Evaluating T cell subsets in neutrophilic asthmatics and in exacerbation models of asthma and COPD

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Abbreviations

ACQ - Asthma Control Questionnaire
ACTH - Adrenocorticotropin hormone
APC - Allophycocyanin
APC - Antigen presenting cell
APC-Cy7 - Allophycocyanin-cyanin 7
APO - Apoptosis antigen
BALF - Bronchial alveolar lavage fluid
C3 - Compliment factor
CCL - Chemokine ligand
CD - Cluster of differentiation
COPD - Chronic Obstructive Pulmonary Disorder
CRP - C reactive protein
CTLA-4 - Cytotoxic T lymphocyte antigen 4
DTT - dithiothreitol
ECP - Eosinophil Cationic protein
Fas - death receptor
FBS - Foetal bovine serum
FEV1 - Forced expiratory volume in 1 second
FITC - Fluorescein isothiocyanate
FoxP3 - Forkhead box 3
FVC - Forced vital capacity
GC - Glucocorticoid
G-CSF - Granulocyte–colony stimulating factor
GINA - Global Initiative for Asthma
GOLD - Global initiative for chronic Obstructive pulmonary Disease
HAT - Histone Acetylase
HDAC - Histone deacetylase
ICOS - Inducible T cell co-stimulator
IL - Interleukin
LPS - Lipopolysaccharide
MAPK - Mitogen Activated Protein Kinase
MHC - Major Histocompatibility complex
MPO - Myeloperoxidase
NFκB - Nuclear factor kappa B
PE - Phycoerythrin
PMA - Phorbol myristate acetate
PMN - Polymononuclear
RORγt - Retinoic acid related orphan receptor
STAT - Signal transducer and activator of transcription
TGF-β - Tumour growth factor beta
Th17 - T helper 17
TLR4 - Toll Like receptor 4
TNF-α - Tumour necrosis factor alpha
Treg - T regulatory
WBC - Whole blood count
Abstract

THE UNIVERSITY OF MANCHESTER
ABSTRACT OF THESIS submitted by Antonia Banyard
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BACKGROUND: Asthma and COPD are both complex inflammatory lung diseases. The hallmark features of these lung diseases are variable airflow obstruction, bronchial hyperreactivity and airway inflammation. T lymphocytes play a central role in the pathogenesis of airway inflammation. It is recognized that disease phenotypes exist, comprising subgroups of patients with distinct clinical or pathological characteristics associated with different prognosis or response to treatment. Neutophilic airway inflammation in asthma and COPD patients is associated with exacerbations (from infection) and limited steroid response. Determining the level of T cell populations within distinct subgroups is essential for developing effective therapy.

The aims of this thesis were: to develop a multi-parametric flow cytometry method to evaluate the subpopulations of Th1, Th2, Treg and Th17 T cell subsets both systemically and within the lung; to compare the response of asthmatic and healthy control Th1, Th2, Treg and Th17 cells (both systemic and lung) in vitro to a T cell specific stimulus; To determine the T cell profile within patients with neutrophilic airway inflammation, namely neutrophilic asthmatics and asthmatics and COPD patients post an inhaled LPS challenge.

METHODS: 1) 6 neutrophilic asthmatics and age matched healthy controls were recruited and underwent a bronchoscopy and blood draw. Th1, Th2, Th17 and Tregs cell expression was measured after PMA/ionomycin ex-vivo stimulation using a multi-parametric flow cytometry method. This method was developed and validated within this study. 2) Flow cytometry was also used to measure Th17 and Tregs in 6 asthmatic patients 0 and 24 hour post LPS inhalation and 11 COPD patients 0 and 4 hours post LPS inhalation.

RESULTS: 1) Th1, Th2 and Th17 cells increased in the lung and the peripheral blood upon ex-vivo stimulation. There was no significant difference between the asthma group and the healthy controls. Treg expression did not increase upon stimulation and there was no significant difference between the asthma and healthy control groups. 2) In asthmatics there was no increase in Th17 or Treg cells 24 hours after LPS inhalation within the lung or the blood. In COPD patients there was a significant increase in Th17 cells in the blood 4 hours post LPS inhalation.

CONCLUSIONS: 1) This cohort of neutrophilic asthmatics did not show signs of favouring a specific T cell phenotype compared to healthy controls upon ex-vivo stimulation. However there a higher expression of T cells cytokines in the lung than the blood suggesting a more reactive environment within the lung. 2) 24 hours post LPS challenge showed no differences in T cell expression in asthmatics in the lung or blood. In COPD patients Th17 cells significantly increased after 4 hours suggesting an innate rapid response in the blood after inhalation of LPS in these patients. This further understanding of the T cell immunology during both stable and exacerbated states may enable better directed therapies for these diseases.
Declaration

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Chapter 1

1.1 Introduction

Asthma and Chronic obstructive pulmonary disorder (COPD) are complex inflammatory diseases that have some similar clinical features but with differences in the patterns of inflammation involving different cytokines and inflammatory cells. Asthma symptoms include prolonged lung inflammation, airway hyper responsiveness and airway remodelling involving damage to the epithelium, goblet cell hyperplasia and basement membrane thickening. This can be coupled with excess mucus production (Manuyakorn et al., 2013). COPD is a progressive disease the symptoms include airway limitations leading to breathlessness and is considered a small airways disease. Over production of mucus also occurs and the pro inflammatory environment eventually causes emphysematic lungs. COPD has many co-morbidities associated with it including heart disease, osteoporosis and depression so requires a multi-faceted approach to treatment (McDonald and Khor, 2013). The diagnosis and classification of severity and the type of asthma is not well defined although there are guidelines from Global Initiative for Asthma (GINA). These are typed from clinical parameters such as airway restriction and reversibility to a methacholine challenge. There are phenotypes such as eosinophilic and neutrophilic and various trigger types such as exercise or allergy. It is becoming more common to describe asthmatics as endotypes to encompass all the different parameters (Xie and Wenzel, 2013). COPD is classified by the Global Initiative for chronic Obstructive pulmonary Disorder (GOLD) system which uses spirometry for classification and is discussed later in detail.

Neutrophilic asthmatics are a relatively recently diagnosed group pertaining to an innate-immunity driven condition (Wood et al., 2012) as opposed to eosinophilic asthma, a T helper cell type 2 condition. This was reiterated by the use of a monoclonal antibody against IL-5 (predominant cytokine released by eosinophils showed limited therapeutic success in asthmatic patients (Leckie et al., 2000) suggesting other cells must have a role to play in asthma. Neutrophilic asthmatics may need different treatments from the classic steroid and bronchodilator
treatments (Louis et al., 2012). To further elucidate the immune system in this patient group this study will investigate the expression levels of T helper subsets involved and whether they differ from age matched controls.

The airways by their very nature are constantly being exposed to pathogens and particulates which will elicit an immune response to clear any infection and to prevent damage. This can become a problem when the inflammatory response becomes dysregulated or dysfunctional causing damage within the airways and ceases to clear infections. Reduced bacterial clearance can lead to increased amounts of lipopolysaccharide (LPS) from the cell walls of gram negative bacteria. LPS can activate an immune response predominantly through the toll like receptor 4 (TLR4) which in turn, through activation of nuclear factor κB (NFκB) and mitogen activated protein kinases (MAPK), increases release of pro-inflammatory cytokines and activates cells such as monocytes and macrophages (Dauphinee and Karsan, 2006). Both asthma and COPD patients can suffer from exacerbations resulting from infection.

Glucocorticoids (GC) are used as an anti-inflammatory treatment during asthma and COPD exacerbations, however there are patients that become resistant to this treatment. It is thought that over exposure of LPS can change the response to GC and promote resistance as increased LPS levels are detected in the bronchial alveolar fluid (BALF) of GC resistant asthmatics (Goleva et al., 2008).

This thesis will attempt to investigate the expression T cell subsets in both lung and peripheral blood using multi parametric flow cytometry analysis in neutrophilic asthmatics compared to controls to determine whether a specific T cell phenotype favours an asthmatic phenotype. Little is known about the role of lymphocytes during COPD and asthmatic exacerbations. This study will investigate the role lymphocytes have in pulmonary and systemic inflammation during these episodes, specifically Th17 and regulatory T cell (Treg) subsets. LPS inhalation has previously been used in healthy subjects to study in vivo the inflammatory response.
This study will utilise the LPS inhalation method to model a COPD exacerbation and to create an in vivo inflammatory environment to determine the response of systemic Th17 and Treg lymphocytes during exacerbation. This study will also follow COPD patients clinically and compare the systemic response observed during a clinical exacerbation to those elicited after an LPS challenge. This study will also utilise the LPS inhalation method to model an asthmatic exacerbation and compare the local and systemic inflammatory responses. Subsequently this study will also investigate whether exposure to LPS has a profound effect on lymphocyte function in relation to possible steroid insensitivity in asthmatics.

1.2 T lymphocyte subsets in the immune system

T lymphocytes originate from haematopoietic stem cells in the bone marrow and expand and divide in the thymus generating a population of immature lymphocytes. These naïve lymphocytes lack the expression of cluster of differentiation (CD)4 or CD8 on the surface. During development the majority of naïve lymphocytes will eventually express either CD4 or CD8, following positive selection by antigen presentation. These cells are then released from the thymus to go on to mature into immunocompetent cells.

T cells are distinguished from other lymphocytes by the expression of T cell receptors (TCR). TCRs along with other co-stimulatory surface markers (such as CD28) are responsible for T cell maturation. T cells are activated when antigen presenting cells (APCs), such as macrophages and dendritic cells, present a unique peptide through the major histocompatibility complex (MHC) and along with co-stimulatory molecules activates the T cell to proliferate and produce the relevant cytokines to the activation. There are several T cell subsets classified from the cytokines produced with unique functions (Figure 1.1).
Figure 1.1: Schematic diagram showing interaction between T cell receptor and MHC class molecules on the APC. LFA-1: Lymphocyte function associated antigen-1, also known as an integrin. This is involved in early stage of weakly binding of T cells to APCs, on TCR activation the conformation changes to prolong the contact to aid T cell proliferation. ICAM-1: Inter-cellular adhesion molecule binds the integrin as above. ICAM is found on leucocytes and endothelial cells. Leucocytes bind to endothelial cells via ICAM-1/LFA-1 and transmigrate into the tissue upon activation. CD80/86: Co-stimulatory signals for T cell activation that work in tandem with each other with CD28 as their ligand, this stabilises the T cell activation inducing T cell proliferation upon antigen representation (depicted as a diamond).

1.2.1 Th1/2 cells

T helper cells (Th) are so named for their ability to assist other immune cells in their function, such as the maturation of B cells into plasma cells and the activation of cytotoxic T cells and macrophages. T cells require two signals to become fully activated. A first signal, which is antigen-specific, is provided through the T cell receptor which interacts with peptide-MHC molecules on the membrane of antigen presenting cells (APC). A second signal, the co-stimulatory signal, is antigen nonspecific and is provided by the interaction between co-stimulatory molecules expressed on the membrane of APC and the T cell.

The CD4+ Th cells become activated after antigen presentation by MHC class II molecules whereas cytotoxic CD8+ T cells are activated by MHC class I molecule. CD8+ T cells are activated by antigen presentation from MHC class I cells directly killing them by release of lytic proteins such as granzymes and
preforins or receptor/ligand interaction of the Fas/APO (apoptotic marker) molecules increasing apoptosis. (Groscurth and Filgueira, 1998)

One of the best characterized costimulatory molecules expressed by T cells is CD28, which interacts with CD80 (B7.1) and CD86 (B7.2) on the membrane of APC. Another costimulatory receptor expressed by T cells is ICOS (Inducible Costimulator), which interacts with ICOS-Ligand.

T cell co-stimulation is necessary for T cell proliferation, differentiation and survival. Activation of T cells without co-stimulation may lead to T cell anergy, T cell deletion or the development of immune tolerance.

1.2.2 Th17 cells

Interleukin-17 (IL-17) was first cloned in 1993 and named CTLA-8 (cytotoxic T lymphocyte associated antigen-8) and was known to have homology to immunologically active genes from the herpesvirus capable of inducing T cell lymphomas (Rouver et al., 1993). Further proteins of similar homology have since been discovered and the IL-17 cytokine family was established. The IL-17 family comprises 6 members (IL-17A-F). IL-17A and IL-17F have the greatest homology and are the best characterised and can form homodimer or heterodimers (Fossiez et al., 1996). CD4+ cells can produce both IL-17A and IL-17F making up the Th17 lineage which is distinct from Th1 and Th2 lineages diverging by a unique profile of effector cytokines independent of the other lineages. It is important to distinguish from the term Th17 and IL-17; the former being the collective name for the lymphocyte immunity the cells exert through producing IL-17A and F, IL-22 and IL-23 and the latter being one of the cytokines that can be expressed.

IL-17 production is not unique to Th17 cells. IL-17 expression has been reported in CD8+ cytotoxic T cells in the lung following influenza challenge (Hamada et al., 2009) and IL-17 mRNA from neutrophils after LPS challenge although secreted protein was not measured (Ferretti et al., 2003).
The most characterised cells that produce IL-17 are the CD4+ cells with antigen specific polarization to a Th17 phenotype, as with the Th1 and Th2 phenotypes. T cells have been historically classified by the effector type cytokines they produce, namely type 1 and type 2, IFN-γ, IL-2 and IL-4 IL-13, respectively (Mosmann and Coffman, 1989). Polarization of Th17 cells as with all the other subsets is dependent on cytokines and antigen co-stimulation (Figure 1.2).
Figure 1.2: Schematic diagram showing the divergence of cytokine driven polarisation. Upon antigen presentation from the APC the naïve T cell has the ability to differentiate into the following T cell subsets. In the presence of IL-12 and IFNγ T cells proliferate and are termed Th1 used mainly for intracellular infections and produce pro-inflammatory cytokines such as TNF-α. In the presence of IL-6 and TGF-β, they predominantly produce Th17 cells and are involved in cell-mediated immunity and produce mainly pro-inflammatory cytokines that also have a positive feedback for example IL-21 to maintain the specific type of T cell subset and method of immunity needed to clear the infection. With IL-10 and TGF-β being present the T cells produce the Treg subset which are involved in immunoregulation and peripheral tolerance to self antigens and this subset produces an anti-inflammatory cytokine milieu. Th2 type T cells are produced in the presence of IL-4 and IL-25 and this subset is associated with allergen challenges and humoral immunity and produces cytokines that enables B cell maturation to produce IgE and degranulation of mast cells (Alcorn et al., 2010)
Th1 is controlled by IL-12 signalling through signal transducer and activator of transcription (STAT) 4 producing a population of cells producing interferon (IFN-γ) mainly expressed on CD8+ T cells, vital for the immunity against microbial infections as a pro-inflammatory cytokine. Determination of the Th2 subset requires IL-4 through STAT6 signalling and produces IL-4, IL-13 and IL-5 (Dong, 2006). It has been shown that CD4 naïve responder cells (CD25 negative) through TCR and tumour growth factor-β (TGF-β) signalling will produce T regulatory cells (Tregs) expressing forkhead box P3 (FoxP3), TGF-beta and IL-10 characterising another subset of T cells (Chen et al., 2003).

In the presence of TGF-β and IL-6 (an acute inflammation phase protein), signalling through the STAT3 and nuclear factor of activated B cells (NF-κB) pathway produce the Th17 phenotype. Th17 cells express Retinoic acid related orphan receptor (RORγt) and produce IL-17A&F, IL-21 and IL-22 (Bettelli et al., 2006). Although it has been shown that there are 2 lineages of transcriptional regulation of Th17 phenotype namely from RORγ and RORα (Yang et al., 2008). It has been shown that IL-6 can inhibit the formation of FoxP3+ cells after they have been induced by TGF-β (Bettelli et al., 2006) suggesting there could be a regulatory relationship between Th17 and Treg populations. It is becoming more apparent that the development of the Th17 phenotype has a degree of plasticity governed by the cytokine milieu present. Depending on the amount of TGF-β, IL-12 and IL-23, Th17 cells can diverge into predominantly IL-17A and F producing cells, IL-17A, IL-22 and IFN-γ producing cells or suppressed IL-17A and F because of a dominance of IL-12 and favouring a Th1 phenotype (Lee et al., 2009).

