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Differential gene expression and cytokine production from neutrophils in asthma phenotypes

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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. This study investigates the activity of neutrophils in asthma phenotypes.

Participants with eosinophilic (n=8), 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 (LPS). Cytokines were measured by ELISA, and gene expression was analysed using Illumina Humanref-8 BeadChips and qPCR.

In non-eosinophilic asthma, blood neutrophils released significantly higher levels of interleukin-8 at rest. Cytokine gene expression and protein production of sputum neutrophils were not different between asthma subtypes. Microarrays demonstrated closely related expression profiles from participants with non-eosinophilic asthma that were significantly distinct from eosinophilic asthma. 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.

INTRODUCTION

The inflammatory response in asthma is heterogeneous involving a well-characterised eosinophilic pathway that is triggered by the inhalation of allergens, and involves activation of T helper (Th) 2 lymphocytes and interleukin (IL)-5 production. Non-eosinophilic asthma represents an alternative asthma phenotype where patients have 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 (TLR) 2, TLR4, and CD14, as well as the proinflammatory 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 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 includes genes

that encode for cytokines, receptors, genes involved in host defense, apoptosis-related genes, transcription factors, and chromatin-remodeling 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. This study investigated the activation of circulating and sputum neutrophils in non-eosinophilic asthma, including the production of innate immune mediators, specifically the proinflammatory cytokines IL-8, IL-1β, TNF-α and oncostatin M (OSM)), the expression of Toll like receptors (TLR)2 and TLR4, and whole genome gene expression using microarrays. We hypothesised that neutrophils would have increased activation in non-eosinophilic asthma compared to eosinophilic asthma demonstrated by increased protein release and gene expression of important innate immune mediators.

MATERIALS AND METHODS

Participants

Non-smoking 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 (AHR) to hypertonic saline. Healthy controls(n=11) had no respiratory symptoms, normal spirometry and airways hyperresponsiveness. Participants were excluded if they 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 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 Research Ethics Committee's 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 minutes was used for all participants. Selected sputum was dispersed using dithiothreitol (DTT). The suspension was filtered, and a total cell count of leucocytes and cell viability was performed. Cytospins were prepared, stained (May-Grunwald Giemsa) and a differential cell count obtained from 400 non-squamous cells.

Asthma Subtype Classification

Based on previous studies [13], participants with sputum eosinophil count of $\geq$1% alone were classified as having eosinophilic asthma and participants with sputum eosinophils <1% were classified as non-eosinophilic asthma . Those participants with increased neutrophils (>63%) and eosinophils (>1%) were classified as non-eosinophilic asthma [13].

Neutrophil Isolation and Culture

Peripheral blood neutrophils were isolated from 50mL of whole blood using 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 [100% (96-100%)] and the sputum neutrophil enriched cell fraction [59% (30-78%) neutrophils; 35% (22-57%) macrophages] were cultured with or without LPS (100ng/mL) for 24 hours. Further details are provided in the online depository.

Detection of Mediators

Cytokine production was assessed from isolated airway and peripheral blood neutrophils at 24 hours of culture. The concentrations of IL-8, IL-1β, tumor necrosis factor (TNF)-α, and oncostatin M (OSM) protein were determined by ELISA (R & D Systems, Minneapolis, MN, USA). The standard curve for these assays ranged from 31.3pg/mL to 2000pg/mL for IL-8, TNF-α and OSM, and 7.8pg/mL to 250pg/mL for IL-1β. Target gene expression was analysed using real time PCR. RNA was prepared and reverse-transcribed to cDNA as described previously [14]. 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 (ABI GeneAmp 7700 cycler, Perkin-Elmer) [14]. The amount of target present was calculated relative to the housekeeping gene 18S and an internal calibrator ($2^{-\Delta\Delta Ct}$).

