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INNATE IMMUNE ACTIVATION IN NEUTROPHILIC ASTHMA AND BRONCHIECTASIS

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Abstract

The role of the innate immune system in the pathogenesis of asthma is unclear. Activation of innate immune receptors in response to bacterial lipopolysaccharide, virus infection and particulate matter, triggers a pre-programmed inflammatory response, which involves IL-8 and neutrophil influx. The inflammatory response in asthma is heterogeneous and this study tested the hypothesis that innate immune activation may be a relevant inflammatory mechanism in neutrophilic asthma where IL-8 levels are increased. Methods: Induced sputum was obtained from non-smoking adults with asthma (n=49), healthy controls (n=13) and a positive reference group with bronchiectasis (n=9). Subjects with asthma were classified into inflammatory subtypes using induced sputum cell counts. Sputum was examined for mRNA expression of the innate immune receptors TLR2, TLR4 and CD14, and inflammatory cytokines. A separate sputum portion was dispersed and supernatant assayed for surfactant protein A, IL-8, soluble CD14 and endotoxin.

Results**:** Expression of innate immune receptors was increased in bronchiectasis and in subjects with neutrophilic asthma compared to other asthma subtypes and healthy controls. There was increased expression of the receptors TLR2, TLR4 and CD14 as well as the pro-inflammatory cytokines IL-8 and IL-1β. Subjects with neutrophilic asthma had higher airway levels of endotoxin compared to other groups studied.

Conclusion: There is evidence of activation of the innate immune system in asthma, which results in the production of pro-inflammatory cytokines, and may contribute to the pathogenesis of neutrophilic asthma.

Introduction

The role of the innate immune system in the pathogenesis of asthma is unclear, but may be relevant to the heterogeneous inflammatory response that occurs in asthma. [1-5] Acquired immune responses in asthma are well characterised and involve allergen-induced Th_2 lymphocyte activation and consequent eosinophilic airway inflammation. Activated eosinophils release potent cytotoxic granules such as major basic protein, and eosinophil cationic protein, which induce airway hyperresponsiveness (AHR) and symptoms. [6, 7] Recently, non-eosinophilic inflammatory subtypes of asthma have been identified [3, 4, 8-16] where symptoms and AHR persist in the absence of increased sputum eosinophils. The mechanisms of non-eosinophilic asthma and more particularly neutrophilic asthma, are not well characterised, however a potential role for neutrophils and IL-8 has been reported. [3, 16] IL-8 mediated neutrophil influx frequently occurs with NF-κB activation, and represents a 'pre-programmed' response that has been conserved throughout evolution, [5] and is typically seen with activation of the innate immune system. [17] This suggests that neutrophilic asthma may involve activation of the innate immune system.

The innate immune system is rapidly activated by pathogen associated molecular patterns (PAMPs). PAMPs such as lipopolysaccharide (LPS) are highly conserved structures common to many microorganisms. They are recognised by pattern recognition receptors such as the toll like receptors (TLR), CD14 and collectins which include pulmonary surfactant proteins. [17] TLR activation triggers a signalling cascade leading to the activation and nuclear translocation of NF-κB resulting in pro-inflammatory cytokine response including TNF-α, IL-8 and IL-1β. [1, 18]

This study questioned whether activation of the innate immune system was a feature of neutrophilic asthma and tested the hypothesis that subjects with asthma and a neutrophilic inflammatory subtype would have activation of the innate immune response characterised by increased expression of innate pattern recognition receptors TLR2, TLR4, SP-A and CD14 and a corresponding cytokine response. In addition, we assessed whether levels of sputum LPS and bacteria were associated with asthma subtype.

Materials and Methods

Subjects and Design

A cross-sectional study design was used. Non-smoking subjects with asthma (n=49, ATS criteria) [19] had a clinical diagnosis of symptomatic asthma and AHR to hypertonic saline. Healthy controls (n=13) without respiratory disease had an FEV_1 >80% of predicted, [20] and normal airway responsiveness. Subjects with bronchiectasis (High Resolution Computed Tomography confirmed (HRCT), n=9) were recruited as a positive reference group. All subjects were stable (no lower respiratory tract infection or exacerbation of respiratory disease in the previous 4 weeks) at the time of assessment. Subjects underwent clinical assessment, spirometry, combined hypertonic saline challenge and sputum induction. [21] Subjects were recruited from the Respiratory Ambulatory Care Service at John Hunter Hospital and by advertisement and gave written informed consent. Subjects with neutrophilic asthma underwent HRCT scans to exclude the presence of co-existing bronchiectasis. The Hunter Area Health Service and The University of Newcastle Research Ethics Committee's approved this study.

Sputum Induction

Spirometry (KoKo PD Instrumentation Louisville CO USA) and combined bronchial provocation testing and sputum induction with hypertonic saline (4.5%) were performed as previously described. [21] Sputum induction was performed using normal (0.9%) saline in 12 (23%) subjects with asthma and 2 (22%) subjects with bronchiectasis where the post bronchodilator FEV_1 was <1.5L. A fixed sputum induction time of 15 minutes was used for all subjects.

Sputum Analysis

100μL of selected sputum was transferred to RNA extraction buffer (QIAGEN, Hilden Germany) and stored at -80°C. RNA was prepared as described previously [16] and below. RNA purity and titre were determined by spectrophotometry (Cary 50; Varian Inc, Palo Alto CA USA) and 100ng of RNA was applied to subsequent RT-PCR reactions.

RNA Preparation: RNA was prepared using the RNeasy Mini Kit (QIAGEN, Hilden Germany). Random primers were combined with sample RNA and a 20μL reverse transcription mix containing 0.5 mM dNTPs, 5mM DTT, 50mM Tris-HCl pH 8.3, 75mM KCl and 2U RNaseOUT™. Following incubation the mix was supplemented with 100U Superscript II™ RNase and incubated at 42°C for 50 min. Following enzyme inactivation the cDNA was stored at –20°C. All RT-PCR reagents other than dNTPs (Promega Corporation; Madison, WS, USA) were purchased from Invitrogen (Carlsbad CA USA).

