL^{-1}	226.0 (172.8–307.4)	241.5 (189.1–437.3)	238.1 (120.6–402.7)	0.82

Data are presented as mean±SD, n (%) or median (interquartile range), unless otherwise specified. FEV_1: forced expiratory volume in 1 s; % pred: % predicted; FVC: forced vital capacity; ICS: inhaled corticosteroid; TCC: total cell count. [#]: 1 μg beclometasone≡1 μg budesonide≡0.5 μg fluticasone. [¶]: $p<0.008$ *versus* healthy controls; [+]: $p<0.008$ *versus* non-eosinophilic asthma (Kruskal–Wallis nonparametric test for significance).

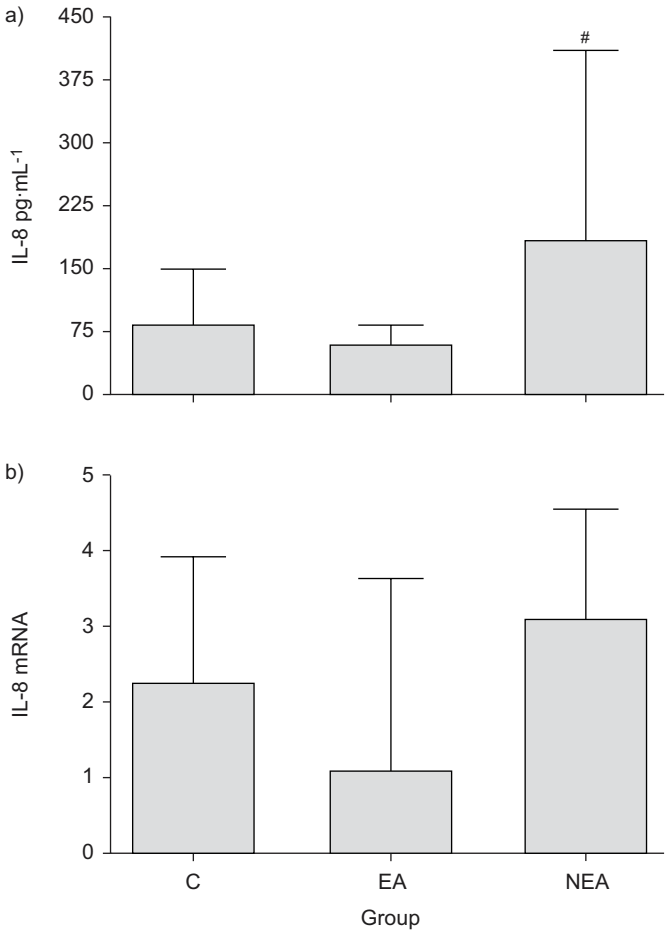

FIGURE 1. Interleukin (IL)-8 in resting blood neutrophils a) protein production and b) gene expression. Data are presented as medians; vertical bars represent the upper quartile. Gene expression is presented relative to the 18S housekeeping gene. C: healthy controls (n=11); EA: eosinophilic asthma (n=8); NEA: non-eosinophilic asthma (n=9). #: $p<0.008$ *versus* eosinophilic asthma.

participants with eosinophilic asthma released significantly less OSM than healthy controls; however, release of IL-8, IL-1β and TNF-α was similar (fig. 2). IL-8, IL-1β, TNF-α, OSM, TLR2 and TLR4 gene expression did not differ significantly between asthma phenotypes in LPS-stimulated neutrophils (table E2 in the supplementary material). TLR2 and IL-1β gene expression was generally lower in the asthma groups than in healthy controls.

Innate immune responses of sputum neutrophils

Minimal changes in sputum neutrophils were seen between the groups. Resting sputum neutrophils from participants with non-eosinophilic asthma released significantly lower levels of TNF-α than did healthy controls; however, this did not differ from eosinophilic asthma (table 2). IL-8 and IL-1β release and IL-8, IL-1β, TNF-α, TLR2 and TLR4 gene expression did not differ significantly between groups in either resting or LPS-stimulated sputum neutrophils, but tended, however, to be lower in both eosinophilic and non-eosinophilic asthma than in healthy controls (table 2). LPS stimulation had no effect on IL-8, IL-1β, TNF-α and OSM release or IL-8, IL-1β, TNF-α, OSM, TLR2 and TLR4 gene expression in sputum neutrophils. OSM was not released at detectable levels from sputum neutrophils and is, therefore, not shown.

