UNIVERSITY OF SOUTHAMPTON

FACULTY OF MEDICINE

Clinical and Experimental Sciences

Expression and function of Programmed Cell Death Protein-1 (PD-1) and ligand PD-L1 in Chronic Obstructive Pulmonary Disease

by

Richard Thomas McKendry

Thesis for the degree of Doctor of Philosophy

September 2014

Abstract

Chronic Obstructive Pulmonary disease (COPD) is an irreversible, progressive disease resulting in a permanent loss of lung function. COPD is characterised by an enhanced inflammatory response to noxious particles in a manner which is harmful to the individual. This inflammatory response alone may cause damage to the lungs, but COPD patients are also susceptible to recurrent respiratory viral infections which cause disease exacerbations and hasten lung function decline. Mechanisms underlying this susceptibility are not understood, however key effectors of the adaptive immune response; CD8+ T cells which clear viral infections, are present in increased numbers in COPD patients. These cells, however, fail to resolve infection and contribute to the immunopathology of the disease. Failure to clear infection may be due to T cell "exhaustion" induced by chronic antigen exposure resulting in the inhibition of cytotoxic function. T cell exhaustion was proposed as a potential mechanism which allows for chronic viral infection in the lungs.

Analysis was performed using blood and lung parenchymal tissue from controls and patients with COPD. Expression of cell surface markers associated with exhaustion, senescence, tolerance and cytotoxicity were measured using flow cytometry and RT-PCR. ELISpot was performed using a monocyte-derived macrophage model of acute infection to measure IFNγ responses. Regulation of responses by corticosteroids, retinoic acid and αPD-L1 blocking antibody was recorded.

T cells in lung were predominantly of a memory phenotype, with a greater proportion of CD8+ T cells in lungs of COPD patients. PD-1 expression is upregulated by T cells in response to influenza infection, but only macrophages, not epithelial cells, upregulate PD-L1. CD8+TEMRA populations appear to express dysfunctional cytotoxic responses to influenza in COPD tissue but not in controls. T cell derived IFNγ is produced in response to X31 infection, but this is abrogated by corticosteroid or retinoic acid treatment. Blocking of PD-1:PD-L1 interactions may upregulate IFNγ responses in the CD8+ T cell population.

This work has established novel experimental techniques to interrogate lung specific immune responses pertinent to live viral infections. It has established a signal of immune dysfunction in COPD which may explain the susceptibility to infection and set up a clear direction for further study to better establish our understanding of this important disease.

Table of Contents

List of Figures

List of Tables

Presentations to Learned Societies

Richard T McKendry, C. Mirella Spalluto, Ben L Nicholas, Karl J Staples, Tom MA Wilkinson (2014)
T cell exhaustion marker expression in the human lung in health and COPD in response to influenza infection
British Association for Lung Research Summer Conference, London, UK
Young Scientist Competition, Finalist – Oral Presentation

Richard T McKendry, C. Mirella Spalluto, Karl J Staples, Tom MA Wilkinson (2013)
Dynamics of viral infection in human lung tissue
European Respiratory Society International Congress, Barcelona, Spain
– Oral Presentation

Richard T McKendry, C. Mirella Spalluto, Karl J Staples, Tom MA Wilkinson (2014)
Modulation of PD-1 and PD-L1 expression in response to acute viral infection of human lung tissue
European Respiratory Society International Congress, Munich
– Poster Presentation

McKendry, RT, Staples, KJ, Spalluto CM, Wilkinson, TMA (2014)
Impact of COPD on PD-1/PD-L1 expression in an ex vivo model of acute influenza virus expression
European Respiratory Society Lung Science Conference, Estoril, Portugal
– Poster Presentation

Richard T McKendry, C. Mirella Spalluto, Karl J Staples, Tom MA Wilkinson (2012)
PD-1 expression on human lung T cells in health and COPD
British Thoracic Society Winter Meeting, London, UK
– Poster Presentation

Academic Thesis: Declaration Of Authorship

I, Richard Thomas McKendry
declare that this thesis and the work presented in it are my own and has been generated by me as the result of my own original research.

"Expression and function of Programmed Cell Death Protein-1 (PD-1) and ligand PD-L1 in Chronic Obstructive Pulmonary Disease"

I confirm that:
1. This work was done wholly or mainly while in candidature for a research degree at this University;
2. 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;
3. Where I have consulted the published work of others, this is always clearly attributed;
4. 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;
5. I have acknowledged all main sources of help;
6. 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;
7. None of this work has been published before submission

Signed:

Date:

Acknowledgements

I wish to thank my supervisors Dr. Tom Wlikinson, Dr. Karl Staples and Dr. C. Mirella Spalluto for their tireless supervision and invaluable guidance. I wish to thank the members of the PI group and the Inflammatory Cell Biology Group – Dr. Ben Nicholas, Dr. Tim Hinks, Doriana Cellura, Dr. Hannah Burke, Dr. Kamran Tariq, Pam Sunder, Josh Wallington and Achika Keys – whose dedication, support and kindness during challenging times will be fondly remembered. Further thanks is in order to Prof. Ratko Djukanović for allowing me the use of his laboratory and the apparatus within. Flow cytometry work could not be performed without the knowledge and effort provided by Richard Jewell and Carolann McGuire to maintain FACS facilities at Southampton at optimum conditions.

I wish to thank research and nursing staff of Southampton Biomedical Research Unit for their successful efforts to recruit patients into my study, for which my work is indebted. Furthermore I wish to express my gratitude toward Dr. Jane Warner and her group – Mel Jannaway, Andy Hutton, Sam Maitland and Jess Rajaram – who also played key roles in the acquisition of human lung tissue.

RT-PCR analysis of sorted CD4+ and CD8+ T cells from tissue was performed by Dr. C. Mirella Spalluto and Miss. Doriana Cellura.

I wish to recognise Dr. Charles McSharry of The University of Glasgow for his encouragement to peruse academic research in the field of lung disease, despite the difficulties that it might bring.

I am immeasurably grateful to the generous participation of all research volunteers involved in my work, especially those who agreed to enter the study while preparing for major surgery.

I wish to thank my parents for putting my education and wellbeing above all else, for giving me the chance to fulfil my potential whatever that may be, and for their love and support. Finally I wish to thank Maria Concetta Zarcone for her continual support and encouragement.

This work was made possible by the MRC for the provision of a PhD stipend to support me during my 4 years of research at Southampton University

List of Abbreviations

APC	Antigen Presenting Cell
BAL	Bronchoalveolar Lavage
BSA	Bovine Serum Albumin
CD	Cluster of Differentiation
COPD	Chronic Obstructive Pulmonary Disease
Ct	Threshold Cycle
CT	Computed tomography
CTLA-4	Cytotoxic T-Lymphocyte Antigen 4
DCs	Dendritic Cells
DNA	Deoxyribonucleic Acid
DPBS	Dulbecco's Phosphate-Buffered Saline
EDTA	Ethylenediaminetetraacetic Acid
ELISpot	Enzyme-Linked ImmunoSpot assay
ETS	Environmental Tobacco Smoke
FACS	Flow Activated Cell Sorter
FEV_1	Forced Expiratory Volume in One Second
FT	Fluticasone
FVC	Forced Vital Capacity
GOLD	Global Initiative for Chronic Obstructive Lung Disease
HCV	Hepatitis C Virus
HIV	Human Immunodeficiency Virus
IFNγ	Interferon Gamma
IgE	Immunoglobulin E
IHC	Immunohistochemistry
IL-	Interleukin
ITIM	Immunoreceptor Tyrosine-based Inhibition Motif
ITSM	Immunoreceptor Tyrosine-based Switch Motif
LAG-3	Lymphocyte-activation gene 3
LCMV	Lymphocytic Choriomeningitis Virus
mAb	Monoclonal Antibody
MACS	Magnetic-activated Cell Sorting

MDM	Monocyte-derived Macrophage
MFI	Mean Fluorescence Intensity
MHC	Major Histocompatibility Complex
MIB	Monocyte Isolation Buffer
MMP	Matrix Metalloproteinase
mRNA	Messenger RNA
NHANES	National Health and Nutrition Examination Survey
NI	Non-infected
NICE	National Institute for Health Care Excellence
NP-1	Influenza Nucleoprotein-1
NTHi	Nontypeable Haemophilus Influenzae
PAMPs	Pathogen-associated Molecular Patterns
PBMCs	Peripheral Blood Mononuclear Cells
PD-1	Programmed Cell Death Protein-1
PD-L1/2	Programmed Cell Death 1 Ligand 1/2
PMA	Phorbol 12-myristate 13-acetate
RA	Retinoic Acid
RAR	Retinoic Acid Receptor
RNA	Ribonucleic Acid
RPMI 1640	Roswell Park Memorial Institute 1640
RSV	Respiratory Syncytial Virus
RT- PCR	Reverse Transcription Polymerase Chain Reaction
RV	Rhinovirus.
SFCs	Spot Forming Colonies
SHP-1/2	Src Homology Region 2 Domain-containing Phosphatase-1/2
SIV	Simian Immunodeficiency Virus
TCR	T Cell Receptor
TGFβ	Tumour Growth Factor Beta
TIM-3	T Cell Immunoglobulin-3
TLR	Toll-like Receptor
Tmem	T memory Cells
TNF-α	Tumour Necrosis Factor Alpha
Treg	T regulatory cell
UVX31	UV-irradiated X31 influenza A virus

Chapter 1

Introduction

Chronic Obstructive Pulmonary disease (COPD) is an irreversible progressive disease resulting in permanent loss of lung function. It is characterised by persistent airflow limitation and innate and adaptive immune cell infiltration into the lungs. COPD individuals experience recurrent viral infections accompanied with lung inflammation resulting in disease exacerbations. Exacerbations of COPD are characterised by a sudden decline in lung function, often require hospitalisation, and may result in death. The recruitment of inflammatory cells, specifically CD8+ T cells, does not correlate with protection from infection, and in fact is associated with a decline in FEV_1. It is proposed that immune regulatory mechanisms designed to limit immunopathology and induce tolerance may prove detrimental to the host, preventing efficient viral clearance and permitting viral persistence. Chronic infection of the lungs may result in T cell "exhaustion", a progressive mechanism of T cell dysfunction. Targeting of this novel immune phenomenon may recover anti-viral T cell function and allow for increased protection of the COPD lung.

This thesis concentrates on improving our understanding of key immune mechanisms pertinent to COPD. It focuses on the responses of CD8+ T cells present in the lung after acute infection. Experimental models and clinical material were utilised to analyse T cell expression of exhaustion markers and functional markers, together with production of Interferon γ (IFNγ) in response to influenza challenge. This introduction will detail the classification of COPD, its disease characteristics and the impact it has upon society. Key cell types implicated in the manifestation, progression and exacerbation of disease will be discussed and the novel phenomenon of T cell exhaustion will be contextualised to disease.