RNA extraction and Reverse Transcription: Primers for TLR2 [22] were custom made (Qiagen, Hilden Germany) and the probe was designed using Primer 3.0 software (http://www.premierbiosoft.com/netprimer/netprimer.html). All other primers and probes were purchased in kit form (Applied Biosystems, Foster City CA USA).

*Semi-quantitative real time PCR :*Multiplex PCR of each target and the endogenous reference (18-S ribosomal RNA) was performed containing 2uL of the sample cDNA, 300nM each of target primers, 100nM of probe and a commercial polymerase solution (Universal PCR Master Mix; Applied Biosystems, Foster City, CA, USA). Each sample was subject to amplification with the following parameters: 2 min at 50°C, 10 min at 95°C and 40 cycles of 95°C for 15 s followed by 1 min at 60°C (ABI GeneAmp 7700 cycler, Perkin-Elmer). The amount of target was calculated relative to a positive calibrator expressing the target of interest and normalised to the endogenous control (18-S). [23]

Specific Target Positive Calibrators: Peripheral blood mononuclear cells and granulocytes were isolate using Percoll separation (Amersham, Castle Hill NSW Australia) and cultured in the presence of LPS (100ng/mL, Sigma St Louis MO USA) or PHA (5μg/mL, Sigma St Louis MO USA) for 6 hours at 37ºC, 5% CO_2. Stimulated cells were stored in RNA extraction buffer at –80ºC for RNA extraction.

An aliquot of sputum was used for bacteriological culture. Chocolate and blood agar plates were inoculated with induced sputum (10μL) and transported with prepared sputum smears for Gram staining and identification to the Microbiology Department of the Hunter Area Pathology Service for culture and reporting. The remaining selected sputum was dispersed using dithiothreitol as described. [21] The suspension was filtered, and a total cell count (TCC) of leucocytes and viability performed. Following centrifugation, supernatant was aspirated and stored at -80°C. Cytospins were prepared, stained (May-Grunwald Geimsa) and a differential cell count obtained from 400 non-squamous cells. TLR protein expression was assessed in sputum cytospins (APAAP technique) with an anti-human TLR2 (Cline TLR2.1) and TLR4 (Clone HTA125)

antibodies (Serotec, Oxford UK) as described [24]. Cytospins were fixed using PLP (paraformaldehyde, l-lysine, periodate) and allowed to air dry before being immersed in sucrose solution for storage. [25] Slides were stored at -20ºC until immunocytochemistry was undertaken. Briefly, slides were thawed and washed in TBS (H+S). Slides were blocked using 20% normal rabbit serum (Dako) for 20 minutes. Primary antibody was applied and slides were incubated at room temperature for 30 minutes. Slides were then washed before application of link rabbit anti mouse immunoglobulins (Dako). Mouse monoclonal APAAP (Dako) was applied after removal of excess link antibody by washing. Excess APAAP was removed by washing before application of liquid permanent red substrate (Dako). A substrate control was included which had TBS (H+S) applied at each step until substrate was added. Slides were observed for colour development and substrate action was stopped by a distilled water wash, when the positive control had developed a strong red colour. A hematoxylin counter stain was applied and slides were air dried before being coverslipped using DPX (Sigma).
Positive control slides: Peripheral blood granulocytes were isolated using Percoll centrifugation (Amersham Uppsala Sweden) and stimulated with 100ng/mL LPS (TLR4) or GM-CSF (TLR2) for 2 hours. Cytospins prepared from the cell suspension were fixed in PLP and allowed to air dry before being immersed in sucrose solution for storage. [25] Slides were stored at -20ºC for subsequent immunocytochemistry.

Asthma Subtype classification
The upper limit of normal for sputum eosinophil and neutrophil counts was taken as the 95th percentile of a healthy control population studied. [16] Subjects with a sputum neutrophil count ≥61% were classified as having neutrophilic asthma, subjects with sputum eosinophils ≥1% alone were classified as eosinophilic asthma and subjects with sputum neutrophils <61% and sputum eosinophils <1% were classified as paucigranulocytic asthma. Based on prior work, subjects with both increased neutrophils and eosinophils were classified as neutrophilic asthma. [16]

Sputum Fluid Phase Mediator Assessment
Determination of IL-8 was by ELISA (R&D Systems, Minneapolis MN USA). Active neutrophil elastase (NE) was measured using a chromogenic substrate specific for human NE, n-methoxysuccinyl-l-alanyl-l-alanyl-prolyl-l-valyl-p-nitroanilide (Sigma, St Louis, MO USA) as previously described. [26] Both IL-8 and NE have been previously validated for assessment in induced sputum. [27] Surfactant protein A (SP-A) concentration was determined by adapting a previously described method. [28] SP-A antigen and antibody were a gift from Dr Ian Doyle, Flinders University SA, Australia. The mean recovery of SP-A (110 ng/mL) added to sputum supernatant processed using DTT dispersion was mean (range) 84% (70% to 99%). There was no effect of DTT on the standard curve. There was good agreement in the SP-A concentrations measured in sputum supernatants diluted 1 in 12 or diluted 1 in 24 with reagent diluent (PBS BSA) (n=8), with all samples within the Bland Altman limits of agreement (mean bias +/- 2SD). The co-efficient of variance was 40.6%. Supernatant measurements were performed using coded samples by a technician blinded to the subject's inflammatory group.

Airway Endotoxin
Endotoxin in sputum supernatant was assayed with a quantitative kinetic chromogenic *Limulus* Amebocyte Lysate (LAL) method (Kinetic QCL no- 50-650 U; Bio Whittaker; LAL Lot no. 3L2360; CSE Lot no. 2L4900 and Lysate Lot no. 3L085D) at 37°C. [29] Inhibition or

enhancement of the LAL assay was not detectable at sample dilutions of 50 times or higher, tested as described. [30] Due to limited supernatant samples, endotoxin levels were assessed in n=6 neutrophilic asthma, n=24 eosinophilic asthma, n=10 paucigranulocytic asthma, n=6 healthy controls and all bronchiectasis samples.

