LPS INDUCED AND AGE RELATED NEUTROPHILIC INFLAMMATION IN ASTHMA

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**List of Abbreviations**

ACQ(-7) – Asthma Control Questionnaire(-7)  
AIA – Aspirin Induced Asthma  
AM – Alveolar Macrophage  
AP-1 – Macrophage Activator Protein-1  
APC – Antigen Presenting Cell  
ATF-1 – Activating Transcription Factor 1  
ATS – American Thoracic Society  
BAL(F) – BronchoAlveolar Lavage(Fluid)  
CBP – p300/CREB Binding Protein  
CCL – Chemokine (C-C motif) Ligand  
CXCL(8) – Cysteine X Cysteine Chemokine Ligand(8)  
CXCR(1,2) – Cysteine X Cysteine Chemokine Receptor (1,2)  
DUSP – Dual-Specificity Phosphatases-2  
ECP – Eosinophilic Cationic Protein  
EGR-1 – Early Growth Response factor-1  
EOA – Early Onset Asthma  
EPO – Eosinophil PerOxidase  
Fc – Fragment, Crystallizable  
FEV$_1$ – Forced Expiratory Volume in 1 second  
FRC – Functional Residual Capacity  
FVC – Forced Vital Capacity  
GM-CSF – Granulocyte Macrophage-Colony Stimulating Factor  
GR – Glucocorticoid Receptor  
ICS – Inhaled CorticoSteroids  
IFN(\(-\gamma\)) – InterFeroN(\(-\gamma\))  
IL – InterLeukin  
IRAK – Interleukin-1-Receptor–Associated Kinase  
IκB – Inhibitor of Kappa-light-chain-enhancer of activated B cells  
LOA – Late Onset Asthma
LPS – LipoPolySaccharide
LTB4 – Leukotriene B4
MAIL – Molecule possessing Ankyrin-repeats Induced by Lipopolysaccharide
MAPK – Mitogen-Activated Protein Kinases
MBP – Major Basic Protein
M-CSF – Macrophage-Colony Stimulating Factor
MGA – Mixed Granulocytic Asthma
MMP – Matrix MetalloProteinase
MPO – MyeloPerOxidase
MyD88 – MYeloid Differentiation factor 88
NE – Neutrophil Elastase
NEA – Non Eosinophilic Asthma
NF-κB – Nuclear Factor Kappa-light-chain-enhancer of activated B cells
NO – Nitric Oxide
PBMC – Peripheral-Blood Mononuclear Cell
PC_{20} – Provocative Concentration causing a 20% fall in FEV_{1}
PCAF – P300/CBP-Associated Factor
PMN – PolyMorphoNucleocytes
SARM – Sterile Alpha and TIR Motif-containing protein
TF – Transcription Factor
TH_{2} – Type 1 T-Helper lymphocyte
TIR – Toll/IL-1 Receptor
TIRAP – Toll-Interleukin 1 Receptor domain-containing Adaptor Protein
TLC – Total Lung Capacity
TLR – Toll Like Receptor
TNF(-α) – Tumor Necrosis Factor(-α)
TNFAIP3 – TNF-α-Induced Protein 3
TRAF(-6) – TNF Receptor-Associated Factor (-6)
TRAM – TIR-domain-containing adaptor inducing InterFeron-β-related Adaptor Molecule
TRIF – TIR-domain-containing adapter-inducing InterFeron-β
Abstract

THE UNIVERSITY OF MANCHESTER
ABSTRACT OF THESIS submitted by: Matthew Richard William Rossall
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BACKGROUND: Asthma is a multidimensional syndrome made up of a range of ‘phenotypes’. Neutrophilic inflammation is one of these phenotypes. Neutrophilic asthma is linked to increased asthma severity, an increased likelihood of exacerbating and a reduced response to therapy.

AIMS: The aim of this project was to investigate the pathogenesis, reproducibility and consequences of neutrophilic inflammation in asthma. The following were investigated: (1) the effect of onset age of asthma on sputum neutrophil counts (2) the reproducibility and validity of sputum neutrophil counts in moderate to severe asthmatics (3) the possible effect of LPS challenge in causing airway neutrophilia in moderate asthmatics, CXCL8 release from alveolar macrophages and possible corticosteroid resistant neutrophil chemotaxis.

METHODS: (1) Clinical and inflammatory data of 19 early onset asthmatics and 19 late onset asthmatics were compared (2) The reproducibility of sputum neutrophils over the long (>2 months) and short term (7±1 days) was investigated in 14 moderate to severe asthmatics (3) Inhaled LPS was administered to 7 moderate asthmatics: Serial clinical measurements were taken to observe the safety of LPS inhalation. Sputum (n=7) and BAL samples (n=7) were analysed to observe the effects of LPS inhalation on the central and peripheral airways. Alveolar macrophages were obtained during bronchoscopy and cultured ex vivo (n=4).

RESULTS: (1) There were increased neutrophil counts in LOA asthmatics compared to those with EOA. LOA subjects had better asthma control representative of increased lung function and reduced ACQ scores (2) sputum neutrophil counts suggested good reproducible value over both long and short term time periods. 90% power calculations were performed for parallel and cross over studies (3) Significant decreases in lung function were observed at 0.5, 1, 2 and 3 hours post LPS, significant increases in temperature post 6 and 8 hours and an observable increase in exhaled nitric oxide levels. There was no associated changes in steroid sensitivity pre and post LPS. Reduced CXCL8 and TNF release was observable (but not statistically significant) post ex vivo LPS stimulation.

CONCLUSIONS: (1) Duration of asthma rather than age of onset of asthma is a key predictor of lung function. Raised sputum neutrophil counts are phenotypic to LOA but they do not indicate it as a biomarker of severe disease as previously described. (2) Sputum neutrophils are a repeatable and reliable endpoint, with good long and short term repeatability. Sputum monitoring during treatment steps could prove more effective based on downstream suppression (3) LPS inhalation has been shown to be a safe inducer of neutrophilic inflammation. No reported changes in steroid response were observed following LPS induced neutrophilia. Alveolar macrophage derived CXCL8 and TNF are tolerant cytokines following ex vivo LPS stimulation.
Declaration

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CHAPTER 1

General Introduction

Asthma is defined as “a common chronic disorder of the airways that is complex and characterized by variable and recurring symptoms, airflow obstruction, bronchial hyperresponsiveness and underlying inflammation” (Kelly, 2007). This highlights that there are no singular mechanisms resulting in asthma pathology and the plausibility of numerous pathways in its development are becoming increasingly recognized.

It has been estimated that approximately 300 million of the global population suffer with asthma - of these - 250,000 people die prematurely each year (Bousquet et al., 2010). This fact alone stresses the clinical implications and value of increasing our understanding of the condition. Symptomatic presentation of asthma is most commonly associated with wheezing, dyspnoea, coughing and chest tightness. The lack of effectiveness in current asthma therapeutics for a significant proportion of asthmatics has increased the pressure to further our understanding of asthma pathogenesis.

The aim of this thesis was to evaluate the presence and development of neutrophils in asthma pathology and determine its pathogenesis, reproducibility and consequences. Previous literature has demonstrated neutrophilia in asthma to be associated with poor asthma control, an increased likelihood of exacerbations, the development of fixed airflow limitation and a reduced response to corticosteroid treatment.

The purpose of the first study was to set out and observe the presence of airway neutrophils in different age related asthma phenotypes. Previous asthma studies into this area of research has been scarce and conflicting, particularly in terms of cellular profiling. This study observed asthma diagnosed after the age of 40 and compared this with those diagnosed before the age of 20, the results were novel. After this
initial study it was then observed whether induced sputum as a method of recognising airway neutrophils was a reproducible and reliable method. Indeed by observing this it would demonstrate the scientific viability of the first study.

After the previous findings, the purpose of this thesis was to explore airway neutrophilic induction in asthmatics via Lipopolysaccharide (LPS) inhalation. Previous studies have used this method as a safe model of inducing airway neutrophilia. Asthmatic airways were stimulated via inhaled LPS to induce a neutrophilic response via macrophage derived CXCL8 and TNF. Previous studies have reported CXCL8 as a corticosteroid resistant cytokine. The purpose of this study was then to observe the effects of steroid on these cytokines and to verify/disprove CXCL8 or TNF as steroid resistant cytokines.
1.1. Inflammation in Asthma

Asthma is a multifactorial syndrome stemming from physiological changes to chronic inflammation in the airways. Prolonged and repeated inflammation can result in a range of structural changes to the airways associated with the onset of asthma pathogenesis. Epithelial cell hyperplasia, smooth muscle hypertrophy and basement membrane thickening are some of the histophysiological observations previously reported in endobronchial biopsies (Behera, 2005). Generally these changes to the airways lie under the term ‘airway remodelling’. Indeed, chronic inflammation has been attributed to the exposure of sensory nerve endings leading to a classical increased susceptibility to extrinsic stimuli, coughing and chest tightness (Barnes, 2003). It is not uncommon in asthmatics to experience a thickening to the basement membrane surrounding the airway lumen. This shift in membrane morphology is contributed with the deposition of suboptimal fibroblasts and/or collagen (type III and V) along with mucous gland and airway smooth muscle hypertrophy resulting in a reduction in the airway wall elasticity (Ward et al., 2001) commonly associated with severe asthma (Benayoun et al., 2003). It is becoming increasingly recognised that a high proportion of pathogenic processes in asthma development can be associated with a direct or indirect result of immediate or long term underlying inflammation.

1.1.1. Helper T Lymphocytes

It is widely accepted that CD4+ T-helper lymphocytes (TH2) are one of the principal cell types in mediating the onset of asthma immunopathology and have been shown to be upregulated in the respiratory tracts of asthmatics when compared to healthy controls (Cantani, 2000, Corrigan et al., 1988). However, singularly, they are not a clear clinical biomarker for asthma alone (Brightling et al., 2002b). Studies have demonstrated that upon presentation with an antigen, TH2 cells release pro-inflammatory cytokines including interleukin (IL)-4, IL-5, IL-9 and IL-13 (Romagnani, 2004, Romagnani, 2006). These cytokines promote characteristic
features of the asthmatic airway including eosinophilia and mucous hypersecretion (Barnes, 2001, Zu et al., 1998). Notably TH2 type mediators – including IL-4, IL-13, and IL-10 inhibit the ‘classically activated macrophage’ pathway and switch to a distinct macrophage phenotype termed ‘alternative macrophage activation’ (Goleva et al., 2008). This could ultimately lead to the development of uncontrolled fibrosis and prolonged chronic inflammation (Duffield, 2003). TH2 has had further links with atopic asthma as studies - in vivo - where subjects were given anti-IL-13 treatment had significantly reduced early- and late-phase allergen responses (Gauvreau et al., 2011).

1.1.2. Mast cells

Mast cells are crucial to the initiation of the atopic response and innate immunity (Rivera and Gilfillan, 2006). There are increased numbers of mast cells localised within the asthmatic airways when compared to healthy controls (Wardlaw et al., 1988). The locality of mast cells within the airway is wide ranged and can be observed within bronchial epithelium, airway smooth muscle and mucous glands (Bradding et al., 1994, Brightling et al., 2002a, Carroll et al., 2002). Upon recognition of pathogenic material including viral and bacterial particulates, intracellular immunoreceptor tyrosine–based activation motifs (ITAMs) begin a rapid process of phosphorylation within the β and γ subunits of FcεRI receptors. This phosphorylation and activation causes the subsequent secretion of pro-inflammatory cytokines (e.g. IL-4, IL-5, IL-13), eicosanoids - including leukotrienes (e.g. LTB4) - and prostaglandins (e.g. PGD2) (Barnes, 2009), closely linked with the onset of asthmatic airway changes. According to recent studies, FcεRI have additionally been observed on the surfaces of dendritic cells, eosinophils and monocytes (Gershwin, 2001) demonstrating an interlinking of inflammatory cell roles.
1.1.3. Primary Antigen Presenting Cells (APCs)

The key antigen presenting cells (APCs) in asthma development are dendritic cells (DCs) (Banchereau et al., 2000, Banchereau and Steinman, 1998, Hammad and Lambrecht, 2008). These cells present peptides via major histocompatibility complex II (MHC-II) to circulating CD4+ cells, thereby promoting a TH$_2$ immune response (Lambrecht and Hammad, 2003). Nevertheless, despite the primary focus on DC’s in allergen recognition, this does not undermine the importance of other APCs including macrophages and their precursors in disease progression.

Indeed, one of the initial cell types which react in response to inflammation are monocytes. The production of these precursor cells is induced by IL-3, macrophage colony stimulating factor (M-CSF) and granulocyte macrophage-colony stimulating factor (GM-CSF) which orchestrates the differentiation into tissue specific macrophages. Upon activation, monocytes are able to secrete a diverse range of mediators including IL-1, IL-6, CXCL8, IL-10, IL-12, IL-15, IL-18, TNF-α and Chemokine ligand 3 (CCL3 or macrophage inflammatory protein-1α) (Kindt, 2007)

Tissue specific monocytes (macrophages) while being potent phagocytes, also exhibit toxic antimicrobial properties against infection. The alveolar macrophage (AM) is the most potent inflammatory cell residing within the airspaces and is thought to be the key orchestrator in eliminating pathogens located within these spaces (Peters-Golden, 2004). Interestingly this pathogen recognition may be more specific than originally thought as alveolar macrophages have been demonstrated to react to gram-negative (Lipopolysaccharide) but not gram positive (Lipoteichoic Acid) bacterial components. They have been demonstrated to be volatile producers of bioactive mediators including nitric oxide (NO), which are induced following exposure with IL-1, interferon-γ (IFN-γ), TNF-α and bacterial components including endotoxin (Rimbach et al., 2000).
Upon stimulation, macrophages can part down two activation cascades, the classical and the alternate pathway. Classically activated macrophages are thought to proliferate upon exposure to IFN-\(\gamma\), endotoxin and another microbial products (Murray and Wynn, 2011) whilst alternate activated macrophages proliferate in response to TH\(_2\) type cytokines including IL-4 and IL-13 (Gordon, 2003). Whilst alternately activated macrophages are generally involved in the processes of tissue repair, classically activated macrophages are thought to be pro-inflammatory in the process of infection resolve (Kumar, 2012).

1.1.4. Eosinophils

Eosinophils originate from the bone marrow which, in the presence of IL-3, GM-CSF or IL-5, differentiate from granulocyte/monocyte progenitor cells (Paul, 2008). It has been widely recognised that eosinophilia is a hallmark of the asthmatic airway found in both atopic and non-atopic asthmatics (Bousquet et al., 1990). Eosinophils release major basic protein (MBP), peroxidase and cationic proteins (EPO and ECP), all which are cytotoxic to the bronchial epithelium (Ayars et al., 1989, Motojima et al., 1989). These mediators also promote fibrosis and airway remodelling. Their existence in the airways can induce bronchial smooth muscle contraction, airway hypersensitivity and increase vascular permeability (Bousquet et al., 2000) all characteristic of asthma pathology.

TH\(_2\) and eosinophilic pathways are partially instigated by the presence of foreign antigens and are therefore responsible for ‘atopic asthma’. Yet despite the TH\(_2\), mast cell and eosinophilic focus on asthma pathogenesis, there are a population of asthmatics who have normal levels of eosinophils, are non-atopic and lack IgE inflammatory dependency. This form of asthma has been contributed to 50 percent of the asthmatic population (Douwes et al., 2002) highlighting the importance of other pathways in the development of asthma.
1.1.5. Neutrophil Biology

Neutrophils are heavily involved in the defence against invading foreign pathogens; they release reactive oxygen species (ROS), proteases, defensins and cytokines (Monteseirin, 2009) all which may play a role in asthma pathogenesis. Indeed, it is the proteases that are deemed responsible for irreversible damage within the airways (Barnes et al., 2002, Snider, 1986, Vignola et al., 2000).

Neutrophilic recruitment is demonstrated to be a direct response to mediators released from macrophages and epithelial cells within the lungs. Airway macrophages induce the expression of adhesion molecules on endothelial cells, which promote ‘extravasation’ (Murphy et al., 2001). Extravasation is caused via the expression of P-selectin on endothelial cells upon exposure to leukotriene B4 (LTB4), histamines or complement subunits including C5a. The upregulation of P-selectins has been attributed to the actions of LPS, TNF-α and IL-1β (Dore and Sirois, 1996, Gotsch et al., 1994, Herlaar and Brown, 1999). LPS and TNF-α also promote the additional synthesis of E-selectin on endothelial cells. Both P-selectin and E-selectin enhance monocyte and neutrophilic adhesion to the vessel wall. Indeed, increased levels of TNF-α may amplify this process by increasing the interaction of leukocyte integrins with other endothelial molecules (Lee et al., 2006). CXCL8 may additionally increase the aggression of this response and thereby upregulate neutrophil recruitment to sites of inflammation (Huber et al., 1991).