Prevalence and Incidence of COPD

COPD is the 3rd highest cause of mortality in the United States of America (Han, 2011), and is predicted to be the 4th leading cause of death worldwide within the next 20 years (Mathers and Loncar, 2006). A meta-analysis by Halbert *et al.* (Halbert et al., 2006) of 62 studies published in journal articles between 1994 and 2000 estimate that between 9-10% of individuals ≥40 years old have COPD globally. The authors detail the limitations of their study due to the non-uniform methods of data collection and analysis by the studies included. Several studies excluded individuals with clinically diagnosed asthma, spirometry was not always performed after post-bronchodilator administration (which does not account for the effect of bronchial hyper-responsiveness from spirometry results on lung function) and

definitions of COPD which were not GOLD standard were included. Furthermore the studies analysed were mainly performed in the United States and Europe, making a reliable global estimate of COPD prevalence difficult. However a global consensus on the importance of this disease is emerging; the Burden of Obstructive Lung Disease (BOLD) Initiative estimate a worldwide incidence of The Global Initiative for Chronic Lung Disease (GOLD) stage II severity or greater COPD in 10.1%, with 11.8% of males and 8.5% of women affected (Buist et al., 2008). This study analysed data from standardised methods from 12 sites throughout the world, with a minimum of 600 participants per site. A secondary objective from the study showed that increased smoking pack years correlated with increased diagnosis of COPD, and this was evident at sites from all socio-economic strata. Pack years is a generalised measurement of smoking quantity of an individual. The number of cigarettes packs smoked (20 cigarettes = 1 pack) is divided by the number of smoking years to determine the degree of smoking. Prevalence of COPD may still be underestimated due to under-reporting (van den Boom et al., 1998), as individuals may be symptomatic in their younger years and be reluctant to contact their physician. These figures show that COPD affects a sizeable number of people, but more concerning is the impact this disease has on the health and quality of life of the individual.

Social and economic burden of COPD

The chronic nature of COPD creates a longitudinal and worsening profile of symptoms and disability. This often requires the use regular medication after diagnosis, and exacerbations of disease may lead to hospitalisation depending on severity. It is difficult to estimate the economic cost of COPD as the impact of direct treatments, loss of earnings and disease severity must be considered for individuals and the medical establishment. Most economic studies focus on the cost of COPD to the United States of America, and a widely accepted estimate of cost is between $20–25 billion per annum from data recorded between 1994 and 2004 (Leigh et al., 2002; Ward et al., 2000; Wilson et al., 2000). Disease exacerbations require additional drugs for their control and potential hospitalisation (Andersson et al., 2002), while the cost of medical care increases with disease severity (Chapman et al., 2006). COPD exacerbations requiring hospitalisation account for the greatest proportion of financial outlay, ranging from 40% to 63% of total medical costs which increases with disease severity (Hilleman et al., 2000). Individuals with COPD are less likely to be in employment (Sin et al., 2002; Thornton Snider et al., 2012), and those that do work lose working days because of

illness attributed to COPD (Halpern et al., 2003). Beyond the working environment the quality of an individual's life decreases as COPD severity increases due to a loss of independence and freedom of mobility (Stahl et al., 2005), but also that the disease affects family and friends who act as carers during all stages of disease (Seamark et al., 2004).

Evolving definition of COPD

It is difficult to pinpoint the real onset of COPD due to the complexity of the condition and the gradual development of pathological changes and associated physiological limitations. This complexity arises as COPD is a term used to encompass several respiratory conditions and its definition has been altered on many occasions since its original inception. Individuals with COPD generally have diseases of lung parenchymal tissue - emphysema, diseases of the small airways, large airway mucosal diseases - termed bronchitis, abnormal respiratory inflammatory responses and dysfunctional epithelial and immune cells within the lung. These factors vary in severity for different individuals, but all contribute to impaired pulmonary function. These conditions arise due to a combination of environmental and genetic factors, but these have not been fully elucidated. COPD does not include diseases such as cystic fibrosis, idiopathic pulmonary fibrosis or bronchiectasis. Thus the definition of "COPD" has evolved with the increasing knowledge of research scientists and clinicians. The detailed description of elements contributing to COPD can be found in literature dating from early in the 19th century. Charles Badham is credited as being the first individual to identify and characterise bronchitis as a distinct respiratory disease, and the condition is described in An Essay on Bronchitis originally published in 1808, with an updated 2nd edition published in 1814 (Badham, 1814). In this work, key characteristics of what would later be termed COPD are described, such as its inflammatory component contributing to disease, mucus hyperplasia and chronic cough without fever. Badham also described the debilitating affect upon individuals with chronic lung disease, and also the potential for disease symptoms to suddenly increase with fatal consequences. Soon after, René Laënnec, inventor of the stethoscope, published work characterising emphysema in the Treatise of Diseases of the Chest in 1821 (Laënnec, 1821). These two works combined form the core of what comprises a modern definition of COPD. The disease mechanisms which are present in COPD have not been easily agreed upon. Despite a gap of only three years, the CIBA Guest Symposium in 1959 (CIBA, 1959) and the American Thoracic Society Committee on Diagnostic Standards in 1962 produced two different definitions of COPD, underlying the complexity of the

disease. Technological developments and utilisation of spirometry has improved global understanding of COPD, and as research unveils new paradigms of disease so too will the definition of COPD evolve in response. The most current and widely accepted definition of COPD was published by The Global Initiative for Chronic Lung Disease in 2014 (GOLD, 2014):

"Chronic Obstructive Pulmonary Disease (COPD), a common preventable and treatable disease, characterised by persistent airflow limitation that is usually progressive and associated with an enhanced chronic inflammatory response in the airways and the lung to noxious particles".

Spirometry as a tool for diagnosing COPD

With several components contributing to the disease, COPD does not have uniform characteristics or progression, creating a phenotypic spectrum of disease (Burrows et al., 1966). Throughout the 20th century COPD was often misdiagnosed as asthma or bronchitis alone, but improved awareness and identification of COPD has seen the incidence of error lowered. COPD is comprised of a number of pulmonary abnormalities which contribute to airway obstruction, including bronchitis, emphysema, and air trapping leading to hyperinflation. Diagnosis and severity of COPD is categorised by the degree of airflow limitation in an individual, and this is mainly measured by spirometry. John Hutchinson is credited for the invention of the spirometer in 1846 (Hutchinson, 1846) and this device has proved vital in the diagnosis of COPD. It took 100 years, however, for this technology to be applied in the context of COPD. The spirometer was utilised by Edward Gaensler to create the air velocity index in 1950 (Gaensler, 1950), allowing for a spirometric definition of COPD to be calculated. Spirometric diagnosis of COPD requires two measurements, the forced expiratory volume in one second (FEV_1) and the forced vital capacity (FVC). FEV_1 is the volume of air which can be forcibly expelled from the lungs by an individual in one second, while FVC measures the total volume which can be expelled irrespective of time after full inspiration. FEV_1 is divided by the FVC of an individual, and if this is < 0.7 the individual displays airway obstruction and associated with a possible diagnosis of COPD. The severity of COPD is measured using the FEV_1 of a patient divided by their predicted FEV_1 calculated by their age, sex and height expressed as a percentage (i.e. percent predicted FEV_1). An example of spirometry in healthy controls and patients with lung disease is shown

in Figure 1.1. The National Institute for Health Care Excellence (NICE) clinical guidelines (2010) categorise mild COPD as $FEV_1 \geq 80\%$, moderate COPD as between 50-79%, severe COPD as 30-49% and very severe COPD as $\leq 30\%$. The FEV_1/FVC ratio is the most widely used method for diagnosis with COPD, but it does have limitations. Lung function naturally declines with age, and Hardie *et al.* (Hardie et al., 2002) have shown that 35% of a cohort of healthy, never-smokers who were ≥70 years old could be characterised as having COPD. Conversely, using a cohort of 20-44 year olds, Cerveri *et al*. (Cerveri et al., 2008) showed that COPD was underdiagnosed in younger people by measuring FEV_1/FVC alone. This is particularly concerning as delay in intervention and treatment could prevent avoidable decline in lung function. Thus age, dyspnea score (Nishimura et al., 2002), productive cough, smoking history, family history of COPD, exposure to risk factors (such as pollution or irritant particles) together with spirometry must be accounted for to ensure correct diagnosis.

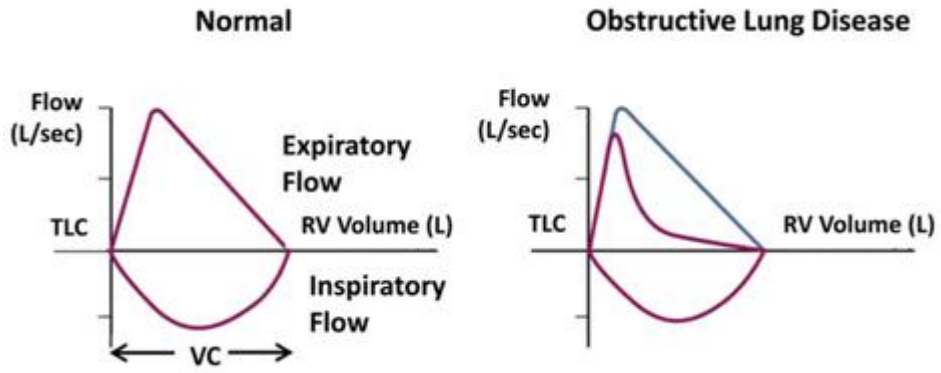

Figure 1.1. Spirometry aids diagnosis of obstructive lung disease. Illustration of flow volume curves comparing spirometry from normal and obstructive lung disease individuals. TLC – Total lung capacity, VC – Vital capacity, RV – Residual volume. Adapted from Miller *et al.* 2005 (Miller et al., 2005).

Chronic Bronchitis in COPD

Although chronic bronchitis may occur in individuals who do not have COPD, it is an important component of the condition which is often poorly controlled and may contribute to decreased lung function and the symptomatic burden of disease. The mechanisms underpinning the development of bronchitis have been studied. The loss of barrier function in the lung epithelia contributes to the development and severity of disease and this is attributed to the breakdown of two key defence mechanisms. The integrity of epithelial tight junctions separating the airways and tissue (Ganesan et al., 2013), and the functionality of the mucociliary escalator to clear inhaled particles are vital in the maintenance of lung health

(Knowles and Boucher, 2002). Abnormalities in these mechanisms can expose the lung tissue to noxious particles or other irritants which can result in further structural damage and inhibited gas exchange (Ganesan et al., 2013; Yao and Rahman, 2011). Exposure of these particles to "unprotected" tissue also results in inflammation of the bronchi which may be recurrent or unresolved (Borchers et al., 1999). The clinical symptoms of chronic bronchitis have been defined as the presence of productive cough for 3 months in two successive years. This productive cough is the result of an inflammatory process in which the thickening of bronchial mucous glands and mucus hypersecretion (due to goblet cell metaplasia) are characteristic. Dunnill *et al.* (Dunnill et al., 1969) quantified the composition of bronchi from healthy controls, asthmatics, patients with chronic bronchitis and emphysematous individuals post-mortem. This study showed that patients with chronic bronchitis had a greater proportion of mucus glands in the bronchi than normal controls, and this difference was not statistically significant for emphysematous individuals. While chronic bronchitis is part of the COPD spectrum, it is not necessarily present in all cases of COPD and the contribution of other lung conditions must be considered.

Emphysema in COPD

Emphysema is a term for the abnormal inflation and expansion of the bronchioles and alveoli, often associated with their destruction (Figure 1.2). Emphysema can affect the entire lung, the upper lobes or subpleural areas (Taraseviciene-Stewart and Voelkel, 2008). It is not clear the precise mechanisms for alveolar destruction, but protease imbalances (Owen, 2005) and immune cell infiltration (Hogg et al., 2004) in the airways has been postulated. Emphysema may also be associated with air trapping due to collapse of the peripheral airways (Mohamed Hoesein et al., 2011; Schroeder et al., 2013). During inhalation air may enter damaged sections of the small airways, which upon exhalation the air cannot readily escape the hyper-inflated lung structure causing airflow obstruction. Computed tomography (CT) technology allows for detailed measurement of emphysema in the lungs of living individuals, and through this method it has been shown that the degree of emphysema in the lungs of heavy smokers can predict their decline in lung function (Mohamed Hoesein et al., 2011). Johannesenn *et al.* (Johannessen et al., 2013) showed that the degree of emphysema is an independent predictor of all-cause mortality, including respiratory mortality to a greater accuracy than the GOLD severity classification. Emphysema alone is not sufficient to diagnose COPD, as it can be present in the lungs of smokers with diagnostically-normal lung

function (Hogg et al., 1994). Therefore the presence of emphysema may allow for phenotypic characterisation of disease and predict disease outcome, but it is not a diagnostic measure alone.