Whole-genome gene expression changes due to LPS stimulation

Dramatic changes in gene expression were apparent between resting and LPS-stimulated circulating neutrophils isolated from participants with asthma. Using the Wilcoxon–Mann–Whitney test, 1,080 genes were identified with a mean expression ratio that was significantly different when comparing resting to LPS-stimulated neutrophils. As expected, the LPS-stimulated gene profile represented a pro-inflammatory state of neutrophil activation, with increases in cytokines (*e.g.* OSM), chemokines (*e.g.* IL-8, CC chemokine ligand 3-like 1 and CXC chemokine ligand 1), signalling molecules (*e.g.* IL-1 receptor-associated kinase (IRAK) 1 and 3), receptors (*e.g.* TLR2, CXC chemokine receptor 4 and CC chemokine receptor 1), molecules regulating apoptosis (*e.g.* growth arrest and DNA-damage-inducible protein (GADD) 45 β, serum/glucocorticoid-regulated kinase (SGK) and CCAAT/enhancer-binding protein β (CEBPB)) and components of the nuclear factor (NF)-κB pathway (*e.g.* NF-κB1, receptor-interacting serine/threonine-protein kinase (RIPK) 2 and TNF receptor superfamily, member 14 (TNFRSF14)). The LPS-regulated genes OSM, TLR2 and IL8 were confirmed to be upregulated *via* real-time PCR (table E3 in the supplementary material).

Whole-genome gene expression changes due to asthma phenotype

Resting blood neutrophils

Using the Wilcoxon–Mann–Whitney test, 317 genes from resting neutrophils were identified as exhibiting significantly different levels of expression between the asthma phenotypes. Construction of a dendrogram containing these 317 genes showed that the gene expression profiles from participants with non-eosinophilic asthma were closely related to, but significantly different from, those from the participants with eosinophilic asthma (fig. 3). In figure 3, columns represent the gene expression in resting neutrophils from each of the subjects with asthma. Downregulation is represented by green, and upregulation by red. The dendrogram at the top of the figure represents the relationship between asthma subtypes (blue branches: non-eosinophilic asthma; red branches: eosinophilic asthma), which were shown to be distinctly different. The horizontal dendrogram shows the relationship between the expression levels of each gene, *i.e.* genes of similar expression across the samples are grouped together.

A considerable number (54%) of genes that were altered in resting neutrophils in non-eosinophilic asthma compared to eosinophilic asthma were also altered by LPS stimulation, suggesting that these genes play a role in neutrophil activation. Altered genes of interest with immune-related functions are shown in table 3. These include important genes relating to neutrophil cell motility, apoptosis and the NF-κB cascade. The expression of several genes shown in table 3 were significantly correlated with percentage predicted FEV_1 (GADD45B: r= -0.70, p=0.036; IRAK3: r= -0.77, p=0.016; G-protein-coupled receptor 109B (HM74): r= -0.72; p=0.030;