1.1.5.1. Interleukin-8 (CXCL8)

CXCL8 has been well established as one of the most potent chemoattractants for neutrophils (Lin et al., 2004, Monteseirin, 2009, Ordonez et al., 2000). In practise, it has been found to be increased in bronchoalveolar lavage (BAL) from asthmatics when compared to healthy controls (Gosset et al., 1997). Current literature demonstrates it to be released directly or indirectly from macrophages (Nakamura et
al., 1995, Palmberg et al., 1998), bronchial epithelial cells (Chanez, 2005, Smith et al., 2000) and fibroblasts (Baggiolini and Clark-Lewis, 1992). The synthesis of CXCL8 by bronchial specific tissue is semi reliant on precursor mediators including IL-1 and TNF-α released primarily from alveolar macrophages and other polymorphonuclear leukocytes (PMNs) (Baggiolini and Clark-Lewis, 1992, Kunkel et al., 1991). CXCL8 can be released through a number of diverse stimuli including LPS or elements released following phagocytosis of yeast particles (Cassatella et al., 1993). Notably neutrophils have been demonstrated to promote autocrine mechanisms by generating their own CXCL8 (Bazzoni et al., 1991, Iho et al., 2003) leading to prolonged survival and differentiation and highlighting the complexity in controlling this form of inflammation. Indeed, CXCL8 induced neutrophil chemotaxis is recognized to be reliant on the activation of CXCR1 and CXCR2 receptors (Nasser et al., 2009) both of which are co-expressed predominantly on the surface of neutrophils (Elgert, 2009)

1.1.5.2. Matrix Metalloproteinases

Matrix metalloproteinases (MMPs) are a group of proteolytic enzymes capable of degrading cellular matrix architecture. The most widely researched regarding asthma pathogenesis is MMP-9, of which neutrophils are the key producers (Takafuji et al., 2003). The initial activation of MMP-9 is dependent on MMP-3 in a double step cleaving process (Ogata et al., 1992). The pre-activation of MMP-3, fundamental to this pathway, is thought to be induced by endopeptidases including elastases released from neutrophils (Ogata et al., 1992). MMP-9 has further been demonstrated to alter the structure of CXCL8 to a more potent form leading to an increase in recruitment of neutrophils to sites of inflammation (Van den Steen et al., 2000). Indeed, levels of MMP-9 have been linked with disease severity in which higher levels are observed in moderate asthma when compared to milder forms (Mattos et al., 2002).
1.1.5.3. Neutrophilic Defensins

Neutrophilic defensins are classically split into α and β families based on the location of cysteines residue pairing. Indeed, each family have a reciprocal characteristic in that they both exhibit antimicrobial activity in response to pathogenic exposure. With this in mind it is generally accepted that neutrophilic defensins are common place within the airways upon exposure to viral elements (Proud, 2006). Interestingly there is an increasing link with neutrophil defensins and airway remodelling, as high volumes of both defensin families have shown to exhibit cytotoxic activity on local epithelial cells (van Wetering et al., 2005). Coincidently these proteins are thought to additionally contribute to excessive mucous production via the activation of MUC5AC and MUC5B genes and mucous cell differentiation (Aarbiou et al., 2004).

1.1.5.4. Neutrophilic Elastase

Other neutrophilic mediators include neutrophilic elastases (NE) which are elastolytic proteases (Zander et al., 2008). In excess, they lead to mucous hypersecretion from goblet/epithelial cells (Agusti et al., 1998, Fischer and Voynow, 2000) which could inhibit phagocytic processes leading to a potential spiralling cascade of asthma pathology. NE degrades airway epithelium and elastin content along with other components of airway architecture (Jameson, 1998). This form of proteolytic enzyme is able to cleave the Fc portion of IgG proteins and elements of the complement cascade highlighting a possible vulnerability to secondary infections of those asthmatics with neutrophilic accumulation. In addition other literature has demonstrated NE to be a potent activator of MMP-9 demonstrating a further positive feedback mechanism in the development of neutrophilic airway accumulation (Ferry et al., 1997).
1.1.5.5. Eosinophilic Cationic Protein (ECP)

Notably, NE is able to induce the release and production of ECP from eosinophils. Yet despite its name, there is a growing level of research demonstrating that environmental ECP can also be directly secreted – albeit not produced – by neutrophils (Byström et al., 2002, Monteseirin et al., 2007, Sur et al., 1998). Neutrophilic ECP secretion (Figure 1.1.5.6-1) is released up to 36 times slower than its eosinophil counterpart demonstrating a reduced role in epithelial cytotoxicity (Monteseirin, 2009). Yet, indeed, this establishes an overlap between atopic and non-atopic asthma phenotypes.

1.1.5.6. IL-17

Relatively recently TH\textsubscript{17} has become part of the T-cell subsets. This lymphocyte family is able to secrete the cytokines IL-6, IL-17 and IL-22 (McKinley et al., 2008). Recent literature has highlighted IL-17 synthesis as a possible protective agent against extracellular bacteria and fungi which TH\textsubscript{2} and TH\textsubscript{1} cells may not be as well adapted for (Tesmer et al., 2008). It is classically able to promote granulopoiesis and neutrophil chemotaxis (Kolls and Linden, 2004) and is increased in the asthmatic airways when compared to healthy controls (Molet et al., 2001). Indeed, IL-25 – a IL-17 family derived cytokine – has shown to orchestrate TH\textsubscript{2} responses through airway epithelial mechanisms via the production of IL-33 and thymic stromal lymphopoietin, suggesting a direct association with innate processes (Gregory et al., 2012). Sputum levels of IL-17 have also been linked to an increase in hyperresponsiveness defined by a response to Methacholine (Barczyk et al., 2003, McKinley et al., 2008). Interestingly murine models lacking the ability to secrete IL-17 have increased susceptibility to gram negative bacteria (Wu et al., 2007, Ye et al., 2001). \textit{In vivo} stimulation with IL-17 has previously caused neutrophilic accumulation for up to 6 hours demonstrating a maintained effect (Hoshino et al., 1999). Relevantly IL-17 driven neutrophilia has also been linked to steroid resistance.
in asthmatics despite the *in vitro* activation of glucocorticoid receptors (McKinley et al., 2008), demonstrating a possible further downstream activation.

**Figure 1.1.5.6-1. Neutrophil mediator release and effects**

A schematic diagram demonstrating neutrophilic development in the airways of an asthmatic. It is worth noting that CXCL8 is able to have multiple circular feedback mechanisms highlighting a difficulty in controlling this cell profile. In addition it presents the other mechanisms orchestrated by MMP-9, ND’s, NE, ECP and IL-17 leading to increased mucous hypersecretion, cytotoxicity and the production of pro-inflammatory mediators.
1.2. Asthma Phenotypes

Asthma is a heterogeneous disease and novel research is progressively gravitating towards new and innovative biomarkers to identify asthma phenotypes. The use of techniques including sputum induction and exhaled nitric oxide has led to the discovery of multiple asthma phenotypes and sub-phenotypes (Haldar et al., 2008, Weatherall et al., 2009, Wenzel, 2012). Previously the phenotypic spectrum was spread across 3 large asthmatic phenotypic families (Wenzel, 2006). These include asthma pathogenesis related to a stimulus or trigger, those who have distinct inflammatory characteristics, and those who don’t follow ‘the norm’ during clinical assessments. Indeed, each of these phenotypes have distinct sub-phenotypes of which there is increasing evidence of overlapping characteristics.

In effort to reduce this overlap there is increasing interest in the use of cluster analysis for the identification of diagnostic categories. This form of phenotype investigation has previously lead to the discovery of multiple obstructive airways disease populations. Variables within cluster populations range from concomitant rhinitis/eczema and degree of airflow obstruction to level of reversibility and smoking history (Weatherall et al., 2009). Previously, similar clusters using separate algorithms have been observed solely in asthmatic populations. Clusters within a concordance between symptom reporting and airway eosinophilia have been found in patients within primary and secondary care asthma. Clustered populations included female/male populations only, presence of atopy and early and late onset asthma. Indeed the study also demonstrated three separate populations based on age of asthma onset each with varying asthma severity, BMI indices, type of inflammation and predominance of gender (Haldar et al., 2008).

1.2.1. Age at Onset and Late Onset Asthma

Age at onset has previously been classified into early onset asthma (EOA) and late onset asthma (LOA). LOA is a poorly defined term which has no agreed age
threshold value for its development. Previously the age of 65 had been used as a cut of point, (Gershwin and Albertson, 2006, Kitch et al., 2000) however an increased likelihood of age related comorbidities can cause confusion in this area and there is little clinical research in this age boundary. Histological differences have been found in asthmatics with a mean onset age of 28 however the study failed to observe a strict criteria for asthma onset (Molina et al., 1977). Studies observing asthma development after the age of 40 have found differences in the genetic variants coding for the RANTES gene (Hizawa et al., 2002), lower blood eosinophil counts, increased symptom free periods along with fewer hospital admissions (Quadrelli and Roncoroni, 1998). This is strongly suggestive of changes in the pattern of inflammation with the later onset of asthma but there is no consensus of where the cut of age should be set for research studies.

Consequently, there are few publications regarding the pathophysiology or pathogenesis of this phenotype. Indeed, LOA can exhibit similar symptoms found in chronic bronchitis and heart failure causing diagnostic confusion. The lack of a clear definition and understanding of the pathogenesis of LOA may have led to under- and mis-diagnosis in a substantial population of asthmatics (Quadrelli and Roncoroni, 1998). Interestingly, asthmatics diagnosed later in life display more evidence of being non-atopic (Dodge and Burrows, 1980, Hizawa et al., 2002) with fixed airflow limitation (Ayres, 1990) signifying a possible link with other cellular profiles (i.e. neutrophilia).

There is evidence that human innate and adaptive immunity changes with age; with decreased peripheral blood eosinophil effector functions (Mathur et al., 2008) changes in lymphocyte function (Weng, 2006, Mund et al., 2001) and changes in the functions of macrophages, neutrophils and dendritic cells (Plackett et al., 2004). These changes caused by ageing, coupled with environmental factors, can lead to the onset of asthma in later life despite no history of childhood asthma. Interestingly, it has been reported that LOA could carry a specific genotype for asthma development
that is activated later in response to infection or exacerbation (Gershwin and Albertson, 2006).

Evidence of similarity in induced sputum cell counts has been demonstrated between those diagnosed later in life and EOA (Papadouli et al., 2006). However the older asthmatics in this study were those diagnosed past the age of 18, which may not be the true threshold age for LOA. In another study, older and younger asthmatics were compared, with no difference with age at onset. They found a significant increase in neutrophils and decrease in macrophages in older asthmatics compared to younger asthmatics (Mathur et al., 2008) yet this may be a pathology associated with typical aging (Thomas et al., 2004). In addition both studies did not exclude the recruitment of smokers or ex-smokers with a low pack year history.
1.2.2. Inflammatory Phenotypes based on Induced Sputum

Induced sputum is a novel and non-invasive biomarker for central airway inflammation (Alexis et al., 2001, Moodley et al., 2000) and is being increasingly used in asthmatic clinical and research trials since its initial use in the 1990s (Pin et al., 1992). There are now three hypotheses to the means in which sputum induction is able to induce a sample 1) it is thought to contribute to the movement of water into the airway lumen by process of increased osmolarity making it easier to expectorate (Jones et al., 2001, Jongejan et al., 1990) 2) it increases mucociliary action that may lead to movement of sputum up the airways (Daviskas et al., 1996) 3) It is able to induce cough and activation of cough receptors (Jones et al., 2001). Indeed, since its initial use, sputum induction has led to the discovery of multiple distinct inflammatory phenotypes.

1.2.2.1. Paucigranulocytic Asthma

Asthma has generally been described as a primarily eosinophilic airway disease, yet there is subgroup of non eosinophilic asthmatics (asthma) (NEA). NEA has been shown to display normal levels of eosinophils within the mucosa (i.e. <3%), a normal thickness of the subepithelial basement membrane along with a decreased level of lymphocytes (CD3+, CD4+, CD8+), mast cells, and macrophages (Wenzel et al., 1999). Simpson et al. (2006) observed induced sputum of 93 asthmatic subjects with varying degrees of asthma severity, 31% of these demonstrated cell profiles similar to healthy controls. This study was well controlled as the majority of subjects had not received any corticosteroid treatment in the previous 12 months, eliminating the causal effect of steroid treatment. This asthma phenotype without any obvious inflammation could be due to abnormal connective tissue (i.e. bronchial mesenchyme), epithelium and/or smooth muscle properties leading to symptom development (Holgate, 2008).
1.2.2.2. Mixed Granulocytic Asthma

The first marked recording of mixed granulocytic asthma (MGA) was by Simpson et al. (2006); they reported a subtype of asthmatics who had both increased levels of percentage eosinophils (>1.01%) and neutrophils (>61%) and were regarded as a mixed inflammatory phenotype. Yet even since its discovery this phenotype has had little investigational consideration, ultimately this gap in our knowledge needs addressing.

1.2.2.3. Eosinophilic Asthma

Sputum eosinophilia is generally regarded as 1.9% of total cells as the upper limit for normal levels based on current literature (Reddel et al., 2009), however lower/upper values than this have also been used (Simpson et al., 2006, Turner et al., 1995). There is a growing confliction of evidence demonstrating eosinophilia as a predictor of asthma severity (Louis et al., 2000, Wardlaw et al., 1988, Woodruff et al., 2001) and this confliction is ever-growing. This has demonstrated a similar picture when associating eosinophils with airway hyper-reactivity (Jatakanon et al., 1998, Grunberg et al., 1997). Nevertheless, there has been a suggestive overlapping with other asthma phenotypes as the level of airway eosinophils has been shown to associate with aspirin induced asthma (AIA) age at onset (Miranda et al., 2004) and occupation (Frew et al., 1995). Eosinophils have been linked with a worsening of asthma control by pulmonary function (Jatakanon et al., 2000, Kodric et al., 2007). There is an increased likelihood of expressed symptoms and an increased risk of exacerbating with an airway presence of eosinophils (Green et al., 2002a), demonstrating a trend towards more severe asthma phenotypes when compared to other inflammatory pathways.

Nevertheless, as stated earlier in this chapter, eosinophilic airway inflammation has only been attributed to 50 percent of the asthmatic population (Douwes et al., 2002) demonstrating an existence of other cellular morphologies.
1.2.2.4. Neutrophilic Asthma

Eosinophilia has for a long period been demonstrated as a classical hallmark of bronchial asthma (Ronchi et al., 1996). Yet there is a growing degree of research suggesting that some subjects present a distinct neutrophilic pathogenesis (Simpson et al., 2006).

Neutrophils are commonplace when observing induced sputum (Ronchi et al., 1996, Thomas et al., 2004) where cut off points for the definition of airway neutrophilia is >61% of total cells (Simpson et al., 2006). Neutrophilia is a more severe phenotype of asthma that is linked to increase asthma severity, reduced response to treatment (Pavord et al., 1999), occupational asthma (Anees et al., 2002) and asthma exacerbations (Fahy et al., 1995). It has previously been demonstrated that approximately 20% of the asthmatic population may have increased neutrophils in the airways (Simpson et al., 2006). This form of inflammatory phenotype has been linked to asthmatics who have acute onset fatal asthma where subjects died within 2 hours than those with more gradual fatalities (Sur et al., 1993). Other studies observing cross sectional areas of the airways have additionally observed increased numbers of submucosal neutrophils in those with ‘fatal asthma’ when compared to control subjects (Carroll et al., 2002).