Gene Expression Profiling

Selected blood neutrophil samples were processed for gene expression analysis including 4 participants with non-eosinophilic asthma that had sputum neutrophils greater than 63% and 5 participants with eosinophilic asthma that had sputum eosinophils greater than 2.5%. RNA was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany) and quantitated using the Quant-iT RiboGreen RNA Quantitation Assay Kit (Molecular Probes Inc, Invitrogen, Eugene, OR, USA). Fluorescence was measured at wavelengths 485nm for excitation and 520nm for emission (FLUOstar Optima, BMG Labtech, VIC, Australia). 500ng of RNA was reverse transcribed into cRNA and biotin-UTP labeled using the Illumina TotalPrep RNA Amplification Kit (Ambion, Texas, USA). 850ng of cRNA was hybridised to the Illumina Sentrix HumanRef-8 v1.1 Expression BeadChips (Illumina, San Diego, CA, USA) using standard protocols (See http://www.illumina.com/pages.ilmn?ID=51 for further details on chip design). 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, San Diego, CA, USA).

Statistical Analysis

Data was analysed by Stata 9 (Stata Corporation, College Station, Texas USA). All data, unless otherwise stated, is non-parametric and reported as the median (Quartile 1-Quartile 3). In the case of age, FEV_1% predicted and FEV_1/FVC data is reported as mean (SD) and significant differences were determined using either the 2 sample Student's t test or the multiple sample test analysis of variance (ANOVA). For all other data significant differences ($p<0.05$) were detected with the 2-sample test Wilcoxon rank sum or the multiple sample test Kruskal-Wallis. For categorical data (Gender and Atopy) Fischer's exact test was applied. Associations between data were determined using Spearman's rank correlation.

For whole genome gene expression, data was normalised using cubic spline in Illumina's BeadStudio 2.0 software (Illumina, San Diego, USA) and exported to GeneSpring 7.3.1 software (Agilent Technologies, Santa Clara, USA) and further normalized to the median. Using the Wilcoxon-Mann-Whitney test, 3 comparisons were carried out, 1) resting to 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 was created 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 statistically significant ($p<0.05$ Wilcoxon-Mann-Whitney test).

RESULTS

Clinical Features and Inflammatory Cells

Clinical details and total and differential inflammatory cell counts from induced sputum samples collected are shown in Table I. Healthy controls (n=11) without respiratory disease or symptoms had an FEV_1 >80% of predicted. All participants with asthma were on inhaled corticosteroid therapy, and 88% (n=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 9 (53%) had non-eosinophilic asthma. 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 for the clinical parameters measured, however sputum eosinophils were increased in eosinophilic asthma and sputum neutrophils were increased in non-eosinophilic asthma. Whole genome gene expression microarrays were performed on selected participants with eosinophilic and non-eosinophilic asthma, and their clinical details are comparable (Table E1 of the online depository).

Innate immune responses of peripheral blood neutrophils

Resting peripheral blood neutrophils from participants with non-eosinophilic asthma released significantly more IL-8 protein compared to participants with eosinophilic asthma (p=0.03, see Figure 1). Resting neutrophils did not release detectable levels of TNF-α, and 93% (n=26) of resting neutrophil samples had undetectable levels of IL-1β and OSM. There was a trend for upregulation of gene expression for IL-8 (Figure 1B) in non-eosinophilic asthma compared to eosinophilic asthma however OSM, IL-1β, TNF-α, TLR2 and TLR4 gene expression was not significantly different between asthma phenotypes (see Table E2 in the online depository).

LPS stimulation induced the release of IL-8, IL-1β, TNF-α, and OSM protein and increased the gene expression of IL-8, IL-1β, TNF-α, OSM, TLR2 and TLR4. LPS stimulated neutrophils isolated from participants with eosinophilic asthma release significantly less OSM compared to healthy controls, however release of IL-8, IL-1β and TNF-α was similar (see Figure 2). Gene expression for IL-8, IL-1β, TNF-α, OSM, TLR2, TLR4 was not significantly different between asthma phenotypes in LPS stimulated neutrophils (see Table E2 in the online depository). TLR2 and IL-1β gene expression was generally lower in the asthma groups compared to healthy controls.