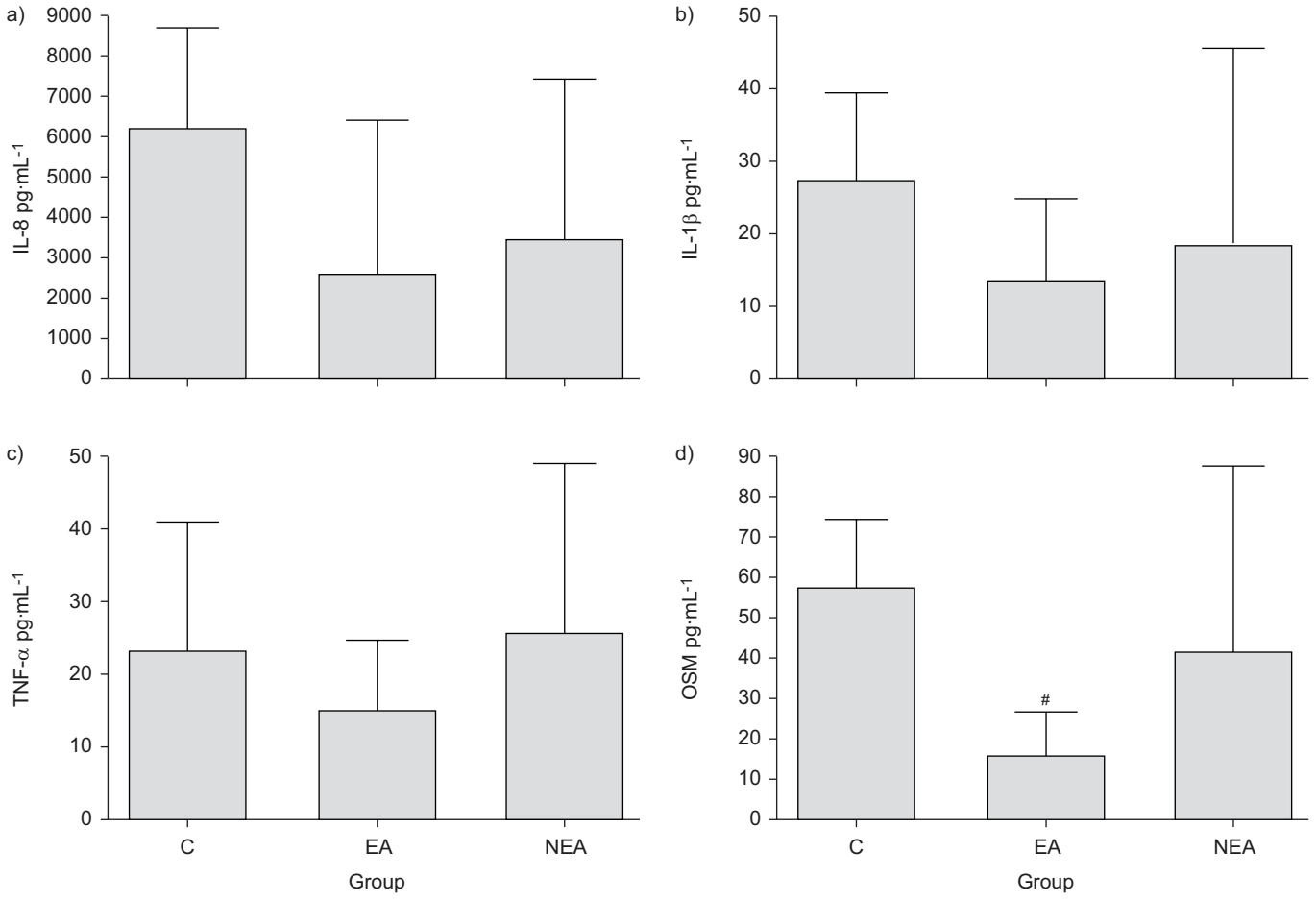

FIGURE 2. Cytokine production from lipopolysaccharide-stimulated blood neutrophils: a) interleukin (IL)-8; b) IL-1β; c) tumour necrosis factor (TNF)-α; and d) oncostatin M (OSM). Data are presented as medians; vertical bars represent the upper quartile. C: healthy controls (n=11); EA: eosinophilic asthma (n=8); NEA: non-eosinophilic asthma (n=9). #: $p<0.008$ *versus* eosinophilic asthma.

molecule possessing ankyrin repeats induced by LPS (MAIL): r= -0.78, p=0.013; protease inhibitor 3, skin derived (PI3): r= -0.83, p=0.005 (n=9)). Further trends for correlation and between gene correlations are reported in table E4 of the supplementary material. The expression of TNFRSF14 and GADD45B were confirmed to be upregulated *via* real-time PCR (table E3 in the supplementary material).

LPS-stimulated blood neutrophils

Using the Wilcoxon–Mann–Whitney test, 221 genes were identified with a mean expression ratio that differed significantly between the asthma subtypes for LPS-stimulated neutrophils. Construction of a dendrogram containing these 221 genes showed that the gene expression profiles from participants with non-eosinophilic asthma were closely related to, but significantly different from, those of the participants with eosinophilic asthma. Selected genes with immune-related functions that were altered in LPS-stimulated neutrophils from participants with non-eosinophilic asthma compared to participants with eosinophilic asthma are listed in table 4. Real-time PCR results testing CC chemokine ligand 23 (CCL23) confirmed this gene to be downregulated in non-eosinophilic asthma; however, plasminogen activator, urokinase (PLAU) was unchanged between asthma subtypes (table E3 in the supplementary material).