Neutrophilic airway disease may be due to cigarette smoke inhalation. A study involving non-asthmatic smokers and asthmatic smokers demonstrated that asthmatic smokers had increased levels of neutrophils and CXCL8 in their induced sputum (Chalmers et al., 2001). Other studies have demonstrated a specific phenotype of asthmatics which have nocturnal symptoms associated with airway neutrophilia (Martin et al., 1991). Interestingly the validity and reliability of sputum neutrophils is not well recognized (Table 1.2.2.4-1).
<table>
<thead>
<tr>
<th>Study Author</th>
<th>Title</th>
<th>Patient Type</th>
<th>Population</th>
<th>Time Between Inductions</th>
<th>Sputum Neutrophil Repeatability (intraclass correlation coefficient)</th>
<th>Additional Repeatability Observations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Simpson J et al.</td>
<td>Inflammatory subtypes in asthma: Assessment and identification using induced sputum</td>
<td>Non-Eosinophilic (mean 0.5%) Asthma</td>
<td>40</td>
<td>28 days (median)</td>
<td>Eosinophils = 0.64†</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>18</td>
<td>5 years</td>
<td>Not Performed</td>
<td>Eosinophils = 0.77†</td>
</tr>
<tr>
<td>Bacic E et al.</td>
<td>Induced sputum is a reproducible method to assess airway inflammation in asthma</td>
<td>Asthma (mixed severity)</td>
<td>29</td>
<td>7 days</td>
<td>Ri = 0.85</td>
<td>Eosinophils = 0.87 Lymphocytes = 0.15 Macrophages = 0.80</td>
</tr>
<tr>
<td>Spanevello A et al.</td>
<td>Induced sputum to assess airway inflammation: a study of reproducibility</td>
<td>Seasonal Rhinitis</td>
<td>77</td>
<td>≤7 days</td>
<td>Ri = 0.84</td>
<td>Eosinophils = 0.98 Lymphocytes = 0.77 Macrophages = 0.85</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>van der Yen J et al.</td>
<td>Repeatability of cellular and soluble markers of inflammation in induced sputum from patients with asthma.</td>
<td>Atopic Asthma (mild)</td>
<td>12</td>
<td></td>
<td>Ri = 0.56</td>
<td>Eosinophils = 0.79 Lymphocytes = 0.11 Macrophages = 0.68</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Atopic Asthma (moderate-severe)</td>
<td>9</td>
<td>4 days (median)</td>
<td>Ri = 0.61</td>
<td>Eosinophils = 0.84 Lymphocytes = 0.68 Macrophages = 0.56</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Atopic Asthma (mild and severe combined)</td>
<td>21</td>
<td></td>
<td>Ri = 0.57</td>
<td>Eosinophils = 0.85 Lymphocytes = 0.36 Macrophages = 0.64</td>
</tr>
<tr>
<td>Pizzichini E et al.</td>
<td>Indices of airway inflammation in induced sputum reproducibility and validity of cell and fluid-phase measurements.</td>
<td>Non-obstructive chronic bronchi</td>
<td>10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Stable asthmatics (moderate-severe)</td>
<td>19</td>
<td>6 days</td>
<td>Ri = 0.81</td>
<td>Eosinophils = 0.94 Lymphocytes = 0.25 Macrophages = 0.71</td>
</tr>
</tbody>
</table>

**Table 1.2.2.4-1. Summary of sputum neutrophil repeatability studies**

It is worthy of note that there is a limit to our knowledge of the reliability of sputum neutrophils over longer time periods.

† kappa statistic
1.3. Toll Like Receptor (TLR) Structure and Function

Toll Like Receptors (TLR’s) are vital in transmembrane signalling against a vast array of bioactive mediators. TLRs are wide ranged and can present themselves in a range of plant and animal species in the defence against foreign pathogens. Ligands for this type of receptor include elements from bacterial cell walls, genomic material, fungal, viral and parasitic products. Their function is essential in the linking between the innate and adaptive immune response.

The structure of TLR’s is made up of two subunits. An extracellular domain comprised of a sequence of leucine repeats and a Toll/IL-1 Receptor (TIR) cytosolic domain. The former is able to initiate downward cascades (Akira and Takeda, 2004) whilst the latter utilises 5 TIR adaptor proteins MyD88, TIRAP, TRIF, TRAM and SARM in cytosolic signalling. Signalling cascades propagated by this class of receptor induce a range of pro-inflammatory mediators, yet may also increase levels of anti-inflammatory molecules (Trinchieri and Sher, 2007). Indeed, these receptors are able to induce a response beneficial to the host, however their role can develop or over express resulting in the initiation of disease pathology.

There are two key pathways which TLR’s utilise. The first pathway is aided by the adapter protein Myeloid Differentiation factor 88 (MyD88) whilst the other pathway is independent of this protein (Gaspari and Tyring, 2008). The MyD88 pathway is chiefly involved in the production of pro-inflammatory cytokines whilst the other pathway, independent of this adapter protein, causes the assembly of Type I interferons (i.e. antiviral peptides). When presented with an appropriate ligand, TLR’s begin an intracellular cascade initiated by the TIR domain. The TIR begins interacting with MyD88 (Figure 1.3.1-1) and as a result tumor necrosis factor receptor–associated factor 6 (TRAF6) and members of the interleukin-1-receptor–associated kinase (IRAK) family present themselves to the TLR locality (Gaspari and Tyring, 2008). This generates two separate cascades, one that activates the inhibitory
factor kappa B kinase (IK-BK) cascade (Faure et al., 2000) and the other which initiates the MAPK pathway.

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>TLR Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Langerhans' Cell</td>
<td>+</td>
</tr>
<tr>
<td>Mast Cell</td>
<td></td>
</tr>
<tr>
<td>Fibroblast</td>
<td>+</td>
</tr>
<tr>
<td>Dendritic Cell</td>
<td>+</td>
</tr>
<tr>
<td>Macrophage</td>
<td>+</td>
</tr>
<tr>
<td>Neutrophil</td>
<td></td>
</tr>
<tr>
<td>T-Regulatory lymphocyte</td>
<td></td>
</tr>
</tbody>
</table>

Table 1.3-1. TLR Expression in common pro-inflammatory cells

An overview of the key cells involved in asthma pathology, portraying the expression of TLRs by immunocells. Notably and uniquely, all the above cells express TLR-4 therefore highlighting its board role in asthma pathogenesis.

There are 11 members of the Toll Like Receptor family of which it is thought that TLR-2 and TLR-4 are the most important for transmembrane signalling.

1.3.1. TLR-4 Activation

The activation of macrophages by bacterial invasion - in particular by LPS present on bacterial surface - has been demonstrated to be reliant on TLR-4 (Beutler, 2000). TLR-4 is the only toll like receptor responsible for activating all 5 of the TIR adaptor proteins (Lu et al., 2008). The initiation of this is heavily reliant of LPS binding to an additional co-receptor - CD14. Indeed, CD14 is expressed upon the surfaces of monocytes, macrophages, neutrophils, whilst also – albeit in less abundance – on the surfaces of epithelial cells and fibroblasts (Anas et al., 2010). CD14 lacks the intracellular domain that TLR-4 has, and therefore singularly is unable to initiate any of the intracellular pathways. Current studies demonstrate that CD14 is able to
become activated via bacterial components including LPS, lipoteichoic acid (LTA) and elements from respiratory syncytial virus (RSV). Upon initial binding to one of these elements, CD14 activates TLR-4 and thus initiates the intercellular pathways including MAPKs and inhibitor of kappa B IκB kinase (Rich, 2010). This results in the activation of transcription factors (TFs) such as nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and p38 MAPK (Figure 1.3.1-1).

Activated TLR-4 upon presentation with bacterial elements is caused by point mutations in the extracellular domain leading to ‘LPS-hyperresponsiveness’, previously demonstrated in murine models (Poltorak et al., 1998). This point mutation may lead to the activation of other TFs, resulting in increased cell survival, proliferation and the release of pro-inflammatory cytokines.
It is worthy of note that this action is also somewhat reliant on the function of CD14 which is present on macrophages, neutrophils and monocytes (Anas et al., 2010). The activation of TLR-4 leads to the recruitment of IL-1 receptor-associated kinases (IRAKs). This associates with MyD88 which in turn recruits TNF receptor-associated factor 6 (TRAF-6). As a result it activates both the MAPKs and IκBK pathways (Lu et al., 2008) leading to the recruitment of numerous transcription factors (TFs) including signal transducers activators of transcription (STAT-1) and activating transcription factor 1 (ATF-1). This can lead to the synthesis of pro-inflammatory mediators including CXCL8, nitric oxide (NO) and TNF-α essential in inflammation and cell proliferation.

**Figure 1.3.1-1. LPS TLR-4 /CD14 complex pathway**
1.3.1.1. **Lipopolysaccharide (LPS)**

LPS is present on the outer walls of gram negative bacteria. A single bacterium can contain approximately $10^6$ LPS particles which is relatively extensive when each bacterium contain $10^7$ of total phospholipids (Varki et al., 2009). The structure of LPS is comprised of three distinct subunits of which there are Lipid A, an inner core region and an O-antigen region. Indeed, it is the Lipid A portion which serves as the key feature in causing bioactive effects in mammals (Khan et al., 1998).

1.3.1.2. **LPS Challenge Models**

Previous studies have found occupational endotoxin exposure to decrease lung function and increase blood neutrophils in cotton mill workers (Rylander et al., 1985). Further studies observed a negative association with endotoxin concentration and FEV$_1$ from endotoxin exposure (Donham et al., 1989, Kennedy et al., 1987). Schwartz et al. (1995) have also observed higher symptom reporting, incidence of cough and a decrease in FEV$_1$ and FEV$_1$/FVC, in those with increased environmental endotoxin levels after adjusting gender, age and cigarette smoking status (Schwartz et al., 1995). With the possibility of LPS exposure being related to airway neutrophilia, there has been a large degree of studies focussing on LPS inhalation, safety and biomarker expression (Table 1.3.1.2-1)
<table>
<thead>
<tr>
<th>Study Author (Year)</th>
<th>Title</th>
<th>Patient Type</th>
<th>Population</th>
<th>Volume of LPS</th>
<th>Clinical Observations</th>
<th>Inflammatory Biomarkers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hernandez M et al. (2012)</td>
<td>Atopic asthmatic patients have reduced airway inflammatory cell recruitment after inhaled endotoxin challenge compared with healthy volunteers</td>
<td>HNS</td>
<td>18</td>
<td>20,000 EU / ~2μg</td>
<td>No reported changes</td>
<td>↑ Percentage sputum neutrophils (p&lt;0.001)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Asthma (atopic)</td>
<td>13</td>
<td></td>
<td></td>
<td>↑ IL-6 and TNF-α from DTT processed sputum supernatants (p&lt;0.05)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>↓ The absolute numbers of airway leukocytes and PMNs</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>↓ TLR-4+ Monocytes (not statistical)</td>
</tr>
<tr>
<td>Kitz et al. (2008)</td>
<td>LPS inhalation challenge: a new tool to characterize the inflammatory response in humans</td>
<td>Healthy</td>
<td>20</td>
<td>100 μg</td>
<td>↑ Body temperature No change in lung function</td>
<td>No change to exhaled nitric oxide</td>
</tr>
<tr>
<td>Kitz et al. (2006)</td>
<td>Systemic and bronchial inflammation following LPS inhalation in asthmatic and healthy subjects</td>
<td>LPS Sensitive Asthmatics</td>
<td>6</td>
<td>100 μg</td>
<td>↓ FEV1 after 90 mins maximum drop after 2 h 3 were symptomatic</td>
<td>↑ Blood CRP LBP TXB2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LPS Non Sensitive</td>
<td>7</td>
<td></td>
<td>↓ FEV1 after 90 mins maximum drop after 2 h 4 were symptomatic</td>
<td>↑ Blood CRP LBP ECP</td>
</tr>
<tr>
<td>Alexis et al. (2003)</td>
<td>Effect of inhaled endotoxin on airway and circulating inflammatory cell phagocytosis and CD11b expression in atopic asthmatic subjects</td>
<td>Asthma (atopic)</td>
<td>10</td>
<td>5 μg</td>
<td>No reported changes</td>
<td>↑ Cell-surface expression of CD14 (membrane-bound CD14) was increased in sputum cells</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>↑ Sputum neutrophils (p&lt;0.002)</td>
</tr>
<tr>
<td>Nightingale et al. (1998)</td>
<td>Effect of inhaled endotoxin induced sputum in normal, atopic, and atopic asthmatic subjects</td>
<td>HNS (atopic)</td>
<td>7</td>
<td></td>
<td>No reported changes</td>
<td>No reported changes</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Asthma (atopic)</td>
<td>8</td>
<td>60 μg</td>
<td>↑ Temperature by 0.6°C (p&lt;0.05)</td>
<td>↑ Percentage sputum neutrophil post 6hrs (p&lt;0.05) and remained high post 24hrs (not statistical)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HNS (non atopic)</td>
<td>11</td>
<td></td>
<td>Symptoms: mild headache</td>
<td>↑ Sputum IL-8 post 24hrs</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>↑ Absolute neutrophil count post 24hrs (p&lt;0.01)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>↑ Sputum IL-8 post 6hrs</td>
</tr>
<tr>
<td>Study Author (Year)</td>
<td>Title</td>
<td>Patient Type</td>
<td>Population</td>
<td>Volume of LPS</td>
<td>Clinical Observations</td>
<td>Inflammatory Biomarkers</td>
</tr>
<tr>
<td>---------------------</td>
<td>------------------------------------------------</td>
<td>--------------</td>
<td>------------</td>
<td>---------------</td>
<td>-----------------------</td>
<td>------------------------</td>
</tr>
<tr>
<td>Thorn and Rylander (1998)</td>
<td>Inflammatory response after inhalation of bacterial endotoxin assessed by the induced sputum technique</td>
<td>HNS</td>
<td>21</td>
<td>40 μg</td>
<td>↓ FEV1 + FVC (p&lt;0.01)</td>
<td>↑ Blood MPO (p&lt;0.001) ECP (not statistical) ↑ Blood % neutrophils (p&lt;0.001) and monocytes (p = &lt;0.01) ↑ Sputum MPO and ECP (p&lt;0.01) ↑ Sputum % neutrophils and monocytes (p&lt;0.01)</td>
</tr>
<tr>
<td>Michel O et al. (1997)</td>
<td>Dose-Response Relationship to Inhaled Endotoxin in Normal Subjects</td>
<td>HNS</td>
<td>9</td>
<td>0.5, 5, and 50 μg</td>
<td>Acute systemic symptoms reported (n=5) ↑ Temperature by 0.7°C at 50 μg LPS (p&lt;0.05) ↓ FEV1 at 50 μg LPS (not statistical)</td>
<td>↑ Blood neutrophils at 5 and 50 μg ↑ Blood CRP at 5 and 50 μg ↑ Urine CRP at 50 μg ↑ Sputum TCC, total and differential neutrophils and monocytes at 5 and 50 μg, lymphocytes at 50 μg (p&lt;0.05) post 6hrs ↑ Sputum MPO post 5 and 50 μg post 6hrs ↑ Sputum ECP and TNF post 50 μg post 6hrs</td>
</tr>
<tr>
<td>Michel O et al. (1989)</td>
<td>Effect of inhaled endotoxin on bronchial reactivity in asthmatic and normal subjects</td>
<td>Asthma</td>
<td>8</td>
<td>22.2 μg</td>
<td>↑ bronchial obstruction (p&lt;0.01)</td>
<td>↑ histamine hyperresponsiveness 5 hafter LPS inhalation (p&lt;0.005)</td>
</tr>
</tbody>
</table>

Table 1.3.1.2-1. Summary table of previous LPS challenge studies including dose used and clinical/biomarker observations.

It is worthy of note that those studies comparing healthy subjects to subjects with asthma have demonstrated increases in temperature but no changes to spirometry.
1.4. Inhaled Corticosteroids (ICSs)

Also termed ‘glucocorticoids’, ICS are the most effective form of therapeutic management for the majority of asthmatics (Barnes, 1998) and cause a reduction of hyperresponsiveness and airway inflammation (Barnes, 1990). A vast array of cells, cytokines, chemokines and other proteins are responsible for the development of airway obstruction, hyperresponsiveness and remodelling. The majority of these cells are controlled by pro-inflammatory TFs including NF-κB and macrophage activator protein-1 (AP-1). NF-κB subsequently binds to promoter regions within DNA and interacts with ‘coactivators’ including p300/CREB Binding Protein (CBP) and P300/CBP-Associated Factor (PCAF) (Barnes and Adcock, 2003). These complexes promote the transcription of inflammatory cytokines such as IL-1, IL-2, IL-6, CXCL8 and TNF-α (Ichiyama et al., 2004).

Glucocorticoid receptors (GRs) are composed of α and β chains. The α chain is associated with chaperone proteins. Corticosteroids bind to the α chains and cause the dissociation of the chaperone proteins. This influences the movement of ‘CS-alpha chain’ complexes which translocate into the nucleus. Subsequently, this complex is able to bind to sections of DNA termed ‘glucocorticoid response elements’ affecting gene transcription. GRs may increase transcription by interacting with the co-activating molecules including PCAF and CBP (mimicking those pathways activated by pro-inflammatory proteins). The increase in gene activation could increase the production of anti-inflammatory peptides, which may include inhibitor molecules including 1kb-α which impedes NF-κB.

Inhaled steroids may also bind to the co-activating molecules CBP and PCAF. This binding ultimately prevents the association with other pro-inflammatory TFs such as NF-κB and therefore prevent the unwrapping of DNA keeping active sites enclosed in a process commonly termed ‘transrepression’
Figure 1.4-1. An illustration of the current understandings behind steroid based inflammatory suppression

Note that corticosteroid action may have multiple pathway targets, both early stream and further downstream. Indeed, it can be observed that the mechanics can be based on the MAPKs and NF-κB. ICSs can induce both negative and positive feedback mechanisms by ultimately suppressing pro-inflammatory processes. By identifying the mechanisms behind ICS action, it aids in the understanding of steroid resistance induced by a significant proportion of asthma sufferers.
1.4.1. Corticosteroid Resistance

It has been established that a significant proportion of asthmatics fail to respond to corticosteroid treatment (Jatakanon et al., 1999, Pavord et al., 1999). Steroid resistance is generally defined when patients are unable to control their asthma, have nocturnal symptoms, demonstrate persistence airflow obstruction (i.e. spirometry $FEV_1$ which is <70% predicted) along with a poor response to steroid therapy (Chan et al., 1998). Yet the exact pathogenic mechanisms in this asthma phenotype are less than clear.