Innate immune responses of sputum neutrophils

Minimal changes were seen in sputum neutrophils between the groups. Resting sputum neutrophils from participants with non-eosinophilic asthma released significantly lower levels of TNF-α protein compared to healthy controls, however this was not different compared to eosinophilic asthma (Table II). IL-8, and IL-1β protein release and gene expression for IL-8, IL-1β, TNF-α, TLR2, and TLR4 was not significantly different between groups in either resting or LPS stimulated sputum neutrophils, however tended to be lower in both eosinophilic and non-eosinophilic asthma compared to healthy controls (Table II). LPS stimulation had no effect on the release of IL-8, IL-1β, TNF-α, and OSM protein or the gene expression of IL-8, IL-1β, TNF-α, OSM, TLR2 and TLR4 in sputum neutrophils. OSM protein 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, 1080 genes were identified with a mean ratio of expression that was significantly different comparing resting to LPS stimulated neutrophils. As expected, the LPS stimulated gene

profile represented a proinflammatory state of neutrophil activation with increases in cytokines (e.g. OSM), chemokines (e.g. IL-8, CCL3L1, CXCL1), signalling molecules (e.g. IRAK1, IRAK3), receptors (e.g. TLR2, CXCR4, CCR1), molecules regulating apoptosis (e.g. GADD45B, SGK, CEBPB) and components of the NF-κB pathway (e.g. NFKB1, RIPK2, TNFRSF14). The LPS regulated genes OSM, TLR2 and IL-8 were confirmed to be upregulated via real time PCR (see Table E3 in the online depository).

Whole genome gene expression changes due to asthma phenotype

Resting Blood Neutrophils

Using the Wilcoxon-Mann-Whitney test, 317 genes were identified as having significantly different levels of expression between the asthma phenotypes, for resting neutrophils. Construction of a dendrogram containing these 317 genes showed that the gene expression profiles from participants with non-eosinophilic asthma were closely related, but significantly different to the participants with eosinophilic asthma (see Figure 3). In figure 3 columns represent gene expression for both resting neutrophils from each of the subjects with asthma. Down-regulation is represented as green, and up-regulation is represented as red. The dendrogram at the top of the figure represents the relationship between asthma subtypes (blue branches: non-eosinophilic asthma; and red branches: eosinophilic asthma), which are shown to be distinctly different. The dendrogram on the left side shows the relationship between the expression levels of the gene, that is, genes of similar expression across the samples are grouped together.

A considerable number of genes (54%) 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 III. These include important genes relating to neutrophil cell motility, apoptosis and the NF-κB cascade. The expression of several genes shown in Table III were significantly correlated with FEV_1% predicted [n=9, GADD45B: r= -0.70; p=

0.036, IRAK3: r= -0.77; p= 0.016, HM74: r= -0.72; p= 0.030, MAIL: r= -0.78; p= 0.013, PI3: r= -0.83; p= 0.005]. Further trends for correlation and between gene correlations are reported in Table E4 of the online supplement. The expression of TNFRSF14 and GADD45B were confirmed to be upregulated via real time PCR (see Table E3 in the online repository).

LPS Stimulated Blood Neutrophils

Using the Wilcoxon-Mann-Whitney test, 221 genes were identified with a mean ratio of expression that was significantly different 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, but significantly different to 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 IV. Real time PCR results testing CCL23 confirmed this gene to be downregulated in non-eosinophilic asthma however PLAU was unchanged between asthma subtypes (see Table E3 in the online repository).

DISCUSSION

This 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 had an enhanced release of IL-8 protein 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 there are novel and distinct gene expression profiles that relate to asthma inflammatory phenotype.

Sputum neutrophil cytokine gene expression and protein production was not different in asthma phenotypes. However, airway neutrophils generally had 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 as 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 a decreased release of TNF-α from airway cells have been previously reported in COPD [15], and the mechanisms of this warrant further investigation.

The development of high-throughput screening and genome wide gene expression by microarrays has allowed 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 have altered expression by real time PCR.