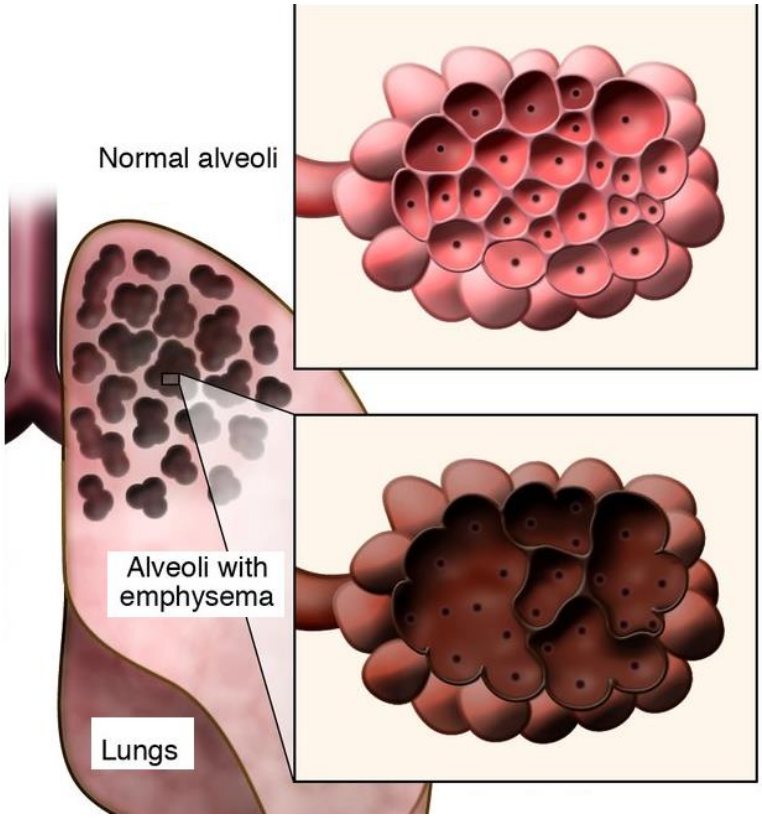

Figure 1.2. Illustration of Emphysematic lung. Comparison of normal lung structure and alveoli structure in emphysema patients. Adapted Taraseviciene-Stewart, Voelkel 2008 (Taraseviciene-Stewart and Voelkel, 2008).

Small airways disease in COPD

Small airways disease is associated with the small airways of the lung and surrounding parenchyma. Obstruction of the airways may lead to small airway closure and associated air trapping (Bommart et al., 2014). Tissue remodelling has been observed in the small airways, measured by fibrosis and smooth muscle hypertrophy (Hogg and Timens, 2009). Abnormal fibroblast responses and expression of Transforming Growth Factor β (TGF-β) have been associated with excessive remodelling of the lower airways (Zandvoort et al., 2008). Cigarette smoke is thought to be a significant promoter of remodelling by inducing oxidative stress, as well as by inducing growth factor production (Churg et al., 2006). The combination

of small airways disease, chronic bronchitis and emphysema all contribute to airway obstruction and progressive decrease in lung function.

Smoking as a risk factor in the development and progression of COPD

Key factors contributing to the development of COPD include age, sex and smoking history. Smoking is the single greatest risk factor for the development of COPD, although smoking alone does not appear to be sufficient for COPD to occur (Celli et al., 2005). There is a natural decline in lung function with aging, but this decline is increased for susceptible smokers (Kohansal et al., 2009). A landmark study by Fletcher and Peto (Fletcher and Peto, 1977) characterised the decline of lung function in never smokers, ex-smokers and lifelong smokers to describe the contribution of smoking on airway obstruction. Susceptible smokers display an irreversible decline in FEV_1 as they age to a much greater degree than never smokers. Furthermore, smoking cessation at any point reduces the severity of the FEV_1 decrease in these individuals. Fletcher and Peto also observed a population of smokers who did not suffer a loss in airway function greater than that of never smokers, suggesting that genetic or other environmental factors contribute to the development of airflow obstruction and not smoking alone (Artigas et al., 2011; Sood et al., 2010). In a study of 2,113 males in the United States of America (Eisner et al., 2005), Environmental Tobacco Smoke (ETS) was shown to be a risk factor in the development of COPD, indicating that passive smoke or smoke in the workplace may also contribute to disease (Hagstad et al., 2014; Mehta et al., 2012; Trupin et al., 2003).

Smoking prevalence has historically been greater in males than females, but smoking amongst females is increasing and may show no statistical difference in the future. Feenstra *et al.* (Feenstra et al., 2001) devised a dynamic multistate life table model to predict changes in smoking patterns and future prevalence of COPD in a Dutch cohort. This model predicts an increased prevalence of COPD by 59% in males and a 123% increase for females between 1994 and 2015. Environmental factors are not solely responsible for the development of COPD. Alpha1-antitrypsin deficiency was first described by Laurell and Eriksson in 1963 (Laurell and Eriksson, 1963) and is the most studied genetic-disorder associated with COPD. Despite this, α1-antitrypsin deficiency is only present in approximately 1.9% of individuals with COPD (Lieberman et al., 1986), and is therefore insufficient to account for the entire susceptible smoker population nor is it likely to be the only genetic factor associated with

COPD. Non-smokers are also at risk of developing COPD with the National Health and Nutrition Examination Survey (NHANES) II suggesting that 19.2% of COPD cases were attributed to the workplace environment, and this proportion was 31.1% in non-smokers (Hnizdo et al., 2004). Certain occupations increase the risk of COPD development in non-smokers, such as farming (Lamprecht et al., 2007) and tunnel workers(Ulvestad et al., 2001), although smokers in these jobs still suffer a greater decline in FEV_1. Fumes from open wood-burning fires (Boman et al., 2006; Orozco-Levi et al., 2006) and other heating and cooking pollutants (Ekici et al., 2005) also contribute to the development of COPD, especially in rural areas (Behera et al., 1994; Perez-Padilla et al., 1996). Smoking is also a significant risk factor in other diseases such as lung cancer. These diseases can occur in combination with COPD, and these co-morbidities can greatly affect the survival outcomes of COPD patients.

Co-morbidities associated with COPD

COPD is a life-threatening and debilitating disease, but it has been associated with many other conditions which impact on quality of life and health outcomes. These conditions include but are not limited to heart failure (Rutten et al., 2005), ischaemic heart disease (Sidney et al., 2005), hypertension (Wang et al., 2005), osteoporosis (Cielen et al., 2014), muscle wasting (Man et al., 2009), metabolic syndrome and depression (Karakurt and Unsal, 2013; Yohannes and Alexopoulos, 2014). Individuals with COPD are more likely to develop lung cancers, but it is unclear as to what risk factors may contribute to both diseases. Smoking is a risk factor for COPD and lung cancer but this alone may not explain their relationship, while genetic associations have been proposed (Schwartz and Ruckdeschel, 2006; Young et al., 2008). A study by Young *et al.* (Young et al., 2009) found that COPD was present in 50% of a lung cancer cohort, but COPD was diagnosed in only 8% of smoking-matched individuals without lung cancer. Furthermore this study showed that lung function declines due to the development of lung cancer, but this is not sufficient to account for the increased prevalence of COPD.

Respiratory Infections

Respiratory infections and COPD are also closely linked, with increased frequency of infection requiring hospitalisation correlating with increased GOLD severity of COPD (Benfield et al., 2008). While respiratory infections occur in healthy individuals, infections in COPD patients are regarded as disease characteristics rather than disease co-morbidities. In

COPD the immune system is ineffective and pathogens may colonise the airways (Marin et al., 2012). These infections induce an inflammatory response and may cause a decrease in lung function and destruction of epithelial tissue, contributing to the progression of COPD. This relationship has been described as a "vicious circle", in which COPD predisposes infection which in turn increases the severity of COPD and allows for further infection (Vestbo and Hogg, 2006; Wilson, 1998). Three bacterial species have been identified in the COPD lung which are implicated in disease exacerbations, *Haemophilus influenzae* (NTHi), *Streptococcus pneumoniae* and *Moraxella catarrhalis* (Sethi et al., 2002; Sethi et al., 2007). Colonisation alone may impact upon lung function, but there is growing evidence that new strains of bacteria may be acquired and induce further exacerbations (Sethi et al., 2002).

COPD exacerbations

COPD exacerbations contribute to the decline of patient lung function, are the primary cause of patient hospitalisation and constitute a large proportion of medical costs (Chapman et al., 2006; Hilleman et al., 2000; Sullivan et al., 2000), but the mechanisms of disease exacerbations are poorly understood. COPD exacerbations are defined as acute events in which patient lung function deteriorates and/or other respiratory symptoms increase in severity beyond daily variations observed in the stable state. The most recognised symptoms of exacerbation are an increased sputum production, a change in colour of sputum produced, increased cough frequency, wheezing, and worsening dyspnoea (Parker et al., 2005). Exacerbations may occur due to a number of factors, such as bacterial infection (Soler et al., 1998), viral infection (Seemungal et al., 2001), concurrent bacterial and viral infection (Papi et al., 2006), pollution or other environmental irritants (Donaldson et al., 1999). An inflammatory immune response is characteristic of disease exacerbations, with increased numbers of neutrophils in bronchial biopsies (Qiu et al., 2003) and eosinophils present in sputum (Papi et al., 2006). Increased cell numbers are likely to be the result of increased levels of the chemotactic mediators CCL5, IL-6, IL-8, CXCL5 and leukotriene (LT)B_4 (Bhowmik et al., 2000; Biernacki et al., 2003; Fujimoto et al., 2005; Zhu et al., 2001). Degradative enzymes, such as matrix metalloproteinase-9 (MMP-9) levels are increased during exacerbation (Mercer et al., 2005), and this may be one mechanism leading to emphysema by degradation of extracellular matrix proteins. The number of disease exacerbations positively correlates with the staging of COPD severity (Hurst et al., 2010), further emphasising their role in disease. The ability to predict and prevent exacerbations

would have both short and long-term effects on disease progression. Exacerbations can be fatal events (Connors et al., 1996) but also individuals with a high frequency of exacerbations have a more rapid decline in FEV_1 (Donaldson et al., 2002). The most severe exacerbations which require hospitalisation may also require mechanical ventilation. These patients have a significantly reduced survival, with only ~57% survival rate 180 days post-exacerbation (Connors et al., 1996). As exacerbations have a significant impact on disease outcome, the ability to predict and prevent exacerbations would greatly improve chances of survival in COPD patients. Hurst *et al.* performed a study analysing the potential of 36 plasma biomarkers to predict COPD exacerbation, but none achieved a statistically significant correlation (Hurst et al., 2006). Subsequent efforts investigating sputum (Saito et al., 2014; Zhu et al., 2014), lipidomics (Telenga et al., 2014) and proteomic (O'Neil et al., 2011) techniques have also been unsuccessful, with new insights into immune functions in COPD required to identify key cells, proteins or mediators of disease.