The lack of efficacy in steroid response has been attributed to two key cellular functions. This can develop through a reduction in GRs or an inability of ligands to bind to these receptors (Moody, 2002). The latter is thought to be caused by IL-2, IL-4 and IL-13 (Barnes and Adcock, 2003, Moody, 2002), cytokines released by TH2 CD4+. More specifically these cytokines are able to stop corticosteroids from binding with GRs (Barnes and Adcock, 2009).

It has been reported that MMP-9 levels are increased in BAL samples in asthmatics who were unresponsive to corticosteroid treatment (Goleva et al., 2007). MMP-9 is primarily secreted by neutrophils and thus potentially highlights an increased role of neutrophils in steroid resistance development. A recent study observed increased levels of TNF-$\alpha$ mRNA and TNF-$\alpha$ proteins in BALF of steroid insensitive asthmatics (Goleva et al., 2008). Barnes and Adcock (2009) state that models which have inhibited p38 MAPK, increase the ability of therapeutic ligands to bind to GRs. It is therefore possible that p38 could cause phosphorylation of GRs.
1.4.2. LPS Exposure and Steroid Insensitivity in Asthmatics

Frequent exposure to pathogenic toxins can often be attributed to occupations involving waste, agriculture, fibreglass and cotton manufacture (Michel, 2003, Milton et al., 1996). Notably, high levels of bioactive LPS have been reported in cigarette smoke which may explain the pathogenesis and steroid resistance of other obstructive diseases including that in COPD (Hasday et al., 1999). Indeed, there may also be increased susceptibility to LPS stimulation in atopic asthmatics (Michel et al., 1989). This is likely due to increased levels of CD14 in atopic asthmatics (Alexis and Peden, 2001). Increased levels of CD14 can be attributed to previous allergen exposure which has been demonstrated in earlier studies (Dubin et al., 1996).

LPS is able to promote the activation and differentiation of macrophages and monocytes respectively, with the ultimate release of pro-inflammatory peptides including TNF-α, IL-1, IL-6, CXCL8, and IL-12 (Raetz, 1990, Cohen, 2002). Indeed, high levels of LPS exposure in macrophages can cause the release pro-inflammatory mediators that may induce septic shock and therefore subsequent damage to surrounding tissue (Hsing et al., 2008, Netea et al., 2003). Upon stimulation with LPS, human alveolar macrophages have been shown to increase CXCL8 production by 15 times at concentrations up to 100μg/ml (Palmberg et al., 1998). Recently, studies have demonstrated that those with corticosteroid resistant asthma have increased markers of classically activated macrophages and decreased markers of alternative macrophages.

Goleva et al. (2008) observed 10 subjects with corticosteroid resistant asthma and 8 with corticosteroid sensitive asthma. Increased levels of endotoxin and associated LPS pathway mediators were observed in BAL studies from asthmatics with steroid resistance when compared to those who were sensitive. Increased levels of early growth response factor-1 (EGR-1), molecule possessing ankyrin-repeats induced by lipopolysaccharide (MAIL), dual-specificity phosphatases-2 (DUSP-2) and TNF-α–induced protein 3 (TNFAIP3) were elevated in subjects with corticosteroid resistant
asthma (Goleva et al., 2008). Parallel literature has further demonstrated increased expression of MMP-9 in response to LPS (Jackson et al., 2009). As discussed earlier in this chapter this is able to promote the potency of CXCL8, recruit neutrophils to the surrounding airway and may contribute to corticosteroid resistance. Relevantly, other studies have demonstrated that p38 may be involved in the production of MMP-9 through the use of other TFs including AP-1 (Simon et al., 2001). The importance of p38 in LPS stimulation has been demonstrated in previous studies involving healthy subjects who were given p38α and p38β inhibitors. The study observed significantly reduced levels of TNF-α, CXCL8, and IL-6 from peripheral-blood mononuclear cells (PBMCs) stimulated with LPS (Parasrampuria et al., 2003), demonstrating a pivotal role of p38 in TLR-4 stimulation.
Figure 1.4.2-1. The proposed pathways leading to steroid resistance.

There is increased likelihood of those cells which contain the CD14 receptor to be involved in this process as LPS stimulation response is amplified. It demonstrates the pathways that may be involved in phosphorylation or nitrosylation of GRs could be orchestrated by the MAPKs or increased nitric oxide. It highlights the production of MMP-9 in this role which has been thought to contribute to steroid resistance via airway remodelling. Notably increased neutrophilia decreases infection control highlighting the range of targets that inhibitory mediators have in impeding this progressive cascade.
CHAPTER 2

2. General Methods

2.1. Subject Selection

Subjects were recruited from a clinical list based at the Medicines Evaluation Unit, University Hospital South Manchester, Greater Manchester. Asthmatic subjects were required to have a previous physician’s diagnosis of asthma for at least 6 months. A physician’s diagnosis included a clinical history alongside suggestive features based on GINA guidelines validated by a GP questionnaire. All patients were required to have been taking medications for asthma for at least 1 year. Exclusion criteria were a smoking history of ≥1 pack years and any other lung disease other than asthma. Subjects reporting a respiratory tract infection in the last four weeks or medicating on oral corticosteroids were also excluded. Subjects were required to have an oxygen saturation of ≥92% on room air. All subjects provided written informed consent and the study was approved by the local ethics committee. Any subjects not adhering to the study restrictions as per protocol were also excluded.

2.2. Induced Sputum

Subjects performed forced expiratory volume in 1 second (FEV₁) manoeuvre after inhaling 200µgs of Salbutamol (Stockport Pharmaceuticals, Stockport, UK) before beginning the procedure on a wedge bellows spirometer (Vitalograph Buckinghamshire, UK). If the FEV₁ was ≥50% of predicted, subjects were permitted to perform the test. Subsequently subjects were asked to inhale 3% sterile hypertonic saline via an ultrasonic nebulizer (Ultraneb 2000, Medix, Harlow, UK) for 5 minutes. If subjects complained of any adverse symptoms the procedure was stopped. Contrary to this, subjects were instructed to breathe in deeply from functional residual capacity (FRC) to total lung capacity (TLC) once per minute. After the initial 5 minutes, subjects were asked to blow their nose and clear their oral cavity with water whilst refraining from swallowing.
Subjects were consequently asked to cough without scraping the palate or sinuses, samples were kept cool on ice to maintain cell viability. Subjects were asked to repeat an FEV<sub>1</sub> manoeuvre, if saline inhalation caused a drop in lung function by <20 but ≥10% from baseline then subjects were asked to repeat an FEV<sub>1</sub> three times. If the levels failed to decrease below a drop of ≥10% FEV<sub>1</sub> from baseline then they inhaled the same concentration of saline. If subjects’ lung function fell by ≥20% then they were given a further 200μgs of Salbutamol and rested for 20 minutes before repeating a FEV<sub>1</sub> prior to being discharged. Accordingly if lung function failed to drop more than 10% then the saline concentration was increased to 4% and then 5%.

2.3. PBS Processed Sputum

Sputum was processed within 2 hours after obtaining initial expectorate. Sputum plugs were isolated from the saliva component and weighed. Phosphate buffer saline (PBS) (Sigma, Poole, UK) was added proportionate to 8 volumes of the initial sputum weight, this was then vortexed (IKA lab dancer, Sigma, Poole, UK) for 10 seconds then rocked (Stuart SRT9, Appleton Woods Ltd, Birmingham, UK) for 15 minutes. Consequently the solution was centrifuged at 790G for 10 minutes at 4°C. 4 volume of PBS supernatant was then aspirated and stored at -80°C for future analyses. 4 volumes of 0.2% Dithiothreitol (DTT) (Sigma, Poole, UK) was subsequently added to the sample which was then vortexed for 10 seconds and rocked for 15 minutes. The sample was passed through 48μm nylon mesh (Sefar Ltd, Bury, UK). 10μl of the filtrate was added to a further 10μls of trypan blue. Viable and non-viable leukocytes were counted on a haemocytometer to determine cell viability, average leukocyte counts and percentage squamous cells. After cell count, cells were repeat centrifuged and DTT supernatants were collected and stored at -80°C for future analyses. Cells were resuspended in PBS to make a final concentration of 0.5 x 10<sup>6</sup> cells per ml for cytopsins and differential cell counts (DCCs). Cells were stained using RapiDiff (Triangle, Skelmersdale, UK) to
determine differential cell counts, which was performed by counting a total of 400 cells.

2.4. Exhaled Nitric Oxide (eNO)

Exhaled Nitric Oxide (eNO) was measured using the standard 50mls/sec flow rate (Niox; Aerocrine; Sweden) indirectly measuring eosinophilic inflammation as previously described (Taylor et al., 2006). Subjects were asked to inhale NO filtered air from the mouthpiece from FRC to TLC. Subsequently subjects were instructed to exhale steadily until a reading could be gathered. Results were recorded in parts per billion based on the 1999 ATS guidelines (ATS, 1999).

2.5. Bronchoscopy

Before beginning the bronchoscopy, a sedative agent (intravenous midazolam) was given. Lignocaine gel was used to provide topical nasal anaesthesia. Lignocaine solution was then applied the vocal cords, trachea and bronchial tree to anaesthetise. The bronchoscope was wedged as distally as possible into a specified lobe. A maximum of 240mls pre-warmed 0.9% saline was instilled into each lung, the mean(SD) of total saline used was 468.6(33.0). All aliquots were collected for culture/analysis. During repeat bronchoscopies, samples were taken from the same lobe but varying segments.

2.6. LPS Challenge

Subjects were given five inhalations of 0.5 mg/ml E.coli LPS (Serotype O26:B6, ref-L2654) (Sigma, Dorset, UK) at 12µl per breath giving a total of 30µgs, which has been used in previous models at the site (Aul et al., 2012). Subjects were instructed to inhale aerosolised LPS from FRC to TLC this was repeated 5 times. This
technique employed a Mefar dosimeter (Markos Mefar, Bresica, Italy) for administration of the LPS. Vital signs including temperature, pulse, blood pressure and lung function and exhaled nitric oxide were monitored prior to the LPS challenge and post hourly for up to 24 hours for subject safety.

2.7. Macrophage Cell Culture

After capturing BAL obtained during bronchoscopy the sample was placed on -20°C ice. The sample was then filtered through a 100μm nylon cell strainer (BD Falcon, Nottingham, UK). 10μl of the filtrate was then added to a further 10μls of trypan blue and a cell count was performed on a haemocytometer. The sample was then centrifuged at 400G for 10 minutes at 4°C. The cell pellet was subsequently resuspended in sufficient RPMI 1460 supplemented with 10% fetal calf serum, 1 mmol/L L-glutamine and 1% penicillin and streptomycin to make a working concentration of 1.0 x 10⁶ cells per ml.

BAL cytospins were then prepared for DCCs to assess peripheral airway inflammation. 40μl-60μls of the cell suspension was added to cytoslides which were subsequently centrifuged at 250G for 6 minutes at room temperature. The remaining cell suspension was placed onto Ficol-paque PLUS (GE healthcare UK Ltd, Buckinghamshire, UK) and spun at 400G for 30 minutes at 25°C, to remove erythrocytic contamination. Once centrifuged, cells were isolated and added to equal volumes of RPMI 1460. The cell suspension was then re-centrifuged at 400G for 10 minutes at 4°C and the supernatant discarded to remove any Ficol-paque PLUS which may be toxic to cells. Subsequently an appropriate volume of RPMI 1460 was added to the cell pellet. A cell count was then repeated as previous so cells could be resuspended to make up 1.0 x 10⁶ macrophages per ml.

100μls of the cell suspension was added to each well of a 96 well polystyrene cell culture flat-bottomed plate, cells were then incubated at 37°C in presence of 5% CO₂.
and left to adhere for 20 hours. The following day the plate was washed twice with RPMI 1460 to remove non-adherent cells. The appropriate conditions were then applied. Cells were treated with either Dexamethasone (Sigma, Dorset, UK) or 0.005% DMSO (vehicle control) for 1 hour before being stimulated with 1μg/ml of 026:B6 LPS (Sigma, Dorset, UK). Cells were stimulated with LPS for 24 hours before being repeat centrifuged at 2000rpm for 10 minutes 4°C for supernatant removal. Supernatants were stored at -20°C for future analysis.

1000 to 0.1nmol/l dexamethasone concentrations were used as previously described (Kane et al., 2009). In addition dexamethasone concentrations of 1000 and 100nmol/l (i.e. 1 to 0.1umol/litre) were used for unstimulated cells as these have been shown to give sufficient levels of cytokine detection in previous studies (Armstrong et al., 2009).

### 2.8. CXCL8 and TNF Analysis

Supernatant concentrations of TNF-α and CXCL8 were determined by enzyme linked immunosorbant assay (ELISA). ELISAs were carried out according to manufacturers’ guidelines (Appendix 1 & 2). Lower limit of detection for CXCL8 and TNF was 31.25 and 15.625 respectively.

3.1. Introduction

Asthma is a heterogeneous disease, comprising subgroups of patients with distinct clinical and pathophysiological characteristics (Wenzel, 2006). The identification and description of phenotypes of asthma may allow targeted management strategies to improve asthma control, in contrast to the “one size fits all” approach. Late onset asthma is a well-known clinical phenomenon, and may represent a distinct phenotype of patients. Studies of this phenotype can be difficult to perform, as there is no accepted cut off definition of the time after which late onset asthma develops (Ayres, 1990), and because of the impact of confounding factors such as smoking history and the development of COPD causing diagnostic confusion. Nevertheless, the need for further characterization of the clinical and pathophysiological features of this group of patients is well recognised (Jones et al., 2011).

A recent study has shown that elderly patients with asthma had significantly more severe exacerbations than younger patients (Haughney et al., 2011). This suggests the possibility that late onset asthmatics are pre-disposed to a more severe phenotype of asthma. Induced sputum is a non-invasive method of assessing airway inflammation that has been extensively used in the phenotyping of asthma. Increased sputum neutrophil counts in asthma are associated with more severe disease (Shaw et al., 2007) and a reduced response to corticosteroids (Green et al., 2002b). The number of airway neutrophils increases with age in healthy subjects (Thomas et al., 2004), but it is not known if late onset asthmatics also have increased neutrophilic airway inflammation. We have investigated the clinical and induced sputum characteristics of patients with late onset asthma, defined as onset after the age of 40, and compared the results to a younger group of patients.
3.2. Methods

3.2.1. Subject Selection

Thirty eight adult patients with a physician diagnosis of asthma for at least 6 months were recruited. Nineteen patients were classified as “late onset asthma” (LOA) diagnosed after the age of 40 years, while 19 were classified as “early onset asthma” (EOA) diagnosed before the age of 20 years. Patients were required to be using inhaled corticosteroids (ICS) with or without a long acting beta agonist. Both groups were matched for ICS use. 10 healthy aged matched controls were taken from historical data. Each were required to have an FEV<sub>1</sub> of >80% predicted and no significant medical or surgical history as deemed by the study physician. Using the between subject standard deviation (23.14) from a larger dataset of asthmatics, it was estimated that this study required a sample size of n=16 to detect a change in neutrophil percentage by 23.76 at 80% power.

3.2.2. Study Design

Subjects attended a 1 day protocol in which they performed an Asthma Control Questionnaire-7 (ACQ-7) (Appendix 3), spirometry with reversibility to 200µg salbutamol, exhaled nitric oxide (eNO) at 50ml/sec (Niox; Aerocrine; Sweden) and sputum induction (Pizzichini et al., 1996). Differential cell counts were performed by counting a total of 400 cells by two blinded observers.

3.2.3. Statistical Analysis

Non-parametric data were either natural log transformed and presented as geometric means with 95% confidence intervals or expressed as medians with ranges. Differences between groups were assessed using unpaired t-tests or the Mann Whitney test. Pearson correlation was applied to assess relationships between clinical data and airway inflammation measurements. All statistical tests were performed using Graphpad version 3 (GraphPad Software, San Diego, CA, USA; http://www.graphpad.com).
3.3. Results

The clinical characteristics are summarised in Table 3.3-1. The mean ages of asthma onset were 7.4 and 49.7 years for EOA and LOA groups respectively, with a significantly longer duration of asthma in the EOA group (23.2 vs 9.7 years respectively, p < 0.0001). EOA had a higher number of patients with atopy, defined by a positive reaction to at least one skin prick test, but the difference between groups was not statistically significant (p = 0.20). There was no difference in ICS usage between the groups. The FEV\(_1\) % predicted was lower in the EOA compared to LOA group (87.6% vs 103% respectively, p = 0.02), and the FVC % predicted was also lower in EOA. The ACQ score was significantly higher in the EOA group (p = 0.03). Percentage reversibility was significantly higher in the EOA group (p = 0.01), which may be due to the lower FEV\(_1\) in this group. The duration of asthma in the whole cohort was significantly associated with FEV\(_1\) % predicted (r = -0.4, p = 0.01), but not ACQ score (r = 0.22, p = 0.17).

