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The Role Of T Cell Subsets In The Airways In Asthma

by

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ABSTRACT

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T-cells are key orchestrators of airways inflammation, but the relative roles of different human T-cell subsets remain unclear. The aim of my PhD was to carry out a detailed investigation of T cell phenotypes in asthma in relation to severity and virus-induced exacerbations, with particular focus on interleukin-17 and TH17 cells, and the recently described mucosal associated invariant T (MAIT) cells, to improve characterisation of severe asthma versus milder forms of asthma.

A role for interleukin-17 secreting TH17 cells in asthma has been suggested by several groups. I used clinical and physiologic phenotyping to compare T-cell subsets in health and a spectrum of different asthma severities. Samples obtained via sputum induction, phlebotomy, and bronchoscopy were phenotyped using 9-colour flow-cytometry/sorting, RT-qPCR and multiplex ELISA. The results of my thesis confirm the pre-eminence of TH2 cells in asthma and provide further evidence of a deficiency of bronchoalveolar Treg in severe asthma, as well as new evidence of a role for CD8+ Tc2 cells in eosinophilic disease. Conversely, the data do not indicate a significant role for TH17 or γδ-17 cells in asthma.

Mucosal immunity is intrinsically linked to the associated commensal or pathogenic microbes. In an exploratory study of these interactions I employed deep-sequencing to characterise the whole microbial and viral metagenome of the airways in asthma and health.

MAIT cells are novel innate-like T-cells which express an invariant TCRα chain and recognise the highly-conserved restriction molecule MR1. I observed a selective deficiency of MAIT cells in asthma, which was not related to age, but exacerbated by systemic corticosteroids and subject to seasonal variation, indicating their possible regulation by vitamin D. I established MAIT cell-lines and observed heterogeneity of cytokine expression profiles. These findings open exciting new avenues for research in this emerging area of T cell biology.

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Staples, K. J., T. S. Hinks, et al. (2012). "Phenotypic characterization of lung macrophages in asthmatic patients: Overexpression of CCL17." J Allergy Clin Immunol 130(6): 1404-1412 e1407.

DECLARATION OF AUTHORSHIP

I, Dr Timothy Stopford Christopher Hinks

declare that the thesis entitled

'The Role Of T Cell Subsets In The Airways In Asthma'

and the work presented in the thesis are both my own, and have been generated by me as the result of my own original research. I confirm that:

- this work was done wholly or mainly while in candidature for a research degree at this University;
- where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;
- where I have consulted the published work of others, this is always clearly attributed;
- where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;
- I have acknowledged all main sources of help;
- where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;
- none of this work has been published before submission

Signed: ……..

Date:…….

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Dr Justus Kenneth Landquist, Dr F Christopher Maddox and Dr Robert J Davies

soli Deo gloria

Definitions and abbreviations

ACQ	Asthma control questionnaire
AHR	Airway hyper-responsiveness
AIM V®	Adoptive Immunotherapy Media V®
APC	Allophycocyanin
APS	Airway provocation system
ATP	Adenosine triphosphate
ATS	American Thoracic Society
β2M	β-2microglobulin
BAL	Broncho-alveolar lavage
BCG	Bacillus Calmette-Guérin
BDP	Beclomethasone dipropionate
BHR	Bronchial hyper-responsiveness
BLAST	Basic Local Alignment Search Tool
β-ME	2-mercaptoethanol (β-mercaptoethanol)
BSA	Bovine serum albumin
BTS	British Thoracic Society
CD	Complementarity determinant
COPD	Chronic obstructive pulmonary disease
cDNA	Complementary DNA
CF	Cystic fibrosis
CRTH2	Chemoattractant receptor-homologous molecule expressed on Th2 cells
CCL	Chemokine (C-C motif) ligand
CXCL	Chemokine (C-X-C motif) ligand
Cy	Cyanine
DC	Dendritic cell
ddH_20	Double distilled water treated with DEPC
DEPC	Diethylpyrocarbonate
$1,25(OH)_2D_3$	1,25-dihydroxy vitamin D(3)
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxyribonucleotide triphosphate
DTE	Dithioerythritol
DTT	Dithiothreitol
EAE	Experimental autoimmune encephalomyelitis

EDTA	Ethylenediamine tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
eNO	Exhaled nitric oxide
ERS	European Respiratory Society
FcγR	Fragment crystallisable gamma receptor
FCS	Foetal calf serum
FE_{NO}	Fractional exhaled nitric oxide (see eNO)
FER	Forced expiratory ratio (FEV1/FVC)
FEV_1	Forced expiratory volume in 1 second
FISH	Fluorescent *in-situ* hybridisation
FITC	Fluorescein isothiocyanate
FOXP3	Forkhead box P3
FSC	Forward scatter
FVC	Forced vital capacity
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GI	Gastrointestinal
GINA	Global Initiative for Asthma
GRO-α	GRO1 oncogene-α (CXCL1)
HBSS	Hank's balanced salt solution
HEPES	N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid]
HRCT	High resolution computed tomography
HRP	Horse radish peroxidase
HSA	Human serum albumin
ICAM	Intercellular adhesion molecule
IFN	Interferon
IFNAR	Interferon-α/β receptor
Ig	Immunoglobulin
IL	Interleukin
IP10	IFN-γ-inducible protein 10 (CXCL10)
iNKT	Invariant natural killer T cell
ITAC	Interferon-inducible T-cell alpha chemoattractant (CXCL11, IP9)
IU	International units
MACS	Magnetic-activated cell sorting
MADscore	Median absolute deviation score
MAIT	Mucosal associated invariant T cell
MHC	Major histocompatibility complex
mL	Millilitres

µL	Microlitres
mM	Millimolar
µM	Micromolar
mRNA	Messenger ribonucleic acid
MS	Multiple sclerosis
MTB	*Mycobacterium tuberculosis*
MxA	Myxoma resistance gene A
NIBSC	National Institute for Biological Standards and Control
OTU	Operational taxonomic unit
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCA	Principal component analysis
PCR	Polymerase chain reaction
PC_{20}	Provocative concentration of methacholine causing a 20% drop in FEV_1 ($PC_{20}FEV_1$).
PD_{20}	Provocative dose of methacholine causing a 20% drop in FEV_1
PE	R-phycoerythrin
PEFR	Peak expiratory flow rate
PerCP	Peridinin chlorophyll-protein
pH	Negative log of hydrogen ion concentration
PHA	Phytohaemagglutinin
PMA	Phorbol 12-myristate 13-acetate
qPCR	Quantitative polymerase chain reaction
RANTES	Regulated and normal T cell expressed and secreted (CCL5)
RMA	Robust multi-array average
RNA	Ribonucleic acid
RNase	Ribonculease
ROR	Retinoic acid-related orphan nuclear hormone receptor
RPM	Revolutions per minute
RPMI	Roswell Park Memorial Institute medium
RSV	Respiratory syncytial virus
RT	Room temperature or reverse transcription
RT-PCR	Reverse transcription-polymerase chain reaction
RV	Rhinovirus
SCFA	Short chain fatty acid
SSC	Side scatter
Src	Sarcoma

Taq	*Thermus aquaticus*
TBE	Tris/Borate/EDTA
Tc	Cytotoxic T cell (CD8+ T lymphocyte)
$TCID_{50}$	Tissue culture infective dose 50
TCR	T cell receptor
TDI	Toluene diisocyanate
TGFβ	Transforming growth factor β
T_H	T helper cell ($CD4^+$ T lymphocyte)
TLCO	Transfer Factor of Lung Carbon monoxide
TLR	Toll-like receptor
TMB	Tetramethyl-benzidine
TNF	Tumour necrosis factor
T_{REG}	Regulatory T cell
T-RFLP	Terminal restriction fragment length polymorphism
Tris	Tris(hydroxymethyl) aminoethane
TTMV	Torque Teno Mini Virus
Tween 20	Polyoxythylenesorbitan monolaurate
UBC	Ubiquitin C
WHO	World Health Organisation
XIAP	X-linked inhibitor of apoptosis
YWHAZ	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide

CHAPTER 1

Introduction

Magna opera Domini esquisira in ornnes coluntares ejrts [1]

[1] Inscription carved into the great oak doors of the Cavendish Laboratory, Free School Lane in Cambridge at the request of Prof James Clerk Maxwell FRS FRSE (1831-1879). Known as the research scientist's text, this was replicated in 1973 over the entrance to the New Cavendish Laboratories. It may be translated 'Great are the works of the Lord; they are pondered by all who delight in them.' [Psalm 111:2, NIV]

The aim of my thesis is to report on a detailed study of T cell phenotypes in asthma in relation to asthma severity and virus-induced asthma exacerbations. My work focuses particularly on two novel T cell subsets: the T helper 17 cell (TH17) and the mucosal associated invariant T (MAIT) cell. Therefore my introduction will provide a brief general review of the nature of asthma and the role of various inflammatory cell types in its pathogenesis, before discussing in much more detail what is known about TH17 cells and the related but functionally antagonistic regulatory T (Treg) cell subset. I will then provide a review of the emerging literature regarding MAIT cells. The activation of these innate and adaptive responses within a mucosal immune system may be related intrinsically to the associated microbial flora which I have therefore also attempted to characterise, and so I will review current knowledge of the nature of the airway microflora. I will conclude this introduction by outlining the specific hypothesis I have undertaken to test.

Asthma: an overview

Definitions of asthma

The Global Initiative for Asthma (GINA) defines asthma as 'a chronic inflammatory disorder of the airways in which many cells and cellular elements play a role. The chronic inflammation is associated with airway hyper-responsiveness that leads to recurrent episodes of wheezing, breathlessness, chest tightness, and coughing, particularly at night or in the early morning. These episodes are usually associated with widespread, but variable, airflow obstruction within the lung that is often reversible either spontaneously or with treatment' ((GINA) 2010).

Asthma a global epidemic

Asthma affects 5 million people in the UK (Holgate 2004) and 150-300 million worldwide and the prevalence is increasing (Cookson 1999; Adcock, Caramori et al. 2008; Anderson 2008). Asthma was uncommon at the start of the 20th century, but in developed countries prevalence particularly of atopic asthma (Upton, McConnachie et al. 2000) has risen dramatically, roughly doubling over the last 20-30 years (Fleming and Crombie 1987; Aberg, Hesselmar et al. 1995) and becoming a true global epidemic (Cookson 1999). Whilst some of the increase may be spurious and due to increased diagnosis (Rona, Chinn et al. 1995), the scale of the epidemiological changes and observations such as tenfold regional differences in prevalence imply some major change or changes in environmental factors (Cookson 1999). Several epidemiological observations are well recognised: asthma and allergic diseases are less common in non-westernized environments, in rural environments, amongst children of livestock farmers, amongst younger siblings, and in households with dogs as pets (Strachan 1989; Cookson 1999). A wide variety of potentially causative environmental factors have been proposed, including early life exposure to house dust mite (Sporik, Holgate et al. 1990), or to infections – 'the hygiene hypothesis' (Strachan 1989; Lewis, Butland et al. 1996; Strachan 2000) - to changes in gastrointestinal microbiome (von Mutius, Fritzsch et al. 1992),

breastfeeding practices (Wills-Karp, Brandt et al. 2004), exposure to paracetamol (Beasley, Clayton et al. 2008), chlorine (Bernard, Carbonnelle et al. 2003) and diesel fumes (Diaz-Sanchez, Proietti et al. 2003), or nutritional factors such as vitamin D and obesity (Chinn 2003; Weiss and Litonjua 2011). As yet no data have provided a decisive explanation for this ongoing epidemic (Cookson 1999).

A historical perspective

Asthma is not a single disease but a spectrum of disorders characterised by airway obstruction that varies spontaneously and with treatment (Barnes, Djukanovic et al. 2003). The term 'asthma' derives from the Greek ἄσθμα – first used in Homer's *Iliad* (Homer) - meaning 'to exhale with open mouth, to pant' and has been used in English since around 1600 (Keeney 1964), although the earliest descriptions of asthma perhaps date back to a Chinese medical textbook c2600BC (Walter and Holtzman 2005). In the first century AD Seneca provided a vivid personal description of asthma, stressing its sudden onset and periodic nature (Seneca 65-65 AD; Panzani 1988). By the 19th century in his classic work the Dorset born physician Henry Hyde Salter defined asthma as 'Paroxysmal dyspnoea of a peculiar character, generally periodic with intervals of healthy respiration between attacks' (Hyde 1860; Sakula 1985). This key element of variability over time has been retained in more modern definitions (Bousquet, Jeffery et al. 2000; (GINA) 2010) and reflects the close link between asthma and underlying allergic airways inflammation.

In 1905 von Pirquet and Schick reported the first clinical observations of anaphylactic reactions in children caused by hypersensitiveness to horse serum (von Pirquet and Schick 1905). The term allergy was introduced by Pirquet a year later to describe the skin reaction following subcutaneous injection of tuberculin in sensitised individuals (Von Pirquet 1906). In 1910 Meltzer suggested that asthma was a manifestation of anaphylaxis, prompted by the earlier studies of Auer, who noted bronchospasm and pulmonary distension in guinea pigs dying of anaphylactic shock (Meltzer 1910). The term 'atopy' was introduced by Coca (1923) to apply to hypersensitivity mediated by an antigen-antibody mechanism, and in which hereditary influences may play an important role (Coca and Cooke 1923), and is now understood to be caused by an exaggerated tendency to mount IgE responses to a wide variety of common environmental allergens (Holgate 1999; Murphy, Travers et al. 2008). This tendency is variably expressed in the distinct but immunologically related conditions of eczema in the skin, allergic rhinitis in the upper airways and, where exposure to aeroallergens triggers airways inflammation, as allergic asthma (Holgate 1999).

Asthma heterogeneity and endotypes

Disease heterogeneity has long been described (Rubin and Rubin 1947) but its relevance to understanding the mechanisms underlying asthma has risen to prominence only lately (Anderson 2008). In recent years the analysis of large, carefully phenotyped cohorts of

asthmatics by the statistical technique of cluster analysis has led to the improved definition of distinct asthmatic clinical phenotypes (Anderson 2008; Haldar, Pavord et al. 2008; Moore, Meyers et al. 2010). Haldar *et al* described two phenotypes of severe refractory asthma both characterised by discordance between symptoms and eosinophilic airway inflammation, which they termed early-onset symptom predominant and late-onset inflammation predominant subsets (Haldar, Pavord et al. 2008). In a larger study of 726 subjects Moore *et al.* identified five phenotypic clusters: 1) early onset atopic asthma with normal lung function; 2) early-onset atopic asthma and preserved lung function with increased medication requirements, 3) older obese women with late-onset non-atopic asthma, moderate reductions in FEV_1 and frequent oral corticosteroid, and 4) and 5) with severe airflow obstruction and bronchodilator responsiveness but differing in their ability to attain normal lung function, age of asthma onset, atopic status and use of oral corticosteroids (Moore, Meyers et al. 2010).

Severe asthma

Arising from these and other studies it is apparent that a subgroup of 5-10% of asthmatics have severe disease despite anti-inflammatory therapy and airway inflammation characterised by neutrophilic infiltration (Wenzel, Szefler et al. 1997; Gibson 2007; Adcock, Caramori et al. 2008). These subjects frequently meet the American Thoracic Society 2000 consensus definition of severe refractory asthma (2000), which requires at least one major criterion and two minor criteria are met, the exclusion of other disorders, the treatment of exacerbating factors and generally good patient compliance:

Major characteristics

- Treatment with continuous or near continuous (≥50% of year) oral corticosteroids
- Need for treatment with high-dose inhaled corticosteroids

Minor characteristics

- Need for additional daily treatment with a controller medication (eg, long-acting β agonist, theophylline, or leukotriene antagonist)
- Asthma symptoms needing short-acting β agonist use on a daily or near-daily basis
- Persistent airway obstruction (FEV_1 <80% predicted, diurnal peak expiratory flow variability >20%)
- One or more urgent care visits for asthma per year
- Three or more oral steroid bursts per year
- Prompt deterioration with ≤25% reduction in oral or intravenous corticosteroid dose
- Near-fatal asthma event in the past

Wenzel et al investigated severe, steroid-dependent asthmatics bronchoscopically and found that the severe asthmatics had higher levels of neutrophils in bronchoalveolar lavage (BAL) and bronchial biopsies than either mild-moderate asthmatics or normal controls, suggesting that

neutrophilic airways inflammation may be at least one mechanism for steroid refractory disease (Wenzel, Szefler et al. 1997).

Asthma exacerbations

The classic variability of asthmatic symptoms is seen most dramatically during acute exacerbations, which are also associated with steroid-refractory inflammation (Grunberg, Sharon et al. 2001). Exacerbations are a major cause of morbidity and mortality (Anderson 2008), as well as conferring a substantial financial cost in terms of healthcare expenditure and lost productivity (Cookson 1999). In severe asthma five risk factors have been identified for recurrent exacerbations: severe nasal sinus disease, gastro-oesophageal reflux, recurrent respiratory infections, psychological affective disorders and obstructive sleep apnoea (Anderson 2008). However, irrespective of these predisposing risk factors, it is now well documented that the direct trigger factors in the vast majority of these exacerbations are viral infections of the upper respiratory tract (Johnston, Pattemore et al. 1995; Johnston, Pattemore et al. 1996). Viruses are detected by PCR in approximately 80% of exacerbations(Johnston, Pattemore et al. 1995) and are associated with airway neutrophilia (Wark, Johnston et al. 2002). Individuals with atopic asthma are not at greater risk of upper airways viral infections than healthy individuals but suffer from more frequent lower respiratory tract (LRT) infections and have more severe and longer-lasting LRT symptoms (Corne, Marshall et al. 2002). Studies of epithelial cultures infected with rhinovirus 16 have demonstrated that the mechanism that explain this susceptibility is a defect in the production of type I (Wark, Johnston et al. 2005) and type III interferons (Contoli, Message et al. 2006), leading to a failure of apoptosis that normally develops as a consequence of virus infection; instead the infected cell undergoes cytolysis when infected, thereby leading to increased viral replication and dissemination within the lower airways (Wark, Johnston et al. 2005).

The pathogenesis of asthma

Whilst diverse mechanisms may underlie the collection of diseases which comprise the syndrome of asthma, common to all are patterns of mucosal inflammation involving activated inflammatory mast cells, eosinophils and T lymphocytes, and with associated altered responses of structural cells in the airways, including epithelial cells, fibroblasts, endothelial cells and smooth muscle cells (Holgate, Lackie et al. 2001; Holgate and Polosa 2006; Holgate 2008). To help place T lymphocytes in the appropriate immunological context I will review briefly what is known of these other key cell types in the pathogenesis of asthma.

Mast cells

Mast cells are found throughout the airways especially within the bronchial epithelium and submucosa (Flint, Leung et al. 1985) but are rare within the lumen. They are key mediators of type-I hypersensitivity reactions in which inhaled aeroallergens cross-link IgE on the surface of mast cells causing rapid degranulation. This releases a variety of pro-inflammatory mediators

including histamine – which directly causes bronchoconstriction, changes in bronchial arterial perfusion and microvascular leakage – as well as the mast cell proteases tryptase, chymase, carboxypeptidase, cathepsin G, elastase, plasminogen activator and matrixmetalloproteinase (MMP)-9 (Macfarlane, Kon et al. 2000). These mediators can exacerbate bronchoconstriction via activation of bradykinin. Activated mast cells also synthesise new mediators including arachidonic acid metabolites such as the leukotrienes which also promote bronchoconstriction and airways inflammation (Laidlaw and Boyce 2012). It is also recognised that mast cell produce a variety of cytokines which had previously been attributed to T cells (Bradding, Roberts et al. 1994).

Eosinophils

Eosinophils have long been associated with asthma by their presence in sputum and the mucosa, their association with clinical responsiveness to steroids and their abundance in the airways in post-mortem studies in asthma(Brightling 2011). Sputum eosinophilia (defined as eosinophils comprising >3% of airway respiratory cells)(Pavord, Brightling et al. 1999; Green, Brightling et al. 2002) is correlated with bronchial hyper-responsiveness and with steroid responsive disease. In turn, airway eosinophilia is correlated with measured levels of exhaled nitric oxide. Eosinophils express the low affinity IgE receptor and are believed to play an important role in the late-phase reaction to inhaled aeroallergens by an IgE dependent mechanism(Durham 1998), releasing oxygen free radicals, leukotrienes and Th2 cytokines, growth factors and MMPs (Wardlaw, Brightling et al. 2000).

Basophils

Basophils are the rarest circulating granulocyte, sharing many functional characteristics with tissue-resident mast cells, and are generally associated with type 2 immune responses (Voehringer 2011). They can leave the circulation to reach tissues where they are able to survive for several weeks. Their role in asthma is the least well defined of all inflammatory cells, but they are known to strongly secrete IL-4 and IL-13 both of which are implicated in atopic disease. Like mast cells they express the high affinity IgE receptor FcεRI and contain basophilic granules, which can produce a wide variety of inflammatory mediators including histamine, platelet-activating factor, leukotriene C4, IL-4 and IL-13. They differ from mast cells in their relative inability to proliferate and perform phagocytosis, their lower responsiveness to complement, and their greater steroid responsiveness (Djukanovic, Wilson et al. 1992). Accumulations of basophils have been found in asthma from bronchial biopsies (Macfarlane, Kon et al. 2000) and in post-mortem tissue (Koshino, Teshima et al. 1993; Kepley, McFeeley et al. 2001). In allergic rhinitis they are the main source of histamine during the late phase response after allergen challenge (Bascom, Wachs et al. 1988).

Neutrophils

Neutrophils are the first cell type to be recruited to the airways during allergen challenge and have been implicated in the pathology of nocturnal asthma, and sudden asthma death (Sur, Crotty et al. 1993). Neutrophilic asthma defined as >61% neutrophils in induced sputum (Belda, Leigh et al. 2000) affects between 20 and 30% of adults with persistent asthma (Green, Brightling et al. 2002; Simpson, Scott et al. 2006), being more common in older people, more severe asthma and those with poor response to corticosteroids (Simpson, Phipps et al. 2009). Neutrophil survival is prolonged within the airways by antiapoptotic factors which are currently unidentified, but known to be quantitatively different in more severe asthma (Uddin, Nong et al. 2010). As neutrophilic inflammation characterises bronchiectasis and is correlated with bacterial load it is likely that bacterial colonisation may be a precursor to persistent airway neutrophilia (Angrill, Agusti et al. 2001). In turn neutrophil products impair mucociliary clearance through induction of mucus hypersecretion (O'Donnell, Breen et al. 2006) and a reduction in ciliary function (Amitani, Wilson et al. 1991), leading to a vicious cycle of airways inflammation (Simpson, Phipps et al. 2009). Neutrophils are recruited to the airways by such chemotactic mediators as IL-8 (CXCL8) and CXCL1 (GRO-alpha) and release mediators such as neutrophil elastase, MMP-9 and oxidative free radicals which can be directly destructive to airway tissue and are likely to contribute to the development of irreversible airflow obstruction. These products are elevated in the airways of neutrophilic asthma, and can recruit and activate further neutrophils in a self-maintaining cycle (Simpson, Phipps et al. 2009). Ironically the mainstay treatments in asthma pharmacotherapy almost certainly contribute to airway neutrophilia, as both corticosteroids (Saffar, Ashdown et al. 2011) and β_2-agonists prolong neutrophil survival (Perttunen, Moilanen et al. 2008).

Macrophages

Macrophages are the predominant immune cells in the airways. Macrophages which secrete type 2 cytokines (IL-4 and -13) and chemokines (the CCR4 ligands CCL17 and CCL22) have been termed alternatively activated (M2) macrophages. Using animal models, M2 macrophages have been implicated in allergic lung inflammation. We have recently shown that although the full M2 phenotype is not seen in human lungs, asthma is characterised by an increased expression of CCL17 in alveolar macrophages and its expression correlates with eosinophilia (Staples, Hinks et al. 2012). Macrophages express the low affinity IgE receptor which is up-regulated in asthma, implying some involvement in atopic allergic responses and expression of eicosanoids, superoxide, platelet activating factor and granulocyte macrophage colony stimulating factor are all increased in alveolar macrophages in asthma (Arnoux, Duval et al. 1980; Godard, Chaintreuil et al. 1982; Damon, Chavis et al. 1983; Capron, Jouault et al. 1986).

Inflammatory mediators

Asthma involves dysregulation of a complex, integrated immune system in which different cell types contribute to an inflammatory network orchestrated by an array of pleotropic and redundant inflammatory mediators. These include leukotrienes, prostanoids, nitric oxide, platelet-activating factor, bradykinin, chemokines and cytokines (Holgate 2011). Leukotrienes are eicosanoid lipids synthesised from arachidonic acid by 5-lipoxygenase; they are potent bronchoconstrictors and have been successfully targeted therapeutically by the leukotriene receptor antagonists zafirlukast and montelukast (Dempsey 2000; Laidlaw and Boyce 2012). Prostanoids are another class of pro-inflammatory eicosanoid, generated by cyclooxygenase and include the prostaglandins, thromboxanes and prostacyclins and may play a role in aspirin sensitive asthma. Nitric oxide acts as a non-adrenergic, non-cholinergic neurotransmitter in the airways, can mediate vasodilation, and is a useful biomarker of airway eosinophilia (Taylor, Pijnenburg et al. 2006). Cytokines are peptide mediators released from inflammatory cells, which are important in signalling between cells (Barnes, Djukanovic et al. 2003; Holgate 2011) and include the interleukins (ILs) which act to stimulate, regulate, or modulate lymphocytes such as T cells (Murphy, Travers et al. 2008). Over 50 cytokines have been identified, which may have pro- or anti-inflammatory roles, or have actions which are context dependent (Murdoch and Lloyd 2010), and interact in complex networks. Much attention in allergy research has focused on the T_H2 cytokines IL-4 (critical for IgE class switching in B cells(Lebman and Coffman 1988)) and IL-5 (important for the terminal differentiation, survival and activation of eosinophils (Sanderson 1992)). Understanding of these inflammatory networks has led to the recent development of two new therapeutic strategies in asthma, ie monoclonal antibodies to IgE (omalizumab) and IL-5 (mepolizumab).

Innate responses

Polymorphisms in toll-like receptors (TLR)s and associated molecules suggest that in addition to the clear role of adaptive immunity, differences also in innate immunity may contribute to asthma pathogenesis (Lazarus, Raby et al. 2004; Moller-Larsen, Nyegaard et al. 2008; Bjornvold, Munthe-Kaas et al. 2009; Bjornsdottir, Holgate et al. 2011). Gene expression profiling of peripheral blood mononuclear cells (PBMC) during asthma exacerbations showed activation of innate pathways including TLR1, 2, 3 and type I IFN (Bjornsdottir, Holgate et al. 2011). DerP2, a major component of house dust mite allergen (HDM), shares structural homology with the lipopolysaccharide (LPS) binding component of TLR4, giving it intrinsic adjuvant properties which may explain the high frequency of HDM sensitisation (Trompette, Divanovic et al. 2009), whilst cockroach frass contains a TLR2 agonist which can directly activate neutrophils(Page, Lierl et al. 2008). Indeed sputum from neutrophilic asthmatics has higher expression of various molecules of innate immunity including TLR2, 4 and IL-8(Simpson, Grissell et al. 2007).

Airways remodelling in asthma

Inflammatory cells do not function in isolation, but interact continually with structural tissues of the airways. Asthma typically involves characteristic changes to the bronchial epithelium including epithelial metaplasia, thickening of the subepithelial basal lamina, increased number of myofibroblasts and other evidence of airway remodeling such as hypertrophy and hyperplasia of airway smooth muscle, mucous gland hyperplasia, angiogenesis and an altered extracellular matrix (Holgate 2008). These features, along with upregulation of epidermal growth factor receptors and reduced makers of cell proliferation, suggest that the asthmatic airway epithelium is chronically injured. Causative factors in this injury include inhaled allergens, viral infections or airway pollution. In response to chronic injury the epithelium can secrete growth factors such as transforming growth factor-β (TGFβ), platelet-derived growth factor, and fibroblast growth factors, which act on surrounding stromal cells to induce the features of airway remodelling such as goblet cell hyperplasia, smooth muscle hypertrophy and myofibroblast differentiation. The interaction between such a susceptible epithelium and T_H2-mediated inflammation, altering communication between the epithelium and the underlying mesenchyme, has led to the concept of the 'epithelial mesenchymal trophic unit' in which these interplays lead to disease persistence, airway remodelling and refractoriness to corticosteroids (Holgate, Lackie et al. 2001). This interplay between mesenchyme and epithelium has been underlined by the recent discovery that smooth muscle contraction alone, induced by methacholine challenge, is sufficient to induce an increase in subepithelial collagen-band thickness, a marker of airway remodelling (Grainge, Lau et al. 2011).

In summary underlying the spectrum of disorders classified as asthma are a wide range of distinct pathological changes, arising from the complex interplay of several intricate biological systems, including chronic injury and activation of structural cells, innate cells and the cells of the adaptive immune system. The preeminent effector and regulatory cells of the cellular adaptive immune system are T lymphocytes.

T lymphocytes (T cells)

T cells are defined by their surface expression of clonally distributed T cell receptors (TCRs) and play a central role in cell mediated immunity. They develop from progenitors that are derived from the pluripotent haematopoietic stem cells in the bone marrow and migrate through the blood to the thymus, where they mature, and it is for this reason that they are called thymus-dependent (T) lymphocytes or T cells (Murphy, Travers et al. 2008). T cells comprise a heterogeneous spectrum of subsets with differing expression of TCR classes – TCRαβ or TCR$\gamma\delta$ -, CD4 and CD8 lineage markers and other surface phenotypes, and very distinct immunological functions. A fundamental dichotomy amongst the major class of TCRαβ+ T cells is determined by expression of either the CD8 co-receptor, enabling these cytotoxic T cells directly to kill cells with intracellular infections, or the CD4 co-receptor defining the T helper (T_H) cell subset which provides essential additional signals to activate B cells or macrophages to

stimulate antibody production or increased cell killing respectively (Murphy, Travers et al. 2008), although there are important exceptions to this general scheme (Mumberg, Monach et al. 1999). Amongst TCRαβ+ CD4+ T cells different cells have differing cytokine secretion profiles which have been used to define T_H1, which activate infected macrophages and provide co-stimulation, and T_H2 cells, which primarily activate naïve B cells to produce antibody (Mosmann, Cherwinski et al. 1986). In recent years many additional T cell subsets have been described including immunoregulatory regulatory T cells (Treg) (Thornton and Shevach 1998), T_H17 cells (Park, Li et al. 2005), innate-like lymphocytes such as iNKT cells (Taniguchi, Koseki et al. 1996), and MAIT cells (Tilloy, Treiner et al. 1999).

The importance of T cells in asthma

T cells are widely recognised as orchestrators of the immune response in asthma. They are increased in asthmatic airways in correlation with activation status (Azzawi, Bradley et al. 1990; Walker, Kaegi et al. 1991; Bentley, Menz et al. 1992; Larche, Robinson et al. 2003). Analysis of sibling pairs revealed genetic linkage between specific IgE responses and a gene in the TCR-α gene complex on chromosome 7 (Moffatt, Hill et al. 1994). Furthermore, T cells can influence the function of many inflammatory cells including mast cells and eosinophils through the production of a group of pro-inflammatory cytokines in the IL-4 gene cluster on chromosome 5q31–33, which tend to exacerbate allergic responses (Holgate 1999). These cytokines define a distinct T cell subset, T helper-2 (T_H2) cells, which in the early 1990s were shown by Robinson *et al* to predominate amongst allergic asthmatics (Robinson, Hamid et al. 1992). The T_H2 cytokines all play key roles in allergic asthma: IL-4 is important for allergic sensitization and IgE production, and IL-5 is crucial for eosinophil survival, whilst IL-13 has pleiotropic effects in the lungs including a central role in the development of airway hyper-responsiveness and tissue remodelling (Holgate 2008; Lloyd and Hessel 2010). Allergen challenge in asthmatics can induce airway recruitment of activated T_H2 cells, with concomitant increase in T_H2 cytokines and eosinophilia(Larche, Robinson et al. 2003). Conversely, interferon (IFN)-γ secreting Th1 cells which antagonise Th2 mediated responses are generally thought not to play a major role in allergic airways inflammation(Holgate 1999).

Beside theoretical considerations and observational associations, what other evidence is there of a causal role for aberrant T cells responses in the pathogenesis of asthma? Till *et al* performed segmental bronchoscopic allergen challenge of house dust mite-sensitive asthmatics and healthy controls. Allergen challenge increased BAL and peripheral T cell proliferation and IL-5 production in asthmatics and these BAL responses correlated with the degree of BAL eosinophilia, implying that allergens induce pathogenic allergen-specific T_H2 responses in the airways(Till, Durham et al. 1998).

Evidence that T cells may be sufficient to provide a trigger for the development of asthma comes from reports of asthma resulting from the adoptive transfer of T cells in autologous bone

marrow transplant (BMT) recipients. Rietz *et al* report two individuals who developed asthma after BMT from a human leukocyte antigen (HLA) identical sibling with asthma, including the acquisition of measureable airflow obstruction, bronchial tissue eosinophilia, and clinical response to inhaled steroids and bronchodilators (Rietz, Plummer et al. 2002). Hallstrand *et al* subsequently followed 5 long-term survivors of BMT received from allergic donors finding high IgE levels frequently persisted and a high rate of new sensitisation occurred, leading to rhinitis and – in 4/5 individuals – asthma (Hallstrand, Sprenger et al. 2004).

Whilst transfer of this phenotype might be caused by transfer of T cells, B cells or hematopoietic stem cells, conversely an interventional trial of anti-CD4 monoclonal antibody (Keliximab) increased lung function in asthmatics and showed slight, albeit non-significant, trends towards improved symptoms, providing intriguing evidence in humans that T cells are at least one necessary component for the development of asthma(Kon, Sihra et al. 1998).

Interleukin-17

Interleukin-17 (IL-17, also called IL-17A) is a cytokine produced by activated memory T cells, and other tissues (Fossiez, Djossou et al. 1996). It was first identified in 1993 by cloning the human homolog of murine cytotoxic T lymphocyte associated antigen (mCTLA8), and was found to be produced on activation of T cells by phorbol myristate acetate and ionomycin(Fossiez, Djossou et al. 1996). It is a disulfide-linked homodimeric glycoprotein of 155 amino acids(Yao, Fanslow et al. 1995), acting as a 35kDa homodimer (Kolls and Linden 2004), and encoded at gene locus 6p12 (Moseley, Haudenschild et al. 2003). IL-17A is now recognised to be the prototypic member of the IL-17 cytokine family which contains 5 further cytokines which were identified by gene database searches, cloned and named IL-17B to IL-17F(Li, Chen et al. 2000; Fort, Cheung et al. 2001; Lee, Ho et al. 2001; Starnes, Robertson et al. 2001; Hurst, Muchamuel et al. 2002). Whilst all six have some degree of structural homology – a common cysteine knot formation (Hymowitz, Filvaroff et al. 2001) – they are otherwise a genetically and functionally divergent group. IL-17A and IL-17F are most related, both being encoded on the same chromosome. As they are located only 45 kBP apart, they are probably co-regulated, indeed both being induced by IL-23(Kolls and Linden 2004). They also share the closest (40-55%) sequence homology (Kolls and Linden 2004) and have functional similarities, as they both induce a neutrophil response (Kolls and Linden 2004).

In contrast to the similar structures and functions of IL-17A and IL-17F, the other family member (IL-17B to E) are more diverse. They have lower (17-29%) sequence homology to IL-17A, and are encoded on four different chromosomes(Kolls and Linden 2004). Neither IL-17B or IL-17C are expressed in the lung (Li, Chen et al. 2000) and IL-17D is expressed on the endothelium rather than epithelium. Whilst IL-17E is expressed in lung tissue, its sequence is the most distantly related to IL-17A, and this cytokine is now better known as IL-25. IL-25 is considered a T_H2-type cytokine (Kolls and Linden 2004) which has been shown to suppress IL-17A

responses (Bettelli, Korn et al. 2008) and induce eosinophilic inflammation(Letuve, Lajoie-Kadoch et al. 2006).

Receptors for IL-17 so far identified include IL-17R, IL-17RH1, IL-17RL (receptor like), IL-17RD and IL-17RE, all of which are type-1 transmembrane receptors whose differences result from alternative splicing (Kolls and Linden 2004). They are ubiquitously expressed on a variety of tissues including lung, but also cartilage, bone, meniscus, brain, hematopoietic tissue, kidney, skin and intestine; and on a variety of cell types including epithelial cells, fibroblasts, B and T cells, myelomonocytic cells and marrow stromal cells (Moseley, Haudenschild et al. 2003). Receptor engagement on stromal cells leads, via the adaptor ACT1/CIKS and TRAF-6, to activation of the transcription factor NF-kB and the Jnk kinases (Schwandner, Yamaguchi et al. 2000), inducing secretion of pro-inflammatory cytokines including IL-6, IL-8 (CXCL8), CXCL2, PGE_2 and G-CSF (Fossiez, Djossou et al. 1996) which are chemotactic for neutrophils (Sergejeva, Ivanov et al. 2005; Fujiwara, Hirose et al. 2007; McKinley, Alcorn et al. 2008), as well as the cytokine IL-22 which in turn induces the antimicrobial peptide human β-defensin 2 (Wiehler and Proud 2007).

The T helper 17 subset

Expression of IL-17, in the absence of IFN-γ, defines a recently described subset of $CD4^+$ T helper lymphocytes called T helper-17 (TH17) lymphocytes (Park, Li et al. 2005). They comprise a distinct T cell lineage, that is not dependent on Th1 and Th2 associated transcription factors T Bet or GATA3 (Park, Li et al. 2005) but on expression of the nuclear transcription factor retinoic acid-related orphan nuclear hormone receptor (ROR)C (or its homolog RORγt in mice), which induce IL-17A and IL-17F (Ivanov, McKenzie et al. 2006). Importantly the predominantly pro-inflammatory TH17 cells share a reciprocal developmental pathway with FOXP3+ regulatory T cells (Treg) implying this dichotomy may have evolved to induce or regulate tissue inflammation (Bettelli, Carrier et al. 2006).

In humans T_H17 can be induced *in vitro* by culture of naïve T cells with IL-21 and TGFβ, or from central memory T cells by IL-1β and TGFβ (Yang, Anderson et al. 2008). Maintenance of the TH17 cell population may depend on the presence of IL-23 (Yang, Anderson et al. 2008).

Emerging animal data suggest reciprocal developmental relationships between T_H17 and Treg (Mucida, Park et al. 2007; Lochner, Peduto et al. 2008) with antagonistic functions of the RORγT and Forkhead box P3 (FOXP3) transcription factors. In the gastrointestinal mucosa T_H17 can induce chronic inflammation(Leppkes, Becker et al. 2008) and it has been suggested that regulation may be influenced by the mucosal microflora (Ivanov, Frutos Rde et al. 2008; Zhou, Lopes et al. 2008).

Interleukin-17, T_H17 cells and asthma

T_H17 cell have been linked to neutrophilic pulmonary inflammation in both human asthma (Molet, Hamid et al. 2001) and mouse models of allergic inflammation (Park, Li et al. 2005). In the airways IL-17 primarily acts on stromal cells to induce cytokines and chemokines including IL-6, -8 (CXCL8), CCL26 (eotaxin-3), CXCL1,and CXCL2 (Fossiez, Djossou et al. 1996; Wang, Voo et al. 2010), which are chemotactic for neutrophils (Sergejeva, Ivanov et al. 2005; Fujiwara, Hirose et al. 2007; McKinley, Alcorn et al. 2008) (See Figure 1.1). Mouse models (Schnyder-Candrian, Togbe et al. 2006; Fujiwara, Hirose et al. 2007; McKinley, Alcorn et al. 2008), human genetic associations (Hizawa, Kawaguchi et al. 2006; Kawaguchi, Takahashi et al. 2006; Chen, Deng et al. 2010; Lluis, Schedel et al. 2011) and studies of protein and messenger RNA (mRNA) expression in sputum or bronchoalveolar-lavage (BAL) (Molet, Hamid et al. 2001; Chakir, Shannon et al. 2003; Bullens, Truyen et al. 2006), have implicated IL-17 in the pathogenesis of asthma and bronchial hyper-reactivity.

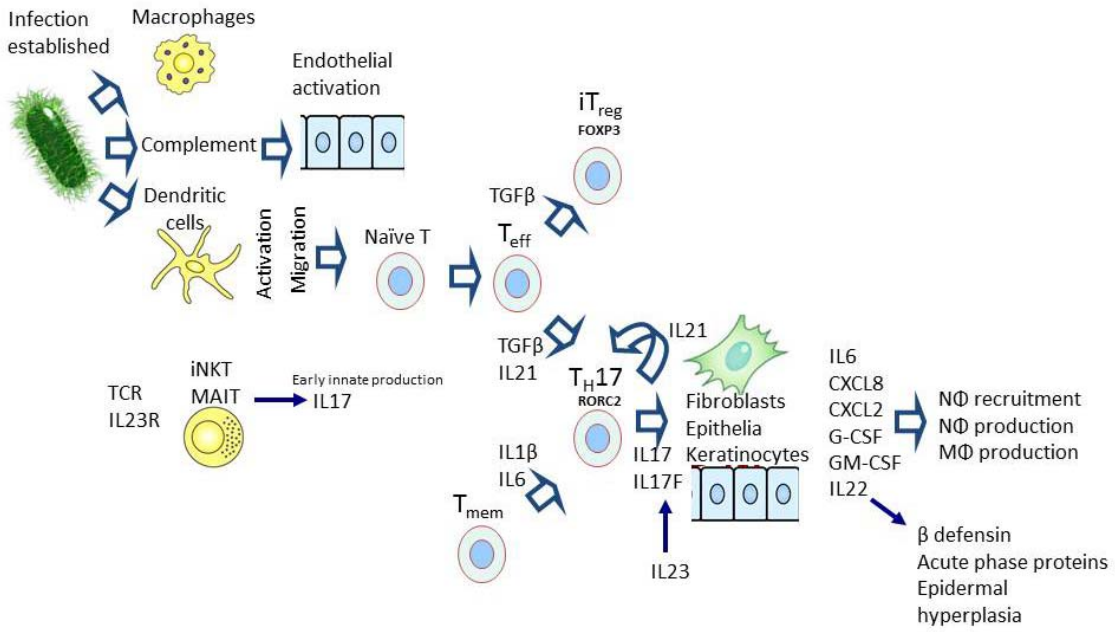


Figure 1.1 IL-17 and T_H17 cells in the immune response

A schematic diagram of the induction of T_H17 cells in an immune response to infection in humans. Infection leads to recognition by the innate immune system: macrophages and complement leading to endothelial cell activation which favours adhesion and extravasation of other immune cells. There is also early recognition and activation of innate-like lymphocytes including invariant iNKT and MAIT leading to early production of IL-17. The adaptive immune system is also triggered, first by the activation of dendritic cells which migrate to draining lymph nodes where they present antigen to naïve T cells. Primed T cells mature into effector T cells (Teff), whose fate is determined by the cytokine milieu. In the presence of TGFβ alone the express the nuclear transcription factor FOXP3, becoming immunoregulatory inducible T reg. By contrast in the presence of TGFβ and IL-21 the transcription factor RORC2 is expressed. This physically interacts with, and displaces FOXP3 from nuclear binding sites, and induces an IL-17+ IFNγ- T_H17 phenotype. T_H17 cells secrete IL-21 which promotes expansion of the T_H17 cell pool in an autocrine manner. The T_H17 population may also be expanded by recruitment of memory T cells (Tmem) under the influence of IL-1β and IL-6. T_H17 cell frequencies are maintained by IL-23. T_H17 cells secrete IL-17(A) and IL-17F which act on stromal cells such as fibroblasts, epithelia and keratinocytes to induce secretion of numerous pro-inflammatory chemokines, including IL-6, IL-8 (CXCL8) and CXCL2 which recruit neutrophils (Nϕ), and G-CSF, GM-CSF which up-regulate production of macrophages (Mϕ). Induction of IL-22 induces further inflammation by induction of acute phase proteins, epidermal hyperplasia, and the antimicrobial peptide β-defensin, which may be considered an end effector molecule T_H17 cells.

Molet reported an increased number of IL-17+ cells in sputum and BAL from six asthmatics (Molet, Hamid et al. 2001), and later the same group reported an increase in IL17+ staining in the submucosa and epithelium of nine moderate-severe asthmatics (Chakir, Shannon et al. 2003), whilst others reported increased numbers of submucosal IL-17 in mild-moderate but not severe asthma (Doe, Bafadhel et al. 2010). Furthermore there have been reports of a correlation between whole sputum IL-17 mRNA and bronchial hyper-responsiveness (Barczyk, Pierzchala et al. 2003), or the presence of asthma (Zhou, Sun et al. 2005; Bullens, Truyen et al. 2006), and also of increased IL-17 mRNA in bronchial biopsies (Vazquez-Tello, Semlali et al. 2010; Howarth 2012).

IL-17 and T_H17 cells in murine models of allergic airways disease

Despite this extensive body of literature regarding IL-17 and T_H17 cells in animal models, very little comparable human data have been obtained to date, and it is currently unknown whether T_H17 cells are involved in human asthma. Indeed, even if IL-17 levels are found to be elevated in asthma, it could have a wide variety of potential cellular sources; thus, not only TCRαβ+ T-cells, but also TCR$\gamma\delta$+ T-cells(Lochner, Peduto et al. 2008), eosinophils (Molet, Hamid et al. 2001) and macrophages (Song, Luo et al. 2008), and even B cells (Vazquez-Tello, Halwani et al. 2012) can potentially secrete IL-17. Moreover it is unknown which putative surface markers (Acosta-Rodriguez, Rivino et al. 2007; Cosmi, De Palma et al. 2008; Pene, Chevalier et al. 2008) identify pulmonary T_H17 cells or whether T_H17 frequencies and functions are associated with distinct asthma phenotypes, such as the neutrophilic forms where it is frequently hypothesised to be significant.

Regulatory T cells

The differentiation of T_H17 cells is closely related to that of the functionally antagonistic regulatory T (Treg) cell subset (Bettelli, Carrier et al. 2006). Tregs have been identified in mice and humans which are believed to be essential for regulating adaptive immune responses, regulating the host response to infection, maintaining self-tolerance and preventing autoimmune diseases (Takahashi, Kuniyasu et al. 1998; Belkaid, Piccirillo et al. 2002). Naturally occurring, thymic derived CD4+CD25+ Treg cells (syn: natural Tregs) inhibit effector functions of other immunocytes, eg CD4+ and CD8+ T cells (Sakaguchi, Sakaguchi et al. 1995; Thornton and Shevach 1998; Baecher-Allan, Brown et al. 2001; Murakami, Sakamoto et al. 2002). Arising from the thymus, they enter peripheral tissues where they suppress the activation of other self-antigen–reactive T cells (Bluestone and Abbas 2003). In murine models, they suppress T-cell responses to several intracellular pathogens, and their depletion *in vivo* leads to increased immune-mediated tissue pathology. Natural Tregs require antigen-specific T cell receptor (TCR) mediated activation, but effector function is non-specific (Liu, Putnam et al. 2006).

Different chemokine receptors determine Treg homing to distinct tissues such as lymphoid or non-lymphoid tissues, or sites of inflammation. Treg act via a number of mechanisms including secretion of IL-10, TGFβ, IL-35, inhibition of dendritic cell (DC) maturation via surface CTLA4, direct granzyme and perforin mediated killing of mature DCs, inhibition of priming effector CD4+ T cells or tumour specific CD8+ T cells, and metabolic inhibition of effector T cells via adenosine and cyclic adenosine monophosphate (cAMP) (Campbell and Koch 2011). Differentiation of Treg can be modulated by cytokines, steroids, sphingolipids and vitamin A and D metabolites. IL-2 plays a particularly important role, signalling in a paracrine fashion via the IL-2 receptor (CD25) to promote Treg survival and proliferation. Treg development can be inhibited by IL-4 with TGFβ, or by IFN-α, and IFN-β. IL-6 inhibits FOXP3 expression and induces T_H17 cells, whilst TNF can potentiate Treg function(Campbell and Koch 2011).

In human disease dysregulation of FOXP3 Treg has been implicated in autoimmunity, lymphoproliferative disease, type I diabetes mellitus, systemic lupus erythematosus, rheumatoid arthritis and multiple sclerosis (Campbell and Koch 2011). Treg have also been studied in a number of chronic infectious diseases such as tuberculosis(Guyot-Revol, Innes et al. 2006), leishmaniasis (Belkaid and Rouse 2005), bacteria, viruses, parasites and fungi (Mills 2004) where they may play a role both in limiting immunopathology, but also in maintaining microbial persistence.

Regulatory T cells in asthma

FOXP3+ Treg have been shown to be present in the bronchial mucosa in infants and were primarily located within isolated lymphoid follicles of bronchus-associated lymphoid tissue(Heier, Malmstrom et al. 2008). Lin *et al* found that asthmatic children had lower FOXP3 levels in peripheral blood(Lin, Shieh et al. 2008), whilst in a longitudinal study of 18 severe asthmatics with frequent exacerbations, peripheral blood and sputum Treg were decreased in frequency and function (blood) during exacerbations in severe asthma (Mamessier, Nieves et al. 2008).

A key effector mechanism for Treg is production of the anti-inflammatory cytokine IL-10. There is evidence that treatment-refractory asthmatics have impaired steroid-induced IL-10 production (Holgate and Polosa 2006), and that blood levels of IL-10 correlate inversely with disease severity in atopic asthma(Matsumoto, Inoue et al. 2004; Hawrylowicz 2005; Matsumoto, Inoue et al. 2008). Furthermore in atopic individuals IL-10 secreting CD25+ Treg can be induced by immunotherapy (Ling, Smith et al. 2004) whilst Treg can suppress allergen-activated IL-4 cells, again via IL-10 and transforming growth factor-β (TGFβ)(Robinson, Larche et al. 2004).

In mouse models of Treg depletion or adoptive transfer Treg numbers correlate negatively with bronchial hyper-reactivity (Hawrylowicz 2005; Kearley, Barker et al. 2005; Lewkowich, Herman et al. 2005; Kearley, Robinson et al. 2008). Treg have also been induced by heat killed *Mycobacterium vaccae* and inhibited AHR via the induction of TGFβ and IL-10 (Zuany-Amorim,

Sawicka et al. 2002; Robinson, Larche et al. 2004). Indeed a trial of *M. vaccae* in humans suggested a trend towards a decrease in the late asthmatic response, although this did not reach significance (Camporota, Corkhill et al. 2003). Likewise murine Treg can also be induced by dendritic cells transfected with DerP2 DNA. These Treg can suppress T_H2 responses, allergen specific CD4+ T cell responses, and AHR (Wu, Bi et al. 2008). In a rat model of chronic aeroallergen exposure Treg are induced in the airway mucosa and inhibit subsequent T cell activation (Strickland, Stumbles et al. 2006).

There have recently been further reports of Treg in peripheral blood in relation to asthma or allergy. McCloughlin *et al* studied Treg frequencies in infants prospectively from birth. Treg frequencies increased over the first two years of life, although their functional capabilities did not change. Treg frequencies at birth did not predict development of subsequent allergy, but by one and two years of age Treg frequencies and suppressive function were associated with reduced allergic sensitization, which appeared to be mediated by IL-10 (McLoughlin, Calatroni et al. 2012). Wang found lower Treg frequencies in peripheral blood in 20 asthmatics (Wang, Lin et al. 2009), and although Provoost *et al* found no difference in $CD4^+25^{Hi}FOXP3^+$cell frequencies in blood between asthma and health, they observed lower FOXP3 expression with these Tregs in adult asthmatics (Provoost, Maes et al. 2009). *Ex vivo* steroid treatment of stimulated PBMC increased the anti-inflammatory ratios of FOXP3/GATA-3, FOXP3/T-bet, and FOXP3/RORC2 (Provoost, Maes et al. 2009).

In summary, a wealth of mouse data suggest a protective role for Treg in asthma, but with the exception of Mamessier's excellent work, published human data are predominantly only from peripheral blood, and a systematic cross sectional analysis of airway Treg, particularly in severe asthma, is currently lacking.

CD8+ T cells and asthma

In order to place T helper subsets in context I have also undertaken an analysis of cytotoxic (CD8+) T cells in asthma. In comparison with CD4+ T cells, there has been much less research on the role of CD8+ T cells in human asthma. Bronchoscopy studies in smokers have reported increased epithelial CD8+ T cell infiltration in subjects with mild airflow limitation compared with those with chronic bronchitis alone (Fournier, Lebargy et al. 1989), and an inverse relationship between CD8+ T cells and FEV_1 in chronic obstructive pulmonary disease (COPD)(O'Shaughnessy, Ansari et al. 1997). A similar increase in CD8+ T cells was found in surgical lung specimens from subjects with COPD compared with healthy smoker s(Saetta, Di Stefano et al. 1998).

A post mortem study of seven subjects who died of asthma found higher frequencies of peribronchial CD8+ T cells, compared with subjects who died of other causes. These cells were activated, expressing CD25, perforin, IL-4 and IFN-γ, with a higher IL4/IFN-γ ratio (O'Sullivan,

Cormican et al. 2001). Given the circumstances of their deaths it is likely these cells were responding to presence of an acute viral infection. Krug *et al* studied BAL T cells before and after allergen challenge in 11 subjects with mild atopic asthma. They found no differences in CD8+ cell frequencies at baseline, but there was a significant fall in the proportion of T cells secreting IFN-γ and IL2 in the asthmatics after allergen challenge, in both CD4+ and CD8+ subsets(Krug, Erpenbeck et al. 2001). Van Rensen *et al* observed a correlation (r=-0.39) between CD8+ T cell frequencies in baseline bronchial biopsies from 32 asthmatics and their subsequent rate of decline in post-bronchodilator FEV_1 (van Rensen, Sont et al. 2005). In 21 infants with wheeze (median age 15.4 months) Arnoux found increased numbers of CD8+ cells in BAL compared with non-wheezing controls (Arnoux, Bousquet et al. 2001). It is not clear whether a viral infection might have induced these responses as all children had a history of an acute viral exacerbation, but the bronchoscopies were performed during a period of clinical stability, and no respiratory viruses were detectable at the time using immunofluorescence.

As with the T_H1/T_H2 dichotomy amongst CD4+ T cells, CD8+ T cells form functionally similar subsets with similar cytokine profiles known as Tc1 and Tc2 (Mosmann, Li et al. 1997). Cho *et al* found increased frequencies of both CD4+ and CD8+ sputum T cells spontaneously secreting IL-4, -5 and IFN-γ in nine subjects with mild-moderate atopic asthma (Cho, Stanciu et al. 2005). This increase was related to disease severity, and this association was stronger for CD8+ than CD4+ cells. Also in peripheral blood Magnan *et al* found an increase in IFN-γ-CD8 cells which was related to asthma severity, to bronchial hyper-responsiveness, to blood eosinophilia and to peripheral blood IL-12 (Magnan, Mely et al. 2000).

In summary little is known about CD8+ T cells in asthma, but what data there are tend to imply a pathological role for CD8+ cells (Betts and Kemeny 2009).

Mucosal Associated Invariant T (MAIT) cells

T cell immunology is a rapidly evolving field with many new T cell subsets identified in recent years (Bluestone and Abbas 2003; Shevach 2006; Schmidt-Weber, Akdis et al. 2007). Perhaps the most exciting of these has been the recognition of distinct classes of innate-like lymphocytes (Arase, Arase et al. 1993) whose role in airways disease has been a subject of some controversy. I have taken the opportunity provided by my focused study on T_H17 cells to undertake the first analysis in the airways of a recently discovered class of innate-like lymphocytes: Mucosal associated invariant T cells (MAIT). This section reviews the current literature on these cells.

Innate-like lymphocytes

Most T cells have diverse T cell receptors (TCRs) due to stochastic recombinations of V, D and J segments, with additional random trimming or addition of nucleotides at the junctions(Tilloy, Treiner et al. 1999). Some “invariant” lymphocyte subsets have more restricted TCRs, namely

B1 B cells, some γδ T cells, and the CD1d restricted invariant natural killer T (iNKT) cells (Tilloy, Treiner et al. 1999). Invariant lymphocytes migrate rapidly to the site of acute inflammation, have a memory phenotype in the absence of deliberate immunization, and respond rapidly to challenge, eg with secretion of massive amounts of cytokines. They are therefore also often called 'innate lymphocytes' (Treiner, Duban et al. 2005). Innate T cells in humans and mice comprise CD1d restricted iNKT and MR1 (MHC related-1) restricted MAIT cells, both of which express "invariant" TCRs that are conserved between species (Treiner and Lantz 2006) and recognise nonpolymorphic antigen presenting molecules (ie CD1d and MR1, respectively) (Porcelli, Yockey et al. 1993). Both iNKT and MAIT cells are believed to react to phylogenetically-conserved antigens, and both subsets are thought to play key regulatory roles in immunity (Treiner and Lantz 2006).

Mucosal associated invariant T cells

The canonical TCR for MAIT cells was first identified in 1993 by Porcelli *et al* who noted that many people expressed an identically rearranged TCRα chain: Vα7.2-Jα33 (Porcelli, Yockey et al. 1993). In 1999 Olivier Lantz *et al* were the first to describe this Vα7.2-Jα33 segment as defining a new subset of T cells, found in humans, mice (which express the homologue Vα19-Jα33), and cattle, with a complementarity determining region (CDR)3 of constant length. MAIT comprise up to 15% of human peripheral blood DN cells (0.1-0.2% of all T cells). They were initially found to predominantly have a double negative (DN, frequency 1/10) or CD8αβ phenotype (frequency 1/50). They have an activated/memory phenotype: $CD45RA^{lo}CD45RO^{+}$ (Tilloy, Triener et al. 1999). They are also $CD27^{+}$ and $CD28^{+}$, NKR-$P1A^{+}$, $\alpha4\beta7^{+}$ $CD56^{-}$ $CD57^{-}$ (Treiner, Duban et al. 2005) $CD95^{Hi}CD62L^{Lo}$ (Dusseaux, Martin et al.). Expression of α4β7integrin (Treiner, Duban et al. 2005) and the chemokine receptor expression pattern $CCR9^{Int}CCR7^{-}CCR5^{Hi}CXCR6^{Hi}CCR6^{Hi}$ (Dusseaux, Martin et al.) enables them to home to the intestine, where they are abundant in lamina propria but virtually absent from epithelium(Treiner, Duban et al. 2005).

MAIT cells have an oligoclonal Vβ repertoire, as the TCRβ chain preferentially uses human Vβ13 and Vβ2 segments, which suggests peripheral expansions (Tilloy, Di Santo et al. 1999). The Vα7.2-Jα33 chain is the product of a single combination event with a CDR3α of defined length and reading frame. That there is some variability in this junction suggests the overrepresentation of this rearrangement is the consequence of selection at the protein level rather than a genetically programmed recombination process (Treiner, Duban et al. 2005).

Greenaway's recent *in silico* analysis of invariant TCRs has suggested the mechanisms by which these limited TCRs can be produced through 'convergent recombination' (Greenaway, Ng et al. 2012). The canonical TCRα amino acid sequences in both iNKT and MAIT cells are encoded by at least one germline-derived nucleotide sequence in all reported species, thus they are not due to random recombinations of V and J segments. Furthermore these sequences use

an overlap between the Vα and Jα genes, in that some of the nucleotides in key CDR codons can come from either the V or J genes, and in some cases palindromic additions are possible, ensuring they are produced by a greater variety of recombination mechanisms.

CD161

MAIT cells have a high surface expression of CD161 (Martin, Treiner et al. 2009; Le Bourhis, Martin et al. 2010; Dusseaux, Martin et al. 2011). CD161 (KLRB1, NKRP1A) is a C-type lectin which is part of the NK complex. Amongst CD8+ cells, high CD161 expression is associated with T_H17 differentiation: expression of IL-17, IL-22, RORγT and IL-23R. CD161 is also expressed by most NK cells as well as $\gamma\delta$T cells, most NKT cells and many tissue-infiltrating T cells (Billerbeck, Kang et al. ; Dusseaux, Martin et al. 2011). For the purposes of this work I have defined MAIT cells using a combination of expression of TCR-Vα7.2 and CD161, consistent with these studies.

MAIT cell restriction

MAIT cell selection and expansion was shown to be dependent on β2-microglobulin (β2M) but not major histocompatibility complex (MHC) II or MHC I, suggesting early on that they were restricted by a non-classical MHC class 1b molecule (Tilloy, Treiner et al. 1999). This class 1b molecule was later found to be 'MHC-related protein 1' (MR1), a highly conserved monomorphic MHCI related molecule. MR1 is encoded on chromosome. With a remarkable 90% sequence identity between mouse and human it is the most highly conserved MCH1 related molecule in mammals (Brossay, Chioda et al. 1998).

MR1 has four isoforms in humans of which only MR1-A is translated and expressed as a heterodimer with β2M. Very stringent conservation of the MR1 amino acid sequence, even distally in the molecule, implies strong evolutionary pressure and the possibility that MR1 is part of a multi-molecular complex or binds to other receptors and co-receptors(Treiner, Duban et al. 2005). It is has long seemed likely that MR1 has an antigen presenting function (Huang, Gilfillan et al. 2005). MR1 mRNA expression seems to be ubiquitous, though it is rarely detectable at the cell surface suggesting it is only surface expressed in the presence of its ligand (Treiner, Duban et al. 2005). This idea has been supported by murine data in which use of a monoclonal antibody to stabilise endogenous MR1 at the cell surface increased MAIT cell activation (Chua, Kim et al. 2011).

MAIT cell ligands

Until recently the ligand for MAIT cells has remained obscure. The presentation pathway of MR1 to MAIT cells is highly evolutionarily conserved (Huang, Martin et al. 2009). MR1 traffics through endocytic compartments, thereby allowing MAIT cells to sample both endocytosed and endogenous antigens(Huang, Gilfillan et al. 2008). Using conformation-dependent monoclonal antibodies to detect surface MR1 Abos *et al* showed MR1 expression was increased at 26°C,

was lost with acid, and independent of the proteasome, suggesting that MR1 binds proteasome-independent ligands (Abos, Gomez Del Moral et al. 2011). Site directed mutagenesis and analysis of the MR1 crystal structure suggested that only two residues, on either side of the MR1 cleft, are essential for TCR activation. This, and the relatively rigid TCR, is characteristic of innate receptors evolved to recognize a very limited range of antigens (Reantragoon, Kjer-Nielsen et al. 2012).

Kjer-Nielsen *et al* have recently shown that B2 vitamin derivatives can occupy, though only partially, the MAIT TCR binding grove. These authors were able to obtain a crystal structure of 6-formyl pterin, a folic acid (vitamin B9) metabolite, bound to MR1, showing the pterin ring sequestered within MR1 (Kjer-Nielsen, Patel et al. 2012). It seems unlikely this is the natural ligand for MAIT as the binding was irreversible, left much of the binding grove unoccupied, and the complex did not activate MAIT cells. However this group have also shown binding of related, bacterially-derived vitamin B derivatives, such as those originating from the bacterial riboflavin (vitamin B2) biosynthetic pathway, which can activate MAIT cells. As many microbes have unique synthetic pathways for vitamins, it seems likely that MAIT cells may recognise microbially-derived products of vitamin biosynthesis as a means of detecting infection.

MAIT cell development

Selection and expansion of MAIT cells depends on B cells, and also on the presence of commensal flora, as MAIT cells are not present in germ-free mice (Sano, Haneda et al. 1999). MAIT cell development is a stepwise process, with an intra-thymic selection followed by peripheral expansion. While MAIT cell development is thymus dependent (absent in nude mice), they are rapidly exported from thymus as they are not readily detectable in thymus by PCR. After birth, MAIT cells acquire a memory phenotype and expand dramatically to 1%-4% of blood T cells (Marks, Ng et al. 2003; Martin, Treiner et al. 2009; Gold, Eid et al. 2012). MAIT cell frequencies are 5 to 10 fold lower in mice than humans, which is the converse of iNKT cells (Treiner, Duban et al. 2005).

MAIT Cell function

A high proportion of transgenic MAIT cells express the natural killer receptor NK1.1, and most have a cell surface phenotype similar to that of Vα14 iNKT cells. They secrete IFN-gamma, IL-4, IL-5, and IL-10 following TCR ligation. There may be two functionally distinct MAIT cell populations; NK1.1+ which can't express IL10 – and are therefore analogous to iNKT cells - and NK1.1- which express high levels of IL10(Kawachi, Maldonado et al. 2006). MAIT cells also produce IFN-γ and Granzyme-B as well as high levels of IL-17 (Dusseaux, Martin et al.).

MAIT cells are believed to play an important role in defence against a range of microbial infections. They can recognise cells infected with bacteria such as *Escherichia coli, Salmonella typhimurium,* and *Staphylococcus aureus*, and mycobacteria, or yeasts, but not viruses (Dong,

Yang et al. 2005; Le Bourhis, Guerri et al. 2011). Gold *et al* showed that even naïve MAIT cells from cord blood can recognise *Mycobacterium tuberculosis* (MTB) infected cells(Gold, Eid et al. 2012), implying they have intrinsic effector function. Gold also showed that MTB-reactive MAIT cells predominate in uninfected individuals, they respond to MTB-infected MR1-expressing lung epithelial cells, decrease in PBMC from subjects with active TB, and were enriched in lung tissue from 2 subjects with pulmonary TB (Gold, Cerri et al. 2010). *In vitro* MAIT cells can inhibit growth of *Mycobacterium bovis* Bacillus Calmette-Guérin (BCG) within macrophages, in a mechanism dependent on IFN-γ (Chua, Truscott et al. 2012). Interestingly in this work MAIT cell responses were not dependent on cognate recognition of MR1 by MAIT cells, but rather on macrophage secretion of IL-12.

MAIT cells in human disease

MAIT cells are abundant in humans and express tissue homing integrins and chemokine receptors. They are common in renal and brain tumours and have been found in a number of inflammatory tissues (Dusseaux, Martin et al. 2011). Their presence in tissues correlates with pro-inflammatory cytokines (Peterfalvi, Gomori et al. 2008).

MAIT accumulate in some lesions in multiple sclerosis (MS), and also in chronic inflammatory demyelinating polyneuropathy (Illes, Shimamura et al. 2004). Whilst iNKT cells are reduced in peripheral blood in MS, Illes *et al* did not find any decrease in MAIT cells, whilst others have observed reduced peripheral MAIT frequencies in MS patients in remission, and particularly in relapse (Miyazaki, Miyake et al. 2011). Conflicting data have been obtained from animal models of experimental autoimmune encephalomyelitis (EAE) with some finding no evidence of MAIT involvement (Yokote, Miyake et al. 2008) and others suggesting that MAIT cells inhibit EAE, and MR1 deficiency increases EAE (Croxford, Miyake et al. 2006).

Some limited data have been obtained from other mouse models of human disease. Data from collagen induced arthritis (CIA), a mouse arthritis model, suggested a pathogenic role of MAIT cells, as knock-out of MR1 ameliorated arthritis, whilst reconstitution with MAIT cells induced severe disease (Chiba, Tajima et al. 2012). Conversely MAIT cells seemed to be protective in a mouse model of inflammatory bowel disease, as adoptive transfer reduced the severity of the colitis (Ruijing, Mengjun et al. 2012).

MAIT cells and the lung

To date MAIT cells have not been studied in the human lung, with two exceptions. Sano *et al* refer to unpublished data from 2003 of RNA from frozen lung biopsies suggested MAIT cells might be present in the lung (Sano, Haneda et al. 1999), whilst Dong *et al* report that MAIT cells have been observed in lung tissue from 2 subjects with pulmonary TB (Dong, Yang et al. 2005). No published data are currently available that characterise MAIT cells in relation to human lung disease.

Vitamin D

Vitamin D is a fat soluble vitamin with pleotropic effects on cell differentiation and function. Vitamin D deficiency has long been known to be associated with increased risk of immune mediated disease or of impaired cell mediated immunity, such as active MTB infection, whilst exogenous 1,25-dihydroxy vitamin D(3) ($1,25(OH)_2D_3$) can suppress T_H1 mediated immune responses (Ooi, Chen et al. 2012). Vitamin D status has been implicated in asthma pathogenesis by genetic associations with the vitamin D receptor and by a number of observational studies, although the results of these have been conflicting. Generally, data suggest that vitamin D is protective against asthma, but firm conclusions will depend on the outcome of prospective clinical trials which are currently ongoing (Paul, Brehm et al. 2012).

Nonetheless, several potential mechanisms have been proposed linking vitamin D with asthma, including direct antiviral properties, enhanced steroid responsiveness and down-regulation of atopy (Paul, Brehm et al. 2012). Of particular relevance to this thesis, it has been shown recently that vitamin D has an effect on the number and functions of innate T cells, specifically iNKT cells. In utero vitamin D deficiency in mice causes a lasting reduction in iNKT cell frequencies in the progeny, due to increased apoptosis of early iNKT cell precursors in the thymus(Yu and Cantorna 2011). Genetic deficiency of vitamin D receptor in mice causes a reduction in iNKT numbers, and impairs the development of experimental airways hyper-reactivity, which can be rescued by adoptive iNKT cell transfer (Yu, Zhao et al. 2011). Vitamin D receptor knockout also affected iNKT cell function, as these cells produced less IL-4, -5, -13 and -17.

These effects have not been investigated in human asthma, and to date there are no published data on the effect of vitamin D on MAIT cell number or function.

The Microbiome

The innate and adaptive responses within a mucosal immune system are intrinsically related to the presence of associated microbial flora. In my thesis I have therefore attempted to characterise these T cell responses in relationship to the airway flora. This sections reviews current knowledge of the nature of the airway microflora.

The term ‘microbiome’ was coined by Joshua Lederberg, to describe the totality of microbes, their genomes, and environmental interactions in a particular environment (Highlander 2012). The emerging use of molecular techniques to identify microbes without the need for traditional culture techniques has led to a recent, intensive effort to characterise distinct microbial flora and anatomical niches of the human microbiome (Costello, Lauber et al. 2009; Nelson, Weinstock et al. 2010). As over 70% of body surface microbes cannot be cultured by standard techniques, culture is no longer considered the gold-standard method for microbial investigation of the

complex microbial populations (Han, Huang et al. 2012). Various molecular techniques have been developed, including fluorescent *in-situ* hybridisation (FISH) with flow cytometry or analysis of terminal restriction fragment length polymorphism (T-RFLP), but these are limited by difficulties in ascribing definitive taxonomies to highly variable communities, or previously undiscovered organisms. Instead the new field of metagenomics depends on high throughput sequencing of entire populations using shot-gun sequencing of whole genomes, a technique which can detect fungi and viruses as well as bacterial RNA (Han, Huang et al. 2012).

The lung microbiome

The use of culture-based techniques led to the traditional teaching that the human lung is sterile in health (Laurenzi, Potter et al. 1961; Pecora 1963). This view has been challenged by the use of culture-independent techniques. Using sequencing of the 16S subunit of ribosomal RNA from bronchial brushings, Hilty *et al* found a mean of 2000 bacterial genomes cm^{-2} in the bronchial tree; a figure comparable to that in the upper small intestine (Hilty, Burke et al. 2010). This group also observed an increased abundance of pathogenic proteobacteria, particularly *Haemophilus* species in asthma compared with health, with a concomitant decrease in bacteroidetes and prevotella species. This study was limited by small numbers (only 13 adult asthmatics), no correlation with clinical or immunological data and the restriction of the technique to the identification of bacterial species only.

A particular challenge to the analysis of the lung microbiome is posed by the relative inaccessibility to direct sampling, compounded by the use of highly sensitive DNA amplification techniques on relatively low biomass samples, leading to a high risk of detecting upper airway or oral contaminants. Charlson *et al* compared the different available sampling techniques using 16S RNA sequencing on oral wash, oropharyngeal swabs, nasopharyngeal swabs, bronchoalveolar lavage and protected bronchial brushing in 6 healthy individuals (Charlson, Bittinger et al. 2011). Their findings suggested that, in contrast to other organ systems, there is no unique lung microbiome in health, but rather bacterial communities are indistinguishable from those of the upper airways, but two to four log lower in biomass. This implies that microbes present in healthy lungs are likely to be the product of microaspiration, rather than the existence of independent communities.

The lung microbiome in cystic fibrosis (CF)

In stark contrast to the situation in health, much research in bronchiectasis, particularly that caused by cystic fibrosis (CF), has shown that complex polymicrobial communities can exist independently in the lung, maintaining remarkable longitudinal stability despite the use of broad spectrum antibiotics. It has long been known from culture techniques that patients with CF acquire infections incrementally over time according to a largely stereotypic sequence, with a relatively limited set of bacterial species, including *Staphylococcus aureus, Haemophilus influenzae* and *Pseudomonas aeruginosa*(Han, Huang et al. 2012). As disease progresses

other opportunistic species such as *Burkholderia cepacia* complex and *Stenotrophomonas maltophilia* are acquired. The use of molecular techniques has revealed a much greater diversity of species, including anaerobic bacteria such as *Prevotella, Veillonella, Propionibacterium* and *Streptococcus milleri*, as well as a majority of unculturable species, with over 60 phylogenetically diverse bacterial genera present. These populations remain fairly unaffected by use of antibiotics (Guss, Roeselers et al. 2011; Daniels, Rogers et al. 2012).

The microbiome in chronic obstructive pulmonary disease (COPD)

In contrast to the microbial diversity observed in CF, the first use of pyrosequencing in BAL and excised lung tissue in COPD showed very limited community diversity (Erb-Downward, Thompson et al. 2011). These data were interpreted to imply a core pulmonary bacterial microbiome including *Pseudomonas, Streptococcus, Prevotella, Fusobacterium, Haemophilus, Veillonella,* and *Porphyromonas*, but as noted this is not consistent with the careful study by Charlson *et al* which used many more sampling techniques and methodological controls (Charlson, Bittinger et al. 2011). Erb-Downard also observed striking micro-anatomic differences in bacterial communities within different areas of the same lung in subjects with advanced COPD (Erb-Downward, Thompson et al. 2011). The longitudinal dynamics of these communities were assessed in a 4 year longitudinal study using molecular typing of sputum from 81 patients with COPD, which revealed that exacerbations were triggered by acquisition of a new strain of *H. influenzae*, *M. catarrhalis*, or *S. pneumoniae*, rather than an increase in absolute bacterial number (Sethi, Evans et al. 2002). It is intriguing to speculate on the source of the microbes, as 16S RNA microarray of cigarettes found 15 different classes of bacteria in cigarettes including many highly pathogenic organisms like *Acinetobacter*, *Bacillus*, *Burkholderia*, *Clostridium*, *Klebsiella*, *Pseudomonas aeruginosa*, and *Serratia* (Sapkota, Berger et al. 2010).

The lung microbiome in asthma

Compared with CF and COPD, much less is known about the microbiome in asthma. The presence of bacteria might be inferred from the innate immune activation seen in neutrophilic asthma (Simpson, Grissell et al. 2007). Using T-RFLP Green *et al* observed *H influenza, Moraxella* or *Streptococcus* in induced sputum from 21/28 severe asthmatics (Green, Kehagia et al. 2008). In the absence of significant upper airways contamination, cultivable bacteria represented only 0.1-20% of species, but these three species were the dominant organism in over half of those colonized. Bacterial colonisation was associated with higher neutrophil count, longer history of asthma and worse lung function. Another study of 42 poorly controlled asthmatics using 16S RNA phylochip sequencing on bronchial brushes found a greater bacterial burden and diversity in asthma compared with health (Huang, Nelson et al. 2011). When these subjects were treated with clarithromycin, greatest clinical response correlated with greater pre-treatment bacterial airway diversity.

By what mechanisms might bacteria drive immunopathology in asthma? In the study by Huang, 100 taxa, mostly proteobacteria, were associated with bronchial hyper-responsiveness. Some of these species might drive asthma through idiosyncratic mechanisms. For instance they observed a *Nitrosomonas* species which can generate nitric oxide, and a *Comamonadaceae* species which can degrade steroids (Huang, Nelson et al. 2011). Likewise, several studies have provided evidence that nasal carriage of *Staphylococcus* may drive IgE mediated inflammation through a super-antigenic effect (Bachert, Gevaert et al. 2003; Bachert, Gevaert et al. 2007), whilst others have implicated *Chlamydia pneumoniae* infection in severe asthma(Black, Scicchitano et al. 2000; ten Brinke, van Dissel et al. 2001; Biscione, Corne et al. 2004; Harju, Leinonen et al. 2006).

In summary, study of the microbiome in asthma is as yet a nascent field, and to date few studies have attempted to apply metagenomic techniques to correlate systematically the microbiome with clinical and immunological metrics across a range of clinical phenotypes. Also, no studies have attempted to integrate flow cytometric or microarray assessment of the innate or adaptive immune system with unbiased analyses of the human lung metagenome.

Objectives

The primary goal of the work presented in this thesis was to elucidate the role of IL-17 and T_H17 cells in relation to asthma severity and virus-induced asthma exacerbations relative to other key $CD4^+$ T lymphocyte subsets, namely T_H1 and T_H2 effector T-cells and regulatory $CD4^+CD25^+FOXP3^+$ Treg, as well as the less-researched cytotoxic T cells and the novel mucosal associated invariant T cell subset (MAIT).

This goal was undertaken with the aim of improving characterisation of severe asthma *versus* milder forms of asthma, thereby facilitating future progress in basic and applied research (Anderson 2008). Moreover it was hoped this would deepen our understanding of the role of IL-17 in the pathogenesis of asthma and host responses to respiratory virus infections, the potential identification of new biomarkers for asthma phenotypes (Gibson 2007) and new targets for pharmacological intervention. This goal was pursued through the investigation of two distinct cohorts in two separate aims.

Aim 1

My initial aim outlined in my successful application for the Wellcome Trust Clinical Training Fellowship, was to provide a detailed phenotypic characterisation of IL-17-producing cells in the airways of mild, moderate and severe asthmatics.

My hypotheses were that:

i) The number of T_H17 cells is raised in more severe forms of asthma.

ii) Dysregulation in the T_H17:Treg balance would be associated with, and help define, the severe neutrophilic asthma phenotype;
iii) Pulmonary TCRαβ+ T_H17 cells would be a major primary cell source of IL-17 in severe human asthma, while TCR$\gamma\delta$+ T-cells would also contribute;
iv) T_H17 cells would be enriched within the lung compartment and be predominantly localised to the bronchial epithelium;
v) T_H17 cells would be correlated with chronic virus infection (Wos, Sanak et al. 2008) and airway bacterial colonisation(Simpson, Grissell et al. 2007; Simpson, Powell et al. 2008).

Aim 2

Within Aim 2, I planned a longitudinal investigation into the dynamics of the T_H17 response during naturally occurring virally-induced exacerbations of asthma, to determine whether IL-17 is induced during naturally occurring asthma exacerbations, leading to neutrophilic infiltration.

In conjunction with a phase II, double-blind, randomised, placebo-controlled trial of inhaled recombinant human (rh)IFN-β1b given at the onset of a common cold to asthmatic patients with the aim of preventing/ameliorating an exacerbations, I undertook longitudinal follow-up of a well characterised cohort of asthmatics with frequent exacerbations. This allowed me to study how T_H17 cells change during virus infections and associated asthma exacerbations as well as to elucidate how treatment with IFN-β influences T_H17 function.

My hypotheses were that:

i) Airway accumulation of T_H17 cells would occur early in infection, leading to neutrophilia, followed by a T_H1 dominant response.
ii) Acute infection would be associated with a decrease in Treg frequency, which would be more marked in asthma.
iii)Administration of inhaled rhIFN-β1b would inhibit the magnitude of the T_H17 response to viral infection measured in PBMC and airway samples.

Aim 3.

My aim was to perform the first analysis of MAIT cells within the human airways by observing their frequencies in peripheral blood and in airway tissues in relationship to disease severity and phenotype and to characterise their functional capabilities.

My hypotheses were that:

i) MAIT cells would be present in the human airways and concentrated in the airway mucosa.
ii) MAIT cells would display pro-inflammatory effector function as judged by their expression of cytokines.

iii) MAIT cell frequencies might be modulated by treatment with exogenous corticosteroids.

iv) MAIT cell frequencies might vary following a seasonal pattern, possibly influenced by variation in levels of vitamin D

Aim 4

My aim was to analyse the microbial metagenome in health and asthma to determine whether asthma, particularly severe, steroid-resistant phenotypes, were associated with increased detection of specific airway bacteria, or increased detection of respiratory viruses. I hypothesised that:

i) Severe, steroid-resistant asthma would be associated with increased detection of pathogenic airway bacteria including *Haemophilus influenzae*, *Streptococcus pneumoniae* and *Moraxella catarrhalis*.

ii) Asthma may be associated with increased detection of viral genomes suggestive of chronic viral infection or delayed viral clearance.