DISCUSSION

The present study investigated activation of sputum and peripheral blood neutrophils in non-eosinophilic and eosinophilic asthma. Although there were minimal differences between groups in release of mediators from sputum cells, there were marked changes in blood neutrophils in non-eosinophilic asthma. Resting blood neutrophils isolated from participants with non-eosinophilic asthma showed enhanced IL-8 release and increased IL-8 gene expression compared to participants with eosinophilic asthma, suggesting that the cells are partially activated or primed for an enhanced response. Further, whole-genome gene expression studies showed that there is a substantial degree of heterogeneity in resting neutrophils from participants with non-eosinophilic and eosinophilic asthma. In non-eosinophilic asthma, there was upregulation of genes involved in neutrophil chemotaxis, neutrophil survival and activation of the NF-κB cascade. This study highlights the ability of microarray technology to define inflammatory gene profiles associated with eosinophilic and non-eosinophilic asthma, and shows that there are novel and

TABLE 2 Relative cytokine production and gene expression in resting and lipopolysaccharide (LPS)-stimulated sputum neutrophils from participants with eosinophilic and non-eosinophilic asthma and healthy controls

	Resting neutrophils				LPS-stimulated neutrophils			
	Controls	Asthma		p-value	Controls	Asthma		p-value
		Eosinophilic	Non-eosinophilic			Eosinophilic	Non-eosinophilic	
Subjects n	7	6	8		7	6	8	
IL-8 pg·mL^{-1}	2457.2 (1262.8–6859.0)	719.8 (191.4–2640.5)	849.3 (363.8–1741.1)	0.13	2963.4 (1147.2–6533.5)	1166.7 (207.6–2511.1)	940.0 (411.3–2656.3)	0.26
IL-8 mRNA	134.4 (77.2–196.7)	40.5 (38.1–43.7)	36.9 (21.8–59.1)	0.06	77.3 (42.5–102.5)	51.1 (48.8–71.0)	62.2 (45.6–113.0)	0.96
IL-1β pg·mL^{-1}	52.5 (6.6–95.7)	3.3 (3.1–7.9)	8.3 (5.0–18.9)	0.07	31.2 (6.5–66.6)	5.2 (3.9–27.6)	9.7 (6.3–21.8)	0.28
IL-1β mRNA	13.5 (6.8–26.5)	1.7 (0.8–1.9)	2.8 (1.0–3.9)	0.07	7.6 (2.0–13.2)	2.2 (1.6–4.3)	4.8 (2.6–5.3)	0.38
TNF-α pg·mL^{-1}	371.7 (116.1–599.0)	28.9 (7.1–74.0)	15.0 (0.0–110.8)$^{\#}$	0.02	257.1 (117.5–550.6)	15.0 (12.4–148.9)	31.5 (1.7–124.3)	0.07
TNF-α mRNA	4.7 (1.6–5.4)	0.98 (0.8–1.5)	1.6 (0.3–3.6)	0.09	3.2 (1.9–5.0)	2.0 (1.3–2.4)	1.1 (0.4–3.5)	0.46
TLR2 mRNA	4.4 (1.3–6.9)	2.0 (0.7–2.9)	2.9 (0.8–4.6)	0.79	1.1 (0.7–2.6)	2.2 (0.7–5.5)	5.2 (2.1–9.5)	0.34
TLR4 mRNA	0.4 (0.4–1.4)	0.2 (0.2–0.2)	0.2 (0.1–0.4)	0.11	0.3 (0.1–0.5)	0.2 (0.2–0.2)	0.3 (0.2–0.3)	0.88

Data are presented as median (interquartile range), unless otherwise indicated. IL: interleukin; TNF: tumour necrosis factor; TLR: Toll-like receptor. $^{\#}$: $p<0.008$ *versus* healthy controls (Kruskal–Wallis nonparametric test for significance).

distinct gene expression profiles that relate to asthma inflammatory phenotype.

Sputum neutrophil cytokine gene expression and protein production did not differ by asthma phenotype. However, airway neutrophils generally showed lower levels of cytokine release in both eosinophilic and non-eosinophilic asthma compared to healthy controls. This only reached significance for TNF-α production in non-eosinophilic asthma. A limitation exists when interpreting these findings since asthma medications, such as inhaled corticosteroids, are regularly used to reduce airway inflammation in asthma. Furthermore, airway neutrophils did not respond to LPS stimulation in healthy controls or eosinophilic and non-eosinophilic asthma. Similar findings of unresponsiveness of airway cells to LPS stimulation along with decreased release of TNF-α from airway cells have previously been reported in chronic obstructive pulmonary disease [14], and the mechanisms of this warrant further investigation.