Activation of the innate immune response including increased expression of the receptors TLR4, TLR2, CD14 and SP-A and cytokines IL-8 and IL-1β has been demonstrated in the airways of participants with non-eosinophilic asthma [6]. Our data shows altered gene expression profiles and increased IL-8 production of resting blood neutrophils in non-eosinophilic asthma. This could both promote the development of non-eosinophilic airway inflammation, and influence existing non-eosinophilic airway inflammation. Many genes (54%) 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* [16] demonstrated that there is a significant correlation between peripheral blood neutrophil phenotypes and the pulmonary response to endotoxin, that is, 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 either enhanced chemotaxis from the blood and/or enhanced survival of these cells. Here we have shown that peripheral blood neutrophils have 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, S100A8), receptors (e.g. CCRL2) and transcription factors (e.g. SRF). These genes are readily expressed in neutrophils and upon neutrophil activation by LPS [17, 18]. Importantly, expression of IL-8 [6], S100A8 [17] 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 the airways.

Circulating levels of IL-8 can also stimulate the bone marrow to release neutrophils into the circulation [19]. Enhanced release of IL-8 has previously been reported in blood neutrophils isolated from patients with cystic fibrosis [20]. 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 needed to elucidate this.

There is a considerable amount of literature demonstrating that cell fate is regulated at the level of gene expression [21], and that these changes are important in the resolution of inflammatory processes [22]. Particular examples of genes whose expression was increased in non-eosinophilic asthma and relate to a delay in apoptosis include GADD45β, HDAC3, HDAC5, SGK, and CEBPB. Several of these genes are thought to increase cell survival through modulation of the NF-κB pathway [23, 24]. RIPK2 is another important signalling molecule involved in the activation of NF-κB through stimulation of numerous innate immune receptors including TLR2, TLR4, NOD proteins, IL-1R, and IL-18R [25]. IRAK-M (IRAK3), a negative regulator of TLR signalling [26] was upregulated in resting neutrophils in non-eosinophilic asthma in this study, and has recently been linked with the pathogenesis of early-onset persistent asthma [27].

The gene expression changes appear to be clinically relevant since many were correlated with the degree of airway obstruction in asthma, including GADD45β, IRAK3, HM74, MAIL, PI3, STX4A, HLAE, HDAC5 and TNFRSF14. These genes have a variety of functions, including cell signaling (GADD45β, and IRAK3), transcriptional regulation (MAIL and HDAC5), receptor activity (HM74, HLAE and TNFRSF14), protease inhibition (PI3) and protein transport (STX4A). Importantly, several of these genes participate in the regulation of NF-κB activity including GADD45β, IRAK3, MAIL, TNFRSF14, and PI3. This further underscores the importance of this pathway in the mechanisms of noneosinophilic asthma.

Differences in the response to LPS may also play a role in the innate immune defense against invading microorganisms, and may contribute to airway inflammation. This study demonstrated significant alterations in gene expression after LPS stimulation in non-eosinophilic asthma compared to eosinophilic asthma. The genes that were altered suggested that there was a potentiation of LPS responses in non-eosinophilic asthma (e.g PLAU and IL-1R1), and further increases in genes relating to cell survival (e.g. PBEF, TNFAIP3, BRAF, PRKDC, SVIL).

In addition to this, we observed a decrease in the production of OSM protein but not mRNA, from LPS stimulated neutrophils in participants with eosinophilic asthma that was significantly different to healthy controls and also lower than non-eosinophilic asthma. OSM, an IL-6 family cytokine, is thought to promote airway remodeling [28], potentially through increasing the proliferation of both fibroblasts and smooth muscle cells [29] and inducing the production of angiogenic factors such as vascular endothelial growth factor [30]. Neutrophils have an intracellular store of OSM and produce large concentrations of the protein upon stimulation with inflammatory triggers such as LPS [31]. Since differences were found in this study between the production of OSM protein but not gene expression, future studies should measure the levels of OSM within the intracellular stores of the neutrophil.

These findings provide further evidence to show 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 protein, indicating differential activation

of neutrophils in asthma phenotypes. This study highlights the importance of neutrophils in the pathogenesis of non-eosinophilic asthma.