CHAPTER 2

Materials and methods

Nullius in verba [2]

[2] Motto of the Royal Society, chosen from Horace's Epistles to signify the Fellows' determination to establish facts via experiments. It may be translated 'Take nobody's word for it'.

The objective of my thesis was to study the role of distinct T cell subsets in asthma in relation to disease severity and virally-induced exacerbations. I therefore needed the following techniques: a range of clinical measurements to obtain detailed clinical phenotyping of subjects, schemas by which to classify participants according to a range of measures of asthma severity and techniques to obtain clinical specimens of peripheral blood, sputum, bronchoalveolar lavage (BAL), bronchial epithelial cells and bronchial biopsies for immunological and microbiological analysis. To enumerate T cells accurately I needed to be able to maintain them in cell culture, stimulate them *ex vivo* and semi-quantify cytokine production using intracellular cytokine staining. In order to characterise the transcriptome of individual cell types I needed to sort cell populations, extract ribonucleic acid from cells and analyse by quantitative polymerase chain reaction (qPCR) and/or microarray hybridisation. In this chapter I will describe the methods used and will highlight those which I first needed to develop in order to conduct the study.

Study design

This study comprised two components. The first component was a cross-sectional study in which healthy volunteers and asthmatic subjects across a range of clinical phenotypes and disease severity were compared by assessment of clinical and immunological parameters, undergoing phlebotomy, sputum induction and bronchoscopy during periods of clinical stability (Figure 2.1). In addition subgroups of subjects underwent repeated sampling after one week of inhaled or oral corticosteroids. The second component was a longitudinal study in which I used the opportunity provided to me by a clinical trial where IFN-β1α was studied for its effects on preventing or attenuating exacerbations caused by upper respiratory tract infections (URTIs)(Figure 2.2). Subjects were sampled by phlebotomy and sputum induction at baseline and at a further 7 time-points from the onset of the next symptomatic URTI.

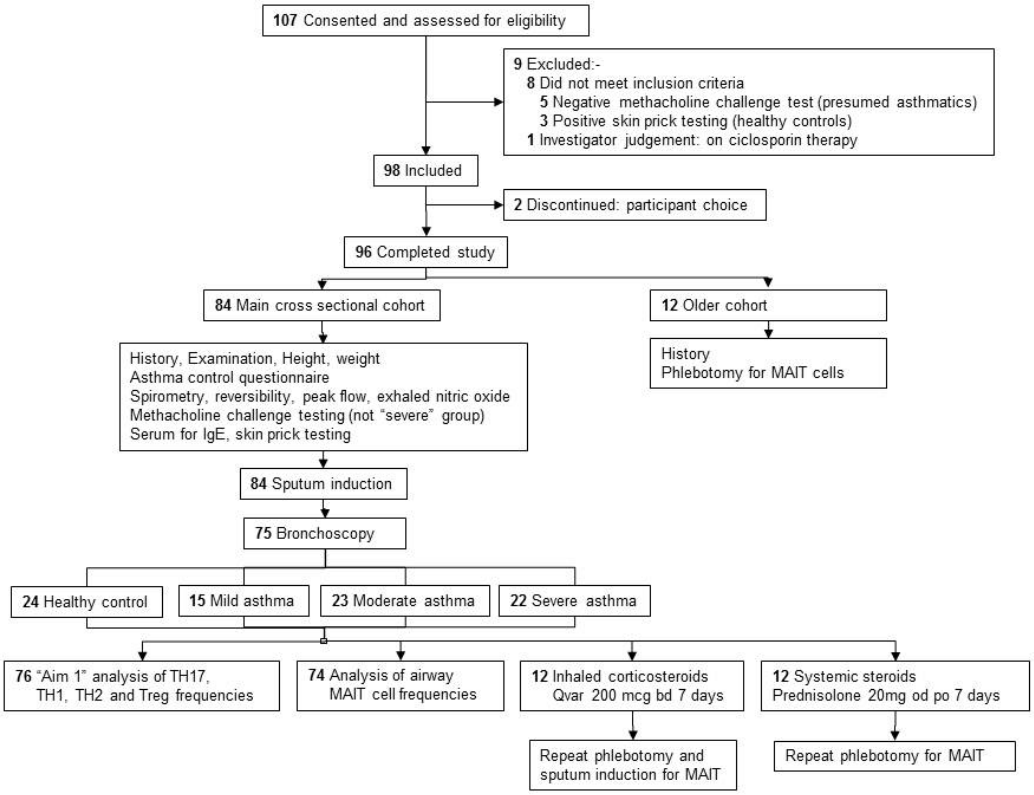


Figure 2.1 Cross sectional study flow diagram

Flow diagram showing study design and recruitment for the cross sectional cohorts (Aim 1).

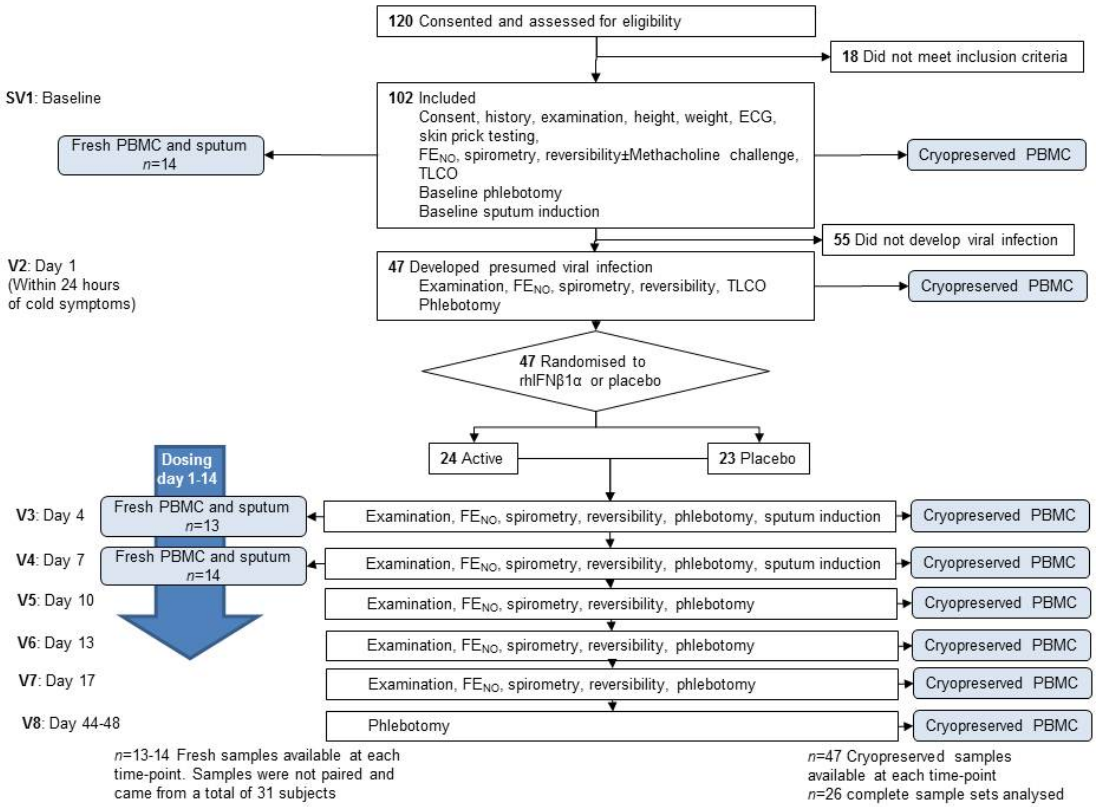


Figure 2.2 Longitudinal study flow diagram

Flow diagram showing study design and recruitment for the longitudinal cohorts (Aim 2). Subjects were recruited from SG005, a multicentre, multinational study, involving 26 sites, but samples gifted to me all came from the Southampton site alone. 120 subjects were screened for eligibility. 47 subjects developed exacerbations of asthma and were randomised to treatment, but of these samples were available only from a subset for immediate ("fresh") analysis of sputum and PBMC at 3 time-points (n=13-14, unpaired samples at each time-point) or analysis of cryopreserved PBMC at 8 time-points (n=26).

Clinical measurements

Peak flow

Peak expiratory flow rates (PEFR) were measured using a European Standard mini wright peak flow meter (EN 13826, Clement Clarke, Harlow, UK), with the subject standing. The highest of three technically acceptable blows were recorded. European Community for Coal and Steel 1993 predicted values were used(Quanjer, Tammeling et al. 1993).

PEFR Variability was measured using twice daily monitoring over 2 weeks and defined as (max PEFR – min PEFR)/max PEFR x 100, expressed as a percentage according to British Thoracic Society (BTS) guidelines (2008); ≥20% variability was considered significant.

Spirometry and reversibility

Spirometry was performed using Vitalograph dry wedge bellows (Vitalograph, Maids Moreton, UK) and the following values calculated: forced expiratory volume in 1 second (FEV_1), forced vital capacity (FVC) and forced expiratory ratio (FER or FEV_1/FVC). The best of 3 technically acceptable manoeuvres were recorded where values were not ≥0.150L different between the largest and the next largest FEV_1/FVC results and were within 5% of each other (whichever was greater), according to European Respiratory Society (ERS) guidelines (Miller, Hankinson et al. 2005).

Bronchodilator reversibility was tested using spirometry before and 12-15 minutes after administration of salbutamol 400 mcg using a pressurised metered dose inhaler (pMDI) with a Volumatic device (Allen and Hanbury's, UK) or 2.5mg salbutamol via oxygen driven nebuliser, according to ERS guidelines (Miller, Hankinson et al. 2005). Reversibility was defined as (post bronchodilator FEV_1 - Pre bronchodilator FEV_1)/Pre bronchodilator FEV_1 x 100, with a 12% increase considered significant(Goldstein, Veza et al. 2001), according to BTS guidelines(2008).

Home monitoring

During the longitudinal study home monitoring of PEFR, FEV_1 and FVC were performed using a Mini-Wright Digital (Clement Clark, Harlow, UK) and downloaded intermittently using the manufacturer's software (MWD Soft 1.73).

TLCO

The transfer factor of lung carbon monoxide (TLCO) was measured using a single breath diffusion method with a Morgan CPL device (Morgan Scientific, Haverhill, MA, USA) and expressed in $mmolmin^{-1}kPa^{-1}$ using the Jones-Mead method with a 10 second (+/- 2 seconds) breath hold time using the machine's standard haemoglobin value and using the European predicted values the mean of the two repeatable TLCO values, within 10% of each other. The TLCO was obtained from the product of the two primary measurements: transfer coefficient (Krogh's K_{CO}) and alveolar volume (V_A) according to Krogh (Krogh 1915).

Exhaled nitric oxide

Exhaled nitric oxide (eNO or FE_{NO}) was measured with a single breath at a flow rate of 50 mL/s using a Niox Mino device (Aerocrine Inc, Princeton, NJ, USA), with an upper limit of normal for adults of <25 ppb.(Taylor, Pijnenburg et al. 2006)

Methacholine challenge testing of airway hyper-responsiveness

Airway hyper-responsiveness (AHR or bronchial hyper-reactivity (BHR)) was measured in all patients except those with severe asthma, or those with recent historical data available. Spirometry and dosing were performed with a Viasys APS system (CareFusion UK, Basingstoke, UK) using a fixed concentration of 32mg/ml methacholine, over the dose range 0.0256 mg – 1.434 mg. Results were reported as the non-cumulative provocative dose of methacholine causing a 20% fall in the FEV_1 ('non-cumulative PD20'), which is automatically calculated using logarithmic interpolation (Schulze, Rosewich et al. 2009).

Historical data were accepted if performed within 1 year for asthmatics or 5 years for healthy controls. In six instances provocative concentration (PC20) data only were available rather than PD20 data and were converted to an approximate equivalent PD20 value by linear regression. In six healthy individuals negative bronchial challenges had been performed with histamine and in each instance a 20% fall in FEV_1 was not achieved with >8mg histamine.

Interpretation: methacholine challenge testing has a specificity of 90 to 95% and a sensitivity of 60-100% for detecting physician-diagnosed asthma (Soysal, Bahceciler et al. 2008). PD20 values were interpreted according to the ATS categorisation of bronchial responsiveness (Schulze, Rosewich et al. 2009) as follows:

Table 2.0

Non-cumulative methacholine PD20 (mg)	**Interpretation**
>1.0	Normal bronchial response
0.6-1.0	Borderline BHR
0.3-0.6	Mild BHR
<0.3	Moderate-severe BHR

Skin prick allergen testing

Skin prick allergen testing (SPT), which is a functional assay of specific IgE responses on mast cells in the skin and is associated with type 1 hypersensitivity (Gould, Sutton et al. 2003), was measured with a panel of common aeroallergens. Allergen solutions used were aspergillus fumigatus, candida albicans, mixed grass pollen, dermatophagoides pteronyssinus, dermatophagoides farina

(respectively European and American house dust mites) feathers, cat and dog (Allergopharma, Reinbek, Germany). Some historical data contained results for mixed tree pollen and for alternaria tenuis. Positive and negative controls were histamine solution and carrier solution respectively. A small drop of each solution was placed on the skin of the volar aspect of the lower forearm after it had been cleaned with water and dried. Disposable sterile lancets (Allergopharma) were used to break the dermis under each drop in turn at a 90˚ angle and the diameter of the weal measured in two perpendicular directions after 15 minutes. A positive result was recorded if the weal was 3mm > result of negative control.

Study populations

The study was approved by the National Research Ethics Service Committee South Central – Southampton B ethics committee (Ref 10/H0504/2). Subjects were recruited who were willing to participate and who met the following specific inclusion criteria, dependent on cohort (see Figure 2.1):

Cross sectional study (Aim 1)

General inclusion criteria

- Able to provide written informed consent.
- Aged 18-70.
- Vital signs; at the discretion of the Investigator.
- Motivation to complete all of the study visits and ability to communicate well with the investigator and be capable of understanding the nature of the research and its treatment including risks and benefits.

Inclusion criteria for healthy, non-atopic, non-asthmatic controls

- All of the general inclusion criteria above.
- Absence of atopy on skin prick allergen testing.
- Absence of bronchial hyper-responsiveness: methacholine PD20 >1.0 mg.
- Not a current smoker.

Inclusion criteria for steroid-responsive mild or moderate asthmatic subjects

- All of the general inclusion criteria above.
- A clinical diagnosis of asthma.
- Presence of atopy on skin prick allergen testing.
- Best described as mild or moderate asthma by the definition in table 2.2.

Inclusion Criteria for Severe Asthmatic Subjects

- All of the general inclusion criteria above.
- A clinical diagnosis of asthma.
- Best described as “severe asthma” by the definition in table 2.2.

Exclusion criteria

- Unable to provide written informed consent.

- Pregnancy either current or planned over the duration of the study.
- Prisoners.
- Children under age 16.
- Lung disease other than asthma.

Additional older healthy controls

Subjects aged 40-70 years, able to provide written informed consent, with no history of atopy, asthma or other lung disease.

Longitudinal study (Aim 2a)

Samples of blood and sputum were gifted to me by Synairgen Research Ltd from the trial SG005 "A randomised, double-blind, placebo-controlled Phase II study, comparing the efficacy and safety of inhaled SNG001 to placebo administered to asthmatic subjects after the onset of a respiratory viral infection for the prevention or attenuation of asthma symptoms caused by respiratory viruses" (NCT01126177)(See Figure 2.2) Full inclusion criteria are published available from the U.S. National Institutes of Health (SynairgenResearchLtd 2012). Briefly, all subjects were aged 18-65 years with a history of asthma for at least 2 years and confirmed by bronchodilator reversibility or bronchial hyper-responsiveness or an exacerbation requiring medical review or hospital admission and who were treated with regular inhaled corticosteroids and had a previous history of virus-induced exacerbations. In addition to these criteria for baseline visits, to be randomised for inclusion in the exacerbation study subjects needed to have been experiencing respiratory virus symptoms within the previous 24 hours, being either cold symptoms (a blocked or runny nose, or sore throat) or influenza-like illness (fever >37.8℃ plus two of headache, cough, sore throat or myalgia).

Clinical classification

Disease heterogeneity is increasingly recognised in asthma, with a recognition that there is a need to elucidate distinct disease endotypes(Anderson 2008). Accordingly I have attempted to analyse data using a variety of dimensions including continuous variables such as lung function (e.g. FEV_1) or symptom scores (e.g. ACQ) or treatment (e.g. step on the BTS treatment algorithm, see Figure 2.3), or level of asthma control, (see table 2.1) or according to inflammatory subtype based on sputum cell differentials (see Definitions of inflammatory subtypes).

Asthma control questionnaire

Subjects were phenotyped along a scale of disease control according to the well validated asthma control questionnaire (ACQ) (Juniper, O'Byrne et al. 2000) which combines subjective self-assessments of disease control with objective assessment of lung function into a global score. in a study of 1323 individuals Juniper *et al* have shown that the crossover point between 'well-controlled' and 'not well-controlled' is close to 1.00 on the ACQ, whilst a cut-point of 1.50 can be used to confidently predict inadequately controlled asthma with a positive predictive value of 0.88 (Juniper, Bousquet et al. 2006).

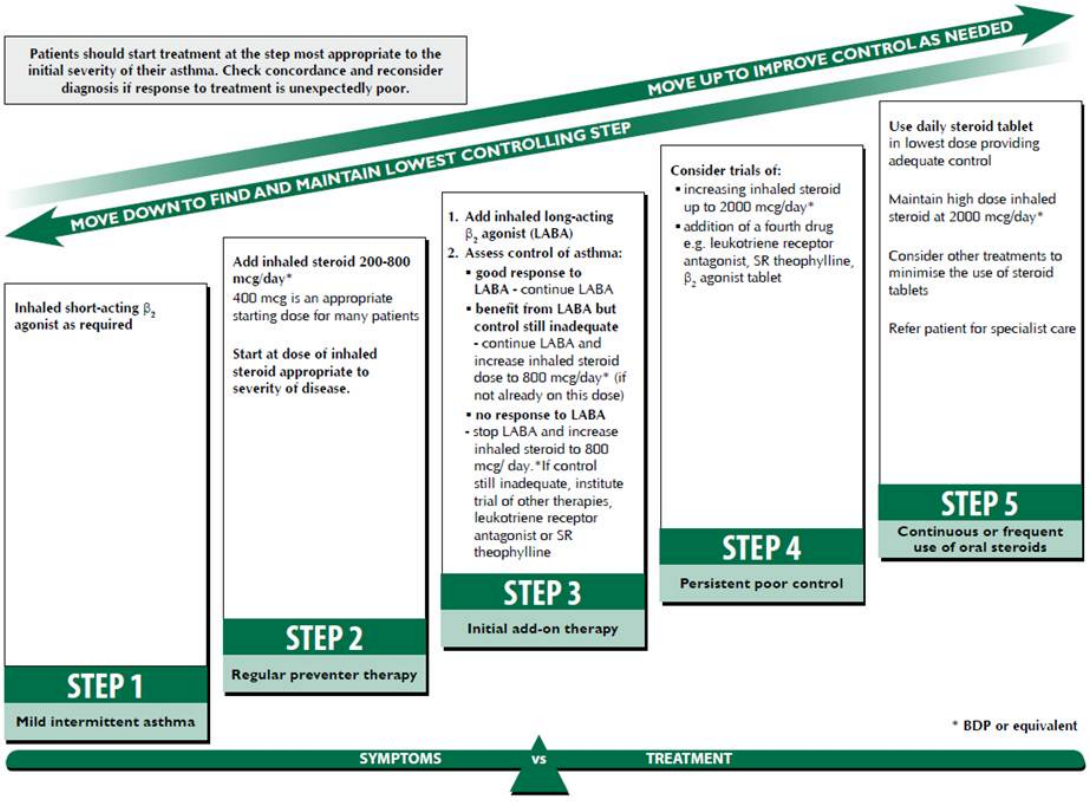


Figure 2.3 BTS treatment algorithm

British Thoracic Society treatment algorithm for asthma in adults. From (**2008**) p iv42 “Summary of stepwise management in adults”.

ASTHMA CONTROL QUESTIONNAIRE

The ACQ consisted of the following questions and instructions:

Circle the number of the response that best describes how you have been during the past week.

1. On average, during the past week, how often were you **woken by your asthma** during the night?	0. Never 1. Hardly ever 2. A few times 3. Several times 4. A great many times 6. Unable to sleep because of asthma
2. On average, during the past week, **how bad were your asthma symptoms when you woke** up in the morning?	0. No symptoms 1. Very mild symptoms 2. Mild symptoms 3. Moderate symptoms 4. Quite severe symptoms 5. Severe symptoms 6. Very severe symptoms
3. In general, during the past week, how **limited were you in your activities** because of your asthma.	0. Not limited at all 1. Very slightly limited 2. Slightly limited 3. Moderately limited 4. Very limited 5. Extremely limited 6. Totally limited
4. In general, during the past week, how much **shortness of breath** did you experience because of your asthma?	0. None 1. Very slightly limited 2. A little 3. A moderate amount 4. Quite a lot 5. A great deal 6. A very great deal
5. In general, during the past week, how much of the time did you **wheeze**?	0. Not at all 1. Hardly any of the time 2. A little of the time 3. A moderate amount of the time 4. A lot of the time 5. Most of the time 6. All of the time
6. On average, during the past week, how many **puffs of short-acting bronchodilator** (e.g.Ventolin) have you used each day?	0. None 1. 1-2 puffs most days 2. 3-4 puffs most days 3. 5-8 puffs most days 4. 9-12 puffs most days 5. 13-16 puffs most days 6. More than 16 puffs most days

Point 7 of the ACQ is completed by the investigator based on pre-bronchodilator FEV_1.
0, 95% predicted; 1, 95–90% 2, 89–80%; 3, 79–70%; 4, 69–60%; 5. 59–50%; 6. 50%

Table 2.1 Levels of asthma control, GINA ((GINA) 2010)

Characteristic	Controlled	Partly controlled	Uncontrolled
Daytime symptoms	None (twice or less/week)	More than twice/week	Three or more features of partly controlled asthma present in any week
Limitation of activities	None	Any	
Nocturnal symptoms / awakening	None	Any	
Need for reliever / rescue treatment	Non (twice or less/week)	More than twice/week	
Lung function (PEF or FEV_1)	Normal	<80% predicted or personal best (if known)	
Exacerbations	None	One or more/year	One in any week

I have also attempted to use cluster analysis to identify distinct endotypes based on a more objective synthesis of all these distinct, interacting factors. I have also stratified subjects into distinct clinical phenotypes by considering asthma as a single disease along a continuous spectrum of severity, according to the following scheme which is based on a global assessment of disease severity and largely derives from the GINA classification of asthma severity ((GINA) 2010).

Table 2.2 Definitions of asthma severity used in this project

Mild intermittent asthma	Mild asthma
Symptoms once a week Nocturnal symptoms not more than twice a month • FEV_1 or PEF ≥ 80% predicted Treatment: • Salbutamol as needed only PD_{20} is needed for these people to ensure correct diagnosis. If borderline, the result has to be interpreted in context with the history.	
Mild persistent asthma	
Symptoms > once a week but < once a day Nocturnal symptoms < twice a month • FEV_1 or PEF ≥ 80% predicted Treatment: • Salbutamol as needed only	
Moderate persistent but well controlled asthma	Moderate asthma
Symptoms <3x/week Nocturnal symptoms <twice a month • FEV_1 or PEF ≥ 80% of predicted or of patient's best Treatment: • Salbutamol as needed only • Low-dose (<800 µg BDP equivalent) inhaled steroids • +/- Long acting beta-2-agonist	
Moderate persistent but not well controlled asthma	
Symptoms >3x/week Nocturnal symptoms >twice a month (some may not have nocturnal symptoms) • FEV_1 or PEF <80% of predicted or patient's best Treatment: • Salbutamol as needed only • Low-dose inhaled steroids • +/- Long acting beta-2-agonist	
Severe asthma	Severe asthma
Symptoms daily Nocturnal symptoms >once a week Daily use of inhaled short-acting ß2-agonist • FEV_1 or PEF <80% of predicted or patient's best Treatment: • High-dose (at least 800 µg of BDP equivalent) inhaled steroids • Long acting beta-2-agonist • +/- frequent or continuous oral steroids	

Notes: where patients do not fit neatly into any category they are considered on an individual basis to make the best possible fit. Not all criteria have to be fulfilled for any of these categories.

Phlebotomy

Serum

Samples for serum were obtained using the 21 gauge BD Vacutainer® Safety-Lok™ blood collection set (367282, BD, Plymouth, UK) into 10 ml BD Vacutainer® serum tubes (367895, BD) and allowed to clot in an upright position at room temperature (RT)RT for ≥30 minutes. Samples were then centrifuged at 1653*g* for 10 minutes and supernatants aspirated and frozen in 1 ml aliquots at -80℃ until required.

Full blood count

Samples for full blood count were taken into 3ml EDTA tubes (367838, BD) and processed in the National Health Service laboratory by automated cytometers.

Peripheral blood mononuclear cell preparation

Blood for isolation of peripheral blood mononuclear cells (PBMC) was obtained into 6 ml lithium heparin tubes (367885, BD). Dulbecco's phosphate buffered saline with no $Mg2^{+}$ or $Ca2^{+}$ (PBS, D8537, Sigma-Aldrich, Gillingham, UK) was warmed and added to the heparinised blood in a 1:1 ratio in 50ml falcons. This mixture was carefully layered over Lymphoprep™ (Nycomed/Axis-Shield PoC, Rodeløkka, Norway) or Ficoll-Paque™ (17-1440-03, GE Healthcare, Uppsala, Sweden) in the ratio 20ml lymphoprep:30ml blood/PBS mixture) and centrifuged at 800*g* for 30 mins at 20°C with the brake off. The buffy coat layer was aspirated using a pastette, into a fresh 50ml falcon and washed twice with 50ml with PBS and centrifugation at 400*g* for 5 mins. 10µL of cells were removed for counting in trypan blue (T8154, Sigma) with a Neubauer haemocytometer (Marienfeld, Lauda-Königshofen, Germany). Cells were then resuspended at appropriate concentrations in culture medium or PBS as required.

Cell preparation tubes

To minimise lab processing time, blood samples from the longitudinal study were taken directly into cell preparation tubes (362780, BD) which contain sodium heparin and Ficoll. Tubes were inverted 8-10 times, then centrifuged at 1650*g* for 20 minutes with the brake off. Using a pastette the top half of plasma layer was aspirated without disturbing the mononuclear cell and platelet layer and the mononuclear cell layer transferred to a fresh 15ml falcon. Samples were washed twice with 15ml warmed Roswell Park Memorial Institute medium 1640 without L-glutamine or phenol red (RPMI, R7509, Sigma-Aldrich, Gillingham, UK or Lonza/Biowhittaker, Basel, Switzerland) and with centrifugation at 400*g* for 5 mins.

Nasal lavage

Nasal lavage was performed during the longitudinal study using reusable metal nasal olives and a 5ml syringe. The subject was asked first to blow their nose, then sit upright with head slightly forward

whilst 2.5ml of normal saline was gently introduced and withdrawn 5 times, before being collected with a funnel into a sterile universal container and repeating in the other nostril with a further 2.5ml.

Sputum induction

Sputum induction protocol

Sputum induction was performed using 4.5% hypertonic saline, except in higher risk patients, according to an established protocol (Djukanovic, Sterk et al. 2002). Baseline spirometry and salbutamol reversibility testing were performed, then subjects inhaled 4.5% hypertonic saline for 5 minutes, using an ultrasonic device (DeVilbiss UltraNeb, Tipton, UK) in an environmental chamber (Protex, Halifax, UK). Subjects were advised to maintain normal tidal breathing and wore a nose clip. After 5 minutes FEV_1 were recorded and any sputum expectorated, before the procedure was repeated a further three times, unless an adequate sample were obtained or FEV_1 fell by 20%. The chance of a successful sputum induction was maximised by encouraging good hydration and using the technique of autogenic postural drainage.

An alternative protocol for high risk subjects was used if post-bronchodilator FEV_1 was < 60% of predicted or <1.5L or there was a history of severe asthma, or highly reactive airways. In this protocol subjects received 0.9% saline for 0.5, 1, 5 mins then 3% saline for 0.5, 1, 2 mins, then 4.5% saline for 0.5, 1, 2, 4, 8 minutes(Djukanovic, Sterk et al. 2002).

Sputum processing

Sputum samples were kept in a petri dish on ice and forceps used to select mucus plugs for transfer into a falcon tube. Samples were weighed and diluted with four volumes of the reducing agent 1,4 dithioerythritol (DTE) in solution (5mM DTE(Sigma), 28.8mM HEPES buffer (Lonza), 30mM NaCl) and placed on a bench rocker for 30 mins, with intermittent homgenisation with a pastette. Mucus was removed with a 100µm filter to remove mucus and filtrate centrifuged at 400*g* for 10 mins to pellet cells. Aliquots of supernatant were ultracentrifuged at 12000*g* for 5 minutes to precipitate bacteria and then frozen at -80℃ and cells resuspended in appropriate medium.

Preparation of cytospins

Twinfrost glass microscope slides (CellPath, Newtown, UK) were pre-coated with poly-L-lysine (Sigma) by immersion in 0.01% (w/v) poly-L-lysine in distilled water for 5 mins, then air dried at RT overnight. Slides were labelled and placed in a cage with a cytofunnel and hole-punched filter paper in a Shandon Cytospin centrifuge. Sputum or bronchoalveolar lavage cells were resuspended at a concentration of $1x10^6$ cells/ml in PBS or culture medium and 70 µL per slide transferred to the cytofunnel, then centrifuged at 450 revolutions per minute (rpm) for 6 minutes.

Slides were then air dried for at least 24 hours and stained with a rapid Romanowsky stain(Jorundsson, Lumsden et al. 1999) (Raymond Lamb, Eastbourne, UK): slides were immersed in methanol fixative for 30 seconds, blotted, stained for 30 seconds in eosin, blotted, stained for 1

minute in methylene blue, then rinsed in running tap water and allow to air dry. Once dry slides were mounted with Pertex (Cell Path) and a coverslip and 400 cells counted manually, recording eosinophils, neutrophils, macrophages/monocytes, lymphocytes and columnar epithelial cells. In addition numbers of squamous cells were noted but not included in the differential. A count of more than 30% squamous cells was suggestive of significant upper airway contamination (Hadjicharalambous, Dent et al. 2004; Singh, Edwards et al. 2010). Cytospin staining and differential counts were performed by my colleague Jon Ward.

Definitions of inflammatory subtypes

For the purposes of this work the following definitions were used for subtypes of asthma.

Inflammatory subtypes	Definition (based on sputum cell differential)
Neutrophilic asthma	≥61% neutrophils
Eosinophilic asthma	≥3% eosinophils
Mixed granulocytic asthma	≥3% eosinophils and ≥61% neutrophils
Paucicellular asthma	<3% eosinophils and <61% neutrophils

This definition of neutrophilic asthma is widely accepted (Simpson, Scott et al. 2006; Haldar and Pavord 2007; Simpson, Grissell et al. 2007; Cowan, Cowan et al. 2010), as is the division of asthma into these four inflammatory subtypes according to sputum cytospin cell differentials. However there is less agreement over the definition of sputum eosinophilia, with different authors choosing cut-offs of 1% (Simpson, Scott et al. 2006), 2% (Jayaram, Pizzichini et al. 2006), and 3% (Green, Brightling et al. 2002; Green and Pavord 2012). I have chosen to use a 3% cut-off because the normal range of sputum eosinophils in adults and children is <2.5% (Spanevello, Confalonieri et al. 2000; Kips, Inman et al. 2002) and because it has been shown that a 3% cut-off identifies individuals with corticosteroid-responsive asthma (Pavord, Brightling et al. 1999; Green, Brightling et al. 2002).

Bronchoscopy

Bronchoscopic technique

Bronchoscopies were performed using Pentax video colour CCD flexible bronchoscope (Pentax UK, Slough, UK) in a purpose-built research endoscopy suite with the assistance of at least 2 nurses and a laboratory technician and in accordance with the BTS guidelines current at the time (2001) and with established research protocols(Jarjour, Peters et al. 1998). Briefly, subjects who had been starved for at least 4 hours underwent routine physical examination and routine measurement of vital signs, spirometry and reversibility(1991), including premedication with 2.5 mg of nebulised salbutamol. An intravenous cannula (Biovalve, Vygon, Swindon UK) was inserted and the procedure performed under light sedation with alfentanyl 0-1000 mcg and / or midazolam 0-10 mg with continuous pulse oximetry. The analgesia and suppression of gag and cough reflexes was achieved with 6-8 sprays (60-80 mg)

of 10% lidocaine orally, 2-5 ml of Instillagel (CliniMed, High Wycombe, UK) nasally, 6 ml of 2% lidocaine to the vocal cords and 5-10 ml of 1% lidocaine to the bronchial tree.

Bronchial brushings were obtained from right and left lower and middle lobe 1^{st}-3^{rd} order bronchi by gentle brushing using sterile 2 mm disposable cytology brushes (BC-202D-2010, Olympus UK, Southend-on-Sea, UK) and samples gently agitated in 5ml of ice-cold PBS. Brushings were taken prior to other samples to minimise contamination with blood which would decrease the cellular purity of samples and can impair growth of primary bronchial epithelial cells *in vitro*.

Bronchoalveolar lavage (BAL) was performed in the right upper lobe, usually in the posterior segment, as the anatomy favours good recovery volumes. Where possible sample contamination from the bronchoscopes was minimised by taking some or all of the BAL through a sterile, 2mm diameter disposable protected catheter (Combicath, ConMed Linvatech, Swindon, UK). After anaesthesia of the relevant lobe the catheter was passed through the bronchoscope and the inner catheter fully inserted then removed, to expel the wax seal. The outer catheter was then placed into the bronchus during the wedge and the lavage taken through the outer catheter using 6 x 20 ml warmed, sterile normal saline, injecting and aspirating with 20 ml syringes. The first 1 ml of BAL recovered was dispensed into a sterile eppendorf and stored at -80℃ for later microbiological analysis. In some instances the complete lavage could not be performed through the Combicath and the procedure was converted to a standard technique at this stage.

Up to 10 bronchial biopsies were taken from 1^{st}-4^{th} order carinae using 1.8 mm alligator cup biopsy forceps (100503, ConMed) and samples gently transferred to ice-cold RPMI.

After the procedure subjects were observed for at least 60 minutes, before having their swallow tested and repeat spirometry performed.

See Figure 2.4 for overview of sample processing.

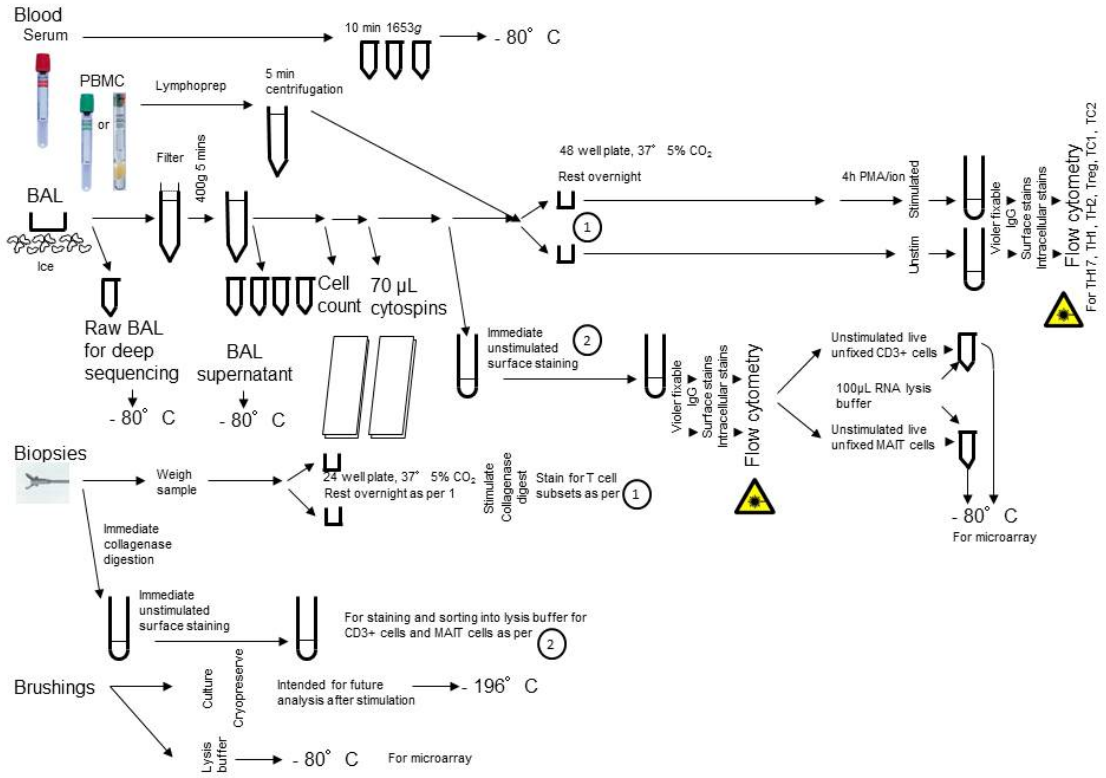


Figure 2.4 Sample processing

Flow diagram showing the processing of samples taken at a bronchoscopy visit in the cross sectional study. Not shown are additional sputum processing steps performed at a separate visit which occurred ≥1 week before or 4 weeks after the bronchoscopy visit.

Processing of BAL

Bronchoalveolar lavage was transferred to the laboratory on ice then, except the aliquot kept for microbiological analysis, was strained through 100µm cell strainer and centrifuged at 400*g*, 5 mins, at 4°C. Aliquots of supernatant were ultracentrifuged at 12000*g* for 5 minutes to precipitate bacteria and then frozen at -80℃ and cells resuspended in appropriate medium. If samples were heavily blood-stained red blood cells were haemolysed by resuspending in 4.5ml sterile water for 30 seconds, followed immediately by addition of 0.5ml 10x Hank's balanced salt solution (HBSS, Gibco) and made up to 50 ml with PBS, before centrifuging again at 400*g* for 5 mins. Next, cells were resuspended in 1ml AIM V, 10 µL removed for viability counts and the concentration adjusted to $1x10^6$ cells/ml for culture or immediate staining for flow cytometry. 2x70 µL of cell suspension were removed to produce cytospins as previously described.

Processing of bronchial biopsies

Biopsies were transferred to the laboratory on ice, washed in fresh RPMI to remove residual blood, weighed in pre-weighed eppendorf tubes and transferred using pastettes either into pre-warmed AIM

V medium for overnight culture or pre-warmed collagenase solution for immediate staining for flow cytometry.

Collagenase digestion of biopsies

Biopsies were dispersed in 2.5ml of type 1 collagenase from *Clostridium histolyticum*, (1mg/ml C0130, Sigma) reconstituted in RPMI for 1hr at 37°C with magnetic stirring. Cells were then passed through a 70μm filter, centrifuged at 400*g* for 5 mins and resuspended in PBS for immediate staining.

This method was originally described for isolation of T cells from adipose tissue, where it was shown that type 1 collagenase is superior to other enzyme preparations - collagenase IV and liberases which are a blend of collagenase I and II with a neutral protease or dispase - with respect to cell viability, yield and preservation of cell surface markers (Hagman, Kuzma et al. 2012). These authors also showed that the optimal time for digest was 60-75 mins because longer digests caused significantly more loss of all surface markers tested(Hagman, Kuzma et al. 2012). This protocol has subsequently been used in our group for dispersion bronchial biopsies (Vijayanand, Seumois et al. 2007; Ganesan 2010) but is complicated by cleavage of the surface CD4 co-receptor (Figure 2.5)(Hagman, Kuzma et al. 2012). For this reason it was necessary in the case of bronchial biopsy derived samples to identify T helper cells by negatively selecting on CD8.

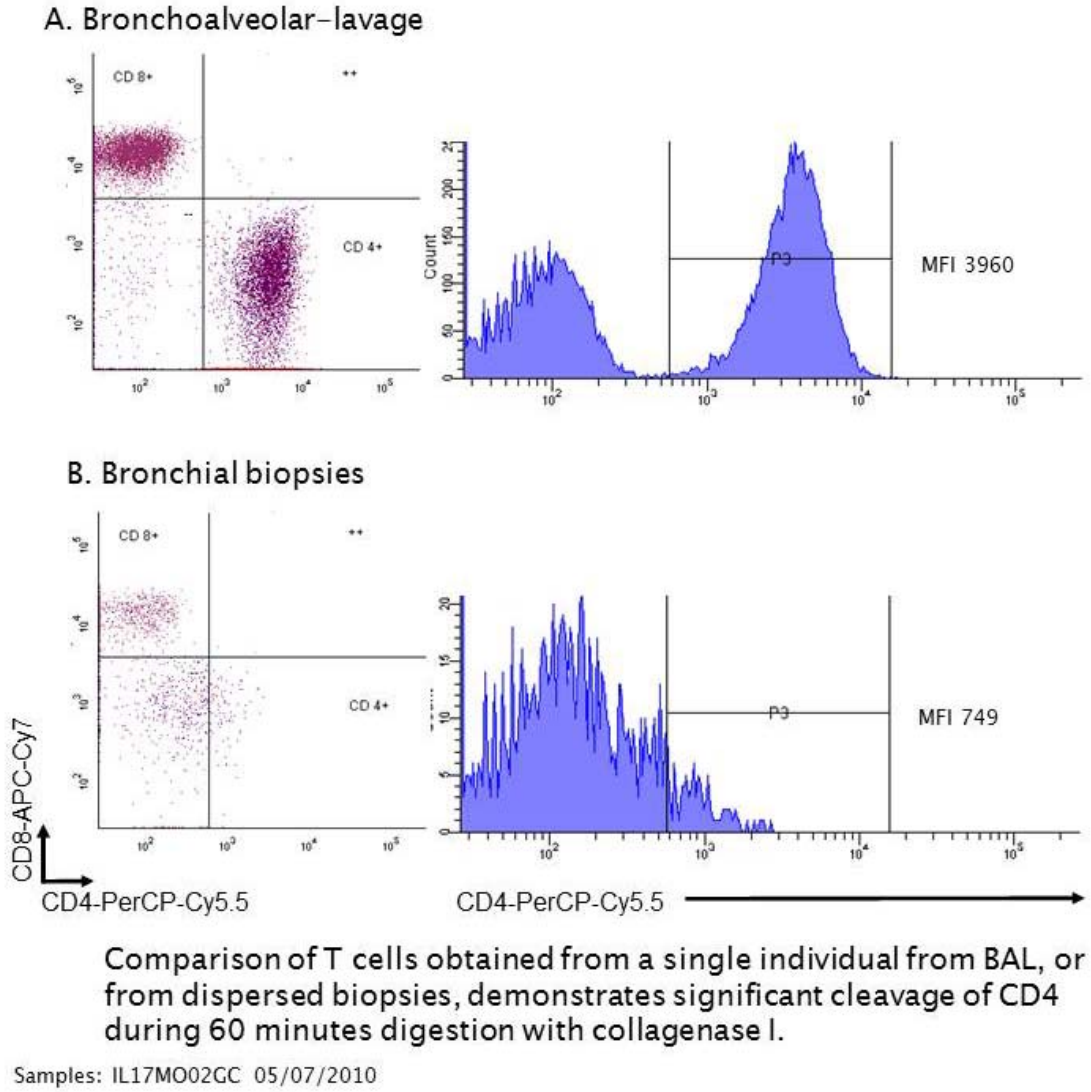


Figure 2.5 Cleavage of CD4 by collagenase dispersion

Surface staining for CD4 and CD8 expression on T cells obtained by either bronchoalveolar lavage (A), or by collagenase dispersion of bronchial biopsies (B), reveals relative preservation of CD8 but dramatic loss of CD4 staining due to cleavage of CD4 by collagenase. Histograms show CD4 brightness, with complete loss of the bimodal distribution of CD4 expression in the case of biopsies. (MFI, Mean Fluorescence Intensity).

Culture media

The following culture media were used for all other experimental work:

RPMI

Roswell Park Memorial Institute medium 1640 without L-glutamine or phenol red (R7509, Sigma or Lonza/Biowhittaker)

Complete serum free medium (AIM V)

AIM V® Medium (Gibco, Life Technologies, Paisley, UK) supplemented with:
0.5 µg/ml Fungizone (Amphotericin B with sodium deoxycholate, Gibco)
2 mM L-glutamine (Gibco)
1 mM sodium pyruvate (Gibco)
100 µg/ml streptomycin (Gibco)
100 U/ml penicillin (Gibco)
0.004% (v/v) 2-mercaptoethanol (β-ME)(Stratagene)

RN10 culture medium with 10% human serum

RPMI 1640 (Sigma or Lonza/Biowhittaker) supplemented with:
2 mM L-glutamine (Gibco)
1 mM sodium pyruvate (Gibco)
100 µg/ml streptomycin (Gibco)
100 U/ml penicillin (Gibco)
50 ml human AB serum (heat inactivated at 56º C for 30 min in water bath)(Sigma)

T cell growth medium

RPMI 1640 supplemented with:
10% (v/v) foetal calf serum
2 mM L-glutamine (GlutaMAX™, GIBCO)
2 mM sodium pyruvate (Gibco)
100 µg/ml streptomycin (Gibco)
100 U/ml penicillin (Gibco)
MEM essential amino acids (M5550, Sigma)
Non-essential amino acids (M7145, Sigma)
400 U/ml rh-IL2 (Proleukin, Prometheus Laboratories, San Diego, CA, USA)
10 ng/ml rhIL-7 (Immunotools Gmbh, Friesoythe, Germany)
10 ng/ml rhIL-15 (Immunotools)

T cell sorting medium

T cell growth medium, as above, supplemented with
2% human serum (Sigma)

0.1 mg/ml Kanamycin
50 μM β-mercaptoethanol

At the time of sorting and intermittently thereafter cells were additionally stimulated with phytohaemagglutanin (PHA-p, Sigma) at 1 μg/ml.

Magnetic-activated cell sorting (MACS) Buffer
2 mM ethylenediamine tetraacetic acid (EDTA, Fluka BioUltra, Sigma) 0.5% (w/v) bovine serum albumin (BSA, Sigma) 0.22 μm filter-sterilised in PBS.

Cryopreservation of cells

PBMC from the longitudinal cohort, as well as cloned cell lines were cryopreserved for long term storage. Fresh cryopreservation solution was made on the day of use (Simione 1998) comprising heat inactivated human serum albumin (Sera Laboratories, Haywards Heath, UK, inactivated at 56º C for 30 min in a water bath) with 10% dimethyl sulphoxide (DMSO, Sigma) and cooled for at least 15 minutes on ice prior to use, as this is an exothermic reaction.

Supernatant was poured off and cells resuspended in residual volume of RPMI by flicking the tubes. They were then resuspended in pre-labelled cryovials on ice, (H.A.N.C. 2011) to a final concentration of 2-10x10^6 cells/ml, in 2 ml ice-cold cryopreservation solution, added drop-wise over at least 2 mins to minimise osmotic shock(Jeurink, Vissers et al. 2008), with intermittent gentle shaking. The cryovials were then transferred immediately to a “Mr Frosty” 5100 Cryo 1°C Freezing Container (Nalgene, Thermo Fisher Scientific, Langenselbold, Germany) containing isopropanol which was pre-warmed to RT. This was placed immediately into a -80℃ freezer which allows approximately 20 minutes for DMSO to equilibrate with the cells whilst they cool at 1℃/min(Simione 1998). The following day samples were transferred to liquid nitrogen (-196 °C) for long term storage.

Human serum albumin was used in preference to foetal calf serum or human AB serum because, where freezing protocols have been compared directly, this provided the best cell viability and retention of lymphocyte function after cryopreservation (Disis, dela Rosa et al. 2006). In addition human serum albumin is less likely to activate cells in functional assays and gives better results in FOXP3 staining (Ganesan 2010). Other factors associated with improved lymphocyte function after cryopreservation include freezing cells at a concentration of 2-4x10^6cells/ml, as lower yields are obtained at lower concentrations((ECACC) ; Simione 1998), gentle handling during cell harvesting and concentration procedures; avoiding vigorous pipetting and high-speed centrifugation and ensuring that cells are defrosted at exactly 37℃ (Disis, dela Rosa et al. 2006). Factors which have been shown not to affect cell viability include using cell preparation tubes or Ficoll Paque (Ruitenberg, Mulder et al. 2006), transferring cells to liquid nitrogen or keeping on dry ice (-78.5℃) for 3 days, thawing in 15ml tube or 50ml tube, centrifuging for 5 or 10mins and at 1200rpm or 1500 rpm during washing or freezing at 10x10^6 cells/ml or 30x10^6 cells/ ml(Disis, dela Rosa et al. 2006).

Thawing cryopreserved cells

Cryovials were removed from liquid nitrogen and immediately warmed at exactly 37ºC in a water bath. As soon as the last ice crystals had disappeared the suspension was pipetted drop-wise into 10ml of pre-warmed RN10 medium, centrifuged immediately at 400*g* for 5 mins, the supernatant removed and cells resuspended in 1ml of RN10 to approximately $1x10^6$ cells /ml. Cells were then rested by incubation overnight in single 0.5ml wells at 37°C, 5% CO_2 to allow them to adapt to culture conditions (Maecker, Moon et al. 2005; Jeurink, Vissers et al. 2008) prior to restimulation for 5 hours in RN10 rather than serum free media.

Enzyme linked immunosorbent assay (ELISA)

Measurement of total immunoglobulin E (IgE)

Frozen serum aliquots were defrosted at RT and transferred into a 96-well round bottomed plate, then assayed using a Platinum ELISA kit (BMS2097, eBioscience, Hatfield, UK) according to the manufacturer's instructions. Briefly 96-well microwell plates pre-coated with monoclonal antibody to anti-human IgE were washed twice with 400 µL of wash buffer (0.05% (v/v) Tween 20 (P-1379, Sigma) in PBS (X6571D, Oxoid, Basingstoke, UK)(pH 7.3)). Duplicate standard curves were prepared in 100 µL of assay buffer (0.05% (v/v) Tween 20 in PBS with 10% BSA), using doubling dilutions of standard IgE protein (500 ng/ml to 7.8 ng/ml). 10 µL of samples were transferred from the round bottom plate and added in single wells to 90 µL of assay buffer in the assay plate. Duplicate blank wells contained 100µL of assay buffer only. Fifty microliters of horse radish peroxidase (HRP)-conjugated anti-human IgE monoclonal antibody were added to all wells and the plates covered with adhesive film and incubated for 60 minutes at RT with vigorous shaking (150 rpm). Wells were then emptied and washed twice with 400 µL of wash buffer and then 100 µL of tetramethyl-benzidine (TMB) substrate solution were added to all wells. The plates were covered and incubated for 25 minutes in the dark. 100 µL of stop solution (1M phosphoric acid) was added to each well and 450 nm absorbance measured on a plate reader (Multiskan Ascent, Agilent Technologies, Wokingham, UK). Concentrations were determined from a standard curve using 5-parameter curve fit. Concentrations in ng/ml were converted to international units (IU)/ml according to the manufacturer's comparison with the WHO Reference Serum (NIBSC code: 75/502, 1 IU/ml corresponds to 2.44 ng/ml).

Measurement of IL-17

Measurement of the concentration of IL17-A protein was attempted in supernatants of sputum, BAL and culture-conditioned media from allergen stimulated biopsies and virally infected parenchymal explants, by ELISA using pre-coated plates (88-7976, eBioscience, clone eBio64CAP17) according to the manufacturer's instructions. Briefly IL17-A standard was diluted in 100 µl assay buffer in duplicate 2-fold serial dilutions over the range 3.9-500 pg/ml. Samples were diluted 1:1 (BAL and culture-conditioned media) or 1:3 (sputum) with assay buffer and 100 µl added to wells in duplicate, plates sealed and incubated at RT for 2 hours. Next wells were aspirated and washed 5 times with 250 µl /

well wash buffer, blotted and incubated for 1 hour at RT with 100 µl/well of detection antibody (eBio64DEC17). Plates were again aspirated and washed 5 times before incubating for 30 mins at RT with 100 µl/well of avidin-horseradish peroxidase, then aspirating and washing a further 7 times, before incubating with 100 µl/well of TMB substrate solution at RT for 15 minutes. The reaction was stopped with 50 µl of stop solution (5.7% phosphoric acid) per well and the plates read at 450 nm.

Meso-Scale Discovery platform

The Meso Scale Discovery multi-array assay platform (MSD) was selected as it is robust and, can be multiplexed with small sample volumes and has have a lower limit of detection than ELISA or Luminex, especially as for IL-17A which quotes a lower limit of detection in serum of 0.4pg/ml. Samples were analysed by MSD according to the manufacturer's instructions using the IL17 ultrasensitive single-plex assay (K151ATC-2, Meso Scale Discovery, Gaithersburg, USA) for IL17-A and the TH1/2 7-cytokine multiplex assay (K15011C-2 Meso Scale Discovery) for IFN-γ, IL-10, IL-12 p70, IL-13, IL-2, IL-4, IL-5, with standard curves made up both in Diluent 2 and in DTE/Diluent 2 1:1 mix. The standard protocol was modified in discussion with the manufacturer according to the sample type.

BAL was first concentrated and a final concentration of approximately 1% bovine serum albumin (BSA) was achieved by addition of 20 µL of 10% BSA to the samples prior to concentration, to act as a carrier protein. 6 x 1ml aliquots of each sample were transferred to Vivaspin 6 centrifugal concentrators (Sartorius Stedim Biotech GmbH, Göettingen, Germany) and centrifuged at 4℃ at 1570-2100*g* for 4-5 hours until the dead-stop volume was approached, to achieve a dilution of 11.3-50 (median 44.7) fold. An additional sample of pooled BAL samples was spiked with 50 pg/ml of cytokine standards. The standard curve for BAL samples was diluted in PBS with 1% (w/v) BSA.

Sputum samples and the DTE standard curve were diluted 1:1 in diluent 2. This was a compromise between the denaturing effect of the reducing agent and the loss of sensitivity with dilution. The manufacturer have data that dithiothreitol (DTT) significantly affects MSD readings at 10 mM concentrations as it denatures antibodies, but this effect is minimal at 1 mM (Yvonne Clements, personal communication). As sputum samples were at a final concentration of 5 mM DTE and we assumed the effect of DTE would be similar to that of DTT. Conversely the manufacturer has previously obtained no detectable cytokines when diluting DTE 5 fold. Spiking recovery was tested in duplicate in an additional four samples at three concentrations: 10 pg/ml, 100 pg/ml and 1000 pg/ml of cytokine standards.

Serum was not diluted beyond the standard protocol. The standard curve was made in Diluent 2.

Method (all incubation steps were at room temperature with vigorous shaking (125 rpm) throughout): plates were incubated for 30 minutes with 25 µl of Diluent 2 per well, then 25 µL of sample diluted as above or standards were added in duplicate and incubated for 2 hours. Plates were washed 3 times

with 0.05% Tween 20 (Sigma) in PBS, incubated for a further 2 hours with 25 µl of detection antibody and washed a further 3 times with PBS-Tween. 150 µl of 2x Read Buffer was added to each well and the plate analysed immediately on a SECTOR Imager (MSD). Data were analysed in PRISM using a 4-parameter logistic model.

RNA extraction and quantitation

TRIzol

Ribonucleic acid (RNA) from MAIT clones was extracted using TRIzol® LS Reagent (Life Technologies). 900-7000 cells were sorted directly into 1ml aliquots TRIzol and stored at -80℃ till later use, at which point samples were defrosted and homogenized by repeated aspiration through a 1 ml filter-tip pipette, incubated at RT for 5 mins before addition of 200 µl of choloroform (Sigma). After a further 5 minutes at RT samples were centrifuged at 16,200*g*, at 4℃ for 30 mins. The upper, aqueous layer was transferred to a fresh tube containing 1 ml isopropanol (Fisher, Loughborough, UK) and 5µg of glycogen and vortexed well, incubated at RT for 10 mins and centrifuged at 16,200*g* at 4℃ for 30 mins to pellet the RNA. The supernatant was carefully removed and the pellet washed with 1ml of 75% ethanol and incubated on ice for 10 mins, centrifuged at 16,200*g* at 4℃ for 5 mins before all the ethanol was removed and the pellet dissolved in 30 µl diethylpyrocarbonate (DEPC) treated water.

Nanoprep

For all other work RNA was extracted using the Absolutely RNA Nanoprep Kit (Stratagene). This kit uses very small (10 µl volume) RNA-binding spin cups containing a silica-based matrix and is optimised for purification of total RNA from very low cells numbers (1-10^4 cells).(Inc 2008) Our group have previously used it successfully to perform PCR on as few as 10 sorted T cells(Vijayanand, Seumois et al. 2007), and made minor modifications to the manufacturer's protocol to maximise sensitivity, specifically reloading samples onto matrix at several steps in the protocol and eluting into a final volume of 15 µl rather than the recommended 10 µl (Vijayanand 2007).

Cells were flow-sorted directly into 100 µl aliquots of Agilent lysis buffer containing the chaotropic salt guanidine thiocyanate to lyse cells and prevent degradation by RNases. Further protection from RNases was provided by an additional 0.7 µl of 14.2M β-mercaptoethanol (β-ME) which reduces disulfide bonds to irreversible denature RNases(Nelson 2005). Samples were vortexed hard for ≥10 s then stored at -80℃ till further use.

Samples were defrosted and thoroughly mixed with an equal volume (100 µl) of 80% sulfolane (Sigma), a water-soluble solvent and centrifuged in the spin-cup at 12,000*g* for 1 minute. Samples were reloaded into the spin cup and centrifuged again at 12,000*g* for 1 minute and the filtrate discarded. DNA was removed by digestion with DNase I in the following manner: spin cups were washed with 300 µl of low-salt wash buffer, centrifuged once at 12,000*g* for 1 min, the filtrate discarded and the spin-cup dried by centrifugation at 12,000*g* for 2 mins, then incubated for 15

minutes at 37℃ with 15 µl of DNase I in digestion buffer solution. The RNA captured on the matrix was then washed once with 300 µl high-salt wash buffer and twice with 300 µl low-salt wash buffer, each time centrifuging at 12,000*g* for 1 min then discarding filtrate. Finally the RNA was eluted from the matrix into a fresh collection tube by incubation for 2 minutes with 15 µl of elution buffer preheated to 60℃, with centrifugation at 12,000*g* for 2 minutes, followed by reloading of the eluate onto the matrix and centrifuging for 12,000*g* for 5 minutes.

Nucleic acid quantitation

Nucleic acid concentration and purity were assessed with a NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, USA). The concentration of nucleic acid was measured in 1 µl of sample and the purity assessed by determination of the ratio of sample absorbance at 260 and 280 nm: pure DNA has a ratio of 1.8 and pure RNA a ratio of 2.0, although the 260/280 ratio is also affected by changes in pH and differing nucleotide mixes in the nucleic acid(ThermoScientific 2008).

Reverse transcription and polymerase chain reaction

Reverse transcription with SuperScript™ III RT kit

RNA from MAIT clones was reverse transcribed (RT) using the SuperScript™ III reverse transcriptase kit (18080-093, Invitrogen) which uses a modified *pol* gene of Moloney Murine Leukemia virus, in a reaction volume of 20 µl. 11 µl of RNA were incubated with 1 µl random primers (predominantly hexamers) and 1 µl deoxyribonucleotide triphosphate (dNTP) mixture at 65℃ for 5 minutes followed by immediate quenching on ice to remove secondary structure of the RNA. A no template control contained 11 µl of ddH_20 in place of RNA. To this was added 4 µl of 5x first strand buffer (250 mM Tris-HCl (pH 8.3 at RT, 375 mM KCl, 15 mM $MgCl_2$), 1 µl 0.1M DTT, 1 µl RNase inhibitor (RNase OUT™) and 1 µl of the RT enzyme. Samples were mixed by pipetting, incubated at RT for 5 mins, then incubated in a Tetrad 2 DNA engine(MJ Research, Bio-Rad, Hemel Hempstead, UK), at 50℃ for 60 mins, followed by enzyme inactivation at 70℃ for 15 mins.

Reverse transcription with Precision nanoScript™ RT kit

RNA from sorted airway T cells was reverse transcribed using Precision nanoScript™ reverse transcription kit (formerly known as qScript, Primer Design, Rownhams, UK), which also uses the same RT enzyme, but for priming used a mixture of both oligo-dT priming and random nonomers. Oligo-dT primers bind to the polyA tail of messenger RNA (mRNA), preferentially targeting the 3'end of mRNA and so reducing transcription of ribosomal RNA (rRNA), which is advantageous for quantitative PCR (qPCR) as it leads to lower threshold cycle (CT) values. The advantage of incorporating random nonomers is increased priming efficiency with partially degraded RNA, which was important to achieve maximum sensitivity for my work on formalin fixed cells(PrimerDesignLtd).

12.5 µl of RNA were incubated at 65℃ for 5 minutes followed by 1 minute on ice. A no template control contained 12.5 µl of ddH_20. To this was added 0.5 µl random nonomers, 1 µl oligoDT, 1 µl dNTP mixture, 2 µl of 100 mM DTT, 2 µl of 10x buffer and ddH_20 to a volume of 20 µl. 1 µl of the RT

enzyme was added to each sample, or 1 µl ddH_20 for the "no enzyme control" to test for genomic contamination. Samples were mixed and incubated in a Mastercycler (Eppendorf, Hamburg, Germany) at 55℃ for 20 mins, then 75℃ for 15 mins.

Polymerase chain reaction (PCR)

For non-quantitative work on MAIT cell clones, RT products were amplified by polymerase chain reaction (PCR) using BioTaq DNA Polymerase (BIO-21040, Gentaur, Brussels, Belgium). The reaction mixture, 50 µL total, contained 1 µl of cDNA, 5 µl 10x NH_4 buffer, 2.5 µl of 50 mM $MgCl_2$, 1µl of 10mM dNTP, 0.5 µl of BioTaq DNA polymerase, 1 µl of forward primer, 1 µl of reverse primer (final concentration of 2 µM in reaction) and 39 µl of ddH_20.

Amplifications were performed on the Mastercycler with the following cycling parameters: 96℃ for 1 min preincubation, then 96℃ for 30 s denaturing, then 65℃ for 30 s annealing, then 72℃ for 2 minutes elongation; repeated for 45 cycles and finished with a 10 min extension at 72℃.

Primers (MWG Biotech AG, Ebersberg, Germany) are shown in table 2.3 and were designed to span the canonical Vα7.2-Jα33 rearrangement of the CDR3α region of the TCRα chain(Porcelli, Yockey et al. 1993; Tilloy, Treiner et al. 1999).

Table 2.3 Oligonucleotide primers used for PCR

Product	Sequence
Vα7.2 TCR	5'-ATA TAT CAT ATG GGA CAA AAC ATT GAC CAG-3' fwd
Jα33 TCR	5'-GCT TTA TAA TTA GCT TGG TCC CAG C-3' rev
IL17-A	5'-CCT CAG ATT ACT ACA ACC GAT CC-3' fwd
	5'-CAC TTT GCC TCC CAG ATC AC-3' rev
FOXP3	5'- CAG CAC ATT CCC AGA GTT CCT-3' fwd
	5'- GCG TGT GAA CCA GTG GTA GAT-3' rev
β-2 microglobulin*	Accession number: NM_004048.2 Anchor nucleotide: 362 Context sequence length: 141bp
YWHAZ*	Accession number: NM_003406.3 Anchor nucleotide: 2585 Context sequence length: 150bp

*The exact sequences are commercially sensitive and not released by the manufacturer.

Gel electrophoresis

10 µL of PCR product premixed with dye was loaded into wells of a 1% (w/v) agarose (Melford, Chelsworth, UK) gel in Tris/Borate/EDTA (TBE) buffer containing 10 µl per 100 ml of Nancy-520 dye (01949, Sigma) and electrophoresed at 80 volts for 60 minutes, before photographing the gel under ultraviolet light.

Quantitative PCR

Quantitative PCR ("qPCR" or "real-time PCR") was used to measure the abundance of mRNA transcripts for IL17-A and FOXP3, as well as the normalising genes B2M and YWHAZ. The reaction mixtures, performed in duplicate 20 µl volumes contained 9 µl of cDNA (or ddH_20 for "no template" control), 10 µl of Precision^{TM} Mastermix (Primer Design, containing a thermostable *Taq* polymerase) and 1 µl of forward and reverse primer mix (6 pmol of each). qPCR analysis was performed using the iCyclerIQ platform (Bio-Rad) with the following cycling parameters: 95℃ for 10 min preincubation, then 95℃ for 10 s, then 50℃ for 30 s, then 72℃ for 10 seconds; repeated for 52 cycles.

PerfectProbe^{TM} primer pairs were designed and tested for amplification efficiency by Primer Design. They are hydrolysis probes in which a quencher molecule at the 3' end of the probe reduces the fluorescence of a fluorophore (FAM-490) at the 5' end of the molecule via fluorescence resonance energy transfer(Holland, Abramson et al. 1991). PerfectProbe differ from this original description in that the quencher and fluorophore are brought into closer proximity by a hairpin loop structure, providing more efficient quenching thus a lower background (PrimerDesignLtd).

Flow cytometry

Surface staining for MAIT cells

Cells were transferred to polypropylene test tubes in + 1 ml of PBS, centrifuged at 400*g* for 5 mins 4℃ and resuspended in 500 µl PBS on ice. Cells were stained with 1 µl of LIVE/DEAD® Fixable Violet Dead Cell Stain for 405 nm excitation (L34955, Invitrogen) for 30 mins, then washed with 2 ml magnetic activated cell sorting (MACS) buffer, centrifuged at 400*g* for 5 mins and resuspended in 200 µl of MACS buffer for surface staining for 30 mins. Cells were then washed with 2 ml of MACS buffer, centrifuged at 400*g* for 5 mins and resuspended in 200 µl of MACS buffer for cytometry the same day. All staining protocols were performed in the dark and on ice, with a centrifuge refrigerated to 4℃.

Table 2.4 Antibodies and fluorochromes used for surface staining

Stain	Clone	Supplier, reference	Ex-MAX / Em-MAX wavelength (nm)	Concentration (µl)	
				PBMC	Other tissues
LIVE/DEAD® Fixable Violet Dead Cell Stain	N/A	Invitrogen, L34955	405/450	1	1
CD3 PE-Cy™7	SK7	BD, 557851	496,546/785	1.5	3
CD4 PerCP-Cy™5.5	L200	BD, 552838	482/695	2.5	5
CD8 APC-Cy™7	SK1	BD, 348813	650/785	2.5	5
TCR Vα7.2 PE	3C10	Biolegend, 351706	496, 546/578	5	10
CD161 FITC	DX12	BD, 556080	494/519	10	10
γδTCR FITC	B1	BD, 61995	494/519	5	5

Table 2.5 Isotype controls

Fluorochrome	Stain for which this is used as control	Isotype class	Clone	Supplier, reference	Concentration (µl)	
					PBMC	Other tissues
FITC	CD161 or γδTCR	Mouse $IgG_1κ$	MOPC-21	BD, 555748	10	10
FITC	IL-13	Mouse IgG_1	11711	R&D Systems, IC002F	10	10
PE	TCR Vα7.2	Mouse $IgG_1κ$	MOPC-21	Biolegend, 400114	0.25	0.5
PE	IL17	Mouse IgG_1	P3.6.2.8.1	eBioscience, 12-4714-42	2.5	2.5
APC	IFN-γ or TNFα	Mouse $IgG_1κ$	P3.6.2.8.1	eBioscience 17-4714-42	0.3	0.3
APC	FOXP3	Rat IgG2α κ	eBR2a	eBioscience, 17-4321	2.5	2.5
Rat serum	N/A	To block nonspecific staining by FOXP3 APC		eBioscience, 245555	2	2

Intracellular cytokine staining

Cells at a concentration of $1x10^6$ cells/ml were first stimulated at 37℃, 5% CO_2 for 4-5 hours (see table 2.6) with 25ng/ml phorbol 12-myristate 13-acetate (PMA, Sigma) which activates protein kinase C and 500ng/ml ionomycin (Sigma) a Ca^{2+} ionophore, in the presence of 2μM monensin (eBioscience), an inhibitor of trans-Golgi function(Nylander and Kalies 1999), leading to intracellular accumulation of cytokines. Paired samples were left unstimulated, without PMA, ionomycin or monensin, both to act as negative controls for the intracellular cytokine staining and also for FOXP3 analysis. Biopsy tissue was stimulated as complete biopsies in 0.5 ml wells of 24-well plates but after 4 hours collagenase dispersed for 1 hour at 37 ℃, before undergoing the same staining as other tissues.

Table 2.6 Stimulation times for each tissue

Tissue	**Duration of *ex vivo* stimulation**
Fresh PBMC	5 hours
Sputum	4.5 hours
Bronchoalveolar lavage	5 hours
Bronchial biopsies	4 hours + 1 hour collagenase digestion
Previously cryopreserved PBMC	5 hours

Next cells were resuspended and transferred to polycarbonate test tubes in + 1ml of PBS, centrifuged at 400*g* for 5 mins 4℃ and resuspended in 500 μl PBS on ice. Cells were stained with 1 μl of LIVE/DEAD® Fixable Violet for 30 mins, then washed with 2ml MACS buffer and centrifuged at 400*g* for 5 mins. Cells were then fix-permeabilised by resuspending in 200 μl of fixation/permeabilization working solution (eBioscience, comprising 1 part Fixation/Permeabilization Concentrate 00-5123 and 3 parts Fixation/Permeabilization Diluent 00-5223) for 30 mins, washed with 2 ml of permeabilisation buffer (00-8333, eBioscience, diluted 1:9 with MACS buffer) and centrifuged at 400*g* for 5 mins. Cells were then resuspended in residual volume, adjusted to total 110 μl volume diluted permeabilisation buffer and incubated with fluorochrome-conjugated antibodies for 45 mins on ice in the dark, before a final wash with 2 ml permeabilisation buffer, centrifugation at 400*g* for 5 mins and resuspension at ≥200 μl volume for flow cytometry.

Table 2.6.1 Antibodies and fluorochromes used for intracellular staining

Stain	Clone	Supplier, reference	Ex-MAX / Em-MAX wavelength (nm)	Concentration (µl)	
				PBMC	Other tissues
IL17A PE	eBio64CAP17	eBioscience, 12-7178-42	496, 546/578	5	5
IL13 FITC	32007	R&D Systems, IC2131F	494/519	5	5
IFN-γ APC	4S.B3	eBioscience, 17-7319-82	650/660	0.3	0.3
FOXP3 APC	PCH101	eBioscience, 17-4776-42	650/660	5	5
TNFα APC	MAb11	eBioscience, 17-7349	650/660	0.5	0.5

Cell sorting and data acquisition

Flow cytometry was performed with a nine-colour FACS Aria^{TM} cell sorter (BD Biosciences) with three lasers at 488, 633 and 407nm wavelengths. Samples other than PBMC were passed again through a 70 µm prior to acquisition on the flow cytometer. Purity was checked using FACS Accudrop beads (BD) and was ≥99% in all cases. Cells were sorted, at 50 µl/min, directly into eppendorfs containing lysis buffer, except PBMC T cells where the sample volume required an additional centrifugation step at 400*g* for 5 mins to pellet cells and remove the supernatant prior ot addition of lysis buffer. Samples were acquired, sorted and analysed using FACS Diva^{TM} 5.0.3 software (BD) and the following conflict resolution settings: yield mask 0, purity mask 32 and phase mask 0.

Gating strategy for MAIT cells

T cells were identified by pulse width-pulse area doublet exclusion, dead cell exclusion by Violet LIVE/DEAD and by side-scatter and CD3-PE-Cy7 staining. MAIT cells were defined as cells double positive for CD161-FITC and the TCR Vα 7.2 chain conjugated to PE (Dusseaux, Martin et al. 2011). Gates were set using isotype controls for IgG1κ-FITC and IgG1-PE (Figure 2.6).

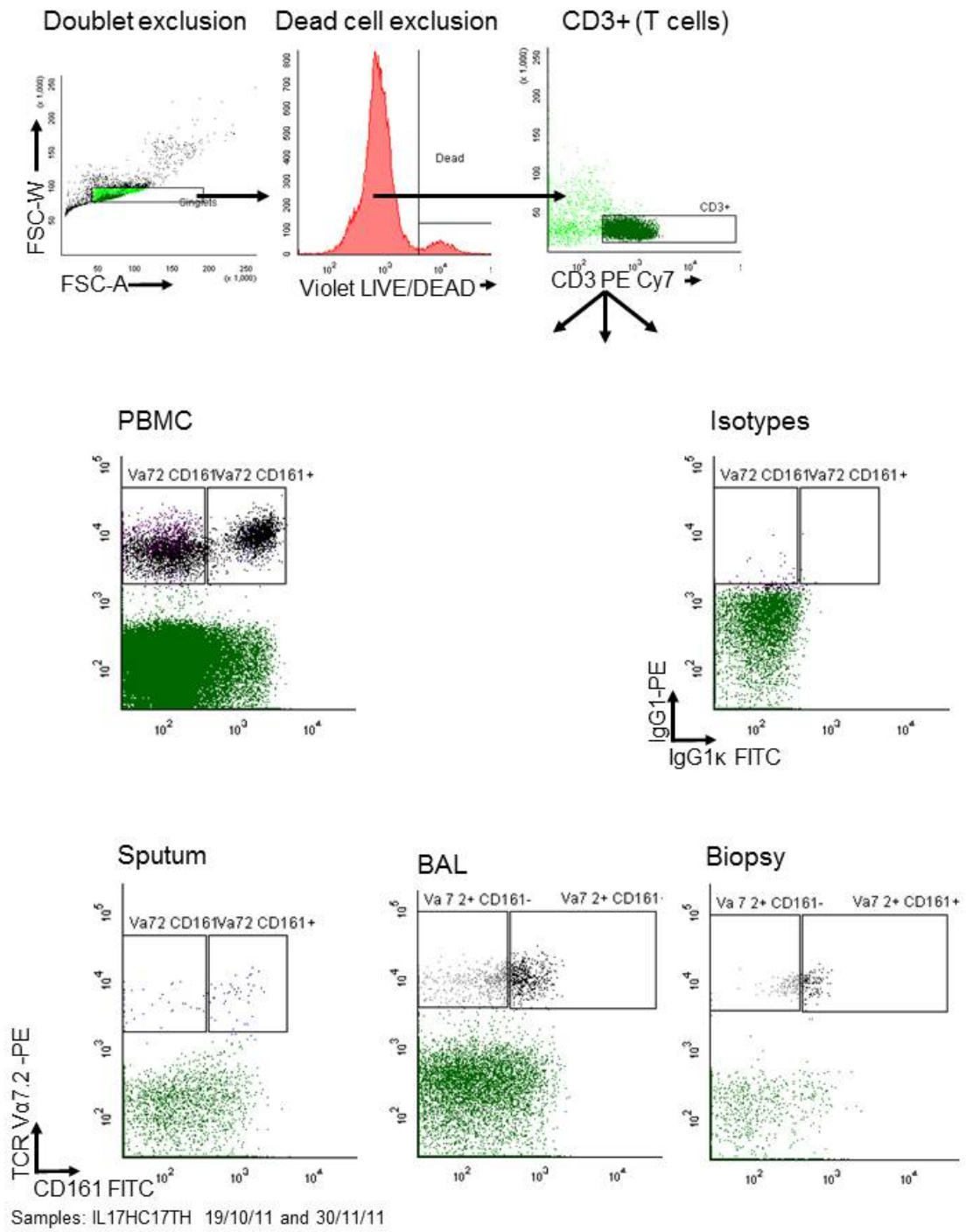


Figure 2.6 Gating strategy for MAIT cells

T cells were identified by pulse width-pulse area doublet exclusion, dead cell exclusion by Violet LIVE/DEAD and by side-scatter and CD3-PE-Cy7 staining. MAIT cells were defined as cells double positive for CD161-FITC and the TCR Vα 7.2 chain conjugated to PE. Gates were set using isotype controls for IgG1κ-FITC and IgG1-PE.

Gating Strategy for T helper cells

Doublets were excluded by gating on forward scatter-area (pulse area) (Figure 2.7, A), which is proportional to the cross sectional area of the cell, versus forward scatter-width (pulse-width), which is proportional to the time taken to pass through the laser beam. Pulse width is greater in doublets, which become aligned perpendicularly to the laser beam in a narrow stream. Dead cells were excluded by their increased uptake of Violet LIVE/DEAD® Fixable (Figure 2.7, B), which permeates compromised membranes of necrotic cells to react irreversibly with free amines intracellularly (Invitrogen 2007). T cells were identified according to surface staining by CD3-PE-Cy7 and side scatter used to exclude large cells and debris (Figure 2.7, C). T cells were further characterised by their surface staining with CD4-PerCP-Cy5.5 and CD8-APC-Cy7 (Figure 2.7, D) and intracellular staining for IL-17PE (T_H17 cells), IFN-γ APC (T_H1 cells) and IL-13 FITC (T_H2 cells)(Figure 2.7, E). Gates for each of these cytokines were set on unstimulated cells using the same stains. Regulatory T cells were identified by intracellular staining for FOXP3 APC.

Controls for flow cytometry

As there is a 50-fold difference in fluorescence between live and dead cells stained with Violet LIVE/DEAD fixable (Invitrogen 2007), two clear fluorescence peaks were always distinguishable to enable confident setting of the dead cell gate, reinforced by the use of an unstained control (Figure 2.8, A). As CD3 is abundantly expressed on the surface of T cells, staining was universally bright and unstained cells could be used as negative controls for the CD3+ gate (Figure 2.8, B).

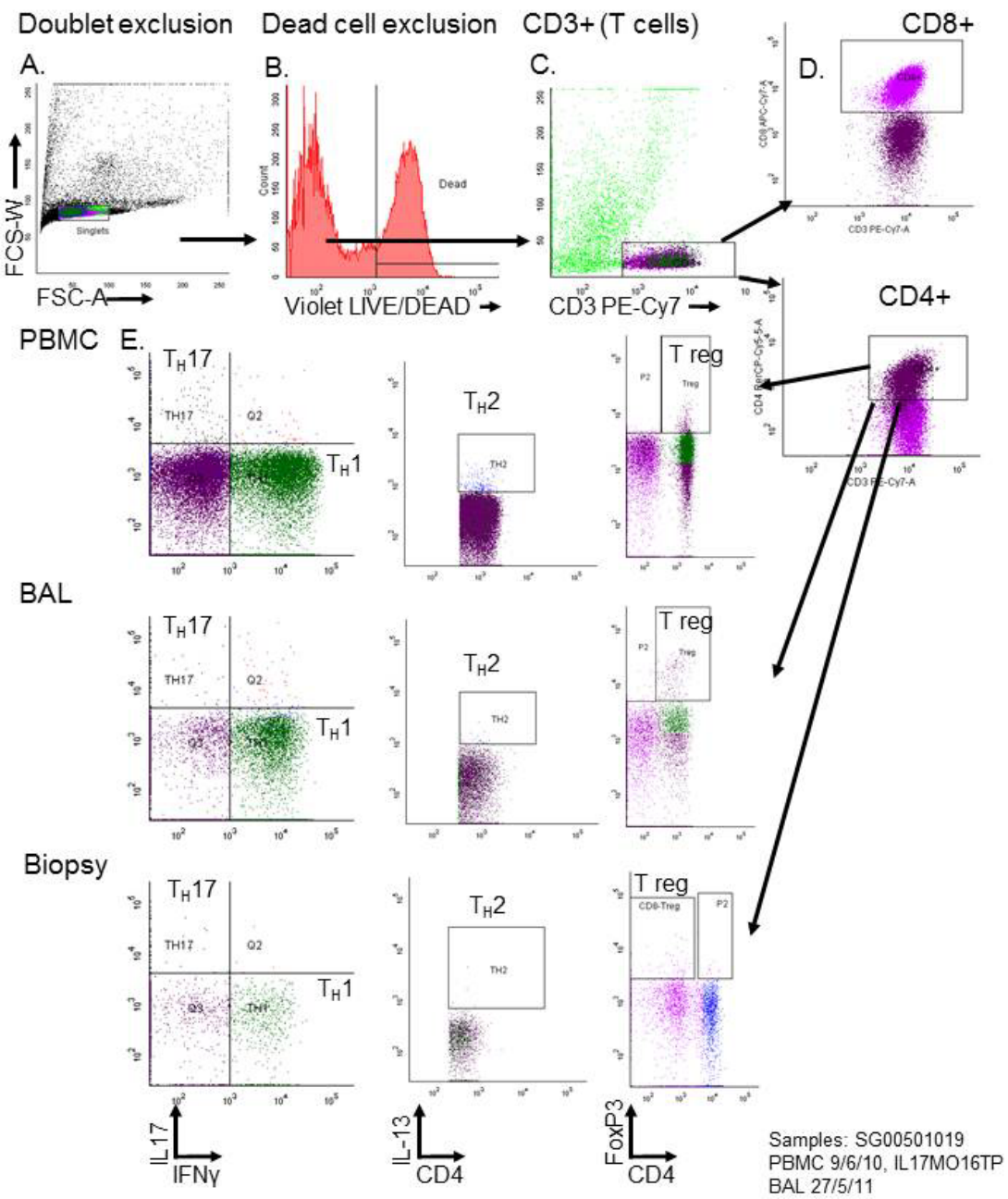


Figure 2.7 Gating strategy T helper cells

Schematic to demonstrate the hierarchical gating strategy employed for flow-cytometric analysis of CD4+ T cells throughout the study. Doublets were excluded by gating on forward scatter-area (pulse area) versus forward scatter-width (pulse-width)(A). Dead cells were excluded by their increased uptake of Violet LIVE/DEAD® Fixable (B) T cells were identified according to side scatter profile and surface staining by CD3-PE-Cy7 (C). T cells were characterised by CD4-PerCP-Cy5.5 and CD8-APC-Cy7 (D), and intracellular staining for IL-17PE, IFNγ APC and IL-13 FITC (E). Gates for each of these cytokines were set on unstimulated cells using the same stains. TREG were identified by intracellular staining for FoxP3 APC.