The development of high-throughput screening and genome-wide gene expression using microarrays has permitted many diseases to be characterised into groups by gene expression profiling. Analysis of the current data suggests that the type of airway inflammation present can separate asthma into subgroups based on altered systemic neutrophil gene expression profiles. Although relatively small groups were studied here, significant differences in gene expression and distinct dendrograms were observed. In addition, genes in peripheral blood neutrophils from asthma phenotypes with known immune-related functions were identified and confirmed to show altered expression using real-time PCR.

Activation of the innate immune response, including increased expression of the receptors TLR4, TLR2, CD14 and surfactant protein A and the cytokines IL-8 and IL-1β, has been demonstrated in the airways of participants with non-eosinophilic asthma [6]. The present data show altered gene expression profiles and increased resting blood neutrophil IL-8 production in non-eosinophilic asthma. This could both promote the development of non-eosinophilic airway inflammation and influence existing non-eosinophilic airway inflammation. Many (54%) genes that were differentially expressed in resting neutrophils in non-eosinophilic asthma were also regulated by LPS stimulation, indicating that these genes play a role in neutrophil activation. ABRAHAM *et al.* [15] demonstrated that there is a significant correlation between peripheral blood neutrophil phenotype and the pulmonary response to endotoxin, *i.e.* the accumulation of neutrophils and the intensity of the immune response in the airways to endotoxin challenge is directly associated with the activation state of circulating neutrophils.

Large numbers of neutrophils are often present in the airways of participants with non-eosinophilic asthma. Increased accumulation of neutrophils in the airways could be due to enhanced chemotaxis from the blood and/or enhanced survival of these cells. Here, it has been shown that peripheral blood neutrophils show increased expression of genes relating to enhanced cell motility and survival. Genes relating to cell motility that were upregulated in non-eosinophilic asthma include proteins (*e.g.* IL-8 and S100 calcium-binding protein A8 (S100A8)), receptors (*e.g.* CC chemokine receptor-like (CCRL) 2) and transcription factors (*e.g.* serum response factor). These genes are readily expressed in neutrophils and upon neutrophil activation by LPS [16, 17]. Importantly, expression of IL-8 [6], S100A8 [16] and CCRL2 [8] have been associated with neutrophilic lung inflammation.

IL-8 is important for many neutrophil functions, including chemotaxis and survival. Enhanced production of IL-8 by blood neutrophils may prime these cells for their migration to