The mean eNO was numerically increased in patients with EOA compared to LOA, but the difference did not reach statistical significance (p = 0.15). The percentage neutrophil counts were higher in the LOA group (p = 0.02), while the macrophage percentage was reduced (p = 0.02) compared to EOA (Figure 3.3-1). There were no differences between groups for other sputum parameters. There was a significant correlation between age and neutrophil percentage (r = 0.39, p = 0.04) and a negative correlation between age and macrophage percentage (r = -0.40, p = 0.04). There was no correlation between duration of asthma and neutrophil (p = 0.12) or macrophage (p = 0.11) percentage cell counts, or between ICS dose and neutrophil percentage (p = 0.4). The analysis of sputum producers only (n=12 LOA and n=16 EOA) did not change the statistical significance of the results in Table 3.3-1, except for ACQ where the numerical difference between groups remained (0.86 vs 1.44), but the reduction in sample size meant that the p value was no longer significant (p = 0.09).
The historical healthy control group (n=10, 4 males / 6 females, mean age 57.8 yrs) was age matched to the LOA group. The mean neutrophil percentage in the healthy subjects was lower compared to LOA patients; 33.9% vs 57.3% respectively, p = 0.01. However EOA had remarkably similar neutrophil counts to the healthy controls where mean percentage neutrophil percentage was 36.6%.

Figure 3.3-1 Induced sputum differential cell counts between Late Onset and Early Onset Asthma

Neutrophils and macrophages are presented with means±SD, eosinophils and lymphocytes are presented as geometric means ±95% confidence intervals. Comparison of cell population between groups were analysed by unpaired t test. A statistical increase and decrease was found in percentage sputum neutrophils and macrophages respectively in those with LOA.
Table 3.3-1. The clinical and induced sputum demographics of the LOA study population

<table>
<thead>
<tr>
<th>Clinical Characteristics</th>
<th>LOA (n=19)</th>
<th>EOA (n=19)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender Male/Female</td>
<td>7/12</td>
<td>9/10</td>
<td></td>
</tr>
<tr>
<td>Daily ICS Dose [BED]^{a} (mcg)^{b}</td>
<td>640 (395 – 1034)</td>
<td>917 (657 – 1281)</td>
<td>p = 0.20</td>
</tr>
<tr>
<td>Atopy</td>
<td>66.6%c</td>
<td>89.5%</td>
<td>p = 0.20d</td>
</tr>
<tr>
<td>Age</td>
<td>59.4 (4.8)</td>
<td>30.6 (5.5)</td>
<td>p &lt; 0.0001</td>
</tr>
<tr>
<td>Age at asthma onset</td>
<td>49.7</td>
<td>7.4</td>
<td>p &lt; 0.0001</td>
</tr>
<tr>
<td>Asthma Duration (years)</td>
<td>9.7 (6.1)</td>
<td>23.2 (6.1)</td>
<td>p &lt; 0.0001</td>
</tr>
<tr>
<td>FEV₁ % Predicted (%)</td>
<td>103 (19)</td>
<td>88 (19)</td>
<td>p = 0.02</td>
</tr>
<tr>
<td>FVC % Predicted (%)</td>
<td>117 (18)</td>
<td>101 (14)</td>
<td>p = 0.003</td>
</tr>
<tr>
<td>Reversibility (mls)</td>
<td>73 (141)</td>
<td>296 (296)</td>
<td>p = 0.01</td>
</tr>
<tr>
<td>Reversibility (%)</td>
<td>3.2 (5.5)</td>
<td>11.8 (12.6)</td>
<td>p = 0.01</td>
</tr>
<tr>
<td>eNO (ppb)</td>
<td>16.3 (12.3 – 21.7)</td>
<td>20.8 (14.0 – 30.9)</td>
<td>p = 0.15</td>
</tr>
<tr>
<td>ACQ-7 Score</td>
<td>0.89 (0.63)</td>
<td>1.46 (0.92)</td>
<td>p = 0.03</td>
</tr>
</tbody>
</table>
### Induced Sputum

<table>
<thead>
<tr>
<th></th>
<th>LOA</th>
<th>EOA</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sputum Total Cell Count (x106)(^c)</td>
<td>1.6</td>
<td>2.3</td>
<td>p = 0.21(^f)</td>
</tr>
<tr>
<td></td>
<td>(0.1 - 8.2)</td>
<td>(0.2 - 8.9)</td>
<td></td>
</tr>
<tr>
<td>Neutrophil %</td>
<td>57.3</td>
<td>36.6</td>
<td>p = 0.02</td>
</tr>
<tr>
<td></td>
<td>(23.2)</td>
<td>(22.1)</td>
<td></td>
</tr>
<tr>
<td>Eosinophil %(^b)</td>
<td>1.3</td>
<td>0.9</td>
<td>p = 0.57</td>
</tr>
<tr>
<td></td>
<td>(0.5 – 3.5)</td>
<td>(0.4 – 2.2)</td>
<td></td>
</tr>
<tr>
<td>Macrophage %</td>
<td>34.3</td>
<td>52.6</td>
<td>p = 0.02</td>
</tr>
<tr>
<td></td>
<td>(20.8)</td>
<td>(19.2)</td>
<td></td>
</tr>
<tr>
<td>Lymphocyte %(^b)</td>
<td>0.3</td>
<td>0.7</td>
<td>p = 0.07</td>
</tr>
<tr>
<td></td>
<td>(0.1 – 0.7)</td>
<td>(0.4 – 1.1)</td>
<td></td>
</tr>
<tr>
<td>Absolute Neutrophil Count (x106/g sputum)</td>
<td>3.5</td>
<td>2.4</td>
<td>p = 0.30</td>
</tr>
<tr>
<td></td>
<td>(3.3)</td>
<td>(2.0)</td>
<td></td>
</tr>
<tr>
<td>Absolute Eosinophil Count (x106/g sputum)(^b)</td>
<td>0.1</td>
<td>0.1</td>
<td>p = 0.73</td>
</tr>
<tr>
<td></td>
<td>(0.02 – 0.2)</td>
<td>(0.02 – 0.1)</td>
<td></td>
</tr>
<tr>
<td>Absolute Macrophage Count (x106/g sputum)(^b)</td>
<td>1.2</td>
<td>2.4</td>
<td>p = 0.14</td>
</tr>
<tr>
<td></td>
<td>(0.6 – 2.7)</td>
<td>(1.4 – 4.1)</td>
<td></td>
</tr>
<tr>
<td>Absolute Lymphocytes Count (x106/g sputum)(^b)</td>
<td>0.02</td>
<td>0.04</td>
<td>p = 0.31</td>
</tr>
<tr>
<td></td>
<td>(0.01 – 0.04)</td>
<td>(0.02 – 0.06)</td>
<td></td>
</tr>
</tbody>
</table>

All parametric data are expressed as means with standard deviations unless stated otherwise. a Beclometasone Equivalent Dose. b denotes geometric mean with 95% confidence intervals. c n = 15. d denotes fisher’s exact test. e denotes median and range f mann-whitney U test. Sputum induction was performed on all subjects, and adequate samples to prepare cytospins were obtained from 12 patients with LOA and 16 patients with EOA.
3.4. Discussion

Patients with LOA in this study had better asthma control, as the FEV$_1$ was higher and ACQ lower than patients with EOA. There was a significant association between duration of asthma and FEV$_1$. This suggests that the onset of asthma later in life is not necessarily associated with more severe disease, but that more severe disease characteristics develop with a longer duration of asthma. This observation is similar to previous studies (Little et al., 2002, Olaguibel Rivera et al., 2007), where the strength of the reported associations (r values of approximately -0.4) have been remarkably similar to this study’s data.

Neutrophil counts have been associated with more severe asthma (Shaw et al., 2007, Green et al., 2002b) but this study observed higher neutrophil counts in LOA patients who had better asthma control. Increased neutrophil counts in LOA are therefore not a marker of more severe disease. Little et al. (2002) reported an association between sputum neutrophil counts and FEV$_1$ in chronic asthma, which contrasts with this study (Little et al., 2002). However, the current study was focused on the phenotype of LOA, and the relationship between sputum neutrophils and FEV$_1$ may vary with the phenotype studied. Furthermore, the findings of Little et al. refer to post oral corticosteroid measurements, which again differ to this study.

An increase in sputum neutrophil counts has been reported in healthy subjects aged > 50 years (Thomas et al., 2004). A previous historical healthy control group had a mean age of 57.8 years, but there was no rise in neutrophil counts. This indicates that neutrophil counts do not increase in all healthy subjects >50 years old, and perhaps greater increases are observed in subjects who are considerably older than 50 years. These findings in LOA therefore appear not to be simply an age related phenomenon, and increased sputum neutrophil counts may be a phenotypic feature of patients who develop LOA. LOA patients had a similar rate of atopy compared to EOA, and so it is possible that other factors are important in driving the neutrophilic airway inflammation observed in LOA, such as environmental pollution or infection which
are both known to cause neutrophilic lung inflammation (Nightingale et al., 1999, Salvi et al., 1999, Nightingale et al., 1998b).

This was a “real-life study” that used a previous physician diagnosis of asthma for which inhaled corticosteroids had been prescribed, although the degree of compliance with medications was not known. This study did not use other arbitrary diagnosis criteria such as reversibility or bronchial hyper-reactivity tests; as such inclusion criteria exclude the majority of patients with true asthma (Travers et al., 2007). The recruitment strategy for this study was via advertising within primary care and thus may have resulted in a selection bias towards subjects who respond to such recruitment methods; further larger studies in different populations are needed to confirm this study’s findings. Additionally, some caution should be applied to the interpretation of association analysis in the limited sample size presented here, as these were not the primary aim of the study and again larger studies are required for confirmation.

LOA patients had better lung function and ACQ scores while using similar doses of ICS. The mean FEV\textsubscript{1}% predicted in the LOA group was >100%, suggesting overprescribing of ICS in this group. GINA guidelines recommend that ICS usage should be titrated according to asthma control (Bateman et al., 2008). It is possible that patients with LOA are prescribed ICS at the correct dose to achieve asthma control, but then the ICS dosage is not titrated downwards. It is difficult in this type of study to match the duration of asthma between groups, as patients with EOA are more likely to have a longer duration.

In summary, the current findings suggest that duration of asthma rather than age of onset of asthma is a key determinant of lung function. Raised sputum neutrophil counts in LOA are a phenotypic characteristic but not a biomarker of severe disease.
4. Long and Short Term Reproducibility of Sputum Neutrophils in Moderate to Severe Asthmatics

4.1. Introduction

Asthma is a chronic condition hallmarked by unstable and persistent inflammation, variable airflow obstruction and bronchial hyperreactivity. Novel biomarkers including cytokines and pro-inflammatory markers are typically at the forefront of contemporary asthma research. Clinical biomarkers have previously been identified in blood, bronchoalveolar lavage and bronchial biopsies, however typically these methods can be invasive, expensive or clinically difficult to perform. Induced sputum is a novel, non-invasive and simple method of detecting airway inflammation in asthmatic patients (Pin et al., 1992). Since its initial use it has been employed and adapted in an extensive number of human trials (Dua et al., 2010, Gibson et al., 1998, Papadopouli et al., 2006, Zhang et al., 2010).

Airway neutrophilia is an inflammatory biomarker which can be detected in induced sputum. Increased airway neutrophils have been previously associated with steroid insensitivity (Jatakanon et al., 1999, Pavord et al., 1999), risk of exacerbation (Fahy et al., 1995) and disease severity (Kamath et al., 2005, Wenzel et al., 1997) in asthmatic studies. Long and short term repeatability of this sputum cellular profile in asthmatic sputum is ill recognised. Previously long and short term studies have shown moderate to strong levels of repeatability in percentage sputum neutrophils in COPD patients (Beeh et al., 2003, Boersma et al., 2007). Indeed this is something that may be expected as prominent neutrophil inflammation is a characteristic of COPD airway pathology (Hogg et al., 2004, Ronchi et al., 1996). Asthmatic airway inflammation could exhibit increased variability due to effects from extrinsic factors including allergen and occupational exposure which are now well recognised and have the potential to cause acute changes in sputum composition (Bettiol et al., 2002, Moscato et al., 2003).
Modern clinical studies use sputum differential cells counts as a key endpoint, these studies either have a cross-over or parallel design. Using intrasubject and within subject standard deviation (SD) it is possible to estimate sample sizes required for these designs. This study sought to observe the short and long term reproducibility of induced sputum neutrophils in moderate to severe asthmatics using the Pin et al. method which were defined as 7 days and >2 months apart respectively. The observations from this study were used to calculate power calculations for future clinical studies.
4.2. Methods

4.2.1. Subject Selection

14 adult patients with a physician diagnosis of asthma for at least 6 months were recruited. Patients were required to be using ICS with a long acting beta agonist. All subjects’ FEV<sub>1</sub> were less than 80% predicted at screen. All had to show present or previous (≤12 months) signs of hyperreactivity by either a change in lung function by ≥12% or 200mls post 200µgs Salbutamol or respond to Methacholine causing a 20% fall in FEV<sub>1</sub> (PC<sub>20</sub> ≤16 mg/ml) based on current guidelines (Bateman et al., 2008). ACQ-7 scores were 1 or greater.

4.2.2. Study Design

Subjects attended a 3 day protocol, all attended a baseline screening visit for medical history, reversibility to 200µg or a Methacholine challenge and sputum induction as previously described (Pin et al., 1992). To assess long term repeatability subjects were required to attend a second visit at least 2 months subsequent (V2) to their initial visit. Subjects were then asked to return 7(±1) days later (V3) to assess short term repeatability.

4.2.3. Statistical Analysis

Parametric data was expressed as means with standard deviations. Analysis of repeated measurements between groups was assessed using repeated measures ANOVA. Differences between individual visits were assessed using paired t-tests. Pearson correlation was applied to assess relationships between clinical data and airway inflammation measurements all analyses were performed on Graphpad version 3 (Graphpad Software, San Diego, CA, USA; http://www.graphpad.com). Differences between individual visits were represented using Bland-Altman analyses where dotted lines represent 95% limits of agreement. Correlation coefficients for two-way, absolute agreement and single measurements were performed using the SPSS version 19 software.
4.3. Results

The clinical characteristics of patients are demonstrated in Table 4.3-1. The mean percentage neutrophil counts for V1, V2 and V3 were 54.1, 59.1 and 64.1 respectively (Figure 4.3-1). Variances between visits 1 to 3 were assessed using repeated measures ANOVA. This demonstrated no differences between the groups (p = 0.30). This study also observed no differences between individual visits using Bland-Altman. The plots illustrate the repeatability between the visits as individual data points lie within the 95% limits of agreement (Figure 4.3-2 & Figure 4.3-3).

Mean differences for both long and short term visits were similar (p ≥ 0.05). Intraclass correlation coefficients showed strong (Ri = 0.72) and moderate (Ri = 0.51) levels of agreement for long and short repeatability (Table 4.3-2 & Table 4.3-3).

This study additionally observed the effects of aging (r = 0.22, p = 0.45) and asthma duration (r = -0.05, p = 0.88) on average percentage neutrophil counts which demonstrated no correlation. In addition FEV\textsubscript{1} % predicted against average percentage neutrophil counts (r = -0.15, p = 0.33) was observed demonstrating no trend. Subjects with increased neutrophils had increased ACQ-7 scores although this did not reach statistical significance (r = 0.37, p = 0.19).
Table 4.3-1 – The clinical demographics of the sputum repeatability study population and duration between visits

<table>
<thead>
<tr>
<th>Clinical Characteristics</th>
<th>n = 14</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender Male/Female</td>
<td>7/12</td>
</tr>
<tr>
<td>Daily ICS Dose [B.E.D](^{a}) (mcg)</td>
<td>1479 (538.1)</td>
</tr>
<tr>
<td>Age</td>
<td>48.0 (14.2)</td>
</tr>
<tr>
<td>Age at asthma onset</td>
<td>12.4 (12.3)</td>
</tr>
<tr>
<td>Asthma Duration (years)</td>
<td>35.6 (12.4)</td>
</tr>
<tr>
<td>Average FEV(_{1}) % Predicted (%)</td>
<td>67.1 (12.5)</td>
</tr>
<tr>
<td>Average FVC % Predicted (%)</td>
<td>87.4 (10.7)</td>
</tr>
<tr>
<td>Average Reversibility (mls)</td>
<td>349.3 (147.8)</td>
</tr>
<tr>
<td>Average Reversibility (%)</td>
<td>19.4 (10.2)</td>
</tr>
<tr>
<td>eNO (ppb)(^{b})</td>
<td>31.0 (25.9)</td>
</tr>
<tr>
<td>ACQ-7 Score</td>
<td>2.09 (0.58)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Visit Intervals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Days between V1 and V2(^{c})</td>
</tr>
<tr>
<td>Days between V2 and V3(^{c})</td>
</tr>
</tbody>
</table>

All data is expressed as means with standard deviations unless stated otherwise.