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Table I: Clinical characteristics and induced sputum inflammatory cell counts of healthy controls and eosinophilic and non-eosinophilic asthma

	Healthy Controls	Eosinophilic Asthma	Non-Eosinophilic Asthma	p
n	11	8	9	
Age years, mean (SD)	56 (18)	53 (20)	65 (9)	0.35
Sex M | F	5 | 6	4 | 4	4 | 5	1.0
Atopy n (%)	6 (55)	7 (88)	7 (78)	0.29
FEV_1 % predicted	98 (17)	77 (19)	66 (18)*	<0.01
FEV_1/FVC %	77 (7)	70 (9)	65 (10)*	0.01
ICS ‡‡ dose (µg) median (IQR)	-	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
Total cell count x 10^6/mL	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 (16-41.1)	58.5 (24.5-72)*	0.08
Neutrophils 10^4/mL	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/mL	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/mL	226 (172.8-307.4)	241.5 (189.1-437.3)	238.1 (120.6-402.7)	0.82

‡‡ ICS dose is calculated 1µg of beclomethasone = 1µg of budesonide = 0.5µg of fluticasone.
*p<0.008 versus healthy controls, ‖ versus non-eosinophilic asthma as determined by Kruskal-Wallis non parametric test for significance

Table II: Relative levels of cytokine gene expression and protein production in resting and LPS stimulated sputum neutrophils isolated from participants with eosinophilic (n=6) and non-eosinophilic asthma (n=8) and healthy controls (n=7).

	Resting Sputum Neutrophils				LPS Stimulated Sputum Neutrophils			
	Healthy Controls	**Eosinophilic Asthma**	**Non-Eosinophilic Asthma**	**p**	**Healthy Controls**	**Eosinophilic Asthma**	**Non-Eosinophilic Asthma**	**p**
IL-8 pg/mL	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	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	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

***p<0.008 versus healthy controls as determined by Kruskal-Wallis non parametric test for significance**

Table III: Selected genes with immune related function that were altered in resting neutrophils from participants with non-eosinophilic asthma compared to eosinophilic asthma.

GenBank	Symbol	Name	p value	Fold Change
Cell Motility				
NM_001456.1	FLNA	Filamin A, α	0.04	+ 5.1
NM_003965.3	CCRL2	Chemokine CC motif receptor like 2	0.04	+ 3.4
NM_003131.1	SRF	Serum response factor	0.01	+ 3.2
NM_002964.3	S100A8	S100 calcium binding protein A8	0.04	+ 2.6
NM_000584.2	IL-8	Interleukin-8	0.04	+ 2.4
NM_006000.1	TUBA1	Tubulin alpha	0.04	+ 2.1
NM_003370.1	VASP	Vasodilator stimulated phosphoprotein	0.04	+ 2.0
Apoptosis				
NM_015675.1	GADD45B	Growth arrest and DNA damage inducible β	0.003	+ 3.1
NM_003883.2	HDAC3	Histone deacetylase 3	0.003	+ 2.7
NM_003821.4	RIPK2	Receptor (TNFRSF) interacting serine-threonine kinase 2	0.003	+ 2.6
NM_139205.1	HDAC5	Histone deacetylase 5	0.01	+ 2.5
NM_014330.2	PPP1R15A	Protein phosphatase 1, regulatory (inhibitor) subunit 15A	0.04	+ 2.4
NM_003820.2	TNFRSF14	Tumor necrosis factor receptor superfamily, member 14	0.003	+ 2.2
NM_003375.2	VDAC2	Voltage dependent anion channel 2	0.003	+ 2.2
NM_005627.2	SGK	Serum/glucocorticoid regulate kinase	0.01	+ 2.0
NM_005194.2	CEBPB	CCAAT/enhancer binding protein (C/EBP), beta	0.01	+ 1.7
NM_005118.2	TNFSF15	Tumor necrosis factor (ligand) superfamily member 15	0.01	- 2.1
Immune Related				
NM_014015.3	DEXI	Dexamethasone-induced transcript	0.04	+ 4.9
NM_007199.1	IRAK3	Interleukin-1 receptor associated kinase-3	0.04	+ 4.3
NM_002638.2	PI3	Protease Inhibitor 3, skin-derived (SKALP)	0.01	+ 3.2
NM_012092.2	ICOS	Inducible T cell co-stimulator	0.04	+ 3.1
NM_006018.1	HM74	G protein coupled receptor 109B	0.01	+ 3.0
NM_000247.1	MICA	MHC class I polypeptide related sequence A	0.01	+ 2.8
NM_013439.2	PILRA	Paired immunoglobulin-like type 2 receptor alpha	0.04	+ 2.4
NM_031419.1	MAIL	Molecule possessing ankyrin repeats induced by lipopolysaccharide	0.04	+ 2.4
NM_002117.3	HLA-C	Major histocompatibility complex class I, C	0.003	+ 2.3
NM_004048.2	B2M	Beta 2 microglobulin	0.01	+ 2.2
NM_000433.1	NCF2	Neutrophilic cytosolic factor 2	0.04	+ 2.0
NM_004604.3	STX4A	Syntaxin 4A	0.003	+ 1.8
NM_005516.3	HLA-E	Major histocompatibility complex class I, E	0.01	+ 1.8
NM_001613.1	ACTA2	Actin α2	0.04	+ 1.8
NM_005729.3	PPIF	Peptidylprolyl isomerase F (cyclophilin F)	0.04	+ 1.7
NM_002697.2	POU2F1	POU domain class 2 transcription factor 1	0.003	- 1.5
NM_133280.1	FCAR	Fc fragment of IgA, receptor for	0.04	- 1.6