Data Analysis

Data were analysed using Stata 7 (Stata Corporation, College Station, Texas USA), with results reported as median and interquartile range unless otherwise indicated. Analysis was performed using the two-sample Wilcoxon Rank Sum test or Kruskal-Wallis test for more than two groups with bonferroni correction. Fischers' exact test was used to analyse categorical data. Associations between data were determined using Spearman's rank correlation. Results were reported as significant when $p<0.05$.

Results

Clinical Features

Seven (14%) of the 49 subjects with asthma had neutrophilic asthma; 26 (53%) had eosinophilic asthma and 16 (33%) had paucigranulocytic asthma. The subjects with asthma had similar clinical characteristics across the 3 inflammatory subtypes (Table 1). The severity of airflow obstruction was similar in subjects with asthma and bronchiectasis, and AHR was present in subjects with asthma, but not in controls or bronchiectasis (Table 1). Three subjects in the neutrophilic asthma group had a mixed granulocytic pattern of inflammation with increased neutrophils and eosinophils.

While not statistically significant, subjects with neutrophilic asthma tended to have a lower FEV_1 % predicted and FEV_1/FVC than subjects with eosinophilic asthma and paucigranulocytic asthma (Table 1).

Table 1: Clinical characteristics of subjects with neutrophilic, eosinophilic and paucigranulocytic asthma, bronchiectasis and healthy control subjects.

	Neutrophilic Asthma	Eosinophilic Asthma	Paucigranulocytic Asthma	Bronchiectasis	Healthy Controls	p*
N	7	26	16	9	13	
Age years, mean (range)	64 (40-72)	54 (24-70)	56 (22-80)	56 (21-72)	41 (21-67)†	0.021
Sex M | F	2 | 5	7 | 19	5 | 11	2 | 7	4 | 9	1.000
Atopy n (%)	7 (100)	21 (81)	11 (69)	6 (67)	7 (54)	0.182
Ex-Smoker n (%)	4 (57)	8 (31)	6 (38)	2 (22)	1(8)	0.176
Pack years, mean (range)	34 (22-42)	42 (32-86)	14.5 (0.5-22)	6.4 (5.7-7)	0.5	0.105
FEV_1% predicted, post bronchodilator mean (sd)	65 (26)	83 (19)	81 (25)	73 (21)	107 (13)‡	<0.001
FEV_1/FVC % mean (sd)	62 (13)	69 (10)	68 (12)	66 (7)	84 (5)‡	<0.001
Dose response slope ¶ median (IQR)	5.1 (0.8-13.9)	3.5 (1.2-12.1)	2.9 (1.3-8.6)	0.9 (0.5-1.2)	0.4 (0.2-0.6)††	<0.001
Duration of Asthma, years mean (range)	38 (14-71)	22 (0.5-57)	33 (3-61)	-	-	
ICS dose µg ‡‡ median (IQR)	1600 (500-2000)	2000 (1000-2000)	1800 (1000-2000)	1300 (800-2000)	-	
Culture positive n (%)	3 (43)	2 (8)	0 (0)	5 (56)	-	<0.001

* Kwallis2 or one way ANOVA (age, FEV_1% predicted, FEV_1/FVC) p value for 5 group analysis. ‡ Bonferroni corrected $p<0.05$ versus asthma and bronchiectasis, † Bonferroni corrected $p<0.05$ versus neutrophilic asthma, †† $p<0.0025$ kwallis2 p value versus eosinophilic and paucigranulocytic asthma. ¶Dose response slope: (%fall FEV_1/mL 4.5% saline), healthy n=12, neutrophilic asthma n=4, eosinophilic asthma n=20, paucigranulocytic asthma n=10, bronchiectasis n=4. ‡‡ICS dose is calculated a 1µg of beclomethasone=1µg budesonide=0.5µg fluticasone.

Inflammatory Cells

Subjects with neutrophilic asthma had an increased total cell count, and an increased proportion and number of neutrophils compared to subjects with eosinophilic asthma, paucigranulocytic asthma, and healthy controls (Table 2). Absolute macrophage counts were similar between groups, indicating that the reduced proportion of macrophages occurred due to the high neutrophil proportion and the expression of the results as a percentage. There was no correlation with sputum neutrophils and age. Those with eosinophilic asthma had an increased proportion and number of eosinophils and increased neutrophil proportion compared to those with paucigranulocytic asthma. Subjects with paucigranulocytic asthma showed no differences in inflammatory cell counts compared to healthy controls.

Table 2: Inflammatory cell counts for subjects with neutrophilic, eosinophilic and paucigranulocytic asthma, bronchiectasis and healthy control subjects. Results shown are median (interquartile range).