In humans it has been shown that TGF-β, IL-1β IL-6 and IL-23 are required for IL-17 production and subsequent IL-6 production from epithelial cells as well as RORγt induction. (Burgler et al., 2009)

The Th17 subset of cells have a receptor family the most known being IL-17RA (Yao et al., 1995). Both IL-17A and IL-17F are capable of binding this receptor but the stoichiometry is not fully known, however IL-17RA is essential for both
IL-17A and F signalling (McAllister et al., 2005). It has been shown that IL-17RA is ubiquitously expressed in the lung and signalling is on the basolateral surface of human bronchial epithelial cells suggesting a specific target area for IL-17 (Huang et al., 2007).

Through IL-17RA signalling there are many other pro-inflammatory cytokines produced (see figure 1.3). Many products are neutrophil chemoattractants such as CXCL1, 2 and 5 and CXCL8 (IL-8) suggesting a direct relationship to IL-17 production and neutrophil increase (Laan et al., 1999)
Pro-inflammatory cytokines and chemokines expressed after IL-17 induction, resulting in increase of neutrophils and macrophages. IL-17A and F and IL-23 act directly on epithelial and fibroblast cells which release a variety of cytokines and chemokines. B-defensin (β-Def): an anti-microbial peptide involved in resistance of epithelial colonisation of gram negative and positive bacteria, viruses and fungi. Lipocalin-2 (Lcn2) is important in the innate immune response to bacteria binding to their sidedorophores and is strongly upregulated during inflammation. S100A is associated with Ca2+ ion release. HCO3- the carbonate levels are important in the ability of anti-microbial peptides to function efficiently and clear inflammation.

From epithelial and fibroblastic cells the following are released: Granulocyte colony-stimulating factor (G-CSF) this stimulates the survival, proliferation and differentiation of neutrophils. Interleukin-6 (IL-6) is a pro-inflammatory cytokine which acts upon neutrophils and augments their cytotoxicity (Johnson et al., 1998). CXCL5 chemokine is produced following stimulation with IL-1 and TNF-α and is a neutrophil chemoattractant. CXCL1 also known as GRO-α and also neutrophil chemoattractant. CXCL2 also known as MIP-2α another chemoattractant. Fibroblast secrete the following chemokines: CCL2 also known as monocyte chemoattractant protein (MCP) recruits monocytes to sites of injury as does CCL7 and CX3CL1 attracts monocytes and lymphocytes and when cell bound promotes lymphocyte adhesion to the endothelium through the CX3CL1 receptor found on macrophages. The result of all these interactions and processes is the increase in matrixmetalloproteinase-9 (MMP9) neutrophil elastase and reactive oxygen species, increasing the inflammatory environment. (Alcorn et al., 2010)
1.2.3 Treg cells

Th17 and Treg cells may have a functional relationship with each other, one being pro-inflammatory and the other having a more suppressive effect being able to control inflammatory diseases, however they share similar induction cytokines, namely TGF-beta.

It has been well established that Treg cells are important in self-tolerance and autoimmunity from mouse studies resulting in numerous auto-immune diseases occurring after CD4+CD25+ cells were removed (Sakaguchi et al., 1995). Its primarily CD4+ CD25+ thymic cells that can are classed as Tregs and exert suppression using cell contact mechanisms on other immune cells, however there are also induced Treg cells which mature in the peripheral system and are termed iTreg, and mediate suppression through cytokine interactions. From multiple studies it is becoming clear that the system of tolerance involves many different types of cells and interactions to maintain the system and these mechanisms have not been completely elucidated. Tregs constitute approximately 5-10% of peripheral CD4 positive cells (Lan et al., 2005). T cells are selected for survival in the thymus based on having intermediate strength of the TCR-MHC interaction and the ability to process the presented antigen, Tregs may differ in their selection having a TCR-MHC interaction strength between the conventional positive and negative T cell selection and avoid being destroyed and develop into specialized regulatory cells (Kronenberg and Rudensky, 2005).

As cited before the T cell gene ontogeny produces recognition of self-antigen which is kept to a minimum by negative selection this is not complete and therefore allows a certain amount of autoimmunity. In order to prevent autoimmune diseases there is tolerance to self antigen.

These immunoregulatory cells are determined usually as CD4+ with high levels of IL-2R (CD25+) peripherally. These CD4+CD25+high population are termed nTregs and are differentiated in response to self antigens in the thymus and maintain the self-tolerance. Its thought there are inducible Tregs termed iTregs which form their phenotype peripherally after environmental stimuli.
Tr1 regulatory cells don’t express CD25 or FoxP3 and are differentiated in the periphery after activation in the presence of IL-10 and act as suppressors by suppression of naïve and memory T cell responses. (Roncarolo et al., 2006) CD4+ cells that express TGF-beta are termed as Th3.

Natural Tregs (nTregs) express the transcription FoxP3 which is essential for their function (Marson et al., 2007). The FoxP3 promoter has increased acetylated histones increasing transcription accessibility and increased FoxP3 expression. It has been shown that FoxP3 expression increased in the presence of histone deacetylase (HDAC) inhibitors and the FoxP3 protein increases HDACs and histone acetyltransferase (HATs) allowing further acetylation of FoxP3 and increases its function (Huehn et al., 2009)

nTregs suppress activated T cells by various cell interactions such as CTLA4 and mTGF-β on the Tregs with the corresponding receptors on the T cells (Nakamura et al., 2001)

Inducible (iTregs) which express the same transcription factor but have been differentiated in the peripheral environment driven by exposure to TGF-beta and has been shown to have partial demethylation of FoxP3 suggesting a less stable condition and exert less suppression compared to nTregs (Huehn et al., 2009) but its thought this inducibility in the periphery maybe a means of generating Tregs specific to an exogenous antigen such as an allergen.

1.3 Asthma

Asthma is a chronic respiratory disease effecting approximately 235 million people worldwide (WHO 2002). Asthma pathophysiology manifests as airway hyper-responsiveness, causing shortness of breath and wheezing with hyper secretion of mucus obstructing the lumen of the airway. The symptoms can be brought on by various triggers including environmental triggers such as pollution, allergens, cigarette smoke, and exercise or having a genetic predisposition. Asthma is an inflammatory disease and thus affects a wide variety of cell types. Bronchial epithelial cells are the first to come into contact with
particles from the environment and as a result release cytokines and chemokines that cause inflammation. Airway smooth muscle shows airway remodelling through hypertrophy and hyperplasia of cells. Endothelial cells play a role in recruiting inflammatory cells from circulating lymphocytes these being a large part which drives and perpetuates the inflammatory response.

1.3.1 Th17 in asthma

There has been extensive research into the involvement of Th2 T cell subset in asthma but there is increasingly more research to suggest a Th17 subset involvement in asthma. Increased IL-17A mRNA correlating well with disease severity and subsequent increase of CXCL-8, a neutrophil chemoattractant, adding more evidence that Th17 cells are involved in neutrophilic asthma as opposed to Th2 in eosinophilic asthma (Bullens et al., 2006). Bullens et al also demonstrated an increase in IL-17A mRNA in sputum samples from asthmatics compared to healthy age matched controls (Bullens et al., 2006). Furthermore increased sputum IL-17 in asthmatics was also associated with bronchial hyperresponsiveness in these patients (Barczyk et al., 2003). There has been cell specific evidence of the Th17 influence in the induction of neutrophils through increased IL-8 release (Al-Ramli et al., 2009). IL-17 has been shown in vitro to increase the release of IL-8 from epithelial and endothelial cells in a dose dependent manner which can be inhibited by an anti-IL17 antibody showing a direct response of IL-17 and IL-8 release and neutrophil recruitment (Laan et al., 1999).

It has been shown that there is an increase of IL-17A T cells in asthmatic patients which increases in proportion to disease severity. There was also increased IL-17A in plasma and activated T cells in asthmatics compared to controls (Zhou et al., 2008). It is well established that the chemokine CCR6 is increased on CD4 cells in peripheral blood of asthmatics (Cosmi et al.). Further studies support the expression of CCR6 and migration of Th17 cells in other inflammatory diseases (Hirata et al.). These cells increase the amount of IL-17A, IL-21 and IL-22 cytokines which characterise the Th17 subset.
IL-17A and IL-18 mRNA have been shown to be elevated in sputum from asthmatics and an increase in neutrophils was higher in moderate-severe than mild asthmatics, suggesting that IL-17A and Th17 cells may cause neutrophilic asthma signalling through IL-8. This hypothesis was substantiated with a further study that showed increased IL-17A and IL-8 in bronchial biopsies (Al-Ramli et al., 2009).

Doe at al showed that there was an increase in IL-17A in the bronchial submucosa of mild and moderate asthmatics compared to the controls and severe asthmatics cases. IL-17F increased in severe asthmatics compared to mild and moderate asthmatics suggesting a role of IL-17A and F but did not find a positive correlation between the increased numbers and increased neutrophilia (Doe et al., 2010).

From this evidence it suggests that Th17 T cells are involved in the pathogenesis of neutrophilic asthma and could be a potential target for future therapies for moderate and severe asthma.

1.3.2 Tregs in asthma

Tregs have been established as balancing immune responses and maintaining peripheral tolerance. Currently it has been shown that asthma has a decreased number of Tregs measured as FoxP3+ cells from peripheral blood suggesting this environment favours Th2 responses leading to the clinical signs of asthma (Provoost et al., 2009). There aren’t many clinical studies performed looking distinctly for FoxP3+ cells in asthmatic patients but a comprehensive study was carried out in childhood asthma by Hartl et al. It was shown that asthmatics had impaired FoxP3+ Treg cells in bronchial alveolar lavage fluid (BALF) and in peripheral blood. CD4+CD25+FoxP3+ cells in asthmatics were shown to have a decreased effect on the proliferation of CD4+ responder cells ex-vivo demonstrating an impairment in their function (Hartl et al., 2007) adding more evidence to enhancing an antigenic specific Treg population as a therapeutic effect for asthma (Venuprasad et al.). Furthermore a recent study has shown that BAL lymphocytes from severe and moderate asthmatics have increased FoxP3
positive cells compared to mild and controls (Smyth et al.) suggesting there is a correlation with disease severity. Together these findings suggest that although the number of Tregs respond to inflammatory conditions they are dysfunctional, leading to an uncontrolled inflammatory response. It has been shown that in asthmatic subjects’ exhaled breath condensates there is a significant increase in IL-4 shown to activate fibroblasts suggesting a pro-inflammatory environment (Moqbel et al., 1995). Also an increase in IL-17 further increasing pro-inflammatory cytokines and chemokines (Molet et al., 2001), along with IL-8 and TGF-β all increasing the pro-inflammatory milieu activating epithelial cells as well as other cell types (Kay et al., 2004).

Th17 and Tregs have been shown to work antagonistically with each other. Tregs have their unique suppressor function due to the expression of FoxP3 transcription factor which has been elucidated by the inhibition of FoxP3 expression abrogates T responder suppression (Hori, 2012). Its suppressive function is not known but FoxP3 expression also prevents cells from expressing IL-17 effector functions by inhibiting RORγt (Zhou et al., 2008), further suggesting a close relationship between Th17 and Treg cells.

1.4 COPD

Chronic obstructive pulmonary disorder (COPD) affects approximately 64 million people and is predicted to be the third biggest killer by 2030 (WHO 2004). Its main aetiological factor is cigarette smoke however indoor and outdoor pollutants can affect people in the same manner. The pathogenesis of this disease has similarities to asthma showing airway obstruction but in the case of COPD its non-reversible and the airway parenchyma damage and alveolar destruction ultimately results in emphysematic lungs. Similar to asthma, COPD is an inflammatory disease showing increases in pro-inflammatory cytokines such as TNF-α and IL-8 but also cytotoxic CD8+ T cells and their receptors (Smyth et al., 2008). COPD severity is classified by the Global Initiative for Chronic Obstructive Lung disease (GOLD) system. Using spirometry there are four stages of disease:
Stage 1, Mild: FEV1:FVC <70% and FEV ≥ 80% predicted, sometimes, but not always associated with cough/sputum production
Stage 2, Moderate: FEV1:FVC <70% and FEV ≥ 50-80% predicted
Stage 3, Severe: FEV1:FVC <70% and FEV1 ≥30-50%
Stage 4, Very Severe: FEV1:FVC <70% and FEV1<30% with signs of respiratory failure
FEV is Forced expiratory volume in 1 second, and FVC is the Forced Volume Capacity which together measures the effectiveness of the lungs.

1.4.1 Th17 in COPD

Th17 activation increases pro-inflammatory cytokines and chemokines from a wide variety of cell types including macrophages and epithelial cells. Cigarette smoke is the main cause of COPD and it has been shown that cigarette smoke upregulates Th17 associated chemokine namely IL-8 with a direct increase in neutrophils in smokers BAL (Mio et al., 1997). The cytokine IL-17A has been shown to be increased in the bronchial sub mucosa of mild/moderate and severe stages of COPD compared to non-smokers (Doe et al., 2010). Cells expressing the Th17 associated cytokines, IL-22 and IL-23, are increased in the bronchial epithelium and sub mucosa suggesting a role in the disease. However there were no differences in the levels of the Th17 transcription factor between stable COPD and smokers suggesting the cytokine differences may be due to smoking (Di Stefano et al., 2009). Released IL-17 protein has been detected in sputum of COPD patients although lower amounts compared to asthmatics. (Barczyk et al., 2003). It has been shown that IL-6 and TNF-α are increased in sputum and BALF in exacerbated COPD patients (Hacievliyagil et al., 2006) and TNF-α is increased due to IL-17A which in turn increases neutrophil recruitment (Feldmann et al., 2001) although this was found in rheumatoid arthritis there are similarities in COPD which some also consider as an autoimmune disease suggesting a possible pivotal role of IL-17 in COPD. The IL-17A and F are the most studied out of the 6 isoforms. It has been shown in humans that there is an increase in IL-17A positive cells compared to IL-17F, specifically from lymphocytes, neutrophils and macrophages in the sub epithelium in the small airway in COPD compared to smoking and non-smoking controls. IL-17F is
increased in epithelia and lymphoid follicles but this is not disease associated (Eustace et al.) adding more evidence to the importance of Th17 cells in COPD. It is known that the inflammatory response in COPD shows an increase in CD8+ cells within the airway wall with increased neutrophils (O'Shaughnessy et al., 1997). Neutrophilia is evident in the airway smooth muscle including increased cytotoxic CD8+ T cells in smokers with COPD compared to controls suggesting a role in the pathogenesis of COPD (Baraldo et al., 2004)

### 1.4.2 Tregs in COPD

There have been many studies on Tregs in inflammatory diseases including COPD. There are differing results depending on the specific target being measured and analytical method used. Lee et al observed a decrease in Tregs in lung cells from COPD emphysematic patients when measuring CD4+CD25+ cells by Fluorescent Activated Cell Sorter (FACS) (Lee et al., 2009). Looking in the large airways it has been shown that Tregs FoxP3 positive cells are increased in COPD and smokers compared to controls but in the small airway FoxP3 positive cells numbers are decreased when using immunohistochemistry (IHC). Other studies using BAL have shown an increase in CD4/CD25+ Tregs in COPD (Smyth et al., 2007) and the same can be seen in lymphoid follicles of COPD subjects even though all control groups showed follicle formation suggesting an altered Treg environment. (Plumb et al., 2009).