NM_000896.1	CYP4F3	Cytochrome P450 family 4 subfamily F polypeptide 3	0.01	- 1.6
NM_031483.3	ITCH	Itchy homologue E3 ubiquitin protein ligase	0.003	- 1.7
NM_147134.1	NFX1	Nuclear transcription factor, X-box binding 1	0.04	- 1.9
NM_178509.3	STXBP4	Syntaxin binding protein 4	0.04	- 1.9
NM_147191.1	MMP21	Matrix metalloproteinase 21	0.01	- 1.9
NM_007038.1	ADAMTS5	A disintegrin like and metallopeptidase with thrombospondin type 1	0.003	- 3.3

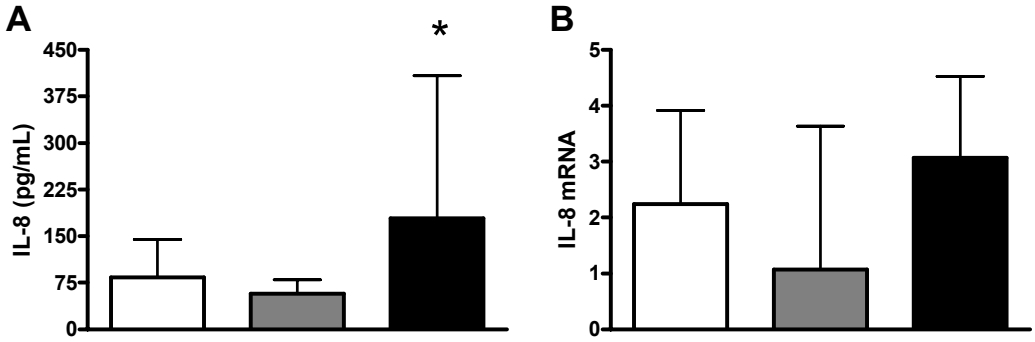
Table IV: Selected genes with immune related function that were altered in LPS stimulated neutrophils from participants with non-eosinophilic asthma compared to eosinophilic asthma.