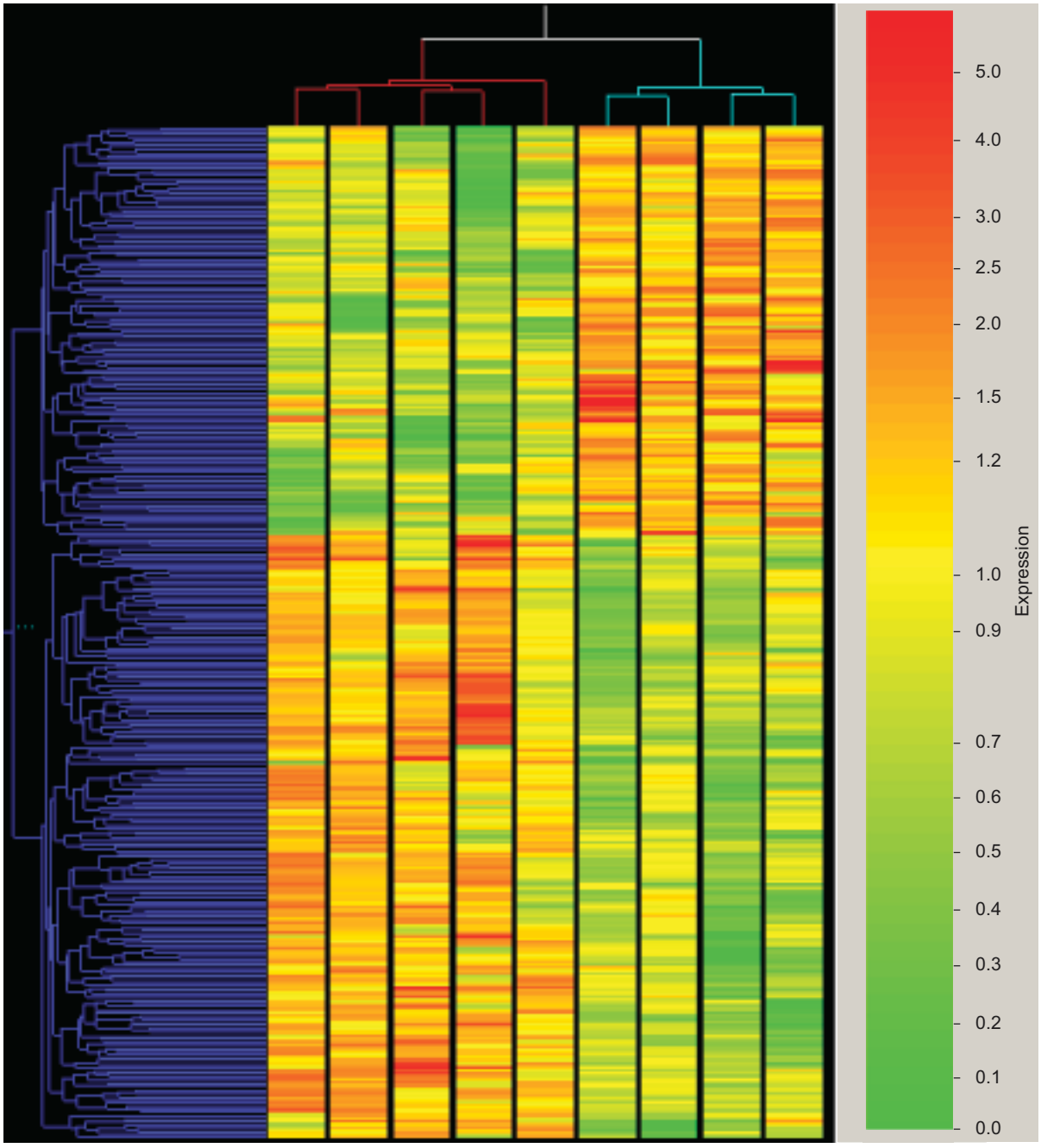

FIGURE 3. Gene expression profiles of resting neutrophils from participants with eosinophilic asthma (n=5) *versus* those with non-eosinophilic asthma (n=4). The vertical dendrogram represents the relationship between participants with non-eosinophilic (blue branches) and eosinophilic asthma (red branches). The horizontal dendrogram represents the relationship between the expression levels of each gene across all of the samples.

the airways. Circulating levels of IL-8 can also stimulate the bone marrow to release neutrophils into the circulation [18]. Enhanced release of IL-8 has previously been reported in blood neutrophils isolated from patients with cystic fibrosis [19]. Enhanced IL-8 release may be due to positive feedback from the leakage of inflammatory mediators from the airways, release of immature neutrophils from the bone marrow or genetic differences, such as IL-8 gene polymorphisms; however, further investigation is required in order to elucidate this.

There is a considerable amount of literature demonstrating that cell fate is regulated at the level of gene expression [20], and that these changes are important in the resolution of inflammatory processes [21]. Particular examples of genes whose expression was increased in non-eosinophilic asthma and relate to a delay in apoptosis include GADD45B, histone deacetylase (HDAC) 3 and 5, SGK and CEBPB. Several of these genes are thought to increase cell survival through modulation of the NF-κB pathway [22, 23]. RIPK2 is another important signalling molecule involved in the activation of NF-κB

TABLE 3 Selected genes with immune-related function that were altered in resting neutrophils from participants with non-eosinophilic asthma compared to eosinophilic asthma