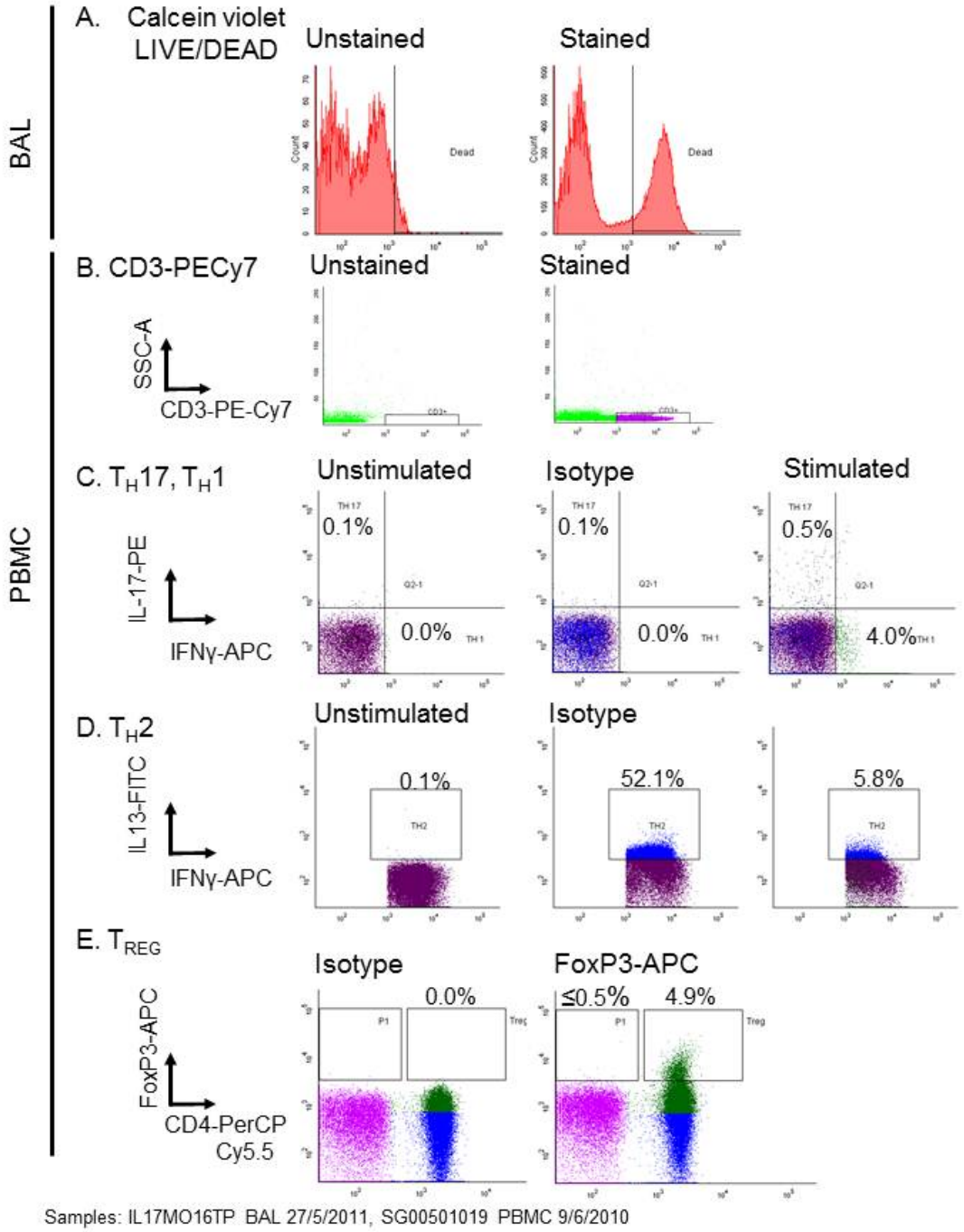


Figure 2.8 Controls for cytometry

With Violet LIVE/DEAD fixable two clear fluorescence peaks were always distinguishable, reinforced by the use of an unstained control (A). CD3-PE-Cy7staining was universally bright with unstained cells as negative controls (B). Gates for TH17, TH1 and TH2 cells were set using unstimulated cells (rested overnight in culture in absence of PMA, ionomycin or monensin), although initially isotype controls were also included (C, D), but could provide spurious results (D middle panel).

Setting of gates for intracellular stains is more challenging as there are rarely clearly demarcated populations within a single specimen. Three main approaches include use of isotype controls, “fluorescence minus one” technique, or unstimulated controls stained with the same antibodies. A number of well recognised problems in the use of isotype controls mean they are frequently of little value, since each antibody and antibody conjugate has very different background staining characteristics (Baumgarth and Roederer 2000). Specifically isotype controls do not have identical tertiary structure, there may be subtle differences because they originate from hybridomas which are not normal cells, the binding of isotypes varies between cell types and their stage of differentiation and variations in purification methods or efficiency of conjugation cause significant lot to lot variation (Keeney, Gratama et al. 1998). For these reasons use of isotypes is particularly problematic for rare events (Keeney, Gratama et al. 1998). These problems can be avoided by the alternative technique of fluorescence minus one the sample is divided into multiple aliquots and each stained with all reagents except for the one of interest (Baumgarth and Roederer 2000; Roederer 2001), but this would not be practicable for this study due to the very small sizes of the tissue samples and the large number of stains. The third approach is to divide a single sample into two equal parts during overnight culture, one of which receives no stimulation with PMA, ionomycin and no Golgi block, preventing any intracellular accumulation of cytokine, but was then stained with an identical panel of stains to the stimulated control. In practice I used this technique which consistently provided reliable negative populations on which to set gates (Figure 2.8, C and D). Initially I also used isotype controls in each case (Figure 2.8, C), but eventually abandoned this as it wasted samples and proved less reliable, frequently providing inaccurate or frankly spurious results, such as higher staining than that seen with the specific antibody (Figure 2.8, D, Figure 2.9).

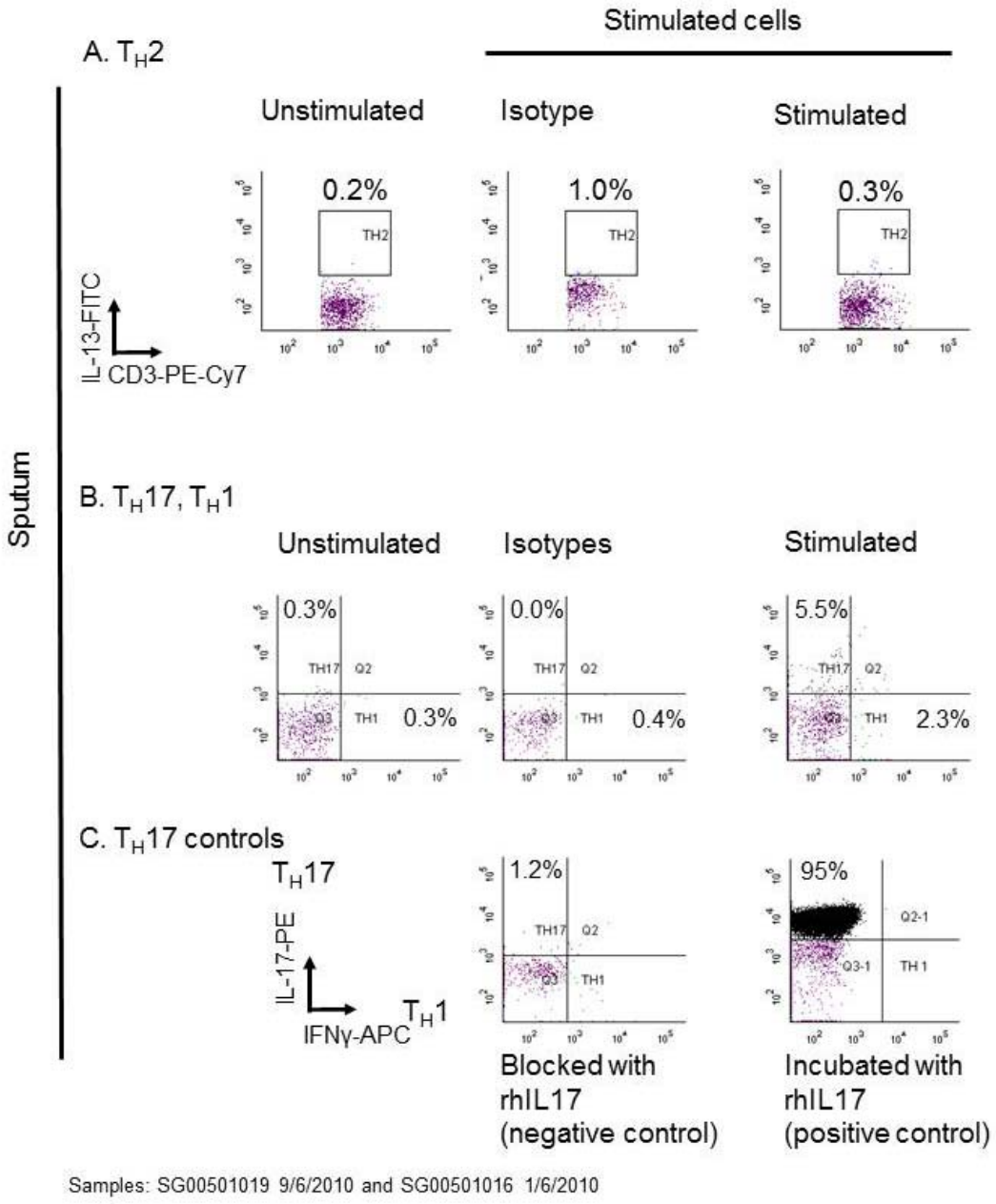


Figure 2.9 Comparison of isotypes and unstimulated cells

A schematic showing additional comparison of the use of isotypes or unstimulated cells as negative controls for determining gating on intracellular cytokine stains. For rare populations such as TH2 cells in sputum it is frequently more accurate to gate on unstimulated cells than using isotype controls, which here would give a negative value for the TH2 cell frequency (A). TH17 and TH1 cells are more abundant and with brighter staining, so isotypes could have been an acceptable alternative (B). As it is theoretically possible that apparent specific IL-17-PE staining might have been an artefact of increased non-specific binding due to the effect of stimulation, additional positive and negative controls are shown in (C). Stimulated cells were stained for IL-17 according to the usual protocol either in the presence of a saturating quantity of recombinant human IL-17 (rhIL-17) to block all specific binding of the antibody (negative control), or after pre-incubation of the cells with rhIL-17 to

cause saturation of the cells' IL-17 receptors (positive control). Together these demonstrate that events with high PE fluorescence do indeed represent true staining for cells expressing IL-17.

Cloning of MAIT cells

As part of my analysis of MAIT cells I sought to clone these cells. This was done with help from a post-doctoral fellow, Dr Salah Mansour, working with Professor Gadola. The method we used was adapted from a protocol developed for cloning iNKT cells (Matulis, Sanderson et al. 2010). PBMC were isolated from a healthy individual and stained with antibodies to detect live $CD3^+CD161^+V\alpha7.2^+$ cells (MAIT cells) which were sorted at 1 cell/well into a 96-well round-bottom plate containing $5x10^4$ autologous irradiated (30 Gy) PBMC feeder cells in 200 µl of T cell sorting medium (see above, contains 10% foetal calf serum, 2 mM L-glutamine, 2 mM sodium pyruvate, 100 µg/ml streptomycin, 100 U/ml penicillin, MEM essential amino acids, non-essential amino acids, 400 U/ml rh-IL2, 10 ng/ml rhIL-7, 10 ng/ml rhIL-15, 2% human serum, 0.1 mg/ml Kanamycin, 50 µM β-ME) and also supplemented with PHA (Sigma) at 1 µg/ml and then cultured at 37℃ 5% CO_2 for several weeks. After 2 weeks culture expanded clones were transferred sequentially into 24-well, 12-well, and 6-well flat-bottom plates in fresh T cell growth medium. Culture medium was refreshed every 2-5 days when a colour change was observed, and at day 29 clones were restimulated with PHA 1 µg/ml. Expanded clones were tested by flow cytometry for surface phenotype and by PCR to confirm the presence of the canonical Vα7.2-Jα33 TCR rearrangement.

Definition of T helper cells for flow cytometry

Accurate enumeration of T cell subsets by flow cytometry depends on accurate definitions of these populations according to consistent and logical setting the gates used in the hierarchical Boolean gating analysis. For the work in my thesis I had to address some specific challenges particular to individual cell populations. Firstly accurately defining CD4+ T cells in the light of stimulation induced downregulation of the CD4 co-receptor and secondly determining the exact definition of a positive FOXP3 gate necessary to define regulatory T cells. I addressed these in turn in the following optimisation experiments.

The problem of CD4 co-receptor downregulation during *ex-vivo* stimulation

T helper lymphocytes are defined by their expression of the CD4 co-receptor (Reinherz, Kung et al. 1980; Reinherz and Schlossman 1980; Bernard, Gay-Bellile et al. 1984). However it is well recognised that stimulation of cells, such as with PMA/ionomycin, causes downregulation of surface receptors, including CD3, CD8 and particularly CD4(O'Neil-Andersen and Lawrence 2002; Hawn, Misch et al. 2007). As the objective of my work was to analyse CD4+ and CD8+ T cell populations using intracellular cytokine staining which depends on *ex vivo* cell stimulation, this effect could have proved problematic. I therefore analysed the effects of stimulation on CD4+ and CD8+ T cell subpopulations in different cellular compartments to address the question of whether it is preferable to define T helper cells by positive selection on CD4 or negative selection on CD8.

Results

Figure 2.10 shows changes in each of the 4 subpopulations of T cells defined by CD4 and CD8 expression before and after stimulation for 4-5 hours with PMA/ionomycin. Changes in the proportion of T cells positive for CD4 are modest, with no significant change in BAL, a significant, but modest decrease in sputum CD4+ cells from 65% (60-74, median and IQR) unstimulated to 46% (37-55) after stimulation (P=0.0005). This is contrasted with a slight increase in CD4+ cells in PBMC from 49% (44-52) to 59% (44-61, P=0.008, see table 2.7), which might perhaps be due to preferential death of CD4+ cells from monensin toxicity(Nylander and Kalies 1999).

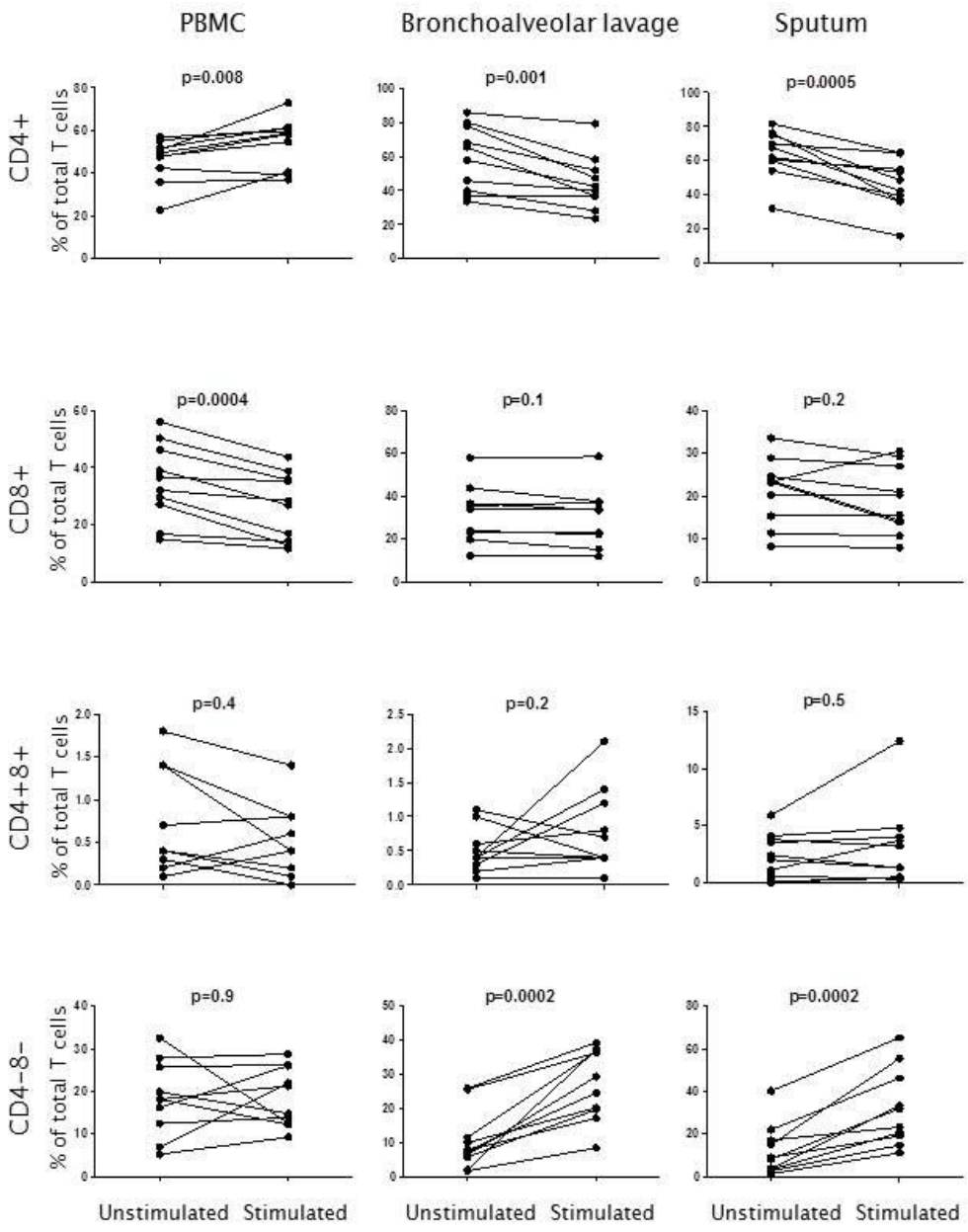


Figure 2.10 Changes in CD4+ and CD8+ populations with stimulation

Changes in each of the 4 subpopulations of T cells defined by CD4 and CD8 expression before and after stimulation for 4-5 hours with PMA/ionomycin. n=10 for each compartment, significance tested with paired t tests, not corrected for multiple comparisons.

The phenomenon of CD4 downregulation is variable between subjects. Whilst it increases over time, being more marked at 5 than 4 hours, Figure 2.11 A,B shows how samples from two individuals differ markedly in the extent of CD4 internalisation. To what degree does CD4 downregulation adversely affect the purity of my T helper cell population if we positively select on CD4? I compared the percentage of cells within the CD4+ gate which were also CD8+ before and after stimulation and found these frequencies were low and did not change significantly with stimulation. The proportions of CD4+ cells also expressing CD8 were 0.75% (0.40-1.7) unstimulated and 0.50% (0.13-1.6) after stimulation in PBMC (p=0.2) and in BAL were 0.65% (0.23-1.4) unstimulated and 0.85% (0.53-2.8) stimulated (P=0.1) and in sputum were 3.6% (1.0-4.5) unstimulated and 2.9 (2.7-6.2) (P=0.2). See Table 2.7.

Table 2.7 The effect of stimulation on relative T cell populations in different tissues.

	PBMC					**BAL**					**Sputum**				
	Unstimulated		Stimulated		P	Unstimulated		Stimulated		P	Unstimulated		Stimulated		P
Purity of CD4 population (%of CD4 +cells which are CD8+)	**0.75**	(0.40-1.7)	**0.50**	(0.13-1.6)	0.2	**0.65**	(0.23-1.4)	**0.85**	(0.53-2.8)	0.1	**3.6**	(1.0-4.5)	**2.9**	(2.7-6.2)	0.2
CD4+ (%)	**49**	(44-52)	**59**	(44-61)	**0.008**	**62**	(41-76)	**41**	(37-51)	**0.001**	**65**	(60-74)	**46**	(37-55)	**0.0005**
CD8+ (%)	**34**	(28-44)	**28**	(15-36)	**0.0005**	**29**	(20-36)	**28**	(17-36)	0.1	**23**	(17-24)	**18**	(14-26)	0.2
CD4+8+(%)	**0.4**	(0.33-1.2)	**0.4**	(0.20-0.75)	0.4	**0.4**	(0.33-0.58)	**0.55**	(0.40-1.1)	0.2	**2.2**	(0.73-3.7)	**2.3**	(0.70-3.9)	0.5
CD4-8- (%)	**18**	(13-24)	**18**	(13-25)	0.9	**7.8**	(6.0-11)	**27**	(20-37)	**0.0002**	**8.8**	(3.63-17)	**27**	(20-43)	**0.0002**
CD4/8 ratio	**1.4**	(1.0-2.0)	**2.1**	(1.2-4.5)	**0.004**	**2.2**	(1.1-3.7)	**1.4**	(1.1-2.9)	**0.009**	**2.9**	(2.2-4.4)	**2.8**	(1.5-3.1)	0.07

Table shows median and IQRs. Uncorrected P values are for paired T tests, with significant values in bold.

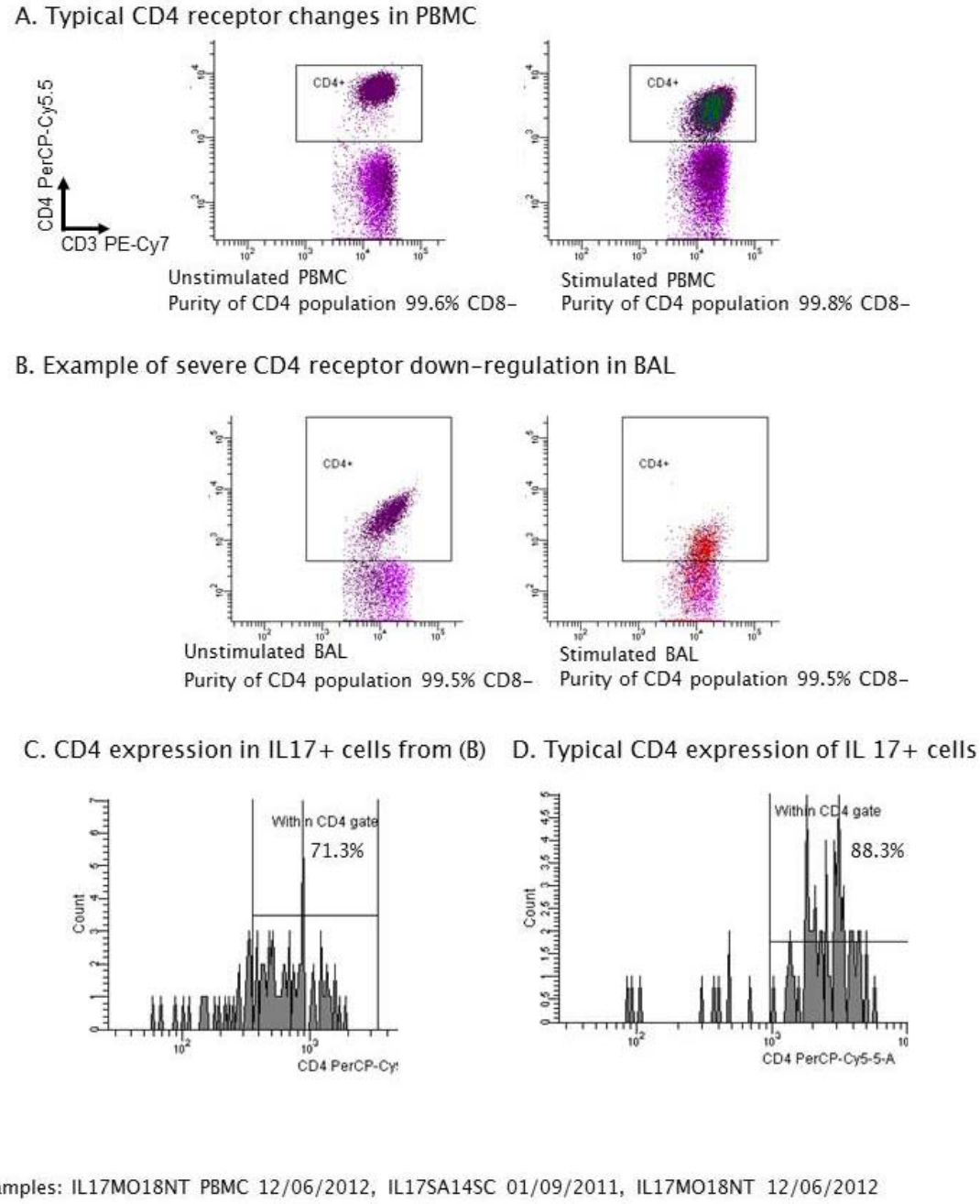


Figure 2.11 CD4 receptor down regulation

CD 4 co-receptor expression before and after 4.5 hours stimulation with PMA/ ionomycin. Despite poorer discrimination of the CD4+ and CD- populations, purity from CD8+ cells is usually preserved.(A) An extreme case of CD4 down-regulation after 5 hours stimulation with PMA/ ionomycin (B). Despite this dramatic change, the CD4 gate retains high purity from CD8- cells. Moreover it can be shown that the majority (71.3% in this case) of IL17+ cells continue to fall within the CD4+ gate (C). A more generally representative plot of CD4 expression amongst IL17+ cells showing that the majority of IL17+ fall within the CD4+ gate: nearly 90% in this case. Thus those cells which move out of the CD4+ gate due to receptor down-regulation are rarely IL17+ cells of interest.

Defining the T helper cell population by negative selection on CD8 would have the benefit of being free from CD4 downregulation. However, it would instead be affected by CD8 downregulation, albeit

to a lesser extent as the receptor is subject to less change of expression. More importantly it would necessarily include the population of CD4-8- cells which comprise 0.4-2.3% of all T cells. This would outweigh the benefit of avoiding use of CD4 as a marker. Furthermore it can be shown that whilst some cells disappear from the CD4 subset during stimulation, these tend not to be those which are strong cytokine producers. See Figure 2.11C. Specifically, because the brightest CD4 cells produce the strongest cytokine secretion, the vast majority of T_H17 cells continue to fall within the CD4+ gate despite CD4 downregulation: nearly 90% in the case shown and in excess of 70% of cells fall within this CD4+ gate even in the most extreme cases of receptor downregulation. See Figure 2.11 B,C.

Conclusion

Positively selecting on CD4+ cells in PBMC, BAL and sputum avoids contamination with double negative T cells, was affected only modestly by CD4 downregulation and did not increase contamination by CD8+ cells. I therefore used this strategy, except in the case of bronchial biopsies, which were additionally affected by collagenase cleavage of CD4.

Definitions of Treg for flow cytometry

Our current concept of regulatory T cells is based on Sakaguchi's 1995 description murine CD4+CD25+ regulatory T cells (Sakaguchi, Sakaguchi et al. 1995). Baecher-Allan later demonstrated that the human equivalent of these murine CD4+25+ cells was the $CD4+CD25^{Hi}$ population (Baecher-Allan, Brown et al. 2001), i.e. a population expressing high levels of CD25 that have the capacity to suppress the function of effector T cells. In 2003 Hori *et al* described the master regulator transcription factor FOXP3 as governing the development and function of Tregs and it is now considered the most reliable marker for natural Tregs (Hori, Nomura et al. 2003). Deficiencies in FOXP3 cause the immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX syndrome) (Hori, Nomura et al. 2003), with autoimmune responses in multiple organs in both humans and mice due to loss of peripheral tolerance (Ostroukhova, Qi et al. 2006). Expression of the *FoxP3* gene in transgenic mice and ectopic expression of *FOXP3* in human cells can genetically reprogram T cells to a regulatory phenotype (Fontenot, Gavin et al. 2003; Hori, Nomura et al. 2003; Khattri, Cox et al. 2003).

Problems with existing markers

CD25

As CD25 is a surface marker, it has proved to be a useful. However, because CD25 is also a marker of activation of T cells, its specificity as a marker of Tregs is limited. (Baecher-Allan, Brown et al. 2001). Furthermore, whilst the CD25hi population is more specific for natural Tregs, it lacks sensitivity because a significant proportion of FOXP3 expressing cells are CD25-. Whilst only 1-2% of peripheral CD4+ cells are CD25hi, up to 8-10% of peripheral CD4+ T cells in humans are FOXP3 positive (Liu, Putnam et al. 2006). Thus the CD4+25hi definition lacks sensitivity and specificity.

CTLA4 and GITR are also reported to be expressed on Treg, but as with CD25 are also expressed on effector T cells (Liu, Putnam et al. 2006).

CD127

In 2006 two groups described CD4+25+CD127lo cells in humans as expressing high levels of FOXP3 and having suppressive functions(Liu, Putnam et al. 2006; Seddiki, Santner-Nanan et al. 2006). These cells are anergic and as suppressive as $CD25^{Hi}$ cells, but are three times as abundant as $CD25^{Hi}$ cells (Liu, Putnam et al. 2006). Liu *et al* also showed an inverse correlation between FOXP3 and CD127 expression and showed FOXP3 interacts with the CC127 promoter.

In healthy adults Seddiki *et al* found CD4+127lo was 87% specific and 84% sensitive for CD25+FoxP3+ cells and that suppression was cell-contact dependent (Seddiki, Santner-Nanan et al. 2006).

In summary, FOXP3 remains the most specific marker for natural Treg (Liu, Putnam et al. 2006). Initially relative *FOXP3* mRNA expression was measured (Guyot-Revol, Innes et al. 2005), but more now antibodies are available, allowing me to use intracellular staining. Nonetheless FOXP3 is only an intracellular marker, so this technique requires fixation and permeabilisation preventing isolation of viable Tregs that are needed in functional studies. For these studies CD127lo is superior to CD25hi cells, but the best surface phenotype is probably CD4+25+127Lo, which comprise 87% of FOXP3+ cells (Seddiki, Santner-Nanan et al. 2006).

Frequencies of Treg

CD4+CD25+ Treg cells comprise approximately 10% of peripheral CD4+ cells(Roncador, Brown et al. 2005). Expression of FOXP3 is highly restricted to Treg populations - indeed genetic transfer of *FoxP3* converts naive CD4+CD25- T cells to a regulatory phenotype – and is highly correlated with CD25 expression in natural CD25+ Treg, so it is a useful specific marker of Treg cells(Wang, Zhang et al. ; Roncador, Brown et al. 2005). Using the phenotype CD3+CD4+FOXP3+ to define Treg the upper range of Treg in peripheral blood is 8-10% of CD4+ T cells in humans (Liu, Putnam et al. 2006; Lin, Chen et al. 2007; Bonelli, von Dalwigk et al. 2008; Bi, Suzuki et al. 2009). The normal range in health has been estimated at 7.5%±2.4% (mean ±SD) (Bi, Suzuki et al. 2009) or 6.5%±1.3%(Bonelli, von Dalwigk et al. 2008). This equates to approximately 4% of CD3+ cells (range 2.5-7.5) (Brusko, Wasserfall et al. 2007)or 1.2% of all lymphocytes(Freier, Weber et al.).

For my work I defined Treg as live, singlet, CD3+, CD4+, FOXP3+ cells. It was not practical to use CD25 as an additional marker due to the small size of tissue samples and use of other cytometer channels for markers for other T cell subsets. Isotype controls for rat IgG2α-APC proved less reliable for setting the FOXP3+ gate than use of the CD4 negative population, which does not express FOXP3, to act as a negative control within the same sample tube (see Figure 2.11). This approach was based on that previously used by my colleague Asha Ganesan (Ganesan 2010). To ensure

consistency it was necessary to define the number of non-specific events which could be found within this empty P1 gate as a proportion of total CD3+ cells. Due to cleavage of CD4 by collagenase, biopsy Treg were defined as the percentage of live FOXP3+ CD8- T cells, using an empty P2 gate set on CD8+ cells.

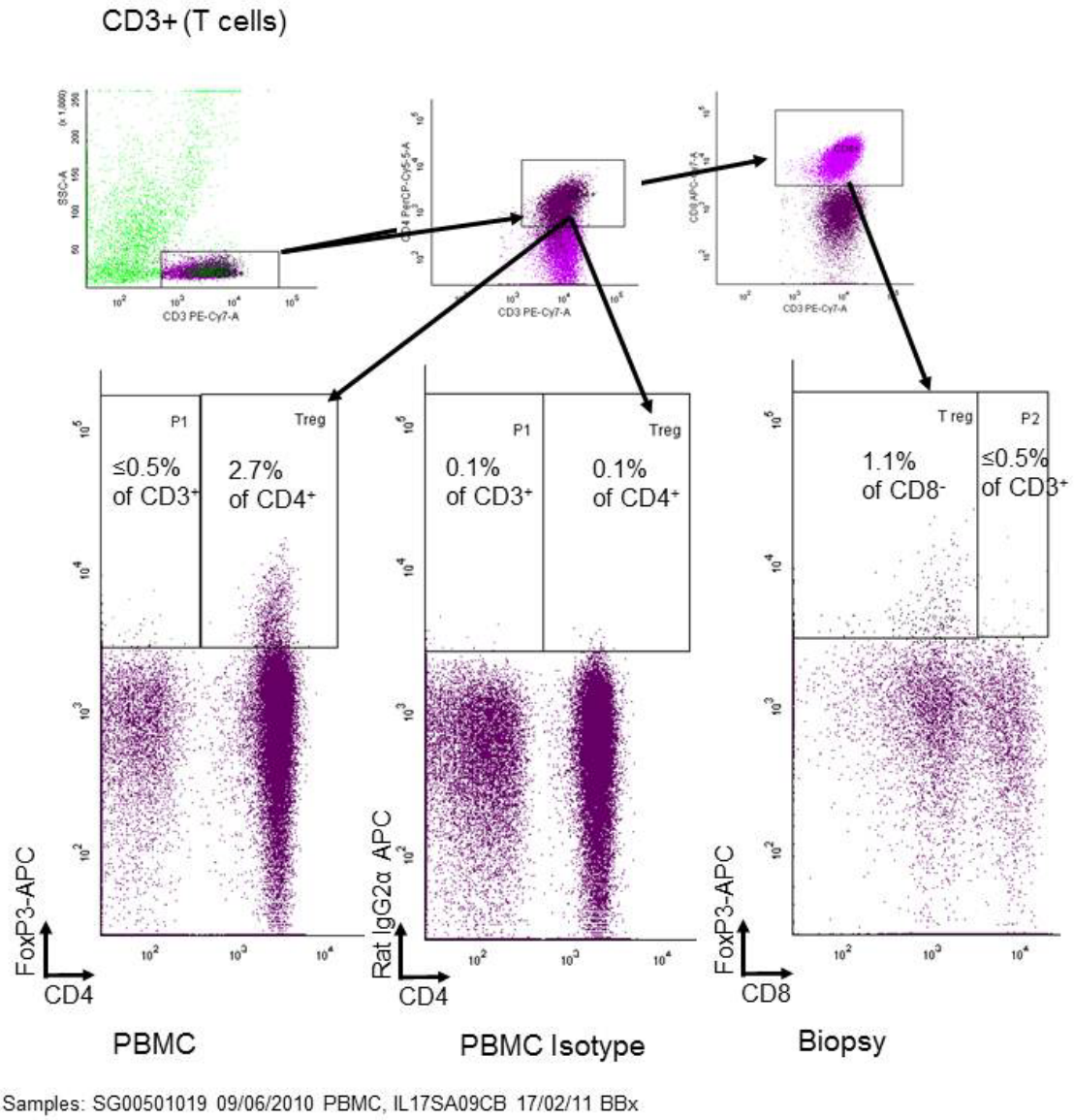


Figure 2.12 Setting of regulatory T cell gates

Schematic of hierarchical gating strategy used to identify Treg, which were defined as percentage of live singlet, CD3+CD4+ cells expressing FOXP3. As FOXP3 was not expressed in CD4- cells this gate was set by the level amongst CD4- cells, such that the P1 gate contained ≤0.5% of CD3+ cells. Biopsy Treg were identified in an analogous manner as percentage of live, singlet CD3+8- T cells expressing FOXP3, according to a CD8+P2 gate containing ≤0.5% of CD3+ cells.

Data analysis to define set-point of FOXP3+ gate

To define this set-point I analysed data I acquired from PBMC from 15 healthy individuals. I analysed T cell frequencies using cut-points of ≤0.5, ≤0.4, ≤0.3, ≤0.2.

Results

Results are shown in Table 2.8

Table 2.8 Results of Treg set-point analysis. PBMC T cell frequencies in n=15 healthy individuals according to definition of P1 gate.

Treg frequency (% of total CD4 T cells)	**P1 cut point (% of total CD3+ cells within gate)**			
	≤0.5	≤0.4	≤0.3	≤0.2
Median	4.5	4.1	3.7	3.4
Interquartile range	(3.8-7.5)	(3.4-6.4)	(3.1-5.6)	(2.4-5.0)
Maximum and minimum range	(1.3-11)	(1.1-11.2)	(0.9-10.5)	(0.6-9.9)

Conclusion

These results imply that adjusting the cut-point has rather little effect on the maximum and minimum values, implying that data-points occurring at the extremes of the Treg range were true values, rather than artefacts of poorly chosen cut-points. This conclusion is supported by the wide ranges seen in the studies cited above.

A cut point of P1 ≤0.5% would be consistent with previous work from our group (Ganesan 2010) and gives a mean (±SD) of 5.2%±2.7% which would be consistent with, but on the low side of the normal ranges for the studies cited above. Therefore I selected a cut-point of up to ≤0.5% in P1 gate for PBMC, sputum and BAL and of ≤0.5% in the P2 gate for biopsy T cells.

Comparison of fresh versus cryopreserved PBMC

The need for cryopreservation

My analysis of viral induced exacerbations was based on samples obtained from the SG005 clinical study already described, in which samples were obtained on a daily basis for a period of 13 months and which therefore made it necessary to cryopreserve peripheral blood mononuclear cells to minimise inter-assay and inter-operator variability. It was however necessary to optimise the methods used for cryopreservation and for subsequent intracellular cytokine staining and also to determine what effect this additional step had on my immunological results. What follows is the optimisation and validation work I conducted.

Cryopreservation has become a widespread technique in recent years (Disis, dela Rosa et al. 2006) and is particularly valuable in longitudinal studies of immunological parameters as it minimises inter-

assay variability inherent in studies, where assays will be affected by changes in unstable reagents, or variability in batches of reagents and by variability in the conduct of an experimental protocol(Weinberg, Song et al. 2009). Secondly it minimises inter-operator variability, as it can enable all assays to be performed by a single investigator, despite sample collection occurring on a daily basis over a long period, by a number of users. Thirdly, the ability to batch process samples greatly reduces workload, making practicable a volume of work which would otherwise be unviable.

Concerns regarding cryopreservation

The method of cryopreservation can have a significant impact on cell viability and functional responses (Betensky, Connick et al. 2000; Weinberg, Wohl et al. 2000; Maecker, Moon et al. 2005), and may increase CD4+ T cell apoptosis (Owen, Sinclair et al. 2007). Moreover, the technique itself may introduce a new source of inter-assay variability. It was thus essential to ensure the technique was optimal prior to collecting longitudinal samples. Nonetheless, with optimal cryopreservation techniques there is evidence of good correlation between results obtained from intracellular cytokine staining with fresh cells and those obtained using cryopreserved cells, with results which did not differ significantly (Maecker, Moon et al. 2005; Jeurink, Vissers et al. 2008; Weinberg, Song et al. 2009). Similarly several groups have demonstrated strong correlation between Treg frequencies in fresh and frozen PBMC (Costantini, Mancini et al. 2003; Elkord 2009).

Method

PBMC were obtained from healthy individuals and either resuspended in RPMI with 10% FCS for overnight culture at 37℃, 5%CO_2, or were immediately cryopreserved at -80℃ in either human serum albumin or foetal calf serum with 10% DMSO, then defrosted at 37℃ into RPMI with 10% human serum and all cells stimulated the next day for 4 hours and stained for intracellular markers.

Results

Typical plots showing direct comparisons of staining are shown in Figure 2.13. Human Treg frequencies (these cells were not stimulated) were well preserved despite cryopreservation, but were superior with HSA compared with FCS, as expected (Disis, dela Rosa et al. 2006). In a large comparison (n=23) of the effects of cryopreservation on PBMC there was no significant effect on Treg frequencies (Figure 2.14 A, Table 2.9), but there was a 56% fall in observed median frequencies of T_H17 cells (P=0.01) and a 62% fall in T_H1 cells (P=0.02). Changes in T_H2 cell frequencies were smaller and not significant (P=0.1).

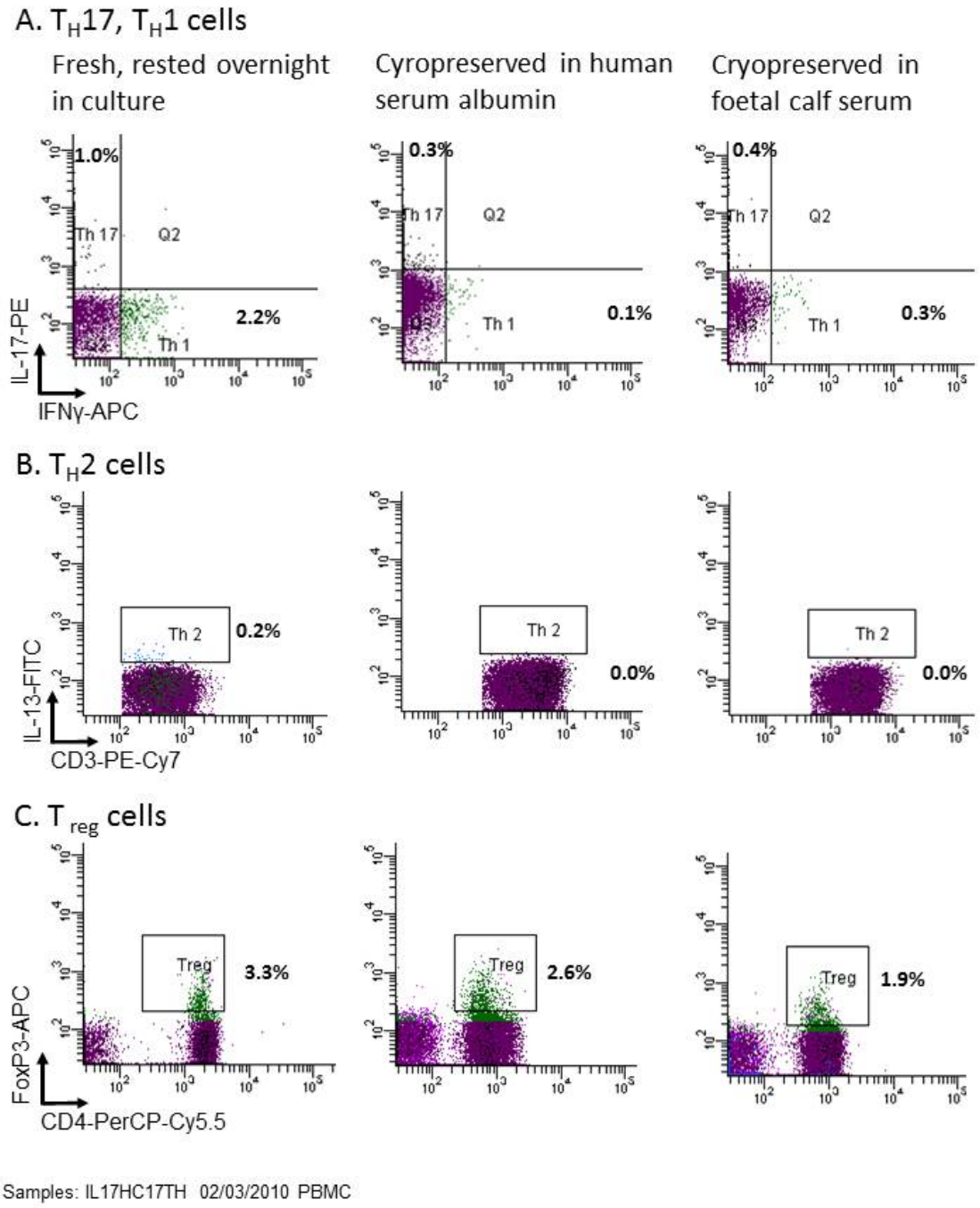


Figure 2.13 Intracellular cytokine staining in fresh and cryopreserved cells

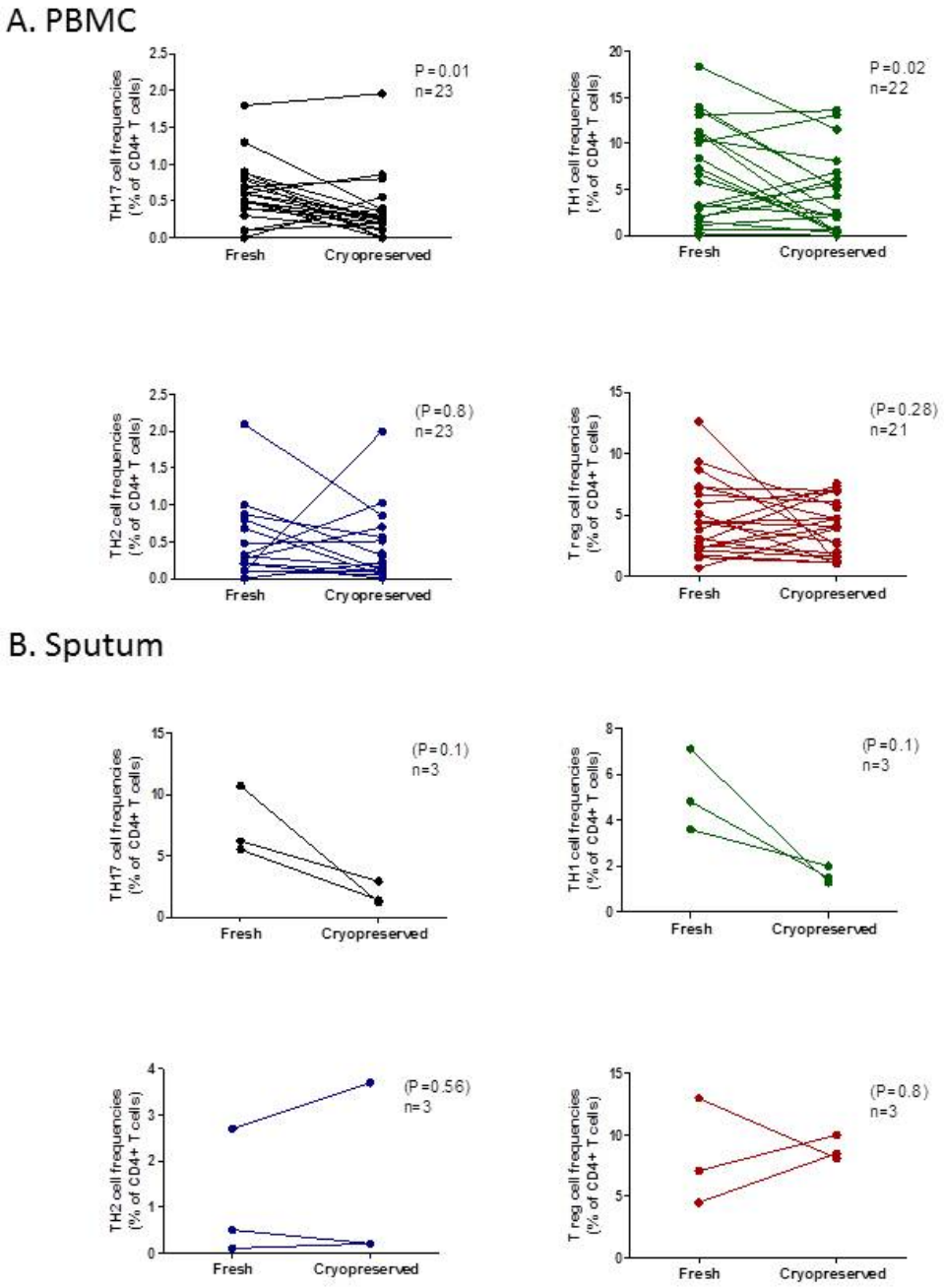
Intracellular cytokine staining in PBMC compared between cells which have been rested overnight in culture and those which have been cryopreserved in human serum albumin or foetal calf serum, then defrosted and stimulated. Plots show frequencies of TH17 and TH1 cells (A), TH2 cells (B) and Treg (C) as a percentage of live CD4+ T cells.

Table 2.9 Median frequencies of T cell subsets assessed by intracellular staining with or without prior cryopreservation, in sputum and blood.

	PBMC, n=23			**Sputum,** n=3		
	Fresh	Cryopreserved	P	Fresh	Cryopreserved	P
Th17	0.55	0.24	**0.01**	6.2	1.4	0.1
Th1	6.3	2.4	**0.02**	4.8	1.5	0.1
Th2	0.15	0.10	0.8	0.50	0.20	0.6
Treg	4.4	4.1	0.3	7.1	8.5	0.8

Table shows median T cell frequencies as percentage of CD4+ T cells. P values are for paired T tests

I performed the same comparison with a much smaller (n=3) set of sputum samples (Figure 2.15 B, Table 2.9). Due to the small sample size differences were not statistically significant, but the trends were similar to those in blood, with no loss of Treg, but a 60-75% fall in frequencies of stimulated T_H17, T_H1 and T_H2 cells.



Samples: Paired PBMC and sputum samples. P values represent paired *t* tests.

Figure 2.14 The effect of cryopreservation on measurement of specific T cell subsets

Median frequencies of T cells in major T cell subsets as measured by intracellular staining in samples of PBMC (A) and sputum (B) which have either been rested overnight in cell culture or have been first cryopreserved at -80℃ or -196℃ in human serum albumin with 10% DMSO then defrosted at 37℃ and rested overnight in tissue culture before stimulation with PMA and ionomycin for 4-5 hours in the presence of monensin. Treg samples were not stimulated.

Conclusion

Cryopreservation has a negligible effect on Treg frequencies, but causes a 33-62% reduction in frequencies of T_H17, T_H1 and $T_H2$cells. For this reason cryopreservation was only used for PBMC

from the longitudinal study where large numbers samples were expected from large numbers of subjects at eight different time points and where immediate analysis of fresh samples daily, over many months would not be feasible. It was anticipated that any loss of 'signal' from these immune assays would be compensated for by large sample numbers.

In the case of sputum samples these data suggest that the effect may be even more dramatic and furthermore many fewer samples were expected. Therefore every effort was made to process every sputum sample immediately, 7 days a week, for the duration of the trial. Results from fresh and cryopreserved samples were never included in the same analysis.

Choice of Golgi blocking agent for cryopreserved samples

I have observed lower frequencies of T_H1 and T_H17 cells after cryopreservation and thawing of PBMC, than in fresh PBMC. One possible explanation for this, suggested by comparison with other workers, was the choice of Golgi blocking agent. Monensin and brefeldin A are inhibitors of intracellular protein transport. Their addition to cell cultures during the last hours of in vitro activation of cells results in enhanced detection of intracellular cytokines. I therefore conducted some experiments to determine which agent might provide the optimal performance in previously cryopreserved cells.

Monensin is an antiprotozoal agent produced by *Streptomyces cinnamonensis*(O'Neil-Andersen and Lawrence 2002). It is a carboxylic sodium ionophore, which inhibits trans-Golgi function (Nylander and Kalies 1999; O'Neil-Andersen and Lawrence 2002). by disrupting intracellular Na+ and H+ gradients it exerts its greatest effects on the regions of the Golgi apparatus that are associated with the final stages of secretory vesicle maturation(Mollenhauer, Morre et al. 1990; O'Neil-Andersen and Lawrence 2002), inducing radical slowing of newly synthesised proteins, proteoglycans and plasma-membrane glycoproteins, inhibiting endocytosis and thereby stopping protein recycling(Karlsson and Nassberger 1995).

Brefeldin A is a naturally occurring macrocyclic lactone antibiotic, produced by a variety of fungi, including *Penicillium brefeldianum* and is synthesized from palmitate (O'Neil-Andersen and Lawrence 2002). It has a number of cellular effects, including inhibition of protein transport between the endoplasmic reticulum and the Golgi (Nylander and Kalies 1999) and transport from the trans-Golgi compartment to the cell surface (Karlsson and Nassberger 1995).

Several publications compare brefeldin and monensin directly in fresh cells and in general suggest that brefeldin A is a more potent, effective and less toxic inhibitor of cytokine secretion than monensin(Nylander and Kalies 1999; Schuerwegh, Stevens et al. 2001; O'Neil-Andersen and Lawrence 2002). There is higher spontaneous intracellular production of IL1β, IL6 and TNFα (Schuerwegh, Stevens et al. 2001), and IFN-y (Caraher, Parenteau et al. 2000) with brefeldin than monensin, but no difference in stimulated cells.

Moreover three separate reports all noted slightly lower viability with monensin(Nylander and Kalies 1999; Schuerwegh, Stevens et al. 2001), and there has been a suggestion that monensin may differentially kill CD4- cells, increasing the relative frequency of CD4+ cells(Nylander and Kalies 1999). Also of relevance to this work, CD4 down-regulation may be pronounced with brefeldin A (O'Neil-Andersen and Lawrence 2002). Interestingly whilst one might consider adding both monensin and brefeldin in combination, Bueno found Brefeldin A alone (10ug/ml) was superior to the combination of brefeldin A and monensin, as it was frequently associated with both a higher percentage of cytokine-positive cells and greater amounts of detectable cytokines per cell(Bueno, Almeida et al. 2001). There is little published literature on the choice of agent in the context of cryopreserved cells.

I therefore conducted several paired comparisons of brefeldin A (at a final concentration of 3.0 µg/ml) and monensin (2.0 µM) on defrosted cryopreserved cells, using a total of 20 different samples of cryopreserved PBMC stimulated for 5 hours in the presence of PMA and ionomycin.

Results

Results are presented in Figure 2.15. Mean frequencies of IFN-γ-producing (T_H1), IL-17-producing (T_H17) and IL13-producing (T_H2) cells tended to be higher with monensin than with brefeldin A and this difference was significant for T_H1 cells (P=0.035).

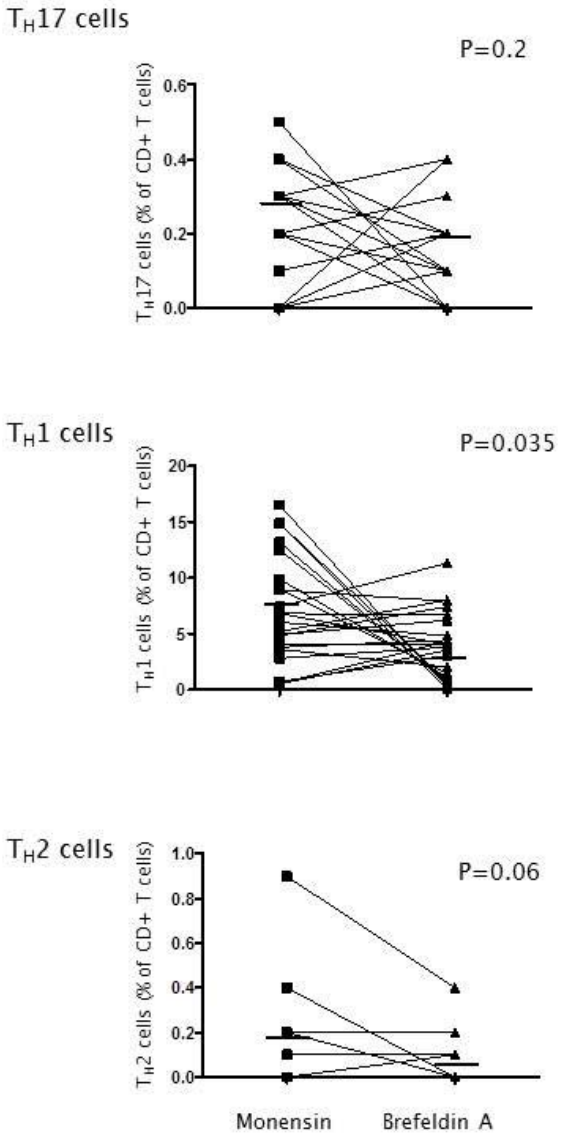


Figure 2.15 Comparison of two inhibitors of Golgi function

Paired comparisons of frequencies of TH17, TH1 and TH2 cells in previously cryopreserved PBMC stimulated with PMA and ionomycin for 5 hours in the presence of either monensin (2.0 μM) or brefeldin A (3.0 μg/ml). p values represent paired t tests, n=23.

Conclusion

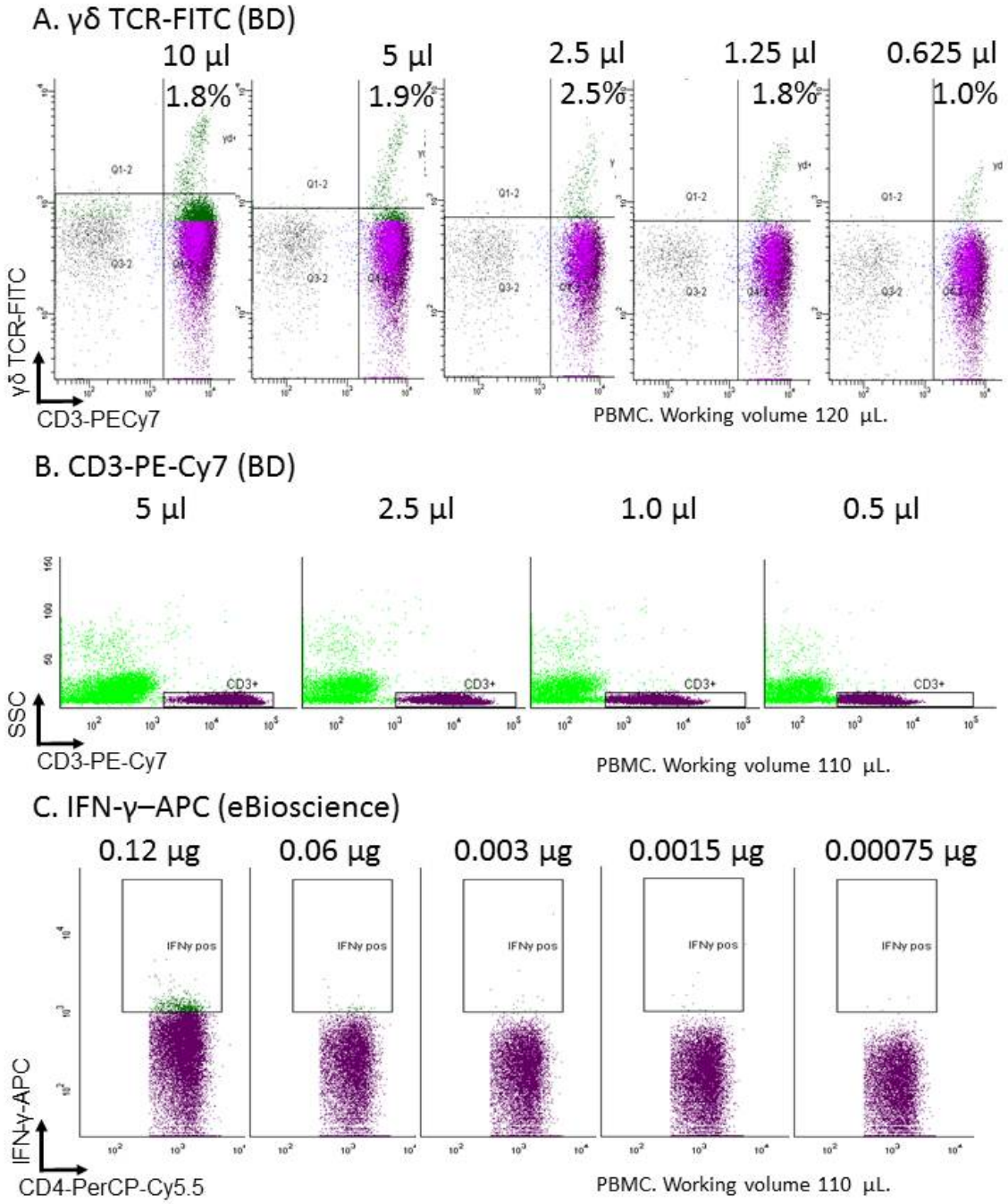
These experiments suggested that with my protocol, monensin gave better results than brefeldin A in previously cryopreserved cells. Therefore and for consistency with my colleagues' prior work and my other work in fresh tissue I chose to continue using monensin rather than brefeldin.

Selection and titration of antibodies

To enumerate the particular T cell subsets of interest to me it was necessary to construct my own panels of fluorescent conjugated antibodies for flow cytometry.

Selection of antibodies was influenced partly by existing protocols within the group, (Ganesan 2010) but I developed these into my own antibody panels according to specific needs, with the aim of optimising performance on small tissue samples by identifying the maximum number of T cell subsets within the minimum number of tubes. CD3-PE-Alexa 610 (MHCD0322, Caltag) performed poorly in tissue compared with acceptable staining in PBMC, so was replaced by CD3-PE-Cy7 (SK7, BD). Treg were defined by FOXP3 alone as a single marker required less sample and I did not attempt functional experiments. To maximise sensitivity for intracellular stains IFN-γ-PE-Cy7 was replaced with IFN-γ-APC as this a brighter fluorochrome.

Antibodies were titrated in blood to determine their optimal staining concentrations (Figure 2.16). Surface stains could generally be used at high dilutions in blood, but at least double the concentration was used for tissue samples due to the higher backgrounds observed. Intracellular stains were used at the same concentrations in blood and tissue.



Samples: A.IL17SA04AS 27/05/2011 PBMC, B.IL17HC02HW 14/05/2010, C.IL17HC05 22/06/2010 PBMC

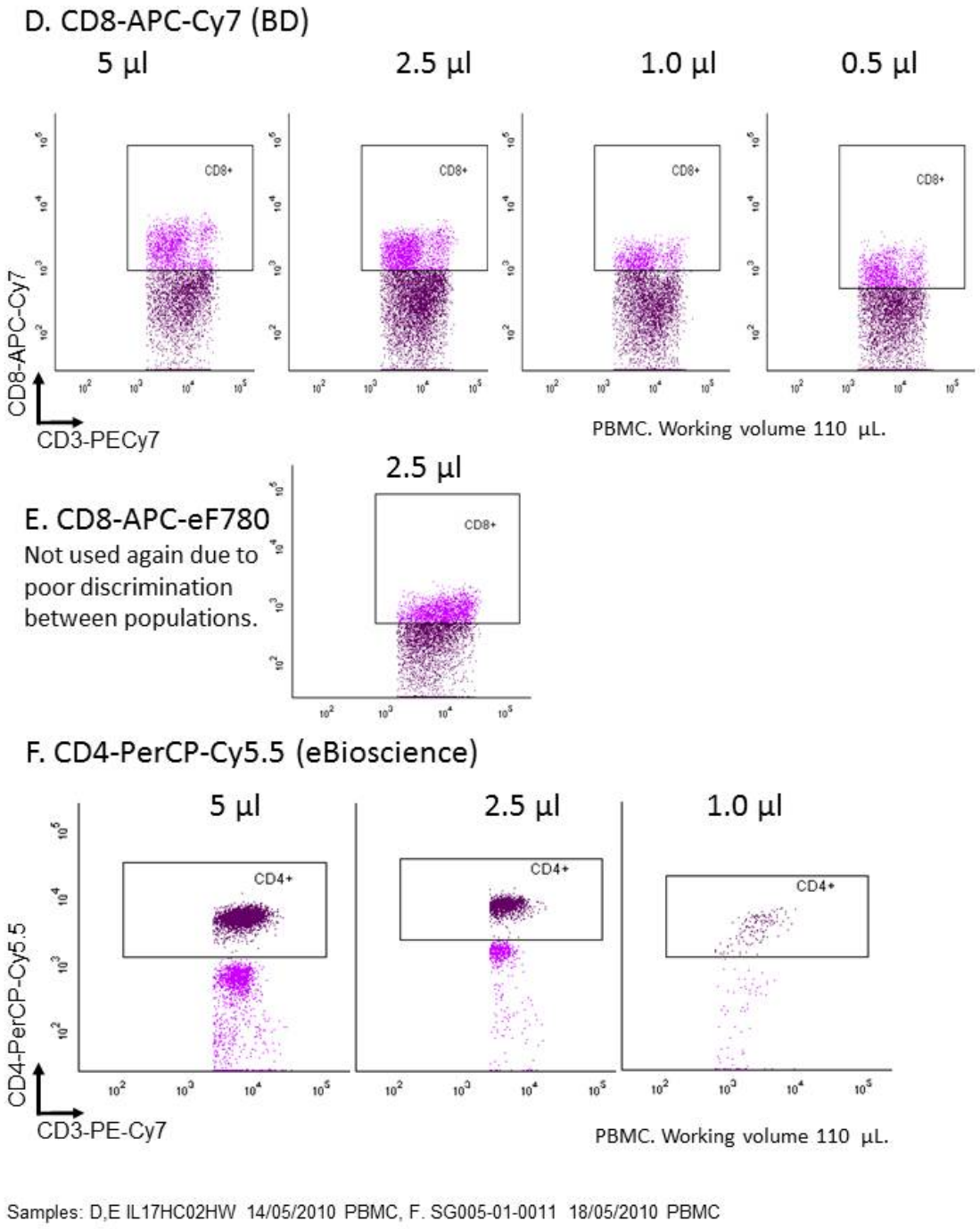


Figure 2.16 Titration of antibodies

Titration of antibodies to determine optimal concentration for use in the project. Titrations were performed in PBMC. Final concentrations chosen are shown in Table 2.4 and 2.5 A. γδ TCR-FITC (555748, clone MOPC-21, BD) B. CD3-PE-Cy7 (557851, clone SK7, BD) C. IFN-γ-APC (17-7319-82, clone 4S.B3, eBioscience) D. CD8-APC-Cy7 (348813, clone SK7, BD) E. CD8-APC-eFlour®780 (47-0087-41, clone SK7, eBioscience) showing very poor discrimination between positive and negative CD8 cells. F. CD4-PerCP-Cy5.5 (552838, clone L200, eBioscience).

Determination of optimum period of stimulation for MAIT cell intracellular cytokine secretion

To enable me to analyse the phenotype of MAIT cells it was necessary to determine the optimum period of cytokine stimulation, in particular to ensure that if a cytokine was not detected this was truly due to the absence of the cytokine, rather than poor performance of the assay. It was conceivable that IL-17 secretion in MAIT cells might not follow the same time course as that seen with CD4+ T_H17 cells. It was also noted that cell surface marker expression is significantly affected by stimulation, which might prove particularly problematic for cells selected on their specific surface marker phenotype. I therefore performed two time-course experiments to determine the period for optimal IL-17 expression and preservation of surface phenotype.

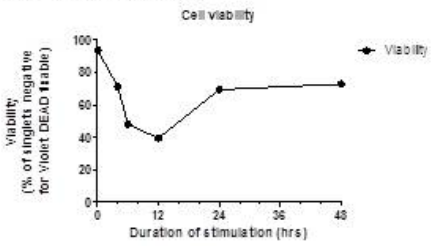
Methods

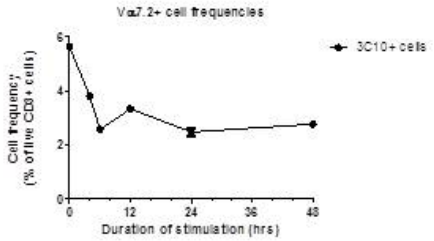
PBMC were obtained from a healthy control then cultured for 48 hours in AIMV media at 37℃, 5% CO_2. 25 ng/ml PMA and ionomycin 500 ng/ml were added for 0, 4, 6, 12, 24 or 48 hours. Monensin (2 μM) were added only for last 2 hours of all experiments to ensure the effect of its toxicity was equivalent in all samples. In a second experiment I added monensin for 5 hours, for consistency with my standard protocol and also performed a timecourse to characterise the toxicity of monensin alone over time.

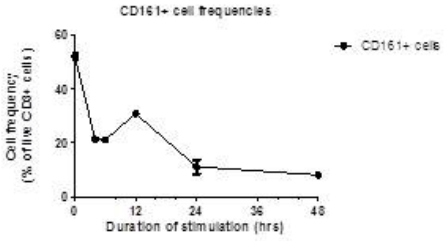
Results

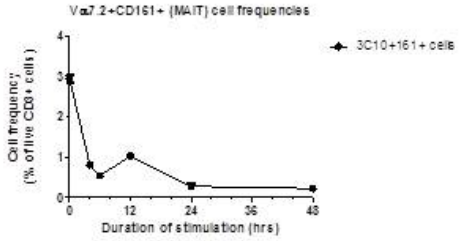
Results are shown in Figure 2.17. The following observations can be made. Firstly cell viability drops with stimulation from nearly 100% initially to 71% at 4 hours and 48% at 6 hours (A). It then increases, presumably due to cell proliferation. This favours the use of shorter stimulation times.

A. Effect of cell stimulation on viability and expression of MAIT cell surface markers

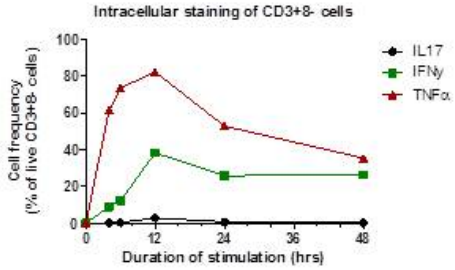


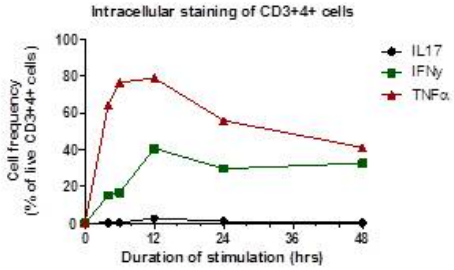


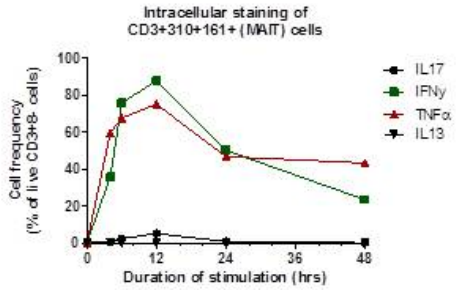


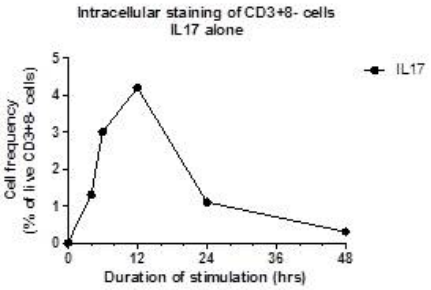


B. Time-course of intracellular cytokine secretion









Samples: IL17HC17TH 02&20/02/09 PBMC

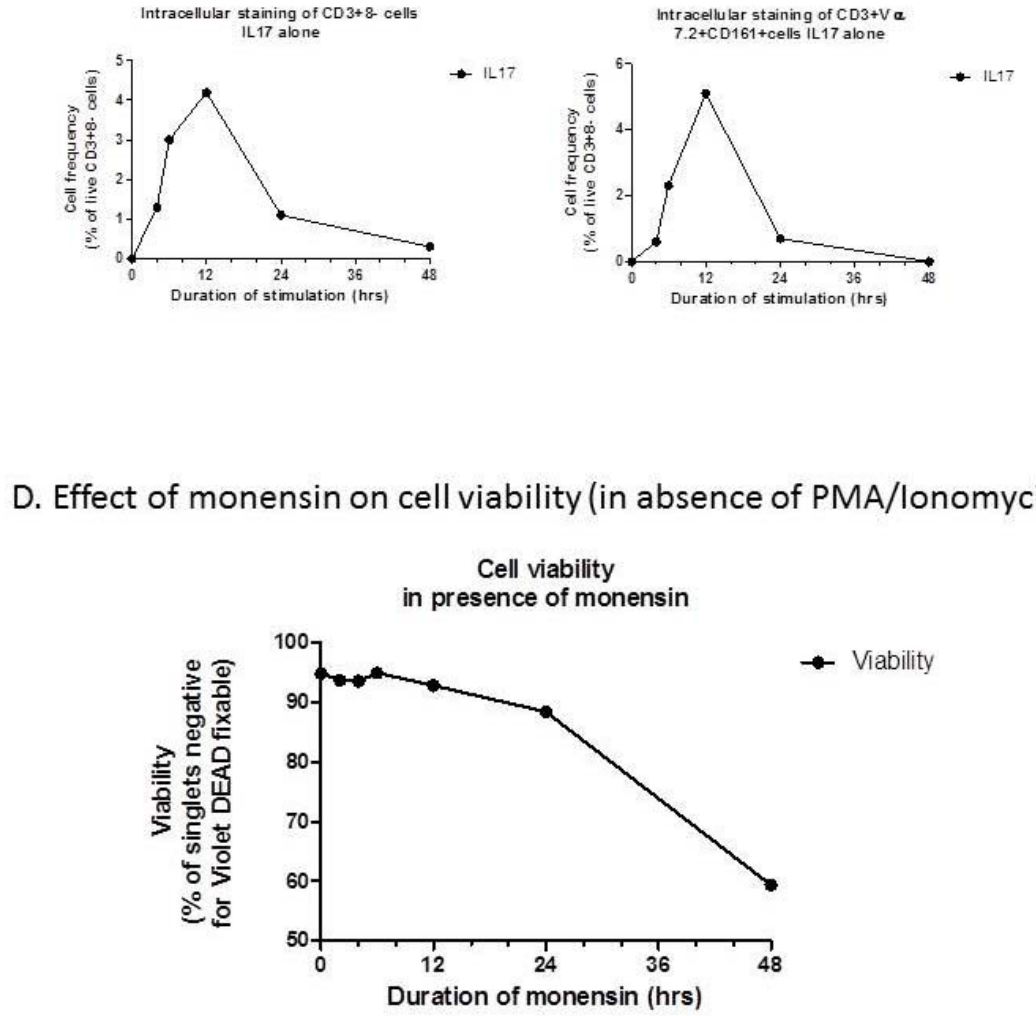


Figure 2.17 Determination of optimum period of stimulation for MAIT cell intracellular cytokine secretion

A. Cell viability – measured by exclusion of Violet LIVE/DEAD Fixable – and frequencies of viable TCR Vα7.2 cells, CD161+ T cells, and MAIT (Vα7.2+CD161+) cells in samples which have been stimulated with PMA and ionomycin for 0 to 48 hours, showing the effects of cell toxicity, receptor down-regulation and also new cell proliferation.

B. Changes over time in measured frequencies of T cells expressing IL-17, IFNγ, and TNFα expressed as a percentage of live CD8- or CD4+ or MAIT cells depending on duration of stimulation. Frequencies of TH17 cells are shown again in greater detail at bottom right.

C. The same time-course pattern is observed for secretion of IL-17 in TH17 cells (left) as with MAIT cells (right).

D. In the same experiment some cells were maintained in culture for 48 hours in the absence of stimulation, but with the addition of monensin for 0 to 48 hours. After 48 hours monensin causes significant cell death. (Note y axis does not start at 0).

Secondly TCR Vα7.2 and CD161 both down regulate rapidly over first 6 hours (A). Again this favours shorter experiments, but it should be noted that even by 4 hours apparent MAIT cell frequencies have fallen by 2/3.

Thirdly (B) TNFα expression is rapid and robust, peaking at 12 hours, but reaching 90% of this peak value by 4 hours. IFN-γ secretion is also rapid and robust, though somewhat slower peaking at 12 hours but reaching only 42% of its peak value by 4 hours. Likewise IL-17 secretion is also rapid and robust, peaking at 12 hours, but reaching only 31% of its peak value by 4 hours. IL-13 production is minimal and bimodally distributed with peaks at 12 hours and then a larger peak at 48 hours, but near the limit of detection.

Fourthly a key observation is that the time-course for IL-17 secretion by MAIT cells specifically was identical to that by CD3+8- cells generally (C). This would support the use of standard published protocols widely used in T cell research – 4 to 5hrs stimulation with PMA/ionomycin, in the presence of monensin - in the investigation of IL-17 secretion by MAIT cells.

When the experiment was repeated, longer use of monensin (5 hours) was associated with greater cell death (not shown). Used alone monensin does not cause significant toxicity until over 24 hours, (D) but in combination with PMA and ionomycin cell viability was very low in this experiment, possibly due to synergistic toxicity. Otherwise the cytokine secretion time-courses were similar.

Conclusions

Cell viability falls progressively with time during in the presence of PMA, ionomycin and monensin. My data support a standard 4-5 hours stimulation with PMA, ionomycin and monensin for my work. Due to the practicalities of differences in tissue handling slightly different durations were used for different tissues, as outlined in chapter 2. Surface CD161 and Vα7.2 are rapidly downregulated with by this stimulation and combined with the effects on cell viability I have shown that it will not be possible to first stimulate cells then sort MAIT cells by surface phenotype. Instead characterisation of MAIT cell function requires sorting of unstimulated cells in the first instance, or the establishment of ex-vivo cell lines (clones), which is the avenue I chose to pursue.

Optimisation and validation of RNA extraction method

To obtain further detailed phenotypic characterisation of T cell subsets I aimed to supplement flow cytometry data with measurement of cytokine production and T cell transcription factors at the mRNA level by RT-qPCR on sorted T cells and epithelial cells. An obstacle to this is that to perform intracellular cytokine staining the samples undergo fixation-permeabilisation (30 mins in eBioscience intracellular permeabilisation buffer containing formaldehyde). This cross-links nucleic acids to proteins, inhibiting RNA extraction(Masuda, Ohnishi et al. 1999). Theoretically this can be overcome by using a proteinase-K digestion step to reverse the cross linking (Masuda, Ohnishi et al. 1999). Such methods have been used for successful RNA extraction from archived formalin-fixed paraffin embedded (FFPE) histological tissue samples in combination with laser capture micro-dissection(Godfrey, Kim et al. 2000; Lehmann and Kreipe 2001).

To determine whether this technique could be applied to fix-permeablised samples I compared RNA extraction and RT-qPCR on two housekeeping genes - YWHAZ and β2microglobulin (β2M) - from unfixed PBMC using the Stratagene Absolutely RNA Microprep RNA extraction kit (400805, Agilent), with fix-permeabilised PBMC extracted using the Stratagene Absolutely RNA FFPE kit (400811, Agilent) involving a proteinase-K digestion step.

Method

In two separate experiments fresh PBMC were obtained, resuspended in PBS or fixation-permeabilisation buffer for 30 minutes, then diluted in 10-fold steps across the range 10 to 600,000 cells in PBS or perm-wash buffer respectively. Cells were then centrifuged at 400*g* for 5 mins, resuspended in 100 µl lysis buffer (Agilent) with 0.7 µl β-ME or proteinase K digestion buffer (Agilent) respectively, frozen to -80℃, then defrosted and RNA extracted according to the manufacturer's protocols. RNA extraction form unfixed samples is described in the methods chapter. Fixed samples were incubated with 10 µl proteinase K (final concentration 1.8 mg/ml) for 3.5 hours at 55℃. Next 0.875 µl β-ME and 125 µl RNA binding buffer were added to each sample on ice, vortex homogenised and diluted with 235 µl of sulfolane 90% (v/v) in RNase-free water, vortexed and RNA extracted using the RNA binding spin-caps according to the manufacturer's protocol.