GenBank	Symbol	Name	p value	Fold Change
NM_002658.	PLAU	Plasminogen activator, urokinase	0.04	+ 3.4
NM_012342.	BAMBI	BMP and activin membrane bound	0.01	+ 3.1
NM_001618.	PARP1	Poly (ADP-ribose) polymerase family member 1	0.01	+ 2.5
NM_006904.	PRKDC	Protein kinase, DNA activated, catalytic polypeptide	0.003	+ 2.4
NM_000181.	GUSB	Glucuronidase β	0.04	+ 2.3
NM_005723.	TM4SF9	Tetraspanin 5	0.04	+ 2.3
NM_005746.	PBEF	Pre B cell colony enhancing factor	0.04	+ 1.9
NM_000877.	IL1R1	Interleukin-1 receptor type 1	0.01	+ 1.9
NM_006290.	TNFAIP	Tumor necrosis factor alpha induced protein 3	0.003	+ 1.8
NM_005063.	SCD	Stearoyl-CoA desaturase (delta-9-desaturase)	0.04	+ 1.7
NM_033405.	PRIC285	Peroxysomal proliferator activated receptor A, interacting	0.04	+ 1.6
NM_006534.	NCOA3	Nuclear receptor coactivator 3	0.04	+ 1.6
NM_002514.	NOV	Nephroblastoma overexpressed gene	0.04	+ 1.6
NM_004333.	BRAF	v-raf murine sarcoma viral oncogene homologue B1	0.04	+ 1.5
NM_021738.	SVIL	Supervillan	0.04	+ 1.5
NM_003153.	STAT6	Signal transducer and activator of transcription-6	0.04	+ 1.5
NM_024535.	FLJ2202	Coronin 7	0.04	- 1.5
NM_005981.	TSPAN3	Tetraspanin 31	0.04	- 1.7
NM_016150.	ASB2	Ankyrin repeat and SOCS box containing 2	0.04	- 1.7
NM_198291.	SRC	V-src sarcoma (Schmidt-Ruppin A-2) viral oncogene	0.003	- 2.4
NM_058171.	ING2	Inhibitor of growth family, member 2	0.04	- 2.8
NM_003265.	TLR3	Toll-like receptor 3	0.04	- 2.9
NM_145898.	CCL23	Chemokine (CC motif) ligand 23	0.003	- 5.9

Figure Legends

Figure 1: IL-8 protein production (A) and gene expression (B) in resting blood neutrophils in non-eosinophilic asthma (black bars, n=9), eosinophilic asthma (grey bars, n=8), and healthy controls (white bars, n=11). Data is displayed as median with the error bar as the 3rd quartile. *p<0.05 versus eosinophilic asthma.