\(^{a}\) Beclomethasone Equivalent Dose \(^{b}\) n=13 \(^{c}\) denotes median and range
Figure 4.3-1. Before-After plot of percentage neutrophil counts over long (V1 vs V2) and short term time points (V2 vs V3)

The above figure is presented with means (±SD). Repeated measures ANOVA demonstrate no difference between the visits p = 0.30

Figure 4.3-2 Bland-Altman analysis of the long term repeatability of percentage sputum neutrophils
Figure 4.3-3. Bland-Altman analysis of the short term repeatability of percentage sputum neutrophils
**Table 4.3-2 Long term repeatability of percentage sputum neutrophils**

Data is representative between visit 1 and visit 2 (long term), mean differences were non statistically different (p > 0.05), with a high intraclass correlation coefficient score, suggesting a good repeatability.

<table>
<thead>
<tr>
<th>Neutrophils</th>
<th>First Sputum Induction Mean (SD)</th>
<th>Second Sputum Induction Mean (SD)</th>
<th>Mean Difference (95% CI)</th>
<th>Within Subject, SD</th>
<th>Intraclass Correlation Coefficient (Ri)</th>
</tr>
</thead>
<tbody>
<tr>
<td>%</td>
<td>54.1 (27.4)</td>
<td>59.1 (25.8)</td>
<td>4.96 (-16.5, 6.54)</td>
<td>p &gt; 0.05</td>
<td>19.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.72</td>
</tr>
</tbody>
</table>

**Table 4.3-3. Short term repeatability of percentage sputum neutrophils**

Data is representative between visit 2 and visit 3 (short term), mean differences were non statistically different (p > 0.05), with a moderate intraclass correlation coefficient score, suggesting a good repeatability.

<table>
<thead>
<tr>
<th>Neutrophils</th>
<th>Second Sputum Induction Mean (SD)</th>
<th>Third Sputum Induction Mean (SD)</th>
<th>Mean Difference (95% CI)</th>
<th>Within Subject, SD</th>
<th>Intraclass Correlation Coefficient (Ri)</th>
</tr>
</thead>
<tbody>
<tr>
<td>%</td>
<td>59.1 (25.8)</td>
<td>64.1 (22.4)</td>
<td>5.00 (-18.9, 8.93)</td>
<td>p &gt; 0.05</td>
<td>24.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.51</td>
</tr>
</tbody>
</table>
4.3.1. **Power Calculations**

Using both the within and between subject standard deviations, power calculations were performed for cross over and parallel study designs. It can be estimated that in long term sputum assessments in cross over studies require sample sizes of $n=12$, $n=43$, $n=167$ for changes in neutrophil percentages by 20, 10 and 5 at 90% power. For the same in short term assessment, sample sizes of $n=16$, $n=62$, $n=245$ are required. For parallel study designs observing long term sputum neutrophil assessments estimated sample sizes of $n=38$, $n=150$, $n=596$ for changes in neutrophil percentages by 20, 10 and 5 and $n=32$, $n=123$, $n=490$ for short term observations.

![Figure 4.3.1-1. Power calculations for parallel and cross over studies](image)

**Figure 4.3.1-1. Power calculations for parallel and cross over studies**

X axis is the sample size required. Y axis is the detectable difference in sputum neutrophils at 90% power.
4.4. Discussion

This present study has observed that long and short term duration between sputum induction procedures in moderate to severe asthmatics shows good repeatable value. This suggests that percentage neutrophil counts obtained from induced sputum is a credible biomarker which can allow for safe and reliable predictions for future short and long term time points within this subject group. This study has performed power calculations for parallel and cross over designs so future investigators may estimate sample sizes for these studies.

These current findings are the first to observe good repeatability of percentage sputum neutrophils in both long and short term studies in these asthma classifications. Previous work similar to this study has reported short term duration between sputum induction procedures (Bacci et al., 2002) presenting higher intraclass correlation coefficient to the ones found in the present study (Ri=85). However this study observed a cohort with a range of asthma severities (Bateman et al., 2008). The lower Ri values found may be a reflection of this sample population where increasing asthma severity demonstrates an associated risk of exacerbation (Thomson and Chaudhuri, 2008). Indeed, other studies have reported similar findings to these, In’t Veen et al. observed induced sputum in moderate to severe asthmatics where short term differences (median duration 4 days) in percentage neutrophils demonstrated Ri values of 0.61 (in ’t Veen et al., 1996).

Determining the duration between sputum inductions is not straightforward as increased neutrophil counts have been observed in short term studies as an effect of the inducing procedure (Holz et al., 1998, Nightingale et al., 1998a). To reduce running the risk of this increase, the current study encouraged a one week gap for the analysis of short term sputum neutrophils. The results demonstrated that this separation was sufficient as there were no detectable changes in neutrophil percentage (p > 0.05). Indeed other studies have identified this separation as sufficient (Boorsma et al., 2007, Spanevello et al., 1997).
Neutrophils in asthma play a role in exacerbation development (Fahy et al., 1995), steroid resistance (Jatakanon et al., 1999, Pavord et al., 1999) and uncontrolled asthma (Maneechotesuwan et al., 2007). The neutrophil counts observed in this study are notably higher than previous healthy control levels, when age is taken into account (Thomas et al., 2004). Repeated sputum assessment during treatment steps could prove more efficient based on downstream suppression.

In summary, percentage sputum neutrophils have good long and short term repeatability. Sputum neutrophils are a reproducible and reliable endpoint.
CHAPTER 5

5. The Effect of LPS Induced Neutrophilia on Steroid Sensitivity in Moderate to Severe Asthmatics

5.1. Introduction

ICS are the cornerstone of anti-inflammatory treatment in asthma (Rottier and Duiverman, 2009). However, many patients remain symptomatic despite high doses of ICS, even when combined with long acting beta agonists (Grove and Lipworth, 1995, Szefer and Leung, 1997). New anti-inflammatory therapies are needed for these patients who show insensitivity to the effects of high-dose corticosteroid therapy. This can be achieved by increasing our understanding of the mechanisms of corticosteroid insensitivity in asthma.

Neutrophils and macrophages are key components of the innate immune response, providing a rapid host defence response against microbial pathogens. Persistent neutrophilic airway inflammation is a characteristic feature of severe asthma, and is associated with a poor clinical response to corticosteroids (Jatakanon et al., 1999, Kamath et al., 2005). The mechanisms underlying neutrophilic asthma may be involved in causing corticosteroid insensitivity.

The airways of many asthma patients are colonised with bacteria, which could result in chronic TLR4 stimulation (Kraft et al., 1998, Martin et al., 2001). Furthermore, the airways of corticosteroid insensitive asthma patients have increased levels of LPS (Wanderer, 2009). This suggests that TLR4 signalling may be involved in corticosteroid insensitivity in patients with asthma. The inhalation of LPS causes airway neutrophilia (Alexis et al., 2004, Kitz et al., 2008, Michel et al., 1997). Inhaled LPS challenges could be used as a model of corticosteroid insensitive neutrophilic airway inflammation in patients with asthma.
Alveolar macrophages release CXCL8 upon stimulation with endotoxin (Dentener et al., 1993, Kane et al., 2009, Palmberg et al., 1998). CXCL8 is a potent neutrophil chemoattractant (Lin et al., 2004, Monteseirin, 2009, Ordonez et al., 2000). Previous “in vitro” work has demonstrated that corticosteroids have a limited inhibitory effect on the release of macrophage derived CXCL8 (Armstrong et al., 2009). It is possible that LPS exposure “in vivo” upregulates CXCL8 release from alveolar macrophages, and this causes corticosteroid resistant neutrophil chemotaxis.

This study used the LPS inhalation model in asthma to study TLR4 driven neutrophil chemotaxis mechanisms; Alveolar macrophages were isolated to observe whether LPS inhalation could affect macrophage derived CXCL8 steroid sensitivity “ex-vivo”.
5.2. Methods

5.2.1. Subject Selection

7 asthmatics adult patients with a physician diagnosis of asthma for at least 6 months were recruited. Patients were required to be using inhaled corticosteroids (ICS) with a long acting beta agonist. Subjects taking oral corticosteroids or leukotriene antagonists were excluded. Subjects were required to have no current or previous history of anaphylaxis/severe type reactions. If female, all subjects had to show a negative result to a pregnancy test. Subjects were required to have a baseline FEV$_1$ of $\geq$70% of predicted. All had to show present or previous (≤12 months) signs of hyperreactivity by either a change in lung function by $\geq$12% or 200mls post 200µgs Salbutamol or respond to Methacholine causing a 20% fall in FEV$_1$ (PC$_{20} \leq$16 mg/ml) based on current guidelines (Bateman et al., 2008).

5.2.2. Study Design

Subjects attended a 4 day protocol, all subjects attended an initial baseline screen whereby they performed lung function, exhaled nitric oxide, physical assessment by an onsite physician, sputum induction, a urine pregnancy test (if applicable) and had medical history taken. Subjects then returned for a second visit to attend a baseline bronchoscopy. 4 weeks subsequent to this, subjects returned for an LPS challenge visit whereby any medical history was updated, urine pregnancy test (if applicable) was performed, lung function, an LPS challenge and serial vital signs, eNO and lung function and a sputum induction. Subjects were asked to return the day after for a post LPS bronchoscopy.

5.2.3. Statistical Analysis

Differences between visits and time points were assessed using paired t-tests. Differences between culture conditions was assessed using unpaired t-test. Group analyses were analysed using repeated measures ANOVA. Non-parametric data were either natural log transformed and presented as geometric means with 95% confidence intervals or expressed as medians with ranges. All statistical tests were
performed using Graphpad version 3 (Graphpad Software, San Diego, CA, USA; http://www.graphpad.com). Column bar graphs are presented with means ± SEM.  
* = P < 0.05
5.3. Results

5.3.1. Clinical Parameters

The clinical characteristics of patients are demonstrated in Table 5.3.1-1. LPS inhalation was well tolerated, statistical decreases in FEV₁ were found at 0.5, 1, 2 and 3 hours post LPS inhalation (Figure 5.3.2-2), increases in temperature was also found at 6 and 8 hours post LPS inhalation (Figure 5.3.2-3). No statistical changes in oxygen saturation and pulse was found from baseline. All clinical assessments returned to baseline values post 24 hours. Exhaled nitric oxide as an indirect measure of eosinophilic inflammation showed increases at 6 (p = 0.083) and 8 (p = 0.098) hours however this was not statistical (Figure 5.3.2-1)
Table 5.3.1-1. The clinical demographics of the LPS inhalation study population

<table>
<thead>
<tr>
<th>Clinical Characteristics</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender Male/Female</td>
<td>3/4</td>
</tr>
<tr>
<td>Daily ICS Dose [B.E.D] (mcg)</td>
<td>1000 (500-1000)</td>
</tr>
<tr>
<td>Age</td>
<td>41.3 (11.3)</td>
</tr>
<tr>
<td>Age at asthma onset</td>
<td>15.7 (11.7)</td>
</tr>
<tr>
<td>Asthma Duration (years)</td>
<td>25.6 (6.97)</td>
</tr>
<tr>
<td>FEV₁ % Predicted (%)</td>
<td>92.0 (22.4)</td>
</tr>
<tr>
<td>Reversibility (mls)</td>
<td>140.9 (13.0 - 649.3)</td>
</tr>
<tr>
<td>Reversibility (%)</td>
<td>7.16 (0.86 - 26.5)</td>
</tr>
<tr>
<td>eNO (ppb)</td>
<td>17.7 (8.25)</td>
</tr>
<tr>
<td>ACQ-7 Score</td>
<td>1.43 (1.02)</td>
</tr>
</tbody>
</table>

All parametric data are expressed as means with standard deviations unless stated otherwise. a Beclometasone Equivalent Dose. b denotes median and range. c denotes geometric mean with 95% confidence intervals. d all subjects on the study were on long term corticosteroid therapy with a long acting beta agonist.
5.3.2. Safety

The most common symptom reported from subjects was tight chest (n=5), other symptoms included cephalalgia (n=3) and myalgia (n=3). Two of the subjects reported shivering and fever, both resolved within 24 hours.
Figure 5.3.2-1. The effects of LPS inhalation on exhaled nitric oxide

Figure 5.3.2-2. The effects of LPS inhalation on airflow limitation

* p = < 0.05.
Figure 5.3.2-3. The effects of LPS inhalation on temperature

Figure 5.3.2-4. The effects of LPS inhalation on percentage blood oxygen saturation
Figure 5.3.2-5. The effects of LPS inhalation on pulse
5.3.3. Sputum and BAL composition

Sputum and BAL composition is presented in Table 5.3.3-1. Sputum total cell count was statistically increased after LPS inhalation where counts at baseline and post LPS were 3.20 and 14.2 respectively (p = 0.02). Similarly the mean percentage neutrophil counts at baseline and post LPS were 38.0 and 85.18 (p = 0.0002). Absolute neutrophil counts (x10⁶/g sputum) were 1.09 and 9.92 demonstrating a statistical increase (p = 0.005). Percentage macrophages decreased proportionately with a rise in neutrophils as this did not affect absolute macrophage counts (p = 0.82). Percentage eosinophils and lymphocytes did not change.
Table 5.3.3-1. The induced sputum characteristics of the LPS inhalation study population

<table>
<thead>
<tr>
<th>Induced Sputum</th>
<th>Baseline</th>
<th>Post LPS (6 hours)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sputum Total Cell Count (x10^6)</td>
<td>3.20 (2.74)</td>
<td>14.2 (7.89)</td>
<td>0.02</td>
</tr>
<tr>
<td>Neutrophil %</td>
<td>38 (17.46)</td>
<td>85.18 (7.90)</td>
<td>0.0002</td>
</tr>
<tr>
<td>Eosinophil % c</td>
<td>0.46 (0.11 - 1.32)</td>
<td>1.07 (0.09 - 5.17)</td>
<td>0.3305</td>
</tr>
<tr>
<td>Macrophage % c</td>
<td>50.8 (35.9 - 71.7)</td>
<td>9.5 (5.37 - 16.6)</td>
<td>0.0005</td>
</tr>
<tr>
<td>Lymphocyte % b</td>
<td>0.25 (0 - 0.5)</td>
<td>0.25 (0 - 0.25)</td>
<td>0.4571</td>
</tr>
<tr>
<td>Absolute Neutrophil Count (x10^6/g sputum)</td>
<td>1.09 (0.86)</td>
<td>9.92 (5.20)</td>
<td>0.005</td>
</tr>
<tr>
<td>Absolute Macrophage Count (x10^6/g sputum)</td>
<td>1.14 (0.49)</td>
<td>1.26 (1.06)</td>
<td>0.8224</td>
</tr>
</tbody>
</table>

All parametric data are expressed as means with standard deviations unless stated otherwise. b denotes median and range. c denotes geometric mean with 95% confidence intervals.
Figure 5.3.3-1. Before-After plot of paired percentage macrophages and neutrophils from post LPS induced sputum.
Figure 5.3.3-2. Before-After plot of paired percentage macrophages and neutrophils from post LPS BAL cytospins
5.3.4. Cell Culture

5.3.4.1. Basal and Dexamethasone treated \textit{in vitro} unstimulated cell culture cytokine release and percentage inhibition (n=4)

Basal (B) alveolar macrophages demonstrated an observable decrease in CXCL8 release post LPS inhalation where levels fell from 28525pg/ml to 13995 (Figure 5.3.4.1-1) however this was not significant (p = 0.22). Similarly this did not statistically effect CXCL8 sensitivity to dexamethasone as presented by percentage inhibition (Figure 5.3.4.1-2). Basal alveolar macrophage derived TNF release also demonstrated no change post LPS inhalation (p = 0.49) and no change to dexamethasone sensitivity (Figure 5.3.4.1-3 & Figure 5.3.4.1-4).
**Figure 5.3.4.1-1.** *In vitro* unstimulated alveolar macrophage derived CXCL8 release at basal (B) and treated with 1000 and 100 nMol/l of dexamethasone. Grey datapoints = 500 mcg BED/24hrs, Black datapoints = 1000 mcg BED/24hrs

**Figure 5.3.4.1-2.** The percentage inhibition of unstimulated alveolar macrophage derived CXCL8.
Figure 5.3.4.1-3. *In vitro* unstimulated alveolar macrophage derived TNF release at basal (B) and treated with 1000 and 100nMol/l of dexamethasone

Figure 5.3.4.1-4. The percentage inhibition of unstimulated alveolar macrophage derived TNF
5.3.4.2. Basal and Dexamethasone treated *in vitro* stimulated cell culture cytokine release and percentage inhibition (n=4)

When observing *in vitro* LPS stimulated macrophages pre and post LPS inhalation, there was a notable decrease in CXCL8 cytokine production post LPS (Figure 5.3.4.2-1). Although not significant, this is particularly evident in macrophages treated with 0.1nmol of dexamethasone where p = 0.18. Similarly this was additionally seen in TNF whereby a reduction in cytokine release can be seen between LPS stimulated wells (p = 0.15) (Figure 5.3.4.2-3).