RNA was quantified by spectrophotometer and reverse transcribed the same day using the Precision nanoScript™ kit (Primer Design). Housekeeping genes and a potential gene of interest – *FOXP3* - were quantified in duplicate by RT-qPCR using PerfectProbe primers (Primer Design).

Results

Optimal PCR signal was obtained for the housekeeping genes from 60,000 to 100,000 unfixed cells (Figure 2.18 A and B). Below, or above this range sensitivity fell, with higher CT values observed at 600,000 cells, which may be due to saturation of the spin cup matrix, as the manufacturer's report it is optimised for 1 cell to $5x10^5$ cells. (AgilentTechnologies 2008) There was virtually no detectable signal from fix-permeabilised cells at any concentration, with CT values near 40 cycles (limit of detection) for

the housekeeping gene and no detection of a gene of interest (*FOXP3*). The threshold for *FOXP3* detection is expected to be higher as the gene is expressed predominantly in T cells (a small proportion of PBMC) and at lower copy number than these abundant housekeeping genes and the primer pair spans a longer fragment (154bp) than β2M (114bp) or YWHAZ (120bp). It is recommended that FFPE is used with fragments less than 100bp (AgilentTechnologies 2008).

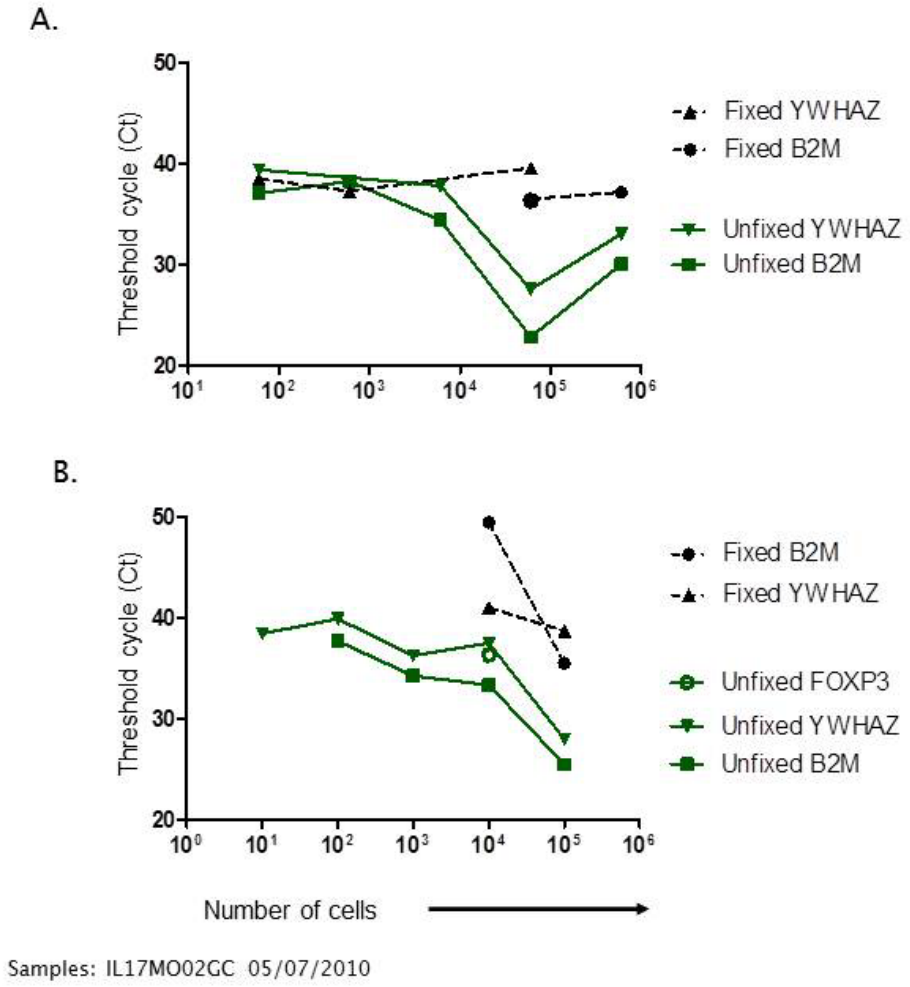


Figure 2.18 Comparison of RT-qPCR on fixed and unfixed T cells

Comparison of cycle threshold (CT) in 2 experiments in which RT-qPCR was used on either fresh PBMC or PBMC which had first been formalin fixed and permeabilised. A range of starting cell numbers were used. Unfixed cells were processed using the Agilent Microprep kit, whilst fixed cells were processed with the Agilent FFPE kit which included a 3 hour incubation step in proteinase K. Cells were reverse transcribed and quantified by RT-qPCR using PerfectProbe primers.

In a third experiment I tried to increase the RNA signal by using a variety of experimental conditions: use of the Agilent Nanoprep kit which uses a smaller spin-cup membrane, optimised for 1 to $1x10^4$ cells) compared with the Agilent Microprep kit (optimised for 1 to $5x10^5$ cells); comparison of proteinase K digestion for a standard 3 hours with shorter digestion for only 1 hour to minimise RNA

degradation; and comparison of 1:5 and 1:10 dilutions of cDNA prior to the PCR step, as there is a trade-off between amount of template and amount of reaction inhibitors. Again I obtained good RNA signal from unfixed cells using the Nanoprep kit (CT 27.65 at 1:5 dilution, CT 28.15 at 1:10 dilution of cDNA) in duplicate, whilst I obtained virtually no signal (CT ≥38.45) under any condition using fixed cells (data not shown).

Conclusion

These data show that it is not practicable to use low numbers of sorted fix-premeabilised cells for PCR, but that using unfixed cells, useable data can be obtained from 10,000 cells using the Nanoprep kit at dilutions of 1:5 or 1:10 of cDNA.

As a result of this work I elected instead to divide each sample into 1/3 for immediate surface staining and cell sorting and 2/3 for overnight culture and intracellular staining for data acquisition only. Moreover, rather than sort epithelial cells cytometrically using antibody to epithelial cell adhesion molecule (EpCAM, CD326), I have taken fresh epithelial cells obtained from bronchial brushings and frozen directly in RNA lysis buffer.

Deep sequencing of the metagenome

Unprocessed samples of sputum and protected BAL were frozen and shipped to the Virgin Laboratory (Department of Pathology and Immunology, Washington School of Medicine St Louis, MO, USA) where they were passed through a 24 μm filter and the metagenome sequenced by pyrosequencing using the Roche/454 next-generation sequencing platform (454 Life Sciences, Branford, CT, USA). Data were analysed using the VirusHunter analysis pipeline (Zhao) in which microbial sequences were identified on the basis of BLAST alignments and the taxonomic classification of the reference sequences to which a read is aligned.

Microarray

To measure whole transcriptome gene expression, pure populations of T cells were sorted directly into 100 μl Agilent lysis buffer with 0.7 μl of β-ME and homogenised, frozen and shipped at -80℃ to Janssen Research & Development (Springhouse, PA, USA) for further analysis as part of a collaboration between the company and my supervisor. RNA was extracted using the Absolutely RNA Nanoprep Kit, reverse transcribed and amplified by *in vitro* transcription with the Ovation Pico WTA System V2 (NuGEN Technologies, San Carlos, USA) and gene expression measured with the GeneChip® HT HG-U133+ PM Array Plate (Affymetrix, Santa Clara, USA) on an Agilent GeneArray Scanner. Samples which passed hybridization signal intensity threshold were robust multi-array average (RMA)/log2 transformed. Additional quality control (principal component analysis, correlation and median absolute deviation score (MADscore)) was performed in ArrayStudio v6.1 software (Omicsoft Corporation, Cary, NC).

Statistical Analysis

Data elaboration and preparation for analysis

Data in the cross sectional study were presented as median (interquartile range (IQR)) and frequency (percentage), unless stated otherwise. Normality of the quantitative (numeric) data distributions was tested by the Shapiro-Wilk test. If the data were not normally distributed they were logarithmically transformed.

Cross sectional study

Data were analysed by descriptive and exploratory statistical methods to compare relationships between variables. The data were analysed using Student's *t* tests (two groups' comparison) and where multiple groups were compared by one-way analysis of variance (ANOVA), the *post hoc* Dunnett's test was also applied (healthy control as reference category). If the groups were ranked according to disease severity, data were tested for linear trend using polynomial contrasts method.

If data were not normally distributed, or the logarithmic transformation generated a loss of many zero values, non-parametric tests were used. Alternatively, a minimal constant of 0.01 or 0.001 was added to each of the values to allow for a logarithmic transformation. The Mann-Whitney-U test was used to compare two groups, while multiple groups were compared by one-way ANOVA using the Kruskal-Wallis method (with *post hoc* Dunn-bonferoni's test applied at the *p*-values of the significant differneces) (Dunn 1964) Linear trend across ranked groups was tested using the pairwise Jonckheere-Terpstra test.

If cyclical patterns were suspected in the variations over time (e.g., seasonality in MAIT cell frequencies), non-linear regression was performed on the log transformed data using a standard sine function, as follows:

Ln (MAIT frequency)=0.0795+0.6024*sin((2π*[Seasonal quarter]/4)+78.27)

The modeling of ELISA and MSD data was performed by standard regression equation with a 5-parameter curve fit.

Exploratory Analyses of Relationships between variables

Additional explorative (hypothesis-generating) investigation was performed to identify possible associations among the demographic, behavioural and clinical parameters in the asthma patients, including the type of asthma (mild, moderate and severe). We used factor analysis (principal component method, or analysis, PCA) by varimax rotation with Kaiser normalisation and generation of scree plots (minimum 80% explained variance by the identified components). Only the variables with a rotated component matrix score >0.50 were considered due to limited sample size (Field 2000). To further check the robustness of identified principal components (clusters), a reliability analysis with computation of Cronbach's alpha coefficient was applied (>0.70 was assumed as acceptable) (Kline

1999). Relationships between pairs of continuous variables were also explored using Spearman's rank correlations.

The data for the longitudinal study were collected and analysed as paired observations per patient over time, for each individual variable, or as averaged, time-series data. Parametric and non-parametric paired tests were used, except for the data on fresh sputum and PBMC as very few samples were present. Analyses were essentially exploratory and descriptive. Analyses were planned a priori including use of ANOVA and t tests to compare baseline (T0) data with data from the time-point of peak difference.

In particular, a number of characteristics (e.g., T_H17 frequency) were measured over time in the groups of patients with rhIFN-β1α and placebo. Repeated measures ANOVA indicated a marginally significant univariate difference between the groups, but due to the lack of normal distribution, a pre-planned analysis further computed the individual areas under the curve (AUC) as summary measures of the overall dynamics for each variable. The AUCs were compared between the two groups by t test according to the method of Matthews (Matthews, Altman et al. 1990).

CHAPTER 3

CD4+ T cell phenotypes in asthma

Let no man think or maintain that anyone can search too far or be too well studied in the book of God's words or in the book of God's works; rather let all endeavour an endless progress or proficience in both.[3]

[3] On page ii of *The Origin of Species*, Charles Darwin FRS (1809-1882) quoted these words from Francis Bacon's *Advancement of Learning* (1605). Darwin, C. (1859). *On The Origin Of Species By Means Of Natural Selection*. London, John Murray.

Introduction

For over a decade many workers have hypothesised a significant role for IL-17 and the T_H17 cell subset in the pathogenesis of human asthma(Molet, Hamid et al. 2001) on the basis of human genetic associations(Hizawa, Kawaguchi et al. 2006; Kawaguchi, Takahashi et al. 2006; Chen, Deng et al. 2010; Lluis, Schedel et al. 2011), murine data (Park, Li et al. 2005; Schnyder-Candrian, Togbe et al. 2006; Fujiwara, Hirose et al. 2007; McKinley, Alcorn et al. 2008) and reports of IL-17 protein and mRNA in airway samples(Molet, Hamid et al. 2001; Barczyk, Pierzchala et al. 2003; Chakir, Shannon et al. 2003; Bullens, Truyen et al. 2006). Despite this there remains a pauctiy of robust human data and there are no studies which have investigated airway T_H17 cells in human asthma. I therefore sought to scrutinise these hypotheses by conducting a comprehensive review of T_H17 cells in the context of other major CD4+ T cell subsets in peripheral blood, sputum, BAL and bronchial biopsies from subjects with a range of asthma phenotypes and healthy controls during periods of clinical stability. This chapter describes my findings of this crosss-sectional study, including an analysis of serum and airway IL-17 protein in the context of other major T cell cytokines complemented by flow cytometry data on different T-helper cell subsets in blood, sputum, BAL and tissues.

Results and comments

Study population

23 healthy subjects and 53 asthmatics (14 mild, steroid-naïve, 17 moderate, treated with low dose inhaled corticosteroids and 22 severe, treated with oral or high dose inhaled corticosteroids) were studied. All had stable symptoms for at least 6 weeks prior to clinical sampling. The study design is shown in Figure 2.1 and clinical characteristics of the study participants are shown in table 3.1.

Table 3.1 Demographic and clinical characteristics of cross sectional cohort for CD4+ and CD8+ T cell analysis

	Healthy controls	Mild asthma	Moderate asthma	Severe asthma
n	23	14	17	22
Demographics				
Sex (M/F)	14 / 9	8 / 6	8 / 9	8 / 14
Age (median [range], years)	28 (20-65)	26 (21-64)	35 (21-56)	53 (23-67)
Pulmonary function				
FEV1 (% predicted)	108 (104-113)	88 (85-101)	99 (86-109)	65 (49-82)
FEV1 reversibility (%)	3.6 (1.8-7.9)	14 (9.9-19)	12 (6.7-19)	13 (2.6-25)
PEFR (% predicted)	108 (97-116)	97 (89-108)	95 (88-99)	70 (53-82)
PEFR variability (%)	15 (N/A)	17 (10-27)	22 (16-34)	17 (12-24)
PD20 (mg methacholine)	Negative	0.18 (0.044-0.48)	0.25 (0.057-0.58)	Not done
Exhaled nitric oxide (ppb, at 50 L/s)	16 (11-21)	56 (30-110)	27 (14-49)	20 (13-38)
Clinical				
Atopy (Skin prick positive, Y/N)	0 / 23	14 / 0	15 / 2	15 / 7
No. of skin prick allergens positive	0 (N/A)	6 (4-7)	3 (2.5-5)	3.5 (0-5.3)
Peripheral eosinophil count (10^9/L)	0.1 (0.1-0.2)	0.1 (0.1-0.6)	0.2 (0.2-0.3)	0.2 (0.1-0.3)
Total IgE (iu/ml)	32 (9.4-62)	173 (62-457)	119 (25-188)	84 (31-669)
Body mass index (kg/m^2)	24.5 (22.3-28.2)	23.6 (22.5-26.7)	25 (22.7-31.5)	31 (27.1-40.9)
Smoking status				
Never	21	13	15	17
Former (Mean pack years)	2 (4)	1 (5)	2 (1.8)	4 (26)
Current (Mean pack years)	0	0	0	1 (49)
Duration of asthma (years)	N/A	18 (15-25)	22 (10-25)	36 (21-49)
ACQ score	N/A	0.65 (0.43-1.3)	1.3 (0.75-1.8)	2.8 (2.2-3.5)
GINA level of control				
Controlled	N/A	7 (50)	3 (18)	0 (0)
Partly controlled	N/A	6 (43)	11 (65)	2 (9.5)
Uncontrolled	N/A	1 (7.1)	3 (18)	19 (90)
Treatment				
Inhaled steroids	No	No	Yes	Yes
Dose (equivalent mcg BDP)	N/A	N/A	400 (400-900)	1600 (1280-2000)
Maintenance oral steroids (Y,N)	No	No	No	6 / 16
Mean dose if taken (mg prednisolone/day)				11
Long acting β agonist (Y/N)	No	No	8 / 9	22 / 0
Leukotriene receptor antagonist (Y/N)	No	No	No	15 / 7
Step on BTS treatment algorithm	N/A	1	2 - 3	4 - 5
Inflammatory subtype (n, %)				
Neutrophilic	4 (25)	2 (14)	2 (14)	10 (48)
Eosinophilic	1 (6.3)	3 (21)	3 (21)	6 (29)
Mixed granulocytic	0 (0)	0 (0)	0 (0)	1 (4.8)
Paucigranulocytic	11 (69)	7 (50)	9 (64)	4 (19)
Sputum cell differential (%)				
Macrophages	52 (31-66)	45 (34-62)	53 (31-65)	30 (19-43)
Neutrophils	31 (11-65)	35 (22-58)	33 (16-56)	61 (32-76)
Epithelial	3.6 (2.0-24)	4.1 (0.83-11)	3.8 (1.1-16)	2.9 (0-7.8)
Eosinophils	0.38 (0-0.94)	1.5 (0.75-1.8)	1 (0.38-1.8)	0.69 (0-6.1)
Lymphocytes	0.1 (0-0.75)	0.3 (0-0.75)	0 (0-0.63)	0.0 (0-0.25)
BAL cell differential (%)				
Macrophages	84 (74-89)	70 (60-80)	81 (73-89)	72 (46-94)
Neutrophils	2.5 (1.0-5.9)	2.5 (1.6-4.8)	3.5 (1.8-6.4)	6.5 (1.4-29)
Epithelial	9.9 (3.9-18)	21 (13-35)	11 (5.6-19)	8.7 (3.3-11)
Eosinophils	0.25 (0.0-0.56)	2.0 (0.75-3.6)	1.0 (0-3.0)	0.1 (0-1.6)
Lymphocytes	1.4 (0.94-2.4)	1.5 (0.38-3.0)	1.3 (0.5-2.3)	1 (0-1.6)
Relevant comorbidities (n, %)				
Allergic rhinitis	0 (0)	11 (79)	8 (47)	10 (46)
Nasal Polyps	0 (0)	0 (0)	1 (5.9)	5 (23)
Eczema	1 (13)	7 (50)	5 (29)	4 (19)
Bronchiectasis (history or CT)	0 (0)	0 (0)	1 (5.9)	1 (4.5)

Values are medians with interquartile ranges, unless stated otherwise. N/A: not available.
Inflammatory subtype is based on sputum differentials using cut-points as per Simpson, J. L., R. Scott, et al. (2006). Respirology 11(1): 54-61 (neutrophilic: >61% neutrophils, eosinophilic: >3%). Percentages are of those with valid data.
ACQ, asthma control questionnaire; BDP, beclometasone dipropionate; BTS, British Thoracic Society; CT, computed tomogram; FEV1, forced expiratory volume in 1 second; FVC, forced vital capacity; GINA, Global Initiative for Asthma; PEFR, peak expiratory flow rate; PD20, provocative dose 20.

Measurement of IL-17 protein by enzyme-linked immunosorbent assay (ELISA)

A previous study has observed increased IL-17 protein levels in sputum samples of asthmatics using ELISA(Barczyk, Pierzchala et al. 2003). Therefore, to determine whether IL-17 protein could indeed be measured in respiratory specimens, I used an ELISA assay (88-7976, eBioscience) to detect IL-17 in a variety of different samples. Samples tested in duplicate included supernatants from sputum (n=15) and BAL (n=15) obtained during periods of clinical stability, and also supernatants from bronchial biopsies with and without *ex vivo* allergen challenge and lung parenchymal samples with or without *ex vivo* challenge with live X31 influenza virus. The assay produced a good standard curve with good replicates over the range 4-500 pg/ml (Figure 3.1 A), but IL-17 was not detected in any of the samples tested, suggesting it is either produced at very low abundance in these samples or very unstable, and that ELISA is not appropriate for this application.

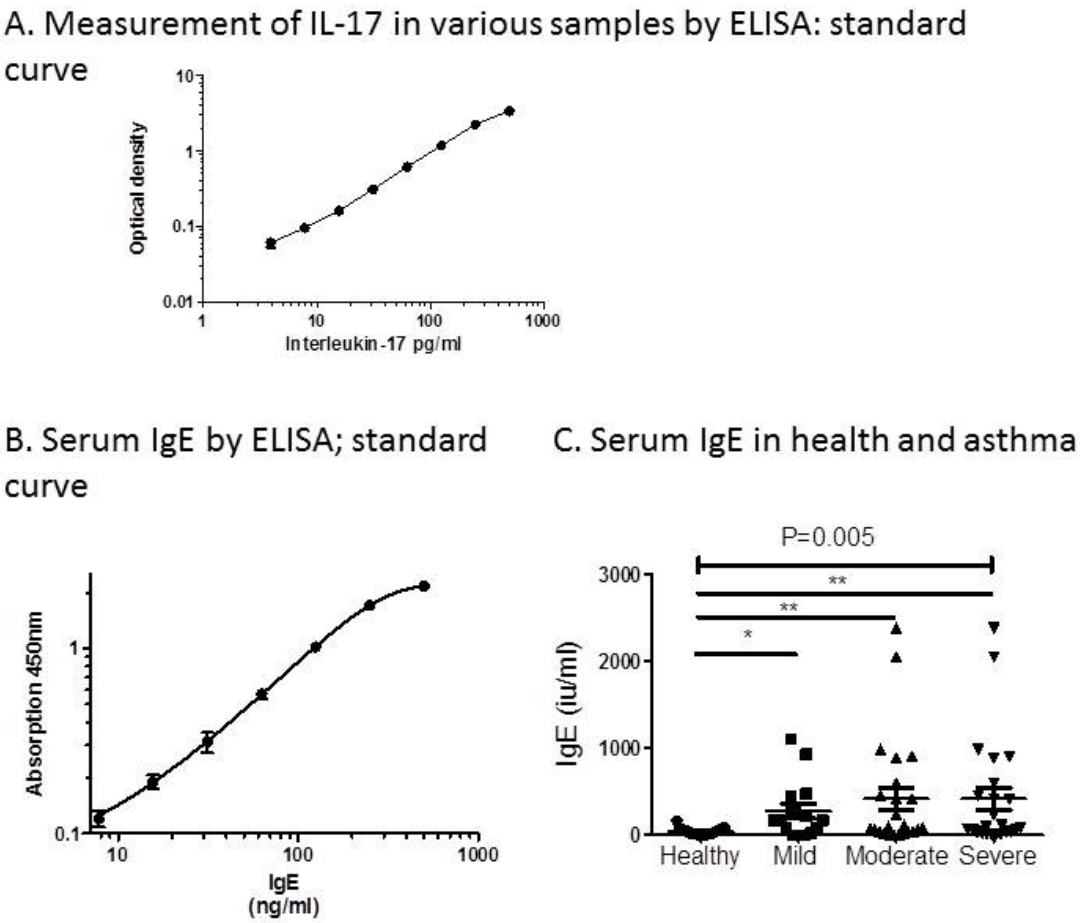


Figure 3.1 ELISA standard curves

Standard curves, using 5 parameter curve-fit, for ELISA experiments performed.

A. IL-17 showing accuracy over the range 4 to 500 pg/ml. (eBioscience 88-7976) Samples assayed included supernatants from sputum and BAL during clinical stability, bronchial biopsy supernatants with and without *ex vivo* allergen challenge and lung parenchymal samples with or without *ex vivo* challenge with live influenza virus.

B. Standard curve of serum IgE measured by ELISA showing accuracy over the range 7.8 to 500 ng/ml. (eBioscience BMS2097)

C. Serum IgE levels in healthy and asthmatic subjects. Differnces are compared by Kruskal-Wallis ANOVA with *post hoc* Dunn's.

Measurement of serum IgE

Serum IgE was measured by ELISA. The limit of detection (LOD) was 7.8 ng/ml and the results are presented in Figure 3.1B and included in table 3.1.

Detection of cytokines by electrochemiluminescence (MSD)

Using standard ELISA with a limit of detection of 4 pg/ml, I was unable to detect IL-17. Of note, others have since reported that IL-17 levels are typically <4 pg/ml in serum(Zhao, Yang et al. 2010) and frequently <2 pg/ml in sputum(Doe, Bafadhel et al. 2010). Therefore I chose to measure serum and airway cytokines using the more sensitive technique of multiplexed enzyme-linked electrochemiluminescent assay using the Meso Scale Discovery (MSD) platform.

According to the MSD manufacturer's reports 10 mM concentrations of dithiothreitol (DTT), a strong reducing agent used for processing sputum samples, denature antibodies thereby reducing assay sensitivity, while this effect has been found to be minimal at 1 mM by researchers (Yvonne Clements, personal communication). To achieve a compromise between dilution effects and antibody denaturation I decided to use dithioerythritol (DTE) at 5 mM. I directly compared assay sensitivity for a range of T cell cytokines diluted either in proprietary diluent or in a 1:1 mix of DTE with proprietary diluent. At this concentration I observed very close agreement for all cytokines tested across a wide range of concentrations from 0.61 to 2500 pg/ml (Figure 3.2 A, Table 3.2).

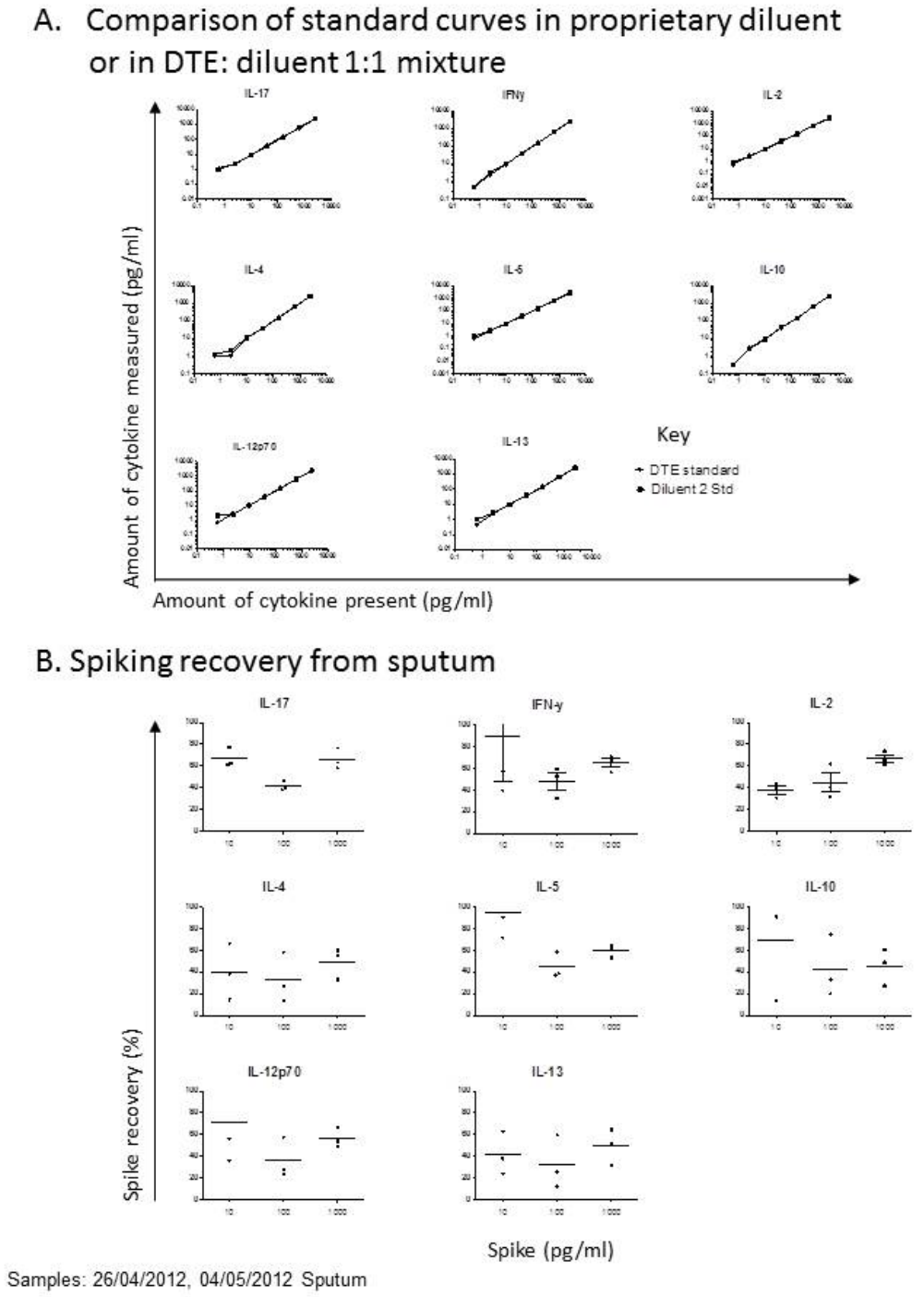


Figure 3.2 Validation of MSD in sputum

Measurement of cytokines by Meso Scale Discovery multiplex ELISA

A. To determine the most appropriate buffer for MSD when analysing sputum samples standard curves were prepared in proprietary diluent and also in a 1:1 mix of DTE with proprietary diluent. Very close agreement was observed across a wide range of concentrations from 0.61 to 2500 pg/ml.

B. Spiking recovery from sputum was tested for each cytokine in duplicate wells, in samples from three different subjects, at three different concentrations: 10, 100 and 1000 pg/ml.

Table 3.2 Percentage of cytokine measured in DTE/diluent 1:1 mix compared with that in proprietary diluent alone across the lower dynamic range (0-39 pg/ml)

Cytokine	IFN-γ	IL-10	IL-12 p70	IL-13	IL-2	IL-4	IL-5	IL-17
Detection in presence of DTE (% of detection in proprietary diluent alone)	92	104	67	75	91	78	87	93

Barczyk reported a three- to four-fold decrease in IL-17 measured by ELISA when using 0.05% DTT (Barczyk, Pierzchala et al. 2003). By contrast my findings suggest that sputum processing with 5 mM (0.1% w/v) DTE does not impair sensitivity of the MSD assay.

Next I tested spiking recovery in sputum samples. Three samples were tested in duplicate wells at 3 different concentrations (10 pg/ml, 100 pg/ml and 1000 pg/ml)(Figure 3.2 B). Average spiking recovery at the 10 pg/ml concentration was 54% but depended on the cytokine being assayed, as shown in table 3.3.

Table 3.3 Average spiking recovery from sputum using 10 pg/ml spikes.

Cytokine	IL-17	IFN-γ	IL-2	IL-4	IL-5	IL-10	IL-12p70	IL-13
Recovery (%)	67.0	89.3	37.7	39.5	94.3	69.4	70.9	41.2

In BAL spiking recovery was generally higher with an average recovery of 66%. As BAL samples underwent centrifugal dialysis the spike was added prior to concentration step. Individual BAL spike recoveries are shown in table 3.4.

Table 3.4 Average spiking recovery from BAL using 10 pg/ml spikes.

Cytokine	IL-17	IFN-γ	I-L2	IL-4	IL-5	IL-10	IL-12p70	IL-13
Recovery (%)	59.1	76.0	56.2	60.6	89.4	50.6	66.5	68.0

Limits of detection for each sample type are shown in table 3.5. The use of MSD and, in the case of BAL samples, additional concentration by centrifugal dialysis allows me to achieve threshold sensitivities an order of magnitude different from prior literature in the field. My assay sensitivity for IL-17 is 70 times greater than that used by Barczyk or Doe in sputum(Barczyk, Pierzchala et al. 2003; Doe, Bafadhel et al. 2010). My threshold sensitivity for serum IL-17 of 0.147 pg/ml is at least 20 times greater than that achieved by others with ELISA (LOD 4-15 pg/ml (Molet, Hamid et al. 2001; Zhao, Yang et al. 2010; Bazzi, Sultan et al. 2011)) or with Luminex® (LOD 3.2 pg/ml(Zhao, Yang et al. 2010)). My combination of MSD and sample concentration makes my measurement of BAL IL-17

1000 fold more sensitive than that achieved by standard ELISA without concentration(Song, Luo et al. 2008).

Table 3.5 Effective limits of detection for cytokines measurement by MSD for each tissue (pg/ml).

Sample type	Cytokine IL-17	IFN-γ	IL-2	IL-4	IL-5	IL-10	IL-12 p70	IL-13
Serum	0.147	1.05	0.289	1.20	0.520	0.887	0.748	1.27
BAL*	0.00395	0.0317	0.0284	0.0730	0.0113	0.0272	0.0223	0.00197
Sputum†	0.216	0.695	0.122	0.615	0.272	0.345	0.510	0.885

*BAL samples were concentrated 50 fold. †Sputum was diluted 2 fold.

Cytokines measured by MSD in serum

Serum concentrations of the following eight cardinal T-cell cytokines were similar between asthma and health: IL-17, IL-2, IL-10, the T_H1 cytokines IFN-γ, IL-12p70 and the T_H2 cytokines IL-4, IL-5 and IL-13(Figure 3.3). Groups were compared by ANOVA on Ln transformed data, and by test for linear trend across groups. Furthermore no significant differences were observed even when all asthmatic subjects were combined (Figure 3_6 A). This finding is at odds with an observation by another group which found plasma IL-17 levels measured by Luminex assay in 12 healthy controls to be uniformly below the limit of detection (3.2 pg/ml), whilst in 29 subjects with allergic asthma they reported a mean level of 12.5 pg/ml (Zhao, Yang et al. 2010). Perhaps differences are due to the differences in method, antibodies or choice of plasma rather than serum. However, MSD is 20 times more sensitive than Luminex, and the data-set analysed in my study is considerably larger and therefore less susceptible to distortion of the mean value by a outliers. Furthermore, in the largest published comparison of serum IL-17 levels to date (Bazzi, Sultan et al. 2011), no significant differences were observed in serum IL-17 levels measured by ELISA between 100 asthmatics and 102 healthy controls, which is fully consistent with my findings.

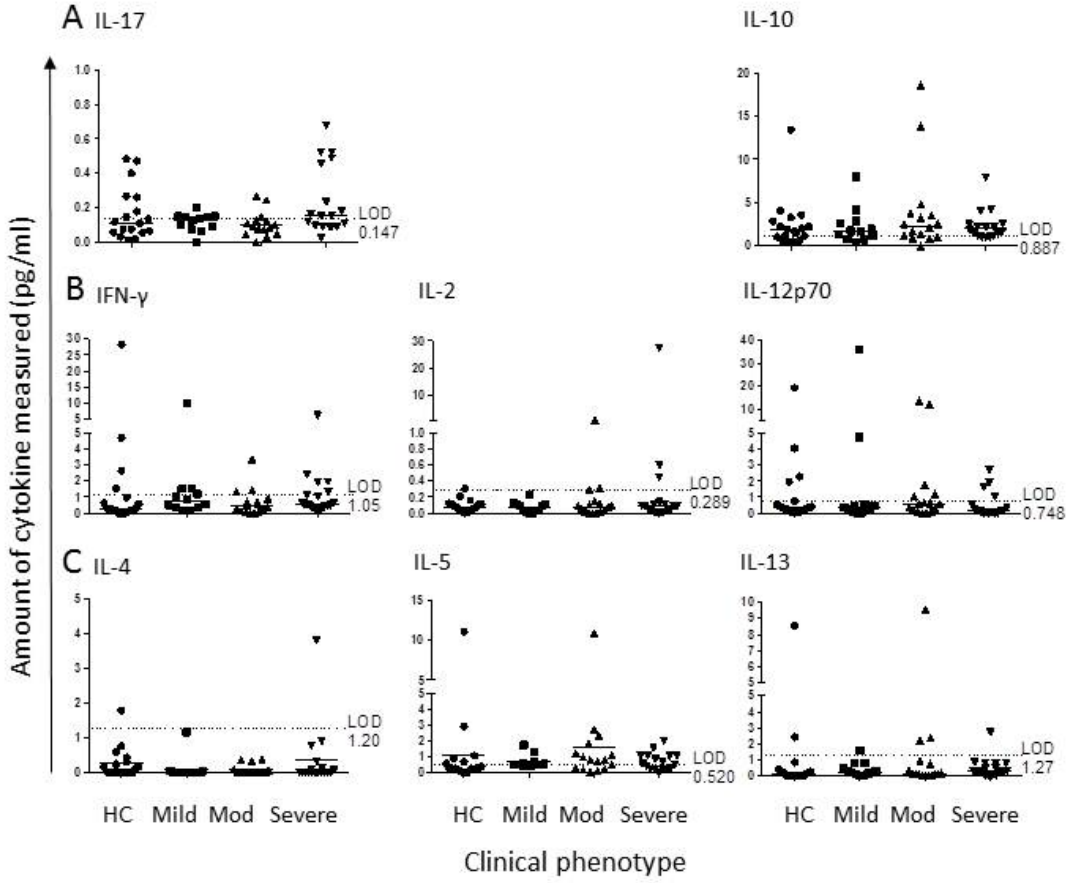


Figure 3.3 Cytokines measured by multiplex ELISA in serum

T cell cytokines measured by multiplex ELISA using the Meso Scale Discovery (MSD) platform from serum samples. Samples were measured in duplicate wells from 64 individuals (18 healthy controls, 12 mild, 16 moderate and 18 severe asthmatics) and are expressed in pg/ml. (A) IL-17 and IL-10. (B) T_H1 cytokines. (C) T_H2 cytokines. No significant differences were observed between groups. LOD, limit of detection (pg/ml); HC, healthy control.

Cytokines measured by MSD in BAL

Interesting data were obtained on cytokine levels in BAL. As expected levels of the T_H2 cytokines IL-5 and IL-13 were markedly elevated in asthma with ANOVA (P<0.0001 and P=0.02 respectively)(Figure 3.4, 3.6 C). This is consistent with the current dogma that allergic asthma is characterised by T_H2 inflammation (Robinson, Hamid et al. 1992; Till, Durham et al. 1998; Larche, Robinson et al. 2003). IL-4, a third T_H2 cytokine, was not detected in BAL, consistent with our own group's previous experience. Furthermore, the elevation of IL-5 and IL-13 was most consistently observed in the subgroup with mild, steroid naïve atopic asthma. Amongst the moderate and severe asthmatic subgroups, BAL T_H2 levels may have been lower in some individuals as a result of treatment (Naseer, Minshall et al. 1997; Richards, Fernandez et al. 2000; Di Lorenzo, Pacor et al. 2002) or due to intrinsic differences in the underlying asthma phenotype (Woodruff, Modrek et al. 2009).

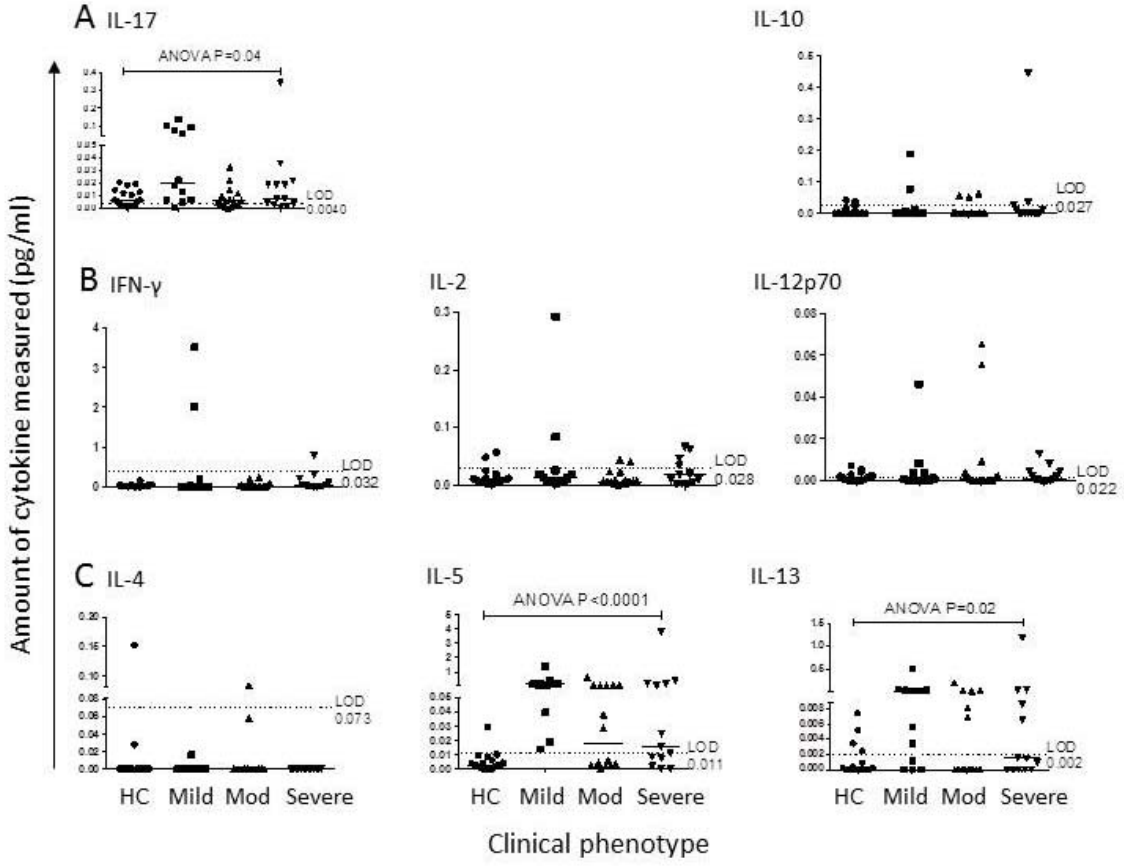


Figure 3.4 Cytokines measured by multiplex ELISA in bronchoalveolar lavage

T cell cytokines measured by multiplex ELISA using the MSD platform from serum samples. Samples were measured in duplicate wells from 59 individuals (18 healthy controls, 12 mild, 16 moderate and 13 severe asthmatics) and are expressed in pg/ml. Samples were first concentrated by centrifugal dialysis. (A) IL-17 and IL-10. (B) T_H1 cytokines. (C) T_H2 cytokines. Distributions were compared by ANOVA on Ln transformed data P values are given where P<0.05. Ln transformed data were also tested for linear trend across groups and significant results are presented. LOD, limit of detection (pg/ml); HC, healthy control.

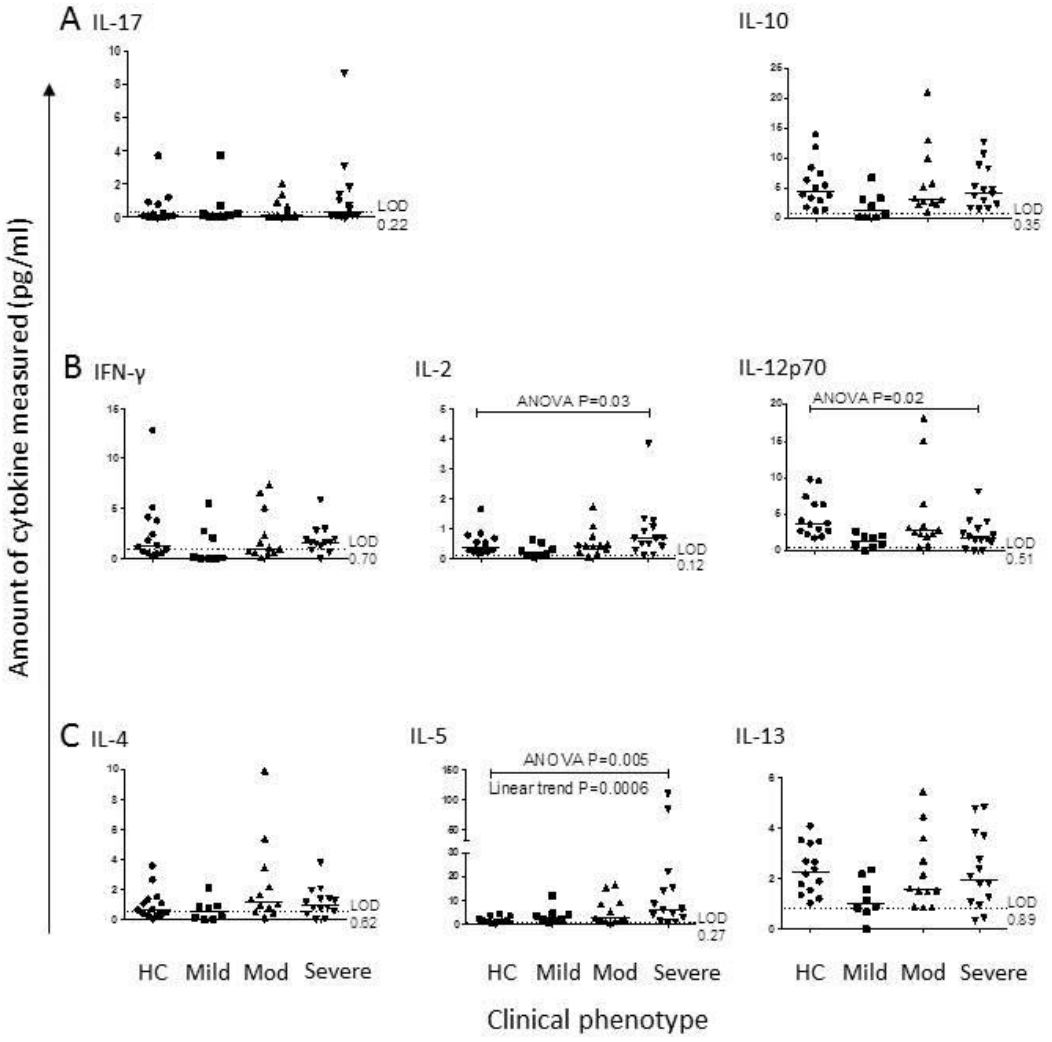


Figure 3.5 Cytokines measured by multiplex ELISA in sputum

T cell cytokines measured by multiplex ELISA using the MSD platform from serum samples. Samples were measured in duplicate wells from 48 individuals (14 healthy controls, 8 mild, 12 moderate and 14 severe asthmatics) and are expressed in pg/ml. (A) IL-17 and IL-10. (B) T_H1 cytokines. (C) T_H2 cytokines. Distributions were compared by ANOVA on Ln transformed data P values are given where $P<0.05$. Ln transformed data were also tested for linear trend across groups and significant results are presented. LOD, limit of detection (pg/ml); HC, healthy control.

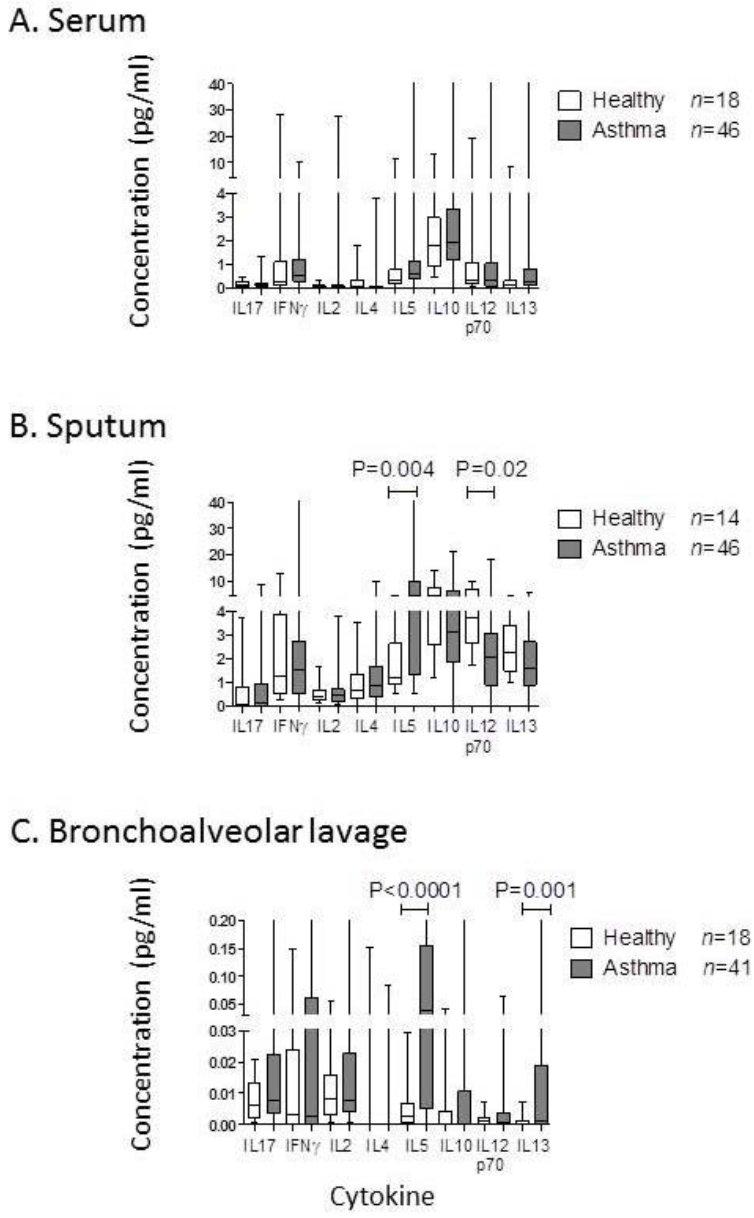


Figure 3.6 Cytokines measured by multiplex ELISA compared between asthma and health

Results from Figures 3.3 to 3.5 stratified to compare health with all asthma combined. P values represent statistically significant differences between asthma and health using unpaired t tests on ln transformed data. After correction for multiple comparisons using $P'=P^*\sqrt{n}$ differences remain significant at $P<0.05$ except for sputum IL-12p70.

Importantly, BAL IL-17 levels did differ between asthma subgroups (ANOVA P=0.04, Figure 3.4), being higher in a subset of mild, steroid naïve asthmatics, although there was no overall difference between health and asthma when all asthmatic subjects were combined (P=0.3, Figure 3.6).

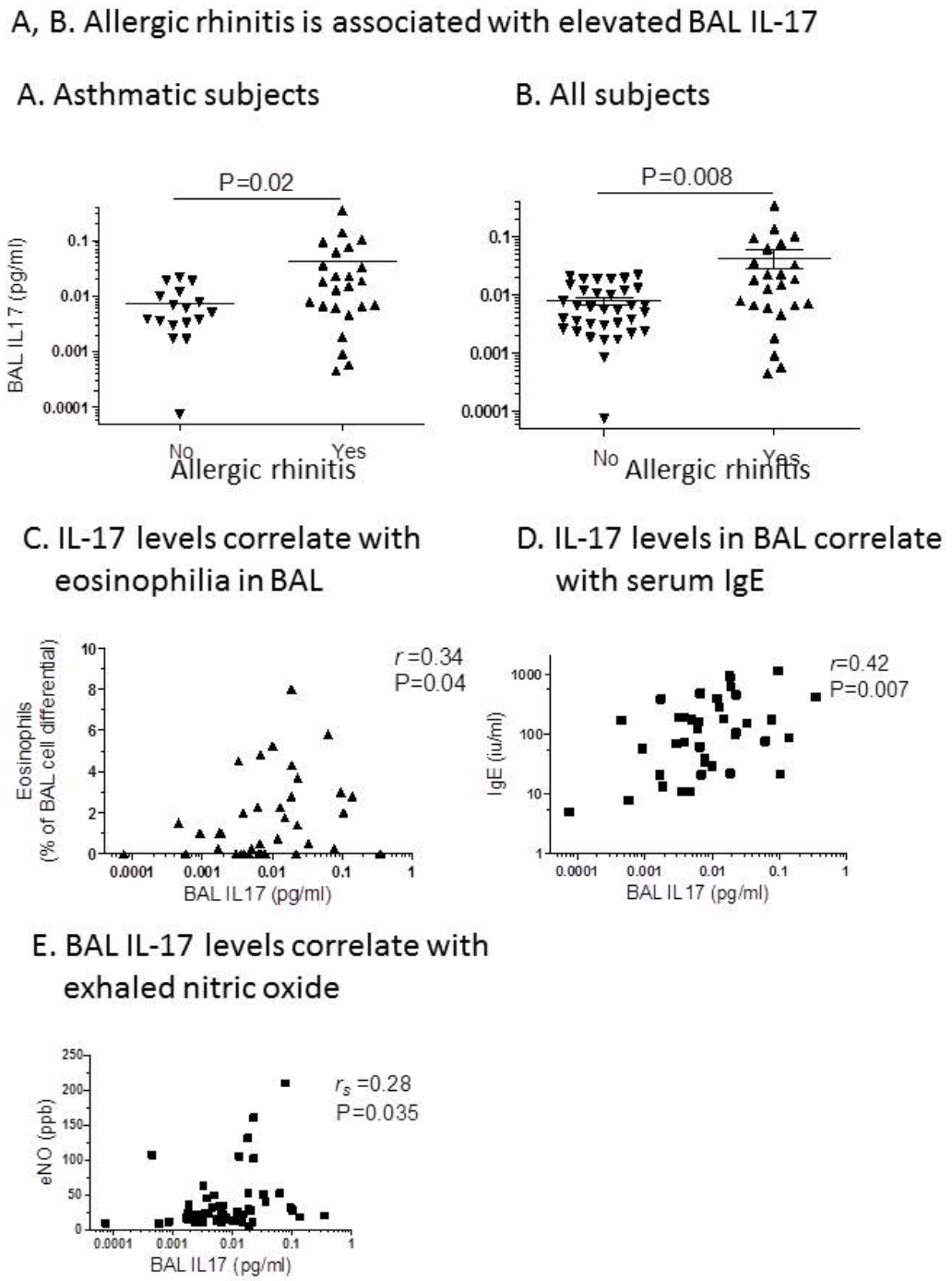


Figure 3.7 Correlates of BAL IL-17 levels

The presence of allergic rhinitis is associated with elevated concentrations of IL-17 in bronchoalveolar lavage in (A) asthmatic subjects and (B) in all subjects combined. P values are for Mann Whitney U test. BAL IL-17 concentrations also correlate with (C) BAL eosinophil counts, with (D) serum IgE levels nad with (E) exhaled nitric oxide. Statistics are for Spearman's correlation.

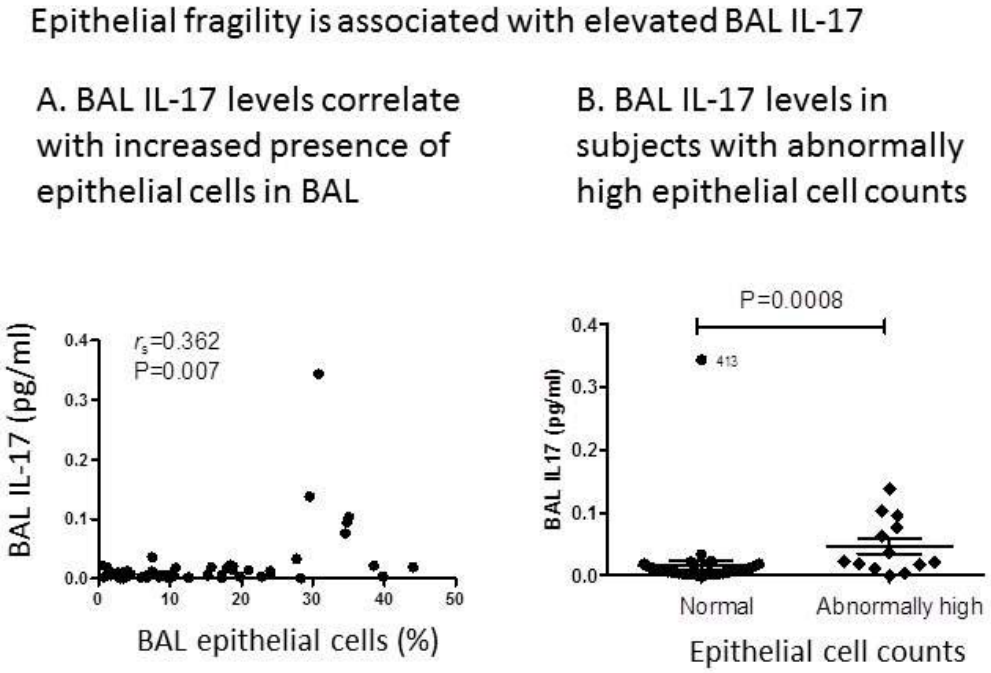


Figure 3.8 Relationship between BAL IL-17 levels and BAL epithelial cells

BAL IL-17 levels are significantly correlated with the proportion of epithelial cells present in the same sample expressed as a percentage of total differential cell count. (A) Relationship is tested by Spearman's correlation (r_s=0.362, P=0.007). As correlations may be misleading where there is an outlying group the data are also presented as BAL IL-17 levels according to whether epithelial cell counts were within the normal range, or abnormally high. (B) The upper limit of the normal range is ≤24% based on the 2.5th-97.5th percentile in my healthy controls. Mean BAL IL-17 levels are 3.6 fold higher in subjects with abnormally high epithelial counts (Mann Whitney P=0.0008).

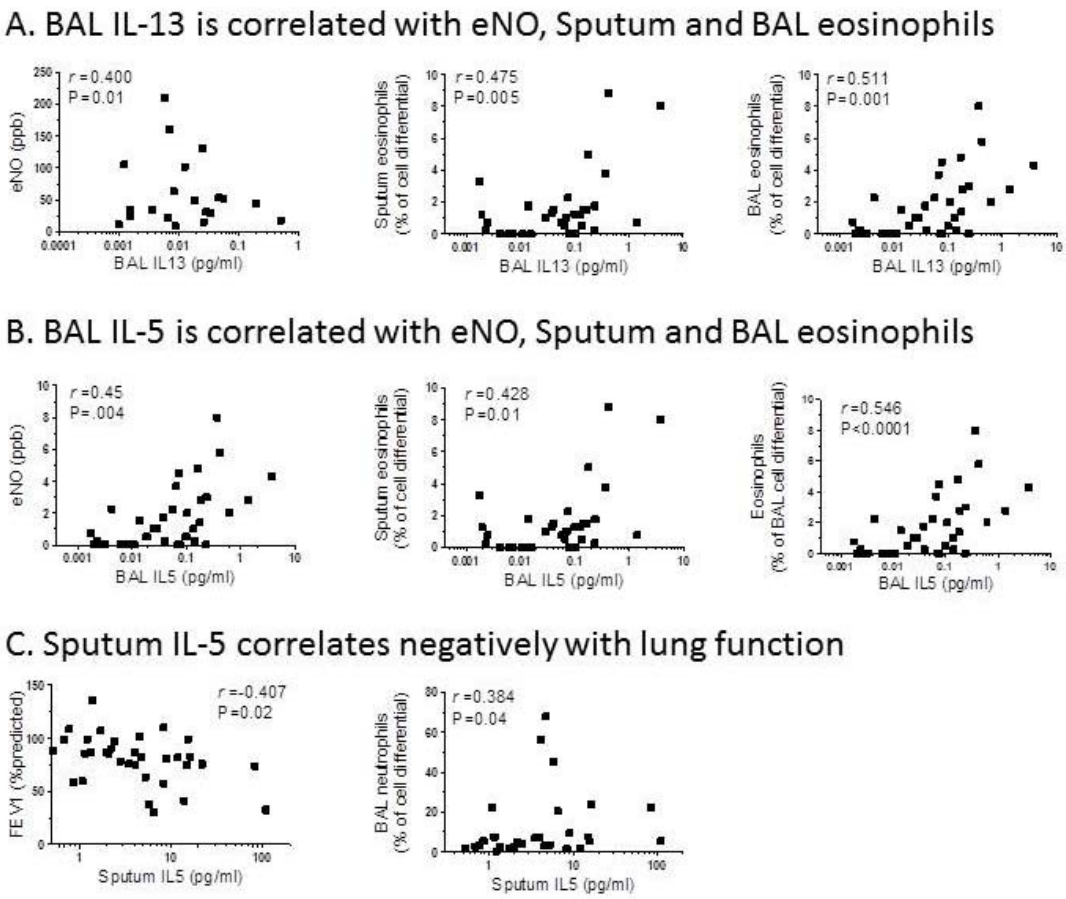


Figure 3.9 Correlates of airway TH2 cytokines

Associations of airway T_H2 cytokine concentrations with markers of eosinophilic inflammation, tested by Spearman's correlations. (A) IL-13 and (B) IL-5 levels in BAL are positively correlated with exhaled nitric oxide and eosinophils in sputum and BAL. (C) Sputum IL-5 levels correlate negatively with lung function, and also weakly correlate positively with sputum neutrophils.

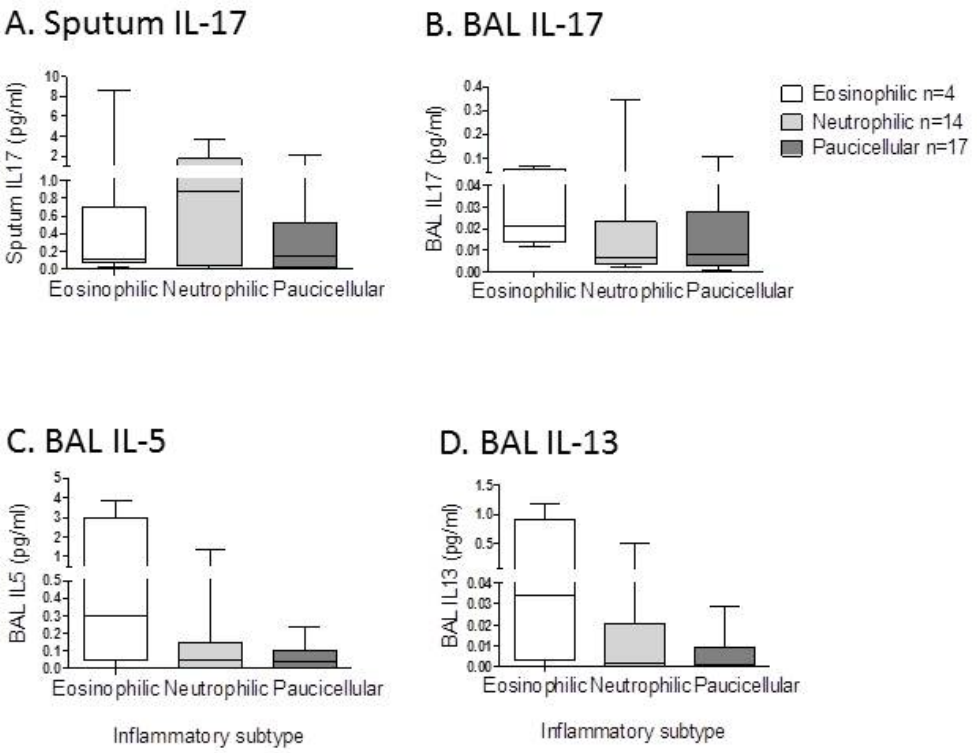


Figure 3.10 Airway cytokines according to inflammatory phenotype

Airway cytokine levels stratified according to asthmatic inflammatory phenotype based on sputum cell differentials: (A) sputum IL-17, (B) BAL IL-17, (C) BAL IL-5, (D) BAL-13. No differences are significant (Kruskall Wallis test), but this is likely to be because of the low number of eosinophilic asthmatics included. Note that many subjects could not expectorate and therefore were not classified according to inflammatory subtype.

Five of the six subjects with high BAL IL-17 levels had mild, steroid naïve asthma. Compared to other mild asthmatics they were older with a mean age of 39 years *versus* a group mean of 25 years (P=0.02). The one severe asthmatic individual with high BAL IL-17 was also older at 63 years of age. High BAL IL-17 levels were also associated with the presence of allergic rhinitis (P=0.02), BAL eosinophilia (r_s=0.34, P=0.04), high serum IgE (r_s=0.42, P=0.007, Figure 3.7) and a tendency towards an eosinophilic sputum inflammatory subtype (NS) (Figure 3.10). In the light of prior literature (Barczyk, Pierzchala et al. 2003) I found no evidence for any association between BHR and IL-17 levels in sputum (r_s=-0.07, P=0.7, *n*=29) or BAL(r_s=0.02, P=0.9, *n*=39).

Elevated BAL IL-17 levels may be associated with epithelial fragility

Epithelial cell derived IL-17 has recently been implicated in nasal inflammation (Semik-Orzech, Barczyk et al. 2009; Saitoh, Kusunoki et al. 2010; Xu, Zhang et al. 2010; Jiang, Li et al. 2011; Quan, Zhang et al. 2012). In light of the above finding of higher BAL IL-17 levels in patients with allergic rhinitis, I wondered whether the bronchial epithelium might be an important source of BAL IL-17. During processing of BAL samples I noted occasional individuals in whom BAL contained sheets of bronchial epithelium. I therefore looked for an association between BAL IL-17 levels and the presence of epithelial cells in the same BAL sample, as measured on BAL cytospins. There was indeed a significant correlation between the number of epithelial cells present in the BAL cytospins and the amount of BAL IL-17 (Spearman's correlation *r*s=0.362, P=0.007)(Figure 3.8 A). All but one of the subjects with high IL-17 levels clustered with very high epithelial cell counts. However, such simple correlations may be misleading. From my data-set, I determined that the upper limit of the normal range for BAL epithelial cell counts is ≤24% based on the 2.5th-97.5th percentile in my healthy controls, which allowed me to dichotomise the subjects into those with normal epithelial cell frequencies and those with abnormally high epithelial cell contamination(Figure 3.8 B). Using this analysis it can be seen that mean BAL IL-17 levels are 3.6 fold higher in subjects with abnormally high epithelial counts (Mann Whitney P=0.0008). Furthermore it should be noted that this cluster of high BAL IL-17 and high epithelial cell counts comprised the same five steroid-naïve, mild asthmatics, whilst the one high IL-17 severe asthmatic subject (413) was again the outlier with normal epithelial cell numbers.

Several mechanism could explain these observations. First, it is conceivable that the cellular source of the BAL IL-17 in the above subset of asthmatics is the inflamed lower airway epithelium, analogous to the situation in the upper airway epithelium (Semik-Orzech, Barczyk et al. 2009; Saitoh, Kusunoki et al. 2010; Xu, Zhang et al. 2010; Jiang, Li et al. 2011; Quan, Zhang et al. 2012). Indeed it has long been recognised that inflammation of the upper airway may be intimately linked with that in the lower airway (Mackenzie 1885) and this is suggested by the association in my data-set between allergic rhinitis and elevated BAL IL-17. Furthermore inflammation of the lower airway epithelium is known to cause loss of epithelial integrity with a selective loss of columnar epithelial cells and disruption to tight junctions with loss of junctional proteins such as ZO-1 and E-cadherin (Swindle, Collins et al. 2009). Such disruption of tight junctions can be promoted by T-cell cytokines including IL-13 and TNF-α

(Swindle, Collins et al. 2009) and IL-17 (Kebir, Kreymborg et al. 2007; Huppert, Closhen et al. 2010; Gutowska-Owsiak, Schaupp et al. 2012; Soyka, Wawrzyniak et al. 2012) leading to epithelial fragility which might predispose to the increased epithelial cell sloughing during bronchoalveolar lavage which I have observed in steroid naïve asthmatics.

Cytokines measured by MSD in sputum

When measured in sputum the T_H2 cytokine IL-5 was again increased in asthma (ANOVA P=0.005, Figure 3.5), although the pattern according to disease phenotype was different from that observed in BAL, with the highest levels correlating with the greatest disease severity (P for linear trend across groups =0.0006). The expression pattern of other cytokines also differed between BAL and sputum, which is typical of the experience of our group, reflecting the different cellular and protein composition of sputum and its more proximal origin.

I observed no significant differences in sputum IL-13 orin sputum IL-17 levels (Figure 3.5) which is contrasts with the findings of Barczyk *et al.* Possible explanations for these differences between Barczyk *et al*. and my own findings includes study size and assay sensitivity; more than twice as many subjects were analysed in my study (48 v 21 sputum samples), and the MSD assay used here is 70 times more sensitive than the Luminex assay employed by Barczyk *et al.*

Sputum IL-2 levels differed between mild and severe asthma (ANOVA P=0.03). IL-2 is produced by activated T_H1 cells and has been found to induce bronchial hyper-reactivity in rats (Barnes, Djukanovic et al. 2003), but the significance of my finding is not clear, particularly as there was no overall difference between asthma and health (Figure 3.6).

Sputum IL-12p70 levels were lower in asthma than in health (P=0.02, Figure 3.5 and 3.6). IL-12 is produced by monocytes and macrophages to promote differentiation of naïve T cells into T_H1 cells(Hsieh, Macatonia et al. 1993), and can inhibit BHR and airway eosinophilia in animal models(Barnes, Djukanovic et al. 2003). Therefore IL-12 might be expected to be deficient in subjects with T_H2 mediated allergic asthma, indeed IL-12 has been reported to be deficient in peripheral blood in allergic asthma(Barnes, Djukanovic et al. 2003).

In summary measurement of eight cardinal T-cell cytokines in a range of tissues revealed a lack of systemic markers of T-cell response in blood, but consistent evidence of T_H2 inflammation in airway samples. My data do not show a generalised increase of IL-17 in asthma, although they suggest that IL-17 may be elevated in a discrete subgroup of mild, steroid naïve asthmatics, which tend to be older with atopic, eosinophilic asthma and allergic rhinitis. These may represent a distinct endotype of asthma. These data do not however identify a specific cellular source of IL-17 in the airways.

Measurement of IL-17 in airway macrophages by RT-qPCR

Airway macrophages constitute one possible cellular source of IL-17(Song, Luo et al. 2008; Park and Lee 2010; Reynolds, Angkasekwinai et al. 2010). I therefore measured IL-17 mRNA by RT-qPCR in live CD45+CD3-HLADR+ sputum macrophages sorted by flow cytometry from 29 subjects comprising 10 healthy controls, 9 mild asthmatics and 10 moderate asthmatics. IL-17 mRNA was quantified in triplicate using PerfectProbe primers for IL-17A and normalised to β2 microglobulin. IL-17 mRNA was detected only from a single mild asthmatic subject (211) at an average cycle threshold (CT) of 37.2 compared with a CT of 14.6 for the house-keeping gene, implying very low transcript abundance (see Figure 3.11). Although airway macrophages have been identified as a source of IL-17 in a murine model of asthma(Song, Luo et al. 2008), my data imply that airway macrophages are not a principle source of IL-17 in the airways in humans.

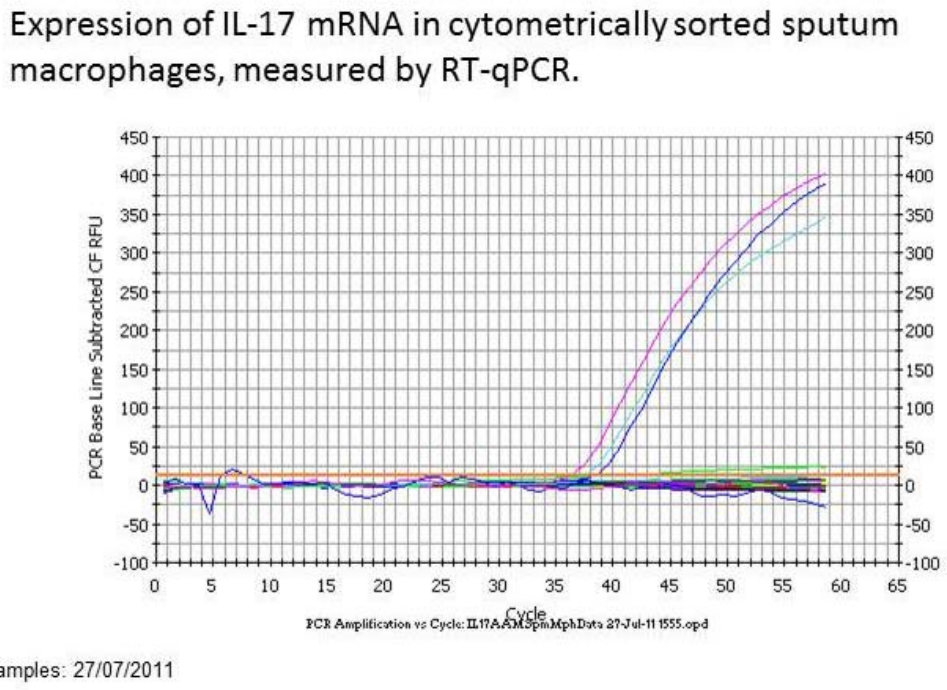


Figure 3.11 Airway macrophage expression of IL-17 mRNA

IL-17 mRNA was measured by RT-qPCR in airway macrophages. Live CD45+CD3-HLADR+ were obtained from sputum and sorted by flow cytometry and mRNA was quantified in triplicate using PerfectProbe primers for IL-17, and normalised to β2 microglobulin. 29 samples were tested from 10 healthy controls, 9 mild asthmatics and 10 moderate asthmatics. IL-17 mRNA was detected only from a single subject, 211, who had mild asthma.

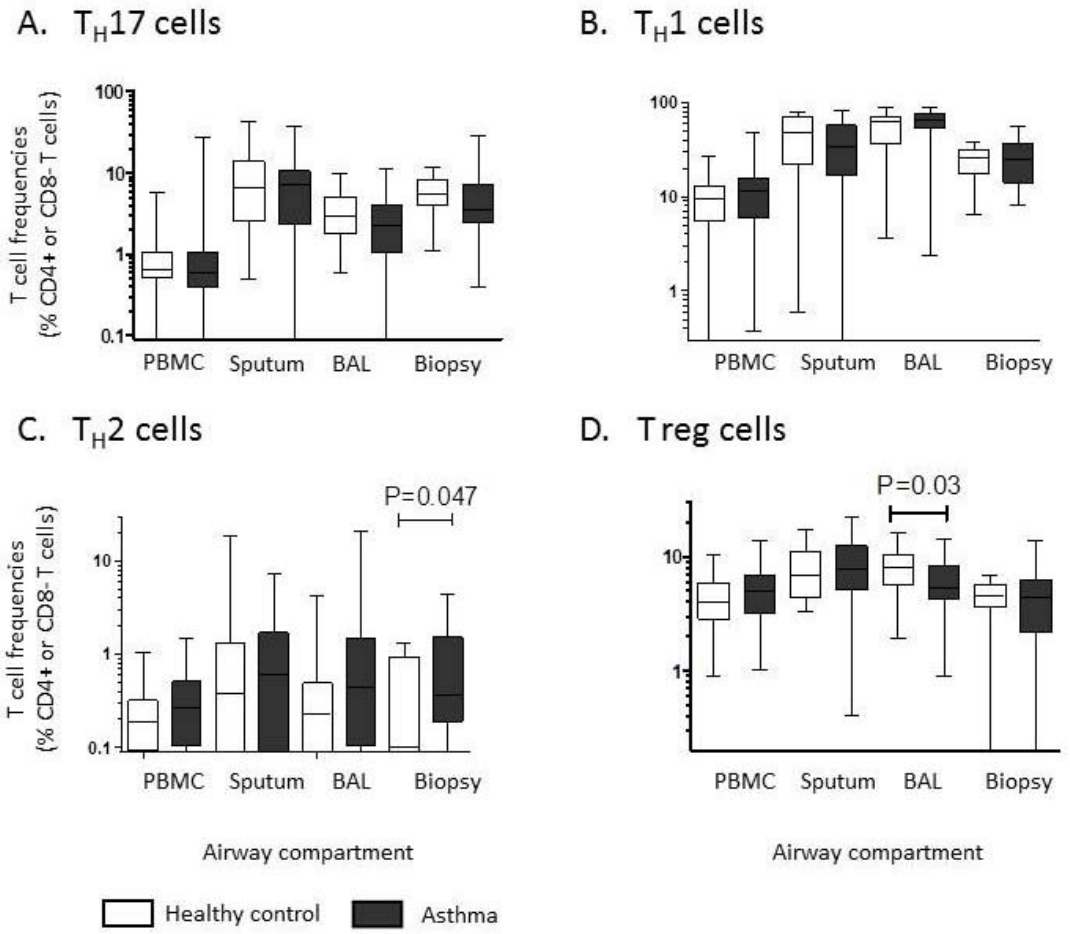


Figure 3.12 Major CD4+ T cell subsets in asthma and health

Frequencies of T cells expressing (A) IL-17 (T_H17 cells), (B) IFN-γ (T_H1 cells), (C) IL-13 (T_H2 cells) and (D) FOXP3 (T reg) in PBMC, sputum, BAL and bronchial biopsies measured by intracellular cytokine staining and flow cytometry. Results expressed as a percentage of live CD3+CD4+ T cells. In the case of bronchial biopsies frequencies are a percentage of CD3+8- T cells. Differences are compared by Mann-Whitney U tests and significances given where $P<0.05$.

□ healthy controls; ■ asthmatic subjects.

Cytometry of major CD4+ T cell subsets in asthma

My next objective was to enumerate key CD4+ T cell subsets in PBMC, sputum, BAL and bronchial biopsies across a spectrum of asthma phenotypes. Flow cytometry is an ideal technique for such work as it combines the high sensitivity and high specificity needed for the accurate detection of rare events with the ability to determine the exact cellular origin of cytokines on a cell-by-cell basis(Baumgarth and Roederer 2000).

Evidence of increased T_H2 cell inflammation, but no differences in T_H17 frequencies in asthma

Figure 3.12 shows data from 23 healthy subjects and 53 asthmatics comparing frequencies of T_H17, T_H1, T_H2 and Treg cells in asthma and health. Contrary to my hypotheses, I found no significant differences in frequencies of T_H17 cells between health and asthma in blood or any tissue compartment (A). The same was true for T_H1 cells (B) and these findings were in clear contrast to my observations of an increase in T_H2 cells in bronchial biopsies (C) with a median 0.36% (IQR 0.19-1.5%) in asthma compared with 0.10% (0.025-1.3%) in health (Mann-Whitney P=0.047, *n*=47), with similar trends in PBMC, sputum and BAL (NS). In addition I also observed a decrement in Treg in BAL (D) in asthmatics at 5.3% (4.3-8.2%) compared with health (8.1% (5.6-10%) P=0.027, *n*=67).

These findings are analysed in greater detail in Figure 3.13 where I have stratified the asthmatic individuals according to disease severity. Again there is clearly no evidence of differences in T_H17 cell frequencies between health or any asthma phenotype (A), whilst the differences in T_H2 cell frequencies are more apparent (C), being most strikingly elevated in mild, steroid naïve asthmatics in PBMC (P=0.003), sputum (P=0.03) and biopsies (P=0.02) with a similar trend in BAL (NS). It can also be seen that the deficiency in BAL Treg correlates with disease severity (P for linear trend =0.02) being most marked in severe asthma with frequencies of 4.4% (3.1-6.1%) compared with 8.1% (5.6-10%) in health (P=0.00).

I further analysed this evidence of a bias towards T_H2 inflammation by comparing ratios of T_H2 to T_H1 cells in each tissue compartment (Figure 3.14). An increase in the T_H2:T_H1 ratio was observed in sputum (Kruskal-Wallis P=0.01), BAL (P=0.049) and bronchial biopsies (P=0.009), with a similar pattern in PBMC (NS). Again this T_H2 bias was most marked in mild, steroid naïve asthmatics.