Prediction Factor	Example
Previous exacerbations	Frequency of exacerbation correlates with disease severity
Bacterial infection	Streptococcus pneumoniae, Haemophilus influenzae, Moraxella catarrhalis
Viral infection	RV, RSV, Influenza virus
Concurrent bacterial and viral infection	See above
Pollution	Wood-burning stoves in poor ventilated areas, poor air quality in industrialised cities

Table 1.1. Predictors of COPD exacerbations.

Cells implicated in COPD manifestation, progression and exacerbation

Many cell types are associated with the innate and adaptive arms of the immune system are implicated in the pathogenesis of COPD. These cells contribute directly to excessive or inappropriate inflammatory responses, but also stimulate or modulate response of other cell types to further enhance disease symptoms. Figure 1.3 illustrates key cells implicated in COPD pathogenesis and how they can directly and directly promote responses from other cells present in the lung.

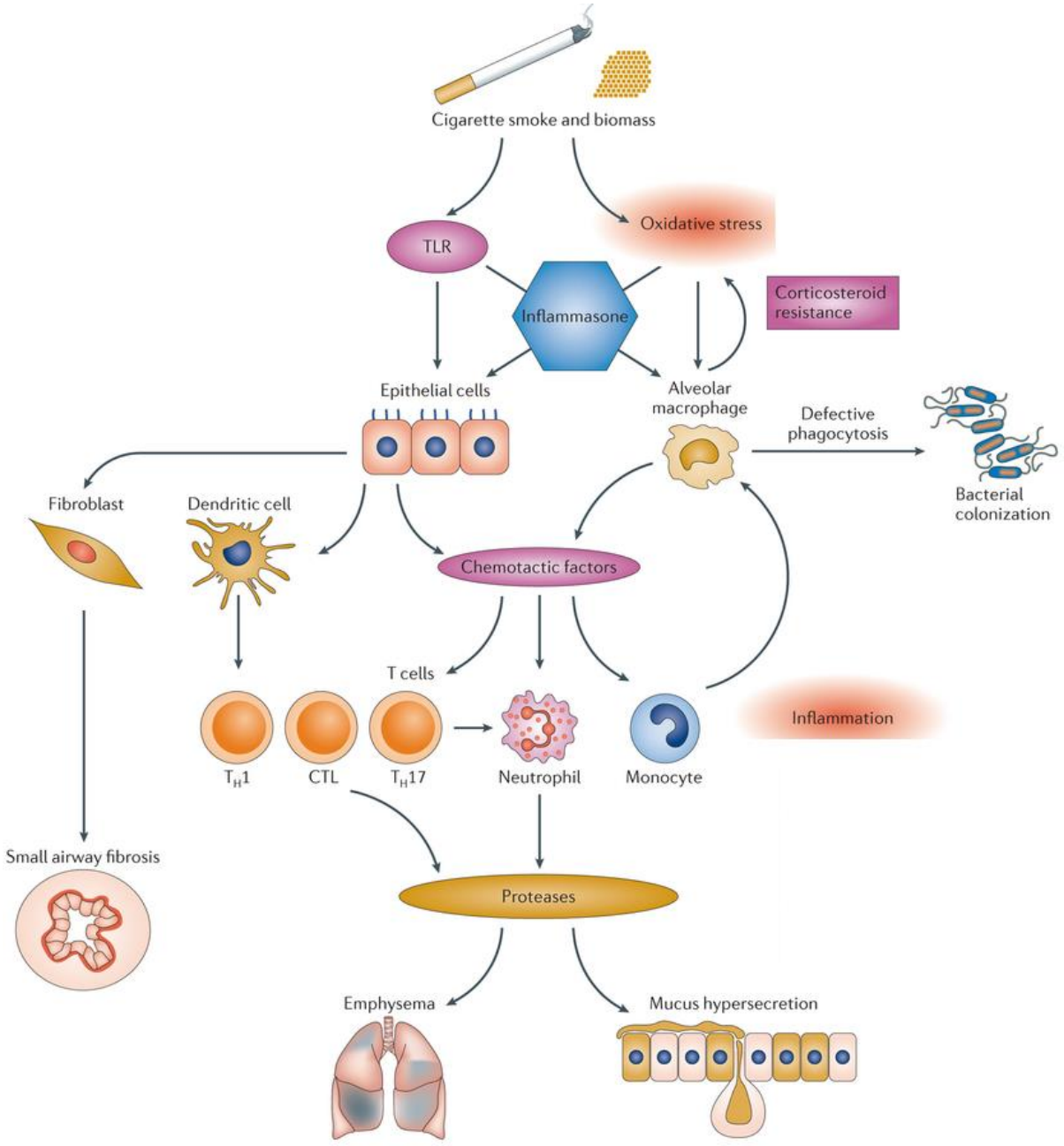

Figure 1.3. Cells of the immune system contribute to COPD pathogenesis. Adapted from Barnes, 2013(Barnes, 2013).

Epithelial cells

The primary function of epithelial cells is to provide a physical and chemical barrier between tissues, pathogens and harmful particulates. This barrier function is compromised in COPD (Polosukhin, 2001; Salazar and Herrera, 2011), but an important secondary function of these cells is to recognise pathogens and stimulate the immune system. Epithelial cells can produce IL-6 and IL-8 in response to oxidative stress and viral infection (Biagioli et al., 1999; Gielen et al., 2010; Kaul et al., 2000), recruiting innate immune cells to the tissue (Abdelaziz et al.,

1995). This is a normal function to protect the lung, but excessive or sustained recruitment can result in tissue damage. Epithelial production of IL-1β can alter structural cell phenotypes and can promote the development of squamous metaplasia (Araya et al., 2007). This change in epithelial cells to a squamous phenotype impairs the protective function of these cells. Epithelial cells derived from COPD patients also produce greater amounts of inflammatory cytokines than those of healthy donors (Schneider et al., 2010). Thus they potentially contribute to lung inflammation in COPD through immune cell recruitment and activation. Epithelial cells are IFN producing cells which are capable of promoting anti-viral responses (Siegel et al., 2011). Epithelial cells in healthy individuals produce IFNβ in response to viral infection, and this is a potent mediator of anti-viral responses (Gaajetaan et al., 2013). In COPD patients, however, IFNβ production is inhibited during Respiratory Syncytial Virus (RSV) infection, and viral load inversely correlates with detected IFNβ in these patients (Mallia et al., 2012). Virally-infected epithelial cells can present antigen to T cells to induce cell-killing and limit viral replication, but they are not professional antigen presenting cells (APCs) (Mulder et al., 2011) (such as dendritic cells or macrophages) and poorly co-stimulate naïve T cells. While they can direct the immune response, it may be APCs which have greater control in regulating immune responses.

Alveolar Macrophages

Macrophages are present in human tissues and skin, but either develop in the foetal yolk sac (Guilliams et al., 2013) or are derived from monocytes which circulate in the blood (Geissmann et al., 2003; Landsman and Jung, 2007; Varol et al., 2007). Tissue-resident macrophages may have diverse functions depending on their location (lung, spleen, gut or lymph nodes), and those which reside in the lungs are termed alveolar macrophages. Resident cells appear to be long lived in the steady-state, but may be replaced by infiltrating monocytes during infection (Maus et al., 2006). Work has been extensively performed in murine models to identify unique macrophage subsets (Dalrymple et al., 2013; Li et al., 2014), but these may not represent macrophage diversity in humans. Key to this is the growing evidence that macrophages are highly plastic multifunctional cells, rather than polarised phenotypes with defined lineages and differentiation pathways (Mosser and Edwards, 2008; Stout et al., 2005). One of the first recognised functions of macrophages was the ability to phagocytise dead cells and cell debris (Erwig and Henson, 2008; Fadok et al., 1998; Mantovani et al., 1972). This is vital to clear apoptotic bodies of dead cells and help prevent

recognition of newly-exposed self-epitopes, which otherwise could result in autoimmunity. Macrophages also detect potential microbial pathogens by a number of innate pathogen-associated molecular pattern receptors (PAMPs), such as Toll-like receptors (TLRs) (Haeberle et al., 2002; Janeway and Medzhitov, 2002). Macrophages may kill these pathogens by release of pro-inflammatory mediators such as reactive oxygen species (Hang do et al., 2011). Furthermore macrophages can recruit and promote microbial killing by other immune cells.

Macrophages are implicated in linking the innate and adaptive arms of the host immune response. Macrophages are professional APCs shown by presentation of antigen and expression of co-stimulatory molecules resulting in induction of anti-viral T cell responses (Evans et al., 2009; Short et al., 2013). Bidirectional interactions between macrophages and T cells can modulate and enhance cytokine responses from both cell types (Chizzolini et al., 1997). Inhibition of monocyte homing to the lung and maturation into macrophages impairs T cell responses in *Mycobacterium tuberculosis* infection (Peters et al., 2001), indicating that these cell are vital during immune responses rather than assisting as accessory cells. Although required for efficient pathogen clearance, interactions between macrophages and T cells may promote immunopathology to the host or sustain autoimmune disease (Agostini et al., 2001; Cruz-Guilloty et al., 2014). Conversely, macrophages can also function as regulatory cells, producing IL-10 to limit inflammation (Sirois et al., 2000) and interact with Treg cells to promote immune tolerance (Mahajan et al., 2006).

In the context of lung disease, macrophages are found at increased numbers in bronchial biopsies (Di Stefano et al., 1998) and lung parenchyma (Hogg et al., 2004) with GOLD stage severity. Cigarette smoke appears to be a factor in the recruitment of monocytes in the blood to migrate into lung, and this may be a source of pulmonary inflammation. The heterogeneity of macrophage populations of the lungs (Duan et al., 2012; Laskin et al., 2001) implicates them in the inflammatory component of COPD and also airway remodelling due to their ability to produce cytokines, oxidants and proteases (Shapiro et al., 1991). Alveolar macrophages for COPD patients secrete the inflammatory cytokine Tumour Necrosis Factor α (TNF-α) (Keatings et al., 1996) and also TGFβ1 which may contribute to fibrosis in the lungs (de Boer et al., 1998). The release of IL-8 (Culpitt et al., 2003) by macrophages recruits neutrophils to the lung, which can release further cytokines, enhancing inflammation.

Neutrophils

Neutrophils are rapid responders to invading pathogens, but they also contribute to inflammation in chronic infections. Neutrophils are present in the bronchial epithelium of COPD individuals in greater numbers than controls (Pesci et al., 1998), and increased numbers of neutrophils are present as airflow limitation increases (Stanescu et al., 1996). Despite increased neutrophil numbers, respiratory infections in COPD patients are increased. Cigarette smoke is implicated in the dysfunction of these cells as it appears to inhibit the phagocytic capabilities of neutrophils (Stringer et al., 2007), preventing pathogen clearance. Neutrophils secrete an array of anti-pathogenic granules upon activation, but these may also result in host tissue damage. This has not yet been shown in the context of COPD, but it is a mechanism which must be considered as a potential contributor to disease progression.