There was an observable increase in steroid sensitivity to CXCL8 in macrophages treated with 0.1nmol of dexamethasone post LPS (p = 0.10) however this was representative of negative percentage inhibition (Figure 5.3.4.2-2). No changes were seen in steroid sensitivity to TNF post LPS inhalation (Figure 5.3.4.2-4).
Figure 5.3.4.2-1. Stimulated (LPS) alveolar macrophage derived CXCL8 release treated with 0.1 to 1000nmol/l of dexamethasone

Figure 5.3.4.2-2. The percentage inhibition of stimulated alveolar macrophage derived CXCL8
Figure 5.3.4.2-3. Stimulated (LPS) alveolar macrophage derived TNF release treated with 0.1 to 1000nmol/l of dexamethasone

* p = < 0.05.

Figure 5.3.4.2-4. The percentage inhibition of stimulated alveolar macrophage derived TNF

* p = < 0.05.
5.4. Discussion

This study used *in vivo* LPS stimulation in asthmatics as an inducer of airway neutrophilia, as previously described in healthy subjects (Michel et al., 1989, Michel et al., 1997, Nightingale et al., 1998b, Thorn and Rylander, 1998). The study has demonstrated LPS inhalation as a safe way of inducing airway neutrophils in asthmatics. Subjects exhibited a local response to LPS where statistical increases in neutrophils were found in both sputum and BAL at 6 and 24 hours respectively. This study observed an associated decrease in cytokine release following *ex vivo* LPS stimulation from alveolar macrophages obtained in BAL.

Increased percentage neutrophils were found in sputum and BAL after LPS inhalation. After correcting for total cell count there was a statistical increase in absolute sputum neutrophils but not sputum macrophages. These finding have been observed previously involving healthy and asthmatic studies using a similar concentration and serotype of LPS (Aul et al., 2012, Thorn and Rylander, 1998). The procedure was tolerated well, symptom reporting and clinical observations in all 7 subjects returned to pre LPS values post 24 hours.

Neutrophilic asthma is widely associated with refractory asthma and steroid insensitivity (Jatakanon et al., 1999, Kamath et al., 2005). It is well recognized that alveolar macrophages play a key role in the initial stages of respiratory pathogen recognition (Peters-Golden, 2004) and extravasation of neutrophils (Murphy et al., 2001, Beck-Schimmer et al., 2005). LPS was administered to illicit the innate response including the activation of macrophages and subsequent proliferation of pulmonary neutrophils. Macrophage derived CXCL8 has been demonstrated to be a corticosteroid insensitive cytokine in subjects with COPD, a neutrophilic model (Armstrong et al., 2009). With this in mind it was hypothesized that *in vivo* LPS induced macrophage activation could not only induced a neutrophilic response but could consequently cause an increase in steroid insensitive CXCL8 from alveolar macrophages.
This study observed no changes to steroid sensitivity representative of percentage inhibition to dexamethasone in alveolar macrophages in both CXCL8 and TNF (Figure 5.3.4.1-2 & 5.3.4.1-4). These findings may be due to the otherwise low levels of basal cytokine release found in non-stimulated macrophages. Indeed this is comparable to previous LPS studies like this one whereby instillation of LPS (4 ng/kg) during bronchoscopy noted similar basal levels of TNF compared to baseline in BALF post 24 hours (O'Grady et al., 2001). Noticeable differences were only found after 6 hours, however the authors failed to measure levels of CXCL8. Notably their serotype of LPS and method of administration is different to this study’s. Nevertheless, in the current study macrophages were isolated and adhered for 20 hours prior to culture, this was performed approximately 24 hours post LPS inhalation. It is therefore likely that the observed low measured basal cytokine levels were due to the long duration between in vivo stimulation and analysis, at which point the innate response may have been controlled. In addition, caution should be taken at the observable effects in TNF as 4 of the baseline dexamethasone treated wells were under the limits of cytokine detection, this was additionally the case for 2 post LPS conditions.

The current study additionally set out to find whether ex vivo LPS stimulation could cause associated steroid insensitive cytokine production. The study was unable to report these findings as post LPS percentage inhibition by dexamethasone were notably similar to baseline values (Figure 5.3.4.2-2 & 5.3.4.2-4). There was a small increase in steroid sensitivity in the percentage inhibition of CXCL8 in response to 0.1nm/l dexamethasone (p = 0.10), however this was representative of negative inhibition.

Interestingly this study has observed a reduction to the LPS response following an additive LPS stimulation (Figure 5.3.4.2-1 & 5.3.4.2-3). This finding is supportive of the hypothesis of endotoxin hyporesponsiveness whereby a noticeable decrease in TNF release (p = 0.15) and to a lesser extent in CXCL8 (p = 0.18) was observed. Similar bronchoscopy studies but using LPS instillation recorded no observable
changes for TNF release following \textit{ex vivo} LPS stimulation (Hoogerwerf et al., 2010) however the study failed to observe CXCL8. Conversely, the authors observed a ‘priming’ of alveolar macrophages following secondary stimulation when observing IL-1B and IL-6 levels. This observation was a direct result of \textit{ex vivo} LPS (TLR-4) and lipoteichoic acid (TLR-2) stimulation. However the authors do not elude the possibility of immune-enhancing effects of their method of macrophage isolation.

Pro-inflammatory mediators produced by macrophages in response to infection are vital to the control of infection and disease pathology. Yet excessive cytokine production can be potentially damaging with the risk of septic shock syndrome (Hsing et al., 2008, Netea et al., 2003). The exact mechanisms involving endotoxin hyporesponsiveness are not fully understood. Yet no observed effects have been found on the surface expression of TLR4, TLR2 or CD14 following secondary stimulation (Hoogerwerf et al., 2010). With respect to the latter it demonstrates a further downstream mechanism leading to the reduction of cytokine release. Further studies have demonstrated that treatment of human monocytes with LPS causes reduced association between TLR-4 and MyD88 when cells are re-stimulated (Medvedev et al., 2002). Indeed, it is additionally possible that secondary exposure to LPS results in an increased expression of repressive p50 NF-κB subunits, TNF receptor II and IL-10 release (Ziegler-Heitbrock, 1995). Yet there is conflicting evidence to support IL-10 as a regulatory cytokine in this process (Hoogerwerf et al., 2010). Nevertheless classically these genes are thought to contribute to a reduction in the pro-inflammatory pathways (Stampfli et al., 1999, Couper et al., 2008, Ziegler-Heitbrock et al., 1994). With this in mind it is observable that endotoxin tolerance is not a result of an exhaustive cellular state but a regulated process hindering excessive inflammatory activation.

We did not use any other form of isolating techniques as other methods may have immuno-enhancing effects (Hoogerwerf et al., 2010). Caution should be taken to the statistical analyses performed in the current study due to the limited sample size (n=7) larger studies are needed to validate these findings. Caution should also be
taken with the cell culture data whereby the lack of sample size (n=4) prevented the use of normality testing. Increasing subject numbers are required to validate the finding observed in cell culture. Due to TNF levels being close to the lower limits of detection in unstimulated culture, future work in this area should consider using high sensitivity ELISA kits.

The earlier chapter focussed on late onset asthma. These patients have higher neutrophil levels. This chapter focussed on the increase of neutrophils caused by LPS as a completely separate mechanism to the age of onset of disease. Therefore these two chapters offer insights into neutrophilic airway inflammation caused by different mechanisms.

This study has demonstrated inhaled LPS as a safe model of central and peripheral neutrophilic airway inflammation. There is no change in steroid response following LPS inhalation. Alveolar macrophage derived CXCL8 and TNF are tolerant following \textit{ex vivo} LPS stimulation.
CHAPTER 6

6. Conclusions, Limitations and Future Work

6.1. Conclusions

The aim of this project was to investigate the pathogenesis, reproducibility and consequences of neutrophilic inflammation in asthmatics. Neutrophilic asthma has been previously associated with increased asthma severities (Shaw et al., 2007), ‘fatal asthma’ (Carroll et al., 2002), an increased risk of exacerbation (Fahy et al., 1995) and refractory asthma (Pavord et al., 1999). The “one size fits all approach” is becoming increasingly overshadowed with the discovery of new and innovative asthma phenotypes. The purpose of this thesis was to broaden the knowledge of airway neutrophils in order to aid future researchers and also improve future patient welfare.

This thesis initially set out to determine whether age at onset of asthma would affect central airway inflammation through the use of induced sputum. The results were intriguing. Inflammatory differences, representative of an increase in airway neutrophils were found in those who were classified as late onset compared to those with early onset asthma. Conversely those with LOA had increased central airway neutrophilia but no relationship with increased in asthma severity, a common association (Carroll et al., 2002, Jatakanon et al., 1999, Little et al., 2002, Shaw et al., 2007). It is therefore a valid postulation that late onset asthma defined as an onset age of 40 should be regarded as phenotype of age at onset.

This report secondly observed that neutrophils counts were repeatable in moderate to severe asthmatics across short and long term time points. This demonstrates that sputum neutrophils can now be recognised as a reliable scientific endpoint. In essence this also verifies the reliability of the endpoints observed in the other two studies. What is appealing is that this findings may hold some clinical value as it demonstrates the potential of sputum induction for clinical biomarker assessment. Using sputum neutrophils as assessment of airway inflammation and as a means to
monitor therapeutic control could increase patient quality of life based on downstream monitoring.

The final part of this project was to observe the effects of LPS as a model of neutrophilic inflammation but also to observe the consequences of neutrophilic airway inflammation on steroid sensitivity. The findings demonstrated that LPS is a safe way to elicit the innate immune response and the activation of neutrophils. In addition, LPS inhalation did not cause an associated decrease in steroid sensitivity. However cytokine release was suppressed upon secondary stimulation with LPS, contributing to the hypothesis of cytokine hyporesponsiveness or tolerance.

In the past decade or so there has been an increasing volume of interest in regards to disease phenotyping. The discovery of phenotypes promotes targeted strategies in line with current treatment methods. This project has refined two asthmatic phenotypes – late onset asthma and LPS inhalation as precursors of neutrophilic inflammation. Late onset asthma has been associated with low symptom reporting and increased FEV$_1$ neutrophilic phenotype. Indeed this thesis has also observed LPS as a trigger related phenotype which demonstrated symptom reporting and a reduction in FEV$_1$. It is therefore possible that these are two sub-phenotypes of airway neutrophilia (Figure 6.1-1)
Figure 6.1-1. Venn diagram of the phenotypes observed in this thesis

Increasing asthma severity is representative of both reported symptoms and airflow limitation.

Indeed, it is also encouraging to think that current observations regarding asthma phenotyping along with findings of this project could indicate the type/origin of airway neutrophilia using subject symptomology and duration of central airway inflammation using the induced sputum procedure.

**Table 6.1-1. Suggested neutrophilia based on subject symptomology and duration of airway neutrophilia on patients on regular ICS use**

<table>
<thead>
<tr>
<th>Neutrophilia</th>
<th>Symptomology</th>
<th>Suggested Origins</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chronic</td>
<td>Limited</td>
<td>Age Related</td>
</tr>
<tr>
<td>Acute</td>
<td>Sufficient</td>
<td>Trigger Related</td>
</tr>
<tr>
<td>Chronic</td>
<td>Sufficient</td>
<td>Treatment Resistant/Uncontrolled</td>
</tr>
<tr>
<td>Acute</td>
<td>Limited</td>
<td>Unknown</td>
</tr>
</tbody>
</table>

The current thesis has highlighted the pathogenesis, reproducibility and consequences of neutrophilic inflammation in asthma. Indeed in the process of doing
so this thesis has highlighted a new clinical phenotype of asthma along with a further trigger related phenotype, via the use of repeatable, novel inflammatory assessment techniques.
6.2. Limitations & Future Work

The bronchoscopy which was performed in the LPS inhalation study was performed approximately 18 hours post sputum induction. Hypertonic saline has been demonstrated to increase levels of prostaglandin E2 (PGE2), leukotriene C4 (LTC4) and histamine from human airway mast cells from bronchoalveolar lavage (BAL) (Gravelyn et al., 1988, Silber et al., 1988). Indeed, other studies have demonstrated repeated sputum induction to cause increased levels of central neutrophils. Nightingale et al. (1998) demonstrated a 26% median increase in sputum neutrophils post 24 hours when compared to baseline values.

The final study in this thesis had a limited subject number. This prevented the determination on whether culture data was parametric or nonparametric and therefore limited the statistical analysis. In regards to macrophage processing this study allowed an adherence time of 20 hours. It is possible that alveolar macrophages in the LPS study became biologically settled and therefore this has an effect on the low levels of basal cytokine release. It is possible that reduced adhesion time for macrophage isolation may detect basal cytokine release when compared to 20 hour, future investigators may wish to consider this.
Appendix

1. Human CXCL8/IL-8 ELISA materials and protocol

1.1.1. Materials Provided

Bring all reagents to room temperature before use.

Capture Antibody (Part 890804, 1 vial) - 720 mg/mL of mouse anti-human IL-8 when reconstituted with 1.0 mL of PBS. After reconstitution, store at 2 - 8°C for up to 60 days or aliquot and store at -20°C to -70°C in a manual defrost freezer for up to 6 months. Dilute to a working concentration of 4.0 mg/mL in PBS, without carrier protein.

Detection Antibody (Part 890805, 1 vial) - 3.6 mg/mL of biotinylated goat anti-human IL-8 when reconstituted with 1.0 mL of Reagent Diluent (see Solutions Required section). After reconstitution, store at 2 - 8°C for up to 60 days or aliquot and store at -20°C to -70°C in a manual defrost freezer for up to 6 months. Dilute to a working concentration of 20 ng/mL in Reagent Diluent.

Standard (Part 890806, 1 vial) - 110 ng/mL of recombinant human IL-8 when reconstituted with 0.5 mL of distilled or deionized water. Allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions. Store reconstituted standard at 2 - 8°C for up to 60 days or aliquot and store at -70°C for up to 6 months. A seven point standard curve using 2-fold serial dilutions in Reagent Diluent, and a high standard of 2000 pg/mL is recommended.

Streptavidin–HRP (Part 890803, 1 vial) - 1.0 mL of streptavidin conjugated to horseradish-peroxidase. Store at 2 - 8°C for up to 6 months after initial use. DO NOT FREEZE. Dilute to the working concentration specified on the vial label using Reagent Diluent.
1.1.2. General ELISA Protocol

1.1.2.1. Plate Preparation

1. Dilute the Capture Antibody to the working concentration in PBS without carrier protein. Immediately coat a 96-well microplate with 100 µL per well of the diluted Capture Antibody. Seal the plate and incubate overnight at room temperature.

2. Aspirate each well and wash with Wash Buffer, repeating the process two times for a total of three washes. Wash by filling each well with Wash Buffer (400 µL) using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each step is essential for good performance. After the last wash, remove any remaining Wash Buffer by aspirating or by inverting the plate and blotting it against clean paper towels.

3. Block plates by adding 300 µL of Block Buffer to each well. Incubate at room temperature for a minimum of 1 hour.

4. Repeat the aspiration/wash as in step 2. The plates are now ready for sample addition.

1.1.2.2. Assay Procedure

1. Add 100µl of sample or standards in Reagent Diluent, or an appropriate diluent, per well. Cover with an adhesive strip and incubate 2 hours at room temperature.

2. Repeat the aspiration/wash as in step 2 of Plate Preparation.

3. Add 100 µL of the Detection Antibody, diluted in Reagent Diluent, to each well. Cover with a new adhesive strip and incubate 2 hours at room temperature.

4. Repeat the aspiration/wash as in step 2 of Plate Preparation.
5. Add 100 µL of the working dilution of Streptavidin-HRP to each well. Cover the plate and incubate for 20 minutes at room temperature. Avoid placing the plate in direct light.

6. Repeat the aspiration/wash as in step 2.

7. Add 100 µL of Substrate Solution to each well. Incubate for 20 minutes at room temperature. Avoid placing the plate in direct light.