	Neutrophilic Asthma	Eosinophilic Asthma	Paucigranulocytic Asthma	Bronchiectasis	Healthy Controls	p*
Total cell count x 10^6/mL	8.4 (6.6-9.7)‡§‖	2.4 (1.0-4.4)	1.4 (1-2.1)	13.1 (3.9-22.2) ‡‖	1.6 (0.8-2.4)	0.0001
Viability	88 (87-97)‡‖	72 (59-80)	50 (28-82)	92 (67-96)	60 (30-80)	0.003
Neutrophils, %	79.8 (70.3-94.3) ‡§‖	29.1 (13.5-36)	9.9 (6.3-25.9)	65.3 (36.1-87.9) ‡‖	13.9 (6.8-22.2)	0.0001
Neutrophils 10^4/mL	632 (589-816) ‡§‖	48.5 (30.7-104) ‖	13.1 (6.3-29.8)	757 (153-1930) ‡‖	16.9 (8.4-33.4)	0.0001
Eosinophils, %	1 (0.3-1.75)	3 (1.5-5) ‡‖°	0.13 (0-0.5)	0.13 (0-0.8)	0 (0-0)	0.0001
Eosinophils 10^4/mL	4.3 (1.6-14.3) ‡‖	4.8 (1.6-25.7) ‡‖	0.1 (0-0.8)	0.4 (0-5.5)	0 (0-0)	0.0001
Macrophages, %	17.5 (5.5-26.2)‡§‖	61.3 (46.8-75.8)	77.2 (59.8-86.4)	31.4 (11.8-53.5) ‡‖	78 (66.9-86.4)	0.0001
Macrophages 10^4/mL	148 (36.1-175)	102 (68.6-306)	80.4 (58-123)	490 (50.2-764)	128 (63.1-200)	0.151
Lymphocytes, %	0.3 (0-0.8)	0.7 (0-1.5)	0.7 (0-1.8)	0.6 (0.3-1.1)	0.6 (0-1.1)	0.835
Lymphocytes 10^4/mL	0.7 (0-6.4)	1.1 (0-4.3)	0.5 (0-1.9)	5.5 (2.7-10)	1.1 (0-3.1)	0.162
Columnar epithelial cells, %	0 (0-0)‡§‖	2.7 (1-7)	6.6 (3.1-12.5)	0 (0-6.5)	3.6 (0.3-14.1)	0.0006
Columnar epithelial cells 10^4/mL	0 (0-0) §‖	4.7 (1.8-13.7)	8.6 (5-13.9)	0 (0-7.7)	0.5 (0.4-16.4)	0.002
Squamous cells, %	1.2 (0-5.2)	1.7 (0.7-10.3)	9.4 (4.9-16.8)§°	1.1 (0.4-6.8)	8.9 (2.9-20.7)	0.003

* kwallis2 p for 5 group analysis. kwallis2 p<0.0025 ‡ versus healthy controls § versus eosinophilic asthma, ‖ versus paucigranulocytic asthma

Innate Immune Receptor Expression

There was increased mRNA expression of TLR2, TLR4 and CD14 in neutrophilic asthma compared to eosinophilic and paucigranulocytic asthma. SP-A levels were also significantly higher in subjects with neutrophilic and eosinophilic asthma compared to healthy controls (Figure 1). Expression of TLR2 and CD14 mRNA were significantly higher in neutrophilic asthma compared to healthy controls. While mRNA expression of TLR2, TLR4 and CD14 was higher in subjects with neutrophilic asthma, these subjects were not statistically different to the positive reference subjects with bronchiectasis, who demonstrated increased TLR2 mRNA and SP-A protein in induced sputum compared to healthy controls ($p<0.05$, Figure 1). There were no differences in soluble CD14 levels between any subject groups studied. TLR immunocytochemistry indicated the presence of TLR2 and TLR4 on sputum macrophages and neutrophils.

Subjects with eosinophilic asthma had increased levels of SP-A compared to healthy controls (Figure 1) but similar levels of all other innate immune receptors and inflammatory markers (Figures 1, 2). Subjects with paucigranulocytic asthma were similar to healthy controls for all of the innate immune activation and inflammation markers assessed here.

Innate Immune Cytokines

Signature cytokines of innate immune activation assessed included IL-8, IL-1β and TNF-α. Subjects with neutrophilic asthma had increased mRNA expression of IL-8, TNF-α and IL-1β as well as increased IL-8 protein compared to paucigranulocytic asthma. While IL-1β and TNF-α mRNA levels were higher in neutrophilic asthma, only IL-8 mRNA levels were significantly increased in comparison to eosinophilic asthma. Fluid phase protein levels and mRNA expression of IL-8 were significantly elevated compared to healthy control subjects in those with neutrophilic asthma and bronchiectasis (Figure 2). IL-1β and TNF-α mRNA expression tended to be higher in neutrophilic asthma and bronchiectasis when compared to healthy controls; however these levels did not reach statistical significance. IL-1β and TNF-α protein levels could not be assessed in the sputum supernatant due to the effect of DTT on measurement of these cytokines.

Neutrophil elastase was detected in 67% of sputum samples from subjects with neutrophilic asthma compared to 4% in eosinophilic asthma ($p<0.002$, Figure 4). No samples from subjects with paucigranulocytic asthma or healthy controls had detectable NE. A similar proportion of subjects with bronchiectasis had NE in their sputum supernatant (67%).

Innate Immune Agonists

Endotoxin and bacterial colonisation were examined as potential activators of the innate immune response. Airway endotoxin levels were very high in neutrophilic asthma (median 5797 Endotoxin Units (EU)/mL, Figure 3) and tended to be higher than subjects with eosinophilic asthma (median 955 EU/mL) and paucigranulocytic asthma (median 729 EU/mL), however these results did not reach statistical significance. Similarly levels were lower in subjects with bronchiectasis (median 1807 EU/mL), and healthy controls (median 738 EU/mL).

There was a significant negative correlation between airway endotoxin levels and airway obstruction (FEV_1/FVC%, r=-0.38, p=0.0024). Also sputum neutrophil proportion (r=0.33,

p=0.035) and IL-8 protein levels (r=0.33, p=0.036) were positively associated with airway endotoxin in subjects with asthma (Table 4b).

Subjects with neutrophilic asthma had a greater prevalence of airway bacterial colonisation (43% with a positive bacteria culture) compared to the other asthma subtypes (8% and 0% eosinophilic and paucigranulocytic respectively, Table 1 and 3). Five of the nine subjects studied with bronchiectasis had evidence of chronic colonisation by common Gram negative respiratory pathogens (*Haemophilus influenzae* and *Pseudomonas aeruginosa*). No subjects returned a positive PCR results for human rhinovirus or respiratory syncytial virus (data not shown).

Table 3: Bacteria identified in subjects with asthma

Patient number	Inflammatory subtype	Bacteria isolated
21	Neutrophilic	*H. influenzae*
27	Neutrophilic	*H. influenzae*
54	Neutrophilic	*H. influenzae*
59	Eosinophilic	*H. influenzae*
98	Eosinophilic	*P. aeruginosa*

Associations

TLR2 mRNA levels were associated with TLR4 and CD14 mRNA (Table 4a) and also correlated with IL-8 mRNA and protein, TNF-α, IL-1β mRNA and neutrophil proportion. TLR4 mRNA levels were strongly associated with CD14 mRNA and TNF-α mRNA (Table 4). Weaker significant correlations were observed with IL-8 mRNA and neutrophil proportion. SP-A levels were significantly associated with total cell count (Table 4) and weakly with IL-8 protein levels (Table 4). IL-8 mRNA levels were significantly associated with IL-8 protein levels, IL-1β and TNF-α mRNA levels (Table 4). There was no correlation between CD14 mRNA levels and soluble CD14 protein levels (p>0.05). Endotoxin levels in sputum supernatant were positively associated with sputum neutrophil proportion, and IL-8 protein levels (Table 4b).