As eluded to previously Th17 and Treg cells may have functional effects on each other furthering the complexity of the four different T cell subsets. Pridgeon et al showed purified Tregs (CD4+CD25+) from peripheral blood monocytes (PBMCs) suppressed Th1, Th2 proliferation and their cytokine secretion whereas Th17 cell proliferation was inhibited but their ability to secrete IL-17 was not. From lung isolated mononuclear cells from patients with chronic lung inflammation it was shown there was no difference in expression of IL-17 between the groups and no correlation between the number of Tregs and IL-17 producing cells, suggesting IL-17 is present in inflammation but not directly because of Treg activity (Pridgeon et al.) Transcriptionally it has been shown that RORC2 (human IL-17 transcription factor) can inhibit FoxP3 messenger and
protein expression suggesting an inverse correlation between the two transcription factors (Burgler et al.)

Together these findings suggest that an intricate relationship between IL-17 and Tregs exists that under certain conditions may lead to a shift in balance resulting in an acute inflammatory response (as observed in COPD exacerbations and asthma attacks) and following a persistent imbalance resulting in chronic inflammatory environment and deterioration in disease.

1.5 Steroid Treatment and Insensitivity

Glucocorticoids (GC) are steroid hormones that have been used as a therapy for lung inflammation since the 1950s and subsequently the advent of a topically inhaled form in the 1970s. They act as an anti-inflammatory and the use in combination with long acting Beta agonists (LABA) to relax the airway smooth muscle has meant a better control of chronic airways disease. GCs including cortisol (naturally occurring in the body) regulates many processes in the body including the immune system. GCs absorb into the body and move into the cytoplasm of cells where it is bound to the glucocorticoid receptor (GR). This complex is then translocated into the nucleus. In the nucleus it binds to glucocorticoid response element (GRE) and activates anti-inflammatory genes by transactivation (Beck et al., 2009) Alternatively the GC-GR complex can bind to DNA through protein-protein interaction with transcription factors such as NFκB and inhibit gene expression of pro-inflammatory cytokines by transrepression.

For most sufferers of chronic lung inflammation steroid treatment is a solution but a small percentage of patients do not respond to this treatment and are classed as steroid resistant (CSR). The mechanism by which steroid insensitivity occurs is not fully known although there is evidence that IL-2, IL-4 and IL-13 which are increased in some CSR cases, can inhibit the affinity of the GC-GR binding (Ito et al., 2006) thus impairing its function. This may be due to the induction of over expression of the GR isoform GR-β which is considered the negative form, over GR-α. GR-α has been shown to be decreased in lungs of COPD patients
(Marwick et al., 2010) and GR-β dominance has been shown in steroid insensitive subjects (Ito et al., 2006). GR-β may have another mechanism of decreasing steroid sensitivity by interfering with GR-α nuclear translocation shown by the knockdown of GR-β in alveolar macrophage increases GR-α and sensitivity to steroids in steroid resistant asthmatics (Goleva et al., 2006). It is well known that IL-2 and IL-4 are increased in asthmatics (Leung et al., 1995) and in vitro they decrease GR translocation and binding capacity in T cells (Ito et al., 2006). This decrease in GR function may be due to phosphorylation of GR by p38 Mitogen activated protein kinase (MAPK) as this affect can be blocked by p38 MAPK inhibitors (Irusen et al., 2002). This is substantiated by p38 MAPK increased activation levels in alveolar macrophages from asthmatics with a decreased response to steroids (Bhavsar et al., 2010). It has been shown in asthmatic alveolar macrophages that CXCL-8 is the least GC sensitive cytokine and acting as a neutrophil attractant could be a mechanism for steroid insensitivity (Kane et al., 2009).

A study showed GR-α expression in biopsies of asthmatics was lower than controls and from epithelial cells there was an increase in GR-β expression upon IL-17A stimulation and IL-17 induced IL-6 levels were not reduced upon treatment with dexamethasone (Vazquez-Tello et al., 2010). Neutrophils also express high levels GR-β and are less sensitive to steroids than T lymphocytes (Strickland et al., 2001). Airway neutrophilia has been seen in asthma and COPD and is associated with steroid insensitivity and Th17 phenotype cells have been associated with this too being a neutrophil attractant provides a good hypothesis that the Th17 phenotype may give rise to airway inflammatory disease and steroid insensitivity.

### 1.6 LPS pathway

Chronic inflammatory lung disease patients can experience exacerbations with their condition. This manifests itself as increased breathlessness and exaggerated symptoms normally associated with bacterial or viral infection (Hayes and Meyer, 2007, Miravitlles, 2007). The main component of gram negative bacteria is lipopolysaccharide (LPS) (Alexander and Rietschel, 2001). This is the agonist
to the toll like receptor (TLR) 4. TLRs (1-9) are a group of receptors referred to as pattern recognition receptors (PRRs). These PRRs recognise conserved molecular patterns known as pathogen-associated molecular patterns (PAMPS). When a recognised ligand (hydrophobic portion of LPS known as lipid A) comes into contact with TLR4 a complex of LPS and LPS-binding protein initiates the signalling pathway. This is aided by the signalling through CD14 which is membrane bound (found on monocytes and myeloid cells as a first defence mechanism. This activation leads to increased cytokine production such as TNF-α through the activation of NFκB (Dauphinee and Karsan, 2006). With this activation and release of cytokines including IL-6 this can activate T cells to increase pro-inflammatory cytokines and perpetuate the inflammatory environment depicted in figure 1.1.

This pathway is integral to exacerbations in both COPD and asthma patients. The main focus has been on polymononucleated cells but T cells work the same environment as these cells and in the past they have been classified in the adaptive immunity but there is increasing evidence of a more innate response too. In the following studies T cell responses were determined in a human LPS inhalation model. These were based on a wealth of previous studies undertaken over the years which are outlined in Table 1.5.

1.6.1 LPS inhalational studies

Neutrophils are key components of the innate immune system and produce an immune response from microbial pathogens. Lipopolysaccharide is the component of bacterial cell wall that elicits the response. Asthmatics show a heavy bacterial load which suggests an increase in LPS levels in these patients and this has been shown in steroid insensitive asthmatics (Goleva et al., 2008). With the increased amount of LPS in the system this could account for bronchial obstruction and symptoms seen in COPD exacerbations. To study the effects of inflammation within the lung various studies have used an LPS inhalation method.
Michel et al looked at asthmatics and healthy individuals and found with a 22.2μg LPS dose there was a slight bronchial obstruction in asthmatics compared to the healthy controls (Michel et al., 1989). Further cellular analysis from inhalation studies showed that in healthy patients after sputum induction following 5μg LPS inhalation there was an increase in polymononuclear cells (PMN) in the blood and sputum showing neutrophilia (Michel et al., 1997).

Evidence from healthy patient studies led the way to looking at asthma patients. Nightingale et al compared normal, atopic and atopic asthmatics patients looking at pulmonary function and clinical observations. None of the groups showed any changes in the spirometry or clinical observations. The normal and asthma groups showed increased neutrophils from induced sputum and increased IL-8 in both these groups (Nightingale et al., 1998). This was confirmed with a study using healthy subjects after LPS inhalation the number of sputum neutrophils increased and demonstrated increased chemotactic capacity for neutrophils with increased IL-8 release (in house data). Further studies have been carried out using higher doses of LPS, 40μg was used in healthy subjects and there were significant number showing a decrease in spirometry and myeloperoxidase (MPO) and neutrophils were increased in the blood. After sputum induction there were significantly more neutrophils and lymphocytes after LPS challenge showing that this amount of LPS causes inflammation which is site specific as well as systemic (Thorn and Rylander, 1998). Other studies have looked at airway inflammation after LPS exposure over time measuring cell numbers in sputum before and after the exposure showing a peak in cell numbers for lymphocytes, neutrophils and macrophages but was down to baseline numbers after 24 hours, showing the inflammation was resolved after 24 hours (Doyen et al.).

There is a good deal of evidence that LPS inhalation studies would be a safe and effective method of determining the pro-inflammatory environment of asthmatics compared to healthy subjects and to progress our knowledge and understanding of the LPS effect upon steroid sensitivity within this disease group. With this study method using mild COPD patients to elucidate further into the inflammatory responses during exacerbations.
1.7 **Aims of the thesis**

T lymphocytes are an increasingly important cell type to study within respiratory disease therefore this thesis proposed to test the following hypotheses:

- That IL-17 is a driving force for neutrophilia within the lung or the periphery in neutrophilic asthmatics. Do the specific phenotype group of neutrophilic asthmatics release more Th17 cytokine than other T cell subsets than the healthy control group?
- That an increased pro-inflammatory environment exists in the lung or the periphery in asthmatics. Do IL-17 and FoxP3 expression increase upon exacerbation simulation using an LPS inhalation model in asthmatics?
- That there is a systemically increased pro-inflammatory environment in COPD. Do IL-17 and FoxP3 expression increase in COPD patients upon exacerbation simulation using an LPS inhalation model in the peripheral blood?
<table>
<thead>
<tr>
<th>LPS Dose</th>
<th>Timepoints</th>
<th>Biomarkers</th>
<th>Clinical outcomes</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy</td>
<td>(100µg) 25µg</td>
<td>Pre and post BAL 3 hours</td>
<td>BAL Neutrophil and lymphocyte count. AMs phagocytosis Fibronectin</td>
<td>↑neutrophils and lymphocytes. ↓phagocytosis in opsonised particles. ↑fibronectin. (Sandstrom et al., 1992)</td>
</tr>
<tr>
<td>Asthmatics</td>
<td>20µg after 7 days of saline inhalation</td>
<td>0/6/24/48</td>
<td>PD20 FEV1 (histamine) C5a, IL-6, TNFa, CRP</td>
<td>↓Lung function 45 minutes post and resolved after 5 hours. ↑TNFa 1 hour post in blood. ↑in blood neutrophils, ↑systemic CRP 24 hours post. (Michel et al., 1992)</td>
</tr>
<tr>
<td>Healthy</td>
<td>20µg</td>
<td>0/5/6/24/48</td>
<td>FEV1 WBC PMN count CRP, TNFa, C3, ACTH</td>
<td>No difference after 6 hours in lung function. ↑WBC 5 hours post ↑PMN 6 hours post PMN activation after 2 hours. ↑CRP, ACTH 24/48h, No change to C3 and TNFa in the blood. (Michel et al., 1995)</td>
</tr>
<tr>
<td>Healthy</td>
<td>0.5, 5 and 50µg dosed weekly</td>
<td>0/6</td>
<td>FEV1 PMN Activation of PMN Blood/urine CRP Induced sputum 6h post</td>
<td>0.5µg saw marginalisation. 5ug ↑CRP and PMN in blood and sputum. 50µg ↑in all markers including temperature. (Michel et al., 1997)</td>
</tr>
<tr>
<td>Atopic asthmatics and healthy</td>
<td>60µg LPS</td>
<td>0, 6 and 24h post LPS sputum induction</td>
<td>FEV1 FVC Obs up to 8 hours post and 24h.</td>
<td>Asthmatics ↑in temperature, neutrophils and CXCL-8. Controls ↑in neutrophils no change in TNFa. (Nightingale et al., 1998)</td>
</tr>
<tr>
<td>Healthy</td>
<td>40µg LPS</td>
<td>0 and 24 hour</td>
<td>FEV Blood Induced sputum MPO, ECP in sputum and blood</td>
<td>↑MPO and blood neutrophils ↓FEV1. ↑sputum eosinophils, neutrophils and MPO. (Thorn and Rylander, 1998)</td>
</tr>
<tr>
<td>Healthy</td>
<td>1-4 ng/kg instilled directly</td>
<td>2/6/24/48</td>
<td>Cell number TNFa, IL-1β, IL-6 and</td>
<td>↑BAL neutrophils 2-6 hours post. ↑in all biomarkers. (O'Grady et al., 2001)</td>
</tr>
<tr>
<td>LPS dose</td>
<td>Timepoint</td>
<td>Biomarkers</td>
<td>Clinical outcomes</td>
<td>Ref</td>
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</tr>
<tr>
<td>Healthy</td>
<td>5µg and 50µg</td>
<td>FEV1 NE, TNFa and CRP</td>
<td>↓Lung function. ↑Sputum Neutrophil elastase and TNFa. ↑systemic CRP.</td>
<td>(Korsgren et al., 2012)</td>
</tr>
<tr>
<td>Healthy</td>
<td>0/4/24/48h</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Healthy</td>
<td>30µg LPS</td>
<td>Measured CXCL-8, IL-6, CCL2. Blood neutrophils in chemotaxis assay using</td>
<td>↑CXCL-8, IL-6 and CCL2 not CXCR1 at 6 and 24h.</td>
<td>(Aul et al., 2012b)</td>
</tr>
<tr>
<td></td>
<td>0/6/24h</td>
<td>CXCR1/CXCR2 antagonist and CXCR2 antagonist.</td>
<td>↑Neutrophil chemotaxis at 24 hours post CXCR1 and CXCR2 dependent.</td>
<td></td>
</tr>
<tr>
<td>Atopic Asthmatics Vs Healthy controls</td>
<td>Induced sputum 0/6/24h</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>20,000 endotoxin units</td>
<td>% neutrophils IL-6, TNFa, IL-1b, IL-18, TLR2 and 4</td>
<td>↑Sputum PMNs in both groups. ↓absolute cell numbers asthmatics. ↑Serum IL-6, TNFa both groups. ↑Serum IL-1b, IL-18 in healthies. ↑TLR2 and 4 in healthies.</td>
<td>(Hernandez et al., 2012)</td>
</tr>
<tr>
<td>Smokers</td>
<td>5µg and 30µg repeated after 2 weeks</td>
<td>IL-6, CRP, CCL-18, SP-D, CC-16, β defensin. Sputum cell counts p65 translocation.</td>
<td>Repeat inhalation tolerated well. ↑sputum neutrophils 6 hours post. ↑p65 translocation. ↑All other biomarkers listed at varying times.</td>
<td>(Aul et al., 2012a)</td>
</tr>
<tr>
<td></td>
<td>Serum 4/8/24 hours post.</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>Induced sputum 0/6/24</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>hours post.</td>
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</table>

Table 1.1 A summary in chronological order of LPS challenge models in humans outlining the biomarkers used and outcomes from the studies.
Chapter 2

2. General materials and methods

2.1 Patient sampling

2.1.1 Subject Recruitment

Subjects were recruited from a clinical list based at the Medicines Evaluation Unit, University Hospital South Manchester, Greater Manchester. All subjects provided full written consent and all research was approved by the local research ethics committee (South Manchester Research Ethics Committee).

Specific inclusion and exclusion criteria are described in the study design section of each chapter. In brief, asthmatics had a physician diagnosis of for at least 6 months, baseline FEV1 of ≥70% of predicted. Show present or previous (≤12 months) signs of hyperreactivity by either a change in lung function by ≥12% or 200ml post 200ug salbutamol or respond to methacholine causing a 20% fall in FEV1 (PC20 ≤16 mg/ml) based on current guidelines (Bateman et al., 2008). COPD was diagnosed based on the Global Initiative for chronic Obstructive Lung Disease guidelines (GOLD).

2.1.2 Lung Function

Using spirometry the subject’s lung function was determined by measuring the total volume expelled after a maximal inspiration. The indices derived from this exhalation are: FVC – Forced Vital Capacity being the total volume of air forcibly expelled in one continuous breath, FEV1 – Forced Expiratory Volume in 1 second, the volume of air expelled in the first 1 second of forced exhalation. The ratio of FEV1/FVC is expressed as a fraction. These parameters are measured in litres and can be expressed as the percentage of predicted values. Predicted values are tabulated from multiple measurements of differences between sex, height, weight, age and ethnicity in Europe these standards are established by the European Community Health and Respiratory Survey (ECHRS). The culmination of these measurements determines the extent of airway obstruction in asthmatics and COPD subjects to classify the severity of disease following GINA (Global Initiative for
Asthma) and GOLD (Global Initiative for chronic Obstructive pulmonary Disease) guidelines respectively.