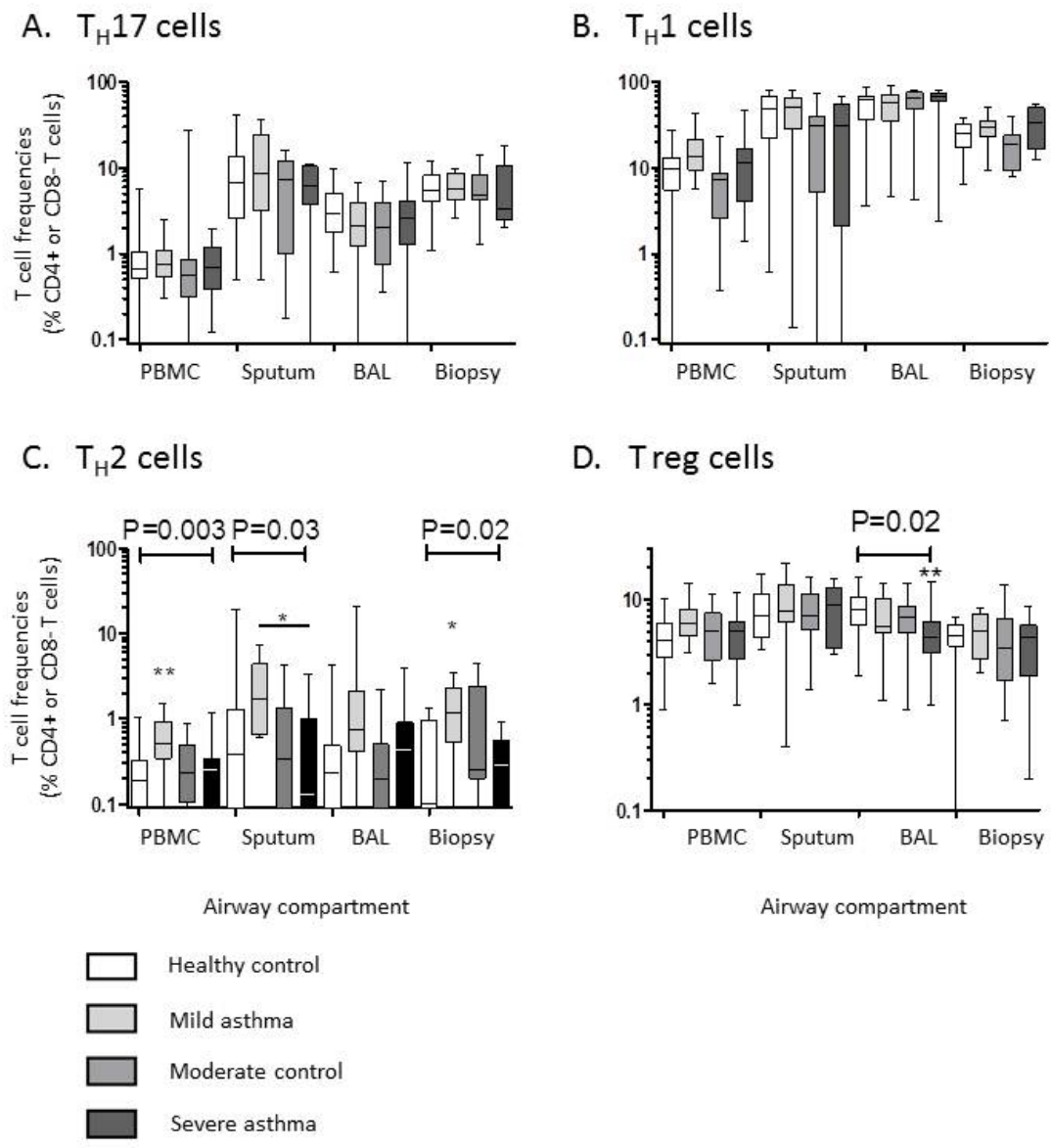


Figure 3.13 Major CD4+ T cell subsets stratified by disease severity

Frequencies of T cells expressing (A) IL-17 (T_H17 cells), (B) IFN-γ (T_H1 cells), (C) IL-13 (T_H2 cells) and (D) FOXP3 (T reg) in PBMC, sputum, BAL and bronchial biopsies measured by intracellular cytokine staining and flow cytometry. Results expressed as a percentage of live CD3+CD4+ T cells. In the case of bronchial biopsies frequencies are a percentage of CD3+8- T cells. Differences are compared by Kruskal-Wallis tests and significances given where P<0.05. Significance *post hoc* by Dunn's compared with health: *P<0.05, ** P<0.01.

healthy controls; mild asthma; moderate asthma; severe asthma.

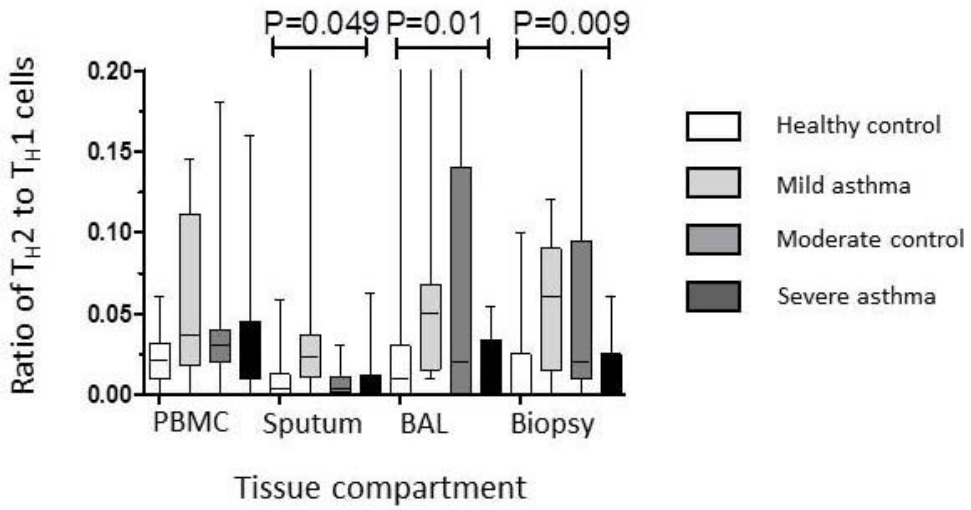


Figure 3.14 Ratio of TH2:TH1 cells in different tissue compartments

Ratios of T_H2 to T_H1 cells in different tissue compartments, measured by intracellular cytokine staining and flow cytometry. Differences are compared by Kruskal-Wallis tests.

healthy controls; mild asthma; moderate asthma; severe asthma.

Peripheral T_H2 responses correlate with atopy and with BAL T_H2 cytokines

This evidence of elevated T_H2 cell frequencies in peripheral blood implies that the T_H2 bias in airway tissues is part of a wider systemic T_H2 bias. Woodruff *et al* used gene expression analysis of airway epithelial cells to identify two subgroups of asthmatics which they termed 'T_H2-high' and 'T_H2-low' and which differed according to biopsy expression of IL-5 and IL-13 as well as AHR, serum IgE and blood and airway eosinophilia (Woodruff, Modrek et al. 2009). I therefore investigated whether similar associations could be replicated in my data-set, and sought to stratify asthmatic subjects into T_H2-high and T_H2-low subjects based on PBMC, sputum, BAL and biopsy T_H2 frequencies. Defining T_H2-high as the top tertile of T_H2 cell frequencies in each tissue type gave the greatest statistical power (compared for instance with dichotomising at the median frequency), and also provided a good differentiation from the normal range observed in healthy controls. For instance 1/3 of asthmatics had a PBMC T_H2 frequency ≥0.44%, whilst this was true for only 9% of healthy controls. I then tested the variables identified by Woodruff using univariate analyses. Asthmatic subjects with high T_H2 frequencies in peripheral blood had higher rates of atopy (100% v 77%, Fisher's exact P=0.04), responded to a greater range of allergens on skin prick allergy testing (P=0.002, Figure 3.15 A) and also had higher BAL IL-5 levels (P=0.02, Figure 3.15 B).

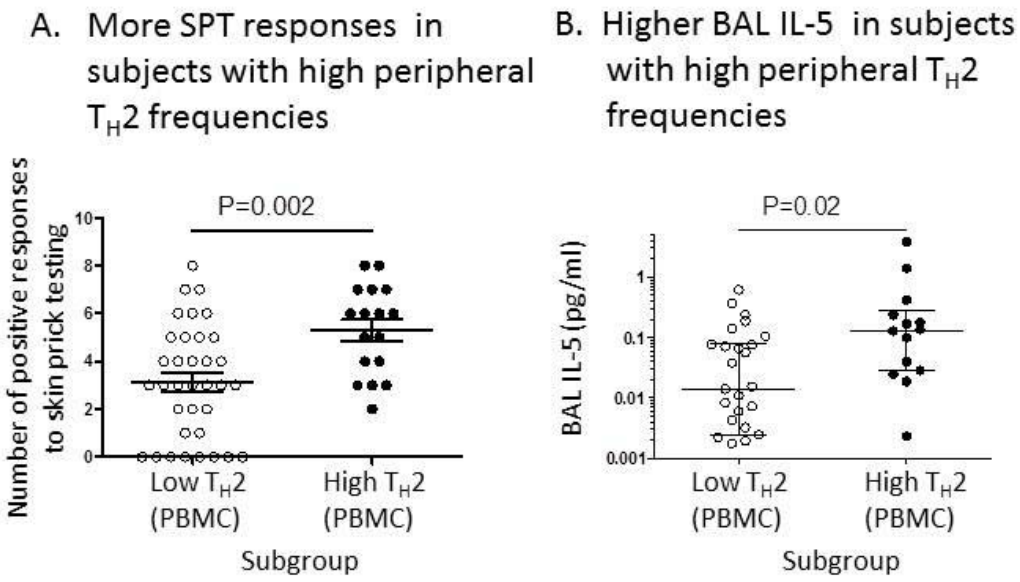


Figure 3.15 Correlates of high peripheral blood TH2 frequencies

Asthmatic subjects stratified according to their peripheral blood frequencies of IL-13+ (T_H2) cells as T_H2 high (the top tertile of PBMC T_H2 frequencies) or T_H2 low (lower two tertiles). (A) T_H2 high subjects tended to respond to more allergens on skin prick testing, t test P=0.002). (B) T_H2 high subjects also tended to have higher IL-5 levels in BAL (t test on Ln transformed data, P=0.02).

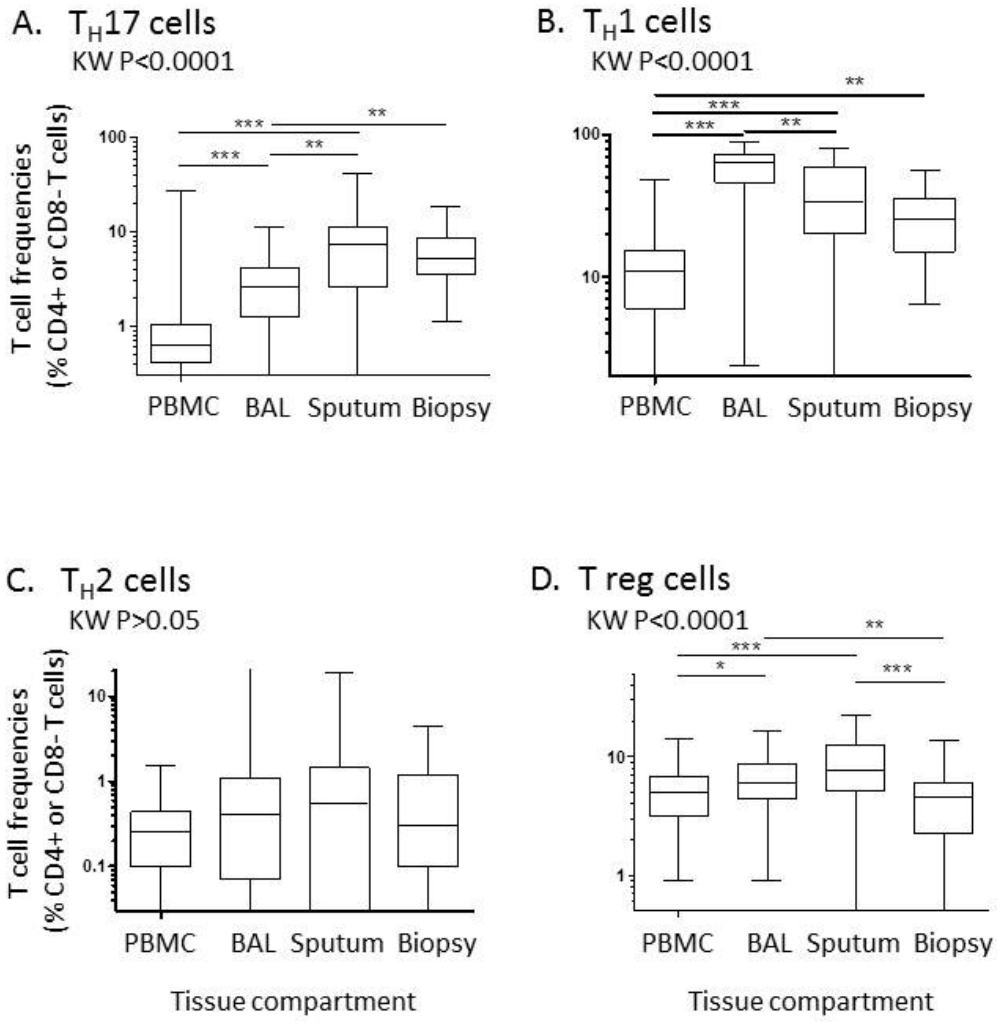


Figure 3.16 Compartmentalisation of tissue CD4+ T cells

Distribution of different CD4+ T cell subsets according to tissue type in all subjects combined. (A) T_H17 cells, (B) T_H1 cells, (C) T_H2 cells, (D) T reg. Groups are compared by Kruskal-Wallis tests with *post hoc* Dunn's. * P<0.05, ** P<0.01, *** P<0.001.

Similarly asthmatic subjects in the top tertile of sputum T_H2 frequencies were more likely to be atopic (P=0.04) and have more bronchial hyper-reactivity (P=0.02), whilst asthmatic subjects in the top tertile of bronchial biopsy T_H2 frequencies responded to a greater range of allergens on skin prick testing (P<0.0005) (data not shown).

Distinct tissue localisation of different T cell subsets

It is apparent from figures 3.12 and 3.13 that different T cell subsets differ in their tissue distributions. This compartmentalisation is analysed in detail in Figure 3.16. Both T_H17 (A) and $T_H1$cells (B) are markedly concentrated in tissue compared with peripheral blood. Highest frequencies of T_H17 cells are observed in sputum and biopsies, whilst T_H1 cells are most strongly localised to the bronchoalveolar compartment. By contrast Treg were found at lowest frequency in biopsy tissue, whilst I did not observe any significant tissue localisation of T_H2 cells. These different tissue

localisations would be consistent with a dominant role of BAL effector / memory T_H1 cells in immunity to viruses (Cautivo, Bueno et al. 2010) or mycobacteria (Silver, Zukowski et al. 2003) whilst T_H17 may be more associated with mucosal immunity against bacterial or fungal invasion of stromal tissue(Veldhoen and Stockinger 2006; Ma, Chew et al. 2008; Michel, Mendes-da-Cruz et al. 2008).

No evidence for a significant role of TCRγδ+ IL-17+ T cells in human asthma

One cell type commonly considered to have a specific association with mucosal tissue is the innate-like γδ-T cell subset (Vanaudenaerde, Verleden et al. 2011). These T-cells are activated via their γδTCRs and toll-like receptors (TLRs) and can provide a rapidly available source of IL-17. A significant role for γδT-cells in allergic airways disease has been implied by animal models (Isogai, Athiviraham et al. 2007; Jin, Roark et al. 2009). It has even been suggested that IL-17 secreting γδT-cells may outnumber T_H17 cells in murine allergic airway inflammation where they seem to be critically involved in injury repair (Murdoch and Lloyd 2010). I therefore analysed IL-17 and IFN-γ secreting γδT-cells in PBMC and BAL from a subset of 9 healthy controls and 24 asthmatics (Figure 3.17), using an antibody specific to all γδTCRs. According to my findings, γδT-cells are rare, comprising only 1.9% (1.3-3.0%, median and IQR) of PBMC and 1.1% (0.65-2.8%) of BAL lymphocytes, with much lower detectable frequencies of cytokine secreting cells. Therefore it was not possible to enumerate γδT-cells in sputum or bronchial biopsy samples. Nonetheless there were no significant differences in IL-17 secreting (A) or dual IL-17/IFN-γ secreting (B) γδT-cells in PBMC or BAL. Although distributions of IFN-γ secreting BAL γδT-cells differed between groups (C), frequencies were not different between health and any asthma phenotype. Thus my data do not provide evidence of appreciable numbers of IL-17 secreting γδT-cells in humans, nor of any association with asthma during periods of clinical stability. However these conclusions are limited by smaller sample sizes than those used for other comparisons.

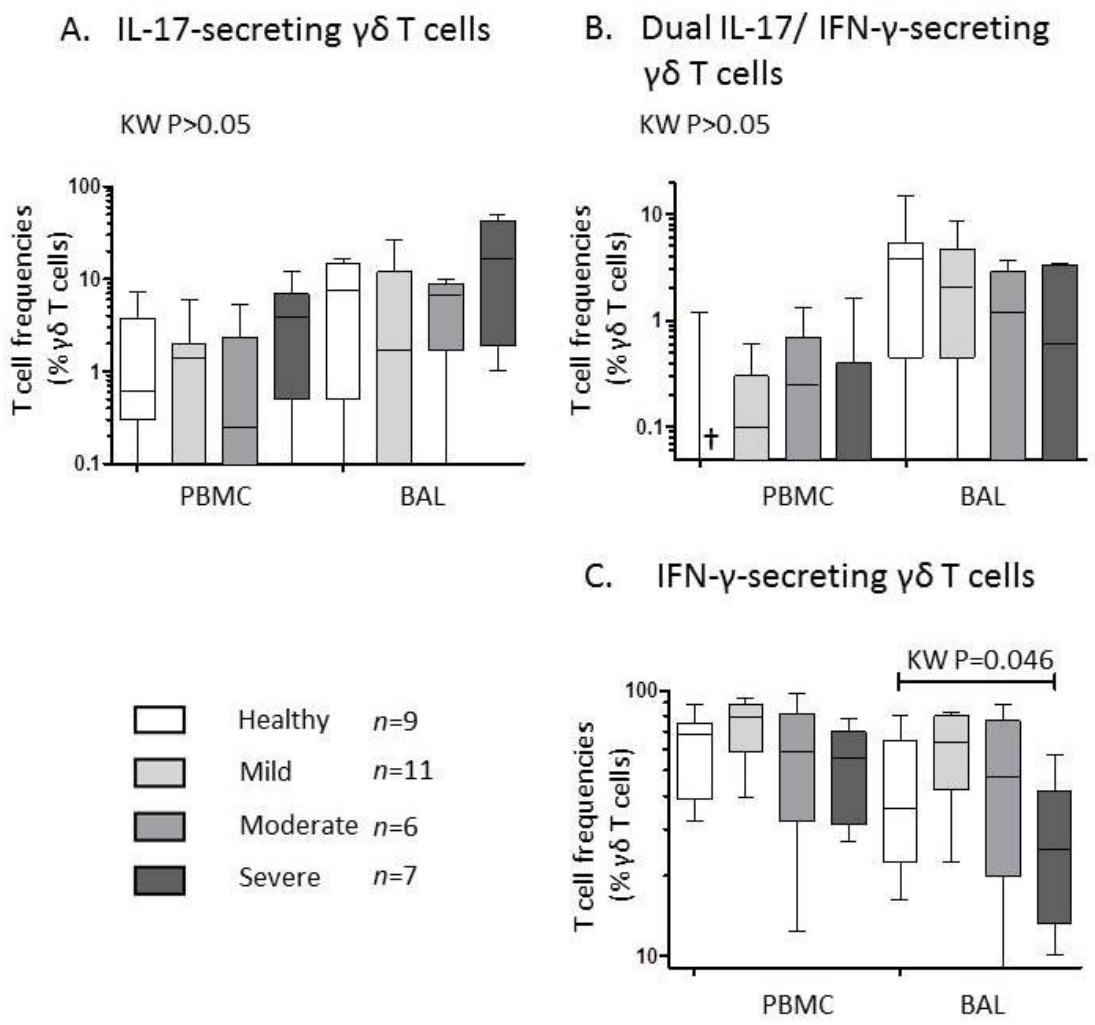


Figure 3.17 γδ T cells in asthma

Frequencies of γδ T cells secreting (A) IL-17, (B) both IL-17 and IFN-γ and (C) IFN-γ as a proportion of total γδ T cells in peripheral blood and in bronchoalveolar lavage. Groups are compared by Kruskal-Wallis tests.

healthy controls; mild asthma; moderate asthma; severe asthma. † median 0, IQR 0-0.05%.

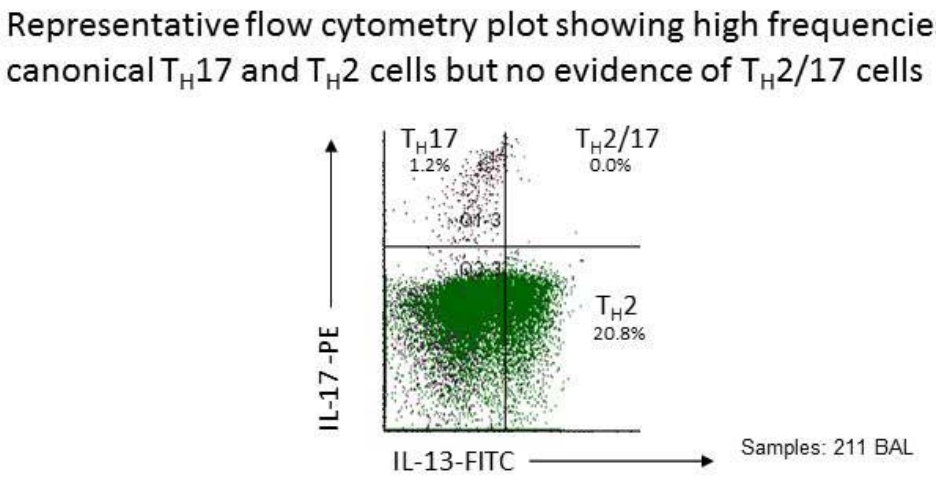


Figure 3.18 No evidence for TH2/17 cells in humans

A representative cytometry plot from subject 211 with moderate allergic asthma showing abundant T_H17 and T_H2 cells, but no evidence of dual-cytokine secreting cells. Some PE bright cells spill into the $T_H2/17$ quadrant due to imperfect compensation.

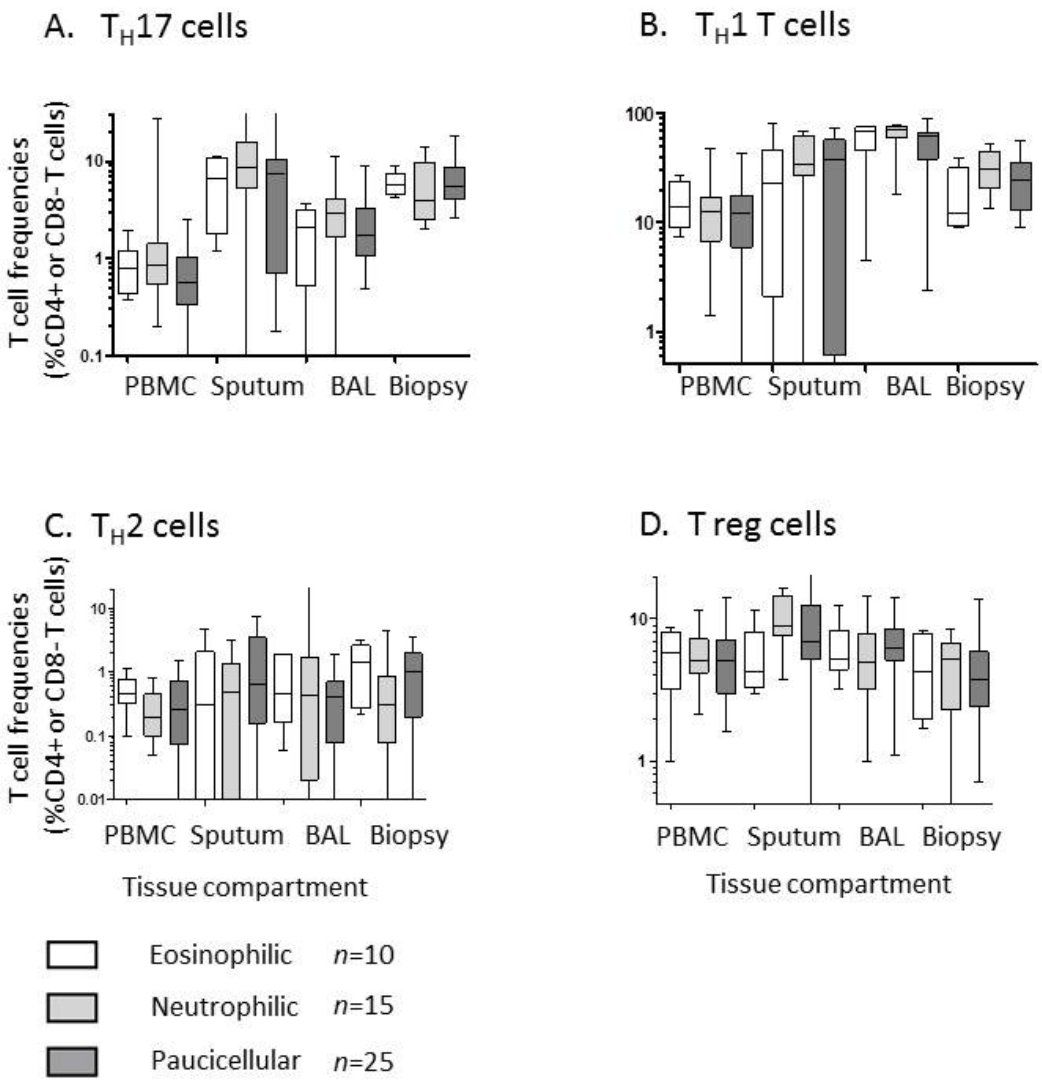


Figure 3.19 CD4+ T cell frequencies stratified by inflammatory cell subtype

(A) T_H17 cells, (B) T_H1 cells, (C) T_H2 cells, (D) Treg cells according to tissue and inflammatory subtype. Subjects have been classified according to sputum cell differentials as eosinophilic, neutrophilic or paucicellular. A single individual had mixed eosinophilic / neutrophilic disease but was classified as eosinophilic as this was the dominant feature. There were no statistically significant differences between groups.

eosinophilic, neutrophilic, paucicellular.

No evidence for IL-17 producing T_H2 cells in human asthma

One group recently reported the existence of IL-17-producing "T_H2 cells" which express both the T_H2 transcription factor GATA3 and the T_H17 transcription factor RORγt. Furthermore they observed increased frequencies of these cells in peripheral blood of subjects with atopic asthma, and presented murine data implicating them in the pathogenesis of experimental allergic airways disease (Wang, Voo et al. 2010). In relation to these findings, I could not detect any evidence for dual IL-17/IL-13 secreting T cells in any tissue compartment. A representative plot is shown in Figure 3.18, where it

can be seen that despite high levels of IL-17 and IL-13 secretion, no dual secreting cells were observed (beyond a minor compensation artefact). I did not however measure surface expression of CCR6 or CRTH2, which were used by Wang *et al* to define the IL-17 secreting T_H2 cells.

Analysis of CD4+ T cells according to inflammatory subtype

An important means to differentiate asthma into distinct endotypes is classification according to inflammatory cell subtype (Simpson, Scott et al. 2006; Anderson 2008). Such fundamentally differing patterns of airways inflammation suggest different underlying immunological processes. Therefore I endeavoured to analyse my clinical and immunological data-sets in asthmatics according to their differing inflammatory subtypes based on the sputum differential cell count. Inflammatory subtypes were indeed related to clinical variables; specifically, logistic regression analysis showed that history of allergic rhinitis was common (65% prevalence) in all subtypes except neutrophilic asthma (14% prevalence, P<0.0001), implying that neutrophilic asthma is not strongly driven by allergic inflammation of the nasal mucosa.

Other factors, whilst statistically significant by logistic regression, were likely to be artefacts of my subject selection. Thus I observed a lower FEV1 in granulocytic asthma (mean 74.7% predicted FEV1 in eosinophilic, neutrophilic or mixed subtypes) compared with pauci-cellular asthma (mean 95.0% predicted, P=0.0009) and a lower FEV1/FVC ratio in eosinophilic asthma (FEV1/FVC=62.6%) compared with other subtypes (73.9%, P=0.0036). I also observed worse symptomatology in neutrophilic asthma (ACQ 2.54 compared with 1.39 in other phenotypes, P=0.0007). However all of these are expected consequences of my targeted recruitment of subjects with severe neutrophilic or severe eosinophilic asthma from the Wessex Severe Asthma Cohort. A valid investigation of whether these specific inflammatory subtypes are associated with worse lung function and symptom scores would require an unbiased assessment of a much larger cohort of unselected asthmatic subjects.

Next I analysed CD4+ T cell frequencies according to inflammatory subtype. Data were available from 10 subjects with eosinophilic asthma, 15 with neutrophilic asthma and 25 with pauci-cellular asthma. A single individual had mixed eosinophilic/neutrophilic disease but was classified as eosinophilic as this was the dominant feature. There were no statistically significant differences between groups for any major CD4+ T cell subset (Figure 3.19).

Cluster analysis to explore relationships between variables

With Dr Borislav Dimitrov I further explored possible associations, or clusters, among the various (*n*=77) demographic, clinical and immunological parameters which described my cross-sectional cohort using principle component analysis (PCA), as described in chapter 2. PCA has two purposes: first to reduce the dimensionality (number of variables) of a high-dimensional data-set into a smaller set of composite variables, much as multiple questions in an ACQ or quality of life score can be reduced to a single summary statistic. The second purpose of PCA is to establish relationships between variables and with the outcome of interest: in this case the presence and severity of asthma.

Using an iterative process we reduced the 77 starting variables to a selected list of 25 variables, which together were reduced by the PCA into 12 components, as shown in table 3.6.

Table 3.6 Principle component analysis of data from the cross sectional study.

	Component											
	1	2	3	4	5	6	7	8	9	10	11	12
Classification Mild/Mod/Sev		**0.577**										
Age												
Gender									0.879			
Allergic Rhinitis								0.689				
Eczema					0.926							
ICS Dose	0.649											
Nasal Polyps				0.808								
Smoking History (PackYears)				0.892								
ACQ		0.763										
BMI												0.803
FEV_1%Pred											-0.789	
GINA class		0.783										
eNO							0.883					
Total IgE										0.951		
PBMC MAIT cells						0.837						
PBMC T_H17/Reg Ratio			0.983									
PTH1								0.917				
PTH17			0.989									
PTH2					0.645							
Serum IFN-γ	0.512											
Serum IL-10	0.987											
Serum IL-13	0.987											
Serum IL-17		0.607							-0.57			
Serum IL-5	0.987											
BAL MAIT cells			0.552			0.641						

Extraction Method: Principal Component Analysis.

Rotation Method: Varimax with Kaiser Normalization.

a. Rotation converged in 21 iterations.

The table is a rotated component matrix. Each column represents a component, ranked from left to right according to the extent to which they explain the variance in the data. Each cell gives a Pearson correlation coefficient for the variable within the component. Only values >0.50 are shown.

What is the interpretation of this analysis? It should be viewed as hypothesis-generating because the data were not all normally distributed and the sample size was small (*n*=31 asthmatic subjects) due to missing data. However further analysis showed the components were robust, which means that the analysis was significantly affected if components were removed. Together these 12 components describe 92.6% of the variance within the dataset.

Component 1 is the most powerful component, explaining 15.7% of the variance alone. This component shows that several T cell serum cytokine levels cluster together with each other and with

the dose of ICS, implying these variables are significantly correlated. Interestingly serum IL-17 clusters separately from these other cytokines in component 2, where it is significantly correlated with three measures of asthma severity: ACQ score, GINA classification of disease control and the primary classification I have used mild / moderate / severe. Although serum IL-17 was not different between health and asthma overall, this result, and Figure 3.3 show that within asthma serum IL-17 levels are higher in more severe disease. This is a different pattern from that observed with BAL fluids, is based on a smaller sample and requires confirmation in a separate validation set. Other components suggest the existence of distinct asthma endotypes such as the association of nasal polyps and smoking in component 4 or the association of allergic rhinitis and blood T_H1 cells in component 8. Of relevance to chapter 5, MAIT cell frequencies in blood and BAL cluster with each other as independent variables, and specifically do not cluster with ICS dose.

Discussion

The fundamental role of T_H2 inflammation in asthma

My data provide the first comprehensive review of T_H17 cells and Treg in the human airways, and set them in the context of the well-characterised T_H1 and T_H2 cell subsets. With respect to the latter I have observed significant increases in T_H2 cells in both peripheral blood and airway tissues, consistent with extensive prior literature (Robinson, Hamid et al. 1992; Anderson and Coyle 1994; Cho, Stanciu et al. 2005; Woodruff, Modrek et al. 2009; Finkelman, Hogan et al. 2010; Lloyd and Hessel 2010). Indeed my data add to the seminal findings of Robinson *et al* by extending the work to a much wider spectrum of asthma. Robinson *et al* studied 15 mild, allergic, steroid naïve asthmatics out of their allergen season, whilst my study has also included subjects on maintenance steroids and subjects with severe asthma requiring high dose inhaled or oral steroids. It is apparent from both the MSD and the cytometry data that the T_H2 bias is less marked in these individuals. This may be due to the effect of steroids in supressing T_H2 responses or due to different underlying pathological processes, or to a combination of the two. It is also apparent that this T_H2 bias is a systemic phenomenon, as it is observable in each tissue compartment tested, although the difference is most marked in the lumen of the airway wall, as the greatest difference in median T_H2 frequencies (a 12 fold difference between health and mild asthma) was observed in bronchial biopsies.

Evidence for a deficiency of regulatory T cells

In addition to the increased airway T_H2 cells, I observed a deficiency of Treg in BAL in asthma which was most pronounced in the most severe asthmatics. Treg share a reciprocal developmental relationship with T_H17 and have evolved to regulate tissue inflammation. The balance of Treg and T_H17 cell differentiation from naïve T-cells is regulated by TGFβ, IL-6, IL-21 (Bettelli, Carrier et al. 2006), Vitamin A and D, and the aryl hydrocarbon receptor (Quintana, Basso et al. 2008). However the T_H2 cytokine IL-4 can also influence Treg frequencies by blocking induction of FOXP3 Treg by TGFβ (Dardalhon, Awasthi et al. 2008) and therefore a deficiency in Treg might be expected in a T_H2 mediated disease such as asthma. In animal models Treg have been shown to suppress T_H2

mediated allergic airway inflammation (Wu, Bi et al. 2008) and to mediate tolerance to chronic aeroallergen exposure (Strickland, Stumbles et al. 2006).

Several authors have studied Treg in peripheral blood in human asthma. Mamessier *et al* studied 18 frequently-exacerbating severe asthmatics and observed lower frequencies of $CD25^{hi}$ Treg in blood compared with 14 healthy controls (Mamessier, Nieves et al. 2008). They also observed a fall in the frequencies and suppressive activity of peripheral Treg during exacerbations. Others have found similar numbers of peripheral $CD25^{Hi}$ cells, but a fall in their FOXP3 expression in asthma (Lin, Shieh et al. 2008; Provoost, Maes et al. 2009). Likewise Want *et al* found lower peripheral $CD25^{Hi}$ Treg in asthma, correlating with higher allergen-induced IL-4 responses, whilst subjects who were atopic but asymptomatic had higher levels of allergen-induced IL-10 implying a protective effect of Treg (Wang, Lin et al. 2009). A key immunosuppressive mechanism for Treg is the production of IL-10 (Belkaid, Piccirillo et al. 2002) which may be important for suppression of AHR (Kearley, Barker et al. 2005) and a deficiency of IL-10 secreting cells in peripheral blood has also been reported in severe compared with mild asthma(Matsumoto, Inoue et al. 2004; Hawrylowicz 2005). These associations are not however straightforward as in paediatric populations others have found increases in IL-10 and FOXP3 associated with presence of allergy (McLoughlin, Calatroni et al. 2012) or of more severe asthma(Lee, Yu et al. 2007). Furthermore recently it has been shown that some FOXP3+ human memory Treg can express RORγt and secrete IL-17 but suppress effector T cells via cell-cell contact (Ayyoub, Deknuydt et al. 2009; Voo, Wang et al. 2009).

To date few studies have examined Treg in the human airways. Heier *et al* demonstrated that some FOXP3+ Treg were present within bronchus associated lymphoid tissue in infants with chronic wheeze, but they did not investigate adults or healthy controls (Heier, Malmstrom et al. 2008). My colleague Asha Ganesan investigated airway Treg in 10 mild asthmatics, 10 moderate asthmatics and 10 healthy controls. She observed a decrease in FOXP3+ Treg in sputum in mild-moderate asthma (mean frequency 7.3% versus 11.8% in health, P=0.001)(Ganesan 2010). She also observed a similar decrease in Treg in BAL, although it was significant only for moderate asthma (mean Treg frequency 9.2% in health, 7.8% in mild asthma, 6.5% in moderate asthma, P=0.04) and found an increase in the sputum T_H17:Treg ratio in asthma.

Although I found no significant differences in peripheral blood Treg frequencies and did not replicate Dr Ganesan's findings in sputum, I did observe a similar deficiency of BAL Treg in asthma. Furthermore, as with Dr Ganesan's data, the difference correlated with disease severity (P=0.02), being most marked in the most severe disease and was of similar magnitude (1.6-1.8 fold). Thus my data constitute an important confirmation of her findings and have extended the observations to a more severe phenotypic group.

Could this Treg deficiency be secondary to steroid treatment? Dr Ganesan's moderate asthmatic cohort were similar to my moderate cohort, with a median FEV1 of 98.1% (IQR 84.0-103) and

receiving a median equivalent of 400 mcg of beclometasone dipropionate per day, whist my severe cohort were receiving 1600 mcg / day equivalent BDP (IQR 1280-2000). However a review of the literature suggests that glucocorticosteroids actually tend to increase Treg frequencies *in vivo* in murine peripheral lymphoid tissue (Chen, Oppenheim et al. 2006) and in human asthma in peripheral blood where FOXP3 mRNA expression is increased by inhaled or oral steroids and correlates with IL-10 mRNA expression (Karagiannidis, Akdis et al. 2004; Robinson, Larche et al. 2004; Provoost, Maes et al. 2009). Whilst Seissler *et al* observed differing effects of steroids on different Treg subsets, they too found that steroids induced the strongest increase in the subset of Tregs which were the most suppressive (Seissler, Schmitt et al. 2012). Whilst each of these human studies sampled only peripheral blood, it seems unlikely that the decrease in BAL Treg frequencies is due to steroids, unless steroids were somehow reducing migration of Treg from blood to BAL.

Absolute frequencies are not the only relevant metric of Treg populations, as there is evidence that steroids can increase the IL-10 production and suppressive activity of Treg(Robinson, Larche et al. 2004), or conversely that T cells from refractory asthmatics may be less able to produce IL-10 in response to dexamethasone(Hawrylowicz, Richards et al. 2002). Therefore future studies need to supplement measurement of Treg frequencies with functional assays of human airway Treg function (Ganesan 2010), preferably before and after steroid treatment. Such work is likely to be worthwhile because of the therapeutic potential of induction of allergen specific Treg by immunotherapy (Robinson, Larche et al. 2004).

The uncertain significance of interleukin-17

With respect to interleukin-17 my findings were at odds with my prior hypotheses and with widespread opinion, as an important role for IL-17 has been hypothesised by many authors in recent years (Linden 2001; Aujla, Dubin et al. 2007; Anderson 2008; Alcorn, Crowe et al. 2010; Lloyd and Hessel 2010; Park and Lee 2010). However whilst the last decade has seen some excellent investigation of IL-17 in animal models (Schnyder-Candrian, Togbe et al. 2006; Wakashin, Hirose et al. 2008; Wilson, Whitehead et al. 2009; Lloyd and Hessel 2010; Murdoch and Lloyd 2010) the case for IL-17 in human asthma has rested on just three papers (Molet, Hamid et al. 2001; Barczyk, Pierzchala et al. 2003; Chakir, Shannon et al. 2003), which together have been cited over 150 times in the literature, and which I must briefly address in the following:

In 2001 Molet *et al* provided the first description of IL-17 in the lungs of asthmatics using ELISA and immunocytochemistry to show an increased number of IL-17+ cells in sputum and BAL from asthmatics (Molet, Hamid et al. 2001). However this was a very small study, including only six asthmatics, and it used an insensitive ELISA (LOD 5 pg/ml). More importantly, as with the other two papers by Barczyk and Chakir, the ELISA technique used could not determine the cellular source of IL-17. Indeed the only co-localisation data presented (by *in situ* hybridization) showed IL-17 production by eosinophils. In 2003 the same group reported further immunocytochemistry showing IL-17+ cells were increased in the submucosa in moderate-to-severe asthma (Chakir, Shannon et al.

2003). Again this was a small study involving only six healthy controls and was unable to determine the cellular source of the IL-17, though the localisation was to the submucosa. In the same year, the above-mentioned paper by Barczyk and colleagues, which has since been widely cited paper, was published (Barczyk, Pierzchala et al. 2003), proposing an association between sputum IL-17 levels and BHR, which is clearly at odds with my data. Concerning the methodology of this study, it included only 10 asthmatics, and the statistical report presented a *post hoc* analysis which may have resulted from subgroup selection. Furthermore the ELISA technique used to measure IL-17 was most likely not wholly appropriate, given a LOD of 15 pg/ml compounded by a reported 3-4 fold decrease in IL-17 levels in the presence of DTT. By contrast, the MSD assay used in my studies had a LOD of 0.22 pg/ml, a more modest effect of sample processing with DTE, and IL-17 levels were below 10 pg/ml in all subjects of my study.

More recently Doe *et al* in Leicester suggested a slightly increased IL17+ submucosal staining in mild-moderate (P=0.04) but, this time, not severe asthma (Doe, Bafadhel et al. 2010). This group used the same IL-17 ELISA as Barczyk and for the reasons I have outlined it is not surprising that all 56 samples were below the limit of detection. The authors went on to measure IL-17 by MSD in 165 asthmatics, but unfortunately they included no healthy controls for comparison (*ibid* Fig 3). This paper did however report more immunohistochemistry data suggesting a slight increase in IL-17+ cells in the submucosa in mild-moderate asthma which would be consistent with Chakir *et al*, and with unpublished immunohistochemistry data from Nivenka Jayasekera showing increased IL-17+ in the epithelium of severe asthma (Jayasekera 2013). Jayasekera found that amongst these severe asthmatics IL-17 correlated negatively with PEF and FEV1, possibly implying a protective role. Furthermore whilst she found no correlation between staining for IL-17A and IL-17F or eNO levels, she did observe an increase in IL-17A in mild asthma after allergen challenge.

How can these findings be brought together into a coherent concept? No group has yet produced compelling data for a significant role for IL-17 in severe neutrophilic asthma, and my data would argue strongly against such a role, at least in stable disease. There is no robust evidence of a relationship between airway IL-17 and bronchial hyper-reactivity and my study provides strong data that there is no such relationship. The work of Chakir, Doe and Jayasekera and others (Vazquez-Tello, Semlali et al. 2010; Howarth 2012) do suggest a modest increase in expression of IL-17 in the cells of the airway mucosa or submucosa in asthma. No agreement exists yet on whether this is predominantly IL-17A or IL-17F (an issue complicated by the use of cross reactive antibodies in humans (Lloyd 2012)) or whether IL-17 is pathogenic or protective (Murdoch and Lloyd 2010), and the exact cellular source of this cytokine is similarly contentious. IL-17 can be produced by a wide variety of cell types including T-cells, NK and NKT cells, tissue inducer lymphocytes, macrophages, and mast cells, eosinophils, neutrophils, and epithelial cells (Molet, Hamid et al. 2001; Reynolds, Angkasekwinai et al. 2010; Saitoh, Kusunoki et al. 2010). My data suggest that in stable asthma neither T-cells nor macrophages constitute a major cellular source. Lack of a correlation with neutrophil numbers would also argue against their being the primary source. It seems much more likely in the light of the

immunohistochemistry that the predominant cellular sources in the asthmatic human airway are either eosinophils or bronchial epithelial cells.

Several investigators have demonstrated IL-17 production by airway eosinophils (Molet, Hamid et al. 2001; Saitoh, Kusunoki et al. 2010). In my data-set BAL IL-17 levels were correlated moderately with BAL eosinophilia and eNO (Figure 3.7) and there was also a tendency towards an eosinophilic sputum inflammatory subtype which was not statistically significant probably only because of the small sample size (*n*=4)(Figure 3.10).

The case can also be made for epithelial cells as the dominant IL-17 producers. Epithelial cells are numerically much more abundant than inflammatory cells of the airway and in my study high IL-17 secretion was strongly associated with unusually high shedding or sloughing of epithelial cells into the BAL. Whilst initial research viewed the epithelial cells as downstream effectors in the IL-17 pathway (Fossiez, Djossou et al. 1996; Linden 2001; Chen, Thai et al. 2003; Huang, Kao et al. 2007; Wiehler and Proud 2007) it has recently been shown in mice (Suzuki, Kokubu et al. 2007; Ishigame, Kakuta et al. 2009) and humans (Xu, Zhang et al. 2010) that airway epithelial cells also constitute a significant source of IL-17F. Furthermore immunohistochemistry has shown epithelial staining for IL-17A (Chakir, Shannon et al. 2003). Jayasekera interpreted this IL-17A staining as predominantly cytoplasmic staining of epithelial cells, implying that it was unlikely to be cytokine bound to surface IL-17 receptors (Jayasekera 2013). The possibility that human bronchial epithelial cells are producing IL-17A is a hypothesis which warrants confirmation, for instance by PCR on pure epithelial cells.

Relegating T_H17 cells

The lack of supporting evidence for pathological relevance of IL-17 in asthma in my studies of blood, BAL, Sputum and tissue goes hand in hand with my central observation that T_H17 cell frequencies do not correlate with any phenotype of asthma. Hence, my findings, whilst confirming the centrality of the T_H2 response in asthma, relegate T_H17 cells to a very minor role. Only few prior published data are available to dispute it. One Chinese group reported differences in peripheral T_H17 cell frequencies in moderate-severe but not mild asthma, but their methods for statistical analysis were unclear, P values not reported and there was a wide overlap between the group distributions (Shi, Shi et al. 2011). Most importantly, airway tissues were not analysed in that study. Others have also looked in peripheral blood but not tissue, such as Wong *et al* who measured peripheral IL-17 secreting cells by ELISA and by surface markers(Wong, Lun et al. 2009). As T_H17 cells are defined by their expression of IL-17 It is not appropriate to enumerate T_H17 cells by surface markers alone, and the marker used in this case – CCR6 – is known to be expressed on both T_H17 and FOXP3 Treg, as well as other T-cell subsets. Bullens *et al* also claimed to have measured T_H17 cells using a different method (Bullens, Truyen et al. 2006). They report an increase in IL-17 mRNA in whole sputum which is moderately correlated (r=0.5) with mRNA for CD3. Given the weakness of the association this is far from an accurate method of enumerating T_H17 cells, and the statistical analysis also raises concerns. Another paper sometimes cited as evidence of T_H17 in asthma is that by Pene *et al* who obtained T cell clones from

bronchial samples (Pene, Chevalier et al. 2008). Whilst this paper proved that T_H17 cells are present in the airway, it included only three subjects, all of whom were asthmatics sampled during an exacerbation, and crucially they included no healthy controls. Finally Al-Ramli *et al* reported an increase in submucosal IL-17+ cells using PCR and immunohistochemistry on bronchial biopsies. However this paper did not present data on co-localisation so again there is no evidence that T cells were the source of the IL-17 (Al-Ramli, Prefontaine et al. 2009).

Others have reported a subset of T_H2 cells, defined as expressing the surface marker chemoattractant receptor-homologous molecule expressed on T_H2 cells (CRTH2), which also expressed IL-17 (Cosmi, Annunziato et al. 2000; Wang, Voo et al. 2010). These cells were found at higher frequencies in peripheral blood of 23 atopic asthmatics(Wang, Voo et al. 2010). While I did not stain for CRTH2, I have been unable to detect any dual IL-13/IL-17 T cells in my dataset, although this may partly be because IL-13 expression was not maximal by 4-5 hours. Cosmi *et al* reported T cell clones which express both IL-17 and IL-4, and found them to be more frequent in asthma (n=11), although they acknowledge these cells are extremely rare, comprising approximately 0.025% of T helper cells in health, and like me they found no difference in T_H17 frequencies between asthma and health(Cosmi, Maggi et al. 2010).

In summary, the data presented in this chapter provide strong evidence that T_H17 cells are not associated with asthma in stable disease. The study subjects did not undergo allergen challenge, although they were sampled throughout the year, so many will have received ongoing exposure to perennial and seasonal allergens. Finally this cohort was not sampled within six weeks of a symptomatic viral illness. It is possible that T_H17 cells might play a more important role during an acute antiviral immune response, and so I present data on T cell responses during natural exacerbations in chapter 7.

γδT-cells

I did not find evidence of high frequencies of 17-secreting γδT-cells associated with asthma. This is perhaps an instance where there is a distinct species difference between the immunology of mice and humans. In mice γδT-cells have been implicated in the pathogenesis of experimental allergic airways disease, being necessary for the IL-4 dependent generation of specific IgE and IgG1, of pulmonary IL-5 and -13 and in recruiting T cells and eosinophils to the airways (Zuany-Amorim, Ruffie et al. 1998; Jin, Roark et al. 2009). Other data suggest they may also play a subsequent role in the resolution of airways inflammation as CD8+ γδT-cells (in rats) (Isogai, Athiviraham et al. 2007) or IL-17 secreting γδT-cells (in mice) (Murdoch and Lloyd 2010) can decrease AHR, the late allergic airway response, eosinophilia and T_H2 responses. In mice the dominant IL-17-producing cells in the spleen are γδT-cells more than T_H17 cells(Stark, Huo et al. 2005) and likewise in a murine OVA challenge model the dominant IL-17 secreting cells in BAL were γδ-17 cells more than T_H17 cells (Murdoch and Lloyd 2010). Conversely in humans I have observed much greater numbers of T_H17 cells than γδ-17 cells in each tissue compartment. In BAL γδT-cells are rare, comprising only 1.1% of lymphocytes, of which a

median 5.6% secreted IL-17, whilst the majority (42.3%) secreted IFN-γ. Perhaps significant species differences in γδT-cells biology are unsurprising as similar differences between mouse and man have been observed with other innate-like lymphocytes, namely the iNKT cells which are found at higher frequencies in mice and MAIT cells which, conversely, are 5-10 fold more abundant in humans (Treiner, Duban et al. 2005). MAIT cells will be the subject of the next chapter.

CHAPTER 4

CD8+ T cells in asthma

The most incomprehensible thing about the world
is that it is comprehensible.[4]

[4] Albert Einstein (1879-1955), cited in Vallentin, A. (1954). Einstein: A Biography. London, Weidenfeld and Nicolson. p24

Introduction

In order to place T helper subsets in context I also undertook a parallel analysis of CD8+ 'cytotoxic' T cells (T_{CYT}). These cells have received much less attention in airways disease, although a few studies have shown that airways disease in COPD may be associated with an increase in epithelial (Fournier, Lebargy et al. 1989) or subepithelial (O'Shaughnessy, Ansari et al. 1997; Saetta, Di Stefano et al. 1998) CD8+ T cells. In asthma CD8+ T cells have been found in increased frequencies and activation state in post-mortem specimens (O'Sullivan, Cormican et al. 2001) and one longitudinal study has shown a modest correlation between bronchial biopsy CD8+ T cells and subsequent rate of decline in lung function (van Rensen, Sont et al. 2005). Furthermore as a primary role of CD8+ T cells is direct antiviral activity and in view of the increasing appreciation of the relevance of respiratory viral infections in asthma, it seems timely to investigate these cells afresh (Johnston, Pattemore et al. 1995; Johnston, Pattemore et al. 1996; Corne, Marshall et al. 2002; Message, Laza-Stanca et al. 2008). As mentioned in chapter 1, CD8+ T cells form functionally distinct subsets known as Tc1 and Tc2 cells, according to their expression of type 1 or type 2 cytokines respectively (Mosmann, Li et al. 1997). One previous studies have found evidence of an increase in the Tc2 subset in asthma in sputum (Cho, Stanciu et al. 2005), and it is this cell type which will be the focus of this chapter.

In this chapter I will present cross-sectional data showing that the Tc2 subset of cells are increased in asthma and are associated specifically with an eosinophilic endotype.

Results and comments

Study population

This analysis was performed on the samples taken from the same population which was described in chapter 3, comprising 23 healthy subjects and 53 asthmatics (14 mild, steroid-naïve, 17 moderate, treated with low dose inhaled corticosteroids and 22 severe, treated with oral or high dose inhaled corticosteroids) were studied. All had stable symptoms for at least 6 weeks prior to clinical sampling. Clinical and demographic characteristics are presented in Table 3.1.

Definitions of T cell subsets

T cells were stimulated *ex vivo* for 4 to 5 hours with PMA and ionomycin and analysed by flow cytometry. Tc1 cells were defined as live CD3+CD8+ T cells expressing IFNγ. Tc2 cells were defined as live CD3+CD8+ T cells expressing IL-13. Frequencies are expressed as a percentage of the total CD8+ T cell population. Analysis using absolute numbers of Tc1 and Tc2 cells, expressed as a proportion of CD3+ T cells, yielded the same findings.

Type 2 cytokine-secreting cytotoxic T cell frequencies are increased in asthma in PBMC and BAL, and correlate with disease severity

I observed significant increases in the relative frequencies of CD8+ T cells secreting IL-13 (Tc2) in asthma compared with health in both peripheral blood (*n*=66, Mann-Whitney P=0.04) and BAL (*n*=60,

P=0.02) with a similar pattern in bronchial biopsies, although the latter did not reach significance, possibly due to the smaller sample size (*n*=48)(Figure 4.1).

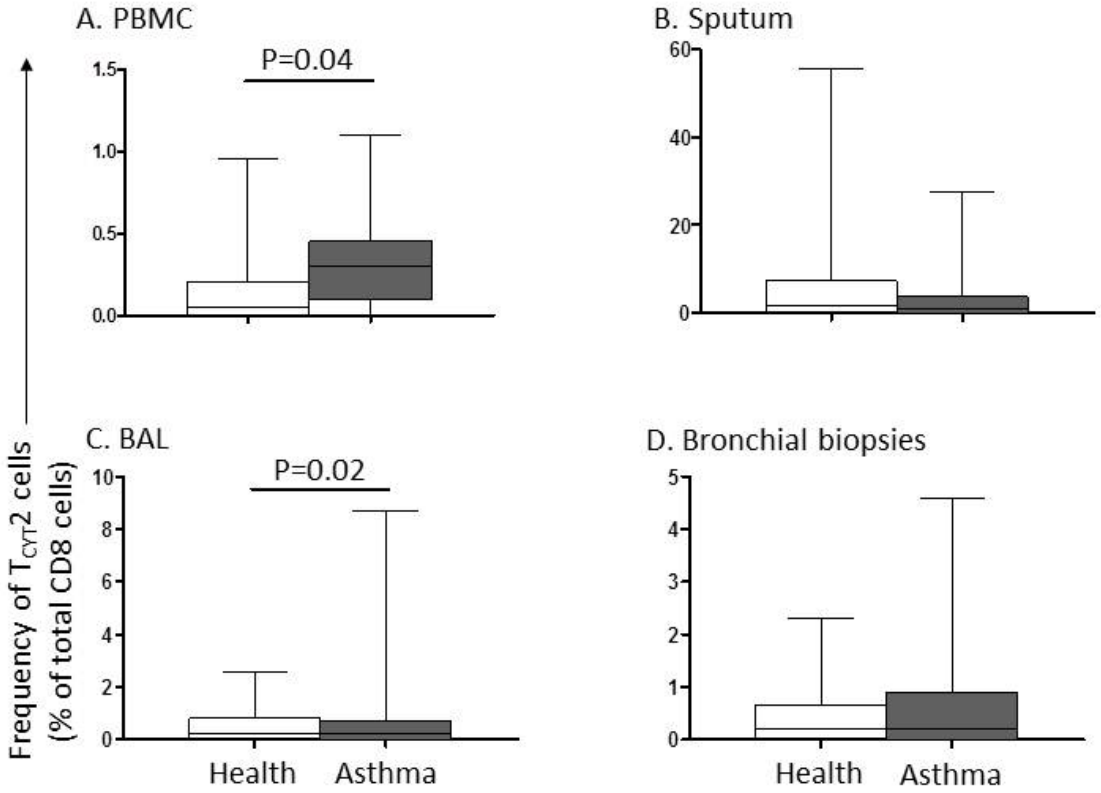


Figure 4.1 Type 2 cytokine-secreting cytotoxic T cell frequencies are increased in asthma in PBMC and BAL

Frequencies of CD8+ T cells which express the type 2 cytokine IL-13 (Tc2 cells) expressed as a proportion of total live CD8+ T cells in peripheral blood, sputum, BAL and biopsies in health and asthma. Box and whisker plots show medians and IQRs. Differences are compared by unpaired Mann-Whitney tests and shown if P>0.05.

healthy controls	*n*=19 PBMC, 12 sputum, 17 BAL, 13 biopsies.
asthma	*n*=47 PBMC, 26 sputum, 43 BAL, 35 biopsies.

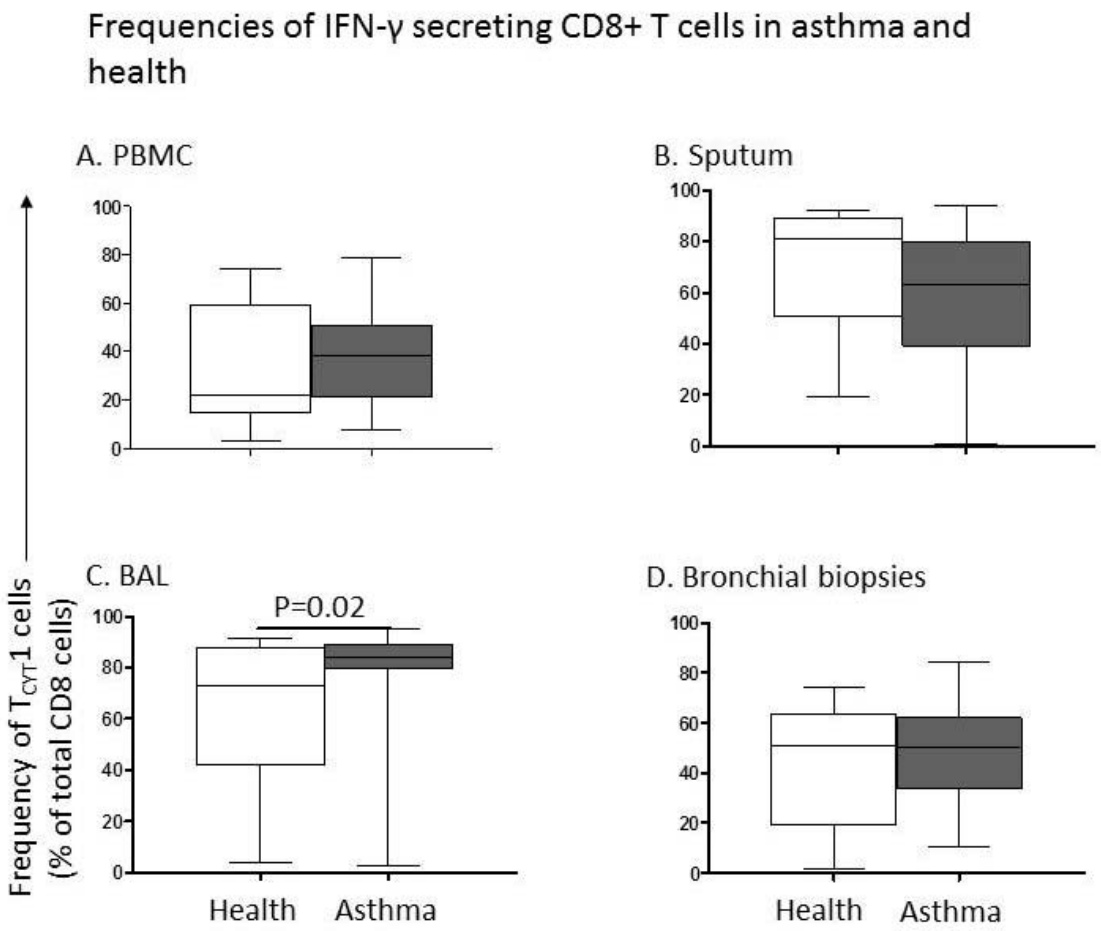


Figure 4.2 Type 1 cytokine-secreting cytotoxic T cell frequencies are increased in BAL in asthma

Frequencies of CD8+ T cells which express the type 1 cytokine IFN-γ (Tc1 cells) expressed as a proportion of total live CD8+ T cells in peripheral blood, sputum, BAL and biopsies in health and asthma. Box and whisker plots show medians and IQRs. Differences are compared by unpaired Mann-Whitney tests and shown if P>0.05.

healthy controls *n*=19 PBMC, 12 sputum, 17 BAL, 13 biopsies.

asthma *n*=47 PBMC, 26 sputum, 43 BAL, 35 biopsies.

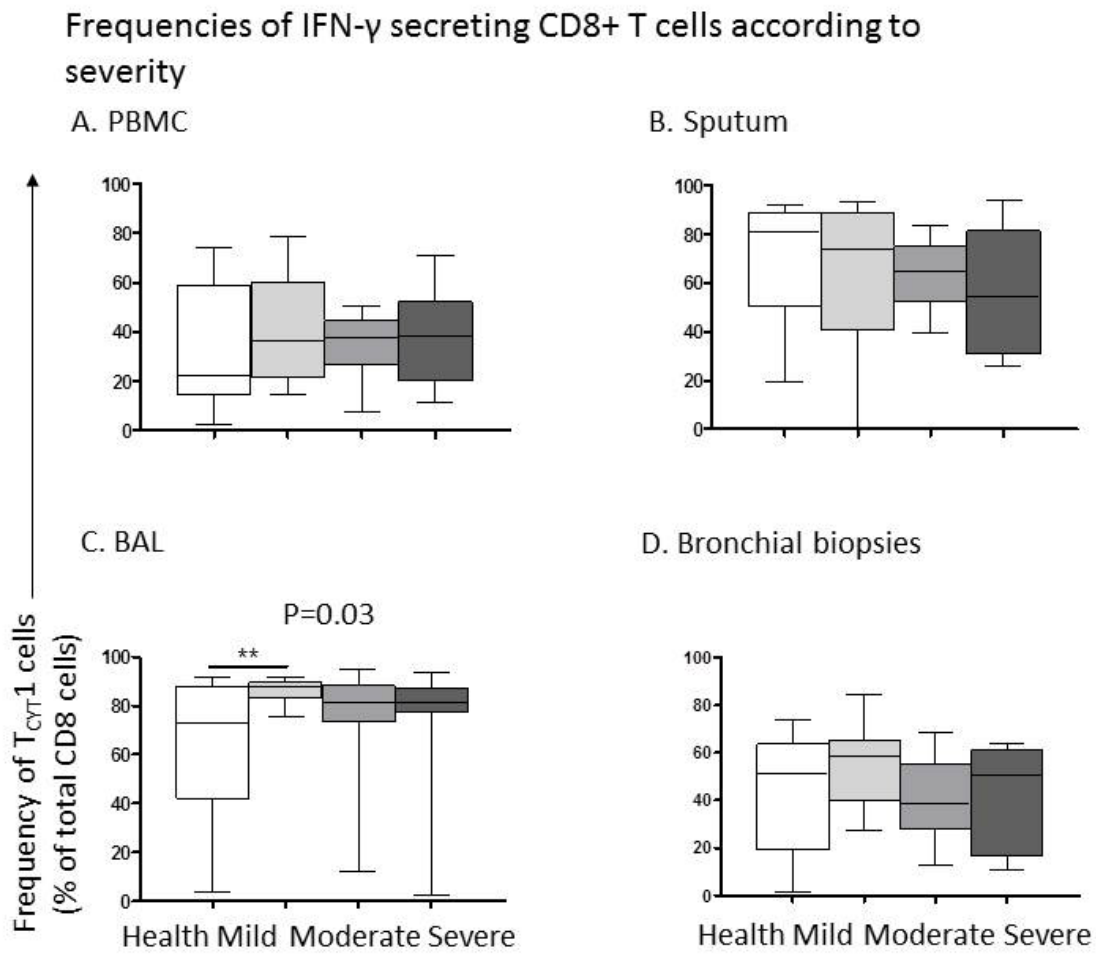


Figure 4.3 Type 1 cytokine-secreting cytotoxic T cells are increase in BAL in mild asthma

Frequencies of CD8+ T cells which express the type 1 cytokine IFN-γ (Tc1 cells) expressed as a proportion of total live CD8+ T cells in peripheral blood, sputum, BAL and biopsies in health and asthma stratified according to disease severity. Box and whisker plots show medians and IQRs. Differences are compared by Kruskal-Wallis tests with *post hoc* Dunn's and are significant only for health v mild asthma in BAL.

healthy controls	*n*=19 PBMC, 12 sputum, 17 BAL, 13 biopsies.
mild asthma	*n*=14 PBMC, 9 sputum, 13 BAL, 13 biopsies.
moderate asthma	*n*=14 PBMC, 8 sputum, 14 BAL, 13 biopsies.
severe asthma	*n*=19 PBMC, 9 sputum, 16 BAL, 10 biopsies.

Type I cytokine-secreting cytotoxic T cell are increased only in BAL, in mild asthma.

IFN-γ-secreting T cells (Tc1) did not differ significantly between asthma and health in PBMC, sputum or bronchial biopsies, but were increased in asthma in BAL with a median frequency of 73% (IQR 80-89%) compared with 84% (42-88%) in health (P=0.02) (Figure 4.2). When subjects were stratified according to disease severity this difference is seen to result from increased frequencies of Tc1 cells in mild asthma (median frequencies in mild asthma 88% (84-90%) compared with health 73% (42-

92%), Dunn's P<0.01, Figure 4.3). This asthma-related increase in IFN-γ-secreting T cells is specific to the CD8+ subset (Tc1) and is not observed in the CD4+ subset (T_H1)(Figure 4.4).

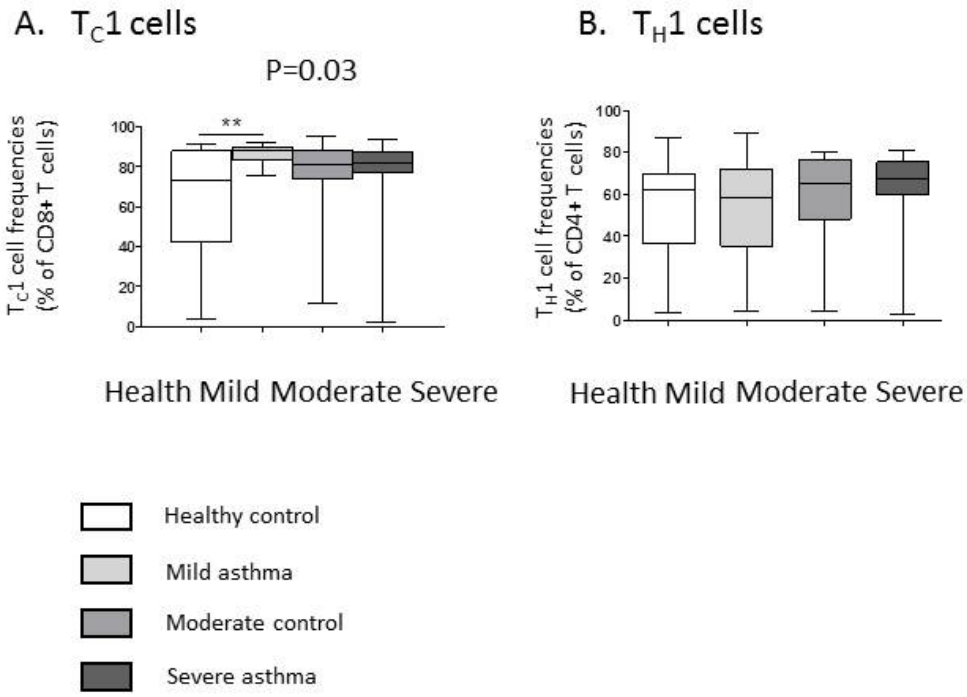


Figure 4.4 A comparison of Tc1 and TH1 cells in BAL

Frequencies of (A) CD8+ Tc1 cells and (B) CD4+ T_H1 expressed as a proportion of total live CD8+ and CD4+ T cells respectively in BAL in health and asthma stratified according to disease severity. Box and whisker plots show medians and IQRs. Differences are compared by Kruskal-Wallis tests with *post hoc* Dunn's and are significant only for Tc1 in health v mild asthma in BAL. By contrast there are no differences in T_H1 cell frequencies in BAL.

healthy controls	*n*=17
mild asthma	*n*=13
moderate asthma	*n*=14
severe asthma	*n*=16

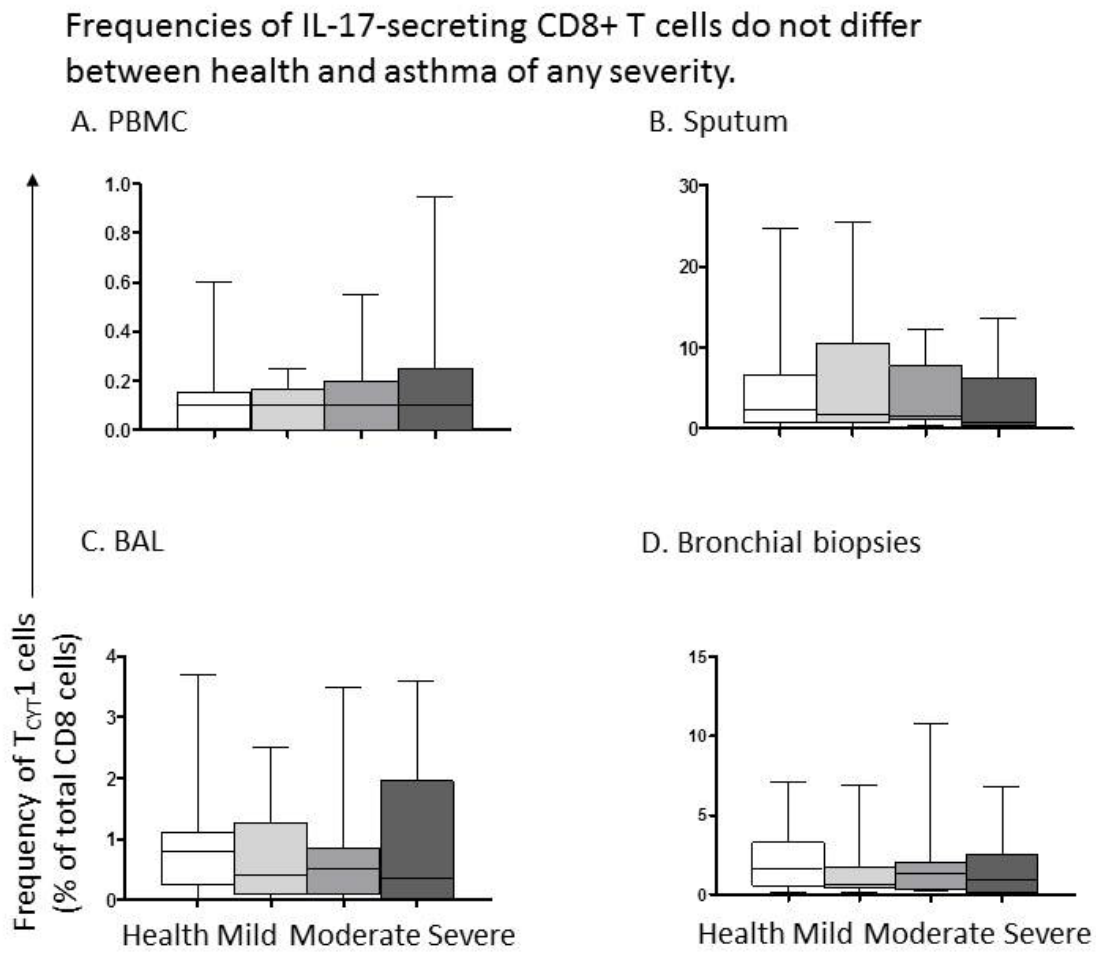


Figure 4.5 Frequencies of IL-17-secreting CD8+ T cell do not differ asthma

Frequencies of CD8+ T cells which express IL-17 (Tc17 cells) expressed as a proportion of total live CD8+ T cells in peripheral blood, sputum, BAL and biopsies in health and asthma. Box and whisker plots show medians and IQRs. No significant differences were observed between asthma and health (Mann-Whitney test P>0.2 in all tissues, not shown) or between health and different phenotypes (Kruskal-Wallis P>0.5 in all tissues) healthy controls.

healthy controls	*n*=19 PBMC, 12 sputum, 17 BAL, 13 biopsies.
mild asthma	*n*=14 PBMC, 9 sputum, 13 BAL, 13 biopsies.
moderate asthma	*n*=14 PBMC, 8 sputum, 14 BAL, 13 biopsies.
severe asthma	*n*=19 PBMC, 9 sputum, 16 BAL, 10 biopsies.

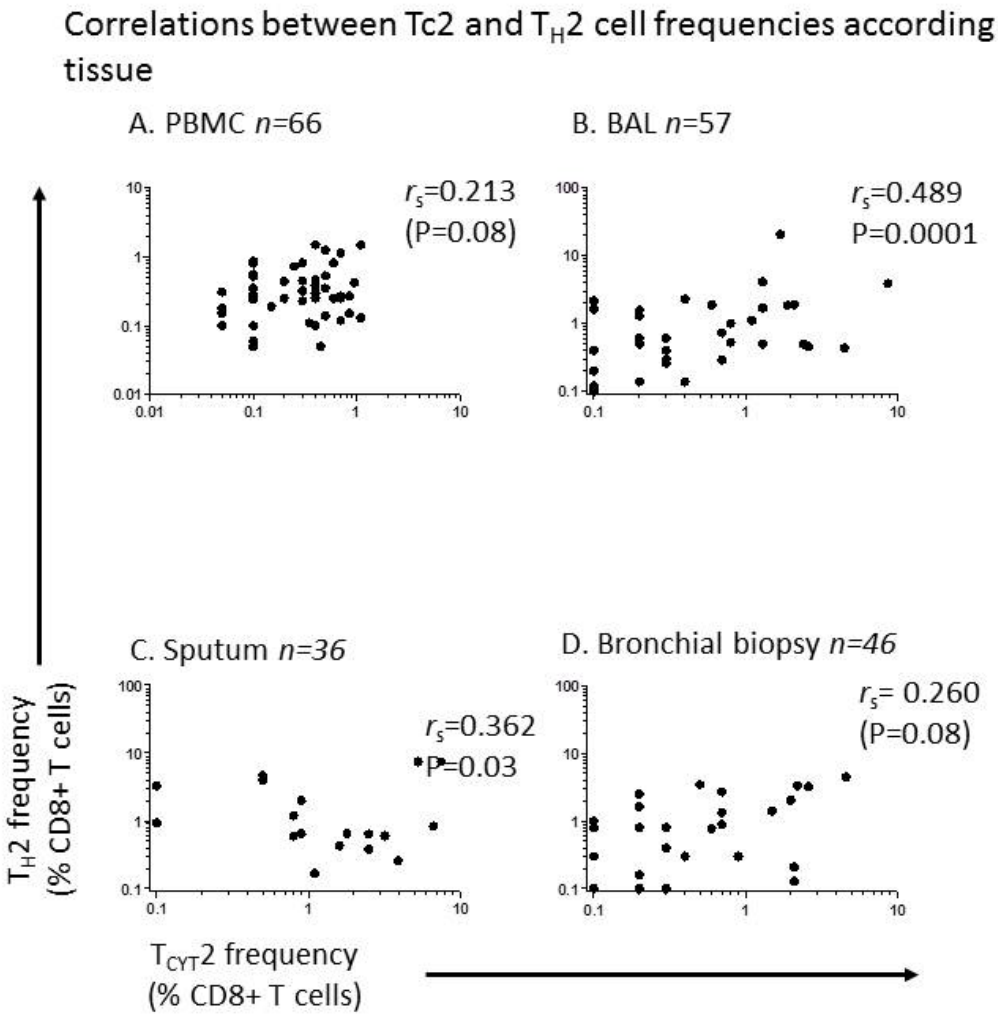


Figure 4.6 Correlations between Tc2 and TH2 cells in tissues

Correlations between frequencies of T_H2 and Tc2 cells in (A) PBMC, (B) BAL, (C), sputum and (D) bronchial biopsies. Spearman's correlations are presented.

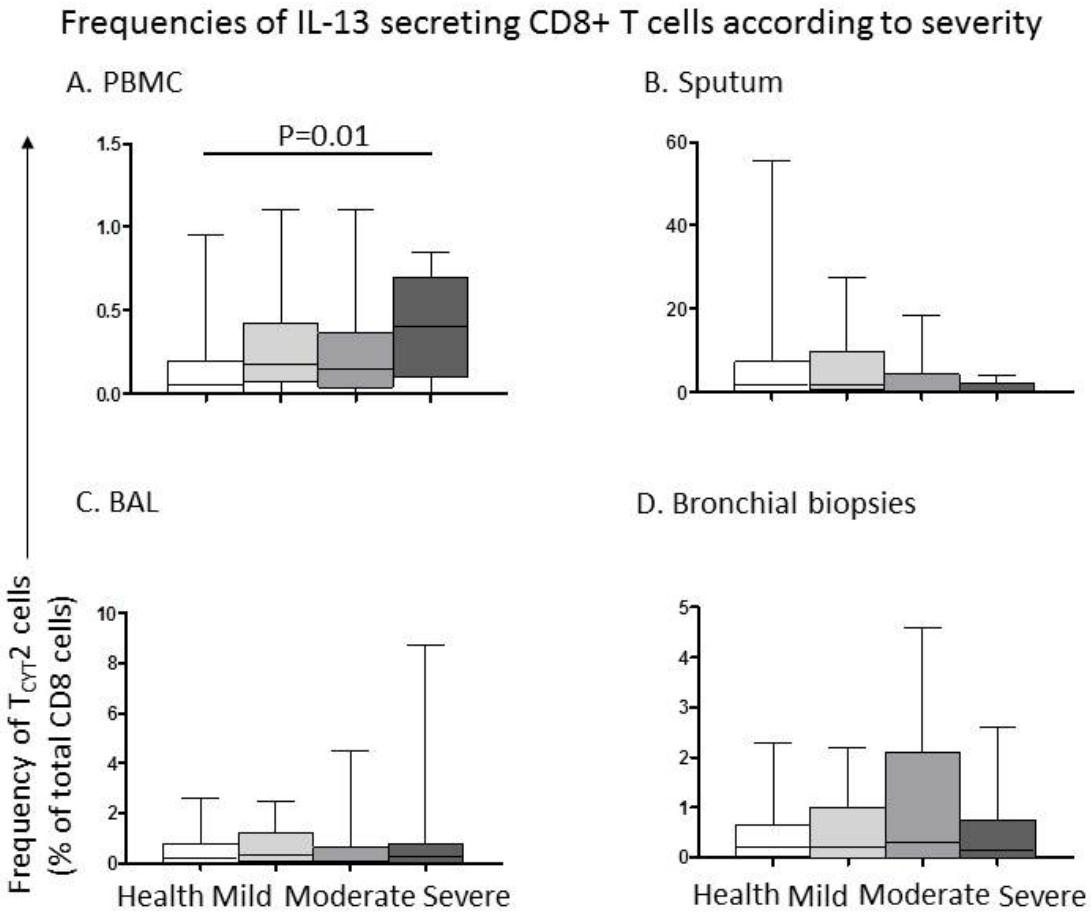


Figure 4.7 Type 2 cytokine-secreting cytotoxic T cell frequencies correlate with disease severity in blood

Frequencies of CD8+ T cells which express the type 2 cytokine IL-13 (Tc2 cells) expressed as a proportion of total live CD8+ T cells in peripheral blood, sputum, BAL and biopsies in health and asthma stratified according to disease severity. Box and whisker plots show medians and IQRs. Linear trends are compared across groups using Jonckhere-Terpstra test and are significant only for PBMC.

healthy controls	*n*=19 PBMC, 12 sputum, 17 BAL, 13 biopsies.
mild asthma	*n*=14 PBMC, 9 sputum, 13 BAL, 13 biopsies.
moderate asthma	*n*=14 PBMC, 8 sputum, 14 BAL, 13 biopsies.
severe asthma	*n*=19 PBMC, 9 sputum, 16 BAL, 10 biopsies.