Table 4a: Correlation matrix for innate immune activation receptors in subjects with asthma

	TLR2 mRNA	TLR4 mRNA
TLR2 mRNA	-	0.41*
TLR4mRNA	0.41*	-
CD14 mRNA	0.39*	0.58*

Table 4b Correlation matrix for inflammatory response markers in subjects with asthma

	TLR2 mRNA	SP-A μg/mL	TLR4 mRNA	IL-8 mRNA	Endotoxin EU/mL
IL-8 ng/mL	0.45*	0.37*	0.10	0.62*	0.34*
Neutrophil, %	0.54*	0.14	0.30	0.71*	0.29*
Total cell count x 10^6/mL	0.25*	0.53*	0.11	0.39*	0.17
IL-8 mRNA	0.65*	0.17	0.32	-	0.41
TNF-α mRNA	0.49*	-0.03	0.51*	0.51*	-0.01
IL-1β mRNA	0.41*	0.17	0.42*	0.67*	-0.15

* Spearman $p<0.05$

Discussion

This is the first clinical study to examine innate immune responses in the airways of subjects with asthma and bronchiectasis. Neutrophilic asthma was associated with an upregulation of the innate immune response. In particular, we found that neutrophilic asthma was characterised by increased expression of several key innate immune receptors: TLR2, TLR4, CD14 and SP-A as well as pro-inflammatory cytokines IL-8 and IL-1β. We also found high levels of airway endotoxin in subjects with neutrophilic asthma. Innate immune activation may therefore be a key mechanism in the development of neutrophilic asthma.

Subjects with bronchiectasis were used in this study to provide a positive reference group, and this represents the first report of innate immune markers in bronchiectasis. These subjects were expected to have innate immune activation due to their chronic bacterial infection and chronic airway inflammation with neutrophils. Persistent bacterial colonisation of the airways in bronchiectasis is due to impaired mucociliary transport and mucus clearance and initiates a vicious cycle of inflammation characterised by activated neutrophils and neutrophil proteases. [31] Upon persistent exposure to PAMPs, we hypothesised and confirmed that innate receptors would be stimulated and activated to induce the production of IL-8 and an influx of neutrophils. Subjects with bronchiectasis had increased sputum mRNA expression of TLR2 and supernatant SP-A compared to healthy controls. There was no difference in TLR4 mRNA expression suggesting that there may be differential expression of toll like receptors in bronchiectasis.

Innate immune activation appears to be an important pro-inflammatory mechanism in neutrophilic asthma. While the pathway leading to eosinophilic asthma is well characterised, the mechanisms of neutrophilic inflammation in asthma are poorly understood. Although neutrophils were thought to be primarily involved in severe asthma, [32, 33] recently increased neutrophil levels have also been shown in stable asthma. [16, 34, 35] Increased neutrophil levels in those studies were not caused by respiratory tract infections since subjects with a reported lower respiratory tract infection in the preceding month were excluded. In the study by Green and co-workers, [35] subjects with neutrophilic asthma were older, had a later onset of disease and were less atopic than subjects with normal levels of neutrophils. Our current study was too small to assess these differences. However it is important to note that neutrophilic asthma has been described in people with mild, moderate and severe asthma, and it is not merely a feature of severe asthma with fixed airflow obstruction. [6,13].

Sputum TNF-α mRNA levels were also highest in subjects with neutrophilic asthma and bronchiectasis and TNF-α mRNA was significantly correlated to both TLR4 and TLR2 mRNA expression. Recently TNF-α has been implicated in the upregulation of TLR2 expression in epithelial cells and therefore may be an important cytokine in the perpetuation of innate immune activation in the airways. [32] Further, a recent randomised controlled trial of a soluble TNF-α receptor in people with severe refractory asthma has shown improvement sin a number of asthma outcomes including asthma control score and AHR, highlighting the potentially important pathogenic role of TNF-a in difficult asthma. [33]

The inflammatory cell counts in neutrophilic asthma were similar to bronchiectasis, and in addition, both groups had an increased frequency of chronic bacterial colonisation of the airways when compared with the other asthma subtypes. This indicates that neutrophilic asthma and

bronchiectasis have a similar pattern of airway inflammation, with evidence of innate immune activation. Sputum endotoxin levels were high in subjects with neutrophilic asthma, which suggests that endotoxin may be the source of PAMPs driving the innate response in this group. The levels were between 6 and 8 fold higher than those observed in the other asthma subtypes studied. In contrast to subjects with bronchiectasis, there was increased mRNA expression of both TLR2 and TLR4 expression in subjects with neutrophilic asthma compared to both eosinophilic and paucigranulocytic asthma. While TLR4 has been identified as the primary receptor for bacterial LPS, recent studies have shown that TLR2 is also capable of responding to LPS. [36] This may explain why both TLR2 and TLR4 were up regulated. Alternatively, other PAMPS or cytokines known to up-regulate TLR2 expression may play a role.

LPS signaling is complex and involves a number of accessory proteins including LPS binding protein and CD14. Subjects with neutrophilic asthma had increased mRNA expression of CD14 and TLR4 compared to healthy controls, subjects with eosinophilic and paucigranulocytic asthma and those with bronchiectasis. The reason for this increase in neutrophilic asthma (which was not observed in bronchiectasis) is unclear, but may be due to the higher levels of endotoxin measured in the sputum supernatant, which may be upregulating the expression of LPS signaling proteins. There was also a strong positive correlation between TLR4 mRNA and CD14 mRNA in subjects with asthma, suggesting that the expression of these proteins is co-regulated.