Asthmatic subjects underwent an Asthma Control Questionnaire (ACQ) which has been devised to assess the amount of impairment suffered and control of symptoms from various treatments offered with an increasing scale with patients scoring 0 representing no impairment and total control to 6 which is a great deal of impairment and no control.

### 2.1.3 Sputum Induction

Sputum was induced following inhalation of increasing concentrations (3, 4, and 5%) of nebulised saline using an ultrasonic nebuliser Ultraneb 2000, (Medix, Harlow, UK) for 3-5 minute periods. Spirometry was performed after each concentration to ensure there was no reduction in FEV1. After each inhalation subjects were asked to rinse their mouth and blow their nose prior to expectorating into a sterile container. Samples were kept on ice prior to processing. Sputum plugs were isolated and weighed and treated with four times volume of 0.1% dithiothreitol (DTT; Sigma Aldrich, Dorset, UK). Sample was then filtered through 48µm sterile nylon gauze (Sefar Ltd, Bury, UK) pelleted and resuspended at a cell density of 0.5 x 10^6/ml in growth medium: RPMI 1460 (Sigma Aldrich) containing 10% foetal calf serum (Fisher, Leicestershire, UK), 1mM/L L-glutamine (Gibco, Paisley, UK) and 1% penicillin/streptomycin (Sigma Aldrich). The sputum supernatants were retained for future cytokine analysis. Cytoslides using 50µl per slide were produced using the cytoslide funnels and centrifuging at 500rpm for 6 minutes in a Boeco C28 centrifuge (Boeckel, Germany) and left to air dry and used for differential cell counts as well as potential cytokine analysis using immunohistochemistry.

### 2.1.4 Blood Processing

Blood was collected into heparin tubes and layered onto Ficoll-PaqueTM (GE Healthcare, Buckinghamshire, UK), using a Pasteur pipette, and then centrifuged at 400g for 30 minutes.
The mononuclear cell layer (PBMC) was carefully harvested using a Pasteur pipette and transferred to a sterile falcon tube, resuspended in growth medium and centrifuged at 400g for 10 minutes to pellet the cells, then resuspended in a known volume of growth medium.

10µl aliquot of the cell suspension was removed and added to 10µl 0.4% Trypan Blue (Sigma, UK) (1:1) and mixed well. Trypan blue is a vital stain and is only taken up by dead cells, live cells prevent the dye from crossing the cell membrane. This was transferred to the chamber of a haemocytometer (Figure 2). Cell number is calculated using the following equations:

1. Average No. cell counted $\times 10^4 = \text{No. cells/ml (x dilution with trypan blue)}$
2. Total No. cells = value from above equation $\times$ volume of resuspended cells.
2.1.5 Lipopolysaccharide (LPS) Inhalation challenge

The Mefar dosimeter MB3 was used to administer 0.5mg/ml LPS (E coli O26:B6 ref L2654 Sigma) to subjects. The dosimeter was set to administer 12µl per breath. Subjects were asked to breathe in fully for 2 seconds and hold their breath for 6 seconds to ensure the LPS reaches the lower airway, and to repeat this five times to ensure they have inhaled a total of 30µg in total in the case of the asthmatics. This dose has already been shown to be well tolerated by smokers and healthy controls with showing inflammatory increases locally and systemically (Aul et al., 2012a) (Aul et al., 2012b). This dose was reduced to 5µg in total for the mild COPD subjects to determine safety parameters. The subjects vital signs were monitored.
every 30 minutes post LPS inhalation. All these procedures were in accordance with Medicines Evaluation Unit SOP Resp 15.02 and Resp 14. FEV1 % predicted was determined at 1, 4, 6 and 8 hours post LPS, with a further sputum induction at 6 hours post LPS to determine cell numbers and cell morphology.

2.1.6 Bronchoscopy

Subjects were sedated with up to 5mg total dose midazolan. The back of the nasal passage and upper airways were anesthetised with 2% lignocaine. The bronchoscope was wedged in the bronchus and a maximum of 4 × 60ml aliquots of pre-warmed sterile 0.9 % saline solution were instilled into each lobe. Aspirated fluid was collected and stored on ice prior to processing.

2.1.7 BALF processing

The BALF was filtered through a 100µm cell sieve (BD, Oxfordshire, UK) into 50ml falcon tubes and then centrifuged at 400g for 10 minutes at 4°C to pellet the cells. Aliquots of the BAL supernatant were stored at -20°C for future biomarker analysis. The BAL cell pellet was then resuspended in growth medium at a cell density of 2 x 10^6/ml. Cell numbers were calculated by trypan blue exclusion method as described in section 2.1.4.

2.1.8 Flow Cytometry

Flow cytometry uses fluorescently labelled antibodies raised against specific targets on cells to differentiate individual cells and their cell markers. It is very important when using this technique that the cells are in a single cell suspension as this fast and effective method registers individual cells. Compensation settings were calculated by acquiring single colour labelled cells and applying DIVA™ compensation software to calculate the amount of compensation needed to allow for spectral overlap between fluorophores such as Fluorescein isothiocyanate (FITC) and Phycoerythrin (PE). Flow cytometry uses the properties of fluorescence in order to distinguish between different targets labelled with fluorophores that are excited by the lasers and emit light at different wavelengths figure 2.3. The emission at different
wavelengths involves a certain degree of spectral overlap which has to be compensated for as shown on figure 2.4.

All flow cytometry was carried out using 2 x 10^6/ml BAL or PBMC cells using 500µl/tube unless otherwise stated. The following antibodies were used to perform the compensation experiment and the calculated compensations are shown in table 1. CD3- Allophycocyanin (APC) (BD cat No. 555335), CD8- Allophycocyanin-cyanin 7 (APC-CY7) (BD cat No. 560179 clone SK1), IL-17- Phycoerythrin (PE) (EBioscience 12-7179-42 clone 1630) FoxP3- Fluorescein isothiocyanate (FITC) (Ebioscience 11-4776-42 clone 468)

Cells were incubated with 20µl/tube manufacturer’s recommendation of Fc block (Ebioscience cat No. 14-9161-73) on ice for 20 minutes to inhibit non-specific binding of the antibodies to the Fc receptors on the surface of the lymphocytes. Cells were incubated with the surface antibodies 5µl of CD3 and CD8 for 30 minutes at room temperature. After permeabilisation of the cells using Ebioscience Fix/Perm buffer (cat No. 00-5523-00) following the staining protocol according to manufacturer’s instructions (Ebioscience) cells were incubated with the intracellular antibodies 20µl and 5µl of IL-17 and FoxP3 respectively or their isotype equivalents at 4°C for 45 minutes. Following washing in stain buffer containing serum, the cells were resuspended in 500µl of PBS ready for FACS analysis.

All samples were analysed by acquiring 100,000 cells with the compensation values applied as shown in table 2.1.
Figure 2.3: Spectral wavelengths of different fluorophores the left hand side of each peak shows the excitation wavelength and the right hand side, the excitation wavelength.

Figure 2.4: Showing the spectral overlap between FITC and Phycoerythrin (PE) fluorophores. Both these fluorophores are excited by the blue laser but with a considerable amount of spectral overlay as shown by the blue arrow. DIVA™ software calculates this overplay so that the two fluorophores will be separate.
<table>
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<th>Fluorophore</th>
<th>Corresponding Fluorophore</th>
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</thead>
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Table 2.1: Calculated compensations for the combinations of fluorophores. Spectral overlap refers to the calculated value to ensure all the fluorophores are seen separately. These compensation values were applied to all subsequent analysis of flow cytometry in these studies unless otherwise stated.
2.1.9 Gating strategy

Gating strategy refers to the method of determining the number of cells of a specific population within the total population when plotting one fluorophore against another. The main gated population were lymphocytes based on forward (cell size) and side scatter (cell complexity) as shown in figure 2.1.9-1. Within this lymphocyte population CD3 is gated upon using APC channel and cell complexity. Using the CD3 population CD4 and CD8 populations were gated upon using a positive and negative gate in the APC-Cy7 channel for CD8 and CD4 respectively (Farrell et al., 2001). Within the CD4 and CD8 populations the intra cellular cytokine IL-17A and transcription factor FoxP3 were gated upon using PE and FITC channels respectively using the isotype control to set the gate as shown in fig 2.1.9-2.

![Figure 2.5: Dot plot showing forward scatter (FSC-A) and side scatter (SSC-A). With increasing size and cellular complexity are the granulocytes and decreasing in size and complexity are the lymphocytes with smaller particles that are debris from the cell preparation.](image)

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Figure 2.6. From the initial FSC vs SSC plot defining the lymphocytes, CD3+ lymphocytes were gated on using APC channel. From this population CD8+ and CD8- (CD4) were separated using the APC-Cy7 channel. Within EACH CD8+/ populaions the intra cellular IL-17 and FoxP3 levels are gated on using PE and FITC channels respectively.
Chapter 3

Method development of multi parametric flow cytometry analysis

3.1 Introduction

Th17 has been associated with inflammatory diseases such as rheumatoid arthritis (Li et al., 2010), multiple sclerosis (Tzartos et al., 2008) and Crohn's disease (Monteleone et al., 2012). It has also been shown in humans to be associated with neutrophilia within the airways of asthma phenotypes (Nakagome et al., 2012). The aim of this study was to determine the role of Th1, Th2, Th17 and T regulatory cells within a specific neutrophilic symptomatic asthma group compared to healthy controls.

The study measured T cell cytokines using multi-parametric flow cytometry on bronchial alveolar lavage fluid (BALF) and blood using peripheral blood monocytes (PBMCs). IL-4, IL-17A and FoxP3 were the candidate targets to represent Th1, Th2, Th17 and T regs respectively. Previous studies have looked at these T cell subsets in blood (Shi et al., 2011) but not in a paired BAL and blood so there was need to validate the labelling method to accommodate both sets of samples. This chapter describes the validation process to optimise the 6 fluorophores used within the experimental system of ex vivo T cell stimulation to determine the level of expression of T cell subsets.

3.2 Method Development

The initial work was based on ex-vivo T cell stimulation to determine whether neutrophilic asthmatics showed different expression levels of T cell phenotypes compared to healthy non-smoking controls. T cells were stimulated using a combination of a phorbol ester, phorbol myristate acetate (PMA) and a calcium ionophore, ionomycin compared to unstimulated cells. The production of intracellular cell mediators which were measured by intracellular antibody labelling with flow cytometry. The flow cytometry involved a 6 colour protocol labelling surface markers for the major lymphocyte population using CD3 with CD4 and CD8.
subsets and intracellular labelling of the, IFN-γ, IL-4 and IL-17A cytokines and FoxP3 transcription factor.

Using the following antibodies purchased from Becton Dickinson (BD™): CD3-APC cat.No. 555335, CD4 or CD8-APC-Cy7 cat.No. 341115 and 348813 respectively, IFNγ-PE cat.No. 562016, IL-4PE-Cy7 cat.No. 560699, IL-17-FITC cat.No. 560488 and FoxP3-PECy5.5 cat.No. 560047 and their isotype equivalents. The labelling optimisation and gating strategy was performed using PBMCs. PBMCs were isolated using the protocol outlined in section 2.4. Using 1 x 10^6 cells/test treatment there were 4 tubes (1 tube with test antibodies and 1 tube with intracellular isotype controls) for both basal and stimulated cells incubated at 37˚C overnight.

3.3 Initial method: cocktail of individual antibodies

The PBMCs were washed by adding 2 ml of FACS wash (phosphate buffered saline (PBS) with 5% foetal bovine serum (FBS)) then centrifuged at 280g for 10 minutes at room temperature. Supernatant was discarded and the excess blotted on a paper towel. The cell pellet was then vortexed until fully dispersed (this system is applied after any washes stated through the protocol). Cells were incubated with 5µl of each surface antibody (manufacturers recommended volume) at room temperature, in the dark, for 30 minutes. Cells were then washed in FACS wash, then fixed and permeabilised using a fix/perm combination buffer (EBiosciences cat. No. 00-5123 and diluent cat. No. 00-5223) at 4˚C in the dark for 45 minutes. Following further washes, cells were incubated with the intra cellular antibodies according to the manufacturers recommended concentration at 4˚C in the dark for 45 minutes. The cells were washed twice then re-suspended in 500µl of PBS before acquiring 100,000 events and analysed on the BD Canto II cytometer.

Using 6 fluorophores requires compensation to overcome the spectral overlap that occurs between them. After this had been set for all the combination of antibodies used in this experiment it was applied to all the tubes before acquiring, these compensation values are applied to all subsequent experiments. The compensation values were determined as described in section 2.1.8 and the calculated compensations are shown in table 3.1
Table 3.1: The calculated compensations for all the spectral overlaps needed to separate the combinations of antibodies within each complex stain this was calculated using DIVA software with single stained controls.

<table>
<thead>
<tr>
<th>Fluorochrome</th>
<th>Corresponding Fluorochrome</th>
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3.3.1 Problem 1: non-specific binding of IL-4 at basal levels

The initial scatter plots displayed good separation and antibody labelling for CD3 and CD4 (CD8 was similar, data not shown) in the basal conditions (medium alone) (Figure 3.3.1 A&B). Low levels of IFNγ (0.1%) and FoxP3 (2.3%) were detected and no IL-17, however almost 100% labelling of IL-4 was observed, suggesting a high degree of non-specific binding of this antibody (figure 3.3.1 D).

Figure 3.3.1: Basal PBMCs with labelling for all six fluorophores: The main population gated on CD3 (A) and the CD4 sub-population within it (B). Within this CD4 positive population intra cellular cytokines of IFNγ (C) IL-4 (D) IL-17 E) and FoxP3 (F) were determined.
3.3.2 Problem 2: non-specific staining of isotypes

In flow cytometry, isotype antibodies of each test antibody are used to help to distinguish between specific and non-specific binding of antibodies to the cells. Non-specific binding can occur due to Fc receptor binding on target cells. The Fc receptor is on the surface of immune cells and is used to recognise antibodies with antigen attachment which drives the cellular immune response. However the receptor can also recognise the antibody of the isotypes and other non-specific targets as seen with the isotype antibodies of IL-17, IL-4 and FoxP3 shown in figure 3.3.2B-D.

It was clear that when combined these antibodies displayed non-specific binding which made it impossible to clearly gate on the cells specifically at basal level (showing similar patterns post stimulation data not shown).

Figure 3.3.2: Basal PBMCs gating on CD3 positive and CD4 positive cells with the intracellular isotype control antibodies showing non-specific labelling for all with IFNγ being the exception (A)
3.3.3 Problem 3: loss of CD4 upon stimulation

Although CD3 labelling remained consistent after stimulation, the CD4 population appeared to decrease following stimulation. As depicted in figure 3.3.3-1B the positive population merged into the negative population resulting in an ambiguous gate being formed. This phenomenon has been reported previously (Kemp and Bruunsgaard, 2001) This was not observed within the CD8 positive population (data not shown) so was specific for CD4. Reduction in CD4 population affected the data obtained for intra-cellular labelling, as these were being measured as a sub-population of CD4 positive and CD8 positive cells shown in figure 3.3.3-1. Without the CD4+ separation, accurately gating the intra-cellular targets was not possible and the isotype antibodies were still showing non-specificity, figure 3.3.3-2.