T cell subsets and functions

An inappropriate innate immune response has been widely recognised as a contributing factor of COPD, but less is known of the role of cells associated with the adaptive immune system. CD8+ T cell numbers are elevated in COPD lung (Saetta et al., 1998), but this does not seem to confer increased protection from viral infection. T cells can dictate adaptive immune responses to generate potent, specific protection against pathogens and cell malignancies. T cells develop in the thymus from bone marrow-derived haematopoietic stem cells. These stem cells develop into thymocytes which undergo positive and negative selection according to their T cell receptor (TCR) specificity, in which autoreactive T cells are destroyed and functional T cells survive (Gascoigne and Palmer, 2011; Morris and Allen, 2012; Starr et al., 2003). T cells express a TCR which recognises a very short peptide sequence, restricting their activation and ensuring that a specific immune response is generated. Upon maturation in the thymus, T cells circulate in the blood and lymphatic networks. T cells will enter a number of lymph nodes in which dendritic cells (DCs) present antigens transported from the periphery. If the TCR recognises antigen presented by Major Histocompatibility Molecules (MHC) Class I or Class II (Stone et al., 2009; Yin et al., 2012), a number of co-stimulatory receptors and ligands are upregulated by the T cell allowing DC-T cell interactions (Dustin, 2008; Henrickson and von Andrian, 2007; Yokosuka and Saito, 2009). CD80 (B7-1) and CD86 (B7-2) are expressed on antigen presenting cells such as macrophages and B cells, and these cell-surface molecules interact with CD28 on the surface of T cells to induce co-stimulatory signals for T cell activation (Riley and June, 2005). CD86 is constitutively expressed on these

cells, while CD80 is upregulated in tandem with antigen presentation by MHC Class I or Class II. Interactions between these molecules will induce the T cell to then migrate to the site of infection where it will interact with other antigen presenting cells (DCs, macrophages, epithelial cells) and perform its effector functions. T cells are key to protection from infections and tumours, but they are also highly regulated cells from their initial development to their activated state. Breakdown in tolerance and the control of T cells can result in auto-immune conditions and severe immunopathology (Ely et al., 2008; Lutz and Kurts, 2009). It has been postulated that an auto-immune component may be involved in COPD pathogenesis (Agusti et al., 2003; Eppert et al., 2013; Packard et al., 2013), but currently there is little evidence of such mechanisms and it is not leading theory for the development of COPD. An illustration of APC and T cell interactions is shown by Figure 1.4.

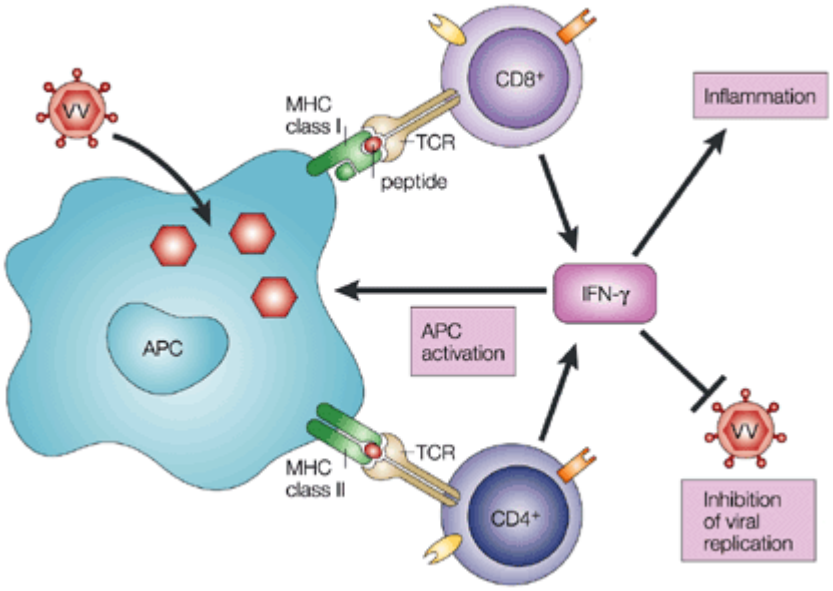

Figure 1.4. T cell responses to virus. Illustration of how macrophages, CD4+ and CD8+ T cells interact to generate an efficient anti-viral response. Adapted from Welsh and Selin, 2002(Welsh and Selin, 2002).

Mature T cells can be divided into CD4+CD8- or CD4-CD8+ subsets which have historically had separate but complementary functions in the adaptive immune system. CD8+ T cells are classically defined as "destroyer" cells which kill virally infected host cells or tumour cells (Masopust et al., 2007), while CD4+ T cells produce cytokines and provide help to CD8+ T cells and B cells (Yamane and Paul, 2012). CD4+ T helper cells are defined by the cytokines they secrete, such as IFNγ, IL-4 or IL-17 which polarise the immune response to protect from a diverse array of pathogens (Zhu et al., 2010).

CD4-CD8- T cells have been identified, and these are comprised of invariant "innate-like" T cells (Kronenberg and Kinjo, 2009). Both CD4+ and CD8+ T cells can be further divided into naïve, central memory and effector memory cells depending on exposure to antigen (Hamann et al., 1997; Sallusto et al., 2004). Effector memory cells are vital in protecting against repeat infections. Unlike naïve T cells, Memory cells require only ligation of TCR by antigen (without the aid of co-stimulatory molecules) to proliferate, kill infected cells or produce inflammatory cytokines. These effector functions generate a potent specific response to pathogens within a relatively short period of time, limiting the spread of infection. Wilkinson *et al.* identified a subset of human CD4+ T cells which had cytotoxic capabilities in response to influenza virus (Wilkinson et al., 2012). These cells predominantly killed target cells by the secretion of perforin, and produced effector cytokines such as IFNγ (both functions associated with CD8+ T cells). Therefore there is a degree of plasticity within T cell subsets, in which function may not be defined by CD4 or CD8 expression. T regulatory cells inhibit T cell effector responses by cell-cell contact mechanisms or production of inhibitory cytokines such as IL-10 (Bettini and Vignali, 2009; Wohlfert and Belkaid, 2008). These cells are also heterogeneous (d'Hennezel et al., 2011), and their specificity of action can allow inhibition of CD4+ and CD8+ effector T cells.

The potent effector mechanisms described above are vital in the ability of the host to defend against viral and bacterial infection. As discussed, COPD exacerbations may be induced and have increased severity due to recurrent or persistent viral infection. T cells have the capabilities to clear infection, but appear to do so poorly in disease. CD8+ T cells are implicated in COPD with their increased frequency in lung parenchyma (Saetta et al., 1998) and their relationship with FEV_1 (O'Shaughnessy et al., 1997). It is unclear whether this inhibition is due to viral evasion mechanisms, or host regulatory mechanisms intended to limit immune pathology.

Regulatory T cells

Regulatory T cells (Treg) (Sakaguchi et al., 1995) can be divided into natural FoxP3+ Tregs (Fontenot et al., 2003) or iTregs which are FoxP3- and differentiate into a regulatory phenotype in the periphery (Walker et al., 2005). Both Treg and iTreg cells are vital in dampening immune responses to prevent autoimmunity or excessive immunopathology (Fulton et al., 2010; Zhao et al., 2011). Treg-dependant inhibition of immune responses,

however, may allow for pathogen survival and persistence, leading to chronic infection (Belkaid et al., 2002; Shafiani et al., 2010). Influenza specific Tregs proliferate and modulate anti-viral CD8+ T effector cells in response to secondary infection, but also play an active role in primary infection (Brincks et al., 2013). Although Tregs function primarily by cytokine production and cell-cell contact with effector T cells, Tregs may also downregulate NK cell (Ralainirina et al., 2007) and macrophage responses (Taams et al., 2005; Zhen et al., 2008). Thus there is a stimulation feedback loop from initial pathogen recognition by epithelial cells and macrophages, NK cell and effector T cell clearance of infection followed by downregulation of responses by Treg cells. Several of these interactions between immune cells are illustrated in Figure 1.3.

Corticosteroid effects on immune responses

Inhaled corticosteroid therapy is the primary treatment to reduce inflammation in the COPD lung. Although corticosteroids may in part relieve exacerbation symptoms (Woods et al., 2014), some patients do not respond to treatment and these drugs do not improve long-term lung function (Soriano et al., 2007) or patient mortality (Walters et al., 2014). Effects of glucocorticoid treatment on inflammatory responses have largely focused on the macrophage population. Steroid treatment of macrophages may induce apoptosis of these cells, with surviving macrophages adopting a regulatory phenotype (Zizzo and Cohen, 2013). Macrophages which are glucocorticoid resistant may promote inflammation in COPD (Chana et al., 2014), although there is evidence to suggest that alveolar macrophages respond in a similar manner to those from healthy individuals (Plumb et al., 2013). Steroid treatment, however, may also inhibit epithelial cell signalling (van den Berge et al., 2014), and patients who do not respond to steroid treatment may do so due to T cell insensitivity to these drugs (Barnes et al., 2006; Barnes, 2011; Kaur et al., 2012). Therefore corticosteroids may modulate immune responses by several cell types, which together could inhibit anti-viral responses.

Retinoic acid effects on immune responses

The importance of Vitamin A for the foetal lung development and maintenance of lung epithelial cell structure has been extensively reported (Biesalski and Nohr, 2003). Vitamin A and its metabolites also appear to play a number of roles in directing the innate and adaptive immune response. Key to this are recognition of metabolites by retinoic acid receptors (RARs)

and the retinoic acid-inducible gene (RIG-1) like receptors (RLRs) (Yoo et al., 2014). Retinoic acid appears to directly promote iTreg differentiation several cell line and mouse model studies (Benson et al., 2007; Denning et al., 2007; Manicassamy et al., 2009). Retinoic acid induces migration of T cells to the gut (Iwata et al., 2004) and this is likely to be a mechanism to sustain tolerance to non-harmful antigen. Conversely, retinoic acid may also induce effector T cells responses. Retinoic acid deficiency interferes with CD4+ T cell development and function (Pino-Lagos et al., 2011). Interactions between retinoic acid and RAR has also been implicated in T cell production of IFNγ by T cells (Hall et al., 2011a). Mice which do not express RAR also recorded a diminished CD8+ T effector memory population, although this may be accounted for by an increased central memory population (Allie et al., 2013). While there is evidence to implicate Vitamin A in COPD exacerbations (Heo et al., 2013), the mechanisms which may drive disease are yet to be elucidated.

T cell exhaustion

Definition and consequences of exhaustion

The majority of infections are cleared by healthy individuals with functioning innate and adaptive immune responses. Failure to resolve infection leads to tissue and organ damage by immunopathology if unchecked, and various cytokines and inhibitory receptors are employed to downregulate immune responses (Gazzinelli et al., 1996; Zhou et al., 2002). T cell exhaustion is defined by the inhibition of effector cell responses (Zajac et al., 1998), such as proliferation, cell killing and inflammatory cytokine production (Wherry, 2011; Yi et al., 2010). This, however, does not appear to be a consequence of a dysfunctional immune system, rather a protective mechanism to reduce potential immunopathology (Keir et al., 2006; Mueller et al., 2007; Okazaki and Honjo, 2006; Sharpe et al., 2007). Exhaustion occurs after a period of chronic T cell activation to limit inflammation when an infection has not been eradicated from the host. T cell exhaustion has been described in detail using Lymphocytic choriomeningitis virus (LCMV)-infected murine models. Using either the Armstrong or clone 13 strains of LCMV, an easily cleared acute infection or a poorly controlled chronic infection can be induced (Wherry et al., 2003). In the acute Armstrong strain infection, T cell exhaustion does not occur and infection is cleared between 6-8 days while mice infected with the chronic clone 13 strain display inhibited T cell function after 7 days (Barber et al., 2006; Wherry et al., 2003). Zajac *et al.* demonstrated the outcomes for epitope specific CD8+ T cells (recognising viral nucleoproteins or glycoproteins) in chronic

LCMV infection in a murine model (Zajac et al., 1998). In this study a population of NP396-specific (nucleoprotein 396) CD8+ T cells were clonally deleted in response to chronic infection while GP33-specific (glycoprotein 33) T cells survived but were unresponsive. Discrepancy between CD8+ T cell outcomes was further investigated by Wherry *et al.* (Wherry et al., 2003) and it was concluded that epitopes expressed at high levels were likely to induce CD8+ T cell death while those expressed at low levels maintained a virus-specific repertoire of non-functional T cells. The authors also observed that exhaustion was a process of many stages. The ability of CD8+ T cells to produce IL-2 was lost rapidly in chronic infection, followed by TNF-α and eventually IFNγ as length of infection and antigen load increased (Wherry et al., 2003) (Figure 1.5). The understanding of T cell exhaustion is not limited to work performed in murine models and has been identified in a number of chronic conditions. Loss of CD8+ T cell functions has been shown in Hepatitis C virus (HCV) (Golden-Mason et al., 2007), Human immunodeficiency virus (HIV) (Peretz et al., 2012; Trautmann et al., 2006) and in some cancers (Ahmadzadeh et al., 2009).