The roles of gene polymorphisms in determining innate immune responses are conflicting. The TLR4 polymorphism Asp299Gly has been associated with hyporesponsivenss to airway challenge with LPS [37] and increased risk of Gram-negative infection [38]. In another study, similar rates of chronic infection were reported between the subjects with a TLR4 polymorphism and wild type TLR4, indicating a role for this polymorphism in risk of acute infection but not in chronic infection [39]. The same TLR4 polymorphism has been associated with asthma in Swedish children [40] but no association was found between TLR4 polymorphisms and a diagnosis of asthma in a large adult study [41]. A polymorphism in the CD14 promoter has been associated with increased soluble CD14 expression and lower IgE levels in serum [42], while polymorphisms of the LPS binding protein gene have been associated with increased risk of sepsis in association with male gender [43]. Regardless of this conflicting data, it is plausible that a polymorphism in innate immune receptors and associated proteins (CD14 and LBP) may explain the variation in immune and inflammatory responses reported.

SP-A functions primarily as an opsonin, identifying targets for phagocytosis and binding of pathogens *in vitro*. In SP-A null mice, exposure to pathogens including viruses results in an increased neutrophilia, epithelial injury and persistence of infection compared to control mice, indicating a protective role for surfactant proteins in lung defence. [44] In this study levels of SP-A were increased in subjects with eosinophilic and neutrophilic asthma compared to healthy controls, indicating host defence activity in these subjects. SP-A did not appear to be a specific marker of innate immune activation as levels were similar between the asthma subtypes. Although levels of SP-A are increased, it is unknown if the SP-A is functional and intact. Neutrophil proteases present in airway fluids degrade SP-A and reduce its antimicrobial and anti-inflammatory functions. SP-A levels are also increased in bronchial lavage from subjects with asthma. [45] Further investigation is required to determine if the integrity of the SP-A from the airways of subjects with asthma is maintained.

In summary, we conclude that persistent activation of the innate immune system in stable asthma results in the production of pro-inflammatory cytokines, which may contribute to the pathogenesis of neutrophilic asthma. This is an important observation that identifies a specific mechanism operating in the neutrophilic subtypes of non-eosinophilic asthma. This adds to our understanding of the heterogeneity of airway inflammation in asthma. The mechanisms of this persistent activation are unclear but may be related to endotoxin exposure or chronic bacterial colonisation of the lower airways.

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Competing Interests
None declared
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Legends

Figure 1: Sputum innate pattern recognition receptors in subjects with neutrophilic asthma, eosinophilic asthma, paucigranulocytic asthma, bronchiectasis and healthy controls. Horizontal bars represent median result. *†‡ kwallis2 $p<0.0025$ versus eosinophilic asthma, paucigranulocytic asthma, and healthy controls respectively.

a) TLR2 mRNA expression
b) TLR4 mRNA expression
c) SP-A protein levels
d) CD14 mRNA expression

Figure 2: Sputum proinflammatory cytokines in subjects with neutrophilic asthma, eosinophilic asthma, paucigranulocytic asthma, bronchiectasis and healthy controls. Horizontal bars represent median result. *†‡ $p<0.0025$ versus eosinophilic asthma, paucigranulocytic asthma, and healthy controls respectively.

a) IL-8 mRNA expression
b) IL-8 protein levels
c) TNF-α mRNA expression
d) IL-1β mRNA expression

Figure 3: Airway endotoxin levels in sputum supernatant from subjects with neutrophilic, eosinophilic and paucigranulocytic asthma, bronchiectasis and healthy controls. Horizontal bars represent median levels.

Figure 4: Neutrophil elastase detected in sputum supernatant from subjects with asthma, bronchiectasis and healthy controls

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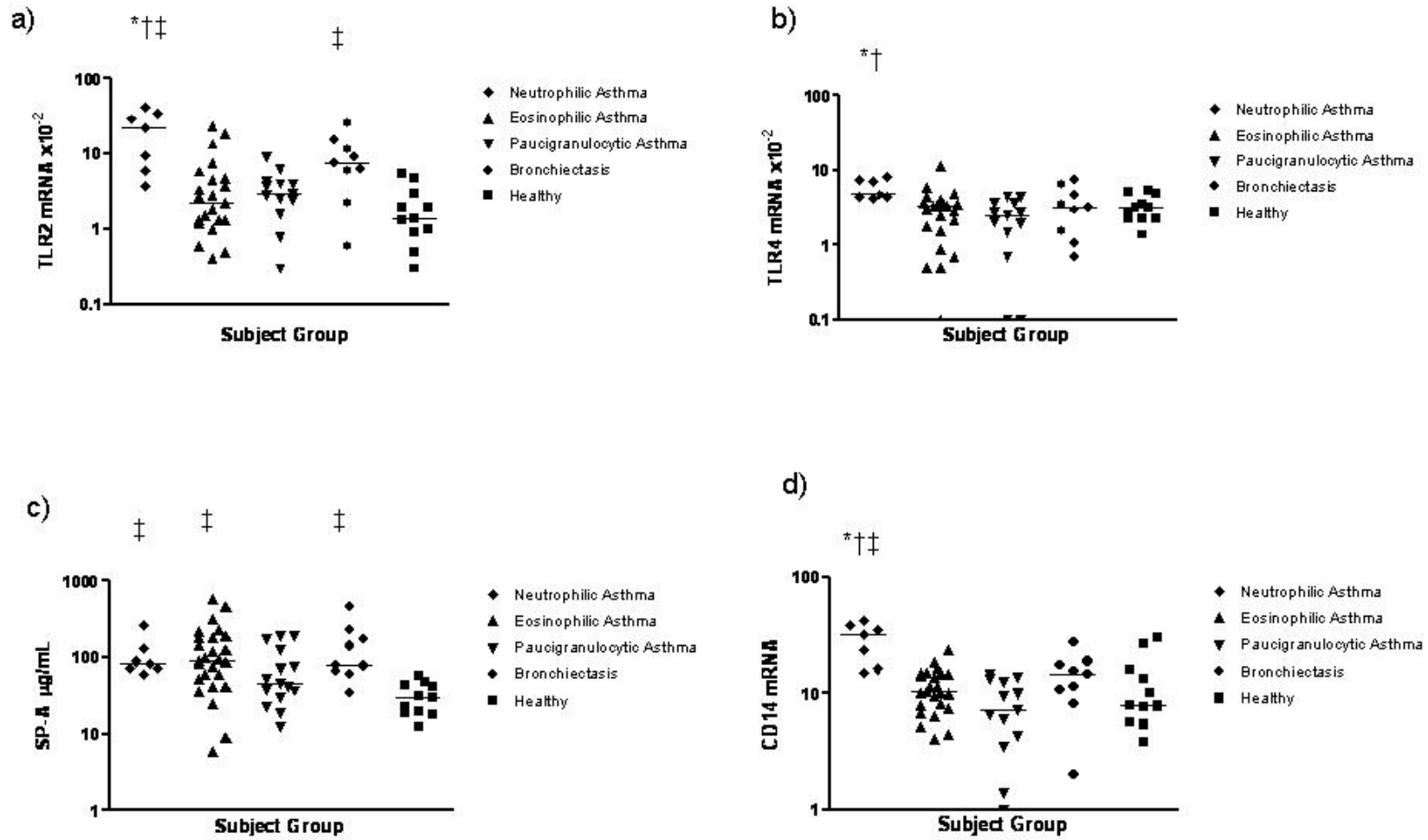
Figure 1