From the initial data it was clear that the antibodies needed optimising when used in conjunction with each other to resolve the three problems outlined.
Figure 3.3.3-1: PMA/ionomycin stimulated PBMCs showing a decrease in specific CD4 labelling (B) and consequentially poor labelling of the intra cellular antibodies (C-F)
Figure 3.3.3-2: PMA/ionomycin stimulated Isotype controls for IFNγ A) IL-4 B) IL-17 C) and FoxP3 D).
3.3.4 Identifying causes: understand the staining profiles of individual antibodies

To investigate problems, the main populations of CD3 and CD4 along with IL-4, IFNγ, IL-17 and FoxP3 were set up in a single label experiment to compare basal and stimulated depicted as single colour histograms. This was to determine which antibodies were causing the non-specificity when in combination with the others. Single staining for the intracellular antibodies showed IL-4-PeCy7 having no discernible labelling differences between basal and PMA/ionomycin stimulation and non-specificity with isotype control (figure 3.3.4-1), also seen within the complex stains, concluding this antibody clone was exhibiting nearly absolute non-specificity to IL-4. IFNγ-PE showed an increase upon stimulation from T cell stimulation however its isotype non-specificity increased slightly too concluding the isotype antibody clone showed non-specificity upon stimulation (figure 3.3.4-2). IL-17-FITC showed a similar level of labelling with its isotype control upon stimulation making it impossible to set the gate (figure 3.3.4-3). FoxP3-PerCP-Cy5.5 showed an increase upon stimulation but nearly 100% labelling with its isotype control (Figure 3.3.4-4).

Figure 3.3.4-1: Histograms of PBMCs single labelled for IL-4 basal A) stimulated B) and isotype control C).
Figure 3.3.4-2: Histograms of PBMCs single labelled for IFNγ basal (A) stimulated (B) and isotype control (C).

Figure 3.3.4-3: Histograms of PBMCs single labelled for IL-17A basal (A) stimulated (B) and isotype control (C).

Figure 3.3.4-4: Histograms of PBMCs single labelled for FoxP3 basal (A) stimulated (B) and isotype control (C).
Upon stimulation the main CD4 positive population decreased negating all the intracellular labels. This phenomenon had been seen previously with certain clones of CD4 antibody (personal observation) perhaps the CD4 epitope is being internalised upon stimulation, clearly the stimulation process was causing the problems. CD3 labelling remained consistent upon stimulation providing a clear population with which to gate on for lymphocytes (Figure 3.3.4-5). It was clear to see that CD4 did indeed show merging of the positive labelled population with the negative population suggesting this clone of CD4-APC-Cy7 would not be suitable for this study (figure 3.3.4-6).

Figure 3.3.4-5: Histograms of PBMCs single label for CD3 basal (A) and stimulated (B).

Figure 3.3.4-6: Histograms of PBMCs single label for CD4 basal (A) and stimulated (B).

From the experiment outlined the following conclusions were made: CD4-APC-Cy7 clone was decreasing upon stimulation, IL-4-PE-Cy7 showed non-specific labelling with basal and stimulated cells, IFNγ-PE showed a specific labelling upon
stimulation but non-specific labelling of the isotype, likewise with IL-17-FITC and FoxP3 isotype.

### 3.4 Selection of new antibodies

The initial fluorophores conjugated to the antibodies were chosen due to availability from the manufacturers but from the recent commercial expansion of the antibody panels available another set of antibodies were chosen to combat the antibody interference. The following were tested: CD3-APC (remained the same as there was no issue with this), CD4-APC-H7 cat.No.560158 clone RPA-T4 utilising a new more stable clone and tandem vector from BD to combat the problem of labelling upon stimulation, this was visualised in the APC-Cy7 channel.

All the intra cellular antibodies were from EBiosciences: IL-17A-PE Cat.No. 12-7179 (IgG1 isotype cat. No. 12-4714) using the brightest fluorophore for the smallest cell population to maximise its visability. IFN-g-PE-Cy5.5 cat. No. 45-7319 (IgG1 isotype cat. No. 45-4714) and IL-4-PE-Cy7 cat. No. 25-7049 (IgG1 isotype cat. No. 25-4714) as there were no alternatives. FoxP3-FITC BDTM cat. No. 560047 (IgG1 isotype cat. No. 557702) which had been used in previous studies and had shown clean labelling (Smyth et al., 2010). A compensation experiment was set up using the new antibody combinations as described in section 2.1.8. The resulting cell profiles using this combination are depicted in figure 2.4.1-1. IFN-γ still showed non-specific binding with its isotype but the rest were clear figure 2.4.1-2.

### 3.4.1 Use of an FcγR binding inhibitor

To reduce any non-specific binding of surface or intra cellular antibodies an FcγR binding inhibitor (pan receptors 1-3) (ebiosciences) was used (5µl/test and incubated on ice and in the dark for 20 minutes) before the surface antibodies. The cells were then permeabilised and fixed following manufacturers instruction with two washes in FACS buffer to remove any unbound antibodies. The resulting antibody profiles for basal and PMA/ionomycin stimulated cells are shown in figure 3.4.1-3 and 3.4.1-2 respectively and isotypes figure 3.4.1-2 and 3.4.1-4. Basally cells displayed low levels for IFNγ, IL-17 and slightly higher for IL-4 and FoxP3 as expected. All biomarkers (except IFNγ) were responding well to the stimulus showing clear specific labelling.
Figure 3.4.1: Basal PBMCs with labelling for all six fluorophores: The main population gated on CD3 (A) and the CD4 sub-population within it (B). Within this CD4 positive population intra cellular cytokines of IFNγ (C) IL-4 (D) IL-17 (E) and FoxP3 (F) were determined.
Figure 3.4.1-2: Basal PBMCs showing intra cellular isotype labelling for IFNγ A) IL-4 B) IL-17 C) and FoxP3 D).
Figure 3.4.1-3: Stimulated PBMCs with labelling for all six fluorophores: The main population gated on CD3 (A) and the CD4 sub-population within it (B). Within this Figure 3.9.3 CD4 positive population intra cellular cytokines of IFNγ (C) IL-4 (D) IL-17 (E) and FoxP3 (F) were determined.
3.4.1-4: Stimulated PBMCs showing intra cellular isotype labelling for IFNγ A) IL-4 B) IL-17 C) and FoxP3 D)
3.4.2 Non-specific binding of the new IFNγ isotype control

Non-specific binding of PerCP-Cy5.5 isotype control for IFNγ remained with and without stimulation.

The results were looking promising having stabilised the CD4 clone and the majority of the intra cellular antibodies showing specificity apart from the IFNγ isotype.

Solution: increase concentration of FcγR binding inhibitor

The FcγR binding inhibitor was increased to the manufacturers maximum recommended amount before inhibitory effects may be seen to 20µl per test incubated on ice for 20 minutes before the permeabilisation and fixation of the cells and the addition of the intra cellular antibodies. This resulted in predicted levels of intra cellular labels and clear gates for all isotype controls upon stimulation, creating a working complex panel of antibodies to measure all 4 T cell subsets in blood (Figure 3.4.1 and 3.4.2).

Figure 3.4.1: Stimulated PBMCs showing intra cellular labelling with FcR inhibitor. Positive labelling of IFN-γ A, IL-4 B, IL-17 C and FoxP3 D.
3.4.2 Stimulated PBMCs showing intra cellular isotype labelling with FcR inhibitor. Isotype labelling of IFN-γ A, IL-4 B, IL-17 C and FoxP3 D.

3.4.3 Sensitivity of the new IL-17 antibody detection

It was becoming increasingly clear through the validation process that IL-17A has a low level of expression in most subjects (1-2% of the CD4 cells after stimulation).

3.4.4 Increase volume of IL-17 antibody used

The antibody used (IL-17-PE) had not been validated in human blood before so to ensure maximum antibody labelling in these cells increased volumes of antibody were applied (5µl to 10 and 20µl) in parallel with equal volumes of the isotype control. Using the same protocol for permabilisation and fixation (in the presence of...
FcγR binding inhibitor) the titrated antibody was added to individual tubes to determine whether the labelling could be maximised without affecting the isotype control labelling maintaining target specificity. Increasing the amount of IL-17A added to the cells increased the amount of specific labelling of the lymphocytes without affecting the isotype labelled cells suggesting the maximum IL-17A labelling could be achieved using 20µl/test, in figure 3.4.3.

Figure 3.4.3: Titration of IL-17-PE adding 5µl (A) 10µl (B) 20µl (C) and 20µl isotype (D) showing 2.3%, 5.7%, 10.1% and 0.1% IL-17 positive cells respectively.

With this completed 3 times for validity in PBMCs this protocol was used in parallel with bronchial alveolar lavage (BAL) cells in the final study as a vital comparison between local and systemic environments (Figure 3.4.4 and 3.4.5)
Figure 3.4.4 Stimulated BAL cells using the optimised set of antibodies of IFN-γ-PerCP-Cy5.5, IL-4-PeCY7, IL-17-PE and FoxP3-FITC.
Figure 3.4.5 Stimulated BAL cells using optimised isotype control antibodies (showing an acceptable amount of non-specific labelling BAL cells are never as clearly defined as blood cells due to the nature of the sample having a tendency to 'clump').

The labelling had been optimised as much as possible although a few concerns remained. Upon stimulation IL-4 still didn’t show discrete labelling from the negative cells as one would expect the only positive cells were highlighted from the gate set rather than an obvious separate population. Although the isotype is clear the pattern from the positive labelled cells is not well defined which could lead to false positives.
3.5 Further method: commercial kit of antibodies

The new Th17 subset and T regulatory subset alongside the classic Th1 and Th2 subsets were of great interest to researchers worldwide and manufacturers were aware of this and had produced an optimised kit to label CD4 with IL-17A, IFNγ, IL-4 (Cat.No. 560751- the components of the kits are: CD4-PerCP-Cy5.5 clone SK3, IL-17A-PE clone N49-653, IFNγ-FITC clone B27 and IL-4-APC clone MP4-25D2). This had been developed using a stimulant referred to as Leucocyte activating cocktail (LAC™ Cat. No. 550583) which is an optimised mixture of PMA and ionomycin. The incubation time was determined at a maximum of 14 hours due to ease of processing time on the day of bronchoscopy and there were no adverse effects on the cells (collaborator’s observations). The cells were cultured with LAC for 14 hours in the presence of the golgi stop (Brefeldin A containing monensin) to prevent any loss of rapidly released cytokines enabling detection of increased levels of intracellular cytokines (figure 3.5.1).

The optimum amount of LAC to be used was determined by titration using 5µl, 4µl, 2µl and 1µl/test which showed there was no appreciable difference in expression levels with the different amounts (figure 3.5.2) but also 1µl showed the greatest release of IL-2 measured by ELISA following R and D manufacturer’s instructions (Figure 3.5.2). A single lot number of LAC could be used for the entire study to eliminate possible batch variation.
Figure 3.5.1: Stimulated PBMCs using 1µ and 4µL LACTM for 14 hour incubation (isotype controls were used in parallel and were clear of non-specific binding)

Figure 3.5.2: Concentration of released IL-2 from 14 hour cultured PBMCs with titrated amounts of LACTM (n=1)
3.5.1 Final method validated

The following protocol was repeated in PBMCs 3 times to test validity: To measure Th1, Th2 and Th17 1 x 10^6 cells/test were incubated in FACS tubes with or without LAC stimulation including brefeldin A for 14 hours at 37°C/5%CO₂. The cells were harvested and washed twice by adding 2ml of PBS stain buffer (cat. No. 554656) and centrifuging at 280g for 7 minutes. The cells were fixed by adding 1ml of cold Cytofix fixation buffer (cat.No. 51-9006613) and incubated for 20 minutes at room temperature. To remove this buffer the cells were washed twice using the PBS stain buffer then the cells were resuspended in 1ml of BD perm wash (10X concentrate Cat. No. 51-2091KZ) and incubated at room temperature for 15 minutes. After the cells were centrifuged the cells were resuspended in 50µl/test of perm/wash and 20µl of antibody cocktail/tube or 20µl isotype control antibodies (cat. No. 560802-the components of the antibody cocktail are: Human CD4 PerCP-Cy™5.5 clone SK3, Mouse mIgG1, κ FITC clone MOPC-21, Mouse mIgG1, κ PE clone MOPC-21, Rat rIgG1, κ APC clone R3-34) and incubated in the dark for 30 minutes. The cells were washed with PBS stain buffer twice and resuspended in 500µl stain buffer/test. The cells were then acquired on BD Canto II flow cytometer setting the total acquisition to 100,000 cells for each tube. An additional gate was set using side scatter width and side scatter area to be able to gate on single cells this is particularly essential when using BAL cells that will contain more granulocytes and will produce cell aggregates after incubation. All the antibodies displayed specific labelling (Figure 3.5.1-1/2). All the isotypes are completely clear for both basal and stimulated cells, this protocol was then used with BALF cells (figure 3.5.1-1).

FoxP3 needed to be measured for this study and this was optimised using the BD Tritest™ which contains CD3-PerCP-Cy5.5, CD4-FITC and CD8-PE and using FoxP3-APC after the cells were permeabilised using eBioscience buffers specifically developed for FoxP3 labelling (cat.No. 00-5523). Using the manufacturers recommended amount of antibodies for each test kit and 20µl FoxP3 the system was validated (figure 3.5.1-3).

All isotypes were completely clear for all experiments and the PBMCs mirrored the plots from the BAL, establishing a protocol to measure 7 different cell populations simultaneously in BAL and PBMCs at basal and after a 14 hour stimulation which
was used to compare the cytokines from neutrophilic asthmatics and healthy controls.

Figure 3.5.1-1: PBMC basal levels of single cells A, CD4 B, IL-17-PE and IFN-γ-FITC C, and IL-4-APC D. LAC stimulated PBMCs levels of IL-17-PE and IFN-γ-FITC E, and IL-4-APC F using the validated commercial antibody staining kit.
Figure 3.5.1-2: BAL cells basal levels of single cells A) CD4 B) IL-17-PE and IFN-γ-FITC C) and IL-4-APC D). LAC stimulated BAL cell levels of , IL-17-PE and IFN-γ-FITC E) and IL-4-APC F) using the validated commercial antibody staining kit
Figure 3.5.1-3: Stimulated BAL cells labelled with CD4 A) and CD8 FoxP3 and CD4 FoxP3 B).
Chapter 4

The role of T cell subsets in neutrophilic asthma using multi parametric flow cytometry

4.1 Introduction

Neutrophilia has been shown in older patients with asthma and those with late onset asthma (Rossall et al., 2012). There is evidence that Th17 cells upon releasing IL-17 attracts neutrophils (Ye et al., 2001) potentially perpetuating neutrophilic inflammation. IL-17 and IL-8 mRNA has been shown to correlate with increased neutrophils levels (Bullens et al., 2006). Th17 cells also induce chemokines such as CXCL-1, GCP-2, CXCL-8 and MCP-1 that can stimulate stromal cells to release further pro-inflammatory cytokines (Fossiez et al., 1996). Neutrophilic asthmatics are a phenotype that don’t respond well to current treatments with corticosteroids (Wang et al., 2010). This study aims to determine the expression levels of Th1, Th2, Th17 and Treg lymphocytes in the lung and peripheral blood using multi parametric flow cytometry comparing neutrophilic asthmatics to healthy age matched controls. By stimulating the T cell receptor on the lymphocytes ex –vivo, the differences between the patient groups can be elucidated and this would predict the in-vivo T cell mediated immunity. This method allows simultaneous measurements of expression levels within the CD4 and CD8 cell populations and analysis of double positive cytokine expressing cells and comparing asthma patients to healthy controls. The patient group was selected on the strict criteria of induced sputum had to contain over 50% neutrophils. Elucidating the role of the Th17 subset and Th1, Th2 and T regulatory subsets in these patients may provide evidence of potential therapeutic indicators targeting the T cell mediated inflammatory response in asthma.

4.2 Methods

4.2.1 Patient selection

Six asthma patients and six healthy age matched controls with normal lung function were recruited from the clinical research database of the Medicines Evaluation Unit, University Hospital of South Manchester. The inclusion criteria for the asthma group
was FEV1 % predicted ≤ 90%, ≥50% neutrophil population from induced sputum and use of relieving inhaler more than twice a week. The healthy controls were age matched never smokers. All subjects provided written informed consent before undergoing a bronchoscopy. The patient demographics were determined and summarised in table 4.1.