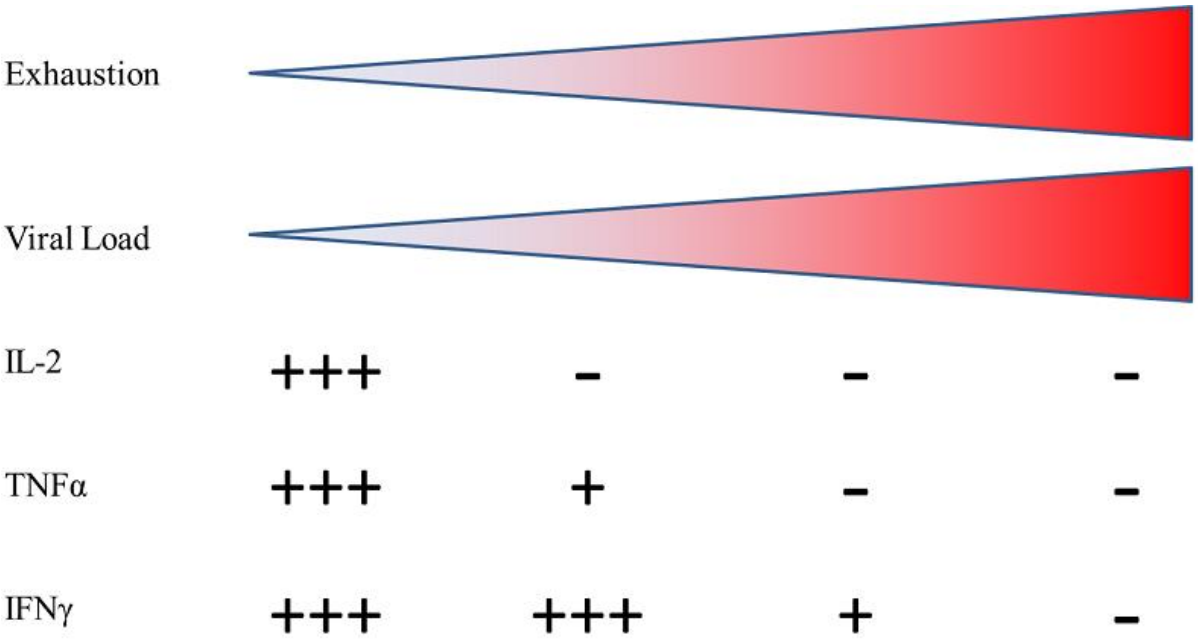

Figure 1.5. Degree of exhaustion determines loss of function on CD8+ T cells. As T cell exhaustion increases (blue-red bar), viral load increases. + and – indicate the degree of cytokine secretion by CD8+ T cells in chronic infection models. +++ indicates functional levels of cytokine expression, + indicates partial inhibition of cytokine expression and – represents no or undetectable levels of cytokine expression. Figure adapted from Yi *et al.* (Yi et al., 2010) and Wherry *et al.* (Wherry et al., 2003).

Cell surface receptors expressed by exhausted T cells

Programmed Cell Death Protein-1 (PD-1) is most well studied receptor associated with T cell exhaustion. PD-1 is an inducible cell surface marker expressed by activated T cells, B cells and macrophages, and is non-detectable or expressed in very low levels of resting T cells (Agata et al., 1996). PD-1 is an inhibitory receptor encoded by the *PDCD1* gene located on chromosome 2 (Shinohara et al., 1994) which is upregulated after TCR-MHC interactions (Youngblood et al., 2011). Its immuno-regulatory properties are displayed in *Pdcd1*$^{-/-}$ mice which develop lupus-like autoimmune disease (Nishimura et al., 1999) or cardiomyopathy (Nishimura et al., 2001). PD-1 is a member of the immunoglobulin gene superfamily and it is comprised of an Ig variable-type extracellular domain for ligand binding, a transmembrane region and a cytoplasmic tail to allow intracellular signalling (Cheng et al., 2013; Ishida et al., 1992). Unlike other members of this family, such as CD28 and CTLA-4, PD-1 is present as a monomer on the surface of cells and has not been observed to dimerise (Zhang et al., 2004). The presence of an immunoreceptor tyrosine-based switch motif (ITSM) and immunoreceptor tyrosine-based inhibition motif (ITIM) in the cytoplasmic region appears to be essential for PD-1 signalling. During T cell activation co-stimulatory and co-inhibitory receptors co-localise with the TCR forming an immunological synapse (Yokosuka et al., 2012). Engagement of PD-1 recruits SHP-1 and SHP-2 to the ITSM (Chemnitz et al., 2004; Latchman et al., 2001) in close proximity to the TCR, and these molecules may attenuate ZAP70 and CD3ζ tyrosine phosphorylation, inhibiting TCR signalling (Sheppard et al., 2004).

SHP-2 may be recruited to the ITSM upon TCR engagement, but inhibitory signalling does not occur without ligation of PD-1. The ligands for PD-1 are Programmed cell death 1 ligand 1 and 2 (PD-L1/PD-L2) and their encoding gene is located on chromosome 9 (Freeman et al., 2000). Freeman *et al.* performed binding experiments between PD-L1, PD-1 and other members of the immunoglobulin gene superfamily (Freeman et al., 2000). PD-L1 was shown to bind to PD-1.Ig but not to CTLA-4.Ig, therefore these inhibitory receptors do not compete for the same ligand and inhibitory signalling pathways may not be shared (Parry et al., 2005). PD-L1 is upregulated on macrophages, T cells and other non-hematopoietic cells such as epithelial cells (Freeman et al., 2000; Ishida et al., 2002; Liang et al., 2003) and expression levels are determined by the tissue microenvironment. PD-L1 mRNA is upregulated on human monocytes and DCs in response to IFNγ treatment, while PD-L1 mRNA is expressed in low levels in unstimulated cells (Freeman et al., 2000). PD-L1 is constitutively expressed

in immune privileged sites requiring strict control of inflammatory cells such as the testes (Cheng et al., 2009) and the eye (Sugita, 2009), emphasising its effectiveness at dampening immune responses. Tumour cells expressing PD-L1 may be positively selected for survival as the level of PD-L1 expression in human tumour cells correlates with disease severity and progression (Iwai et al., 2002; Nakanishi et al., 2007). The role of PD-L2 is less well understood. PD-L1 and PD-L2 share the same receptor, but these ligands cannot bind simultaneously and they compete for PD-1 (Ghiotto et al., 2010). PD-L2 has a greater binding affinity to PD-1 (Youngnak et al., 2003), but the functional relevance of this is unknown. PD-L2 can inhibit T cell proliferation and cytokine production in vitro, but this is restored in the presence of CD28 (Latchman et al., 2001). The use of monoclonal antibodies to block PD-L2 on DCs ex vivo increases T cell proliferation and cytokine production, and this was increased further when PD-L1 was also blocked (Brown et al., 2003). While PD-1 is classically associated with anti-tumour and viral responses, PD-L2 may play a role in Th2 type responses, as PD-L2 has displayed and ability to regulate CD4+ T cells in helminthic infection models (Ishiwata et al., 2010). More work is required to elucidate the role of PD-L2 in a variety of diseases, but PD-L1 is a key molecule in the control of T cells responses to chronic viral infections.

Interactions between PD-1 on T cells and PD-L1 on APCs and target cells inhibit T cell functions. *In vitro* experiments have shown that the addition of a PD-L1 monoclonal antibody (mAb) greatly reduced the capability of the T cells to proliferate and inhibited the production of IFNγ and IL-10 in vitro (Freeman et al., 2000). This work has been expanded to show that blocking of PD-1:PD-L1 interactions can restore the effector functions of exhausted T cells. Barber *et al.* (Barber et al., 2006) utilised an LCMV murine model of chronic infection to show that PD-1 expression and T cell exhaustion correlated with an increased viral load. Injection of an anti-PD-L1 blocking antibody into these mice restored CD8+ T cell proliferation, IFNγ and TNF-α secretion, and cytotoxic potential which resulted in the clearance of viral infection. Aubert *et al.* have also described an increased production of IFNγ, TNF-α and IL-2 by CD4+ T cells using an anti-PD-L1 blocking antibody in a similar LCMV model (Aubert et al., 2011). The PD-1 pathway is therefore a potential therapeutic target and research is currently being performed to restore immune responses in SIV (Velu et al., 2009) and cancer (Brahmer et al., 2012; Lipson et al., 2013; Ott et al., 2013; Topalian et al., 2012).

Another inhibitory receptor gaining interest in T cell exhaustion is T cell immunoglobulin-3 (TIM-3). PD-1 may be expressed in populations of functional activated T cells (Duraiswamy et al., 2011), but the co-expression of TIM-3 and PD-1 on T cells indicates exhaustion (Baitsch et al., 2011; Fourcade et al., 2010). Furthermore TIM-3 expressing cells have been implicated in HIV (Jones et al., 2008) and cancer (Sakuishi et al., 2010) in a similar manner to PD-1. Dual blockade of TIM-3 and PD-1 in an LCMV model (Jin et al., 2010) and blocking TIM-3 alone in Hepatitis C infection can restore exhausted CD4+ and CD8+ T cell function (Golden-Mason et al., 2009) suggesting shared functionality between these inhibitory receptors.

Respiratory viral infections and T cell exhaustion

T cell exhaustion has been described in chronic viral infections such as LCMV and HIV, but characteristics of exhaustion have also been identified in response to acute respiratory viral infections. RSV infection of tracheal (NCI-H292), bronchial (BEAS-2B), and alveolar (A549) epithelial cell lines induced upregulation of PD-L1 and PD-L2 (Stanciu et al., 2006). Levels of expression on these cells could be enhanced with the addition of IFNγ, implicating the cytokine environment in regulation of the immune response. Functional impairment of CD8+ T cells during RSV (Chang and Braciale, 2002) and influenza A (Vallbracht et al., 2006) infection has been described in murine models. Erickson *et al.* elucidated potential mechanisms for this impairment by identifying PD-1 expression on virus-specific T cells and PD-L1 upregulation in response to infection (Erickson et al., 2012), and Telcian *et al.* showed restoration of CD8+ T cell cytokine secretion in response to PD-L1 blocking antibody during RSV infection (Telcian et al., 2011). Current work has been performed on murine models and in human cell lines. Analysis of PD-1:PD-L1 interactions with T cell in human parenchymal or bronchial tissue is required to further understand exhaustion in respiratory viral infections, particularly in the context of chronic airways disease.

T cell senescence

T cell senescence is a phenomenon in which T cells become less responsive to pathogens or other activating stimuli (Effros, 2004). T cell senescence is mainly identified in elderly individuals, and these cells can be characterised by short telomeres (Akbar and Vukmanovic-Stejic, 2007), suggesting they have undergone multiple proliferative cycles. Although senescent T cells are poorly protective in a manner similar to T cell exhaustion (such as loss

of cytokine production), senescent T cells appear to be distinct from exhausted T cells or T cells in a state of immune tolerance (Akbar and Henson, 2011). CD57 is a human marker commonly used to detect senescent T cells, although this marker can also be used to identify highly cytotoxic T cells. Furthermore as COPD is a disease of the elderly, care must be taken to ensure non-responsive T cells are indeed exhausted rather than senescent (a process independent of disease).