8. Add 50 µL of Stop Solution to each well. Gently tap the plate to ensure thorough mixing.

9. Determine the optical density of each well immediately, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.
2. **Human TNF-α/TNFSF1A ELISA materials and protocol**

2.1.1. **Materials Provided**

Bring all reagents to room temperature before use. Capture Antibody (Part 840119, 1 vial) - 720 mg/mL of mouse anti-human TNF-α when reconstituted in 1.0 mL of PBS. After reconstitution, store at 2 - 8°C for up to 60 days or aliquot and store at -20°C to -70°C in a manual defrost freezer for up to 6 months. Dilute to a working concentration of 4.0 mg/mL in PBS4, without carrier protein.

Detection Antibody (Part 840120, 1 vial) - 45 mg/mL of biotinylated goat anti-human TNF-α when reconstituted with 1.0 mL of Reagent Diluent. After reconstitution, store at 2 - 8°C for up to 60 days or aliquot and store at -20°C to -70°C in a manual defrost freezer for up to 6 months. Dilute to a working concentration of 250 ng/mL in Reagent Diluent.

Standard (Part 840121, 1 vial) - 340 ng/mL of recombinant human TNF-α when reconstituted with 0.5 mL of Reagent Diluent. Allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions. Store reconstituted standard at 2 - 8°C for up to 60 days or aliquot and store at -70°C for up to 6 months. A seven point standard curve using 2-fold serial dilutions in Reagent Diluent, and a high standard of 1000 pg/mL is recommended.

Streptavidin-HRP (Part 890803, 1 vial) - 1.0 mL of streptavidin conjugated to horseradish-peroxidase. Store at 2 - 8°C for up to 6 months after initial use. DO NOT FREEZE. Dilute to the working concentration specified on the vial label using the Reagent Diluent.
2.1.2. General ELISA Protocol

2.1.2.1. Plate Preparation

1. Dilute the Capture Antibody to the working concentration in PBS without carrier protein. Immediately coat a 96-well microplate with 100 µL per well of the diluted Capture Antibody. Seal the plate and incubate overnight at room temperature.

2. Aspirate each well and wash with Wash Buffer, repeating the process two times for a total of three washes. Wash by filling each well with Wash Buffer (400 µL) using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each step is essential for good performance. After the last wash, remove any remaining Wash Buffer by aspirating or by inverting the plate and blotting it against clean paper towels.

3. Block plates by adding 300 µL of Reagent Diluent to each well. Incubate at room temperature for a minimum of 1 hour.

4. Repeat the aspiration/wash as in step 2. The plates are now ready for sample addition.

2.1.2.2. Assay Procedure

1. Add 100 µL of sample or standard in Reagent Diluent, or an appropriate diluent, per well. Cover with an adhesive strip and incubate 2 hours at room temperature.

2. Repeat the aspiration/wash as in step 2 of Plate Preparation.

3. Add 100 µL of the Detection Antibody, diluted in Reagent Diluent, to each well. Cover with a new adhesive strip and incubate 2 hours at room temperature.
4. Repeat the aspiration/wash as in step 2 of Plate Preparation.

5. Add 100 µL of the working dilution of Streptavidin-HRP to each well. Cover the plate and incubate for 20 minutes at room temperature. Avoid placing the plate in direct light.

6. Repeat the aspiration/wash as in step 2.

7. Add 100 µL of Substrate Solution to each well. Incubate for 20 minutes at room temperature. Avoid placing the plate in direct light.

8. Add 50 µL of Stop Solution to each well. Gently tap the plate to ensure thorough mixing.

9. Determine the optical density of each well immediately, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.
3. Asthma Control Questionnaire(-7) (Juniper et al., 1999)

1. On average, during the past week, how often were you 
   woken by your asthma during the night?

   0 Never
   1 Hardly ever
   2 A few minutes
   3 Several times
   4 Many times
   5 A great many times
   6 Unable to sleep because of asthma

2. On average, during the past week, how bad were you 
   asthma symptoms when you woke in the morning?

   0 No symptoms
   1 Very mild symptoms
   2 Mild symptoms
   3 Moderate symptoms
   4 Quite severe symptoms
   5 Severe symptoms
   6 Very severe symptoms

3. In general, during the past week, how limited were you in 
   your activities because of your asthma?

   0 Not limited at all
   1 Very slightly limited
   2 Slightly limited
   3 Moderately limited
   4 Very limited
   5 Extremely limited
   6 Totally limited

4. In general, during the past week, how much shortness of 
   breath did you experience because of your asthma?

   0 None
   1 A very little
   2 A little
   3 A moderate amount
   4 Quite a lot
   5 A great deal
   6 A very great deal

5. In general, during the past week, how much of the time did 
   you wheeze?

   0 Not at all
   1 Hardly any of the time
   2 A little of the time
   3 A moderate amount of the time
   4 A lot of the time
   5 Most of the time
   6 All of the time

6. On average, during the past week, how many puffs of short-
   acting bronchodilator (e.g. Ventolin or Bricanyl) have you 
   used each day?

   0 None
   1 1-2 puffs most days
   2 3-4 puffs most days
   3 5-8 puffs most days
   4 9-12 puffs most days
   5 13-16 puffs most days
   6 More than 16 puffs most days

7. To be filled in by technician/physician:

   FEV₁ pre-bronchodilator: ..........................
   FEV₁ predicted .................................
   FEV₁ % predicted ..............................

Score = Total / 7
4. Individual data plots for clinical parameters

Figure 4-1. Individual data plots of change in exhaled nitric oxide from baseline to up to 8 hours post LPS inhalation.

Figure 4-2. Individual data plots of change in body Temperature from baseline to up to 24 hours post LPS inhalation.
Figure 4-3. Individual data plots of FEV1 from baseline to up to 8 hours post LPS inhalation.

Figure 4-4. Individual data plots of Oxygen Saturations from baseline to up to 24 hours post LPS inhalation.
Figure 4-5. Individual data plots of diastolic (Dia) and systolic (Sys) blood pressure from baseline to up to 24 hours post LPS inhalation.

Figure 4-6. Individual data plots of change in pulse (in beats per minute) from baseline to up to 24 hours post LPS inhalation.
5. Paper Published from Thesis

A Comparison of the Clinical and Induced Sputum Characteristics of Early- and Late-Onset Asthma

Matthew Rossall • Paul Cadden • Umme Kolsum • Dave Singh

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Abstract

Background There are few studies describing the phenotype of late-onset asthma (LOA). We sought to investigate the clinical and induced sputum characteristics of patients with LOA.

Methods Nineteen patients with LOA diagnosed after the age of 40 years and 19 patients with early-onset asthma (EOA) diagnosed before the age of 20 years were recruited. Subjects performed lung function, reversibility, asthma control questionnaire (ACQ), exhaled nitric oxide (NO), and sputum induction.

Results The FEV1 % predicted was lower in EOA compared to LOA (87.6 % vs. 103 %, respectively, \( p = 0.02 \)), while ACQ scores were significantly higher in EOA (1.46 vs. 0.89, respectively, \( p = 0.03 \)). NO was not different between the groups, but the percentage neutrophil counts were lower in the EOA group compared to the LOA group (36.6 vs. 57.3, respectively, \( p = 0.02 \)). Asthma duration, but not age, was negatively associated with lung function (\( r = -0.4, p = 0.01 \)). Neutrophil counts in healthy age-matched controls (\( n = 10 \)) were similar to EOA and lower than LOA.

Conclusion Raised sputum neutrophils in LOA are not an indicator of severe disease and could be a characteristic feature of this asthma phenotype. Duration of asthma influences lung function.

Keywords Late onset asthma • Sputum neutrophils • Duration of asthma • Asthma phenotypes • Phenotype

Asthma is a heterogeneous disease comprising subgroups of patients with distinct clinical and pathophysiological characteristics [1]. The description of phenotypes may allow targeted management strategies to improve asthma control, in contrast to the “one size fits all” approach. Late-onset asthma (LOA) is a well-known clinical phenomenon. Studies of this phenotype are difficult to perform because there is no accepted cut off definition of the time after which LOA develops [2], and because confounding factors such as smoking history and the development of COPD may cause diagnostic confusion. Nevertheless, the need for further characterization of this phenotype is well recognized [3].

Increased sputum neutrophil counts in asthma are associated with more severe disease [4] and a reduced response to corticosteroids [5] The number of airway neutrophils increases with age in healthy subjects [6], but it is not known if LOA is also associated with increased neutrophilic airway inflammation. We have investigated the clinical and induced sputum characteristics of patients with LOA and compared the results to a younger group of patients.

Thirty-eight adult patients with a physician diagnosis of asthma for at least 6 months were recruited by advertising within primary care practices. Nineteen patients were classified as having LOA diagnosed after the age of 40 years, while 19 were classified as having early-onset asthma (EOA) diagnosed before the age of 20 years. The history of asthma was checked in the primary care records. Patients were required to be using inhaled corticosteroids (ICS) with or without a long-acting \( \beta \)-agonist. Both groups were matched for ICS use. Exclusion criteria were a smoking history \( \geq 1 \) pack-year, use of oral corticosteroids, montelukast, or omalizumab, and a respiratory tract infection in the previous 4 weeks. We also used historical
data from older healthy subjects who had previously provided induced sputum in our research unit as a control group. All subjects provided written informed consent and the study was approved by the local ethics committee. Asthma Control Questionnaire-7 (ACQ-7); spirometry with reversibility to 200 µg salbutamol; skin prick testing using house dust mite, cat, and grass allergens; exhaled nitric oxide (eNO) at 50 ml/s (Niox, Aerocrine, Sweden); and sputum induction [7] were performed.

The study had 80% power to detect a 23.7% difference in sputum neutrophil percentage between groups with 16 subjects per group using a between-subject SD of 23.1%. This sample size is similar to other studies that used induced sputum in asthma [8, 9]. Nonparametric data were either natural log transformed and presented as geometric means with 95% confidence intervals or expressed as median (range). Differences between groups were assessed using unpaired t tests, Mann–Whitney U tests, or Fisher’s exact test. Pearson correlation was used to assess relationships between measurements.

The clinical characteristics of the patients are summarised in Table 1. The mean ages of asthma onset were 7.4 and 49.7 years for EOA and LOA groups, respectively, with a significantly longer duration of asthma in the EOA group (23.2 vs. 9.7 years, respectively, p < 0.0001). EOA had a higher number of patients with atopy, defined by a positive reaction to at least one skin prick test, but the difference between groups was not statistically significant (p = 0.20). There was no difference in ICS usage between the groups. The FEV1 % predicted was lower in the EOA than in the LOA group (87.6% vs. 103%, respectively, p = 0.02), and the FVC % predicted was also lower in EOA. The ACQ score was significantly higher in the EOA group (p = 0.03). Percentage reversibility was

<table>
<thead>
<tr>
<th>Clinical characteristics</th>
<th>LOA (n = 19)</th>
<th>EOA (n = 19)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender male/female</td>
<td>7/12</td>
<td>9/10</td>
<td></td>
</tr>
<tr>
<td>Daily ICS dosea (mcg)b</td>
<td>640 (395–1034)</td>
<td>917 (657–1281)</td>
<td>0.20</td>
</tr>
<tr>
<td>Atopy</td>
<td>66.6 %c</td>
<td>89.5 %</td>
<td>0.20d</td>
</tr>
<tr>
<td>Age</td>
<td>59.4 (4.8)</td>
<td>30.6 (5.5)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Age at asthma onset</td>
<td>49.7 (5.6)</td>
<td>7.4 (6.4)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Asthma duration (years)</td>
<td>9.7 (6.1)</td>
<td>23.2 (6.1)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>FEV1 % predicted (%)</td>
<td>103 (19)</td>
<td>88 (19)</td>
<td>0.02</td>
</tr>
<tr>
<td>FVC % predicted (%)</td>
<td>117 (18)</td>
<td>101 (14)</td>
<td>0.003</td>
</tr>
<tr>
<td>Reversibility (ml)</td>
<td>73 (141)</td>
<td>296 (296)</td>
<td>0.01</td>
</tr>
<tr>
<td>Reversibility (%)</td>
<td>3.2 (5.5)</td>
<td>11.8 (12.6)</td>
<td>0.01</td>
</tr>
<tr>
<td>eNO (ppb)b</td>
<td>16.3 (12.3–21.7)</td>
<td>20.8 (14.0–30.9)</td>
<td>0.15</td>
</tr>
<tr>
<td>ACQ-7 score</td>
<td>0.89 (0.63)</td>
<td>1.46 (0.92)</td>
<td>0.03</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Induced sputum</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Sputum total cell count (x10⁶)</td>
<td>1.6 (0.1–8.2)</td>
<td>2.3 (0.2–8.9)</td>
<td>0.21e</td>
</tr>
<tr>
<td>Neutrophil (%)</td>
<td>57.3 (23.2)</td>
<td>36.6 (22.1)</td>
<td>0.02</td>
</tr>
<tr>
<td>Eosinophil (%)b</td>
<td>1.3 (0.5–3.5)</td>
<td>0.9 (0.4–2.2)</td>
<td>0.57</td>
</tr>
<tr>
<td>Macrophage (%)</td>
<td>34.3 (20.8)</td>
<td>52.6 (19.2)</td>
<td>0.02</td>
</tr>
<tr>
<td>Lymphocyte (%)b</td>
<td>0.3 (0.1–0.7)</td>
<td>0.7 (0.4–1.1)</td>
<td>0.07</td>
</tr>
<tr>
<td>Absolute neutrophil count (x10⁶/g sputum)</td>
<td>3.5 (3.3)</td>
<td>2.4 (2.0)</td>
<td>0.30</td>
</tr>
<tr>
<td>Absolute eosinophil count (x10⁶/g sputum)b</td>
<td>0.1 (0.02–0.2)</td>
<td>0.1 (0.02–0.1)</td>
<td>0.73</td>
</tr>
<tr>
<td>Absolute macrophage count (x10⁶/g sputum)b</td>
<td>1.2 (0.6–2.7)</td>
<td>2.4 (1.4–4.1)</td>
<td>0.14</td>
</tr>
<tr>
<td>Absolute lymphocytes count (x10⁶/g sputum)b</td>
<td>0.02 (0.01–0.04)</td>
<td>0.04 (0.02–0.06)</td>
<td>0.31</td>
</tr>
</tbody>
</table>

Sputum induction was performed on all subjects, and adequate samples to prepare cytopsins were obtained from 12 patients with LOA and 16 patients with EOA.

All parametric data are expressed as mean (standard deviation) unless stated otherwise.

a Beclometasone equivalent dose

b Geometric mean with 95% confidence interval
c n = 15
d Fisher’s exact test
e Median and range

f Mann–Whitney U test
An increase in sputum neutrophil counts has been reported in healthy subjects aged >50 years [6]. Our historically healthy control group had a mean age of 57.8 years but there was no rise in neutrophil counts. This indicates that neutrophil counts do not increase in all healthy subjects >50 years old; perhaps greater increases are observed in subjects who are considerably older than 50 years. Our findings in LOA patients therefore appear not to be simply an age-related phenomenon, and increased sputum neutrophil counts may be a phenotypic feature of patients who develop LOA. LOA patients had a similar rate of atopy compared to EOA patients, and so we speculate that other factors are important in driving the neutrophilic airway inflammation observed in LOA, such as environmental pollution or infection which are both known to cause neutrophilic lung inflammation [12–14].

This was a “real-life study” that used a previous physician diagnosis of asthma for which inhaled corticosteroids had been prescribed, although the degree of compliance with medications was not known. We did not use other arbitrary diagnosis criteria such as reversibility or bronchial hyperreactivity tests; as such, inclusion criteria exclude the majority of patients with true asthma [15]. Our recruitment strategy of advertising within primary care may have resulted in a selection bias towards subjects who respond to such recruitment methods; further larger studies in different populations are needed to confirm our findings. Additionally, some caution should be applied to the interpretation of association analyses in the limited sample size presented here, as these were not the primary aim of the study and again larger studies are required for confirmation.

LOA patients had better lung function and ACQ scores while using similar doses of ICS. The mean FEV1 % predicted in the LOA group was >100 %, suggesting the overprescribing of ICS in this group. GINA guidelines recommend that ICS usage should be titrated according to asthma control [16]. It is possible that patients with LOA are prescribed ICS at the correct dose to achieve asthma control, but then the ICS dosage is not titrated downwards. It is difficult in this type of study to match the duration of asthma between groups, as patients with EOA are more likely to have a longer duration.

In summary, our findings suggest that duration of asthma rather than age of onset of asthma is a key determinant of lung function. Raised sputum neutrophil counts in LOA are a phenotypic characteristic but not a biomarker of severe disease.

Conflict of interest M. Rossall, P. Cadden, and U. Kolsum have stated that they have no conflict of interest. D. Singh has received lecture fees, research grants, consultancy fees and support conference attendance from various pharmaceutical companies including AstraZeneca, GlaxoSmithKline, Chiesi, Boehringer Ingleheim and Roche.
References

References

N = 223