a)
*†‡
‡
TLR2 mRNA x10^-2
100
10
1
0.1
Subject Group
Neutrophilic Asthma
Eosinophilic Asthma
Paucigranulocytic Asthma
Bronchiectasis
Healthy
b)
*†
TLR4 mRNA x10^-2
100
10
1
0.1
Subject Group
Neutrophilic Asthma
Eosinophilic Asthma
Paucigranulocytic Asthma
Bronchiectasis
Healthy
c)
‡
‡
‡
SP-A µg/mL
1000
100
10
1
Subject Group
Neutrophilic Asthma
Eosinophilic Asthma
Paucigranulocytic Asthma
Bronchiectasis
Healthy
d)
*†‡
CD14 mRNA
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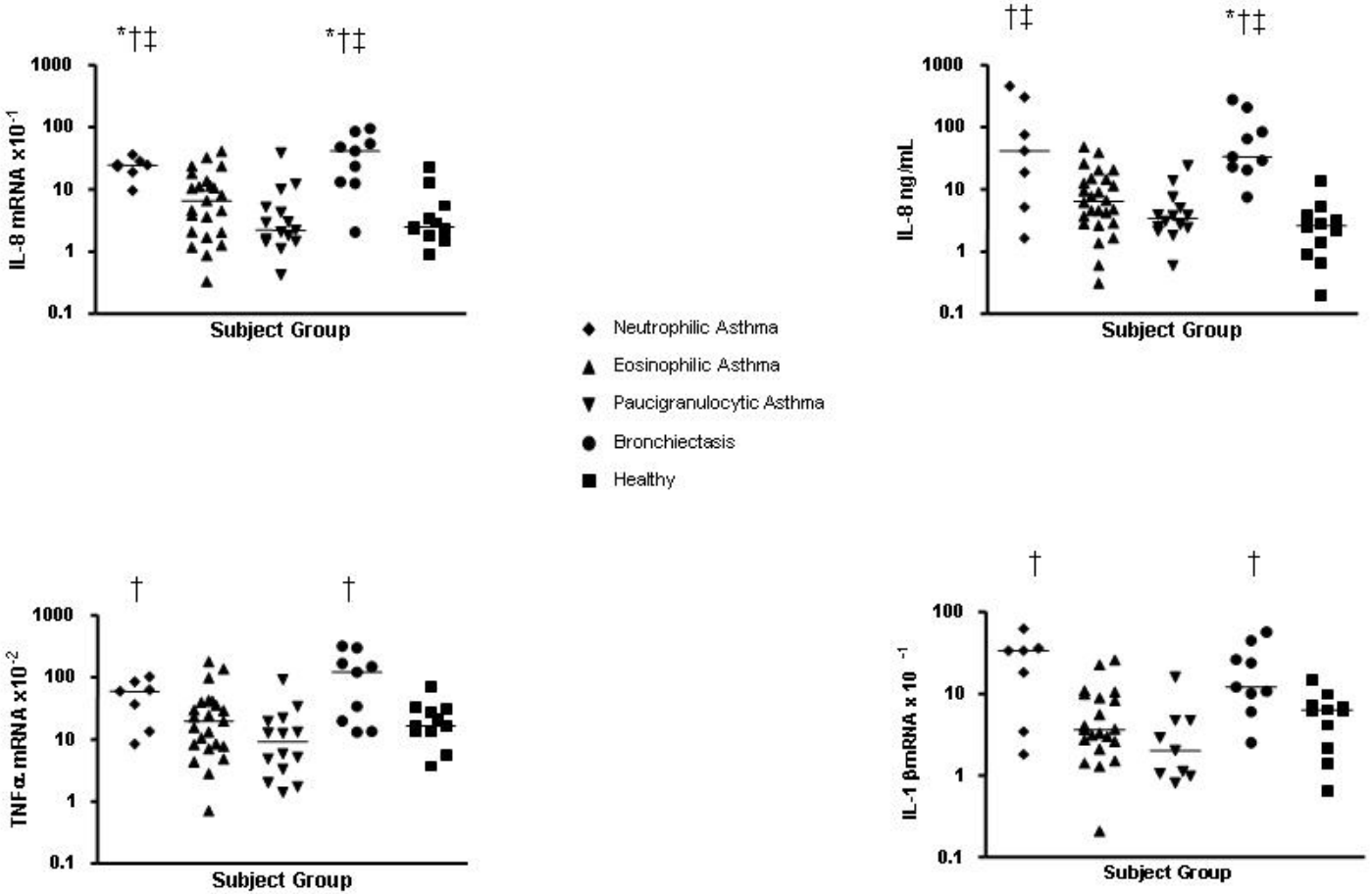
10
1
Subject Group
Neutrophilic Asthma
Eosinophilic Asthma
Paucigranulocytic Asthma
Bronchiectasis
Healthy