Accession No.#	Symbol	Name	Fold change	p-value
Cell Motility				
NM_001456.1	FLNA	Filamin A, α	5.1	0.04
NM_003965.3	CCRL2	Chemokine (CC motif) receptor-like 2	3.4	0.04
NM_003131.1	SRF	Serum response factor	3.2	0.01
NM_002964.3	S100A8	S100 calcium-binding protein A8	2.6	0.04
NM_000584.2	IL8	Interleukin-8	2.4	0.04
NM_006000.1	TUBA1	α_1-Tubulin	2.1	0.04
NM_003370.1	VASP	Vasodilator-stimulated phosphoprotein	2.0	0.04
Apoptosis				
NM_015675.1	GADD45B	Growth arrest and DNA-damage-inducible β	3.1	0.003
NM_003883.2	HDAC3	Histone deacetylase 3	2.7	0.003
NM_003821.4	RIPK2	Receptor (TNFRSF)-interacting serine-threonine kinase 2	2.6	0.003
NM_139205.1	HDAC5	Histone deacetylase 5	2.5	0.01
NM_014330.2	PPP1R15A	Protein phosphatase 1, regulatory (inhibitor) subunit 15A	2.4	0.04
NM_003820.2	TNFRSF14	Tumour necrosis factor receptor superfamily, member 14	2.2	0.003
NM_003375.2	VDAC2	Voltage-dependent anion channel 2	2.2	0.003
NM_005627.2	SGK	Serum/glucocorticoid-regulated kinase	2.0	0.01
NM_005194.2	CEBPB	CCAAT/enhancer binding protein (C/EBP), beta	1.7	0.01
NM_005118.2	TNFSF15	Tumour necrosis factor (ligand) superfamily, member 15	-2.1	0.01
Immune-related				
NM_014015.3	DEXI	Dexamethasone-induced transcript	4.9	0.04
NM_007199.1	IRAK3	Interleukin-1 receptor-associated kinase 3	4.3	0.04
NM_002638.2	PI3	Protease inhibitor 3, skin-derived (SKALP)	3.2	0.01
NM_012092.2	ICOS	Inducible T-cell co-stimulator	3.1	0.04
NM_006018.1	HM74	G-protein-coupled receptor 109B	3.0	0.01
NM_000247.1	MICA	MHC class I polypeptide-related sequence A	2.8	0.01
NM_013439.2	PILRA	Paired immunoglobulin-like type 2 receptor alpha	2.4	0.04
NM_031419.1	MAIL	Molecule possessing ankyrin repeats induced by lipopolysaccharide	2.4	0.04
NM_002117.3	HLA-C	MHC, class I, C	2.3	0.003
NM_004048.2	B2M	β_2-Microglobulin	2.2	0.01
NM_000433.1	NCF2	Neutrophilic cytosolic factor 2	2.0	0.04
NM_004604.3	STX4A	Syntaxin 4A	1.8	0.003
NM_005516.3	HLA-E	MHC, class I, E	1.8	0.01
NM_001613.1	ACTA2	α_2-Actin	1.8	0.04
NM_005729.3	PPIF	Peptidylprolyl isomerase F (cyclophilin F)	1.7	0.04
NM_002697.2	POU2F1	POU domain class 2, transcription factor 1	-1.5	0.003
NM_133280.1	FCAR	Fc fragment of IgA receptor	-1.6	0.04
NM_000896.1	CYP4F3	Cytochrome P_{450}, family 4, subfamily F, polypeptide 3	-1.6	0.01
NM_031483.3	ITCH	Itchy homologue E3 ubiquitin protein ligase	-1.7	0.003
NM_147134.1	NFX1	Nuclear transcription factor, X-box binding 1	-1.9	0.04
NM_178509.3	STXBP4	Syntaxin binding protein 4	-1.9	0.04
NM_147191.1	MMP21	Matrix metalloproteinase 21	-1.9	0.01
NM_007038.1	ADAMTS5	A disintegrin-like and metallopeptidase with thrombospondin type 1 motif, 5	-3.3	0.003

MHC: major histocompatibility complex #: GenBank.

through stimulation of numerous innate immune receptors, including TLR2, TLR4, nucleotide-binding oligomerisation domain proteins, IL-1 receptor (IL-1R) and IL-18R [24]. IRAK-M (IRAK3), a negative regulator of TLR signalling [25] was upregulated in resting neutrophils in non-eosinophilic asthma in the present study, and has recently been linked to the pathogenesis of early-onset persistent asthma [26].

The gene expression changes appear to be clinically relevant since many were correlated with the degree of airway obstruction in asthma, including GADD45B, IRAK3, HM74, MAIL, PI3, syntaxin 4A (STX4A), major histocompatibility complex, class I, E (HLA-E), HDAC5 and TNFRSF14. These genes exhibit a variety of functions, including cell signalling (GADD45B and IRAK3), transcriptional regulation (MAIL and

TABLE 4 Selected genes with immune-related function that were altered in lipopolysaccharide-stimulated neutrophils from participants with non-eosinophilic asthma compared to eosinophilic asthma