Influenza infection in COPD

Influenza is a common respiratory viral infection which occurs in a mild seasonal epidemic or a severe pandemic form. Seasonal flu causes an acute infection which has no long-term effects in healthy individuals, but a similar infection in the young, elderly or immuno-compromised can be fatal. Influenza is the term given to a group of single-stranded RNA virus' classified by their expression of hemagglutinin and neuraminidase proteins on the viral envelope surface (Bouvier and Palese, 2008). Key to the survival of influenza is the concept of antigenic drift and antigenic shift. Antigenic drift occurs due to numerous mutations within one influenza strain which results in loss of epitopes recognised by an individual. This individual will no longer have protection from this mutated strain and may be re-infected. Antigenic shift occurs when gene segments from one influenza strain are acquired by another strain, generating new antigenic proteins. These novel proteins are not recognised by the host immune system which may lead to poor control of infection, and this process is responsible for the pandemic forms of disease. Vaccines to protect against infection have been largely successful, but these are not effective for all influenza strains(Epstein et al., 2005). Global surveillance of influenza is required to ensure that annually developed vaccines confer protection to strains most likely to be present during the winter months. Despite measures to immunise at-risk individuals, influenza infection in individuals with COPD reduces lung function and may induce disease exacerbations (Rohde et al., 2003). In a study by Rohde, et al., influenza A was detected 25% of exacerbations in a COPD cohort, implicating the virus in disease. The degree to which a dysfunctional immune system enables influenza infection is yet to be fully elucidated, but T cells are required to protect against disease.

In 1978 Yap *et al.* showed that cytotoxic T cells played a protective role during influenza infection (Yap et al., 1978). Since then the mechanisms by which CD8+ T cells kill infected cells has been expanded to include a number of death-inducing pathways involving cell-cell

interactions such as FasL, perforin (Topham et al., 1997) or TRAIL (Brincks et al., 2013). Primary influenza infection in mice can be eradicated by CD8+ T cells without CD4+ T cell help but these mice display a poor CD8+ T cell memory response upon re-infection (Belz et al., 2002). CD4+ T cells play a role in clearing influenza infection via IFNγ production (Brown et al., 2012), provision of B cell help to generate anti-viral antibodies (Brown et al., 2006) or kill infected cells directly using perforin and granzyme (Wilkinson et al., 2012), but CD8+ T cells are considered the key effector cells. The potential of influenza virus infection to induce T cell exhaustion has been shown in murine models, but often it has been overlooked in favour of LCMV infection. Bucks *et al.* analysed anti-viral T cell populations in mice infected with influenza virus on a single occasion or repeatedly infected mice (Bucks et al., 2009). In this model virus-specific CD8+ T cells from chronically stimulated mice produced significantly less IFNγ than single-stimulated controls. Although a greater proportion of these CD8+ T cells expressed PD-1, blocking PD-L1 did not restore T cell functions suggesting other signalling pathways may be involved in this model. The host-virus relationship is most widely studied in murine models, although some influenza challenge studies have been performed in humans. A weakness in animal models is that mice do not naturally contract influenza, and infection may occur in an artificial method via i.v. or i.p. injection rather than intra-nasally. Thus the development of infection models using human cells and tissues may yield novel mechanisms of disease which are not displayed in animals.

Work within the group has concentrated on development of human tissue models of acute viral infection. Human bronchial and parenchymal tissues from healthy, COPD and asthmatic patients have previously been infected with influenza and RSV. Epithelial cells and macrophages have been identified as cells susceptible to infection, while T cells and B cells do not appear to be infected (Nicholas et al., 2013). Using these models of infection has allowed for a number of different cell–based outputs to be characterised and quantified. Cells from tissue can be labelled with fluorescent antibody for flow cytometry and cells can be sorted by FACS in order to yield enriched cell populations. Sorted cells can be harvested for their RNA to allow for gene expression protocols, such as RT-PCR, to be performed. Supernatants for infection can also be analysed for viral shedding and cytokine production by ELISA or Luminex methods. These methods combined can illustrate viral responses by a number of different cells in a physiologically relevant context to elucidate mechanisms of the anti-viral immune response.

Methods utilised to characterise cellular dysfunction in COPD

Identification of immune cells and mediators from peripheral blood is a non-invasive and widely used method to diagnose, analyse severity and progression of many diseases. Lymphocyte counts from blood can diagnose chronic lymphocytic leukaemia (Rai et al., 1975), elevated levels of IgE has been shown to be a key component of allergic asthma (Dullaers et al., 2012) and testing of C-peptide levels aids diagnosis of diabetes mellitus type 1 (Wang et al., 2012). Cells retrieved from peripheral blood, however, may display altered characteristics than cells residing in tissues, at the site of infection or injury (de Bree et al., 2007; de Bree et al., 2005). Bronchoalveolar lavage (BAL) and sputum analysis are commonly performed in studies of lung disease (Wen et al., 2010). Immunohistochemistry methods have been used to assess cell location and surface markers in tissue sections, but a lack of standardisation in sample preparation (Grube, 2004), the variability of human judgement (Rhodes et al., 2000) and semi-quantitative dat a(Taylor and Levenson, 2006) call for alternative forms of analysis.

Analysis of human lung parenchyma

COPD is a complex heterogeneous disease with a multitude of cells and mechanisms associated with disease manifestation, progression and severity. Analysis of cells derived from tissue may better represent the microenvironment of affected organs compared to methods sampling the periphery. This is demonstrated in a recent study of T resident memory cells (TRM) in human lungs by Purwar *et al.* (Purwar et al., 2011). Lung TRM cells proliferated in response to influenza virus, but T cells from blood of the same patients did not respond to this stimulus. Furthermore, TRM cells from the skin also did not respond, suggesting site-specific functional responses by cells from different tissues. Lung parenchyma can also be infected to measure responses of cells within the tissue to virus and susceptibility to infection (Nicholas et al., 2013). Work performed in lung parenchyma is proposed as a physiologically relevant challenge model, with which a variety of different mechanisms may be studied. Analysis of human lung parenchyma will provide a further model of analysis to elucidate mechanisms of disease associated with COPD.

Collagenase digestion of lung parenchyma

Flow cytometry is now a standard and validated method for the identification of cell surface and intracellular markers (Maino and Picker, 1998; Stewart, 1992). While this technology allows for multiple parameters on individual cells to be measured, it requires a single cell suspension which is easily achieved in blood as density gradient centrifugation allows for a single cell suspension of peripheral blood mononuclear cells (PBMCs) to be acquired. Isolation of cells from organs is more difficult, as they may be retained within the complex cellular matrix of the tissue. In order to retrieve cells of interest in sufficient numbers, several protocols have focused on digesting tissue with various combinations of enzymes to degrade extracellular matrix proteins and adhesion molecules (Abuzakouk et al., 1996). Many of the enzymes used are derived from bacterial products which contribute to pathogenesis during infection, and as such have evolved to disrupt tissue structure. A key consideration is that although enzymatic digestion increases cell yield compared to mechanical breakdown alone (Novelli et al., 2000), some enzymes may cleave cell-surface markers which are vital for cell identification by flow cytometry (Abuzakouk et al., 1996; Hagman et al., 2012; Van Damme et al., 2000). Depending on the enzymes used, their concentration and the length of digestion time, cell-surface markers may be preserved. Great effort has been made to balance cell yield, cell viability and preservation of cell markers, but currently there are no universally optimal conditions for all tissue types or cell populations. Collagenase is the most routinely used enzyme for digestion for a variety of tissues (Grange et al., 2011; Holt et al., 1986; Novelli et al., 2000), but dispase (Abuzakouk et al., 1996), liberase (Hagman et al., 2012), trypsin (Williams et al., 1995), pronase (Hackett et al., 2008), DNase (Sperr et al., 1994) and elastase (Chen et al., 2004) have also been studied, along with combined enzymatic digestion protocols (Pilgaard et al., 2008). Work from Pilgaard *et al.* (Pilgaard et al., 2008) concludes that collagenase digestion alone is insufficient to optimally obtain stem cells from adipose tissue, suggesting that digestion must be tailored to yield cells of interest from different tissues. The conclusions from tissue digestion studies are often contradictory, but this may be due to variations between protocols preventing direct comparison. Hagman *et al.* (Hagman et al., 2012), however, reasoned that preserving the desired cell populations is more important than yield and that certain cell types may require less severe enzymatic treatment for their extraction. Thus the degree of tissue digestion required appears to depend on the study, and strict adherence to a defined protocol is vital to obtain reliable data.

Summary

The lungs of healthy individuals can confer robust anti-viral immune responses in the event of infection. This is achieved through a number of cell-mediated mechanisms to direct innate and adaptive arms of the immune system. In COPD, the lungs are less effective at clearing pathogens. Defects in the responses of many cell types have been implicated in disease progression and severity, but none of these have been translated into effective therapeutics. The importance of CD8+ T cells to provide anti-viral immune responses has been established through murine and human models of infection. The mechanism of T cell exhaustion has been studied for a decade in models of chronic viral infection and in cancer, but little work has been performed to investigate this phenomenon in the context of respiratory infections. Elucidating the potential T cell inhibition induced by exhaustion in COPD may reveal mechanisms which may be altered to restore T cell anti-viral functions.

Hypothesis

The hypothesis of this study was that T cells in the COPD lung express an exhausted phenotype compared to cells derived from control lungs which accounts for poor responses to viral infection that may lead to COPD exacerbations.

Aims

Aim 1: To identify and quantify T cell populations in human parenchymal tissues.
This will be achieved by flow cytometric identification of CD4+ and CD8+ T cells by in digested lung parenchyma controls and COPD patients. T cell populations will also be identified by their naïve/memory phenotype. T cell populations in tissue and blood will be compared.

Aim 2: To assess T cell exhaustion by expression of PD-1 and TIM-3.
With T cell populations identified, flow cytometry will be performed to measure the expression of the key exhaustion markers PD-1 and TIM-3 in blood and tissue.

Aim 3: To investigate the dynamics of PD-1 and PD-L1 expression after acute influenza infection of lung parenchyma.
This will be achieved by utilising the lung parenchyma influenza infection model. PD-1 on T cells and PD-L1 on epithelial cells and macrophages will be measured by flow cytometry from non-infected and X31-infected tissue samples. This will provide baseline measurements of these markers and their degree of upregulation after infection.

Aim 4: To assess IFNγ responses of T cells when blocking the PD-1:PD-L1 pathway.
IFNγ responses will be measured using a MDM model of acute influenza infection. Infected MDMs will be co-cultured with autologous T cells to promote IFNγ production which will be measured using an ELISpot assay. αPD-L1 blocking antibody can be added to this model to investigate its ability to enhance IFNγ responses.

Aim 5: To analyse the effect of corticosteroids and retinoic acid supplementation on T cell production of IFNγ.
The MDM infection model will be supplemented by fluticasone or retinoic acid to elucidate their potential regulation of IFNγ responses. This will be measured using ELISpot.