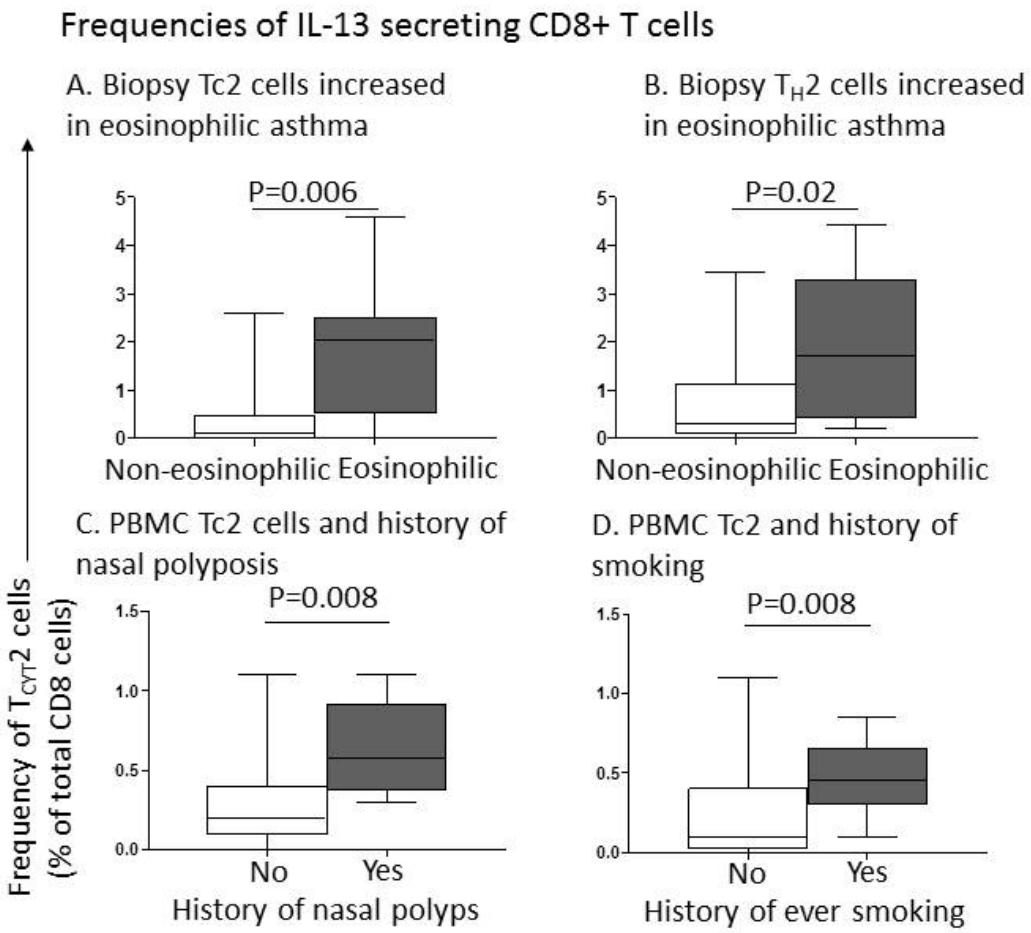


Figure 4.8 Type 2 cytokine-secreting cytotoxic T cell frequencies according to inflammatory subtype, nasal polyposis and history of smoking

(A) Frequencies of Tc2 cells in bronchial biopsies correlate strongly with the presence of eosinophilic asthma (based on sputum inflammatory cell type)(Kruskal-Wallis P=0.03 across eosinophilic, neutrophilic and paucicellular subgroups, *post hoc* Mann-Whitney P=0.006). (B) Biopsy T_H2 cell frequencies are also associated with eosinophilic asthma, but the association is less strong (Mann-Whitney P=0.02). In peripheral blood Tc2 cell frequencies are associated with (C) a history of nasal polyposis (Mann-Whitney P=0.008) and a history of ever smoking (Mann-Whitney P=0.008). Box and whisker plots show medians and IQRs.

Frequencies of IL-17 secreting cytotoxic T cells are not associated with asthma

I observed no differences in frequencies of IL-17-secreting CD8+ T cells between asthma and health (P>0.2 in all tissues) or between health and any category of disease severity (P>0.5 for all tissues, Figure 4.5).

Clinical correlations with Tc2 cell frequencies

Tc2 cell frequencies correlated with Th2 cell frequencies in BAL (r_s=0.489, P=0.0001, *n*=57), and weakly in sputum (r_s=0.362, P=0.03, *n*=36), Tc2 and Th2 frequencies were not significantly correlated in blood or biopsies Figure 4.6).

Peripheral blood Tc2 frequencies were higher in more severe disease (Jonckhere-Terpstra test, P=0.01), with a similar pattern in BAL (ns)(Figure 4.7).

Type 2 cytokine-secreting cytotoxic T cell frequencies according to inflammatory subtype, nasal polyposis and history of smoking

I observed a wide range of Tc2 frequencies, particularly in biopsies, suggesting that these patterns may result from a large increase of Tc2 cells in a specific subset of individuals. I therefore explored relationships with clinical characteristics in univariate analyses. Frequencies of bronchial biopsy Tc2 cells differed significantly between different inflammatory subtypes (Kruskal-Wallis P=0.03). This difference was due to a striking 20 fold increase in Tc2 cells in eosinophilic asthma (median 2.1%, IQR 0.53-2.5%) compared with other subtypes (0.10%, 0.0-0.48%, Mann-Whitney P=0.006)(Figure 4.8 A). For comparison in my data-set this difference is of greater magnitude than the much better documented phenomenon of increased biopsy CD4+ T_H2 cells in eosinophilic asthma (eosinophilic asthma 1.7%, 0.4-3.3% *versus* other subtypes 0.3%, 0.1-1.1%, P=0.02)(Figure 4.8 B). This phenomenon can also be observed as a much lower ratio of bronchial biopsy Tc1 to Tc2 cells in eosinophilic asthma (median 27% (15-43%) than paucigranulocytic asthma (median 117% (86-472%, P<0.05, Figure 4.9).

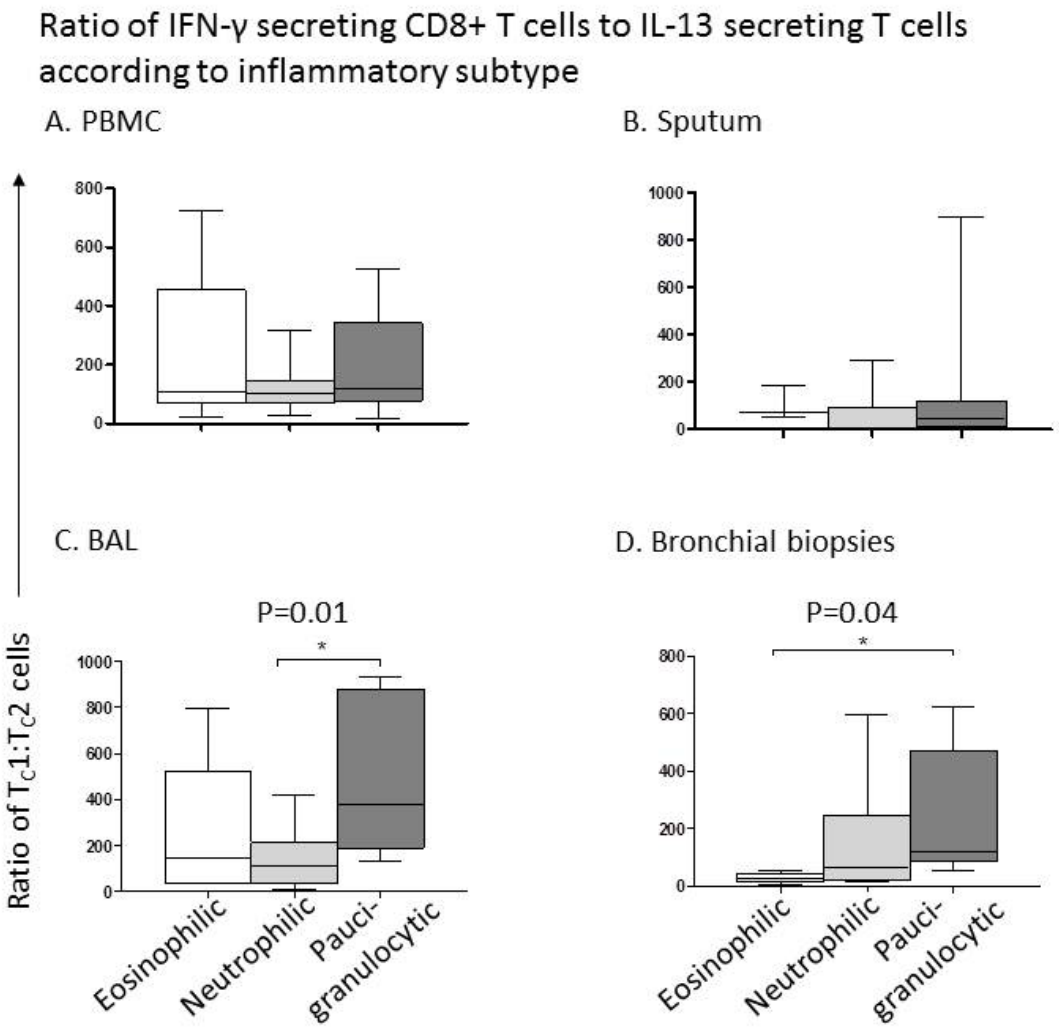


Figure 4.9 Ratio of TC1:TC2 T cells according to inflammatory subtype

The ratio of Tc1 to Tc2 cells in peripheral blood, sputum, BAL and biopsies in asthma stratified according to sputum inflammatory cell subtype. Box and whisker plots show medians and IQRs. A single subject with mixed subtype was classified as eosinophilic for this analysis as this was the predominant feature. Differences are compared by Kruskal-Wallis tests (P values given) with *post hoc* Dunn's (denoted with * for P<0.05) and are significant in BAL and bronchial biopsies.

Eosinophilic	*n*=9 PBMC, 3 sputum, 6 BAL, 5 biopsies.
Neutrophilic	*n*=12 PBMC, 6 sputum, 12 BAL, 9 biopsies.
Paucigranulocytic	*n*=14 PBMC, 10 sputum, 10 BAL, 6 biopsies.

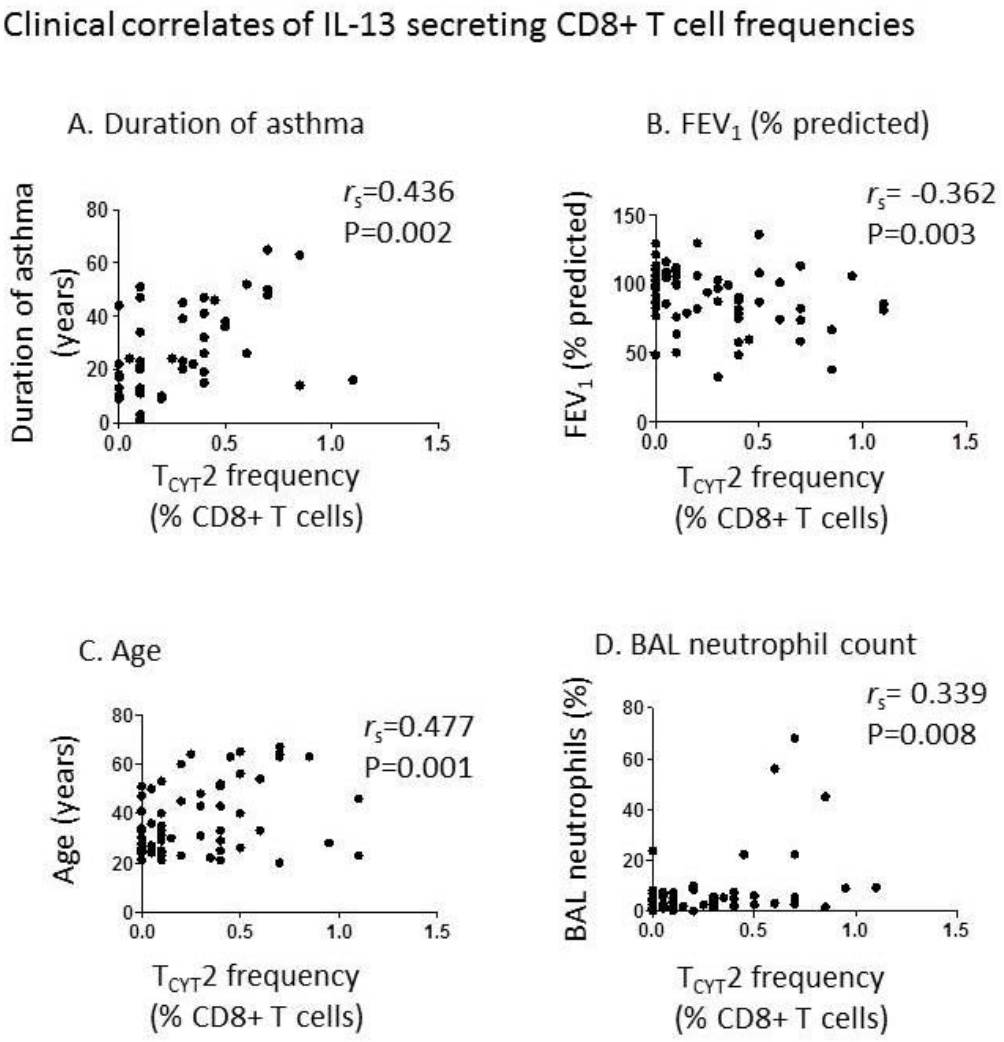


Figure 4.10 Clinical correlates of peripheral blood Tc2 cell frequencies

Frequencies of IL-13-secreting CD8+ T cells as a percentage of total CD8 cells are correlated positively with (A) duration of asthma in years, (C) age in years and (D) BAL neutrophilia and correlated negatively with FEV_1% predicted. Spearman's correlations are presented.

Clinical correlates of peripheral blood Tc2 cell frequencies

Univariate analyses of peripheral blood Tc2 frequencies, showed higher frequencies were associated with a history of nasal polyposis (P=0.008) and a history of ever smoking (P=0.008, Figure 4.8 C,D).

Using univariate Spearman's correlations frequencies of Tc2 cells were correlated positively with duration of asthma (r_s=0.436, P=0.002, Figure 4.10 A), with age (r_s=0.477, P=0.001, Figure 4.10 C) and more weakly with BAL neutrophil count (r_s=0.339, P=0.008, Figure 4.10 D). Finally Tc2 frequencies were correlated negatively with FEV_1 (% predicated).

Preliminary analysis of the T cell transciptome is supportive of a role for CD8+ T cells in asthma

In the discussion chapter I will outline my plans for a comprehensive analysis of the T cell the transcriptome in pure populations of sorted CD3 T cells from blood and airway tissues. Numbers of samples which were successfully hybridised are presented in table 4.1. Such comprehensive analysis has not yet been performed, but I will briefly present the most preliminary analysis of this data-set, as it is relevant to this chapter.

Table 4.1 Numbers of successful microarrays performed and passing quality data quality control

Tissue	Healthy control	Mild asthma	Moderate asthma	Severe Asthma	Total
PBMC	12	9	11	10	**42**
BAL	14	14	10	8	**46**
Sputum	5	7	4	8	**24**
Sputum post ICS	6	6	N/A	N/A	**12**
Epithelial cells	12	12	10	8	**42**

Figure 4.11 shows a network analysis of peripheral blood CD3+ T cells revealing severe asthma is associated with a significant down-regulation in T cell associated networks. Furthermore these genes were not found to correlate with ICS dose. These data provide further evidence of the importance of T cells in the pathogenesis of asthma, and imply that such responses are steroid resistant.

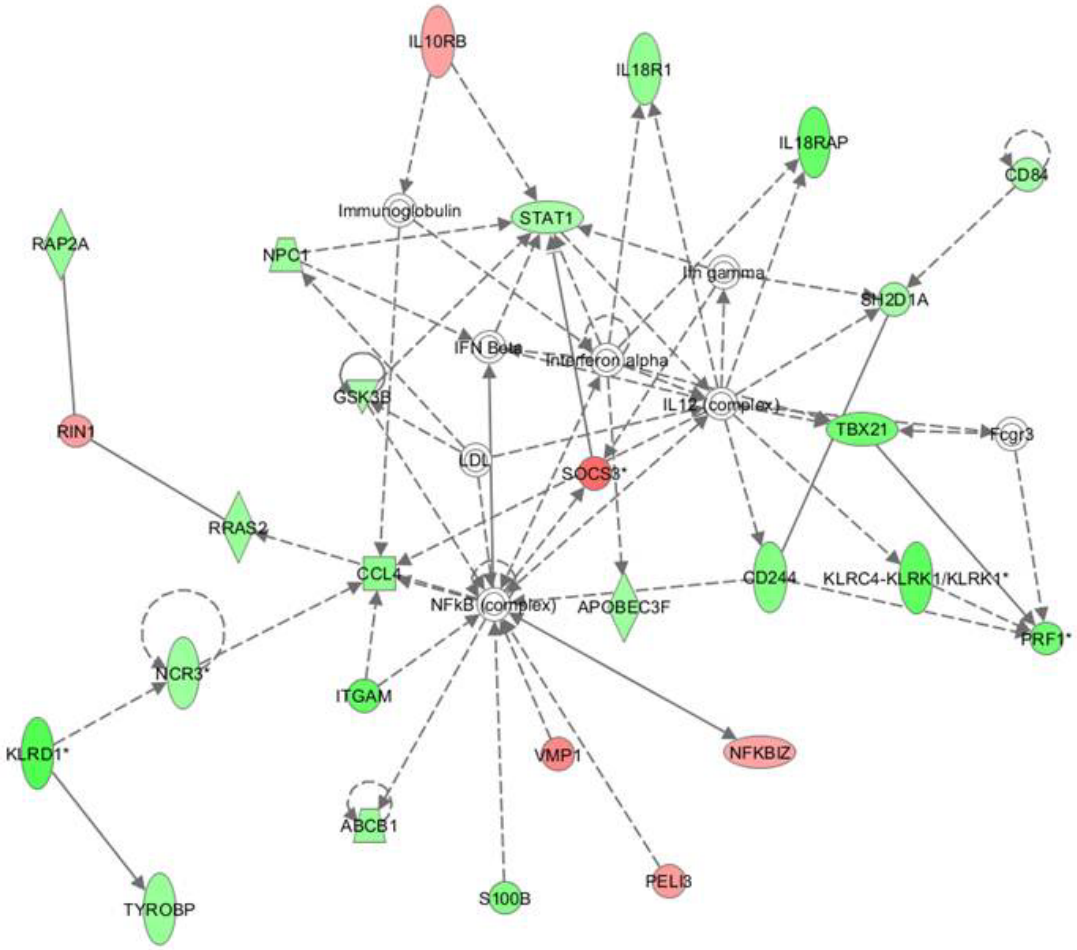


Figure 4.11 T cell associated networks are down-regulated in severe asthma

Transcriptomic data from sorted peripheral blood CD3+ cells reveal that severe asthma is associated with a significant down-regulation in genes associated with T cell networks. These genes did not correlate with ICS dose.

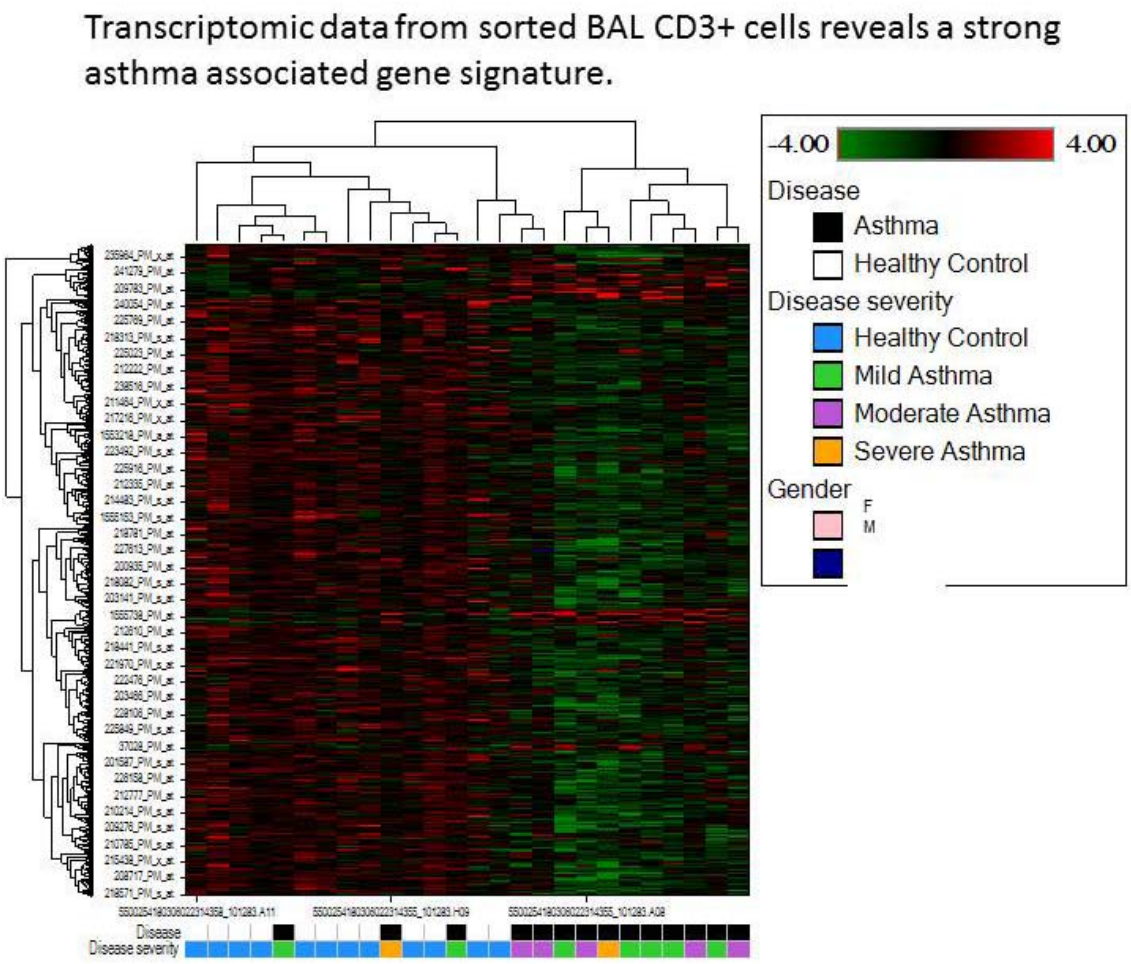
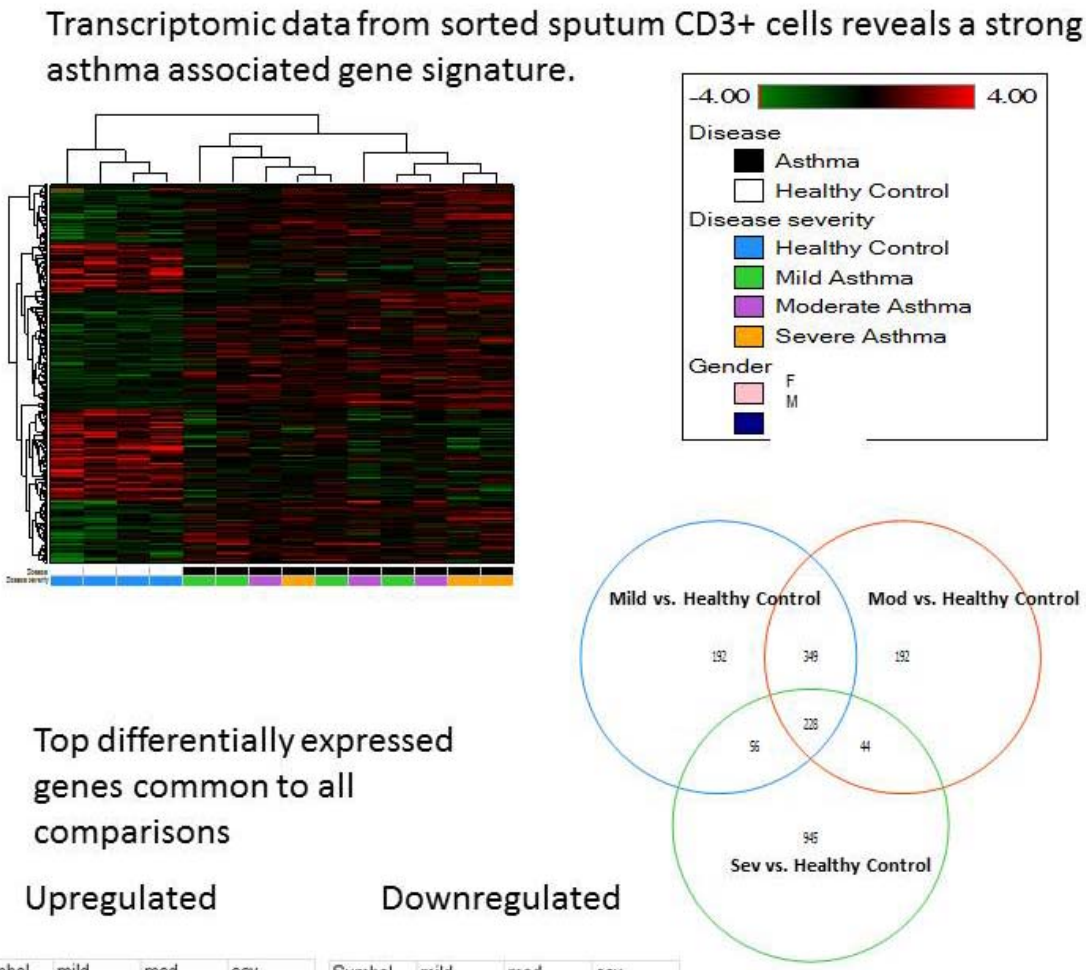


Figure 4.12 Hierarchical clustering of asthma v health in BAL T cells reveals a strong asthma-associated gene signature

Hierarchical clustering was performed on gene lists from asthma v healthy with greater than ±1.5 fold, P≤0.05 difference in gene expression (875 probe set IDs). Only male subjects were included because of a strong gender effect.



Symbol	mild	mod	sev
CCL18	6.5	4.9	11.8
S100A8	3.9	7.4	11.6
MARCKS	7.9	8.5	9.9
FCGR2A	5.4	7.1	9.2
BCL2A1	6.2	7.6	9.0
IL1RN	10.1	4.4	8.4
SPSB1	3.3	4.1	6.3
HSPA6	2.5	2.6	6.3
LILRB2	4.7	4.0	5.9
SAT1	2.4	3.1	5.8
LYN	3.8	3.2	5.3
ARNTL	3.9	7.1	4.6
GSN	4.2	2.0	4.3
MS4A4A	4.0	5.4	4.2
CTSB	3.4	2.7	4.2

Symbol	mild	mod	sev
USP48	-7.8	-12.3	-0.7
N4BP2L2	-5.3	-6.4	-1.0
B3GAT3	-1.5	-2.5	-1.6
LIX1L	-2.1	-1.9	-1.6
NAGLU	-1.8	-2.2	-1.6
DOK4	-1.7	-1.7	-1.7
S1PR2	-2.1	-2.2	-1.8
RAB26	-1.6	-1.9	-1.9
VPS13A-A	-1.7	-1.8	-1.9
TNFRSF12	-2.3	-2.3	-1.9
EIF4EBP2	-1.7	-1.7	-1.9
ARHGAP1	2.6	-2.3	-2.1
NFATC2	-2.0	-2.2	-2.1
PPIA	-3.8	2.4	-2.1
EXOSC6	-4.2	-3.9	-2.1

Figure 4.13 Hierarchical clustering of asthma v health in sputum T cells reveals a strong asthma-associated gene signature

Hierarchical clustering was performed on gene lists from asthma v healthy with greater than ±1.5 fold, P≤0.05 difference in gene expression. Only male subjects were included because of a strong gender effect.

Hierarchical clustering of genes differentially expressed (±1.5 fold, P≤0.05) between asthma and health in BAL Figure 4.12 (875 genes) and sputum Figure 4.13 (1181 genes). Further comparisons are given in Figure 4.13 (health *versus* mild asthma, health *versus* moderate asthma and health *versus* severe asthma in sputum). The highest upregulated gene was CCL18 which is a chemotactic for T cells and expressed at high levels in the lung. Pathway analysis identified 'cytotoxic T

lymphocyte mediated apoptosis of target cells' as the 5th most differentially expressed pathway in asthma, providing further evidence of the importance of CD8+ T cells in asthma.

Thus this very brief report of T cell transcriptomics demonstrates that asthma is associated with significant changes in the function of T cell networks, including cytotoxic T cells, which will merit detailed investigation in the future through data-sets such as this (see chapter 8).

Discussion

In this brief cross-sectional investigation of CD8+ T cells I have observed a specific increase in the Tc2 cell subset in asthma in PBMC and BAL, which is associated with increasing disease severity and which is most striking in a subset of eosinophilic asthmatics. This subset were further characterised as subjects who tended to have a history of smoking, of nasal polyposis, and older age.

What is known of a link between CD8+ cells and eosinophils in asthma?

Perhaps one reason that CD8+ cells have received so little attention is that several early studies have using allergen challenge in humans found no increase in airway CD8+ T cells after allergen challenge (Aalbers, Kauffman et al. 1993; Aalbers, Kauffman et al. 1993; Bentley, Meng et al. 1993) but instead reported significant increases in airway eosinophils in BAL and bronchial biopsies. However these studies did not compare asthmatics with healthy controls and focussed on different asthma endotypes. Furthermore more recent studies have analysed not just absolute numbers of T cells, but also their activation status and cytokine expression and this has led to different conclusions. Walker *et al*. focussed on eosinophilic lung diseases, including allergic asthma and did find significantly increased numbers of activated CD4+ and CD8+ T cells in BAL compared with health, observing a close correlation between numbers of activated T cells, eosinophils and IL-5 levels (Walker, Bauer et al. 1994). Using immunohistochemistry Ying *et al*. found that biopsy CD8+ cells as well as CD4+ cells expressed IL-4 and -5 in asthma (Ying, Humbert et al. 1997).

Increases in CD8+ T cells have also been observed in other forms of asthma. Frew *et al.* compared atopic asthma with red cedar asthma, and found the latter was characterised by a 4-fold greater increase in biopsy T cells and 2.5 times greater increase in biopsy eosinophils than atopic asthma (Frew, Chan et al. 1995). They specifically found increases in biopsy CD8+ T cells in a subset of individuals with red cedar asthma. Another subtype of asthma is induced by toluene diisocyanate (TDI) (a chemical intermediate in the production of polyurethane) and Finotto *et al*. observed that TDI challenge in sensitised individuals induced a 56% increases in CD8+ T cells at 8 hours, followed at 24 hours by a 2.5 fold increase in eosinophils (Finotto, Fabbri et al. 1991).

Together these studies suggest a close relationship between CD8+ T cells and eosinophils. It is likely that this is mediated, as least in part, by IL-4 and IL-5. Till *et al*. found asthma was associated with increased IL-5 production in CD8+ T cell lines derived from BAL (Till, Li et al. 1995). Cho *et al*. recently reported increased production of IL-4 and IL-5 by unstimulated sputum CD4+ and CD8+ cells

in asthma, which was more closely related to disease severity in CD8+ than CD4+ T cells (Cho, Stanciu et al. 2005).

Data from animal models has given further insight into the mechanisms underlying these observations, and suggests that viruses play an important role in the link between eosinophils and CD8+ T cells. Allergen challenge in guinea pigs caused an increase in mucosal T cells which were almost entirely CD8+ and were strongly correlated with eosinophils at 6 hours (Frew, Moqbel et al. 1990), but CD8+ cells are not pathogenic in all animal models (Huang, MacAry et al. 1999; Isogai, Athiviraham et al. 2007; Jiang, Wang et al. 2009). These apparent inconsistencies seem to depend on the interaction between viruses and allergen. In a mouse model of allergic airways disease bystander allergen specific T_H2 responses, mediated by IL-4, could re-programme virus-specific CD8+ T cells to produce IL-5 and recruit eosinophils to the airways (Coyle, Erard et al. 1995). Furthermore when the mice were re-challenged with virus specific peptides they responded by IL-5 production recruiting further eosinophils to the airways. Likewise mice infected with respiratory syncytial virus (RSV) developed lung eosinophilia and AHR which could be prevented by prior depletion of CD8+ cells (Huang, MacAry et al. 1999; Schwarze, Cieslewicz et al. 1999). Similarly CD8+ cells were essential for induction of eosinophil degranulation and AHR in guinea pigs infected with parainfluenza (Adamko, Fryer et al. 2003). In this model it was allergen sensitisation which increased the number of eosinophils in close relation to airway nerves, but the CD8+ cells mediated the release of major basic protein by degranulation of eosinophils which induced AHR by blocking M2 muscarinic receptors. Enomoto has shown that perforin (and thus cytotoxicity) is necessary for allergen-specific CD8+ cells to modulate allergic inflammation (Enomoto, Hyde et al. 2012). Finally Sawicka *et al.* used adoptive transfer experiments in mice to show it was the Tc2 cells, not Tc1 cells, which induced the eosinophilia and AHR (Sawicka, Noble et al. 2004).

Together these animal data suggest a synergy between allergens and viruses leading to eosinophilic AHR. This would explain the strong association in my data-set between increased numbers of Tc2 cells and eosinophils in biopsies. Indeed there is some evidence of such synergy occurring in humans. Calhoun *et al*. performed segmental allergen challenge before and after experimental infection with Rhinovirus type 16 (RV16) in 7 patients with allergic rhinitis and 5 healthy controls (Calhoun, Dick et al. 1994). They found that allergen challenge induced greater histamine release and eosinophil recruitment associated with RV16 infection and persisting for a month afterwards. This may explain why inhaled corticosteroids, which potently decrease airway eosinophils, also reduce the frequency of exacerbations in persistent asthma (Kelly and Busse 2008).

I also observed an association between peripheral blood Tc2 cells and BAL neutrophilia. Neutrophils are the predominant inflammatory cell during exacerbations (Dougherty and Fahy 2009) and perhaps the neutrophils I observed in subjects with Tc2 inflammation were a residual effect of previous exacerbations, although none of my subjects had viral symptoms within the preceding 6 weeks and

viruses were not detectable in the BAL at the time of sampling. It would be informative to study the time course of Tc2 responses during viral infection and correlate these with airway inflammatory cells.

The role of Tc1 cells in asthma

I have also presented data on other CD8+ T cells. Whilst I did not observe significant differences in IL-17 secreting CD8+ T cells in any tissue, I did observe an increase in Tc1 cell frequencies in asthma. This difference was only observed in the BAL tissue compartment, and was significant only in mild asthma. Moreover this difference in IFN-γ secreting T cells was observed only amongst CD8+ cells and not in the CD4+ T helper subset.

These observations may help reconcile apparently conflicting data in previous literature. Whilst much type 2 cytokine secreting T cells have received much more attention in asthma, there are a handful of previous reports regarding IFN-γ secretion in BAL cells in human asthma. Increased spontaneous release of IFN-γ has been observed from BAL leukocytes in BAL (Cembrzynska-Nowak, Szklarz et al. 1993). Krug *et al.* further characterised these cells, observing increased IFN-γ secreting T cells in the BAL from 10 asthmatic subjects (Krug, Madden et al. 1996). These subjects all had mild, steroid naïve atopic asthma with a mean FEV_1 of 100.4% predicted. Krug *et al*. subsequently reported that allergen challenge caused a decrease in the proportion of BAL T cells secreting IFN-γ (Krug, Erpenbeck et al. 2001). These findings were not in accord with the findings of Del Prete *et al.* who reported that the majority of CD4+ T cell clones produced only low quantities of IFN-γ (Del Prete, Maggi et al. 1988). Crucially, however, the papers by Krug *et al.* did not co-stain for CD4 and CD8, and so were unable to distinguish between cytokines secreted by Tc1 and T_H1 cells. My data would suggest that the source of the increased IFN-γ in these studies of human asthma was the Tc1 cell population.

The effect of IFN-γ in human asthma is unknown. It has been suggested, based on animal data, that Tc1 cells may be capable of moderating inflammation and suppressing AHR (Betts and Kemeny 2009). In mice IFN-γ can inhibit airway eosinophilia and production of mucus and chitinases, as well as inhibiting eosinophil production in the bone marrow (Sawicka, Noble et al. 2004; Mitchell, Provost et al. 2011). Others have shown that IFN-γ can be induced 60 fold in CD4+ and CD8+ T cells by factors produced by mast cell lines (de Pater-Huijsen, de Riemer et al. 2002). IFN-γ may have other effects which are less beneficial in airways inflammation. Experiments in which allergen-specific T_H1, T_H2 cells or both were adoptively transferred in mice showed that T_H1 cells alone could induce airway inflammation and lymphocyte recruitment and further that T_H1 cells could facilitate T_H2 cell recruitment, to synergistically induce a more vigorous, eosinophilic inflammatory response (Randolph, Stephens et al. 1999). This paper did not examine CD8+ cells, but one group have reported on Tc1 cells in a murine model. Allergen challenge of mice injected with Tc1 cells induced neutrophilic airway inflammation but no induction of AHR, which was in contrast to the eosinophilia and AHR induced by Tc2 cells (Sawicka, Noble et al. 2004). In contrast to this I did not observe an association between BAL Tc1 frequencies and neutrophilic inflammation (Figure 4.9 C).

Therefore my data suggest that asthma, particularly mild asthma, is associated with an increase in BAL Tc1 cells which it is likely may have mixed effects on airway inflammation, though these are likely to include inhibition of cardinal features of T_H2 inflammation including airway eosinophilia, mucus hyper-secretion and AHR.

Conclusion

Compared with T helper cells, CD8+ T cells have been the subject of little attention from asthma researchers in recent years. My data suggest they may well play an important role in asthma, particularly in a subset of individuals with eosinophilic inflammation of the airway mucosa. These subjects tend to have distinct clinical features – namely a history of smoking, of nasal polyposis and are older – suggesting they may represent a distinct clinical phenotype of asthma. There is likely to be an important interaction between virus and allergen-induced inflammation with animal data suggesting allergen-specific IL-4-mediated T_H2 responses may re-programme virus-specific IL-5 mediated Tc2 cells to induce pathogenic airway eosinophilia and AHR. The role of Tc2 cells merits further work, which should investigate the specificity of these cells, for example using tetramers, ELISpot, or ELISpot arrays and should delineate their role in viral infections in asthma.

CHAPTER 5

MAIT cells – new players in asthma

The presence of bronchial asthma is much more easily ascertained than is the cause.[5]

[5] Rubin, E.H. and M. Rubin, *Diseases of the Chest* 1947, Philadelphia and London: W.B. Saunders

Introduction

Having investigated the roles of both the major adaptive T cell subsets – CD4+ T helper cells and CD8+ cytotoxic T cells – I wish now to turn to the role of an emerging class of innate-like T cells: the mucosal associated invariant T (MAIT) cell. The discovery in the last decade of innate-like T cells which are restricted by MHC-like molecules and can respond rapidly to non-peptide antigens has been an exciting development in our understanding of T cell biology (Kronenberg and Kinjo 2009), but the role of one such cell – the iNKT cell - in the human airways has been an issue of some controversy (Umetsu and Dekruyff 2006; Vijayanand, Seumois et al. 2007; Djukanovic and Gadola 2008; Meyer, DeKruyff et al. 2008). The more recent discovery of MAIT cells is of great interest. MAIT cells share with iNKT cells the distinction of expressing conserved αβTCRs and being restricted by a MHC class I-like nonpolymorphic molecules, i.e. MR1 (Treiner and Lantz 2006). MAIT cells are relatively frequent in human peripheral blood, and they are five to tenfold more frequent in humans than in mice, while iNKT cells seem to be more abundant in mice (Treiner, Duban et al. 2005). MAIT cells are specifically activated by molecule MR1 which is the most highly conserved MCH class 1 related molecule in mammals, implying a functional role of key evolutionary importance (Brossay, Chioda et al. 1998). Yet to date no data have been published on MAIT cells in relation to human lung disease.

In this chapter I will describe the first analysis of MAIT cells within the human airways, in which I have made the novel finding of a deficiency of MAIT cells in asthma. I will show how this deficiency correlates with various clinical characteristics. I will then present exploratory research into the biology of these cells including a characterisation of the cytokine expression profile of MAIT cell clones and evidence of their potential to be modulated by corticosteroids and perhaps influenced by systemic levels of vitamin D3.

Results and comments

Study population

Twenty healthy subjects and 54 asthmatics (15 mild, steroid-naïve, 22 moderate, treated with low dose inhaled corticosteroids (ICS) and 17 severe, treated with oral or high dose inhaled corticosteroids) were studied. All had stable symptoms for at least 6 weeks prior to clinical sampling. The study design is shown in Figure 2.1 and clinical characteristics of the study participants are shown in table 5.1.

Table 5.1 Clinical characteristics of MAIT cell study population

	Healthy controls	Mild asthma	Moderate asthma	Severe asthma
n	20	15	22	17
Demographics				
Sex (M/F)	12 / 8	8 / 7	9 / 13	7 / 10
Age (median [range], years)	28 (24-43)	26 (22-33)	36 (24-47)	53 (42-63)
Pulmonary function				
FEV_1 (% predicted)	107 (95-113)	88 (86-103)	99 (87-108)	64 (49-79)
FEV_1 reversibility (%)	2.9 (1.8-8.0)	13 (11-19)	10 (2.4-18)	14 (4.2-26)
PEFR (% predicted)	105 (97-114)	98 (89-107)	96 (85-101)	70 (58-82)
PEFR variability (%)	N/A	17 (10-25)	21 (16-32)	19 (11-24)
PD20 (mg methacholine)	Negative (>1.5)	0.19 (0.05-0.79)	0.25 (0.06-0.73)	Not done
Exhaled nitric oxide (ppb, at 50L/s)	15 (10-24)	52 (27-107)	29 (15-51)	19 (13-38)
Clinical				
Atopy (Skin prick positive, Y/N)	0 / 20	15 / 0	19 / 3	12 / 5
No. of skin prick allergens positive	0 (N/A)	6 (4-7)	3 (1.8-5)	4 (0-6)
Peripheral eosinophil count (10^9/L)	0.1 (0.1-0.2)	0.1 (0.1-0.6)	0.2 (0.1-0.3)	0.2 (0.1-0.2)
Total IgE (iu/ml)	19 (10-57)	172 (21-451)	112 (45-189)	96 (24-526)
Body mass index (kg/m^2)	25.6 (23.5-29.1)	23.6 (22.7-26.5)	25.1 (23.2-31.2)	33 (27.6-41.3)
Smoking status				
Never	17	14	18	12
Former (Mean pack years)	3 (6.5)	1 (6.7)	4 (5.6)	4 (27)
Current (Mean pack years)	0	0	0	1 (49)
Duration of asthma (years)	N/A	18 (15-26)	22 (11-29)	41 (17-51)
ACQ score	N/A	0.6 (0.45-1.3)	1.0 (0.53-1.5)	2.7 (2.2-3.4)
GINA level of control				
Controlled	N/A	8 (53)	4 (18)	0 (0)
Partly controlled	N/A	6 (40)	15 (68)	1 (5.9)
Uncontrolled	N/A	1 (6.7)	3 (14)	16 (94)
Treatment				
Inhaled steroids	No	No	Yes	Yes
Dose (equivalent mcg BDP)	N/A	N/A	400 (200-500)	1600 (1280-2040)
Maintenance oral steroids (Y,N)	No	No	No	4 / 13
Mean dose if taken (mg prednisolone/day)				8.3
Long acting β agonist (Y/N)	No	No	9 / 13	17 / 0
Leukotriene receptor antagonist (Y/N)	No	No	1 / 21	13 / 4
Step on BTS treatment algorithm	N/A	1	2 - 3	4 - 5
Inflammatory subtype (n,%)				
Neutrophilic	3 (21)	3 (23)	2 (12)	9 (5.3)
Eosinophilic	1 (7.1)	2 (15)	3 (18)	4 (26)
Mixed granulocytic	0 (0)	0 (0)	0 (0)	1 (5.9)
Paucigranulocytic	10 (71)	8 (62)	12 (71)	3 (18)
Sputum cell differential (%)				
Macrophages	52 (31-69)	49 (35-64)	49 (30-63)	30 (18-41)
Neutrophils	24 (8.0-64)	34 (22-54)	33 (15-51)	68 (39-78)
Epithelial	4.9 (2.0-28)	4.3 (1.7-10)	5.3 (1.4-22)	1.1 (0.0-6.3)
Eosinophils	0.38 (0.0-1.0)	1.5 (0.75-1.8)	0.75 (0.25-1.6)	0.69 (0.0-4.8
Lymphocytes	0.1 (0.0-0.81)	0.25 (0-0.75)	0 (0.0-0.58)	0.0 (0.0-0.25)
BAL cell differential (%)				
Macrophages	79 (74-89)	70 (60-80)	81 (72-88)	72 (46-95)
Neutrophils	2.8 (1.0-6.0)	2.5 (1.6-4.8)	3.5 (1.8-7.0)	5.5 (1.3-22)
Epithelial	10.3 (4.0-19)	21 (13-35)	12 (7.0-19)	6.0 (2.8-11)
Eosinophils	0.5 (0.0-0.75)	2.0 (0.75-3.6)	1.0 (0.6-3.3)	0.0 (0.0-1.0)
Lymphocytes	1.5 (1.0-3.0)	1.5 (0.38-3.0)	1.5 (0.57-2.4)	1 (0.0-1.5)
Relevant comorbidities (n,%)				
Allergic rhinitis	0 (0)	12 (80)	10 (45)	7 (41)
Nasal Polyps	0 (0)	0 (0)	1 (4.5)	4 (24)
Eczema	2 (10)	7 (46)	6 (27)	3 (18)
Bronchiectasis (history or CT)	0 (0)	0 (0)	1 (4.5)	1 (5.9)

Values are medians with interquartile ranges, unless stated otherwise. N/A: not available.
Inflammatory subtype is based on sputum differentials using cut-points as per Simpson, J. L., R. Scott, et al. (2006). Respirology 11(1): 54-61 (neutrophilic: >61% neutrophils, eosinophilic: >3%). Percentages are of those with valid data.

ACQ, asthma control questionnaire; BDP, beclometasone dipropionate; BTS, British Thoracic Society; CT, computed tomogram; FEV_1, forced expiratory volume in 1 second; FVC, forced vital capacity; GINA, Global Initiative for Asthma; PEFR, peak expiratory flow rate; PD20, provocative dose 20.

Analysis of MAIT cells in human asthma

First, I studied the frequencies of MAIT cells in peripheral blood, induced sputum, BAL and bronchial biopsies using flow cytometry. As described in the methods chapter, MAIT cells were defined as CD3+ live lymphocytes (i.e. live T-cells) expressing the NK marker CD161 and the TCR Vα7.2 chain (Figure 2.6). Whilst canonical MAIT cells are defined by their expression of the invariant Vα7.2-Jα33 TCR rearrangement, it has previously been shown that in humans surface expression of either CD161 or IL-18R, together with the Vα7.2 segment unequivocally identifies MAIT cells in both peripheral blood and tissues (Martin, Treiner et al. 2009; Le Bourhis, Martin et al. 2010; Dusseaux, Martin et al. 2011). This definition enabled me to enumerate and to sort live MAIT cells based on their surface staining properties alone.

MAIT cells are deficient in human asthma and correlate with disease severity

Vα7.2+CD161+ (MAIT) cells were abundant in airway tissues, comprising a median 1.8% (IQR 0.73-3.0%) of T cells in health in blood, sputum, BAL and biopsy. There was no evidence of specific tissue compartmentalisation (Kruskal-Wallis P=0.7). Frequencies of MAIT cells were lower in asthma than in health in blood (P=0.005), in sputum (P=0.002) and in bronchial biopsies (P=0.02), with a similar pattern in BAL (ns) (Figure 5.1). Furthermore when asthmatic subjects were stratified according to three categories of disease severity - mild, moderate or severe asthma – there was a strong linear trend across groups in PBMC (P<0.0001) and sputum (P=0.006, Figure 5.2), implying that this deficiency correlated with disease severity.

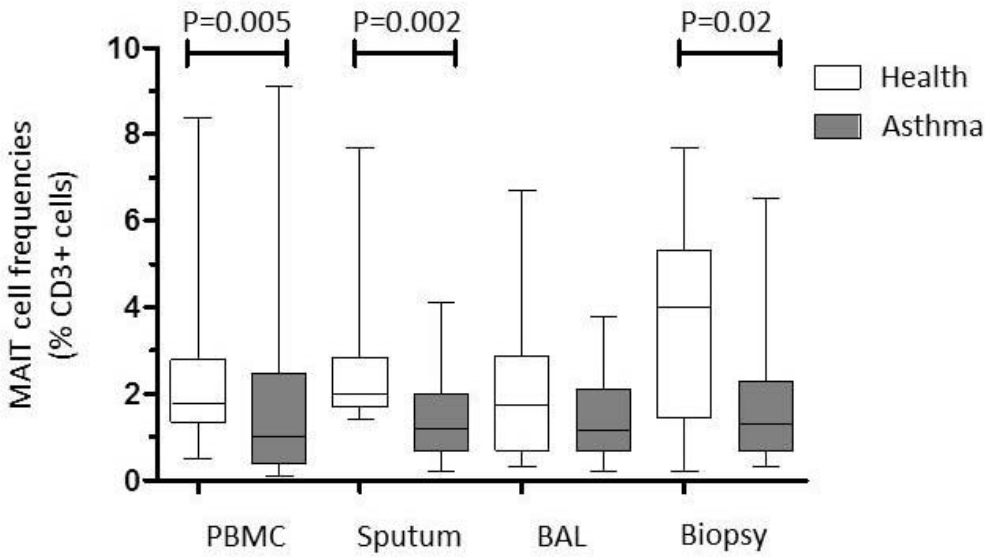


Figure 5.1 MAIT cells are deficient in asthma

Frequencies of Vα7.2+CD161+ (MAIT) cells as a proportion of total live CD3+ T cells in peripheral blood, sputum, BAL and biopsies in health and asthma. Box and whisker plots show medians and IQRs. Differences are compared by unpaired t tests on Ln transformed data.

healthy controls *n*=21 PBMC, 13 sputum, 20 BAL, 14 biopsies.

asthma *n*=48 PBMC, 31 sputum, 40 BAL, 27 biopsies.

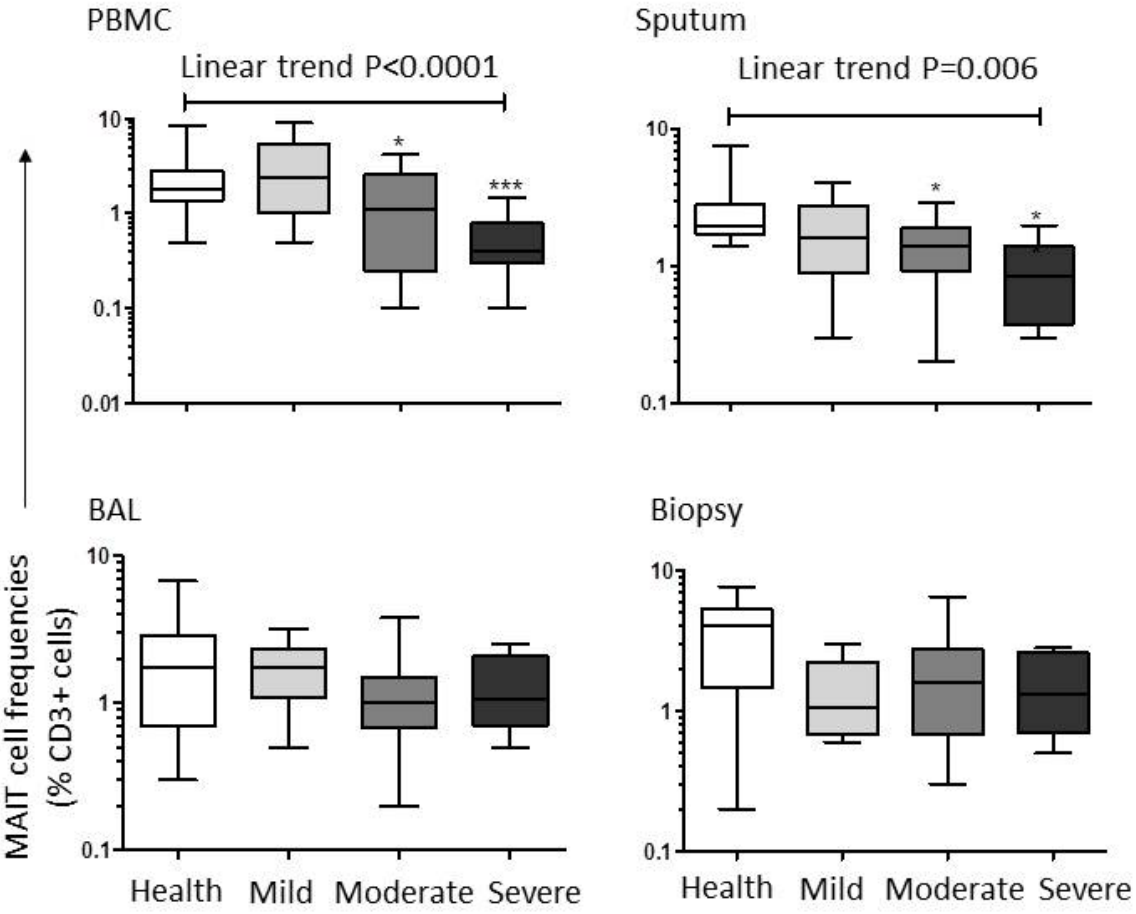


Figure 5.2 MAIT cell deficiency correlates with asthma severity

Frequencies of Vα7.2+CD161+ (MAIT) cells as a proportion of total live CD3+ T cells in peripheral blood, sputum, BAL and biopsies stratified according to disease severity. Box and whisker plots show medians and IQRs. Linear trends are compared across groups using residuals on Ln transformed data. (Not significant for BAL or biopsy).

healthy controls	*n*=21 PBMC, 13 sputum, 20 BAL, 14 biopsies.
mild asthma	*n*=14 PBMC, 11 sputum, 14 BAL, 10 biopsies.
moderate asthma	*n*=17 PBMC, 10 sputum, 14 BAL, 10 biopsies.
severe asthma	*n*=17 PBMC, 10 sputum, 12 BAL, 7 biopsies.

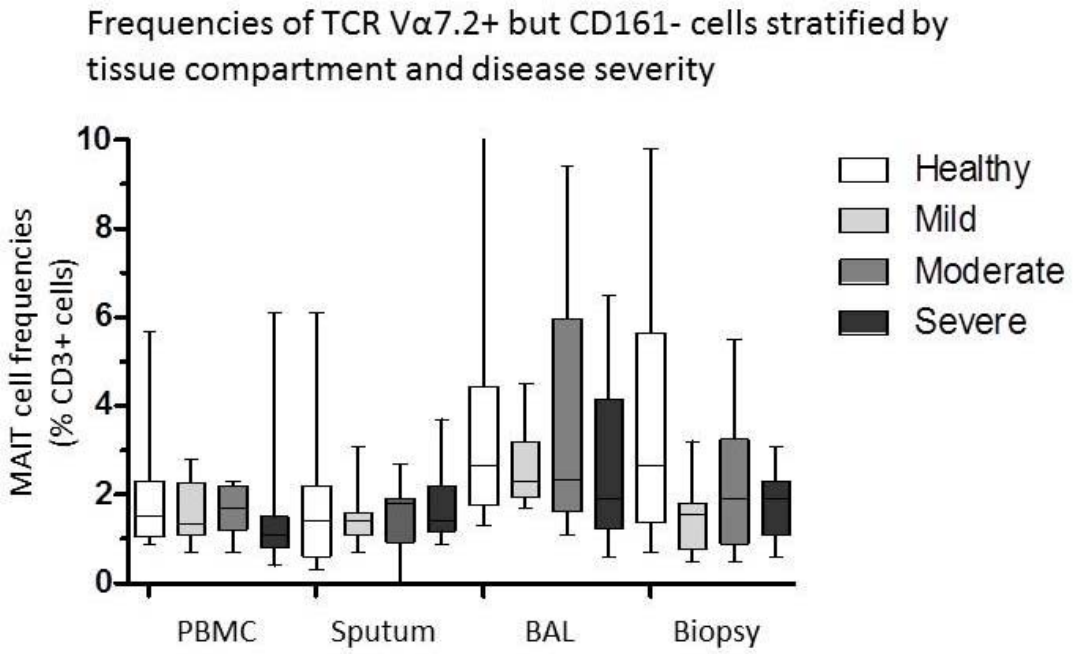


Figure 5.3 Frequencies of a non-MAIT T cell subset do not differ in asthma

Frequencies of non-MAIT T cell subset Vα7.2+CD161- as a proportion of total live CD3+ T cells in peripheral blood, sputum, BAL and biopsies stratified according to disease severity. Box and whisker plots show medians and IQRs. No differences between groups were statistically significant.

healthy controls	*n*=21 PBMC, 13 sputum, 20 BAL, 14 biopsies.
mild asthma	*n*=14 PBMC, 11 sputum, 14 BAL, 10 biopsies.
moderate asthma	*n*=17 PBMC, 10 sputum, 14 BAL, 10 biopsies.
severe asthma	*n*=17 PBMC, 10 sputum, 12 BAL, 7 biopsies.

By contrast, the frequency of Vα7.2+ CD161- T-cells in blood or tissue showed no correlation with either the presence or the severity of asthma (Figure 5.3). These “non-MAIT” cells represent conventional adaptive T cells which use differently rearranged TCR Vα7.2 segments, but not the invariant Vα7.2-Jα33 rearrangement that is unique to the MAIT TCR. Hence, these results show a selective reduction in both peripheral blood and tissue MAIT in asthma and they suggest that the reduction in MAIT cells correlates with asthma severity.

MAIT cell frequencies are not related to age

In the light of this association with disease severity I investigated whether MAIT cell frequencies might correlate with other clinical factors. One important factor to consider was the effect of age as iNKT cell frequency in peripheral blood decreases with age, especially after the age of 45-50 years, in humans. Whilst I had selected healthy controls which were age-matched to the mild and moderate asthmatic subgroups, the severe asthmatic cohort in my study tended to be older as a result of my specific interest in severe neutrophilic asthma. To confirm or refute the hypothesis that MAIT cell frequencies

fall with advancing age I recruited a further cohort of 12 older healthy controls with a median age of 53 years (IQR 48-57) and enumerated MAIT cells in peripheral blood. There was no difference in MAIT cell frequencies when compared to the younger healthy controls (median age 27, IQR 24-34 years, n=15)(P=0.4, Figure 5.4).

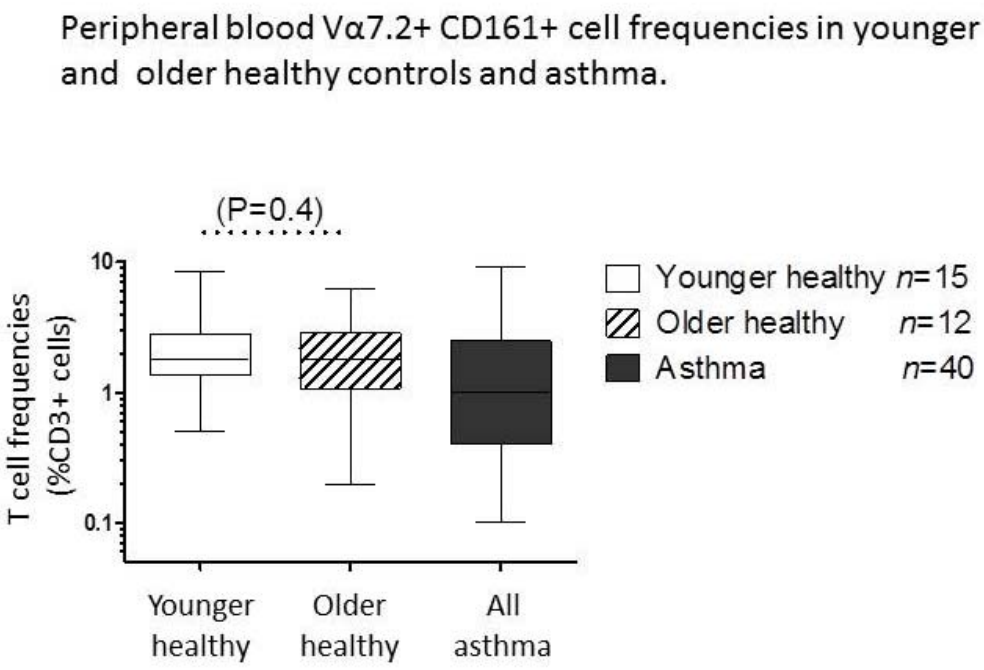
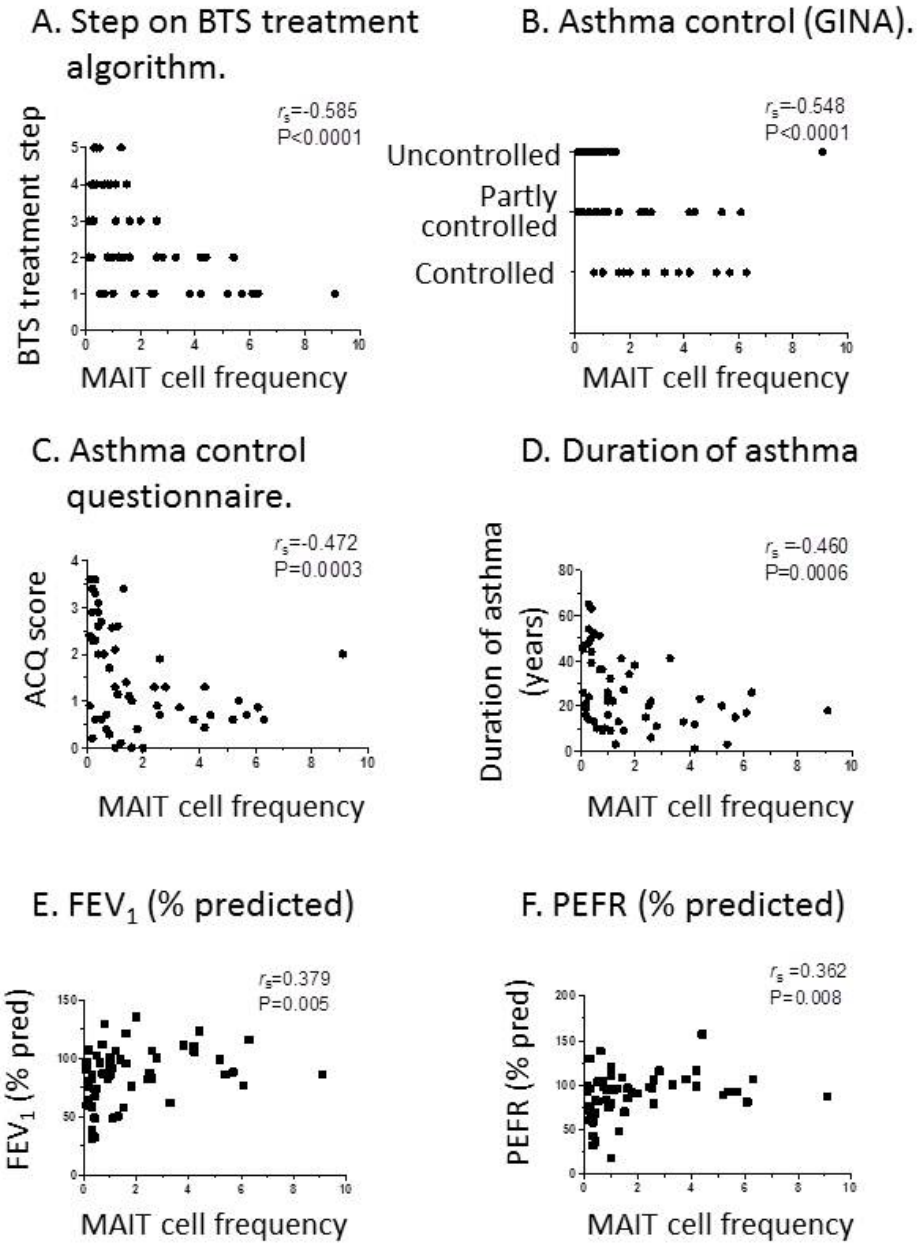


Figure 5.4 MAIT cell frequencies are not related to age

Peripheral blood frequencies of Vα7.2+CC161+ MAIT cells compared in younger healthy individuals (median age=27 years (IQR 24-34), *n*=15), older healthy individuals (53 years (48-57), *n*=12) and all asthmatics (38 years (25-51), *n*=40). MAIT frequencies did not differ between the younger and older healthy controls, (unpaired t test, P=0.4).


A. Step on BTS treatment algorithm.
BTS treatment step
r_s=-0.585
P<0.0001
MAIT cell frequency
B. Asthma control (GINA).
Uncontrolled
Partly controlled
Controlled
r_s=-0.548
P<0.0001
MAIT cell frequency
C. Asthma control questionnaire.
ACQ score
r_s=-0.472
P=0.0003
MAIT cell frequency
D. Duration of asthma
Duration of asthma (years)
r_s =-0.460
P=0.0006
MAIT cell frequency
E. FEV_1 (% predicted)
FEV_1 (% pred)
r_s=0.379
P=0.005
MAIT cell frequency
F. PEFR (% predicted)
PEFR (% pred)
r_s =0.362
P=0.008
MAIT cell frequency

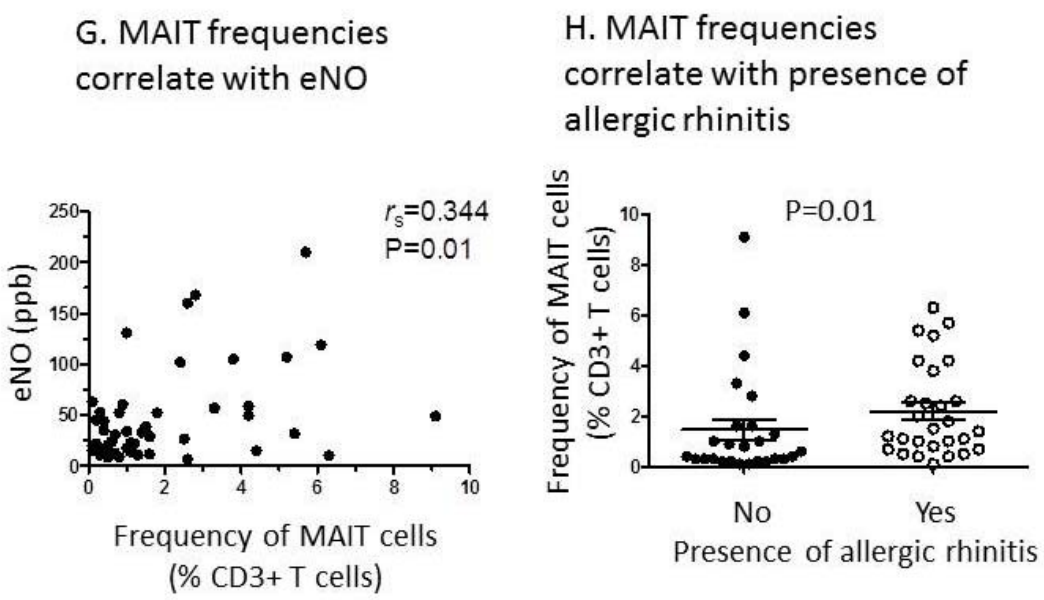


Figure 5.5 Clinical correlates of peripheral blood MAIT cell frequencies

Peripheral blood frequencies of Vα7.2+ CD161+ (MAIT) cells are correlated negatively with (A) treatment step on BTS treatment algorithm, (B) level of asthma control according to GINA classification, (C) level of asthma control according to asthma control questionnaire score, (D) duration of asthma; and are correlated positively with lung function including (E) FEV_1 and (F) PEFR as percentages of predicted values. Figures present Spearman's correlations. MAIT cell frequencies are expressed as a percentage of total live CD3+ T cells. Frequencies of Vα7.2+ CD161+ (MAIT) cells are correlated positively with (G) exhaled nitric oxide levels and (H) with the presence of allergic rhinitis. (H) P value is for a Mann-Whitney test.

Clinical correlations with MAIT cell frequencies

Next I investigated other potential clinical correlates of MAIT cell frequencies with univariate analyses using Spearman's correlations. Amongst asthmatic subjects MAIT cell frequencies correlated negatively with the advancement of treatment steps on the BTS treatment algorithm, being lowest in step 4 and 5 subjects (Figure 5.5 A) (r_s= -0.585, P<0.0001). MAIT cell frequencies also correlated negatively with level of asthma control according to the GINA classification (Figure 5.5 B), being lowest in uncontrolled asthma (r_s= -0.548, P<0.0001), or according to score on the asthma control questionnaire (Figure 5.5 C) (r_s= -0.472, P=0.0003).

MAIT frequencies also correlated negatively with duration of asthma, being lowest in those with the most long-standing asthma (Figure 5.5 D) (r_s= -0.460, P=0.0006). Patients with the longest duration of asthma tended to be older. However, as explained above, age alone as a potential confounding factor is unlikely to explain these differences.

MAIT cell frequencies correlated positively with lung function including percentage of predicted FEV_1 (r_s=0.379, P=0.005, Figure 5.5 E), and percentage of predicted peak expiratory flow (r_s=0.362, P=0.008, Figure 5.5 F), although the strength of the correlation was lower than the above mentioned correlations. These findings are consistent with the observed inverse correlation of MAIT cell frequency with disease severity.

Furthermore, I found weak positive correlations between MAIT frequencies and exhaled nitric oxide (eNO; r_s=0.344, P=0.01, Figure 5.5 G), and also with the presence of allergic rhinitis (P=0.01, Figure 5.5 H). This suggested the possibility that MAIT deficiency might relate to specific endotypes, i.e. eosinophilic or neutrophilic subtypes of asthma, but this hypothesis was not supported by either sputum or BAL cell differentials. The weak positive correlation of MAIT frequencies with allergic rhinitis is therefore more likely to be a consequence of the relatively higher rates of allergic rhinitis in the milder asthmatic subjects (Table 5.1).

Modulation of MAIT cell frequencies by corticosteroids

Whilst the associations I have observed with disease severity are strong, they may be confounded by an effect of steroid therapy. The possibility that steroids may negatively modulate frequencies of MAIT cells is also raised by the association with BTS treatment step and possibly also by correlation with eNO, as high eNO levels are associated with lower corticosteroid use (McNicholl, Stevenson et al. 2012). I therefore correlated MAIT cell frequencies with the doses of inhaled corticosteroids (ICS) in all subjects. MAIT cell frequencies were indeed negatively correlated with the dose of ICS in both PBMC (r_s= -0.584, P<0.0001, Figure 5.6 A), and to a lesser extent in BAL (r_s= -0.315, P=0.048, Figure 5.6B). However, a causal link cannot be proven by simple correlation, so to confirm directly whether corticosteroids can modulate MAIT cell frequencies I conducted an additional sub-study in which I analysed MAIT cell frequencies before and after introduction of either low-dose inhaled corticosteroids or higher-dose systemic corticosteroids.

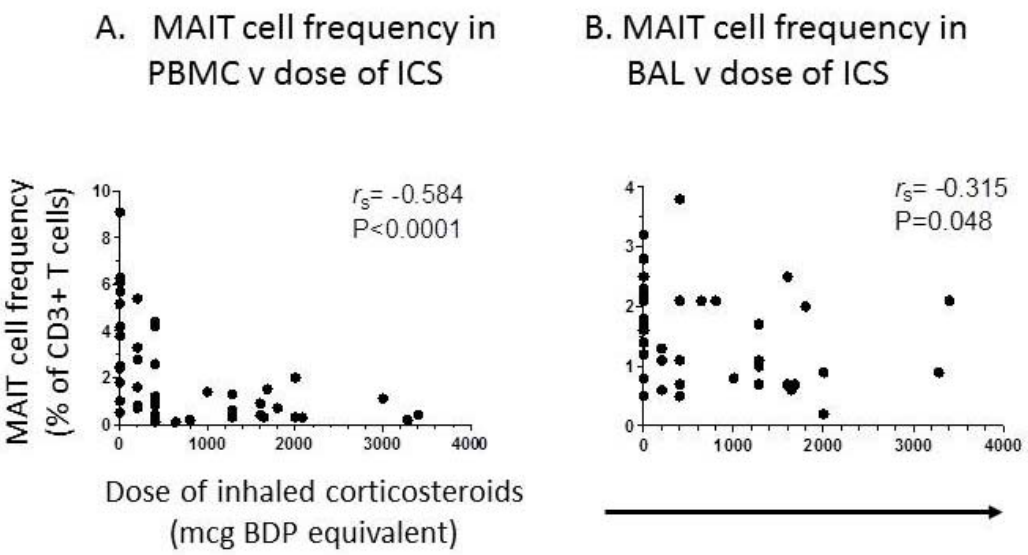


Figure 5.6 MAIT cell frequencies and use of inhaled corticosteroids

Frequencies of Vα7.2+ CD161+ (MAIT) cells are correlated negatively with daily dose of inhaled corticosteroids (ICS) in (A) peripheral blood and (B) BAL. Figures present Spearman's correlations. BDP, beclometasone dipropionate.

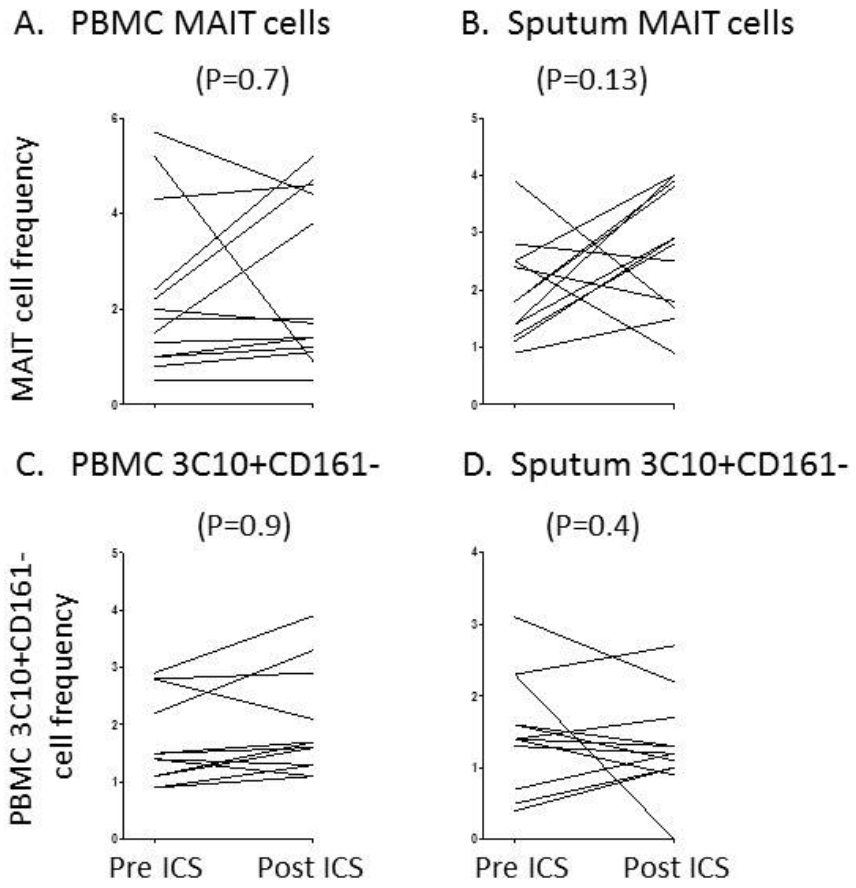


Figure 5.7 MAIT cell and non-MAIT cell frequencies before and after inhaled corticosteroids
Frequencies of MAIT (Vα7.2+CD161+) cells and a non MAIT cell population (Vα7.2+CD161- cells) in 12 steroid-naïve subjects before and after 7 days of treatment with 200 mcg bd inhaled Qvar. No differences are significant by paired t tests.

Inhaled corticosteroids

I administered 200 mcg of inhaled ultrafine particle hydroluoroalkane-134a (HFA) beclometasone dipropionate (Qvar) twice daily for 7 days to 12 steroid-naïve subjects with mild asthma. No significant differences were observed after ICS therapy in frequencies of MAIT cells or a non-MAIT cell population (Vα7.2+CD161- cells) in either peripheral blood or in sputum (Figure 5.7).

Oral corticosteroids

I hypothesised that modulation of MAIT cell frequencies might occur only with higher, systemic doses of corticosteroids and so I conducted a second sub-study in which I measured peripheral blood MAIT cell frequencies before and after 7 days treatment with 20 mg prednisolone once daily, orally. At this dose there was a significant 23% decrease in median MAIT cell frequencies over the week (P=0.03)(Figure 5.8 A). This implies that steroids can modulate frequencies of MAIT cells.

Furthermore this modulation is specific to MAIT cells, as there was no change in the frequencies of the comparator non-MAIT cell population (Figure 5.8 B).

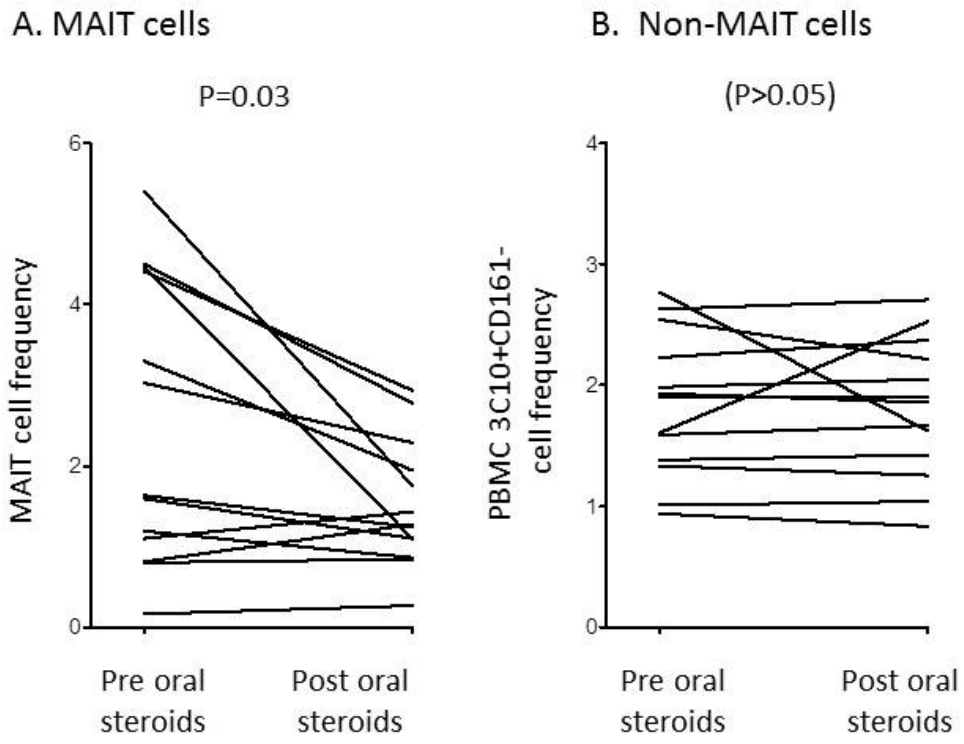


Figure 5.8 MAIT cell and non-MAIT cell frequencies before and after oral corticosteroids
Frequencies of MAIT (Vα7.2+CD161+) cells and a non MAIT cell population (Vα7.2+CD161- cells) in 12 moderate asthmatic subjects, usually controlled on inhaled corticosteroids, before and after 7 days of treatment with 20 mg od oral prednisolone. Frequencies are a % of live CD3+ T cells. P values are for paired t tests.

Although it cannot be excluded that this steroid effect might be the sole explanation for the deficiency of MAIT cells in more severe asthma, it is unlikely: In the majority of subjects the main route of steroid administration was by inhalation, and yet the correlation between ICS dose and MAIT cell frequencies was much stronger in PBMC than in BAL (Figure 5.6) or biopsy or sputum (correlations are not shown as they were not statistically significant). If steroids were the sole driver for MAIT cell suppression then it would be expected that the effect would be most marked on airway cells. Furthermore the data presented in Figure 5.5 show that the correlation of MAIT frequencies with measures of asthma control (B, C) is just as strong as their correlation with treatment (Figure 5.5 A and Figure 5.6 A), which would not be expected if steroids were the only modulating factor. In the following section I will present evidence for at least one other factor which could modulate MAIT cell frequencies *in vivo*.