Accession No.#	Symbol	Name	Fold change	p-value
NM_002658.1	PLAU	Plasminogen activator, urokinase	3.4	0.04
NM_012342.1	BAMBI	BMP and activin membrane-bound inhibitor homologue	3.1	0.01
NM_001618.2	PARP1	Poly(ADP-ribose) polymerase family, member 1	2.5	0.01
NM_006904.6	PRKDC	Protein kinase, DNA-activated, catalytic polypeptide	2.4	0.003
NM_000181.1	GUSB	β-Glucuronidase	2.3	0.04
NM_005723.2	TM4SF9	Tetraspanin 5	2.3	0.04
NM_005746.1	PBEF	Pre-B-cell colony enhancing factor	1.9	0.04
NM_000877.2	IL1R1	Interleukin-1 receptor, type 1	1.9	0.01
NM_006290.2	TNFAIP3	Tumour necrosis factor-α-induced protein 3	1.8	0.003
NM_005063.3	SCD	Stearoyl-CoA desaturase (Δ^9-desaturase)	1.7	0.04
NM_033405.2	PRIC285	Peroxisomal proliferator-activated receptor-α-interacting complex 285	1.6	0.04
NM_006534.2	NCOA3	Nuclear receptor coactivator 3	1.6	0.04
NM_002514.2	NOV	Nephroblastoma overexpressed gene	1.6	0.04
NM_004333.2	BRAF	v-*raf* murine sarcoma viral oncogene homologue B1	1.5	0.04
NM_021738.1	SVIL	Supervillin	1.5	0.04
NM_003153.3	STAT6	Signal transducer and activator of transcription 6	1.5	0.04
NM_024535.1	FLJ22021	Coronin 7	-1.5	0.04
NM_005981.3	TSPAN31	Tetraspanin 31	-1.7	0.04
NM_016150.3	ASB2	Ankyrin repeat and SOCS box-containing 2	-1.7	0.04
NM_198291.1	SRC	v-*src* sarcoma (Schmidt-Ruppin A-2) viral oncogene homologue	-2.4	0.003
NM_058171.2	ING2	Inhibitor of growth family, member 2	-2.8	0.04
NM_003265.2	TLR3	Toll-like receptor 3	-2.9	0.04
NM_145898.1	CCL23	Chemokine (CC motif) ligand 23	-5.9	0.003

BMP: bone morphogenetic protein; SOCS: suppressor of cytokine signalling. #: GenBank.

HDAC5), receptor activity (HM74, HLA-E and TNFRSF14), protease inhibition (PI3) and protein transport (STX4A). Importantly, several of these genes participate in the regulation of NF-κB activity, including GADD45B, IRAK3, MAIL, TNFRSF14 and PI3. This further underscores the importance of this pathway in the mechanisms of non-eosinophilic asthma.

Differences in the response to LPS may also play a role in the innate immune defence against invading microorganisms, and may contribute to airway inflammation. The present study demonstrated significant alterations in gene expression following LPS stimulation in non-eosinophilic asthma compared to eosinophilic asthma. The genes that were altered (*e.g.* PLAU and IL1R1) suggested that there was a potentiation of LPS responses in non-eosinophilic asthma, and further increases in genes relating to cell survival (*e.g.* pre-B-cell colony enhancing factor (PBEF); TNF-α-induced protein 3 (TNAIF3); v-*raf* murine sarcoma viral oncogene homologue B1 (BRAF); protein kinase, DNA-activated, catalytic polypeptide (PRKDC) and supervillin (SVIL)).

In addition to this, a decrease in the production of OSM but not OSM mRNA from LPS-stimulated neutrophils was observed in participants with eosinophilic asthma, which was significantly different from that in healthy controls and also lower than in non-eosinophilic asthma. OSM, an IL-6 family cytokine, is thought to promote airway remodelling [27], potentially through increasing the proliferation of both fibroblasts and smooth muscle cells [28] and inducing the production of angiogenic factors, such as vascular endothelial growth factor [29]. Neutrophils have an intracellular store of OSM and produce large concentrations of the protein upon stimulation with inflammatory triggers, such as LPS [30]. Since differences were found in the present study between the production of OSM and its gene expression, future studies should measure the levels of OSM within the intracellular stores of the neutrophil.

These findings provide further evidence that neutrophils are transcriptionally active cells that are responsive to environmental stimuli and capable of a complex series of late transcriptional changes. We have identified specific gene profiles associated with non-eosinophilic and eosinophilic asthma, providing further validation that these phenotypes of asthma involve very different molecular mechanisms of disease pathogenesis at the systemic level. There is altered production of both IL-8 and OSM, indicating differential activation of neutrophils in asthma phenotypes. This study highlights the importance of neutrophils in the pathogenesis of non-eosinophilic asthma.

STATEMENT OF INTEREST

None declared.

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