4.2.2 Study Design

Asthmatic patients and the healthy controls attended a bronchoscopy to obtain bronchial alveolar lavage (BAL). Paired BAL and blood cells 1 x 10⁶ cells/test were incubated at 37°C for 14 hours with and without 1µl Leucocyte Activating Cocktail LAC™ (stimulated and basal respectively) with 0.5µl Brefeldin A. LAC is an optimised combination of PMA and ionomycin. PMA passively crosses the cell membrane and stimulates the T cell receptor driving the production of cytokines through the protein C pathway. Ionomycin raises the intra cellular calcium levels also stimulating cytokine production. The multi parametric antibody labelling protocol used for these patient samples is described in detail in section 3.13.

4.2.3 FACS analysis

Th1, Th2, Th17 and Treg lymphocyte subsets were analysed measuring IFN-γ, IL-4, IL-17 and FoxP3 respectively in paired BAL and PBMC basal and stimulated samples. The analysis was completed using BD DIVA™ software acquiring 100,000 CD3 positive cells. From this population CD4 was gated upon and from this gated on IFNγ, IL-4, IL-17A and FoxP3 for the intra cellular cytokines, using isotype equivalents as controls. The gating strategy is shown in detail in chapter 3.

4.2.4 Statistics

Comparison between basal and stimulated cells for the control and asthma groups used a paired T test (with parametric distribution) and Wilcoxon comparisons (non parametric data) were made as indicated. The data distribution was calculated using GraphPad software. Further unpaired comparisons were made between control and
asthma groups for basal and stimulated samples. Statistical significance was achieved when P≤ 0.05(*) or ≤0.01 (**). Statistical analysis of the data was conducted using GraphPad Instat version 5 (GraphPad Software, Inc, San Diego, CA, USA).

4.3 Results

4.3.1 Patient Demographics

There was a significant difference between the FEV1 values from healthy controls (mean value of 3.55L) and asthmatics (mean value of 2.74L) (p=0.04 unpaired T test) (table 4.1) suggesting the asthmatics have less lung capacity. The FEV1 % predicted for healthy controls was 101% and for asthmatics, 80.1 % (p=0.01). The mean percentage of sputum neutrophils of 65.1% further characterised the asthmatic cohort.

4.3.1 BAL T lymphocyte cells

LAC stimulation induced a Th17 response in BAL lymphocytes in both the control and asthma group showing an increase in the mean CD4 IL-17 expression from 0.3% to 7% (p=0.03) in the controls and 1.6% to 5.6% (p=0.06) in asthma group (figure 4.3.1a). There was no difference in CD4 IL-17 expression between the groups at the basal level or stimulated (p=0.35 Basal, p=0.41 stimulated). LAC produced a large increase in CD4 IFNγ in BAL cells in both groups showing a Th1 response. In the control group it ranged from 0.017% to 62.9% (p=0.03) upon stimulation. In the asthma group from 0.03% to 56.9% (p=0.03) but again no differences between groups (p=0.54 basal, p=0.1) (figure 4.3.1b). CD4 IL-4 expression representing Th2 phenotype increased upon LAC stimulation in both groups although there was no significant difference in the asthma group, from 0.3% to 2.4% (p=0.04) 0.7% to 3.1% (p=0.21) in control and asthma groups respectively (figure 4.3.1c). FoxP3 expression denoting T regulatory phenotypes showed no significant increase in expression on BAL lymphocytes upon stimulation. The expression levels from basal to stimulated were 3.4% to 4.1% (p=0.22) and 2.7% to 3.03% (p=0.37) in the control and asthma group respectively (figure 4.3.1d). The percentage expression of the
main lymphocyte populations namely CD3, CD4 and CD8 showed no differences
details shown in table 4.2.

4.3.2 Blood T lymphocyte cells

LAC stimulated PBMCs showed a significant increase in expression of CD4 IL-17
for both groups. Means increased from 0.01% to 0.8% (p=0.03) and 0.08% to 0.71%
(p=0.03) in control and asthma groups although the absolute expression level was
lower than BAL lymphocytes. There was no significant difference between groups
(p=0.99 basal, p=0.29 stimulated) (figure 4.3.2a). CD4 IFNγ expression significantly
increased in both groups from basal 0% to 9.7% (p=0.03) in the controls and 0% to
11.8% (p=0.03) (figure 4.3.2b). CD4 IL-4 expression in PBMC lymphocytes showed
a very significant increase with LAC stimulation in the control group from 0.23%
rising to 1.28% (p=0.007) and from 0.1% to 1.28% (p=0.01) in the asthma group
(figure 4.3.2c)
CD4 FoxP3 expression did not change upon LAC stimulation and ranged from 1.3%
to 0.95% (p=0.29) and 1.9% to 2.1% (p=0.62) in the control and asthma group
respectively (figure 4.3.2d). The percentage expression of the main lymphocyte
populations namely CD3, CD4 and CD8 showed no differences upon stimulation or
between groups details shown in table 4.2.
Table 4.1 Patient demographics. The data are represented as mean (SD). F=female, M=male. *cell counts from sputum differential counts. n/a=insufficient sputum produced for cell counts. n/d =not done.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Healthy (n=6)</th>
<th>Asthmatics (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex (F/M)</td>
<td>4/2</td>
<td>1/5</td>
</tr>
<tr>
<td>Age (years)</td>
<td>36 (10.9)</td>
<td>39.6 (9.1)</td>
</tr>
<tr>
<td>FEV1 (Litres)</td>
<td>3.55 (0.4)</td>
<td>2.74 (0.6)</td>
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<tr>
<td>FEV1 % predicted</td>
<td>101.2 (8.6)</td>
<td>80.14 (3.7)</td>
</tr>
<tr>
<td>FEV1/FVC (82)</td>
<td>82 (8)</td>
<td>0.67 (0.04) (p=0.007**)</td>
</tr>
<tr>
<td>Saline instilled (ml)</td>
<td>480 (0)</td>
<td>475 (450-480)</td>
</tr>
<tr>
<td>BALF recovered (ml)</td>
<td>279.3 (240-394)</td>
<td>196.8 (104-280)</td>
</tr>
<tr>
<td>% recovery</td>
<td>58.2 (47.1-82.1)</td>
<td>34.4 (32.1-58.3)</td>
</tr>
<tr>
<td>Total cells recovered (x 10^6)</td>
<td>29.3 (13.2-63.8)</td>
<td>21.1 (9.2-39)</td>
</tr>
<tr>
<td>Cells/ml recovered (x 10^6)</td>
<td>0.1 (0.05-0.16)</td>
<td>0.13 (0.06-0.38)</td>
</tr>
<tr>
<td>ACQ Score</td>
<td>n/d</td>
<td>1.4 (1.14-1.71)</td>
</tr>
<tr>
<td>Reversibility (ml)</td>
<td>201 (90-490)</td>
<td>303 (150-530)</td>
</tr>
<tr>
<td>% Neutrophils*</td>
<td>n/a</td>
<td>65.1 (54-82)</td>
</tr>
<tr>
<td>% Macrophages*</td>
<td>n/a</td>
<td>29.9 (15.5-48.5)</td>
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<tr>
<td>% Eosinophils*</td>
<td>n/a</td>
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<tr>
<td>% Lymphocytes*</td>
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<td>0.54 (0.0-1.0)</td>
</tr>
<tr>
<td>% Epithelial*</td>
<td>n/a</td>
<td>2.54 (0.25-8.7)</td>
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Table 4.2  Mean percentage of the main lymphocyte populations of CD3, CD4 and CD8 for control and asthma groups, basal and stimulated.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Lymphocyte label</th>
<th>Control</th>
<th>Asthma</th>
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<tr>
<td></td>
<td>Basal</td>
<td>Stimulated</td>
<td>Basal</td>
</tr>
<tr>
<td>BAL</td>
<td>CD3</td>
<td>66.68</td>
<td>63.6</td>
</tr>
<tr>
<td></td>
<td>CD4</td>
<td>58.1</td>
<td>54.5</td>
</tr>
<tr>
<td></td>
<td>CD8</td>
<td>31.6</td>
<td>30.2</td>
</tr>
<tr>
<td>PBMC</td>
<td>CD3</td>
<td>57.6</td>
<td>56.3</td>
</tr>
<tr>
<td></td>
<td>CD4</td>
<td>62.4</td>
<td>54.9</td>
</tr>
<tr>
<td></td>
<td>CD8</td>
<td>24.3</td>
<td>22.2</td>
</tr>
</tbody>
</table>
Figure 4.3.1 Expression levels of CD4 IL-17A in BAL lymphocytes compared using Wilcoxon paired test A), CD4 IFNγ BAL lymphocytes compared using Wilcoxon paired test B) CD4 IL-4 expression in BAL lymphocytes compared using a paired T test C). CD4 FoxP3 expression in BAL compared using a paired T test D).
Figure 4.3.2  Expression levels of CD4 IL-17 in PBMCs compared using Wilcoxon paired test A). CD4 IFNγ expression compared using Wilcoxon paired test B). CD4 IL-4 expression in the control group compared using paired T test and asthma group compared using Wilcoxon paired test C). CD4 FoxP3 expression in PBMCs compared using paired T test D).
Figure 4.3.3  Expression levels of IFNγ-IL-17A double positive cells in the control group comparing BAL and PBMC using Wilcoxon paired test A) and the asthmatic group B). Comparing BAL expression levels of IFNγ-IL-17 between the controls and asthma group using Wilcoxon paired test C). Comparing PBMC expression of the double positive cells using the same test D).
4.4 Discussion

This study has shown that using LACTM as a T cell stimulus on lymphocytes increases the expression of Th1, Th2, Th17 but not in the Treg cells in healthy controls and asthmatics in the blood and the lung. This particular cohort of asthmatics were neutrophilic. The immune system uses IL-17 to induce neutrophil migration to sites of inflammation (Oppmann et al., 2000) to clear pathogens. However IL-17 has been associated with asthma (Molet et al., 2001, Hayes and Meyer, 2007, Al-Ramli et al., 2009, Doe et al., 2010) so may be a contributor to chronic inflammation and a feature of this phenotype. Upon stimulation with LAC there were no differences in the expression of IL-17 between the controls and asthmatics (figure 4.3.1) suggesting that within the time period of 14 hour T cell stimulation IL-17 expression levels are similar in asthmatics to healthy controls. Many researchers use a smaller time frame between 4 and 6 hours for stimulation (Zhao et al., 2010) but from the optimisation of the flow cytometry detailed in chapter 3 we saw no differences in T cell expression at 4 hours stimulation and 14 hour stimulation, for logistical reasons 14 hour stimulation was used. It is important to remember that most of the previous studies conducted, measured IL-17 in induced sputum and blood whereas these results shown here are from BAL. This study measured the cytokine environment within the lung showing higher expression levels for all the cytokines in the lung compared to the blood suggesting sequestration of highly reactive T lymphocytes to the lung. There was no significant difference between the patient groups which may suggest that there is no direct correlation with neutrophilic asthmatics and increased cytokine release compared to healthy controls, even when they are symptomatic asthmatics. Other studies have looked at PMA/ionomycin stimulated BAL from clinical patients and have shown a wide range of IL-17 expression per individuals ranging from 30% to 0.2% (Miravitlles, 2007). The amount of IL-17 seen in our cohort is similar confirming that IL-17 expression is variable in the lung. Potentially the asthma group may express more IL-17 being in an inflammatory state, although all the asthma patients had ICS treatment s this may account for no differences between groups. It would be advantageous to recruit more severe cases of asthma as severity has been linked to increase in IL-17 (Zhao et al., 2010), potentially linking neutrophilic asthma and IL-17.
If this could be linked, IL-17 would be a good target for a therapeutic treatment especially with steroid resistant patients which neutrophilic patients can be (Alexander and Rietschel, 2001).

CD4 IFN-γ expression in BAL increased dramatically in the controls and asthmatics upon LACTM stimulation (figure 4.3.1b). For both groups the basal levels were virtually 0 increasing to over 59% suggesting a strong Th1 response in both patient groups (Mosmann and Coffman, 1989). Bearing in mind the antibody kit used to label the BAL cells although having been optimised in PBMCs has never been used in BAL before but the individual patient values were consistent assuring the results are real. There doesn’t appear to any bias to either Th17 or Th1 lineage in either groups in the lung.

CD4 IL-4 expression in BAL increased upon stimulation in both groups, although it was not statistically significant in the asthma group (figure 4.3.1c). IL-4 is considered a Th2 lineage cytokine and has been associated with asthma (Manuyakorn et al., 2013, McDonald and Khor, 2013). There was a range of IL-4 expression in the asthma group making the mean slightly higher than the control group but not statistically significant. This may be due to the small sample size so would be prudent to increase this to ascertain if IL-4 is higher in asthmatics compared to the healthy controls. Potentially IL-13 may have been a more relevant cytokine to represent Th2 lineage as this is responsive to PMA stimulation and it also closely associated with asthma (Xie and Wenzel, 2013, Louis et al., 2012, Wood et al., 2012).

CD4 FoxP3 expression in BAL showed no statistically significant difference between basal and stimulated or between healthy controls and asthmatics (figure 4.3.1d). T regulatory cells are a heterogeneous population with varying biomarkers delineating them. Historically CD4+CD25+FoxP3+ cells were considered as T regulatory cells (Lan et al., 2005) but more recently CD4+CD25+CD127- (CD127 of IL-7 receptor is down regulated upon cell activation) cells were considered T regulatory predominantly used in cell sorting as all the markers are surface markers. Within this study I decided to use CD4+FoxP3+ labelling based on previous studies.
showing an increase in asthmatics (Smyth et al., 2010) and all cells FoxP3+ will be suppressing cells.

By their function of maintaining inflammation and self-tolerance their expression was not affected by this ex-vivo stimulation. It would be worth assessing patients with increased disease severity to see whether the chronic increased inflammation would have an effect on the lung T regulatory cells. The same pattern of expression of T cells with LAC™ stimulation was seen in the PBMCs. CD4 IL-17, IFN-γ, IL-4 all significantly increased upon stimulation in both groups (figure 4.3.2a-c) although there were no differences between the control group and asthmatics. Of note the expression levels are lower than in the BAL suggesting that within the lung the cells are more reactive to ensure sufficient protection from their environment.

Analysis of the only double positive cells seen in this study upon stimulation, namely IL-17/IFN-γ cells showed that there were significantly more in BAL than blood in the control group (figure 4.3.3a) and in the asthma group (figure 4.3.3b). There were no statistical differences between the groups in BAL (figure 4.3.3.c) or in PBMCs (figure 4.3.3d). The difference in the BAL could be because of the large increase in IFN-γ positive cells within the BAL so more likely to see double positive cells. Even though Th17 cells are a distinct lineage from Th1 (Harrington et al., 2005) they also use the same p40 subunit when signally through the IL-23 receptor or IL-12 receptor respectively (Oppmann et al., 2000) showing a certain plasticity among these lineages and perhaps with more severe disease the emphasis may favour Th17.

The outcome of this study has shown there are no significant differences in the T cell subsets between these two patient cohorts. However it is worth noting that this is the first study to use these techniques in measuring complete T cell subsets in different cell compartments. For future studies non-neutrophilic asthmatics could be used to better determine any differences directly attributed to the neutrophilia in the lung and no other cause such as atopy.
Chapter 5

Using an LPS inhalation model to mimic exacerbation states in asthma and to determine the role of Th17 and T regulatory cells in the lung and peripheral blood.