Seasonal variation in MAIT cell frequencies

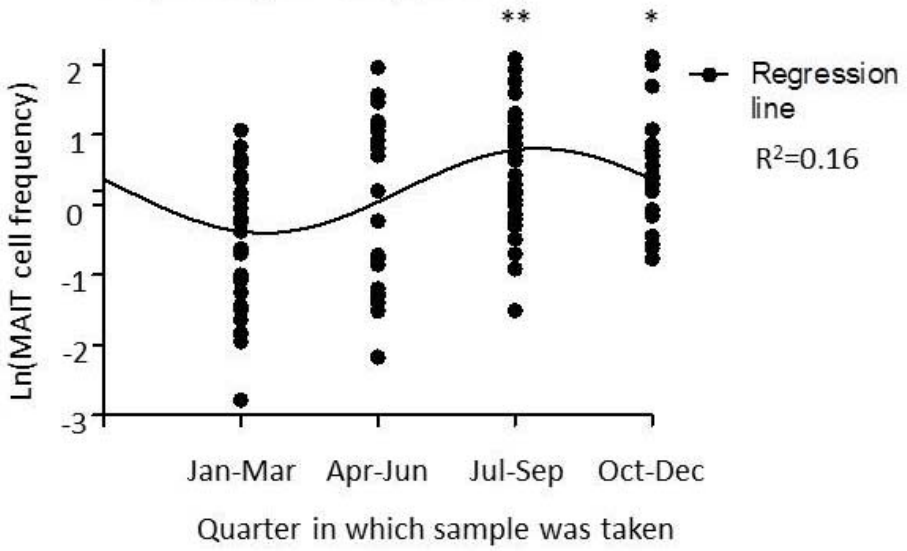
Rationale for investigating seasonal variation

I carefully inspected my data-set to search for evidence of other modulating factors. It has recently been documented that vitamin D3 is important for development and function of iNKT cells, another innate-like T cell. The main supply of active Vitamin D3 for the body is provided through sunlight-induced synthesis of Vitamin D in the skin, while food-related uptake of Vitamin D plays a very minor role. Deficiency of vitamin D *in utero* in mice results in a significant reduction in iNKT which persists life-long and is refractory to later vitamin D supplementation because it results from increased apoptosis of early iNKT cell precursors in the thymus (Yu and Cantorna 2011). Furthermore, this modulation has been linked to experimental airways hyper-reactivity (AHR) in mice, as vitamin D receptor (VDR) knock-out prevents the development of AHR in a manner which can be rescued by adoptive transfer of VDR competent cells (Yu, Zhao et al. 2011). To date, there have been no publications relating vitamin D and MAIT cells. I therefore wondered whether there was any evidence in my dataset of an association between peripheral blood MAIT cell frequencies and either the month of subject birth (if there were a long lasting effect) or the month in which study samples were taken (in case there were a short term effect on vitamin D levels).

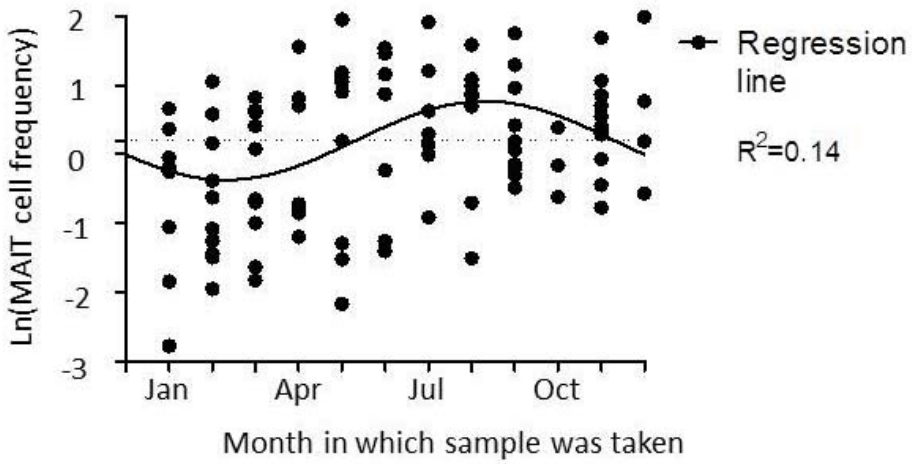
Seasonal variations in MAIT cell frequencies

There was a strong association between the season in which phlebotomy was performed and the MAIT cell frequency (ANOVA on Ln transformed data, $P<0.001$, Figure 5.13 A). MAIT cell frequencies peaked in August with a nadir in February (Figure 5.13 B) and *post-hoc* tests revealed both summer and autumn frequencies differed significantly from the Jan-Mar quarter (Figure 5.9 A), which was consistent with my hypothesis that the lowest MAIT frequencies would be observed in the winter when vitamin D levels would are at their lowest. This effect was specific to MAIT cells as it was not observed with a non-MAIT population (Vα7.2+CD161- cells, ANOVA $P>0.05$).

A. Peripheral MAIT cells according to date of sample acquisition (quarter).



B. Peripheral MAIT cells according to date of sample acquisition (month).



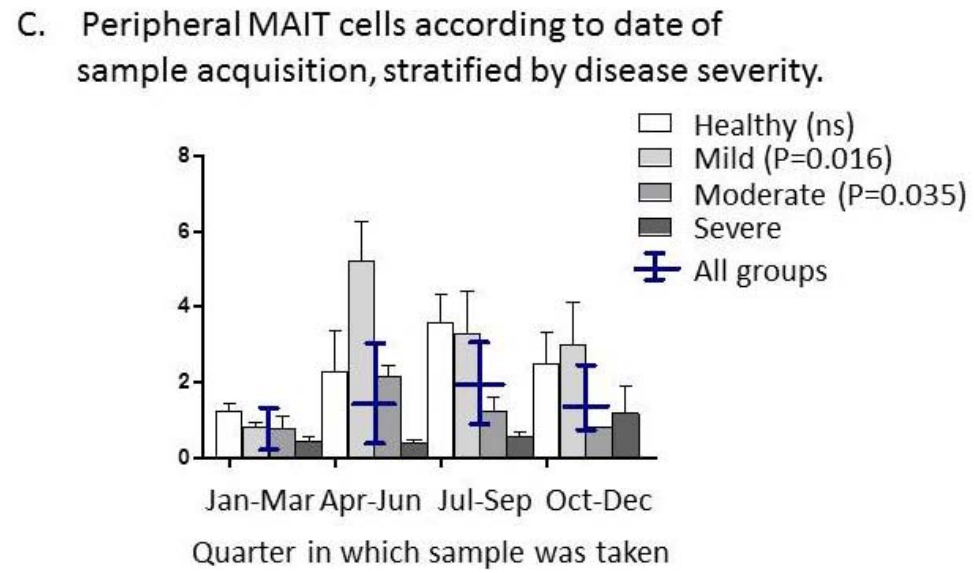


Figure 5.9 Annual variation in MAIT cell frequencies

Peripheral blood MAIT cell frequencies vary over the course of the year and are highest in the summer months. Figure shows log transformed Vα7.2+CD161+ cell frequencies from healthy and asthmatic subjects according to the quarter in which phlebotomy was performed. (A) A sinusoidal regression line is fitted, with a value for R^2 of 0.16. ANOVA P<0.0001 with *post hoc* Dunnett's * P<0.05 and **P<0.01 compared with Jan-Mar. (B) The same data as A but presented according to month in which sample was taken. R^2=0.14. (C) The same data as A stratified by disease severity. Plots show medians and IQR. P values are for ANOVA on Ln transformed data.

Sinusoidal regression yielded a value for R^2 of 0.16 which means that 16% of the variance in MAIT cell frequencies is attributable to this seasonal variation, which is impressive given the likelihood that many other factors are also likely to modulate frequencies of MAIT cells.

I investigated whether this effect could be a result of confounding by non-random sampling of my population: perhaps I sampled more severe asthmatics in winter months and healthy individuals in summer months. This turned out not to be the case because when the data were stratified by disease severity there was no evidence of such a systematic error, and I observed the same pattern in healthy, mild and moderate individuals. This effect was significant in mild (P=0.016) and moderate (P=0.035) subjects despite the smaller subgroup sizes (Figure 5.9 C).

Development and characterisation of MAIT cell clones

The data presented above suggest that MAIT cells may be very relevant to respiratory immunology, being abundant in the airways, related to airways disease and modulated by asthma therapies. I have, therefore, begun to further investigate the biology of MAIT cells by establishing cell lines (clones) which in turn enabled me to begin to investigate the functional capabilities of these cells. I will describe the cloning technique, the validation of the TCR sequence of these clones and preliminary data regarding the cytokine expression profile of these clones.

Cloning technique

With help from a post-doctoral fellow, Dr Salah Mansour, working with Professor Gadola, I adapted a protocol developed for cloning iNKT cells (Matulis, Sanderson et al. 2010) and established 7 cell lines from peripheral blood MAIT cells. This is the first time MAIT clones have been established in this manner, although Gold *et al.* have generated MAIT cell clones in the presence of dendritic cells infected with live MTB and rhIL-2(Gold, Cerri et al. 2010). The details of the protocol I used are described in the methods section. Cloning efficiency was low at 1/80. I investigated whether cloning efficiency would be improved by the addition of the Src family tyrosine kinase inhibitor dasatinib, which can prevent activation-induced TCR and co-receptor down-regulation without inducing apoptosis (Weichsel, Dix et al. 2008). Cloning efficacy was unaffected by dasatinib: clones were established in 3/240 wells in the presence of dasatinib and 3/240 in its absence.

The surface phenotypes of these MAIT cell clones are shown in Figure 5.9. All clones expressed the Vα7.2 TCR and CD161, but varied in their expression of the CD4 and CD8 co-receptors. The original description of MAIT cells suggested that they were predominantly CD4-8- or CD8αα cells(Tilloy, Di Santo et al. 1999), but others have subsequently shown them to be more commonly CD8+ (Turtle, Swanson et al. 2009; Gold, Cerri et al. 2010; Walker, Kang et al. 2012) and to also include CD4+ subsets. Of the clones I established four were CD4+, two were CD8+, and one was CD4-8-.

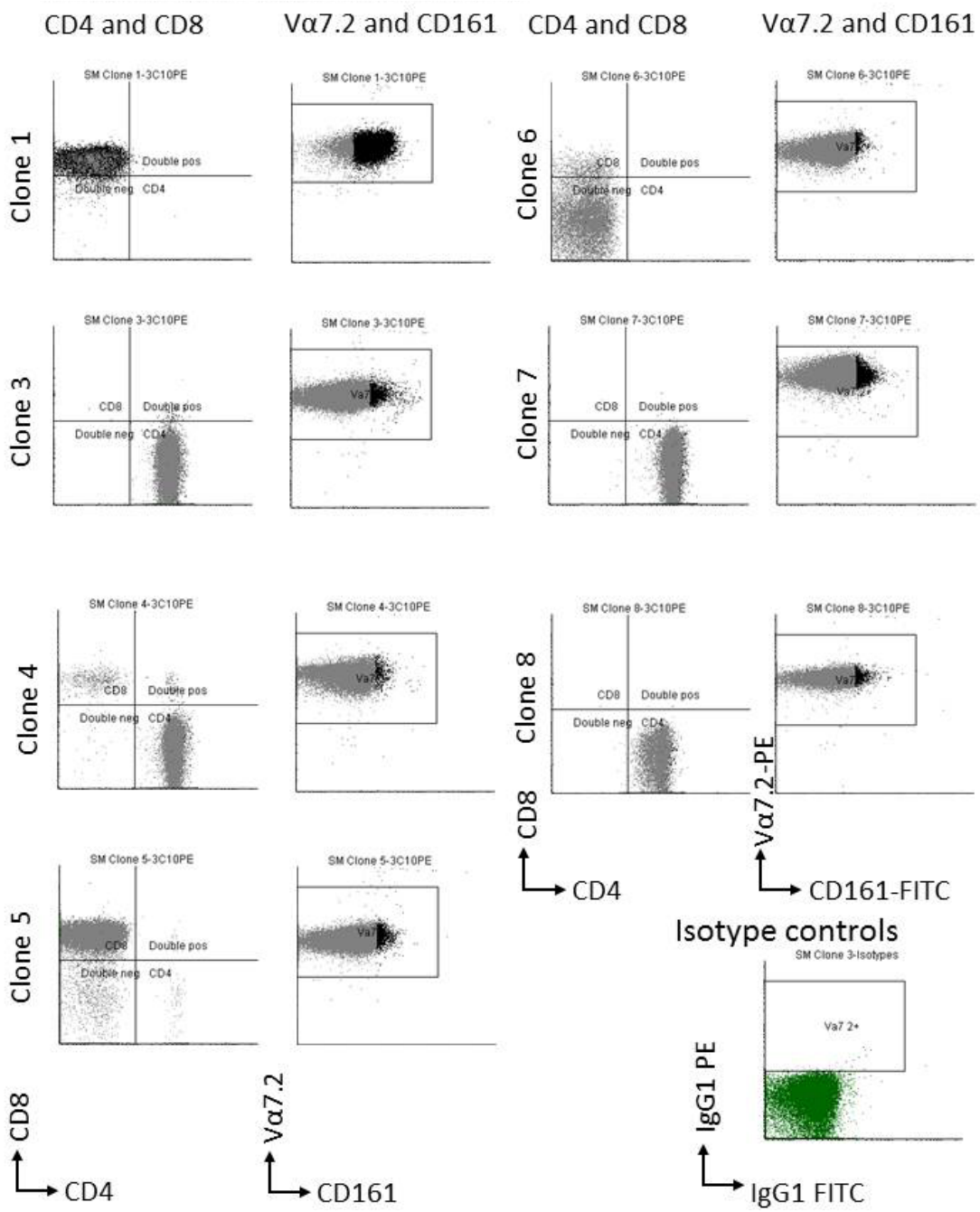


Figure 5.10 Surface phenotype of MAIT clones

Surface expression of CD4, CD8, CD161 and TCR Vα7.2 on the first 7 successful MAIT cell clones. All clones are Vα7.2+ and CD161+, although *ex vivo* stimulation causes significant CD161 downregulation in many cells. Clones differ in their CD4 and CD8 expression profiles.

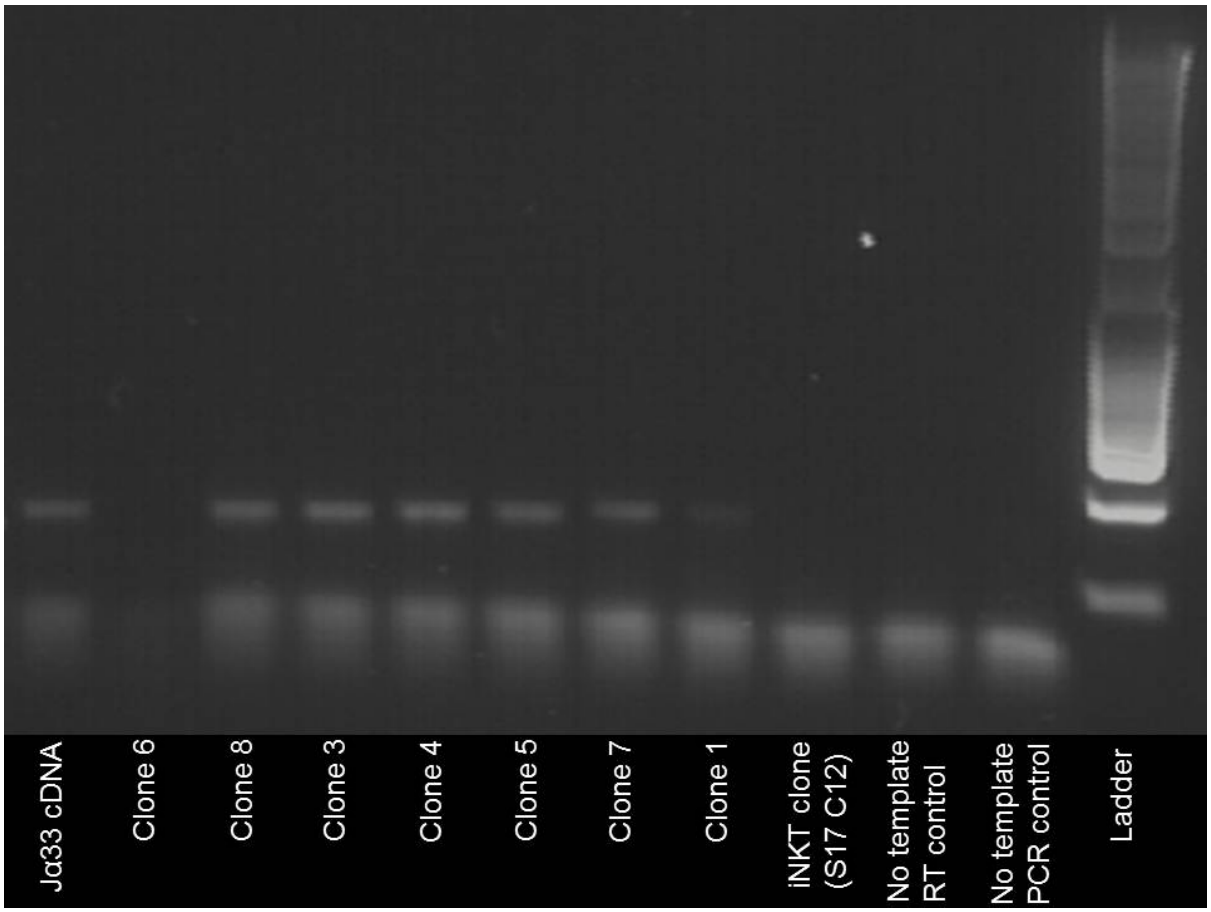


Figure 5.11 Confirmation that MAIT clones express the invariant Vα7.2-Jα33 TCR rearrangement

Clones which were selected for expression of TCR Vα7.2 and CD161 were analysed by PCR and gel electrophoresis. Growing clones were resuspended, washed twice with PBS RNA extracted using chloroform, reverse transcribed using qScript kit and cDNA amplified by PCR using the Bioline Taq kit and primers specific for the canonical Vα7.2-Jα33 TCR rearrangement. cDNA was run on a 1.1% agarose gel at 80 Volts for 30 minutes. A clear product of appropriate length over 400bp is seen in the lanes for clones 1,3,4,5,7 and 8 implying these are true MAIT cells expressing the invariant Vα7.2-Jα33 rearrangement. From left to right: positive control Jα33 cDNA, clones 6, 8, 3, 4, 5, 7, 1, cDNA from an iNKT clone as negative control, no template RT control; no template PCR control, ladder.

Confirmation of MAIT clones by PCR

To confirm that these established TCRVα7.2+ cell lines expressed the full Vα7.2-Jα33 TCR rearrangement, I used RT-PCR to measure expression of mRNA for the MAIT TCR using primers specific for the full Vα7.2-Jα33 segment. Gel electrophoresis of the PCR product revealed distinct bands of over 400 base pairs from 6 of the clones (Figure 5.11) confirming presence of the canonical rearrangement. Positive controls included cDNA for the Jα33 segment and also PBMC from a subject known to have very high frequencies of MAIT cells. Negative controls included no template controls from the RT and PCR steps. An iNKT clone was also included as a negative control.

Clone phenotype

Finally I carried out an initial investigation into the functional capacity of these putative MAIT clones by measuring staining for intracellular cytokines after 4 hours *ex vivo* stimulation with PMA and ionomycin (see representative FACS plots in Figure 5.12). All clones were strong producers of TNFα but differed in their secretion of other cytokines (Figure 5.13). Some clones were strong producers of the T_H17 cytokine IL-17, others of the T_H1 cytokine IFNγ, whilst other clones produced neither cytokine but instead produced the T_H2 cytokine IL-13. This suggests that peripheral blood MAIT cells, similar to peripheral blood iNKT cells, are a functionally heterogeneous population.

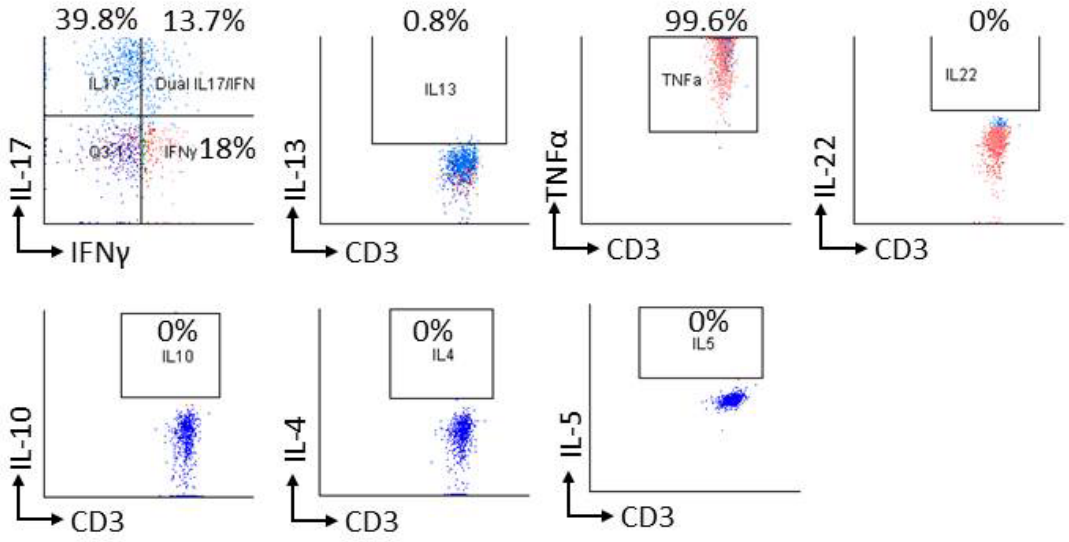


Figure 5.12 Typical intracellular cytokine expression by a stimulated MAIT clone

Representative example of intracellular cytokine staining for IL-17, IFNγ, TNFα, IL-4, -5, -10, -13 and -22 on the MAIT clone 3 after 4 hours *ex vivo* stimulation with PMA and ionomycin.

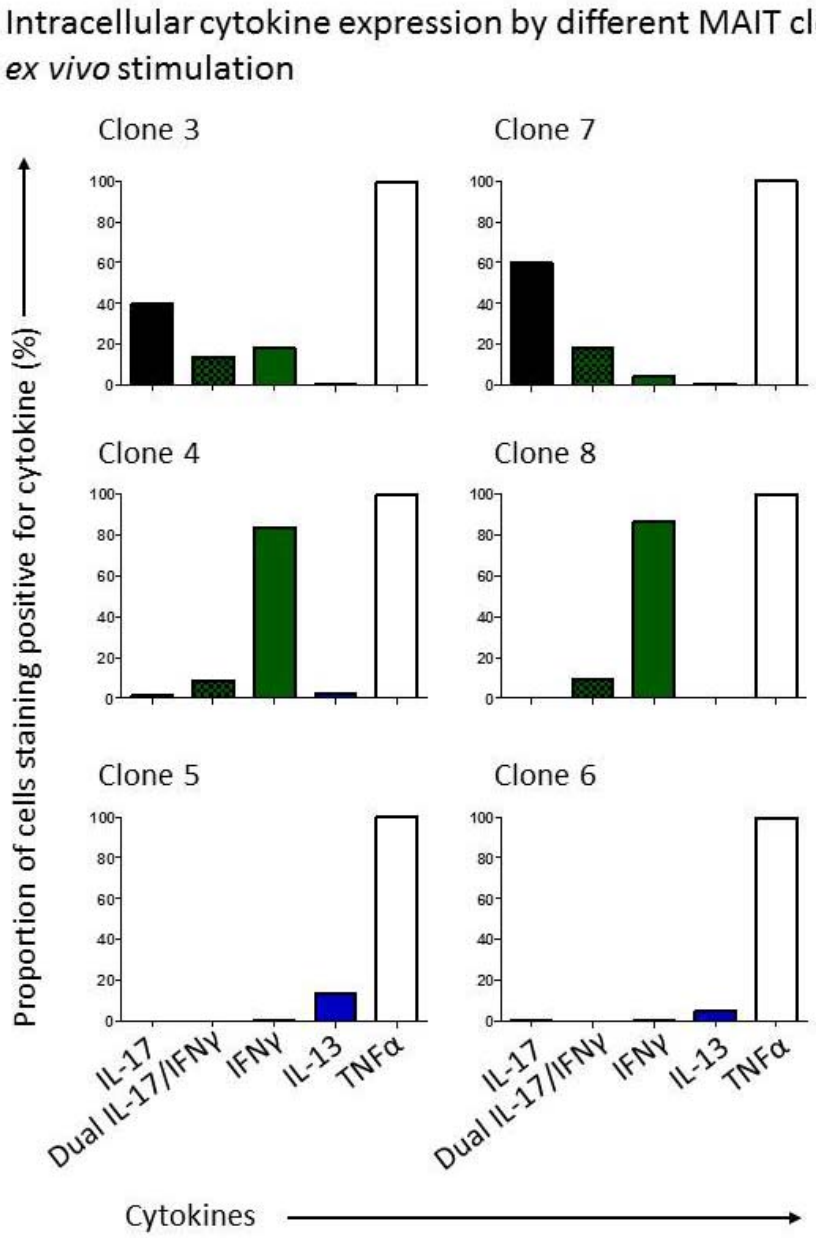


Figure 5.13 Heterogeneity of cytokine expression profile of MAIT clones

Differing cytokine expression profiles in 6 MAIT clones stimulated *ex vivo* for 4 hours with PMA and ionomycin measured by intracellular staining for IL-17, IFNγ, TNFα, IL-13. Plots show proportions of cells staining positive for each cytokine as a percentage of total live CD3+ cells.

Discussion

The data presented in this chapter comprise the first description of human MAIT cells in a respiratory disease. Prior research on MAIT cells has focussed on peripheral blood or the GI mucosa where they were originally described and, to date, the only published data on MAIT cells in the human lung was the report that CD8+Vα7.2+ cells were detectable in the lymph nodes and lung parenchyma of organs from 2 individuals which had been rejected for organ transplantation (Gold, Cerri et al. 2010). Here, I have characterised these T-cells systematically in 74 subjects, in peripheral blood and a range of airway tissues, and this led to the discovery that MAIT cells are deficient in asthma. I have explored clinical correlates of these immunological findings in a clinical cohort whose clinical phenotypes have been characterised in great detail, and explored the association of MAIT frequencies with therapeutic use of corticosteroids in two intervention studies. Finally, my studies have revealed a strong seasonal variation of MAIT cell frequency suggesting a role for Vitamin D3 in MAIT cell development.

Although MAIT cells have never been studied in asthma before, an association between MAIT cells and asthma would not be unexpected. MAIT cells share many similarities with another invariant T cell subset, iNKT cells (Treiner and Lantz 2006), which have been implicated in allergic airways disease in several murine models (Hachem, Lisbonne et al. 2005; Meyer, DeKruyff et al. 2008; Pichavant, Goya et al. 2008; Wingender, Rogers et al. 2011; Yu, Zhao et al. 2011). However, it has not been possible to extrapolate these findings to human asthma (Mutalithas, Croudace et al. 2007; Vijayanand, Seumois et al. 2007; Thomas, Chyung et al. 2010), which may be related to the much lower abundance of iNKT cells in humans compared with mice (Treiner, Duban et al. 2005). Conversely the much higher abundance of MAIT cells in humans (Treiner, Duban et al. 2005) suggests that MAIT cells may perhaps fulfil a corresponding role to the murine iNKT.

Whilst I have demonstrated that steroids can modulate MAIT cell frequencies, as I have argued above, it is unlikely that use of ICS is the sole mechanism for MAIT deficiency in asthma. The correlation between ICS dose and MAIT frequency is strongest in peripheral blood rather than the airway tissues where drug delivery occurs. MAIT cell frequencies correlated just as strongly with measures of asthma control as with steroids doses. Furthermore, it remains to be determined whether the steroid-induced suppression of MAIT cells is beneficial or detrimental to the integrity of the mucosal immune system. In a murine model of inflammatory colitis MAIT cells have been demonstrated to play a protective, anti-inflammatory role in the GI mucosa (Ruijing, Mengjun et al. 2012). Moreover, current understanding of MAIT cells suggests that they are an important mechanism in preventing bacterial and mycobacterial infections (Gold, Cerri et al. 2010; Le Bourhis, Martin et al. 2010; Kjer-Nielsen, Patel et al. 2012). Therefore, steroid-induced suppression of MAIT cells might underlie the increased risk of invasive pneumococcal disease associated with severe asthma (Talbot, Hartert et al. 2005; Klemets, Lyytikainen et al. 2010) and increased risk of pneumonia in subjects with COPD receiving inhaled fluticasone (Calverley, Anderson et al. 2007; Crim, Calverley et al. 2009; Welsh, Cates et al. 2010). Given the very widespread use of ICS, this possibility certainly warrants

further investigation. Such investigations should also explore potential deficiencies in MAIT cell function.

My finding of a link between seasonality and MAIT cell frequencies also warrants further investigation. This was a novel, hypothesis-driven observation and is consistent with what is already known about the existence of mechanisms by which invariant T cell number and function can be modulated by vitamin D (Yu and Cantorna 2011; Yu, Zhao et al. 2011). If this link were confirmed then it would add to our understanding of the relationships between asthma and vitamin D, which is currently an area of controversy. Studies in humans have provided evidence that vitamin D can modulate T cell immunity *in vivo.* Vitamin D levels are positively correlated with peripheral blood T_H1/T_H2 ratios and to a lesser extent with Treg frequencies in asthma (Chambers, Nanzer et al. 2012; Maalmi, Berraies et al. 2012). *In vitro* free $25(OH)_2$ vitamin D_3 influences the balance between inflammatory and regulatory T cell responses by its effect on dendritic cells (Jeffery, Wood et al. 2012). However large scale clinical studies have not yet provided a clear understanding of the significance of these findings. Children with asthma seem to be at increased risk of vitamin D deficiency and there are associations between low vitamin D and worse asthma symptoms, disease severity, frequencies of exacerbations and poorer lung function (Gupta, Bush et al. 2012). However, most data come from case control studies which have provided conflicting results and are affected by selection bias (Paul, Brehm et al. 2012). Furthermore, the vitamin D effect may be weaker in adults (Goleva, Searing et al. 2012). Several longitudinal studies are now ongoing, the first of which found that low vitamin D levels were associated with increased risk of asthma exacerbations in over 1000 children with mild-to-moderate asthma (Wu, Tantisira et al. 2012).

The seasonal variation I have observed could be secondary to factors other than sunlight exposure-related vitamin D levels. In particular, it could be that the winter nadir in MAIT cells is due to more frequent viral exacerbations or increased use of oral steroids at that time of year. Arguing against this would be the observation that the seasonal variation was most marked in the mildest subjects, who were the least likely group to suffer from viral exacerbations or require oral steroids. To resolve this uncertainty I have identified 87 serum samples which are temporally paired with the peripheral blood MAIT cell data and in which vitamin D metabolites are currently being measured by mass spectrometry by my collaborators Prof Alan Jackson and Dr Steve Wootton in the Southampton Nutrition BRC (see acknowledgements list).

The ability to establish and maintain MAIT cell lines is a valuable tool for the next steps in investigating MAIT cell biology. My work provides proof of concept that clones can be established without the need for infection of dendritic cells with live MTB, the only published method to date (Gold, Cerri et al. 2010). The low cloning efficiency will need to be addressed in future work, which might initially compare the efficiency of stimuli other than PHA, such as anti-CD3 OKT, microbial ligands or heat killed BCG. However a possible explanation for this low cloning efficiency can be inferred from a very recent publication which found that MAIT cells have a strong predisposition to apoptosis due to

high expression of caspases 3 and 7 (Gerart, Siberil et al. 2012). These authors made this observation by studying X-linked lymphoproliferative syndrome, a primary immunodeficiency caused by mutation in the X-linked inhibitor of apoptosis (XIAP) which is a physiological inhibitor of caspases 3, 7 and 9 (Eckelman, Salvesen et al. 2006). This genetic disorder leads to a deficiency in frequencies of iNKT and MAIT cells leading to a susceptibility to Epstien-Barr virus infection, haemophagocytic lymphohistiocytosis and hypogammaglobulinaemia (Pachlopnik Schmid, Canioni et al. 2011; Gerart, Siberil et al. 2012). Gerart *et al.* showed that this pro-apoptotic tendency can be reversed by inhibition of the transcription factor PLZF/ZBTB-16 (Gerart, Siberil et al. 2012); thus potential avenues for increasing this cloning efficiency might include inhibition of PLZF/ZBTB-16 or caspases 3 and 7. On the other hand, addition of the Src family tyrosine kinase inhibitor dasatinib, which inhibits TCR-mediated cell activation, did not improve cloning efficacy. However, it is possible, that – in the absence of selective MAIT antigens – using the nonspecific mitogen PHA, which potentially crosslinks many different glycosylated surface receptors, overrode the inhibitory effect of dasatinib on T-cell activation.

Conclusions

The high abundance of MAIT cells - which I have found comprise an average of 2% of T cells in peripheral blood and 4% of bronchial biopsy T cells in health – and their remarkable homology across diverse mammalian species indicates that they serve an important function within the immune system. As yet this function and the factors triggering a specific MAIT response (Kjer-Nielsen, Patel et al. 2012), remain poorly understood. My results are consistent with an important role of MAIT cells in the airways, and together with published studies it is likely that they are key players during respiratory host defence, including resistance to pneumonia, invasive bacterial disease, bronchiectasis and opportunistic infections such as opportunistic mycobacterial infections. My data indicate that MAIT cell homeostasis is markedly disturbed in asthma, particularly more severe forms and that this disturbance is exacerbated by therapeutic use of corticosteroids. It remains to be seen whether this has beneficial or detrimental consequences for the integrity of the respiratory mucosal immune system, and whether or not seasonal variations in MAIT frequencies are directly related to Vitamin D metabolism.

CHAPTER 6

Deep sequencing of the airway microbiome

Deus ex operibus cognoscitur [6]

[6] Sir Isaac Newton PRS MP (1642-1727) Translated 'God is known from his works'. Isaac Newton, Cambridge University Library MS Add. 3695, section 13

Introduction

Having systematically characterised both adaptive and innate-like T cell populations in asthma, I undertook to investigate the presence of potential airway microbial stimuli which might be driving these immune responses. The airways were once thought to be sterile in health (Laurenzi, Potter et al. 1961; Pecora 1963), but there is accumulating evidence for the presence of bacteria in the airways (Charlson, Bittinger et al. 2011) and perhaps even a commensal airway microbial community (Hilty, Burke et al. 2010; Huang, Nelson et al. 2011). However, only two studies have been conducted to date, and so the relevance of finding microbes in disease and health remains unknown. Some studies suggest that bacteria or fungi may drive the recruitment of neutrophils to the airways in neutrophilic airways disease (Simpson, Grissell et al. 2007; Green, Kehagia et al. 2008; Simpson, Powell et al. 2008; Essilfie, Simpson et al. 2012).

It is also important to consider the role of respiratory viruses in asthma. A strong causal link has now been established between impaired innate response to acute viral infections and the development of acute asthma exacerbations (Wark, Johnston et al. 2005; Contoli, Message et al. 2006; Message, Laza-Stanca et al. 2008). Others have postulated that viruses may play a further aetiological role in the pathogenesis of asthma, either through early life infections (Jartti, Paul-Anttila et al. 2009), or possibly through impaired viral clearance leading to chronic viral persistence (Kling, Donninger et al. 2005; Harju, Leinonen et al. 2006; Wos, Sanak et al. 2008).

As introduced in chapter 1, the microbiome constitutes the totality of microbes, their genomes, and environmental interactions in a particular environment (Highlander 2012). The emerging use of high throughput molecular techniques to identify microbes without the need for traditional culture techniques has transformed our ability to characterise the microbial flora in distinct anatomical niches of the human microbiome. Until recently analysis of the respiratory tract flora has depended on traditional culture-based microbiological techniques, which tended to suggest the airways were sterile (Laurenzi, Potter et al. 1961; Pecora 1963). However, only 70% of body surface microbes (Han, Huang et al. 2012) and only 1% of all known microorganisms can be cultured by such techniques (Staley and Konopka 1985). Furthermore culture-based techniques are biased towards selecting for organisms which grow on the chosen culture media, they typically excluded organisms normally present at high levels in the upper respiratory tract, or restricted analysis to potential pulmonary pathogens, and depended on arbitrary quantitative thresholds for clinically significant numbers of organisms (Charlson, Bittinger et al. 2011).

More recently culture-independent techniques have been developed for microbial analysis with several advantages. Rather than detecting a handful of species, they can instead characterise entire microbial populations, involving much less bias and providing accurate measurements of relative abundances of species(Charlson, Bittinger et al. 2011). To date studies of the human airway microbiome have used PCR based methods to analyse bacterial 16s rRNA. In cystic fibrosis (Harris, De Groote et al. 2007; Guss, Roeselers et al. 2011; Daniels, Rogers et al. 2012) and COPD (Han,

Huang et al. 2012; Sze, Dimitriu et al. 2012) these studies have shown that a much broader range of species is present in the airways, many of which cannot be cultured or are anaerobic species not previously thought to survive in the airways. Furthermore, they have identified associations between distinct bacterial community compositions and particular disease phenotypes (Sze, Dimitriu et al. 2012). However, to date, only two published studies have used this technique to analyse the airway microbiome in health(Hilty, Burke et al. 2010; Charlson, Bittinger et al. 2011), of which only one compared the airway microbiome in health and asthma(Hilty, Burke et al. 2010). The careful analysis of the airway microbiome in health by Charlson et al. has suggested a continuity of the lower airway microbiome with bacterial communities found in the upper respiratory tract; this was in contrast to the conclusion drawn by Hilty *et al.* of the existence of a core pulmonary microbiome comprising distinct microbial communities. Hilty *et al.* also reported an increase in the presence of *Haemophilus* species in asthma using bronchial brushings. This single study in asthma was small, involving only eight healthy adults and 11 asthmatics, and may not be representative of most cases of asthma. In addition, it did not account for recent antibiotic usage. Furthermore, 16s rRNA sequencing only detects prokaryotic organisms (bacteria and archaea), but not viruses or fungi. Similarly, to date no one has attempted to correlate the analysis of the airway microbiome in asthma with immunological read-outs.

In contrast to these studies, and in order to measure the broadest possible range of species and to minimise bias towards specific taxa, I elected to analyse microbial RNA and DNA in respiratory samples by whole genome shot-gun sequencing ('deep sequencing') using the Roche/454 next-generation sequencing platform. I chose this technique because, unlike the technologies mentioned above, this can detect not only bacterial, but also fungal and viral genomes including those incorporated into the human genome. The technique has very high sensitivity, and is ideal for detecting previously unknown species as it does not require prior knowledge of the organisms expected. Currently there are no publications in the literature using this technique on respiratory samples.

Data were analysed using the VirusHunter analysis pipeline(Zhao) in which microbial sequences were identified on the basis of Basic Local Alignment Search Tool (BLAST) alignments and the taxonomic classification of the reference sequences to which a read is aligned. A 'read' is a short sequence generated by high-throughput sequencing and typically <1000 base pairs in length. In phylogenetic analysis a unique organism is referred to as an operational taxonomic unit (OTU). An OTU is defined by the National Centre for Biotechnology Information (NCBI) as a 'taxonomic level of sampling selected by the user to be used in a study, such as individuals, populations, species, genera, or bacterial strains'(Blaxter, Mann et al. 2005).

Pyrosequencing is a technique of massively parallel DNA sequencing capable of sequencing roughly 400-600 megabases of DNA per 10-hour run. RNA is extracted from cells and reverse transcribed into cDNA. Genomic and cDNA are ligated to adapters and fixed to small DNA-capture beads in a water-

in-oil emulsion. The DNA fixed to these beads is then amplified by PCR. Each DNA-ligated bead acts as a separate microreactor in which parallel DNA amplifications are performed, yielding approximately 107 copies of a template per bead (Margulies, Egholm et al. 2005). Each bead is then placed by centrifugation into a 29 μm well on a fibre optic chip with smaller beads carrying a mix of enzymes including DNA polymerase, ATP sulfurylase, and luciferase(Voelkerding, Dames et al. 2009). Sequencing is based on the detection of pyrophosphate released during DNA synthesis, using a cascade of enzymatic reactions in which visible light is generated in proportion to the number of nucleotides incorporated and detected using a charge-coupled device(Ronaghi 2001; Margulies, Egholm et al. 2005). Pyrosequencing differs from Sanger sequencing in that it detects incorporation of pyrophosphate rather than chain termination with dideoxynucleotides, and has the advantages of greater accuracy, parallel processing and automation (Ronaghi 2001).

Samples were collected by me but were sequenced and analysed by the Virgin Laboratory, Washington School of Medicine at St Louis (see acknowledgment list).

Results and comments

Participants

Forty-seven BAL and 39 sputum samples were obtained from 55 individuals during periods of clinical stability and at least 6 weeks after the end of the last known respiratory infection. These individuals were a subset of the population reported in chapter 3 and comprised 9 with mild asthma, 16 with moderate asthma, 15 with severe asthma and 15 healthy controls. Samples were sequenced and analysed in two separate batches, generating a pilot data-set of 9 BAL samples and 8 sputum samples, and a subsequent main data-set of 38 BAL samples and 39 sputum samples. The results section is, therefore divided so as to present the bacterial and viral analyses separately. The first part of the bacterial analyses comprises results from a) pilot study and b) main study. Further parts c) and d) report on the pilot and main analyses conducted on sputum samples. The second section reports on viral analyses and is also structured as pilot and main study.

Results section I

a) Bacterial species in BAL from the pilot study

Potentially significant bacterial sequences from the pilot data-set are presented in Table 6.1. Bacterial OTU identified by only a single read, or those with poor homology to the reference sequence, have been excluded.

Table 6.1 Bacterial OTU identified from BAL in the pilot dataset.

Subject ID	Classification	OTU (species)	Number of reads	Sequence homology (range, %)
104	Healthy control	*None*		
321	Moderate asthma	*Escherichia*	2	98.6 - 99.5
403	Severe asthma	*Streptococcus mitis*	2	61.8 – 92.1
		Prevotella melaninogenica	2	84.6 – 97.8
404	Severe asthma	*None*		
406	Severe asthma	*None*		
407	Severe asthma	*None*		
409	Severe asthma	*None*		
412	Severe asthma	*Haemophilus influenzae*	38	90.6 – 100
		Leptotrichia buccalis	7	76.5 – 93.1
		Environmental *Eubacterium*	6	97.9 - 100
422	Severe asthma	*Tropheryma whipplei*	35	80.3 – 100
		Rothia mucilaginosa	3	97.6 – 99.5

As seen in Table 6.1, in 4/9 individuals, including the healthy control, there was no evidence of bacterial colonisation. In the other five individuals, there was evidence of the presence of bacteria typical of those obtained from the oral cavity or upper respiratory tract: the gram-positive cocci *Rothia mucilaginosa* and *Streptococcus mitis* and the anaerobic gram-negative bacilli *Prevotella melaninogenica* and *Leptotrichia buccalis.* These would be consistent with microaspiration from the upper respiratory tract as suggested recently (Gleeson, Eggli et al. 1997; Charlson, Bittinger et al. 2011). As with the study by Charlson *et al.* (Charlson, Bittinger et al. 2011) it is not clear whether these sequences are derived from live or dead bacteria.

Two organisms were identified by ≥35 separate reads, implying much higher abundance, and are of more specific interest. The gram-negative bacillus *Haemophilus influenzae*, which is an opportunistic respiratory pathogen, was identified in one subject (412), a 63 year old male with severe neutrophilic asthma and frequent exacerbations. In this particular instance, because of high clinical suspicion, the lavage fluid was also sent for routine microbiological culture, which yielded heavy growth of *H influenza* sensitive to amoxicillin, doxycycline and erythromycin. High resolution computed

tomography (HRCT) revealed mild bronchial wall thickening of the lower lobes with insufficient evidence of bronchiectasis, whilst the sample originated from the right upper lobe which was unaffected. There was marked neutrophilia in both induced sputum (71%) and BAL (68%). As a consequence of the culture result, the subject was initiated on long term amoxicillin, to which he responded well. Specifically in the 12 months prior to the bronchoscopy he had experienced 20 exacerbations requiring oral steroids. He was using reliever inhaler 3-4 per day and reported waking 3-4 times per night. After initiation of long term antibiotics he experienced a 1.1 point fall in ACQ, suffered only one exacerbation in 18 months and reported in a typical day requiring no reliever medication and having no nocturnal awakenings.

Deep sequencing has not previously been used as a clinical tool in respiratory medicine, but this case study provides an interesting proof of principle that data obtained by culture-independent techniques can correlate with both traditional culture results and with the clinical picture.

In the same individual the T_H17 cell frequency in BAL was strikingly elevated at 11.3%. This was the highest BAL T_H17 cell frequency recorded in the whole study (n=60) and was well above the normal range I have observed: median 2.59% (IQR 1.28-4.03%). By contrast peripheral T_H17 frequencies were not elevated, but at 0.47% were in the bottom quartile: median 0.58, (IQR 0.38-0.77%). This is consistent with the hypothesised role of T_H17 migrating out of peripheral blood and into, and differentiating within, sites of early or ongoing microbial infection (Veldhoen and Stockinger 2006; Torchinsky, Garaude et al. 2009). Thus, although this is a single case, I was able for the first time to correlate immunological and metagenomic data.

The second organism identified at high abundance in this dataset was *Tropheryma whipplei* from subject 422. This was detected in 35 separate reads with 99% nucleotide and 100% amino acid homology to the reference database over their full length. BAL cytospins contained foamy macrophages which are typical in Whipple's disease but are non-specific. PAS staining of cytospins and bronchial biopsies and standard culture of BAL were negative, so a definitive diagnosis of pulmonary Whipple's disease has not yet been made.

T whipplei is a gram positive actinobacteria which can cause a serious but rare systemic bacterial infection affecting virtually any organ, with a wide variety of clinical presentations. Isolated lung disease is rare(Urbanski, Rivereau et al. 2012), although recognised manifestations include chronic cough, pleural effusions, hilar lymphadenopathy and pulmonary infiltrates including nodular shadows and basal parenchymal interstitial infiltrates(Ratnaike 2000). Whipple's disease is associated with immune dysfunction including defects in intracellular killing by monocytes and macrophages, defects in the interleukin-12 axis and alterations in lymphocyte populations(Ratnaike 2000; Schinnerling, Moos et al. 2011) such as an increase in the T_H2/T reg ratio(Biagi, Badulli et al. 2012).

Interestingly subject 422 has evidence of a longstanding abnormality of cell mediated immunity. She is a 23 year old female who presented first to tertiary services at age 11 with frequent infective exacerbations of severe eczema. She has since suffered from recurrent skin infections including breast abscesses and chronic genital yeast infections, and has a long-term extremely elevated IgE, with levels of over 46000 IU in the past. She is atopic with a history of peanut allergy. Her asthma developed in early infancy and is again characterised by neutrophilia (sputum neutrophils 74%), without bronchiectasis, and her predominant symptom is chronic cough. Although this clinical presentation does not meet diagnostic criteria, (Woellner, Gertz et al. 2010) it is reminiscent of hyper IgE syndrome (Job's syndrome), which is associated with mutations in signal transducer and activator of transcription (STAT)3 leading to insufficient expression of RORγt and consequent deficiency of T_H17 cells (Holland, DeLeo et al. 2007; Ma, Chew et al. 2008; Milner, Brenchley et al. 2008). Subject 422 had normal T_H17 cell frequencies in blood (0.87% compared with a study median of 0.52%, IQR 0.31-0.90%) but she had low T_H17 frequencies in bronchial biopsies (2.3% compared with a study median 3.3%, IQR 2.3-6.8%) and very low BAL T_H17 frequencies at 0.1% (study median 2.6%, IQR 0.85%-4.0%) which is the second lowest value I have recorded. This raises the possibility that the subject was predisposed to acquisition of *T whipplei* as a consequence of a primary pulmonary T_H17 cell deficiency, for instance due to a defect in T_H17 specific chemokines. Thus I have again provided anecdotal evidence of a correlation between immunological and metagenomic data.

b) Bacterial species in BAL from the main study

Next sequencing of BAL was conducted on samples from a further 38 subjects. Unfortunately, my collaborators unexpectedly added an additional processing step of passing the defrosted samples through a 24 μm filter with the aim of increasing the relative abundance of viral sequences. This may have decreased the relative number of bacterial and fungal reads making it impossible directly to combine the pilot and definitive data-sets. Nonetheless, many bacterial reads were detected - typically 500 sequences but in some cases over 5000.

The proportions of bacterial taxa in each BAL sample are presented in Figure 6.1, in which subjects have been arranged by hierarchical cluster analysis to emphasize taxa with similar abundance patterns. It is apparent from this figure that no specific pattern emerges within the hierarchical clustering of the subjects implying no evidence of association between disease and bacteria in this data-set. One sample from a moderate asthmatic contained sequences from *Acinetobacter* and *Moraxella* which, again, might represent microaspiration as these are recognised upper airway flora. Hilty *et al.* reported an increase in the presence of proteobacteria particularly *haemophilus* species in asthma using bronchial brushings (Hilty, Burke et al. 2010). I did not replicate this observation.

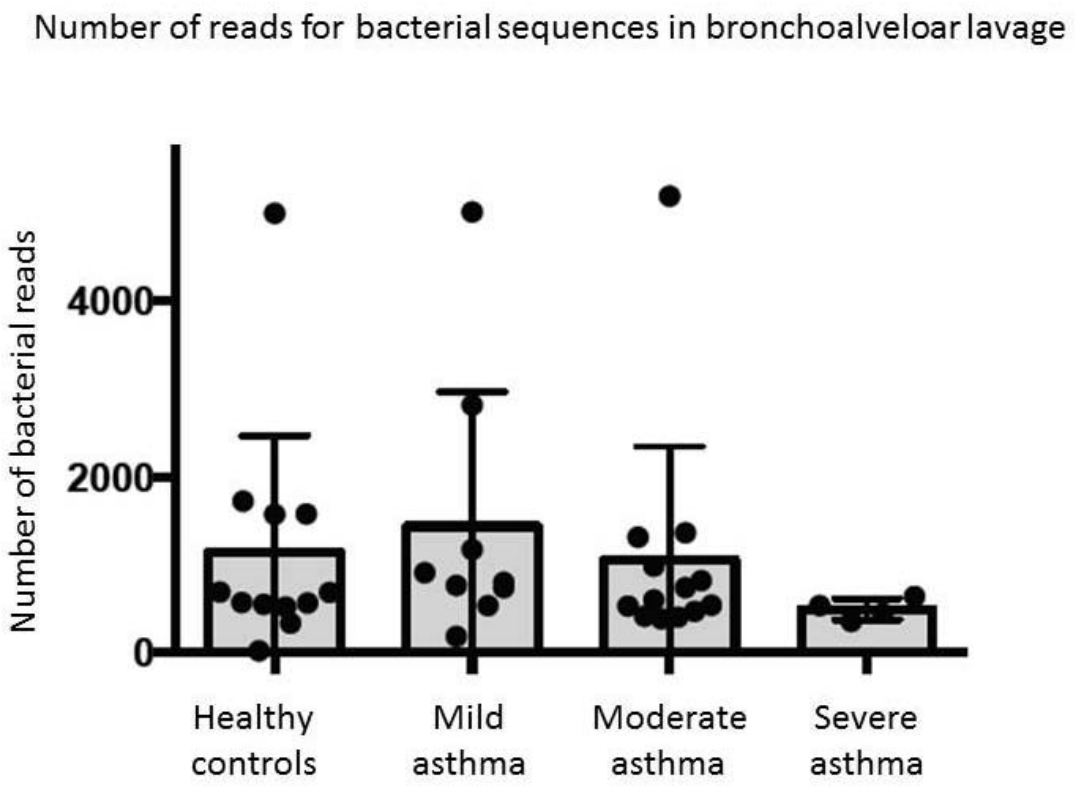


Figure 6.1 Proportions of bacterial taxa in each bronchoalveolar lavage sample

Proportions of bacterial taxa in each sample inferred from pyrosequence data. Each column corresponds to a specific bacterial order (A), family (B), or genus (C). Each row corresponds to an individual subject. Columns and rows have been subjected to hierarchical cluster analysis to emphasize taxa with similar abundance patterns. The proportional representation (relative abundance) of each family is represented by the colour code. Subjects are identified by a 3 digit number. The first number corresponds to the major disease classification: 1xx, healthy control; 2xx, mild asthma; 3xx, moderate asthma; 4xx, severe asthma. It can be seen, therefore, that no specific pattern emerges within the hierarchical clustering of the subjects, implying no evidence of association between disease and bacteria in this data-set. Abundance was low for most taxa, with the following exceptions (see text): Erysipelotrichales Erysipelotrichaceae *Coprobacillus* and Archoleplasmatales Archoleplasmataceae *Phytoplasma*. In addition one sample (subject 313, a moderate asthmatic) contained reads for *Acinetobacter* and *Moraxella*).

Otherwise the main finding was that abundance was low for most taxa, with the exceptions of two: Erysipelotrichales *Erysipelotrichaceae Coprobacillus* and Archoleplasmatales *Archoleplasmataceae Phytoplasma*. These taxa are not recognised respiratory flora. *Coprobacillus* are gram negative bacilli found in human faeces(Lyra, Rinttila et al. 2009; Park, Kim et al. 2011), whilst *Phytoplasma* are plant pathogens (Strauss 2009; Gasparich 2010) whose DNA might well be detectable in human faeces. In the light of this and given the detection in nearly all BAL samples it is possible that these are contaminants which have been transferred to the bronchoscopes during the cleaning process. Whilst I

took samples through a sterile BAL catheter (Combicath) to minimise contact between bronchoscopes and lavage fluid, BAL fluid can still contact the tip of the scope where this forms the 'wedge'. Subsequent investigation revealed that the bronchoscopes were cleaned in a central facility which also handle lower gastrointestinal endoscopes and which would be likely to carry a very high biomass of faecal bacteria. It is possible that microbial DNA could have survived cleaning with the acidic oxidising agent peracetic acid and been transferred to the bronchoscopes. Similar contamination with a low background of soil- and water-associated organisms was noted by Charlson *et al.* in their pre-bronchoscopy channel specimens (Charlson, Bittinger et al. 2011).

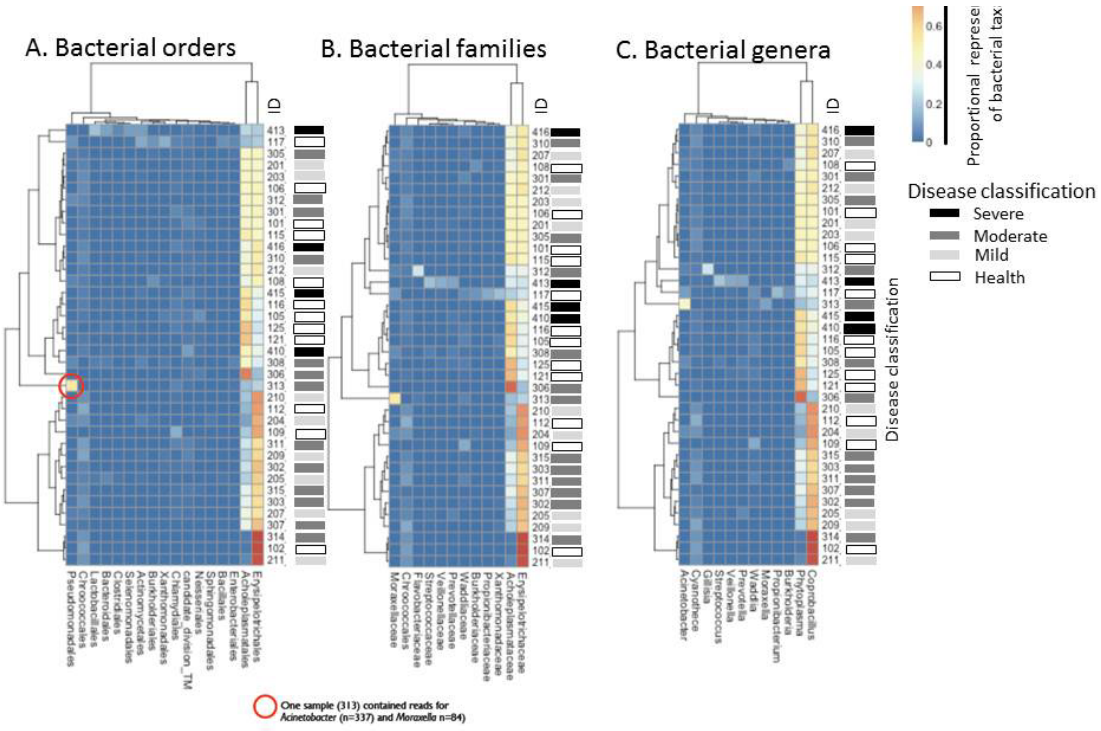


Figure 6.2 Bacterial abundance in bronchoalveolar lavage

Plot of raw number of bacterial sequence reads obtained from each bronchoalveolar lavage sample, grouped according to disease classification (x) axis. Number of sequences obtained ranges from 5 to over 5000, and is related to relative bacterial abundance. No significant differences were observed between the distributions according to disease severity, even if outliers were removed. Note that these plots do not contain data from an initial pilot data-set, which is why severe subjects are under-represented.

Figure 6.2 shows the relationship between the raw number of bacterial sequences (which can be considered a marker of relative bacterial abundance (Charlson, Bittinger et al. 2011)) obtained from each bronchoalveolar lavage sample, according to disease classification. There were no statistically significant differences between bacterial abundance and either presence or absence of asthma, or disease severity within the asthma phenotype.

c) Bacterial species in sputum from the pilot study

Deep sequencing was also conducted on a pilot set of nine sputum samples from subjects with moderate asthma taken during acute upper respiratory tract infections. This revealed multiple bacterial genomes in each of the exacerbation sputum samples as shown in Table 6.2. These are all recognised dental or upper respiratory tract flora from the same microbial communities as those observed at lower frequencies in the lung, notably *Rothia mucilaginosa, Veillonella parvula, Actinomyces, and Enterococcus,* which are all typical oral flora(Vaccher, Cordiali et al. 2007; Bizhang, Ellerbrock et al. 2011; Brittan, Buckeridge et al. 2012) or and bacteria such as *Streptococcus pneumoniae* and *Neisseria meningitides* which colonise the upper airways(Brittan, Buckeridge et al. 2012). This similarity of bacteria between upper and lower airways would be expected from mixing with saliva during sputum induction, or potentially from colonisation of central airway mucous by bacteria micro-aspirated into the airways. In this study it is not possible to distinguish bacteria arising from the airways from those which were present in saliva. Each of these bacterial families were also observed by Charlson *et al*., namely *Veillonella, Enterococcus, Neisseria* spp,, Actinomycetales micrococcaceae which includes *Rothia mucilaginosa*, Actinobacteria Actinomycetaceae which includes the *Actinomyces,* and Firmicutes Lacobacillales Streptococcaceae which includes *Streptococcus pneumoniae* (Charlson, Bittinger et al. 2011).

Matched samples from a single individual at two visits 3 days apart have been analysed from subject 53. Three out of 4 species detected on symptom day 7 were detected again on symptom day 10, reflecting the consistency of the technique in detecting what are presumably fairly stable polymicrobial populations of commensal flora.

Table 6.2 Bacterial OTU identified by more than one read from sputum samples collected during acute viral upper respiratory tract infections

Subject ID	Day of sample collection (symptom day 1 to 10)	OTU (species)	Number of reads	Sequence homology (range, %)
10	7	*Rothia mucilaginosa*	321	80.0 - 100
		Streptococcus sp	213	65.3 - 100
		Actinomyces sp	169	74.2-100
11	4	*Veillonella parvula*	5	70.8 - 96.3
		Neisseria meningitides	5	86.8 - 99.4
		Streptococcus pneumoniae	14	82.6 - 98.1
		Streptococcus mitis	15	61.9 - 97.4
27	1	*Prevotella melaninogenica*	119	67.5 - 100
		Rothia mucilaginosa	45	72.0 - 100
		Streptococcus sp	31	74.1 - 100
		Veillonella parvula	28	69.8 - 99.3
		Streptococcus mitis	25	70.0 - 99.1
30	7	*Rothia mucilaginosa*	20	91.4 - 100
		Streptococcus sp	4	93.8 - 100
		Streptococcus oralis	2	93.2 - 99.3
35	10	*Rothia mucilaginosa*	359	79.5 - 100
41	1	*Prevotella melaninogenica*	156	39.3 - 99.7
		Veillonella parvula	55	63.6 - 99.7
		Rothia mucilaginosa	29	45.0 - 100
		Streptococcus sp	28	73.4 - 100
		Rothia dentocariosa;	24	45.6 - 100
43	1	*Haemophilus parainfluenzae*	36	85.8 - 99.4
		Rothia mucilaginosa	32	83.7 - 100
		Streptococcus sp	26	68.6 - 100
		Streptococcus pneumoniae	19	78.0 - 98.9
53	4	*Streptococcus sp*	101	67.2 - 100
		Rothia mucilaginosa	96	82.9 - 100
		Veillonella parvula	89	71.5 - 99.6
		Actinomyces sp	31	79.8 - 99.1
53	7	*Rothia mucilaginosa*	211	71.6 - 100

Streptococcus sp	98	85.4 - 100
Enterococcus sp	33	76.4 - 96.4
Veillonella parvula	22	84.3 - 100

d) Bacterial species in sputum from the main study

Thirty-nine sputum samples were obtained from the same cohort of patients. These samples were filtered through a 24 μm filter after thawing. Bacterial genomes were detected with higher abundance in sputum samples, but indicated the presence of the same microbial communities as those observed at lower frequencies in the BAL, notably *Rothia mucilaginosa, Veillonella parvula, Actinomyces, Enterococcus,* which are all typical oral flora(Vaccher, Cordiali et al. 2007; Bizhang, Ellerbrock et al. 2011; Brittan, Buckeridge et al. 2012) and similar to those species observed by Hilty (Hilty, Burke et al. 2010). Given the similarity to the BAL data a detailed analysis of individually annotated taxa was not performed.

Summary

In summary, these analyses of the bacterial flora in BAL and sputum show the presence in the lower airways of typical microbes of the oral and upper-respiratory tract, but have not shown evidence of distinct lower airway microbial communities. Cladistic analysis does not suggest general differences in microbial communities between asthma and health, with the exception of some individuals who may have colonisation with a specific respiratory pathogen, whose presence may correlate with clinical and immunological features.

Results section II

Viral species in sputum and BAL

Figure 6.3 shows a representation of the relative abundance of viral sequences in BAL according to virus family and disease phenotype. All samples in this data-set were passed through a 24 μm filter with the aim of enriching the abundance of viral sequences relative to those of human or bacterial genomes. Retroviral sequences were almost universally present, which is expected due to the high frequencies of endogenous retroviruses incorporated into the human genome. These comprise up to 8% of the human genome and do not indicate actively replicating viruses (Belshaw, Pereira et al. 2004; Bizhang, Ellerbrock et al. 2011). Similarly, the presence of low numbers of sequences identified as poxvirus, phycodnavirus and iridovirus families is unlikely to be significant because these sequences probably belong not to viruses but to the orthologous host sequence. This sequence similarity can occur due to viral hijacking of host genes or due to the presence of shared repetitive motifs (Handley 2012). As can be seen from this figure I observed no association between the presence of viral sequences and the presence of asthma or the severity of asthma.

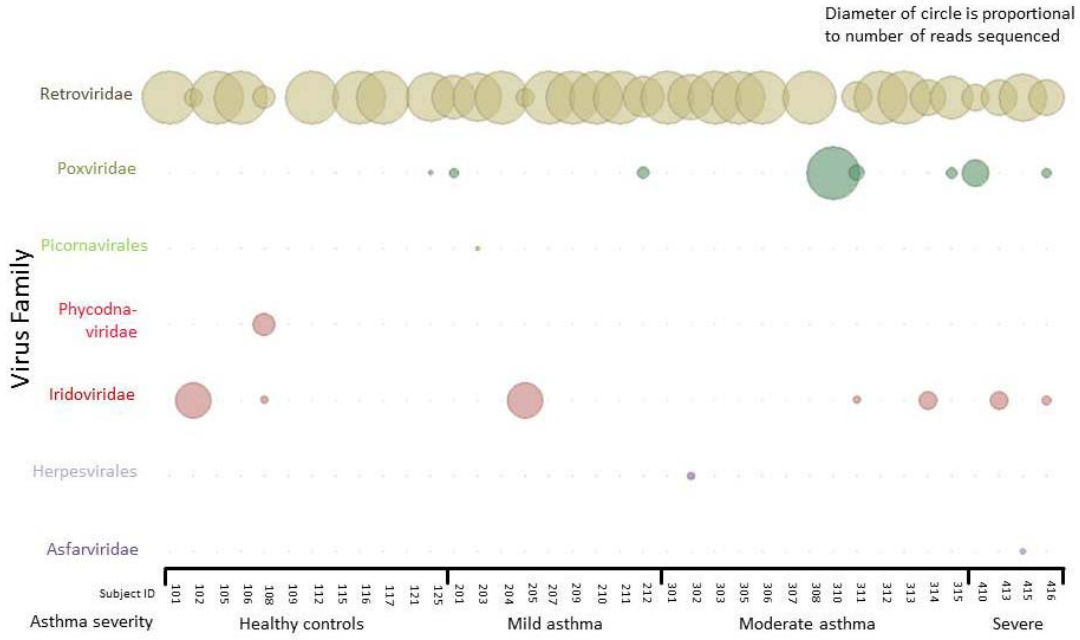


Figure 6.3 Viral taxa in bronchoalveolar lavage samples

A plot of numbers of viral sequence reads for each individual in bronchoalveolar lavage, arranged according to viral family. The diameter of each circle is proportional to the number of reads sequenced in each individual. Individuals are arranged along the x axis, grouped according to disease classification.

Despite using a sample preparation and data analysis pipeline which has been developed and validated for the detection of novel viruses (Zhao), the analysis failed to detect evidence of any particular virus at significant copy number in either BAL or sputum, from these cross sectional samples.

One virus, *Betatorquevirus*, was repeatedly detected at low copy number in BAL from 2 subjects with severe neutrophilic asthma receiving oral (subject 409) or high dose inhaled (subject 422) corticosteroids, as well as 3/9 sputum samples from subjects with suspected acute viral exacerbations of asthma. The significance of this finding is not clear. These samples were taken over a 5 month period from one geographical area and it is possible one virus might have been circulating in the community. Little is known about *Betatorquevirus,* also known as Torque Teno Mini Virus (TTMV), which is a single stranded DNA anellovius discovered only recently. However it is believed these viruses replicate in the respiratory tract (Maggi, Pifferi et al. 2003), and one very recent report has identified *Betatorque* virus in children with parapneumonic effusions and demonstrated that it is able to infect and replicate within alveolar epithelial cells and induce innate immunity (Galmes, Li et al. 2012), potentially implicating it as a respiratory pathogen.

Discussion

I have found similar bacterial species in BAL to those found in sputum, although the abundance was higher in the latter sampling technique, suggesting a continuity of microbial communities between the proximal and lower airways as well as the upper respiratory tract where expectorated sputum might get contaminated during sample collection. I have not observed any general differences between asthma and health in the composition of these microbial communities. However, I have identified two individuals with high abundance of potential respiratory pathogens in BAL and it may be that future larger studies will confirm these subjects to be representative of a subset of individuals with asthma who have long term bacterial colonisation of the airways. Furthermore, these two individuals have demonstrated proof of the concept that relationships can be identified within individual subjects between airway microbial colonisation and local activation of the mucosal immune system. I have also not found evidence of chronic persistent infection of the airway epithelium with respiratory viruses in asthma.

Microbial modulation of the respiratory immune system

My aim in this analysis was to investigate the presence of potential microbial stimuli which might be driving the immune response in asthma and to explore their relationship with the immunological data that I generated in my cross-sectional study. Microbes might drive an aberrant immune response in asthma in several ways: by providing ongoing stimulation of immune-pathology through local activation of the mucosal immune system (Simpson, Grissell et al. 2007; Simpson, Powell et al. 2008; Huang, Nelson et al. 2011), or by modulating the immune system systemically(Vael, Vanheirstraeten et al. 2011), or they might play a significant role in the initial development of asthma. This latter concept is sometimes termed the 'hygiene hypothesis'(Strachan 2000): the suggestion that there is a preventive effect of early childhood infections on the risk of allergic sensitisation, based on relationships between risk of asthma and childhood family size (Strachan 1989; Strachan 1997; Strachan 2000; Cullinan 2006), attendance at day care (von Mutius 2007), childhood bacterial infections (von Mutius 2007), exposure to non-viable microbial products (Riedler, Braun-Fahrlander et al. 2001) or to greater environmental microbial diversity (Ege, Mayer et al. 2011). These relationships are particularly strong if the exposure occurs in the first year of life (Ege, Bieli et al. 2006; Loss, Bitter et al. 2012). This may be related to the acquisition of different types of microbial flora early in life (Bisgaard, Hermansen et al. 2007; Thavagnanam, Fleming et al. 2008; Roduit, Scholtens et al. 2009), which in turn could be influenced by antibiotics (Wickens, Pearce et al. 1999; Droste, Wieringa et al. 2000; Noverr, Noggle et al. 2004; Russell, Gold et al. 2012).

Antibiotics may be modulating asthma risk by their effects on the faecal microbiome (Bisgaard, Li et al. 2011; Vael, Vanheirstraeten et al. 2011; Han, Huang et al. 2012). Gastrointestinal (GI) flora may play an important role in the induction of tolerance to airway allergens (Maeda, Noda et al. 2001; Noverr, Noggle et al. 2004) mediated by Treg to down-regulate airway T_H2 responses to the same antigens (Noverr, Noggle et al. 2004). Experimental allergic airways disease is exacerbated in germ-free mice compared with normal, and this exaggeration can be reversed by GI recolonisation with

normal commensal flora (Herbst, Sichelstiel et al. 2011). Commensal gut microbes can also modulate the generation of virus-specific T cells (Ichinohe, Pang et al. 2011) or produce anti-inflammatory short chain fatty acids by fermentation of dietary fibre (Maslowski, Vieira et al. 2009).

In summary microbes may play a key role in initiating asthma and also act later in life by driving activation of the airway mucosal immune system either through the distant immunomodulatory effects of the GI microbiome or more directly through the local presence of an airways microbiome.

Low bacterial frequencies argue against a significant airways microbiome

The key observation from my data is that in BAL from 47 subjects across a spectrum of health and asthma I did not find evidence of a complex commensal airways microbiome in health or asthma. In general the number of sequences (reads) detected was low and it was rare to find multiple sequences from a single OTU in any given sample. These observations argue against the existence of a consistent and distinct microbiome in the airways or healthy or asthmatic subjects. Of note, my study included severe asthmatics in whom on might expect that microbes could be playing a more important role, especially in neutrophilic forms.

This would contrast with the conclusions drawn of Hilty *et al.* (Hilty, Burke et al. 2010) who studied 11 adult asthmatics and eight healthy controls by sequencing DNA for the bacterial 16S RNA genes found on protected airway brushings. These authors suggested that the 'bronchial tree contains a characteristic microbial flora that differs between health and disease'. The numbers in their study are small, and the method was different, quantifying DNA by semi-quantitative PCR and sequencing DNA after cloning in bacteria. It is not clear that these conclusions are valid from the data presented, because they report cladistic analysis showing that bronchial microbial communities clustered with oropharyngeal in health. This would rather support the conclusions of Charlson *et al.* that 'bacterial populations in the healthy lower respiratory tract (LRT) largely reflect upper respiratory tract (URT) organisms, likely resulting from transient entry rather than independent communities with indistinguishable structure (Charlson, Bittinger et al. 2011). Furthermore, unlike the work by Charlson *et al*., the study by Hilty *et al.* did not present separate analysis of upper airway microbiota, specify the route of intubation, nor include environmental controls. Hilty *et al*. report a finding of 2000 genomes cm^{-2} of bronchial surface, but they do not present a separate analysis between asthma and health, rather basing this figure on a mixed population comprising healthy controls, asthmatic individuals and subjects with COPD. It is very unlikely that bacterial counts in COPD would be similar to those found in health as chronic bacterial colonisation of COPD is well documented(Hill, Campbell et al. 2000; Sethi, Evans et al. 2002; Wilkinson, Patel et al. 2003; Hurst, Wilkinson et al. 2005). It is therefore misleading to present a global estimate of airway colonisation as a single figure from such a mixed population. It is also inappropriate to present a mean value when the bacterial genome copy numbers measured ranged from 62 to 210,000, suggesting a data-set which is highly skewed, most likely by the inclusion of these heavily colonised COPD subjects.

Charlson *et al.* present a very careful analysis of airway microflora from multiple sites within each individual's respiratory tract; nasopharyngeal swabs, oropharyngeal swabs, oral washes, two swabs from the tip of the bronchoscopes, three bronchoalveolar lavage samples (Charlson, Bittinger et al. 2011). Furthermore upper airway contamination was minimised by the use of oral intubation and 2 sequential bronchoscopes, whilst environmental contamination was controlled by also analysing a wash taken from each bronchoscope prior to the procedure. These results convincingly supported their conclusions that in health 'the lung does not contain a consistent distinct microbiome, but instead contains low levels of bacterial sequences largely indistinguishable from upper airway flora'. As mentioned above I would argue that a correct interpretation of the data of Hilty *et al*. would also support this conclusion.

Unique contributions from this thesis

Can my data add to the work by Charlson *et al*.? Charlson examined only six healthy controls, and the logical extension was to compare health with asthma, which I have done. However, my data have several limitations. First, they did not include the environmental controls used by Charlson. This would have conclusively detected the suspected contamination of the bronchoscopes. In future work I would include these controls, but in the event the pattern and nature of the contamination could be clearly deduced and do not detract from the key findings. Second, I have sampled only sputum and BAL. However the work by Charlson *et al.* has now demonstrated that continuity of microbial communities between different airway samples means that the same communities may be detected by BAL or by brush or oral wash, and so the exact choice of sampling technique is not critical, and it is rational to select a single endobronchial sampling technique. Third, I used only a single bronchoscope, and in some individuals this was by nasal intubation. I have however used a different method of minimising upper airway contamination: using a sterile double lumen BAL catheter which was sealed with a wax plug and was introduced only after the bronchoscope lumen had in effect been washed with 10-16 ml of lidocaine solution. This technique is probably comparable in sterility to the use of protected brushes as they are not actually sealed with a wax plug, but as I have noted a small area of the bronchoscope tip which forms the wedge will contact the BAL fluid.

Notwithstanding these limitations, my work also has several strengths, principally the use of whole-genome sequencing. To date this technique has not been applied to airway samples in asthma and has the unique advantage of also detecting organisms which do not express bacterial 16S RNA, namely viruses and fungi. Furthermore, whole-genome sequencing can also identify entirely novel pathogens, so is the method of choice for pathogen discovery, which is of particular use in detecting novel viruses causing acute exacerbations of asthma.

My description of the subject with *H.Influenzae* infection provides proof of concept for the use of shot-gun metagenomics in clinical care. However as Charlson has noted, unlike quantitative thresholds empirically determined for diagnosis of pneumonia, there are no validated criteria for defining colonization or identifying normal microbial populations of the lower airways(Charlson, Bittinger et al.

2011). Determination and validation of such thresholds will require large scale clinical studies using highly standardised protocols and preferably linked prospectively to therapeutic treatment decisions.

The detection of *T whipplei* in one individual adds to the emerging evidence(Bousbia, Papazian et al. 2010; Charlson, Bittinger et al. 2011; Fenollar, Ponge et al. 2012; Urbanski, Rivereau et al. 2012) that this poorly understood bacterium may be distinguished by an unusual ability to achieve long term colonisation of the airways, perhaps only in immunologically susceptible individuals, and perhaps functioning as an opportunistic pathogen. On the other hand, it might be argued that given the high sensitivity of PCR methods, this could have been an incidental finding of no pathological significance. PCR techniques have shown high rates of *T whipplei* carriage in asymptomatic individuals in saliva and faeces (Ratnaike 2000; Rolain, Fenollar et al. 2007; Fenollar, Laouira et al. 2008; Fenollar, Trape et al. 2009). If present in saliva, it is feasible that this bacterium might have arrived by microaspiration of saliva. Nonetheless, with the tools now available there are increasing reports of *T whipplei* being identified solely in the respiratory tract, either in patients with symptoms of pulmonary Whipple's (Fenollar, Ponge et al. 2012) or culture-negative pneumonia (Bousbia, Papazian et al. 2010). It is intriguing to note that in the study by Charlson *et al.* (Charlson, Bittinger et al. 2011) using deep sequencing in healthy individuals, of 3431 total OTU identified, there was only one which was present at high abundance (32 sequences) in all lower respiratory tract samples and absent from all upper respiratory tract samples of the same individual and this was *T whipplei* (Charlson, Bittinger et al. 2011). This was considered to represent a genuine detection of a bacterium, which supports the hypothesis that the 35 sequences we detected also represent genuine presence of the bacterium. There is certainly much that is unknown about *T whipplei*. It is interesting to note that as awareness of the condition has increased over the last decade, due to the application of PCR techniques, and the number of samples sent for testing has increased dramatically, there has been no change in the positive ratio of tested samples (Edouard, Fenollar et al. 2012), which implies that *T whipplei* remains a markedly under-diagnosed condition. It is plausible that the current intensive investigation of the lung microbiome will identify the *T whipplei* as one of a small number of bacteria – along with the phylogenetically related mycobacteria - capable of achieving long term colonisation of this unique anatomical niche, perhaps only in immunologically susceptible individuals(Lagier, Fenollar et al. 2011).

No evidence of chronic respiratory viral infection in asthma

My failure to detect evidence of any particular virus at significant copy number in either BAL or sputum from these cross sectional samples argues against the presence of chronic active viral infection in the respiratory tract as a pathogenic mechanism in asthma.

The metagenome includes all organisms that live on us or in us. By extrapolation from seroprevalence studies it is estimated that humans are chronically infected with 8-12 viruses, such as herpesviruses, cytomegalovirus, anelloviruses, Epstein-Barr virus (EBV), and JC and BK polyomaviruses(Virgin, Wherry et al. 2009). The high species specificity of polyomaviruses, which infect 72-98% of humans,

suggests a prolonged period of coevolution with humans. Viruses can maintain latent infection in three ways: continuous replication (e.g. human immunodeficiency virus, hepatitis B and C), latency and reactivation (e.g. EBV) and invasion of the genome (e.g. endogenous retroviral elements which are transmitted vertically). Many viruses maintain latency by subversion of immunity which may have a number of general effects on the immune system including persistent secretion of proinflammatory cytokines and potential skewing of T cell response towards effector rather than memory phenotypes(Virgin, Wherry et al. 2009). Of particular relevance to this thesis is the observation in a murine model for respiratory syncytial virus (RSV) that prolonged presence of Sendai virus, is associated with an iNKT mediated activation of IL-13 secretion from macrophages which contributes to allergic airways disease (Kim, Battaile et al. 2008). This occurs even though the virus is cleared to non-infectious trace levels by day 12 yet the IL-13 secreting macrophage phenotype does not develop till day 21 post infection. Thus infections that are generally considered harmless or unimportant play a role in shaping the normal immune response, at the cost of introducing immunopathology in susceptible individuals (Virgin, Wherry et al. 2009).

Is there evidence of this occurring in human asthma outside the setting of an acute exacerbation? The possibility for chronic persistence of human rhinovirus (RV) has been demonstrated in immunosuppressed transplant recipients by Kaiser *et al.* who recurrently isolated viable RV of the same strain from 2 lung transplant recipients over a 12 month period(Kaiser, Aubert et al. 2006). In asthmatics Harju *et al* detected RV by PCR in sputum more often than in health and noted that asthmatics positive for RV had worse symptoms and poorer lung function(Harju, Leinonen et al. 2006). Using immunohistochemistry Wos *et al.* found RV more in 9/14 (63%) of bronchial biopsies from asthmatics but only 2/6 (33%) of controls, whilst using *in situ* PCR she found RV in 73% of asthmatics and 22% of control biopsies ($P<0.001$) and again presence of RV correlated with poorer lung function and worse eosinophilic inflammation(Wos, Sanak et al. 2008). Malmstrom *et al.* detected RV by PCR in 45% of bronchial biopsies from infants with persistent wheeze and again presence of RV correlated with worse lung function (Malmstrom, Pitkaranta et al. 2006). The fundamental problem with all these studies is that RV replication persists much longer than, and also precedes, the period of upper respiratory tract symptoms, and so it is very hard to determine that detection of RV is not associated with a recent acute viral infection, or even one which is about to occur. Jartti *et al.* found that 16% of asymptomatic healthy children were positive for RV, of which 38% developed symptoms in the subsequent week, and found that RV takes at least 5-6 weeks (Jartti, Paul-Anttila et al. 2009) to become undetectable by PCR, perhaps longer(Kling, Donninger et al. 2005). None of the studies in asthma mentioned above allowed for this: Wos sampled at least three weeks after an exacerbation, Harju four weeks, and Malmstom had no period of quarantine, and therefore noted higher rates of RV detection in subjects who had suffered a symptomatic URT infection within the previous six weeks. Furthermore it is recognised that RV secretion persists for longer in asthmatics (Corne, Marshall et al. 2002) due to defects in the induction of type I and III interferons (Wark, Johnston et al. 2005; Contoli, Message et al. 2006).