Figure 2


*†‡
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1000
100
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IL-8 mRNA x10-1
Subject Group
†‡
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IL-8 ng/mL
Neutrophilic Asthma
Eosinophilic Asthma
Paucigranulocytic Asthma
Bronchiectasis
Healthy
†
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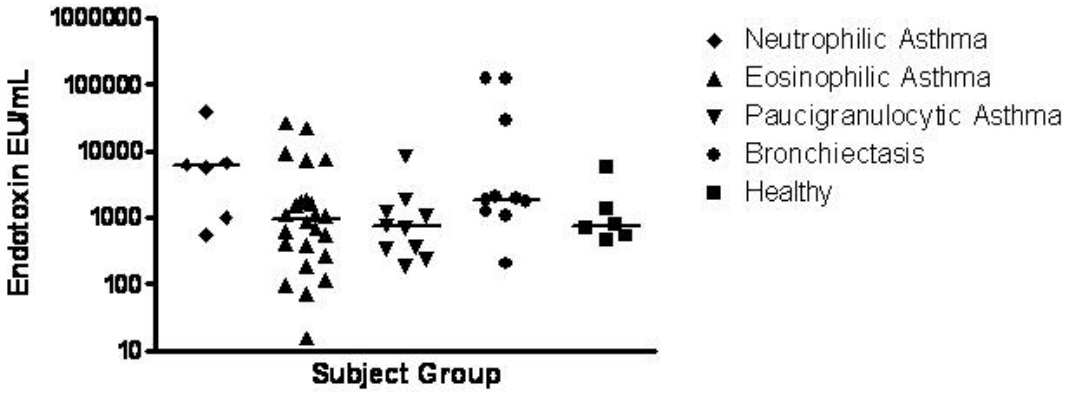
TNFα mRNA x10-2
†
†
IL-1 βmRNA x 10 -1

Figure 3


1000000
100000
10000
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Endotoxin EU/mL
Subject Group
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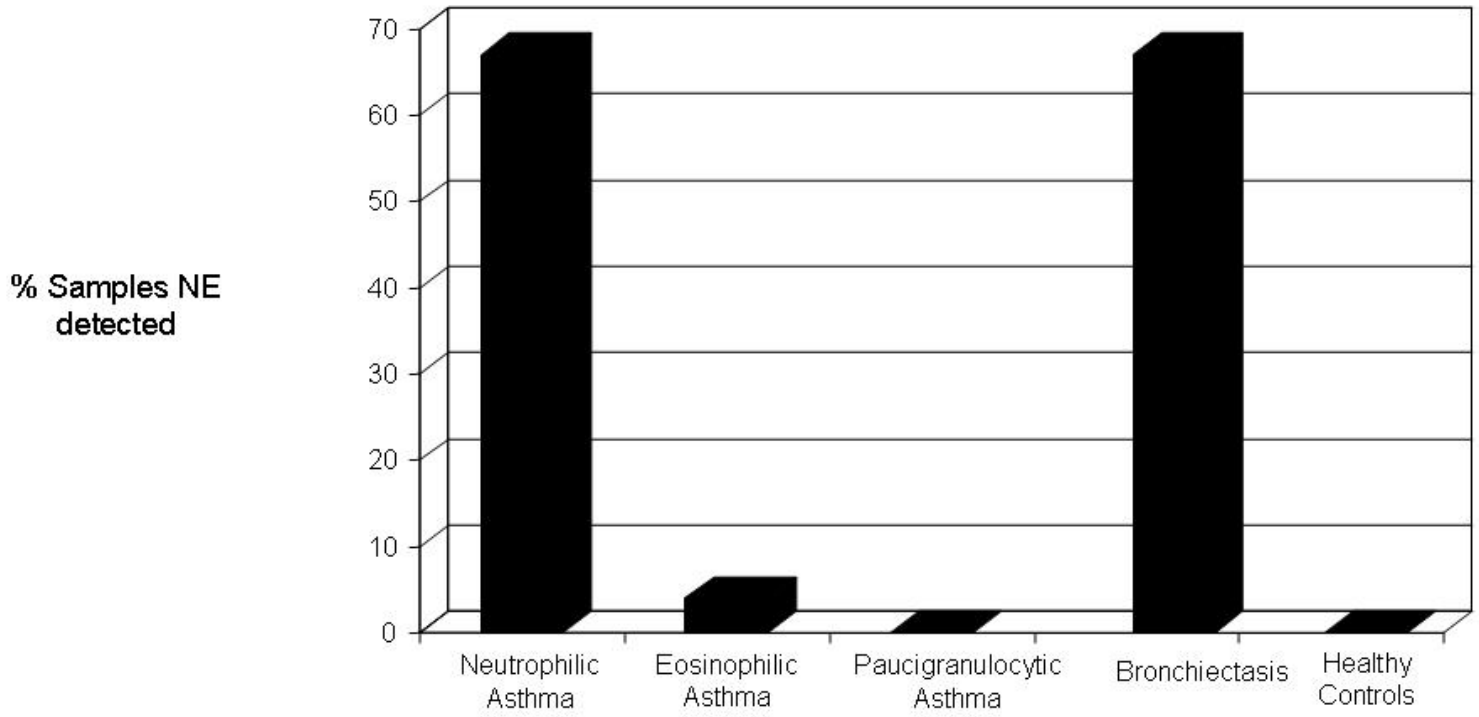
Eosinophilic Asthma
Paucigranulocytic Asthma
Bronchiectasis
Healthy

Figure 4


% Samples NE detected
70
60
50
40
30
20
10
0
Neutrophilic Asthma
Eosinophilic Asthma
Paucigranulocytic Asthma
Bronchiectasis
Healthy Controls