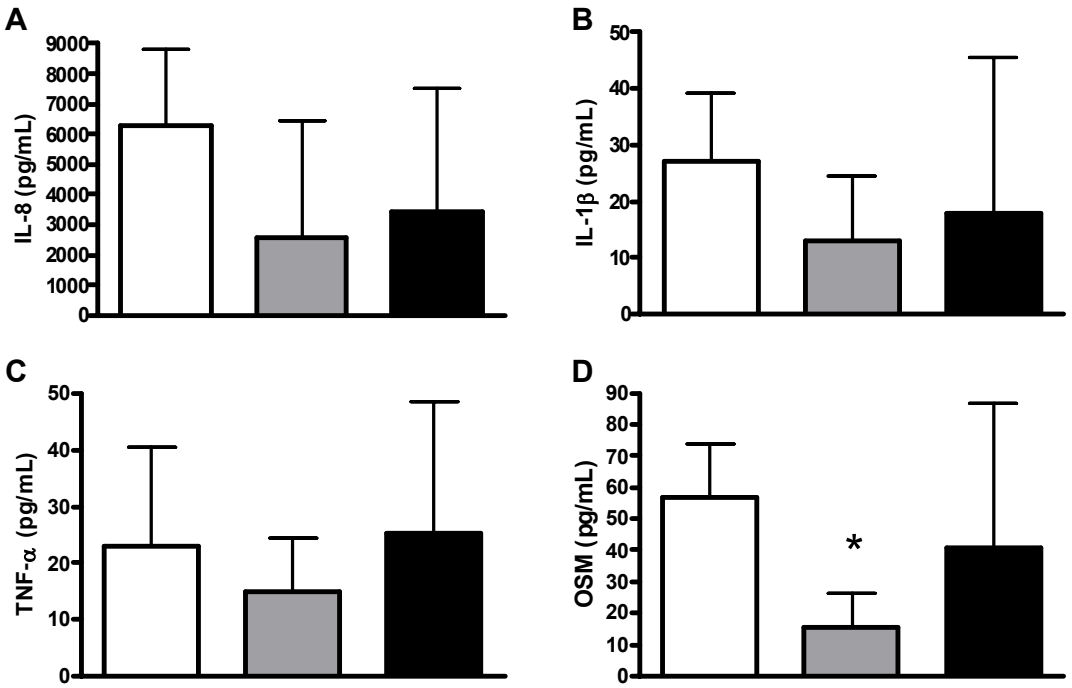
Figure 1



*p<0.008 versus eosinophilic asthma.

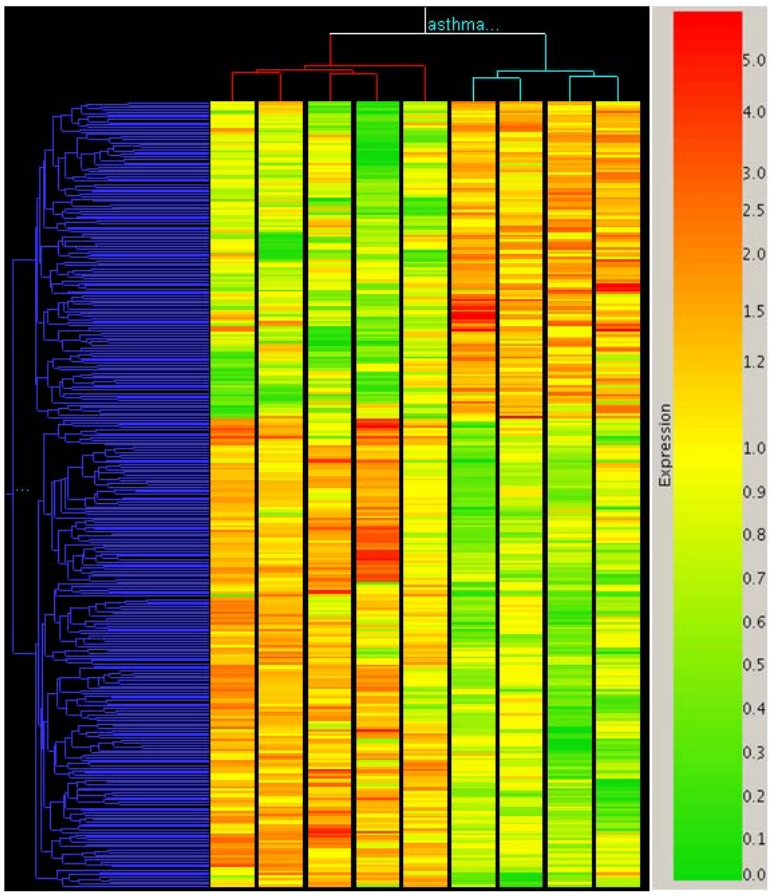
Figure 2: Cytokine production (A: IL-8, B: IL-1β, C: TNF-α, D: OSM) from LPS stimulated blood neutrophils in non-eosinophilic asthma (black bars, n=9), eosinophilic (grey bars, n=8), and healthy controls (white bars, n=11). Data is displayed as median with the error bar as the 3rd quartile.
*p<0.05 versus eosinophilic asthma.

Figure 2



*p<0.008 versus healthy controls

Figure 3: Gene expression profiles of resting neutrophils from participants with eosinophilic asthma (n=5) versus those with non-eosinophilic asthma (n=4). The dendrogram at the top of the figure represents the relationship between participants with non-eosinophilic (blue branches) and eosinophilic asthma (red branches). The dendrogram on the left side represents the relationship between the expression levels of each gene across all the samples.


asthma...
Expression
5.0
4.0
3.0
2.5
2.0
1.5
1.2
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0