5.1 Introduction

The inflammation with asthma is chronic and patients can suffer from episodic exacerbations following bacterial (Papadopoulos et al., 2011) or viral (Johnston and Sears, 2006) infection which can lead to increased morbidity and possible mortality with late stage disease cases (Masoli et al., 2004). Little is known about the immune status during these episodes but there are an increasing number of studies attempting to elucidate the mechanisms using an LPS inhalation model which I have outlined in table 1.5 in the introduction. The LPS inhalation method was used as a model of bacterial driven exacerbation in moderate asthmatics subjects. This study is predominantly interested in the role of T cells in particular Th17 and T regulatory cells during and after LPS exposure in both the lung from BAL and peripheral blood. There have been many studies looking at the innate response to LPS concentrating on granulocytes (Michel et al., 1997, Nightingale et al., 1998) but increasingly there is more interest in the role of T cell mediated immunity (Loh et al., 2006). Th17 cells have been associated with asthma (Wong et al., 2009) (Sun et al., 2005). IL-17 plays a critical role in host defence against intra cellular and extra cellular bacteria by promoting the homing of neutrophils to the site of inflammation (Wu et al., 2007, Lu et al., 2008, Kolls and Linden, 2004). IL-17 is known to be a pro-inflammatory cytokine produced predominantly from CD4+ lymphocytes (Park et al., 2005) although it is becoming apparent that many other cell types release this cytokine (Kondo et al., 2009, Peng et al., 2008). In this study CD4 and CD8 delineated IL-17 cells were determined pre and post LPS inhalation. Increased numbers of IL-17 positive cells have been shown in asthmatics mainly in the sub-epithelium (Al-Ramli et al., 2009) suggesting asthma patients may be primed to have a higher number due to the chronic inflammation seen with this condition. Th17 cells have also been categorised as a potential source to mediate between innate and adaptive immunity (Bullens et al., 2013). Using the LPS inhalation model I wanted to determine if there would be an increase in IL-17 producing lymphocytes following LPS in asthmatics.
Potentially showing an innate form of protection after TLR activation from LPS with a rapid IL-17 response. T regulatory cells have also been associated with asthma although with conflicting results (Provoost et al., 2009, Yuksek et al., 2011, Hamzaoui et al., 2011). T regulatory cells phenotypes are heterogeneous comprising of 3 subpopulations, resting Tregs, suppressive Tregs and cytokine producing Tregs which activate T cells (Vanaudenaerde et al., 2011) varying phenotypes depending on their origin and cytokine milieu which can potentially account for the differing results found. I chose to determine the number of total T regulatory cells using their transcription factor FoxP3 as the main biomarker, thereby ensuring the total Treg population. It has been shown that there is some plasticity between the lineages of Th17 and Treg populations (Koenen et al., 2008) depending on the stimulus so I wanted to see if there would be any interplay between these T cell lineages after LPS exposure potentially mimicking an exacerbation in asthmatics.

5.2 Methods

5.2.1 Patient Selection

7 patients with a physician diagnosis of asthma for at least 6 months were recruited. Subjects were required to have a baseline FEV1 of ≥70% predicted and 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 FEV1 (PC20 ≤16 mg/ml) based on current guidelines (Bateman et al., 2008). Patients were taking either inhaled corticosteroids (ICS) and long acting β agonists (LABA) or both. Patients were excluded from the study if they were taking oral steroids and leukotriene antagonists (relatively new treatment with limited use in exacerbations (Sadatsafavi et al., 2013). All patient demographics are summarised in table 5.3.1

5.2.2 Study Design

A baseline bronchoscopy along with a 20 ml of blood draw was undertaken 24 hours after an initial baseline medical screen, where lung function, sputum induction, a physical assessment and urine pregnancy tests (if applicable) were performed and their medical history documented. 4 weeks later subjects returned for their LPS
challenge. At this point serial vital signs, lung function, exhaled nitric oxide, sputum induction and a urine pregnancy test (if applicable) were performed and their medical history updated and blood drawn 4 hours post challenge. Subjects then returned 24 hours later for a post LPS bronchoscopy and another blood draw. All seven subjects underwent bronchoscopy and blood draw with 4 subjects undergoing additional blood draw 4 hours post challenge.

5.2.2 Bronchoscopy

The asthmatic patients underwent a bronchoscopy to obtain BALF and blood was taken for PBMCs. These subjects were then rested for 4 weeks to return for the second visit and completed the LPS inhalation as described in section 2.1.5. Post inhalation clinical observations were taken hourly for the subsequent 12 hours and for a subset of subjects (n=4) blood was taken 4 hours post LPS. 24 hours post LPS the subjects underwent a further bronchoscopy and bloods were taken. The cells were phenotyped immediately after being isolated as described in section 2.1.4 and 2.1.7 for PBMCs and BALF respectively using multi parametric flow cytometry analysis protocol outlined in section 2.1.8.

5.2.5 FACS analysis

Th17 and Treg lymphocyte subsets were analysed pre and post LPS inhalation challenge in paired BAL and PBMC samples (6 out of the 7 patients produced enough samples for this analysis). The analysis was completed using BD DIVA™ software acquiring 100,000 CD3 positive cells, gating on CD4 or CD8 within this with IL-17A and FoxP3 for the intra cellular cytokines, using isotype equivalents as controls.

The flow analysis showed the percentage of the intra cellular antibody labels of CD4 and CD8 populations respectively within the total CD3 population.

5.2.6 Statistical Analysis

The cellular expression of IL-17 and FoxP3 within CD3/CD4 cells or CD8 cells - pre (0h) and post (24h) LPS challenge were compared by paired T test assuming normal Guassian distribution unless otherwise stated. The time course was analysed by one
way ANOVA with Bonferroni correction (assuming parametric distribution) $P \leq 0.05$ was considered significant. Statistical analysis was conducted using GraphPad Instat version 5 (GraphPad Software, Inc, San Diego, CA, USA).

5.3 Results

5.3.1 Patient Demographics

The mean FEV1% predicted was 92% which is fairly high but there was a wide range (SD=22.4) from these patients and this is not the only predictor of asthma. The mean FEV1/FVC was 70.5% showing airflow obstruction. The mean reversibility was 7.16% with a wide range. Not all patients will be reversible and may need to be repeated (GINA), but these patients already had a physician’s diagnosis of asthma.

5.3.2 Lymphocyte expression in the lung

Total cell numbers in the BAL increased 24h post LPS challenge from 0.09 to 0.2 ($x10^6$) ($p=0.06$, figure 5.3.2.1) however this numerical difference was not, statistically significant ($p=0.06$). This figure is not statistically significant possibly due to one of the patients not responding to LPS and their cell numbers decreased post LPS. Cellular increase post LPS is well documented (O’Grady et al., 2001) so this is unexpected. The total number of alveolar macrophages increased significantly from 0.04 to 0.1 ($x10^6$) ($p=0.02$, figure 5.3.2.2), however there was no significant increase in BAL lymphocyte numbers ($p=0.34$, figure 5.3.2.3) post LPS inhalation. There was no significant change in the expression levels of CD4 IL-17 and FoxP3 24 hours post LPS inhalation in the BAL cells (figure 5.3.2.4A) or CD8 with equivalent IL-17 and FoxP3 expression (figure 5.3.2.4B).

5.3.3 Systemic expression of lymphocytes

There was no significant change in the total lymphocyte numbers in the blood post LPS inhalation ($p=0.34$ figure 5.3.2.3). The percentage expression of blood CD4 IL-17 at basal level (0.75%) did not significantly change 24 hours post LPS (1.03%) $p=0.28$ figure 5.3.2.5A. This was also true for the expression of CD4 FoxP3. 5.7% expressed basally and remaining there after LPS ($p=0.14$ figure 5.3.2.5A). CD8 IL-17 showed a decrease after LPS treatment from 3.5% to 1.37% ($p=0.057$ figure
5.3.2.5B) although not significant this may be due to small sample size. CD8 FoxP3 showed a significant decrease after LPS treatment starting at 13.3% to 3.7% (p=0.04 figure 5.3.2.B).

There was a significant increase of CD4 IL-17A from 0.89% to 3.6% (p≤0.01) 4 hour post LPS inhalation. These expression levels decreased 24 hours post LPS down to 0.81% (p≤0.01) showing an acute CD4 IL-17 response to LPS. There was an increase in CD8 L-17 4 hours post LPS from basally 3.3% to 5.4% 4 hours post, dropping to 1.06% 24 hours post LPS but this was not significant (p=0.1) which may be due to the small sample size as the CD8 IL-17 seems to mirror the CD4 IL-17 (figure 5.3.2.5A).

CD4 FoxP3 showed no significant changes over time post LPS. The mean expression levels were 8.02% to 9.1% to 6.3% at 0 hour, 4 hour and 24 hour post LPS respectively (p=0.67 figure 5.3.2.5A). CD8 FoxP3 showed no significant difference over time (p=0.43 figure 5.3.2.5B). The main CD3, CD4 and CD8 populations showed no significant changes post LPS showing no fundamental changes in the antigen recognition capabilities of the cells in either the BAL or blood. These data are summarised on table 5.3.2.
Table 5.3.1 Asthma patient demographics pre LPS challenge showing lung function. All data is shown as mean (SD). * denotes geometric mean with 95% confidence limits.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Asthmatics n=7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex (F/M)</td>
<td>4/3</td>
</tr>
<tr>
<td>Age (Yrs)</td>
<td>41.3 (11.3)</td>
</tr>
<tr>
<td>FEV1 (L)</td>
<td>2.9 (0.9)</td>
</tr>
<tr>
<td>FEV1 % Predicted</td>
<td>92 (22.4)</td>
</tr>
<tr>
<td>FEV1/FVC (%)</td>
<td>70.5 (6.9)</td>
</tr>
<tr>
<td>ICS users</td>
<td>7</td>
</tr>
<tr>
<td>Reversibility %</td>
<td>7.16 (0.86-26.5)*</td>
</tr>
<tr>
<td>ACQ Scores</td>
<td>1.43 (1.02)</td>
</tr>
</tbody>
</table>
Figure 5.3.2.1 Comparing total BAL cell number/ml pre and post LPS inhalation.

![Graph comparing total BAL cell number/ml pre and post LPS inhalation.](image)

Figure 5.3.2.2 Comparing alveolar macrophage cell number/ml pre and post LPS inhalation.

![Graph comparing alveolar macrophage cell number/ml pre and post LPS inhalation.](image)
Figure 5.3.2.3 Comparing lymphocyte cell numbers in the BAL/ml pre and post LPS inhalation

![Graph showing comparison of lymphocyte cell numbers in BAL/ml pre and post LPS inhalation. The x-axis represents hours post LPS, the y-axis represents lymphocyte cell number x 10^6/ml BAL. The graph shows a decrease in lymphocyte cells from 0 to 24 hours post LPS inhalation with a p-value of 0.34.]
Figure 5.3.2.4 Gating strategy and example of the analysis of data. CD3 gating A) CD4 and CD8 gating B) CD4, CD8-IL-17 and FoxP3 gating pre LPS C) CD4, CD8-IL-17 and FoxP3 gating 4 hours post LPS D) and CD4, CD8-IL-17 and FoxP3 gating 24 post LPS E).
Figure 5.3.2.5 Showing CD4 IL-17 and FoxP3 positive lymphocytes in the BAL pre (0) and post (24) LPS inhalation (A) BAL CD8 IL-17 and FoxP3 positive lymphocyte pre and post LPS inhalation (B).

A

B
Figure 5.3.2.6 Showing CD4 IL-17 and FoxP3 positive lymphocytes in PBMCs pre (0) and post (24) LPS inhalation (A). CD8 IL-17 and FoxP3 positive cells from the same conditions (B).
Figure 5.3.2.7 Showing the subset of 4 patients out of the total of 6 patients PBMCs drawn at 0, 4 and 24 hours post LPS the percentage expression of CD4 IL-17 and FoxP3 (A) and CD8 IL-17 and FoxP3 (B).
Table 5.3.2 Mean expression levels shown as percentage of gated lymphocytes, of CD3, CD4 and CD8 in BAL and PBMCs over time post LPS inhalation.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Lymphocyte label</th>
<th>0 hour (n=6)</th>
<th>4 hour (n=4)</th>
<th>24 hour (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAL</td>
<td>CD3</td>
<td>14.25</td>
<td></td>
<td>17.5</td>
</tr>
<tr>
<td></td>
<td>CD4</td>
<td>68.41</td>
<td></td>
<td>76.63</td>
</tr>
<tr>
<td></td>
<td>CD8</td>
<td>23.98</td>
<td></td>
<td>23.76</td>
</tr>
<tr>
<td>PBMC</td>
<td>CD3</td>
<td>38.30</td>
<td>37.22</td>
<td>32.72</td>
</tr>
<tr>
<td></td>
<td>CD4</td>
<td>61.26</td>
<td>71.79</td>
<td>65.29</td>
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<tr>
<td></td>
<td>CD8</td>
<td>30.65</td>
<td>28.11</td>
<td>33.46</td>
</tr>
</tbody>
</table>
5.4 Discussion

The asthma patients in this study showed an increase in total cell count in the lung after the inhaled exposure of LPS suggesting a local inflammatory response. This was clarified further by the significant increase in alveolar macrophages. LPS being the main component of gram negative bacterium would elicit an innate immune response targeting TLR4 and CD14 signal transduction pathway on granulocytes and monocytes and their mobilisation to the site of inflammation. This proved the LPS was reaching the lower airways and the site of interest. There was no significant change in BAL lymphocyte numbers. This may be because the time point of 24 hours post LPS exposure was not optimum for lymphocytes as they may react quicker than this (Sandstrom et al., 1992).

This may account for the percentage of CD4 IL-17 BAL cells not significantly changing post LPS. Although on an individual basis 2 out of the 6 patients that were measured showed an increase, warranting the need for further patients to increase the sample size. However mean values suggests that a trend exists between CD4 IL-17 positive cells and lymphocyte numbers post LPS suggesting an increase may be related to more lymphocyte infiltration and not increase expression on lymphocytes. The percentage of CD4 FoxP3 cells showed no change post LPS however this may be due to this cohort already having elevated number of Tregs (Smyth et al., 2010) or again an optimum time after LPS may have seen differences. Tregs are a heterogeneous cell type having different biomarkers for different type of cells. For this study CD4+ FoxP3+ cells were determined so this would include all phenotypes of T regulatory cells, so perhaps differentiating between the native and inducible and resting Tregs may have shown different reactions to LPS in the lung.

The percentage of CD8 IL-17 cells showed no differences after LPS exposure but there was a significant decrease in CD8 FoxP3 positive cells. Tregs have traditionally been seen as a predominantly CD4 cell but CD8 cells can also have the suppressive function (Cosmi et al., 2003). LPS exposure seems to be diminishing the CD8 Treg cells this may suggest that the cytotoxic Treg cells are impaired after LPS because the CD4 IL-17 and Treg cells are the dominant cells cleared the and attracted the phagocytes.
In the peripheral blood there were no significant differences in the CD4 IL-17 and FoxP3 expressing populations. This could be attributed to the 24 hour time point or the LPS inhaled did not have a systemic affect. Again the CD8 IL-17 and FoxP3 expressing populations showed a significant decrease post LPS suggesting the cytotoxic Tc17 and regulatory cells were not being utilised after the LPS exposure. With the inclusion of 4 hours post LPS blood draw CD4 IL-17 increased significantly and then returned to similar expression levels at the start. This showed a rapid lymphocyte Th17 response to LPS, suggesting innate immunity. This was not seen for any other cytokine measured. This suggested that Treg cells are not affected at any time point after the exposure of LPS, perhaps because of their elevated levels from these patients being asthmatics and taking ICS (Karagiannidis et al., 2004). There were no differences in expression of the main lymphocyte populations suggesting LPS did not have a prolonged systemic affect.

This study has shown that intra cellular biomarkers IL-17 and FoxP3 can be routinely measured in patient’s BAL and blood. However the time point denoted to this study was not optimum to determine the lymphocyte reaction to inhaled LPS this was apparent with the rapid increase of IL-17 in the blood after 4 hours with resolution at 24 hours. Potentially the procedure of a bronchoscopy to establish baseline measurements may have an impact on the T cells initiating a pro-inflammatory response. This may be masking the effects of LPS in these T cell population and perhaps leaving a longer time period between the two bronchoscopies may elucidate more T cell function.