I ensured that subjects had been free from symptoms of respiratory infection for at least six weeks prior to sampling, which may explain why I found no evidence of chronic respiratory viral infection. The most consistent interpretation of all these studies is that RV does not cause chronic infection in asthmatic airways during periods of clinical stability, but rather is frequently detectable by sensitive molecular methods several weeks after an overt or occult acute upper respiratory tract infection, and this finding is more frequent in asthmatic people due to the well understood defect in antiviral innate immunity.

Conclusion

In summary, I have presented a novel application of whole-genome shot gun sequencing to the analysis of airway microbial samples. My data argue against the existence of a distinct airway microbiome in health or in asthma, and support the conclusion that microbes within the lung are in general a transient result of microaspiration of upper airway flora. Conversely in specific cases chronic low grade infection with opportunistic pathogens or true pathogens may drive the immunopathology of asthma, and perhaps this is particularly true in neutrophilic phenotypes. In a single BAL sample I have directly shown the presence of an active infection with a true pathogen inducing an exuberant BAL T_H17 response, and in another individual a deficiency of BAL and bronchial T_H17 cells appears to be linked, perhaps causally, to the presence of an opportunistic pathogen. Finally, I have found no evidence to support the hypothesis that chronic persistent viral infection drives airways inflammation in asthma during periods of clinical stability, although my data do lend weight to the emerging evidence that the recently described *Betatorquevirus* may be a respiratory pathogen.

Future work

There would be much value in conducting future studies to further explore the relationship between asthma and the human microbiome, particularly in addressing the following questions.

I. Whilst there is no evidence of a commensal airway microbiome in most individuals, are there subsets of asthmatics in whom chronic colonisation with airway microbes occurs, and does this respond to directed antibiotic therapy? This may be particularly relevant in patients who have bronchiectasis associated with asthma. Patients with associated chronic rhinosinusitis might also be a subgroup of asthma in whom microbes could play a more important role and a study of the microbiome in these could provide more information than could be obtained by simple microbial culture.
II. If such individuals exist, what is the mechanism by which they have become colonised: is this attributable to genetic or epigenetic defects in innate immunity, or is it perhaps a result of the immunopathology of chronic asthma causing impairment of mucosal immune function, or is it a consequence of deficiency of adaptive immunity, such as MAIT cells? Is such a deficiency a primary phenomenon, or is it secondary to therapy such as with corticosteroids?
III. What is the nature of the relationship between the gut microbiome and the airway mucosal immune system in humans?

IV. Can the use of whole genome sequencing be validated and developed for clinical application in respiratory medicine?

Such studies need careful design. My data would suggest the importance of highly standardised protocols for collection of specimens, the priority of collecting controls for environmental contamination and the value in collecting simultaneous upper airway and stool samples for metagenomics. Bronchoscopes used should be handled and cleaned in a dedicated facility which does not process endoscopes used in the GI tract or other sources of high biomass contamination. Airway sampling could be best achieved either by using wax plug protected brushes or by bronchial lavage. In the latter case, samples should ideally be concentrated by ultracentrifugation to compensate for the very low biomass present in the human airways.

CHAPTER 7

T cell phenotypes during natural cold-induced asthma exacerbations

I see no reason to call it by its Greek name, difficulty in breathing being a perfectly good way of describing it. Its onslaught is of very brief duration – like a squall, it is generally over within the hour. One could hardly, after all, expect anyone to keep on drawing his last breath for long, could one?...doctors call it a 'rehearsal for death', since eventually the breath does what it has often been trying to do. [7]

[7] The Stoic philosopher Lucius Seneca's (4 BC-AD 65) vivid description of his own symptoms, perhaps the earliest known personal description of asthma. Seneca, *Epistulae Morales ad Lucilium*, c.AD 62-5

Introduction

The data presented in earlier chapters of this thesis all concerned samples taken during periods of clinical stability. However temporal variability of symptoms and lung function is a cardinal feature of asthma (Hyde 1860; Bousquet, Jeffery et al. 2000) and this is manifest most clearly during an acute exacerbation. As T cells are part of a complex immune system perpetually responding to dynamic changes in antigenic stimulation I will present in this chapter the results of a longitudinal investigation into the dynamics of CD4+ T cell responses during naturally occurring acute exacerbations. I will first briefly define the concept of an exacerbation and present a summary of what is known about the dynamics of the associated immune response, particularly with respect to IL-17 and T_H17 cells, the subject of this thesis and also IFN-β1α, which was administered to some of the participants.

The nature of asthma exacerbations

The definition of an exacerbation is still a subject of discussion (Dougherty and Fahy 2009; Fuhlbrigge, Peden et al. 2012), but acute exacerbations are defined in the GINA guidelines as 'episodes of progressive increase in shortness of breath, cough, wheezing, or chest tightness, or some combination of these symptoms, accompanied by decreases in expiratory airflow that can be quantified by measurement of lung function' or also as an 'acute and severe loss of control that requires urgent treatment' ((GINA) 2010).

Whilst exacerbations can be triggered by a variety of factors including allergens, pollutants, emotional stress and drugs ((GINA) 2010), the triggers in the majority of exacerbations are acute viral infections of the upper respiratory tract (Johnston, Pattemore et al. 1995; Johnston, Pattemore et al. 1996). Many asthmatic individuals suffer from increased and more severe lower respiratory tract symptoms during these infections due to a defect in the production of type I (Wark, Johnston et al. 2005) and type III (Contoli, Message et al. 2006) interferons. Viruses are detected by PCR in approximately 80% of exacerbations (Johnston, Pattemore et al. 1995) and are associated with airway neutrophilia (Wark, Johnston et al. 2002).

The immune response to rhinovirus

Approximately 2/3 of these viruses are identified as rhinovirus (RV)(Kelly and Busse 2008). RV infection induces inflammation, and airway recruitment of neutrophils, eosinophils, mast cells, $CD4^+$ and $CD8^+$ T cells, via increased IL-6, -8, -16, eotaxin, IFN-γ-inducible protein 10 (IP10, CXCL5), and regulated and normal T cell expressed and secreted (RANTES, CCL5). Murine models have shown that RV infection induces T_H1 and T_H2 cytokines, and exacerbates T_H2 response to allergen challenge (Bartlett, Walton et al. 2008).

Regarding the dynamics of the T cell response to rhinovirus, *in vitro* analysis of human tonsillar tissue shows that RV evokes a dose-dependent, CD4-dominant T cell response, with a peak of IL-2 secretion at 24 hours and IFN-γ at 3 days (Wimalasundera, Katz et al. 1997). A study of

children with tracheostomies found virus-specific and bystander $CD8^+$ cells migrated to the lungs during acute respiratory viral infection, accompanied by a reciprocal fall in peripheral antigen-specific T cells and transient increase in the CD8:4 ratio (Heidema, Rossen et al. 2008), consistent with similar findings in mice (Levandowski, Ou et al. 1986).

T_H17 cells may play an important role in the antiviral host response as they induce human β-defensins, which are antiviral and can recruit memory T cells via CCR6 (Wolk, Kunz et al. 2004) and may infiltrate bronchial mucosa during an asthma exacerbation (Pene, Chevalier et al. 2008). In healthy humans IL-17 modifies the responses of *in vitro* cultured epithelial cells to rhinovirus, enhancing virally-induced synthesis of IL-8 and β-defensin and consequent neutrophilic inflammation, whilst suppressing induction of the eosinophilic chemokine regulated and normal T cell expressed and secreted (RANTES)(Wiehler and Proud 2007). In mice pulmonary viral infection is associated with T_H17 recruitment (Lochner, Peduto et al. 2008), higher IL-17 expression and mucus hyper-secretion (Hashimoto, Graham et al. 2004; Hashimoto, Durbin et al. 2005). However human data on the dynamics of anti-viral T_H17 responses are lacking.

Whilst I have not observed differences in T_H17 immunity during clinically stabile asthma, it was necessary to investigate the possibility that asthma exacerbations might be associated with aberrance in the dynamics of the T_H17 response during acute exacerbations. In conjunction with a controlled trial of inhaled recombinant human (rh)IFN-β1α for the prevention of asthma exacerbations during the common cold I undertook longitudinal follow-up of a well characterised cohort of asthmatics with frequent exacerbations with the aim of studying how T_H17 cells change during virus infections and associated asthma exacerbations. Furthermore, as *in vitro* (Ramgolam, Sha et al. 2009; Wenink, Santegoets et al. 2009; Zhang, Jin et al. 2009) and animal data (Guo, Chang et al. 2008; Martin-Saavedra, Gonzalez-Garcia et al. 2008; Orgun, Mathis et al. 2008; Shinohara, Kim et al. 2008; Chen, Chen et al. 2009) have suggested IFN-β may influence T_H17 differentiation, I was able also to investigate how treatment with IFN-β1α influences T_H17 function *in vivo*.

Study design

Interferon beta study

I undertook longitudinal follow-up of subjects with moderate asthma and a history of frequent exacerbations who were participating in a phase II, double-blind, randomised, placebo-controlled trial ("SG005", NCT01126177 (SynairgenResearchLtd 2012)) of inhaled recombinant human (rh)IFN-β1α given at the onset of a common cold to asthmatic patients with the aim of preventing/ameliorating an exacerbations (Figure 2.2). Subjects were screened at baseline then recalled for a second study visit within 24 hours of developing symptoms of an upper respiratory tract infection. At this stage subjects were randomised to receive 6 MIU rhINF-β1α or placebo once daily for 14 days via an I-neb Adaptive Aerosol Delivery device (Philips Respironics, Guildford, UK)). Subjects returned for scheduled visits on days 4, 7, 10, 13, 17 and days 44-48 after the first onset of symptoms. At all visits subjects underwent clinical assessment and phlebotomy. In addition sputum induction was performed on the baseline visit (V1) and visits 3 and 4 (days 4 and 7 respectively) (see study schedule, Table 7.1). Subjects also performed home lung function monitoring and Asthma Index score (Sorkness, Gonzalez-Fernandez et al. 2008) reporting twice daily, Jackson Cold score (Jackson, Dowling et al. 1958) reporting once daily, and shortened-Asthma Control Questionnaire (ACQ (Juniper, Svensson et al. 2005)) reporting weekly throughout the treatment phase.

I was an active member of the team conducting this study in the capacity of (honorary) clinical research fellow along with 3 other clinical fellows and I also participated in the processing of the PBMC along with other members of the laboratory team. I conducted all the processing of sputum on the samples gifted to me and all processing of PBMC subsequent to the initial isolation, or cryopreservation of PBMC (see acknowledgements).

Table 7.1 Study schedule for longitudinal study

Visit Number	**V1**	**V2**	**V3**	**V4**	**V5**	**V6**	**V7**	**V8**
	Pre-treatment phase	**Treatment phase**						
Assessment days	**Screening**	**Day 1 (Within 24h of cold symptoms)**	**Day 4**	**Day 7**	**Day 10**	**Day 13**	**Day 17**	**Day 44-48**
Consent	**X**							
Medical history	**X**							
Physical examination	**X**	**x**	**x**	**x**	**x**	**x**	**x**	
Vital signs	**X**	**x**	**x**	**x**	**x**	**x**	**x**	
Height and weight	**X**						**x**	
12 lead ECG	**X**							
Skin allergy test	**x**							
FE_{NO}	**x**	**x**	**x**	**x**	**x**	**x**	**x**	
FEV_1, FVC, PEFR	**x**	**x**	**x**	**x**	**x**	**x**	**x**	
TLCO	**x**	**x**					**x**	
PD_{20}	**x**							
Home monitoring		**x**	**x**	**x**	**x**	**x**	**x**	**x**
Urinalysis	**x**	**x**					**x**	
Nasal lavage		**x**	**x**	**x**				
Dose administration		**x**	**x**	**x**	**x**	**x**		
Phlebotomy (cryopreserved)	**x**	**x**	**x**	**x**	**x**	**x**	**x**	**x**
Phlebotomy (fresh)	**x**		**x**	**x**				
Sputum induction (fresh)	**x**		**x**	**x**				

ECG, electrocardiogram; FE_{NO}, fractional exhaled nitric oxide; FEV_1, forced expiratory volume in 1 second; FVC, forced vital capacity; PEFR, peak expiratory flow rate; PD_{20}, provocative dose 20; TL_{CO}, transfer factor carbon monoxide.

Immunological samples

Two types of sample were available to me:

i) 'fresh' samples of paired peripheral blood and induced sputum at visits 1 (baseline), visits 3 (day 4) and visits 4 (day 7). Not all subjects successfully produced induced sputum at each visit, and so these samples were only available on a subset of 31 subjects (see Figure 2.2). PBMC were only analysed freshly if there was an adequate paired sputum sample from that visit. Adequate samples were obtained from 14 subjects at V1, 13 subjects at V3 and 14 subjects at V4.

ii) cryopreserved samples of peripheral blood mononuclear cells from every subject at every study visit. Complete paired sets of sample from all 8 study visits have been analysed from 26 of these subjects, 13 who were randomised to rhIFN-β1α and 13 who were randomised to placebo.

As the process of cryopreservation significantly affected the phenotype of these cells as measured by intracellular cytokine staining (ICS), immunological data from these two sets of samples will be presented separately, in separate results sections.

In addition some PCR data were obtained from samples gifted to me from a pilot study called 'SG007'. This was a single-group, unblinded rhinovirus challenge study in which 11 moderate asthmatics were challenged with 100 tissue culture infectious dose 50 (TCID50)/mL) of human RV16 (Parry, Busse et al. 2000; Adura 2013). Phlebotomy, sputum induction and nasal lavage were performed at baseline and at 6 follow-up visits over the following 14 days (Adura 2013).

First I will present data from this pilot study SG007 (Results I), then I will present an analysis of fresh samples from SG005 (Results II), then I will present data from the SG005 cryopreserved samples (Results III).

Study populations

Pilot RV16 challenge study

Forty-four non-smoking, moderate asthmatics were screened for serological evidence of humoral immunity to RV16. Eleven subjects had no humoral immunity to RV16 and received viral inoculation. They comprised 4 male and 7 female subjects with a median age of 38 years (range 20-53 years) with a median baseline FEV_1 98.0% of predicted (IQR 85.5-105.5). All were receiving ICS with a median dose of 400 mcg BDP equivalent (400-900). All 11 subjects developed active infection, as evidenced by a) sero-conversion (>4-fold increase in anti-RV16 neutralising antibodies in serum during convalescence) (*n*=8/11), b) shedding of virus in the nose detected by qPCR of nasal lavage (*n*=10/11) and c) virus detection by qPCR in the lower respiratory tract as measured in sputum (*n*=6/11) (Adura 2013). The cDNA from these samples was gifted to me and I had no part in prior collection or processing of the samples (see acknowledgements).

Interferon-beta study longitudinal cohorts

The study was approved by the Southampton and South West Hampshire Research Ethics committee A (REC number 10/H0502/14). Subjects were aged 18 to 65 with symptoms of asthma for at least 2 years confirmed by medical history and ≥12% and 200mL bronchodilator reversibility or evidence of BHR. Subjects were on maintenance ICS. Subjects had a history of virus-induced exacerbations of asthma with at least one exacerbation in the last 24 months (but not within the last 1 month) requiring oral steroids or antibiotics and answered 'Yes' to the question 'Does a cold make your asthma worse?' Baseline clinical characteristics of those subjects included in the analysis of fresh and of cryopreserved samples are shown in Table 7.2 and Table 7.3 respectively.

Additional criteria for entry to randomisation and the treatment phase included a history of respiratory virus symptoms that had developed within the last 24 hours, defined as either:

> Cold symptoms (specifically a blocked or runny nose, and a sore or scratchy throat) *or*
> Influenza-like illness (Fever >37.8 °C plus two of the following: headache, cough, sore throat and myalgia)

As shown in figure 2.2, 120 subjects were assessed for eligibility and consented at Southampton, of which 102 successfully completed the baseline screening visit and satisfied all inclusion and exclusion criteria. During the period of the study (March 2010 - December 2011) 47 subjects developed symptoms of an acute URTI and were randomised. Of these fresh samples were obtained from 31 subjects (Table 7.2) and complete series of paired cryopreserved samples were obtained from 26 subjects (Table 7.3).

Table 7.2 Clinical characteristics of the longitudinal cohort (fresh samples)

	All subjects
n	31
Demographics	
Sex (M/F)	12 / 19
Age (median [range], years)	30 (19-63)
Pulmonary function	
FEV1 (% predicted)	106 (89-112)
FEV1 reversibility (%)	5.7 (1.9-8.7)
PEFR (% predicted)	101 (84-108)
PEFR reversibility (%)	4.6 (2.5-11)
PD20 (mg methacholine)	0.13 (0.082-0.45)
Exhaled nitric oxide (ppb, at 50 L/s)	22 (15-34)
Clinical	
Atopy (Skin prick positive, Y/N)	18 / 13
Peripheral eosinophil count (10^9/L)	0.2 (0.1-0.4)
Body mass index (kg/m^2)	26.8 (24.4-30.5)
Smoking status	
Never / Former / Current	25 / 5 / 1
Duration of asthma (years)	23 (19-28)
ACQ score	0.71 (0.50-1.4)
GINA level of control (n, %)	
Controlled	0 (3.8)
Partly controlled	27 (87)
Uncontrolled	4 (13)
Treatment	
Inhaled steroids	Yes
Dose (equivalent mcg BDP)	400 (400-900)
Maintenance oral steroids (Y,N)	No
Long acting β agonist (Y/N)	17 / 14
Leukotriene receptor antagonist (Y/N)	1 / 30
Antihistamine	6 / 25
Step on BTS treatment algorithm	2 - 4
Exacerbation history (last 2 years)	
Courses of antibiotics	2 (1-4)
Numer of exacerbatoins	4 (2-7)
Hospital admissions	0 (0-0)
Courses of oral steroids	2 (1-2)
Relevant comorbidities (n, %)	
Allergic rhinitis	20 (65)
Eczema	7 (65)

Values are medians with interquartile ranges, unless stated otherwise. N/A: not available.

ACQ, asthma control questionnaire; BDP, beclometasone dipropionate; BTS, British Thoracic Society; CT, computed

Table 7.3 Clinical characteristics of the longitudinal cohort (cryopreserved)

	All subjects	Active rhIFN-β1α	Placebo
n	26	13	13
Demographics			
Sex (M/F)	4 / 22	4 / 9	0 / 13
Age (median [range], years)	30 (19-58)	30 (22-55)	27 (19-58)
Pulmonary function			
FEV1 (% predicted)	103 (87-111)	109 (86-118)	96 (89-106)
FEV1 reversibility (%)	6.5 (2.9-8.8)	5.7 (2.8-8.9)	7.7 (4.0-8.6)
PEFR (% predicted)	102 (85-109)	108 (84-120)	100 (87-107)
PEFR reversibility (%)	4.2 (0-5.5)	3.0 (0.0-4.3)	4.8 (2.9-11)
PD20 (mg methacholine)	0.15 (0.046-0.26)	0.24 (0.12-0.36)	0.09 (0.032-0.15)
Exhaled nitric oxide (ppb, at 50 L/s)	23 (15-37)	29 (17-41)	18 (12-26)
Clinical			
Atopy (Skin prick positive, Y/N)	16 / 10	8 / 5	8 / 5
Peripheral eosinophil count (10^9/L)	0.2 (0.1-0.3)	0.2 (0.1-0.3)	0.2 (0.1-0.3)
Body mass index (kg/m^2)	27.5 (24.4-32.5)	25.1 (24.3-29.9)	31.2 (27.4-34.4)
Smoking status			
Never / Former / Current	21 / 4 / 1	10 / 3 / 0	11 / 1 / 1
Duration of asthma (years)	20 (15-26)	20 (12-23)	23 (15-29)
ACQ score	0.86 (0.43-1.3)	0.86 (0.43-1.3)	0.9 (0.57-1.57)
GINA level of control (n, %)			
Controlled	1 (3.8)	0 (0)	1 (7.7)
Partly controlled	21 (81)	12 (92)	9 (69)
Uncontrolled	4 (15)	1 (7.7	3 (23)
Treatment			
Inhaled steroids	Yes	Yes	Yes
Dose (equivalent mcg BDP)	400 (400-800)	400 (200-400)	800 (400-800)
Maintenance oral steroids (Y,N)	No	No	No
Long acting β agonist (Y/N)	16 / 10	9 / 4	7 / 6
Leukotriene receptor antagonist (Y/N)	1 / 25	0 / 13	1 / 12
Antihistamine	6 / 20	4 / 9	2 / 11
Step on BTS treatment algorithm	2 - 4	2 - 4	2 - 4
Exacerbation history (last 2 years)			
Courses of antibiotics	2 (1-4)	2 (1-4)	1 (1-3)
Numer of exacerbatoins	5 (2-6)	5 (3-7)	3 (2-6)
Hospital admissions	0 (0-0)	0 (0-1)	0 (0-0)
Courses of oral steroids	2 (0-3)	2 (0-4)	2 (1-2)
Relevant comorbidities (n, %)			
Allergic rhinitis	18 (69)	8 (62)	10 (77)
Eczema	8 (31)	3 (23)	5 (38)

Values are medians with interquartile ranges, unless stated otherwise. N/A: not available.
ACQ, asthma control questionnaire; BDP, beclometasone dipropionate; BTS, British Thoracic Society; CT, computed tomogram; FEV1, forced expiratory volume in 1 second; FVC, forced vital capacity; GINA, Global Initiative for Asthma; PEFR, peak expiratory flow rate; PD20, provocative dose 20.

Results I Analysis of pilot data from RV challenge cohort

Induction of IL-17 mRNA in sputum during experimental RV infection

In a pilot experiment to determine whether IL-17 was induced during acute respiratory viral infections and if so what the magnitude of this induction was, I initially measured IL-17A mRNA in whole sputum obtained from the SG007 pilot RV16 challenge study. Of 44 subjects who were screened for inclusion in the study, 11 were challenged with virus and developed evidence of infection. From these subjects only 4 provided usable sputum samples at more than one time-point. Acute infection was associated with only modest induction of IL-17A mRNA (Figure 7.1). The mean maximum fold-induction relative to baseline was 3.7, which occurred on day 3 post-inoculation. By comparison there was a much greater, 2-3 log fold-induction of IFN-β in the same subjects, with 1-2 log fold increases in other antiviral genes including interferon gamma-induced protein 10 (IP-10) and myxoma resistance gene A (MxA) (Adura 2013).

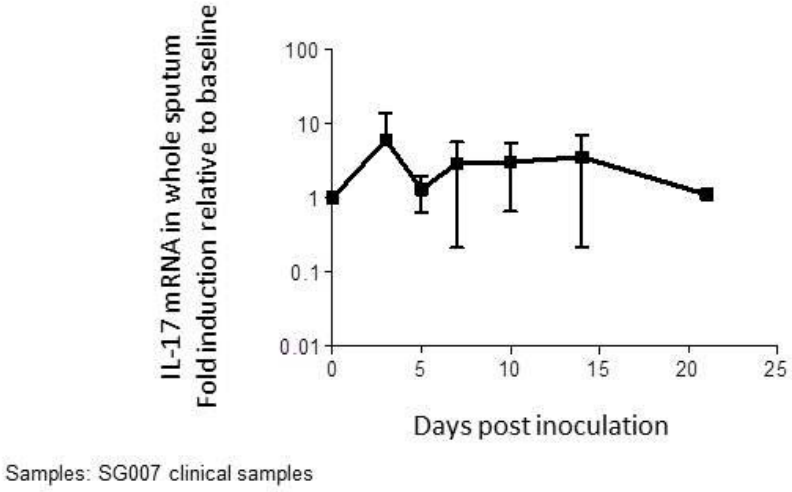


Figure 7.1 Sputum IL-17 mRNA during experimental RV infection

IL-17 mRNA measured in whole sputum by RT-qPCR in 4 subjects undergoing experimental infection with 100 $TCID_{50}$ of rhinovirus 16 as part of clinical study SG007. Samples were normalised to the housekeeping genes UBC and GAPDH. Graphs show means ± SD.

These preliminary data challenged the hypothesis that viral infections would produce a major induction of IL-17A. It is possible that this was partly due to the nature of RV16 which is known to produce a fairly mild clinical syndrome (Fleming, Little et al. 1999; Grunberg, Timmers et al. 1999). Nonetheless, all subjects did experience a significant increase in URTI symptoms (mean increase in Jackson Cold Score of 8.6, $P<0.001$) and in asthma symptoms ($P<0.001$), with concomitant falls in FEV_1 and PEFR (Adura 2013). Furthermore they provided data needed to inform sample size calculations for a proposed viral challenge study, which ultimately was not considered feasible.

Results II Analysis of fresh samples from longitudinal cohort

Next I analysed data obtained from 'fresh' samples processed immediately *ex vivo* from the longitudinal study of naturally-occurring viral infections.

T cell frequencies in peripheral blood and sputum during acute viral infection

Frequencies of T cells in peripheral blood and sputum from all 31 subjects combined are presented in Figure 7.2. Unfortunately very few subjects produced usable samples from sputum induction at the baseline visit (*n*=14 samples out of 102 attempted inductions, Table 7.4) and these were therefore not well paired with the samples obtained at symptom days 4 and 7, so paired statistical tests could not be used and sample sizes overall were small (*n*=13-14 at each visit). Therefore no statistically significant differences were observed in T cell frequencies over time (ANOVA P>0.15), except for T_H17 cell frequencies in sputum, where differences of borderline statistical significance were observed. Mean frequencies of sputum T_H17 cells increased 1.8-fold from 6.2% at baseline to 11% at symptom day 7 (ANOVA P=0.087, *post-hoc* t test P=0.034).

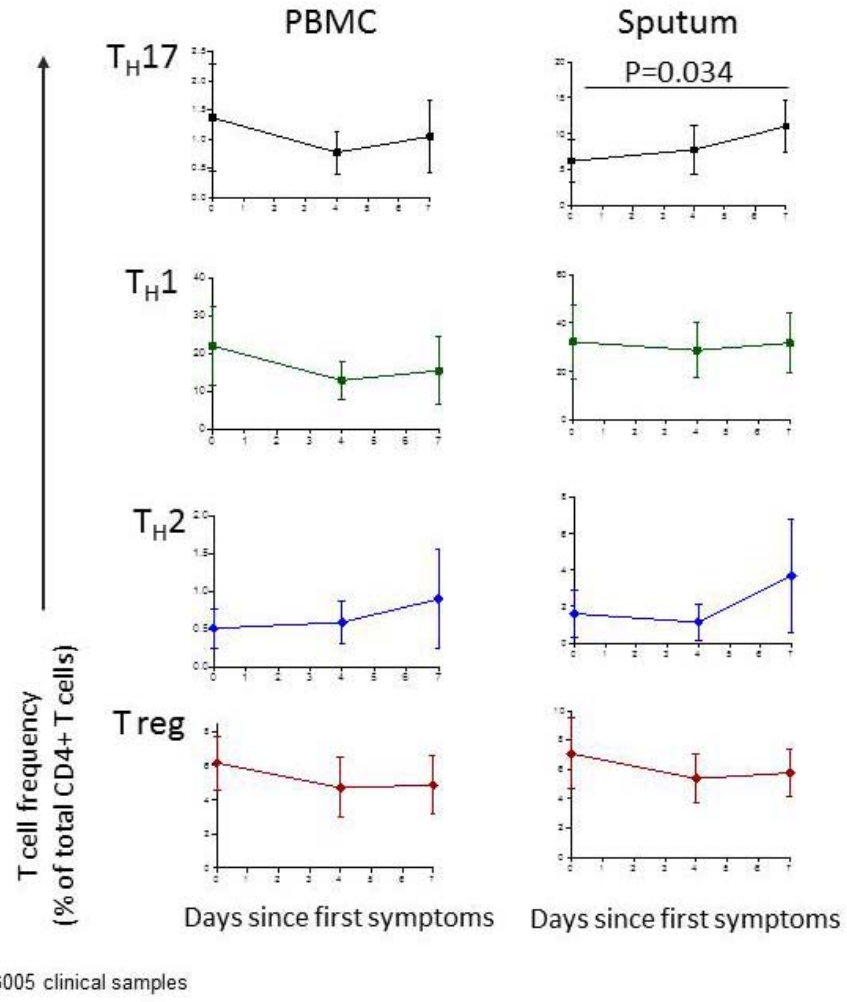


Figure 7.2 T cell frequencies in peripheral blood and sputum during acute viral infection
Frequencies of T cells in peripheral blood and induced sputum during acute upper respiratory tract infections measured by intracellular cytokine staining and flow cytometry on samples which had not been cryopreserved. Day 0, baseline screening visit (*n*=14); day 4, symptom day 4 (*n*=13); day 7, symptom day 7 (*n*=14). Plots show means±95% confidence intervals (CI). Most data are not paired. No differences were significant by ANOVA for any comparison.

Table 7.4 Rates of successful sputum inductions during longitudinal study

Study visit	Number of subjects attending visit	Number of successful sputum inductions	Rate of successful sputum induction (%)
1. Screening	102	14	14
3. Symptom day 4	47	13	28
4. Symptom day 7	47	14	30

I had hypothesised that I would observe a dramatic early increase in sputum T_H17 frequencies, followed later by an increase in T_H1 cells, mirrored by reciprocal falls in peripheral blood T_H17 and T_H1 frequencies. It is not possible to draw firm conclusions from this data-set, but whilst there is evidence of the increase in sputum T_H17 cell frequencies which I had expected, it is unlikely the magnitude of such an increase would much exceed a doubling in T_H17 cell frequencies at most.

The effect of IFN-β1α on T cell frequencies in blood and sputum

Next I stratified these data from fresh samples according to allocation to IFN-β1α or placebo (Figure 7.3). Statistical analysis was not possible because lack of pairing of data prevented comparison of areas under the curve (AUC) between active and placebo, the data are not corrected for baseline differences or adjusted for covariates and samples sizes were small.

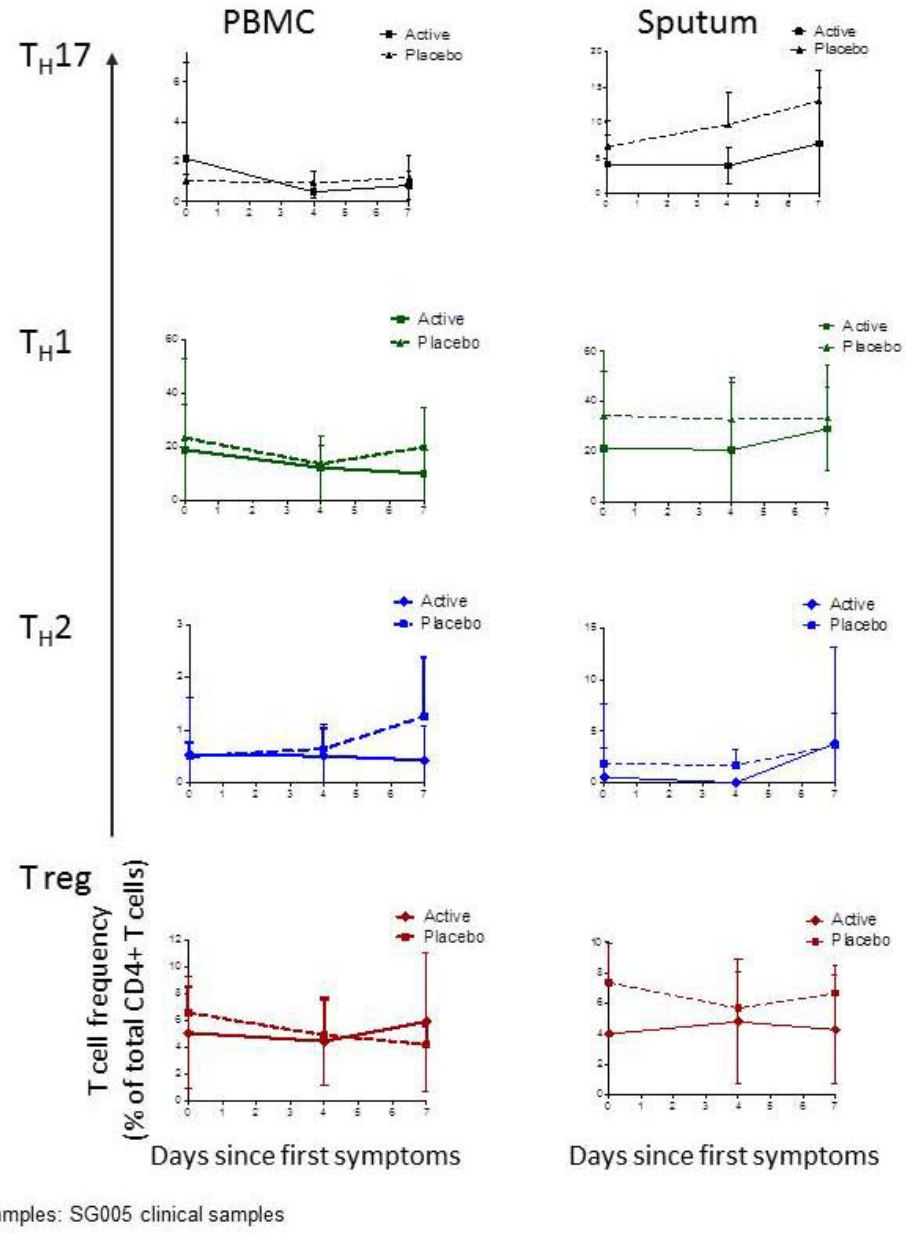


Figure 7.3 T cell frequencies in peripheral blood and sputum stratified by treatment group

Data presented in Figure 7.2 stratified by whether subjects received inhaled active rhIFNβ1α (continuous lines *n*=4-6) or placebo (broken lines *n*=10-8). Samples had not been cryopreserved. Plots show means ± 95% CI. Most data are not paired. No significant differences were observed between groups.

Results III Analysis of cryopreserved PBMC samples from longitudinal cohort

In addition to these samples I also obtained much larger numbers of cryopreserved PBMC at 8 different time-points which provided a more extensive characterisation of the dynamics of the T cell responses during acute exacerbations, and allowing more informative statistical analyses.

Frequencies of T_H17, T_H1, T_H2 and Treg cells in PBMC are presented in Figure 7.4 for 26 subjects at 8 visits. T cell frequencies did not differ significantly over time by ANOVA in any T cell subset. There was considerable inter-individual variation in T cell frequencies, which is apparent when frequencies at multiple time-points a plotted separately for each subject Figure 7.5.

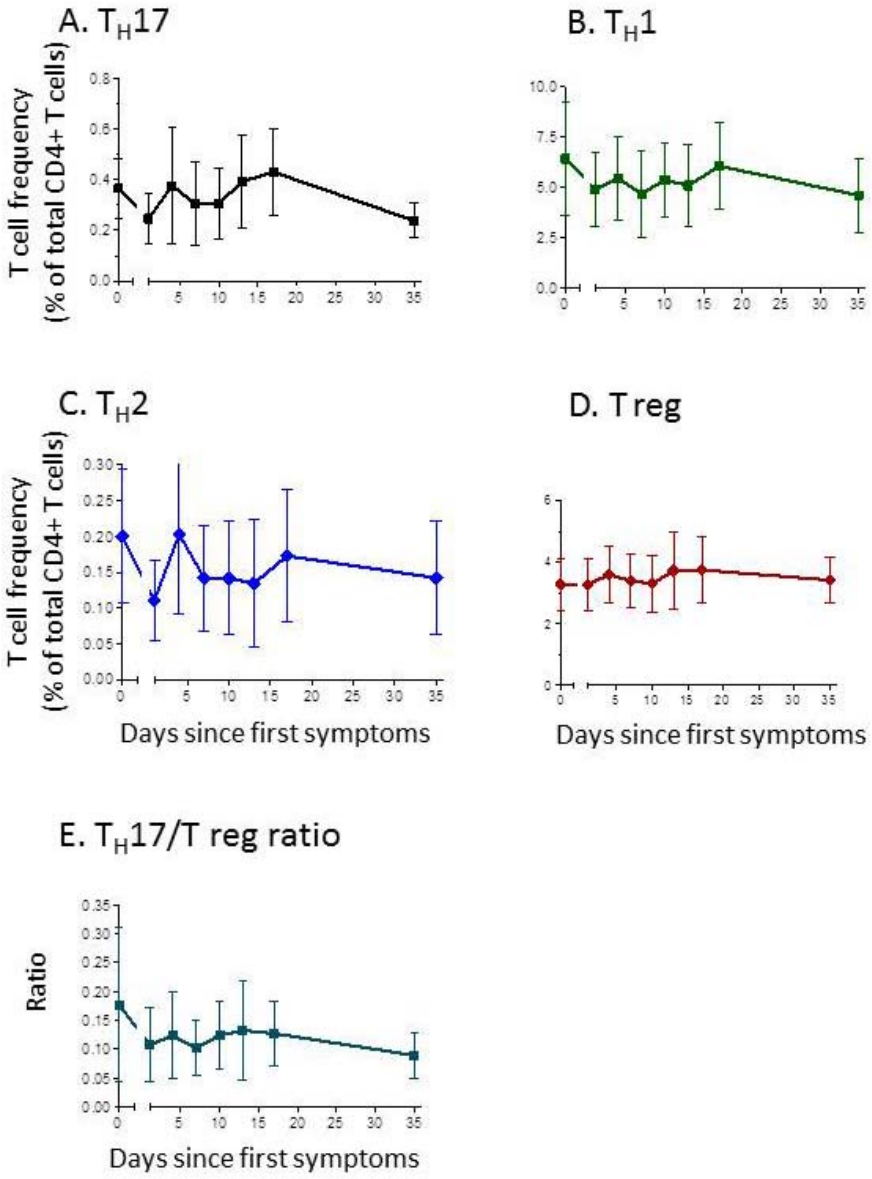


Figure 7.4 T cell frequencies in cryopreserved peripheral blood during an acute viral infection

Frequencies of major T cell subsets in peripheral blood during an acute upper respiratory tract infection. T cells which were cryopreserved at baseline and 7 time-points from the onset of symptoms were enumerated by intracellular cytokine staining and FACS. Plots show mean and 95% confidence intervals for *n*=26 individuals. Day 0, screening visit. Day 1, visit occurring within 24 hours of developing first upper respiratory tract symptoms. Other visits occurred on days 4, 7, 10, 13, 17 (each ±1 day) and day 30-35. (A) T_H17 cells, (B) T_H1 cells, (C) T_H2 cells, (D) Treg cells as % of total CD4+ T cells. (E) ratio of T_H17 to Treg cells.

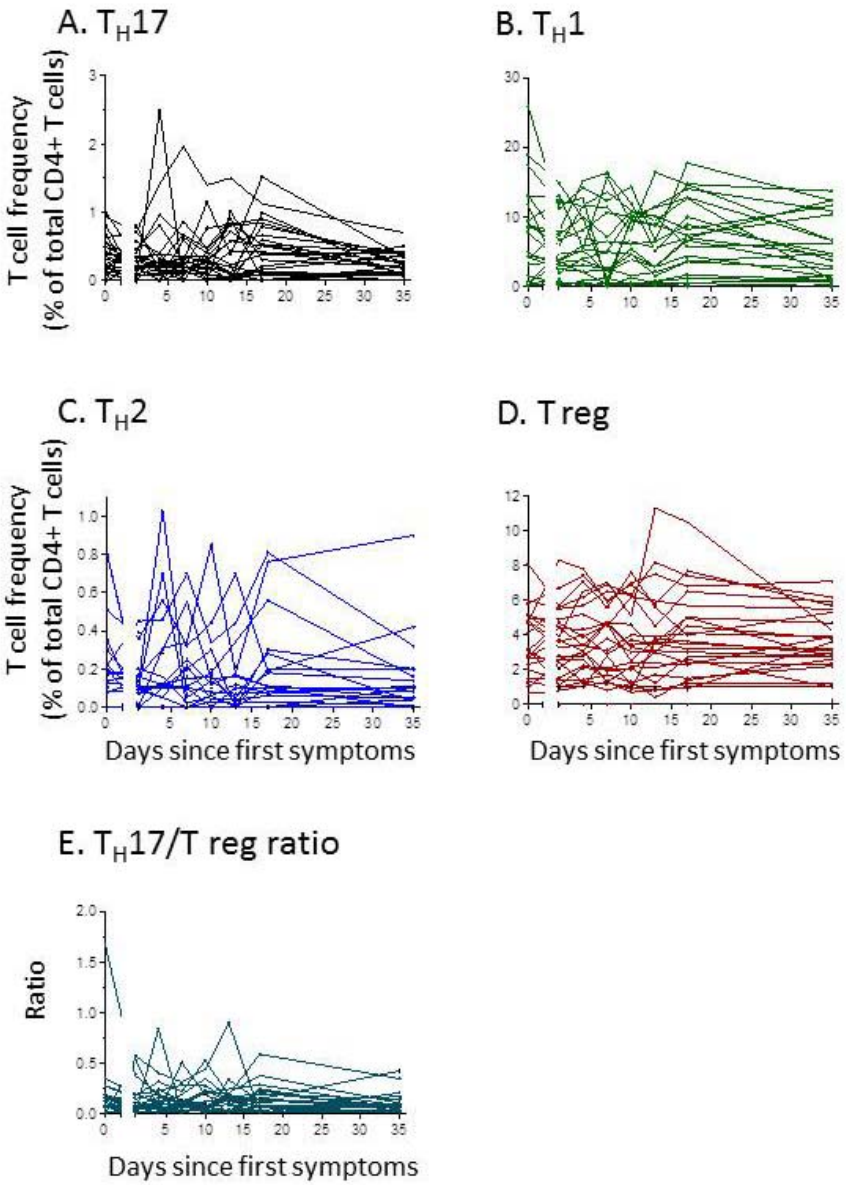


Figure 7.5 T cell frequencies in cryopreserved peripheral blood during an acute viral infection: showing individual subjects separately

The same data as shown in Figure 7.4 but plotted to show results from individual subjects separately. (A) T_H17 cells, (B) T_H1 cells, (C) T_H2 cells, (D) Treg cells as % of total CD4+ T cells. (E) ratio of T_H17 to Treg cells.

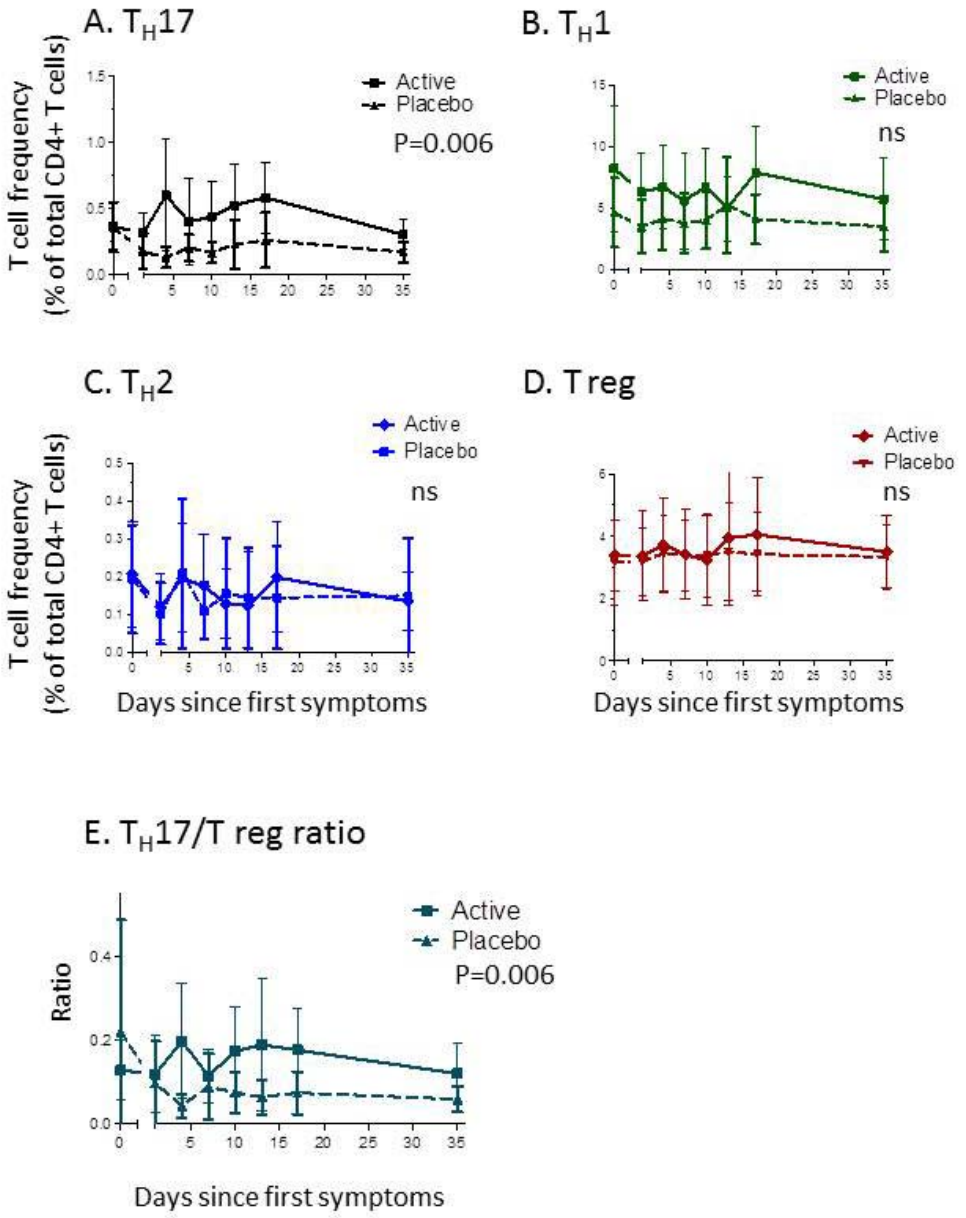


Figure 7.6 Peripheral blood T cell subsets according to treatment group

A. Peripheral blood T cell frequencies plotted over time and stratified by whether subjects received inhaled active rhIFNβ1α (*n*=13, continuous lines) or placebo (*n*=13, broken lines). Plots show mean ±95% confidence interval. (A) T_H17 cells, (B) T_H1 cells, (C) T_H2 cells, (D) Treg cells as % of total CD4+ T cells. (E) ratio of T_H17 to Treg cells. Differences in the areas under the curves for treatment groups were compared by t tests, and were significant for T_H17 cells (P=0.006) and for the T_H17/Treg ratio (P=0.006) only.

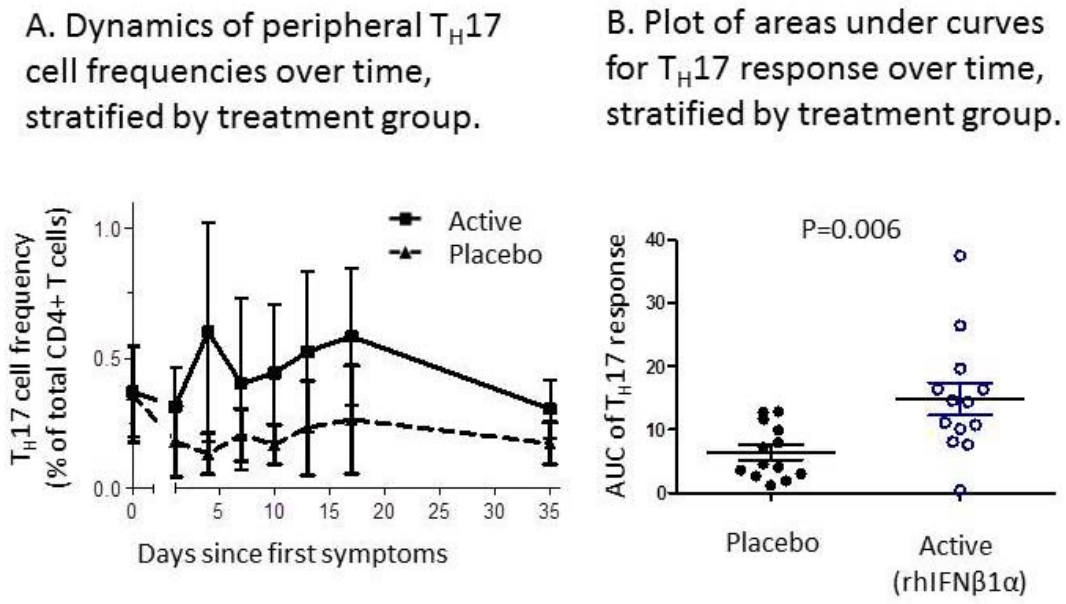


Figure 7.7 Peripheral blood TH17 response according to treatment group

A. Peripheral blood T_H17 cell frequencies measured by FACS plotted over time and stratified by whether subjects received inhaled active rhIFNβ1α (*n*=13, continuous lines) or placebo (*n*=13, broken lines). Plots show mean ±95% confidence interval.

B.Plot of areas under the curve for peripheral T_H17 response over time, stratified by treatment group. P=0.006 for unpaired t test.

T_H17 cell frequencies in peripheral blood are elevated during treatment with inhaled rhIFN-β1α

When subjects are stratified according to study randomisation, differences emerge between those allocated to active IFN-β1α (*n*=13) and placebo (*n*=13) (Figure 7.6). Both the T_H17 cell frequencies and the ratio of T_H17:Treg were significantly higher in subjects receiving active treatment than placebo (P=0.006 for AUC comparison (Matthews, Altman et al. 1990)). The comparison of the dynamics of the T_H17 response is shown in more detail in Figure 7.7 where the individual AUC for each subject are presented (Figure 7.7 B). The mean AUC was 2.2 fold greater in subjects receiving IFN-β1α. As T_H1, T_H2 and Treg frequencies did not differ significantly between treatment groups the change in the T_H17:Treg ratio is attributable to differences in T_H17 cells alone.

T_H17 cell frequencies in peripheral blood are according to whether subjects suffer an asthma exacerbation

Could these IFN-β1α-induced differences in T_H17 frequencies be due to drug treatment preventing exacerbations? To address this possibility I stratified subjects instead according to

whether or not they developed an acute exacerbation of their asthma defined as a 0.5 point fall in ACQ between screening and symptom day 7 (*n*=16 exacerbated, *n*=10 did not). The mean magnitude of the AUC was 1.6 fold greater in subjects who exacerbated, but this did not reach statistical significance (P=0.2 for AUC of T_H17 response and P=0.12 for AUC of T_H17:Treg ratio)(Figure 7.8).

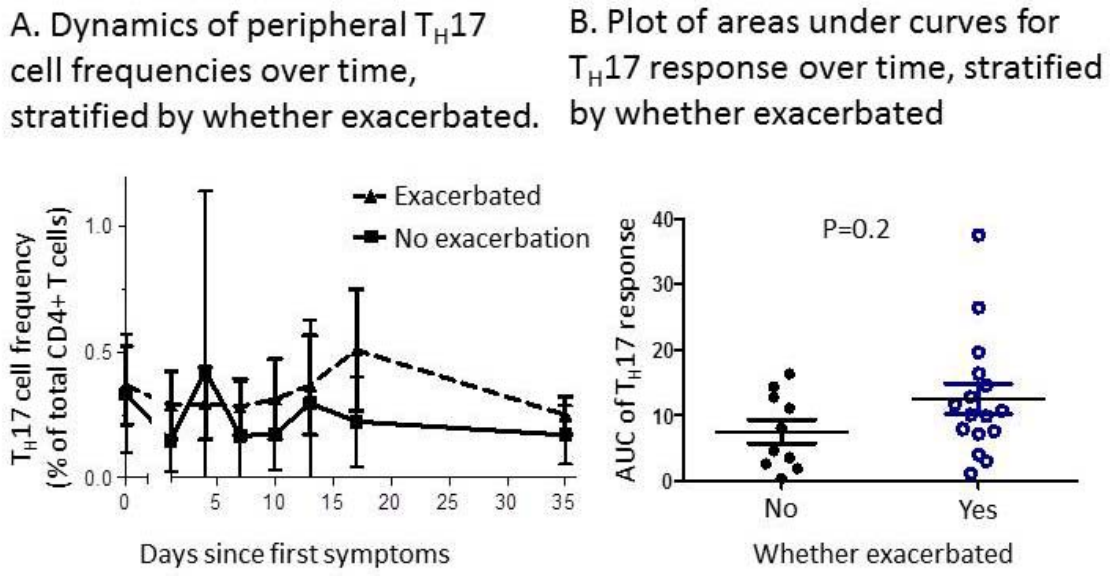


Figure 7.8 Peripheral blood TH17 response according to whether exacerbated

A. Peripheral blood T_H17 cell frequencies measured by FACS plotted over time and stratified by whether subjects experienced an exacerbation of their asthma defined as a 0.5 point fall in ACQ between screening and symptom day 7 (*n*=16 exacerbated, *n*=10 did not). Plots show mean ±95% confidence interval.

B.Plot of areas under the curve for peripheral T_H17 response over time, stratified by whether experienced an exacerbation. P=0.2 for unpaired t test.

Discussion

These longitudinal investigations into the dynamics of the immune response were undertaken with the aim of determining whether IL-17 and T_H17 cells are induced during naturally occurring asthma exacerbations, and elucidating how treatment with a type-I interferon influences T_H17 function. Two main conclusions can be drawn from the data presented in this chapter with respect to these aims and the challenges of investigating airway T cell response *in vivo* in humans.

Respiratory virus infections are not associated with a T_H17 response

I hypothesised that airway accumulation of T_H17 cells would occur early in infection, leading to neutrophilia, followed by a T_H1 dominant response. T_H17 cells are induced early in immune responses such as in early transplant rejection (Loeuillet, Martinon et al. 2006) or immunity to mycobacterial infections (Khader, Bell et al. 2007; Umemura, Yahagi et al. 2007; Khader and Cooper 2008) where they are present 3 days sooner than T_H1 cells (Khader, Bell et al. 2007). Indeed IL-17 can be produced even earlier by several innate-like cells such as iNKT cells (Rachitskaya, Hansen et al. 2008), MAIT cells (Dusseaux, Martin et al. 2011) and γδ T cells (Umemura, Yahagi et al. 2007; Khader and Cooper 2008). As the immune response matures and subsequently polarises into T_H1 or T_H2 cells these can then rapidly suppress the more transient T_H17 response (Khader and Cooper 2008). Furthermore T_H17 cells have been implicated in viral exacerbations because of their ability to recruit neutrophils (Linden 2001; Hellings, Kasran et al. 2003; Prause, Bozinovski et al. 2004; Oda, Canelos et al. 2005; Wiehler and Proud 2007), which are the predominant cell type in the airways during exacerbations (Message and Johnston 2001).

However, despite small numbers, my data do not show an early induction of a T_H17 response. In contrast to the 10-1000 fold induction of other anti-viral genes, I observed a mean maximum 3.7 fold induction of IL-17 in the RV16 challenge study. Whilst this study was small, challenge studies have the particular strengths of a well-defined pathogen and precise knowledge of the temporal course of the infection.

Similarly data from the study of naturally occurring exacerbations did not produce strong evidence of major changes in the T_H17 cell frequencies in peripheral blood in the cohort as a whole. Although T_H17 frequencies did increase in sputum, this change was of borderline statistical significance (ANOVA P=0.087, *post-hoc* t test P=0.034) and modest magnitude (1.8-fold). In addition IL-17 was not detectable at baseline or symptom day 4 in the serum from 58 asthmatics on BTS treatment step 4 and 5 in the SG005 study using a Luminex based assay (Multi-Analyte Profiling, Myriad RBM, Austin, TX, USA)(Monk 2012). Furthermore there were no significant differences in T_H17 responses between subjects who did and who did not develop an exacerbation. I did not include a group of healthy controls for comparison with asthma, but arguably the comparison between asthmatics who did or did not exacerbate is as informative.

Subjects who developed exacerbations in this study tended to be those who had more severe asthma consistent with previous studies which have shown that patients with poorer baseline control are more likely to experience exacerbations (Bateman, Bousquet et al. 2008). However it is interesting that the mean baseline T_H17 frequencies were identical amongst subjects who did or did not exacerbate (Figure 7.8) implying that baseline T_H17 cell frequencies are not a factor determining the risk of an exacerbation. Thus together these data challenge the hypothesis that asthma exacerbations might be characterised by a major dysregulation of IL-17 immune responses.

Perhaps this lack of a strong antiviral T_H17 response is because the predominant role of IL-17 is immunity against bacteria and fungi. A deficiency of T_H17 humans with heterozygous mutations in STAT3 is associated with increased susceptibility to *Staphylococcus aureus, Candida albicans* and bacterial pneumonias (Ma, Chew et al. 2008; Milner, Brenchley et al. 2008; Woellner, Gertz et al. 2010) rather than viral infections. Whilst IL-17 can induce molecules like β-defensin which has antiviral as well as antibacterial functions (Wolk, Kunz et al. 2004), it is not known how important T_H17 cells are for viral immunity *in vivo*. Indeed in an animal model T_H17 cells promoted persistent of a viral infection by inhibiting virus-induced apoptosis (Grajewski, Hansen et al. 2008). Probably the greater relevance of T_H17 cells to respiratory viruses is that virus induced suppression of IL-17 mediated by type I IFN may contribute to susceptibility to secondary bacterial and fungal infections, as has been demonstrated in a murine model of influenza infection (Kudva, Scheller et al. 2011).

Administration of inhaled rhIFN-β1α is associated with increased T_H17 frequencies in peripheral blood

It is because of this link between type I IFNs and IL-17 that the comparison of T_H17 responses between active- and placebo-treated groups is of interest. I hypothesised that administration of inhaled rhIFN-β1α would inhibit the magnitude of the T_H17 response to viral infection measured in PBMC and airway samples. Contrary to this hypothesis I observed the opposite: evidence of an increased T_H17 cell response in peripheral blood. In the context of the potential therapeutic use of rhIFN-β1α to prevent virus induced exacerbations of asthma, this is a reassuring finding.

Respiratory virus infections predispose to bacterial super-infections (Morens, Taubenberger et al. 2008; Weeks-Gorospe, Hurtig et al. 2012) and in the case of rhinovirus this may be through both disruption of epithelial barrier functions (Sajjan, Wang et al. 2008) and inhibition of T cell function (Gern, Joseph et al. 1996). Moreover asthmatics are at particularly high risk of these effects as severe asthma is a risk factor for invasive pneumococcal disease (Talbot, Hartert et al. 2005; Klemets, Lyytikainen et al. 2010) and inhaled steroids are associated with increased risk of pneumonia in subjects with airways disease (Calverley, Anderson et al. 2007; Crim, Calverley et al. 2009; Welsh, Cates et al. 2010). Therefore it would be a concern if rhIFN-β1α specifically impaired T_H17 cell responses.

Hypothetically T_H17 cells might be suppressed by IFN-β. In animal models type I IFN favours T_H1 (Orgun, Mathis et al. 2008; Shinohara, Kim et al. 2008) or T_H2 (Martin-Saavedra, Gonzalez-Garcia et al. 2008) differentiation over T_H17 and defects in the type I IFN receptor (IFNAR) lead to increased IL-17 levels (Guo, Chang et al. 2008). There is less known in humans, although there are some data related to the use of rhIFN-β1α intravenously for treating multiple sclerosis (MS). *In vitro* IFN-β (Durelli, Conti et al. 2009; Ramgolam, Sha et al. 2009) or supernatant from IFN-β-treated dendritic cells (Ramgolam, Sha et al. 2009) decreases T_H17 frequencies in PBMC, as well as decreasing RORC and IL-17A gene expression (Zhang, Jin et al. 2009). However the situation may be different *in vivo.* IFN-β can increase the survival of CD4 cells (van Boxel-Dezaire, Zula et al. 2010) and longitudinal follow up of 36 patients with MS found that treatment with IFN-β was associated with decreased mRNA for IFNγ and T-bet but no fall in IL-17 or RORC in peripheral blood (Drulovic, Savic et al. 2009). Given the difficulties of extrapolating to humans data from *in vitro* analysis of PBMC or from animal models due weight should be given to my data, which are the first to compare the effect on T_H17 cells of IFN-β compared with placebo *in vivo* in humans.

Conclusion

In summary I have supplemented my data from my cross-sectional analyses of T cell subsets with data on the dynamics of T_H17 cell frequencies, showing that virus-induced exacerbations of asthma are not associated with major fluctuations in T_H17 cell frequencies. Furthermore amongst subjects with URTIs I did not observe differences of significant magnitude in either the baseline T_H17 frequencies or the dynamics of the T_H17 response between subjects who developed an asthma exacerbation and those which did not. The use of rhIFN-β1α is not associated with a suppression of T_H17 cell immunity, but rather with a potentially beneficial increase in peripheral T_H17 cell frequencies.

These studies have highlighted the considerable challenges to investigation of the dynamics of the airway immune responses *in vivo*. The rates of successful sputum inductions were low in both studies, consistent with other asthma studies (Boniface, Koscher et al. 2003; Yoshida, Watson et al. 2005; Papadopouli, Tzanakis et al. 2006; Mamessier, Lorec et al. 2007; Mamessier, Milhe et al. 2007; Mamessier, Nieves et al. 2008) This is particularly problematic for the study of rare cell populations and for intracellular cytokine staining which induces high rates of apoptosis. These limitations constitute significant obstacles for longitudinal studies which are especially sensitive to missing data (Matthews, Altman et al. 1990). Finally an additional obstacle to the conduct of challenge studies with rhinovirus is the high prevalence of pre-existing, cross-reactive humoral immunity.

These findings support the conclusions from my cross-sectional study that IL-17 and T_H17 cells do not play a significant role in the pathogenesis of human asthma.

CHAPTER 8

Discussion

I was merely thinking God's thoughts after him. Since we astronomers are priests of the highest God in regard to the book of nature, it benefits us to be thoughtful, not of the glory of our minds, but rather, above all else, of the glory of God.[8]

[8] Johannes Kepler (1571-1630)

My primary aim was detailed investigation of T cell phenotypes in asthma in relation to severity and virus-induced exacerbations, with a particular focus on interleukin-17 T_H17 cells and MAIT cells. My intent was to translate advances in basic science and animal models into humans *in vivo* and to improve characterisation of severe asthma versus milder forms of asthma, thereby facilitating future progress in basic and applied research. In this final chapter I will summarise the findings of this present work and their implications for our understanding of asthma in a wider context. I will then discuss their implications for future research, some of which is in progress already.

The fundamental role of T_H2 inflammation in asthma

Recent decades have witnessed a rapid expansion in our understanding of the variety of different innate and adaptive T cell subsets (Shevach 2006; Lloyd and Hessel 2010), yet the cross-sectional data presented in chapter 3 have again highlighted the pre-eminent role of T_H2 cells in asthma, which remains unchallenged 20 years after their initial recognition (Robinson, Hamid et al. 1992). In extending such investigations to a wider spectrum of asthma phenotypes, my work has revealed diversity in the patterns of T cell responses underlying distinct endotypes, such as the lower peripheral blood T_H2 bias in non-atopic asthma, or the deficiencies in BAL Treg, or sputum and biopsy MAIT cells in more severe asthma. The study of T_H2 cells in asthma is therefore likely to continue to prove fruitful. Future work should focus on the mechanisms which can influence the persistence of a T_H2 response, such as epigenetic effects (Vijayanand, Seumois et al. 2012) which are potentially amenable to pharmacological modulation or, immunotherapy (Robinson, Larche et al. 2004; Larche 2007; Roncarolo and Battaglia 2007).

The history of interleukin-17 and T_H17 cells in asthma highlights research pitfalls

Using a number of techniques in a wide range of subjects and clinical samples I found little evidence to support the now widely hypothesised role of IL-17 in asthma, nor have I found evidence that T_H17 cells could be central players in chronic disease. These findings will, I hope, focus the attention of other researchers away from this particular avenue. My data do suggest that IL-17 may be elevated in a subset of mild, steroid naïve asthmatics, who suffer from allergic rhinitis and perhaps represent a distinct endotype. My data also do not negate the findings by others that IL-17 mRNA or protein may be raised in various samples of the asthmatic airways, so any further investigation of IL-17 should address this specific phenotype and should focus on epithelial cells or eosinophils within the respiratory mucosa as these are the most likely sources of the cytokine (Chakir, Shannon et al. 2003; Doe, Bafadhel et al. 2010; Vazquez-Tello, Semlali et al. 2010; Howarth 2012; Jayasekera 2013).

IL-17 is frequently considered as a mediator of immune pathology, because of its importance in inducing pro-inflammatory cytokines (Fossiez, Djossou et al. 1996) and in recruiting neutrophils (Sergejeva, Ivanov et al. 2005; Fujiwara, Hirose et al. 2007; McKinley, Alcorn et al. 2008). However this concept is an oversimplification as IL-17 has also been shown in animal studies to have protective roles, functioning as a negative regulator of established inflammation (Schnyder-Candrian, Togbe et al. 2006; Braun, Ferrick et al. 2008; O'Connor, Kamanaka et al. 2009; Murdoch and Lloyd

2010). Therefore future studies should include investigation of the dynamics of IL-17 in immune responses such as acute allergen challenge and also investigation of the functional effects of IL-17 *in vivo* perhaps by correlating with down-stream effects on immunological networks, such as by analysis of the transcriptome of epithelial cells and other effector cells.

The negative findings from my studies of T_H17 and γδ-17 cells constitute a refutation of what is at present a popular hypothesis. This eventual failure of the T_H17 hypothesis raises some general issues and in particular underscores two pitfalls in asthma research. Firstly there is a danger in pursuing hypotheses founded on clinical data based on animal models and relatively small human studies. As I have outlined in the discussion of chapter 3 more than a decade of basic science and animal research has been founded on evidence from just three clinical studies which have together been cited over 150 times in the literature (Molet, Hamid et al. 2001; Barczyk, Pierzchala et al. 2003; Chakir, Shannon et al. 2003). These studies were of small size, with two enrolling only 6-10 asthmatics and the other only including 6 healthy controls. They did not use the best techniques available at the time - such as multi-colour flow cytometry (Krug, Madden et al. 1996) – nor robust statistical analysis (Barczyk, Pierzchala et al. 2003) and crucially the data were never subsequently confirmed by other investigators or even by data from the same groups.

The absence of robust human data has led in this case to another pitfall, that of overreliance on animal models (Lloyd and Hessel 2010; Holmes, Solari et al. 2011) (Schnyder-Candrian, Togbe et al. 2006; Wakashin, Hirose et al. 2008; Wilson, Whitehead et al. 2009; Lloyd and Hessel 2010; Murdoch and Lloyd 2010). Whilst these animal studies have been conducted to high standards and provided fascinating insights into general T cell biology it can often be very difficult to extrapolate their findings into a complex and uniquely human disease such as asthma. No animals, except perhaps cats or horse, are known to suffer from asthma (Holmes, Solari et al. 2011) and even within a species there are significant differences in outcomes between different strains and different immunisation protocols. These protocols depend on unphysiological sensitisation procedures such as intraperitoneal injection of high dose allergen in the presence of adjuvants (Zosky and Sly 2007), which are very unlike the natural history of human asthma. Furthermore the allergic airways inflammation which ensues is arguably more reminiscent of allergic alveolitis than asthma (Zosky and Sly 2007). Finally, few models take account of the agents which usually trigger asthma exacerbations in humans such as infections, air pollution, diet, tobacco smoke, drugs and other chemicals (Holmes, Solari et al. 2011).

An alternative approach is the development of *ex vivo* human models of airway immunology. Such models are becoming more complex with the development of air-liquid interphase cultures of primary bronchial epithelial cells (Swindle, Collins et al. 2009) which can retain important genetic and epigenetic features of the human source and which are currently being developed to include interactions with stromal cells and matrix, as well as the use of micro-fluidics to emulate the dynamics of inflammatory cell influx (Swindle and Davies 2011) which will potentially enable modelling of dynamic T cell-epithelial cell interactions. An alternative to this synthetic approach to modelling is the

use of whole tissue explant cultures which have the advantage of maintaining all the tissues cell types of the mucosa in their entirety without destroying their functional networks (Nicholas, Staples et al. 2013). These models provide a potential platform for exploring truly integrated systems biology.

It is, also, likely that the application of a systems biology approach to assess whole interacting networks of cytokines and inflammatory cells is going to be necessary to advance our understanding of complex diseases such as asthma beyond what can be understood from a conventional reductionist study of individual cells or cytokines (Sabroe, Parker et al. 2007; Cookson and Moffatt 2011; Zhang, Moffatt et al. 2012).

In conclusion, further research into the mechanisms, aetiology and clinical phenotypes of asthma must always be driven by observations arising first from high quality, large scale studies in humans, supplemented with novel, disease relevant *ex vivo* human models and the application of systems biology.

A renewed interest in CD8+ T cells in asthma is warranted

Data presented in chapter 4 demonstrated an important relationship between CD8+ T cells and asthma, and are a timely reminder that these somewhat neglected cells should merit further research. Such investigation should focus on the specific clinical endotypes in which they are implicated by my data: subjects with eosinophilic asthma and a history of nasal polyps and smoking. The relationship with smoking and nasal polyps suggests potential mechanisms which might lead to the development of Tc2 inflammation, such as smoking-related oxidative stress (Pierrou, Broberg et al. 2007), or nasal colonisation with pathogenic bacteria such as *S.aureus* which produces staphylococcal enterotoxins associated with allergic rhinitis, nasal polyps and asthma (Bachert, Gevaert et al. 2007). An important issue to research is the nature of the antigen specificity of these CD8+ cells to determine whether their primary specificity is to respiratory, to colonising bacteria or to aeroallergens. Such work should also include functional studies exploring the potential to reverse the T_H2-induced reprogramming of virus-specific CD8+ T cells which may contribute to their pathogenic effects (Coyle, Erard et al. 1995; Chatila, Li et al. 2008). Already the potential for CD8+ T cell-mediated immunotherapy has been demonstrated in animal models in which antigen conjugated to cationic liposome-DNA suppressed AHR, eosinophilia and goblet cell metaplasia through the induction of allergen-specific Tc1 cells (Takeda, Dow et al. 2009), giving hope that human studies may be an imminent prospect.

The need for the application of deep sequencing to the study of asthma

The development of high throughput sequencing technologies (Margulies, Egholm et al. 2005) has enabled a step-change in our ability to characterise complex microbial communities. This method was rapidly translated from its original applications in the analysis of marine ecology to the characterisation the human oral (Zaura, Keijser et al. 2009) and gastrointestinal (Willing, Dicksved et al. 2010) microbiome, generating new insights into the mechanisms of complex diseases such as inflammatory bowel disease (Willing, Dicksved et al. 2010), diabetes (Serino, Luche et al. 2012) and

obesity (Henao-Mejia, Elinav et al. 2012). Yet the respiratory community have been very slow to adopt these tools. Indeed this year the Human Microbiome Project Consortium published data from 4788 specimens from 242 phenotyped adults, providing a reference atlas of human ecology covering 18 anatomical niches including oral, skin, lower GI and urogenital tracts but no respiratory samples were included(Peterson, Garges et al. 2009; Nelson, Weinstock et al. 2010; 2012). To date only one study has been published in asthma and this used the older technology of 16sRNA sequencing (Hilty, Burke et al. 2010) rather than the whole genome sequencing approach I have used. Given the emerging evidence implicating both the acquisition of commensal flora in early life (Bisgaard, Hermansen et al. 2007; Thavagnanam, Fleming et al. 2008; Roduit, Scholtens et al. 2009) and the composition of the faecal microbiome (Maeda, Noda et al. 2001; Noverr, Noggle et al. 2004; Maslowski, Vieira et al. 2009; Bisgaard, Li et al. 2011; Ichinohe, Pang et al. 2011; Vael, Vanheirstraeten et al. 2011; Han, Huang et al. 2012) in the pathogenesis of asthma there is an urgent need for respiratory researcher to catch up with the rate of progress being made in other fields.

My data demonstrate the power of whole genome metagenomics to characterise the airway viral and microbial flora in their entirety. The data argue against a hypothesised role for chronic viral persistence in asthma (Wos, Sanak et al. 2008) and against the proposed existence of a core airway commensal microbial community in health or in asthma (Hilty, Burke et al. 2010). Instead they suggest that a minority of individuals with severe asthma may be suffering from chronic infection with specific respiratory pathogens or opportunistic infections. As a consequence future research should aim to apply this technique on a larger scale to a wide spectrum of asthmatic subjects with the aim of defining which are the common causative organisms in this chronic infections, what are the causal risk factors for development of these infections and what might be biomarkers to identify such individuals in clinic in routine practice. Although some of the patients that I studied had severe asthma, it is likely that different results, i.e. a larger microbiome, may be found in sub-phenotypes of severe asthma, e.g. patients with chronic expectoration, patients with bronchiectasis and smoking asthmatics who likely have elements of chronic bronchitis. Once such a comprehensive, unbiased survey has been completed the data could then be used to produce simpler diagnostics for focussed sets of identified pathogens, such as multiplexed PCR kits. These should then be validated in prospective clinical trials which include antibiotics as interventions and are linked to clinically important outcomes. Such work would likely to be of significant benefit to a small subset of subjects with severe asthma. However, in addition community prescription of antibiotics is widespread. Subjects in the exacerbation study had received a median 2 (IQR 1-4) doses of antibiotics in the preceding 2 years and thus an additional and related priority for future research should be the development of an evidence base for the rational use of antibiotic prescription for the treatment of asthma exacerbations in the community. Again this should include the use of biomarkers and be linked to a prospective interventional trial. My results show that raw BAL fluid has a low biomass so future bronchoscopy studies should use either brushings or consider using ultracentrifugation to concentrate the microbial content prior to sequencing. Finally future research into the respiratory

microbiome should include carefully planned controls for environmental contamination (Charlson, Bittinger et al. 2011) as well as obtaining paired samples of the faecal microbiome.

MAIT cells as a priority for future research

This study is the first to investigate the role of MAIT cells in the human lung. I observed a selective deficiency of MAIT cells in asthma, which was not related to age, but was exacerbated by systemic corticosteroids and was subject to seasonal variation, indicating their possible regulation by vitamin D. I established MAIT clones which allowed me to observe the heterogeneity of cytokine expression profiles and also represent proof of concept for the ability to develop MAIT clones which will constitute a key tool for future MAIT cell research. The high degree of evolutionary conservation of the MR1 restriction molecule (Brossay, Chioda et al. 1998; Treiner and Lantz 2006) implies these poorly understood cells perform some key immunological functions, which are yet to be defined. The recent discovery that the MR1 binding grove can recognise microbially-derived vitamin B metabolites (Kjer-Nielsen, Patel et al. 2012) and their association with mucosal surfaces (Treiner, Duban et al. 2003; Ruijing, Mengjun et al. 2011) provides strong evidence that the role of these cells is related to the interaction between the immune system and microbes at mucosal surfaces. It is therefore likely that they will be of relevance to airway host defence in conditions such as acute pneumonia, invasive bacterial infection, pulmonary tuberculosis and bronchiectasis, in addition to their relationship with severe asthma.

Future work

To conclude this thesis I will discuss future research questions, beginning with projects which I have initiated already.

Deep sequencing of the microbiome during exacerbations

Whilst the deep sequencing data presented did not identify respiratory viruses during clinically stable disease, such analysis of samples from subjects with symptomatic viral infections will certainly yield very different results. Through the collaboration I have established with Prof Virgin (Washington University School of Medicine, Saint Louis, MO) I am arranging the sequencing of samples of sputum and nasal lavage obtained from the longitudinal study of acute viral exacerbations. Samples will be available from all centres which participated in this multi-centre trial. Amongst 134 subjects with clinically confirmed URITs (with a Jackson cold score >14 on two consecutive days), a virus was not detected in 37% of nasal lavage samples tested by PCR for 21 common respiratory viruses. These samples may contain rare or previously undiscovered pathogens. The use of an unbiased whole-genome approach using 454 pyrosequencing or the newer Illumina HiSeq platform, coupled with a data analysis pipeline tailored to virus pathogen discovery (Zhao) will enable characterisation of these previously undiagnosed viral illnesses.

An integrated systems biology approach to the analysis of transcriptomic data obtained from microarray of epithelial cells and pure T cell populations

I have obtained epithelial brushings and sorted pure populations of T cells and MAIT cells from PBMC, sputum, and BAL which have been kept in RNA lysis buffer and sent to my collaborators Janssen Research & Development (Springhouse, Pennsylvania) for RNA extraction and transcriptomic analysis. To date 166 T cell samples (*n*=42 epithelial cells, 42 PBMC, 46 BAL, 24 sputum and 12 sputum samples after ICS) of cDNA have been extracted, passed quality control thresholds and been successfully hybridised to the Affymetrix GeneChip Arrays. I will shortly be able to analyse the results in collaboration with Janssen Research & Development, using pathway analysis tools and the tranSMART knowledge management platform. This large set of paired samples from highly phenotyped individuals will provide a powerful data-set in which to explore the distinct activation signatures of the innate (epithelial cell), adaptive (CD3+ T cell) and innate-like (MAIT cell) immune systems in asthma. Furthermore the pairing of the samples will enable me to explore the interactions between these different cell types, for example the relationship between T cell cytokines and their down-steam induction of effector pathways, and these different tissue compartments. I wish to maximise the potential for using a fully integrated systems biology approach to generate new hypotheses from these data-sets. Hence, through the use of the tranSMART knowledge platform I would aim further to explore relationships between these transcriptomic data and the associated immunological data (such as multiplex ELISA data I have already obtained from paired serum, BAL and sputum samples) to correlate analyses at the transcriptomic and protein-levels. A further aim would be to attempt to identify asthma endotypes through an unbiased statistical analysis of these transcriptomic data. Together these aims constitute an ambitious undertaking, but I believe are the essential next step in deepening our understanding of complex diseases.

A characterisation of the function of MAIT cells in human lung diseases

The data I have presented on MAIT cells provoke several questions which I wish to address in the future.

1. Do serum levels of vitamin D3 influence MAIT cell frequencies in peripheral blood? The seasonal variation in MAIT cell frequencies and inferences from iNKT biology (Yu and Cantorna 2011; Yu, Zhao et al. 2011) suggests a possible relationship between serum vitamin D3 levels and MAIT cell frequencies. I am currently awaiting the analysis by mass spectrometry of vitamin D3 levels in 86 serum samples paired to the PBMC MAIT cell frequencies, which may provide definitive evidence to test this hypothesis. If the data are suggestive then it would be interesting to explore the effect of vitamin D3 on MAIT cell lines *in vitro*.
2. I have found that MAIT cells are deficient in asthma. Is this due to MAIT cells migrating into the lung during inflammation and then undergoing activation-induced apoptosis? Can these cells recover during periods of clinical stability? If so, as these cells are readily detectable in peripheral blood, might they be useful as a biomarker of disease activity?

3. Are MAIT cells involved in barrier immunity in the lung? The discovery that microbial-derived vitamin B metabolites may act as ligands for MR1 implicates MAIT cells in antimicrobial defense. Therefore it is pertinent to investigate associations between MAIT frequencies in chronic inflammatory lung diseases such as chronic infection, COPD and bronchiectasis. Clinical observations could be complemented by *ex vivo* modeling of MAIT cell responses to bacteria in explanted tissue. Could MAIT-targeting approaches be used therapeutically in such chronic diseases?
4. I have found that high dose steroids can reduce MAIT cell frequencies. What is the dose-response relationship? Can chronic low-dose ICS have a similar effect? Do steroids affect not just the frequency, but also the function of MAIT cells?
5. What are the other functions of MAIT cells? Are they present in lung tumours, and if so, what role do they play? Are they deficient in some patients with idiopathic bronchiectasis? Are MAIT cells in the upper airway mucosa associated with invasive pneumococcal disease?

I have the tools necessary to investigate these cells in greater depth including antibodies for MAIT cells and MR1 and the ability to clone MAIT cells. With the recent description of a vitamin B metabolite 6-formyl pterin as a ligand for MR1 (Kjer-Nielsen, Patel et al. 2012) one of my supervisors, Prof Gadola, is already developing the protocols to refold MR1 round 6-formyl pterin and other possible ligands, which could lead to the development of another essential tool: tetramers for MAIT cells. Thus MAIT cells constitute an emerging research area in T cell biology with the potential to rapidly expand and we are well placed to be at the forefront of this stimulating field of research.

References

The fat boy stood by him, breathing hard.

"My auntie told me not to run," he explained, "on account of my asthma."

"Ass-mar?"

"That's right. Can't catch me breath. I was the only boy in our school what had asthma," said the fat boy with a touch of pride.[9]

[9] The protagonist - Piggy's – childhood asthma, which was considered a rare condition at the time, famously distanced him from his peers in Lord of the Flies, for which William Golding won the 1983 Nobel Prize for literature. Golding, W. (1954). Lord of the Flies, Faber. p9

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