There were only LPS inhalation asthmatics within this study with no saline inhalation controls. Judging from the many LPS inhalation studies performed by other groups (table 1) saline did not show any significant effects, therefore the temporal differences of T cell cytokines within the LPS model was deemed to not need controls.
Chapter 6

Using an LPS inhalation model to mimic exacerbation states in mild COPD and to determine the role of Th17 and T regulatory cells in peripheral blood.

6.1 Introduction

COPD is a progressive obstructive respiratory disease with declining lung function during exacerbations from bacterial or viral infections. These exacerbations manifest in decreased lung function with the number of exacerbations and FEV1 values negatively correlated. (Burge and Wedzicha, 2003). Patients experience breathlessness, persistent cough and over production of mucus. Patients are normally prescribed either oral or inhaled glucocorticoid steroids plus antibiotics and may require hospitalisation at the later stages of the disease. There is an increase in the pro-inflammatory cytokines IL-6 and IL-8 in sputum from frequent exacerbaters compared to infrequent (Burge and Wedzicha, 2003) suggesting a prolonged inflammatory response. There is evidence of sustained bacterial colonisation in COPD patients lungs possibly due to dysfunction of the alveolar macrophages as the first line of defence to infection and this could lead to an exacerbation. Neutrophils are also an important cell in the lungs defence system. In a well-functioning environment the neutrophils will clear extra cellular pathogens by phagocytosis. Neutrophils have a reactive time span of a few days and are cleared by the macrophages which in turn clears the inflammation and pathogen. In the COPD lung it has been shown that CXCL-8 is upregulated (Schulz et al., 2003). There is evidence that IL-17 released from activated T cells within the lungs stimulate stromal cells (epithelial) and other reactive cells to release more IL-8 which is a chemoattractant of neutrophils (Fossiez et al., 1996). With the increase in neutrophils within the lung this can prolong the inflammatory environment and can cause cellular damage and death (Strydom and Rankin, 2013). Potentially this could see opportunistic pathogens such as Haemophilus or Respiratory syncytial virus (RSV) to cause further infection resulting in an exacerbation. Little is known about the immune status during exacerbation episodes but there is an increasing number of studies attempting to elucidate the mechanisms using an LPS inhalation model which I have outlined in table 1 in the introduction.
In this study the LPS inhalation method was used as a model of bacterial driven exacerbation in mild COPD subjects. This study is interested in the expression of Th17 and Treg cells during and after LPS exposure in peripheral blood. Using IL-17 and FoxP3 to measure Th17 and Treg cells respectively. Th17 cells have been associated with COPD (Chang et al., 2011, Doe et al., 2010). The main cytokine, IL-17 is known to be a pro-inflammatory cytokine enabling clearance of extra cellular pathogens and neutrophil attractant (Fujie et al., 2012). However if there is dysregulation in the production of IL-17 chronic inflammation can occur (Tan and Rosenthal, 2013). Exacerbating patients may produce more IL-17 because of the inflammatory environment and opportunistic subsequent infections seen in the COPD patient cohort. Treg cells have a suppressive effect on proliferating CD4 cells in order to control the pro-inflammatory system (Cederbom et al., 2000). Treg cells can be induced by TGF-β as an anti-inflammatory cytokine, however in the presence of IL-6 (pro-inflammatory cytokine) these cells can be induced to Th17 cells (Bettelli et al., 2006). This study looked at the expression levels of FoxP3 in Treg cells and their relationship with IL-17 cells.

6.2 Methods

6.2.1 Patient Selection

Recruited 12 patients with a physician diagnosis of mild COPD based on the GOLD guidelines with FEV1 values of less than 80% predicted and FEV1/FVC of less than 70%. Patients were excluded if they had any other significant respiratory conditions or had experienced an exacerbation 6 weeks prior to the start of the study.

6.2.4 Study Design

After the patients were screened for suitability of the study 20ml of blood was drawn before LPS inhalation as described in section 2.1.5. 4 hours and a subset of 4 patients at 24 hours post inhalation a further 20ml of blood was drawn. PBMC cells were isolated and counted as described in section 2.1.4. 1 x 10^6cells/tube were labelled immediately for multi parametric flow cytometry analysis as described in section 2.1.8. Eleven patients out of the twelve recruited produced enough cells for the flow cytometry analysis.
6.2.5 FACS analysis

Th17 and Treg phenotypes in PBMCs were analysed pre and 4 hours post LPS inhalation using flow cytometry method detailed in 2.1.8. Acquired 100,000 CD3 positive cells and gated on CD4 and CD8 with IL-17 and FoxP3 for both CD4 and 8, using isotype equivalents as controls. Gating strategy is outlined in section 5.3.2.4. The flow analysis showed the percentage expression of IL-17 and FoxP3 from CD4 and CD8 populations within the CD3 population and analysed using DIVA™ software.

6.2.6 Statistical Analysis

Th17 and Treg phenotype expression at 0 hour and 4 hour were compared using a matched paired T test unless otherwise stated. P≤0.05 was considered significant. The same phenotypes in the timecourse were compared using the non-parametric Kruskal Wallis test, where P≤0.05 was deemed significant. Statistical analysis of the data was conducted using GraphPadInstat version 5 (GraphPad Software, Inc, San Diego, CA, USA).

6.3 Results

6.3.1 Patient Demographies

The mean FEV1 % predicted was 85.3% equating to mild COPD with reference to the G.O.L.D. standards. The mean FEV/FVC was 60.4% confirming the persistent airflow obstruction confirming the COPD diagnosis. There was a large range of pack years in the smoking history and 8 out of 12 patients took inhaled corticosteroids.

6.3.2 Systemic expression of lymphocytes

In COPD patients CD4 IL-17 expression increased significantly from basal expression of 0.32% to 4.3% 4 hours post LPS (p=0.02 figure 6.3.1.1). This was mirrored with CD8 IL-17 showing a significant increase 4 hours post LPS ranging from 0.99% to 4.06% (P=0.04 figure 6.3.1.2). CD4 FoxP3 expression increased after LPS inhalation from 7.7% basally to 10.25% (P=0.01 figure 6.3.1.3). CD8 FoxP3 expression increased from 2.66% basally to 5.7% 4 hours post LPS (P=0.02 figure 6.3.1.4). The
subset of 5 patients within the cohort with 24 post LPS data showed no significant difference when compared using Kruskal-Wallis test (figure 6.3.1.5). It transpires that this subset included predominantly low responding patients 4 hours post LPS reiterating the need for a larger sample size. This was the case for CD8 IL-17 (figure 6.3.1.6), CD4 FoxP3 (Figure 6.3.1.7) and CD8 FoxP3 (Figure 6.3.1.8). The main CD3, CD4 and CD8 lymphocyte populations did not change expression post LPS the means are summarised in table 6.3.2.
Table 6.3.1  COPD patient demographics showing lung function. All data is shown as mean (SD). * denotes mean with range.

<table>
<thead>
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<th>Characteristic</th>
<th>COPD n=12</th>
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<tr>
<td>Sex (F/M)</td>
<td>2/10</td>
</tr>
<tr>
<td>Age (Yr)</td>
<td>62.2 (4.6)</td>
</tr>
<tr>
<td>FEV1 (L)</td>
<td>2.6 (0.7)</td>
</tr>
<tr>
<td>FEV1 predicted (%)</td>
<td>85.3 (9.3)</td>
</tr>
<tr>
<td>FEV/FVC (%)</td>
<td>60.4 (6.3)</td>
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<tr>
<td>Smoking history (Pack years)</td>
<td>38.75 (11-122)*</td>
</tr>
<tr>
<td>ICS users</td>
<td>8</td>
</tr>
</tbody>
</table>
Figure 6.3.1.1 CD4 IL-17 positive lymphocytes in PBMCs pre and 4 hours post LPS inhalation.

Figure 6.3.1.2 CD8 IL-17 positive lymphocytes in PBMCs pre and 4 hours post LPS inhalation. Comparison was performed using a paired T test with parametric distribution.
Figure 6.3.1.3 CD4 FoxP3 positive lymphocytes in PBMCs pre and 4 hours post LPS inhalation. Comparison was performed using a paired T test with parametric distribution.

Figure 6.3.1.4 CD8 FoxP3 positive lymphocytes in PBMCs pre and 4 hours post LPS inhalation. Comparison was performed using a paired T test with parametric distribution.
Figure 6.3.1.5 CD4 IL-17 expression 0, 4 and 24 hour post LPS inhalation using a subset of 5 patients from the cohort. Comparisons were analysed using Kruskal-Wallis test.

P=0.93

Figure 6.3.1.6 CD8 IL-17 expression 0, 4 and 24 hour post LPS inhalation using a subset of 5 patients from the cohort. Comparisons were analysed using Kruskal-Wallis test.

P=0.79
Figure 6.3.1.7 CD4 FoxP3 expression 0, 4 and 24 hour post LPS inhalation using a subset of 5 patients from the cohort.

Figure 6.3.1.8 CD8 FoxP3 expression 0, 4 and 24 hour post LPS inhalation using a subset of 5 patients from the cohort.
Table 6.3.2 Mean expression shown as percentages of gated PBMC lymphocytes, CD3, CD4 and CD8 pre and post LPS inhalation.

Table 9.3.2

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Lymphocyte label</th>
<th>0 hour</th>
<th>4 hour</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBMC</td>
<td>CD3</td>
<td>42.1</td>
<td>43.6</td>
</tr>
<tr>
<td></td>
<td>CD4</td>
<td>70.3</td>
<td>71.4</td>
</tr>
<tr>
<td></td>
<td>CD8</td>
<td>29.5</td>
<td>28.5</td>
</tr>
</tbody>
</table>
6.4 Discussion

The COPD patients in this study showed a significant increase in expression of Th17 (CD4 IL-17) cells 4 hours post LPS inhalation in peripheral blood (figure 6.3.1.1) showing a systemic T cell mediated response. COPD patients are known to have increased neutrophils and macrophages in BAL (Larsson, 2008). The Th17 phenotype became an interest when it was shown that cells releasing IL-17 was associated with the recruitment of neutrophils and macrophages (Curtis et al., 2007). With the exposure to LPS in the lung this will stimulate the resident monocytes and macrophages via the TLR4 and CD14 receptor (Peri et al., 2010). This results in the translation of NFkB driving the release of pro-inflammatory cytokines, including IL-1β and TNFα promoting the migration of inflammatory cells such as neutrophils (Michel et al., 1997, Barnes, 2004). The temporal expression of IL-17 suggests an innate response to the LPS stimulus. Producing a rapid response from the lymphocytes suggests an antibody independent T cell mediated response. IL-17 in COPD patients has not been measured before in LPS inhalation studies and the increase correlates well with previous studies showing neutrophilia after 6 hours in smokers (Aul et al., 2012a).

CD8 IL-17 expression also significantly increased (figure 6.3.1.2). These cells are termed as Tc17 cells having CD8 (recognising MHC class 1) and IL-17 and behave as cytotoxic T cells. It has been shown in rheumatoid arthritis (RA) that Tc17 expression is raised in active disease and a greater ability of cells to produce IL-17 (Henriques et al., 2013). RA is an inflammatory autoimmune disease and COPD has been considered as similar this may explain the huge increase in IL-17 production (much more than seen with TCR stimulation-from chapter 4). Perhaps COPD patient have T cells primed for the rapid release potentially showing a dyregulation of systemic IL-17 in this disease.

CD4 T regulatory cells (T regs) suppress T cell proliferation and were first classified as the main T cell to maintain self-tolerance (Sakaguchi et al., 2006). In this study there was a significant increase in expression of CD4 FoxP3 cells 4 hours post LPS inhalation. There has been a relationship between Th17 and T reg cells depending on the cytokine milieu and levels of TGF-β T regs can become Th17 cells (Bi et al.,
2011). Also in COPD patients it has been shown that even with increased Tregs inhibit Th17 proliferation (to control the increased IL-17 being released) they do not decrease their ability to release IL-17 (Pridgeon et al., 2011). This study confirms this showing an increase in both IL-17 and FoxP3 and that this could be exaggerated during exacerbation (although cell proliferation was not measured in this study).

CD8 FoxP3 positive cells increased 4 hours post LPS (Figure 6.3.1.8). Little is known about this cell type but functionally these cells may suppress the cytotoxic T cell proliferation which may be important in COPD because of the increase in CD8 expression in this disease (Saetta et al., 1999). Interestingly it has been shown that CD8+ cells from COPD patients have increased expression of TLR4 (Freeman et al., 2013). This may account for the increased suppressing CD8 cells after LPS stimulation needing to control the pro-inflammatory response as these patients would already have chronic inflammation.

For all the different cell phenotypes in this study when looked at over time in a subset of the final cohort showed no significant change in expression (figures 6.3.1.5-6.3.1.8). This is an example of the need for a sufficient cohort size in which to draw conclusions due to patient variation. The 5 patients used to measure Th17 and Treg cells at 0, 4 and 24 hours post LPS did not respond significantly. However when analysed in total there were changes.

Table 6.3.2 shows the mean expression percentages of CD3, CD4 and CD8. There are no changes in expression pre and post LPS suggesting antigen recognition patterns are not changed after LPS exposure.

This study has provided evidence that IL-17 and FoxP3 positive cells are reactive to LPS in COPD patients. Within 4 hours post LPS exposure they both increased significantly within the blood suggesting an innate rapid response to potential bacterial infection within the lung. However COPD patients are already in a pro-inflammatory state and further assault from pathogens enables an exaggerated and chronic inflammatory state. Potentially if the IL-17 pro-inflammatory stimulus was blocked or modulated this may be an ideal candidate for therapeutic intervention for frequent exacerbating patients.
Chapter 7

7.1 Conclusions, limitations and further work

This thesis set out to determine whether the phenotype of neutrophilic asthmatics showed a different T cell subset favouring Th17 because of its main cytokine, IL-17 and its ability to attract neutrophils (Ye et al., 2001). However from this study looking at 6 individuals compared to controls there were no significant differences. This was a pilot study and calculating the power of the study post completion performed on all cytokine and compartment comparisons using Graphpad statmate (GraphPad Software, Inc, San Diego, CA, USA) using the SD and delta between groups, this yielded a 30% confidence in seeing a difference between groups. Using the same software to see 95% confidence limits in a difference it would be advised to sample 30 in each group. Clearly from the limited data from this clinical study no differences were seen between asthmatics and controls and therefore no definitive conclusions can be drawn but the multi parametric analysis can be applied to any clinical study to add invaluable intra cellular information on individual cells.

This thesis set out to determine the effect of LPS inhalation in T cell subsets of Th17 and Tregs within the lung and circulating blood. To test the hypothesis that IL-17 could be a driver of the pro-inflammatory environment in the presence of bacteria but may be prolonged in asthmatics causing damage to the lung. However within this clinical study, using 6 asthmatics exposed to inhaled LPS there were no differences in the T cell subsets pre and post LPS exposure. Calculating the power of the study using the SD and delta between groups yielded a 20% confidence and calculating the predicted number of samples needed to see a difference was 90, clearly showing this was an underpowered study and therefore would have to reject the hypothesis.

This same hypothesis was assessed within mild COPD patients. After 4 hours there was a significant increase in IL-17 and FoxP3 in peripheral blood therefore the hypothesis that IL-17 and Treg cells are increased after LPS exposure can be accepted. The main limitations of this study are that BAL cells were not collected to be compared as in the asthma study, so now showing that systematically these T cells are increased may warrant the need to look within the lung itself in the future.
Other pro inflammatory conditions such as psoiasris and Crohns (Baeten and Kuchroo, 2013) are seeing success with IL-17 inhibitors and with the evidence of IL-17 increase systemically after LPS, an IL-17 inhibitor could be developed to alleviate the symptoms and underlying inflammation of exacerbations in COPD patients.


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