Chapter 2

Methods

Introduction

The aims of this thesis were to characterise T cells in COPD individuals and their responses to influenza A viral challenge. Clinical samples of blood and explanted lung parenchymal tissue were required to compare inflammatory cell populations at the site of disease and in the periphery. Lung parenchymal tissue was acquired form control and COPD patients as these samples were representative of cell types implicated in disease which cannot be analysed in blood, sputum or BAL. Investigation of inflammatory cells in COPD lung tissue has been limited, and novel methods for analysis were developed and optimised during this study. Flow cytometry is a high-throughput technique allowing for detailed characterisation of individual cells and populations of cells, but requires a single-cell suspension solution for analysis. Lung parenchyma required digestion and disaggregation to generate single cell suspensions for FACS analysis, while maintaining the expression key cell markers of cells of interest. Furthermore infection of tissue epithelial cells and macrophages could be quantified by FACS. T cells from tissue required the extraction of RNA and analysis of their gene expression, thus RT-PCR techniques were employed. The number of cells yielded from tissue was too low to perform validated T cell functional assays, therefore a blood model of influenza infection to study T cell responses was required. IFNγ production was measured in response to infection of MDMs by ELISpot.

Patient Recruitment

The collection of blood was approved by and performed in accordance with the ethical standards of the Southampton and South West Hampshire Research Ethics Committee, REC no: 13/SC/0416. Blood was taken from healthy individuals for use in T simulation experiments and ELISpot work.

Lung tissue was obtained from patients undergoing airway re-sectioning surgery at Southampton General Hospital. The collection of tissues was approved by and performed in accordance with the ethical standards of the Southampton and South West Hampshire Research Ethics Committee, LREC no: 09/H0504/109.

Patient Phenotype

Lung parenchymal tissue samples were received from individuals undergoing airway re-sectioning surgery. Patients with a FEV_1/FVC ratio of < 0.7 were classified as COPD. Those with a FEV_1/FVC ratio of ≥ 0.7 were defined as Control patients. Individuals with other inflammatory lung conditions (cystic fibrosis, asbestosis), patients with prior lung infections (tuberculosis) and individuals with autoimmune conditions (rheumatoid arthritis) were excluded from the study. One pack year was defined as 20 cigarettes smoked per day for one year. Smoking status and pack years was determined by consultation with the patient. Details of lung function, potential co-morbidities and current medication were taken from patient bedside notes. Additional data was obtained by NHS records (Equest, Southampton General Hospital) with assistance from Dr. Hannah Burke, but these were not regularly available and often did not provide data for more specific patient phenotyping.

Numbers of control (n = 24) and COPD (n = 31) individuals was similar, and groups were age matched (Table 2.1). Proportions of males and females were similar between groups. Pack years appeared to be greater in COPD patients than controls (45.98 ± 8.31 SD vs. 27.2 ± 11.16 SD) but this was not statistically significant (p=0.053). FEV_1% was significantly lower in COPD than controls (98.79 ± 14.71 SD vs. 77.26 ± 14.57). The FEV_1/FVC ratio was significantly lower in COPD than controls, but this was expected as this measurement determined whether patients were allocated to the control or COPD group.

	Control	COPD	p Value
N	24	31	-
Age (years)	67.5 (62.79 – 72.72)	67.29 (64.24 – 70.34)	0.9
Gender M/F	12 / 12	15 / 16	-
Smoker (Never/Ex/Current)	6 / 15 / 3	1 / 20 / 10	-
Pack Years	27.2 (14.53 – 39.87)	45.98 (32.04 – 55.91)	0.053
Cancer Diagnosis (Yes/Unknown)	15 / 9	18 / 13	-
FEV_1%	98.79 (92.43 – 105.2)	77.26 (71.72 – 82.8)	< 0.0001
FEV_1/FVC ratio	0.772 (0.752 – 0.792)	0.599 (0.573 – 0.625)	< 0.0001

Table 2.1. Patient phenotypes of individuals recruited to the study. Individuals were defined as COPD by spirometry if FEV_1/FVC <0.7. Mean and IQR shown. Unpaired t test performed.

Preparation of monocytes and T cells from peripheral blood

Blood was obtained from human volunteers in BD Vacutainer heparinised blood collection tubes (BD Biosciences, Oxford, UK). Blood was diluted 1:1 with Dulbecco's Phosphate-Buffered Saline **(**DPBS) (Sigma, Poole, UK) and layered upon Ficoll-Paque Plus (GE Lifesciences, Little Chalfont, UK) for density gradient centrifugation at 800 g, 20ºC for 30 min. Approximately equal volumes of blood/DPBS and Ficoll-Paque Plus were used for cell separation. Cells were removed from the interface, washed in DPBS then counted using a haemocytometer. Live cells were identified by exclusion of Trypan blue (Sigma, Poole, UK). In order to isolate monocytes from the PBMC suspension, 10µl anti-CD14 MACS beads (Miltenyi Biotech, Surrey, UK) were added per 1 x 10^7 cells suspended in a Monocyte Isolation Buffer (MIB – sterile DPBS, 0.5% BSA and 0.5 M EDTA). This solution was incubated on ice for 30 min. Cells were centrifuged at 400 g, 4ºC for 5 min and the pellet was resuspended in MIB. This cell solution was added to a LS MACS Separation Column (Miltenyi Biotech, Surrey, UK) attached to a magnetic stand in order to isolate monocytes from other blood lymphocytes. The column was removed from the magnet and MIB was added to yield monocytes retained in the column. This was repeated with anti-CD4 and anti-CD8 beads (Miltenyi Biotech, Surrey, UK) to isolate pure T cell populations from the lymphocyte filtrate. Both monocytes and monocyte-depleted lymphocyte cell suspensions were counted using a haemocytometer and centrifuged at 400 g, 4ºC for 5 min. Monocyte pellets were resuspended to a desired cell concentration in RPMI 1640 (Sigma, Poole, UK) supplemented with 1% penicillin/streptomycin (Gibco Life Sciences, Paisley, UK), 250 ng/ml fungizone (Gibco Life Sciences, Paisley, UK), 10% Foetal Calf Serum (Sigma, Poole, UK), 2 mM L-glutmine (Gibco Life Sciences, Paisley, UK), and 2 ng/ml GM-CSF (R&D Systems, Abbinton, UK) and were cultured overnight or for 12 days. Lymphocytes were resuspended to a desired cell concentration in RPMI 1640 (Sigma, Poole, UK) supplemented with 1% penicillin/streptomycin (Gibco Life Sciences, Paisley, UK), 250 ng/ml fungizone (Gibco Life Sciences, Paisley, UK), and 10% Foetal Calf Serum (Sigma, Poole, UK) and were cultured overnight or stimulated. T lymphocytes were also suspended in BSA supplemented with 10% DMSO and at -80ºC using a Mr. Frosty Freezing Container.

Monocyte Derived Macrophages (MDMs) were prepared by culturing isolated monocytes in RPMI supplemented with 1% penicillin/streptomycin (Gibco Life Sciences, Paisley, UK), 250 ng/ml fungizone (Gibco Life Sciences, Paisley, UK), 10% Foetal Calf Serum (Sigma,

Poole, UK), 2 mM L-glutmine (Gibco Life Sciences, Paisley, UK), and 2 ng/ml GM-CSF (R&D Systems, Abbinton, UK) for 12 days. T cells were resuspended in 90% FCS and 10% DMSO for freezing at -80ºC. A schematic representation of MDM preparation for ELISpot is shown in Figure 2.1.

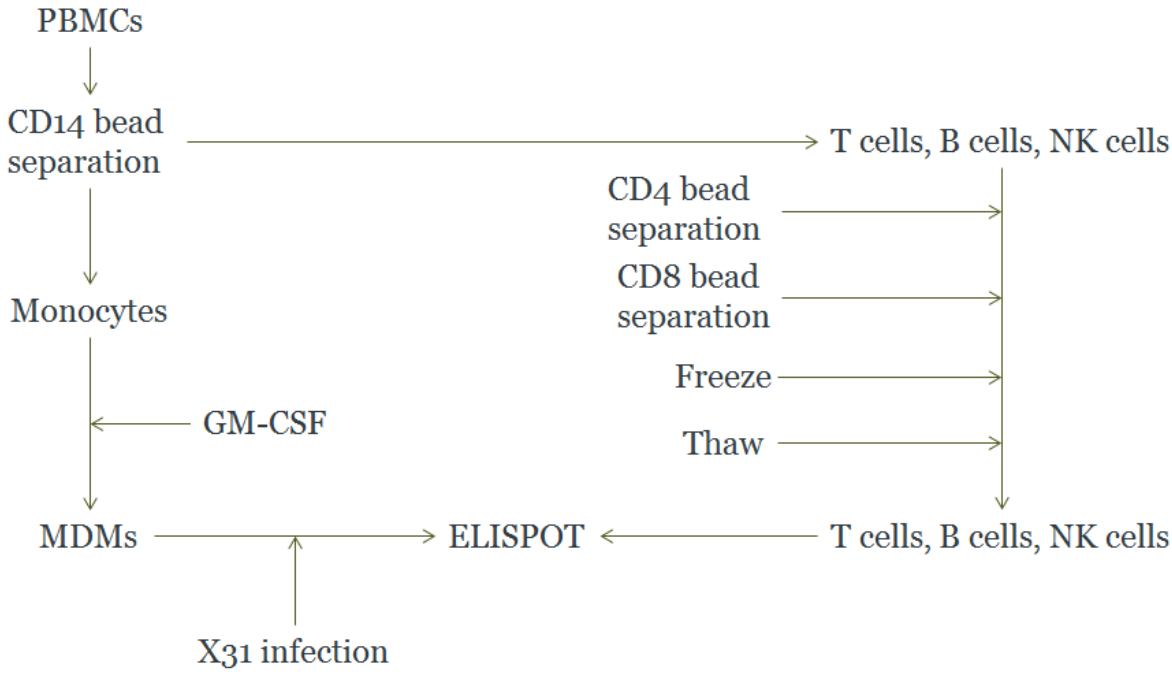

Figure 2.1. Isolation of Monocytes and T cells from PBMCs and differentiation to Monocyte Derived Macrophages. PBMCs were obtained from the interface layer of blood density centrifugation separation. PBMCs were incubated with anti-CD14 beads and passed through a magnetic separation column to yield monocytes through positive selection. The effluent was incubated with anti-CD4 beads and was passed through a fresh column to yield CD4+ T cells. Finally, effluent was incubated with anti-CD8 beads and was passed through a fresh column to yield CD8+ T cells. Fresh supplemented RPMI with 2 ng/ml GM-CSF was added to monocytes every 3 days for 12 d to yield MDM. T cells were frozen at -80ºC using a Mr. Frosty Freezing Container and thawed for use with differentiated MDMs.

Preparation of lung parenchyma for tissue digestion

The sections of lung tissue used were distal to any tumours present in the lung as judged by the pathology department. Bronchial tissue was removed from the sample, and for this study only lung parenchymal tissue was investigated. A novel protocol used to digest tissue was adapted was optimised from Holt *et al*. (Holt et al., 1986) and Dr. Ben Niclolas (Nicholas et al., 2013). Tissue was cut into 1 mm^3 sections and added to a 24-well flat-bottomed culture plate (Sigma, Poole, UK) before washing with DPBS. Washing of the tissue was performed by removing DPBS from the wells and replacing it with fresh DPBS, followed by

unsupplemented RPMI and finally RPMI supplemented with 1% penicillin/streptomycin (Gibco Life Sciences, Paisley, UK) and 1% gentamycin (PAA, Somerset, UK). Tissue was then incubated overnight at 37ºC and 5% CO_2. The wet weight of tissue was recorded. Tissue was added to a solution of pre-warmed unsupplemented RPMI and 0.5 mg/ml collagenase type I (Sigma, Poole, UK) for digestion (unless different concentration is stated) at 37ºC for the times indicated. A magnetic stirrer was added to the solution in order to mechanically disaggregate tissue. For non-enzymatic tissue disaggregation, a GentleMACS Dissociator (Miltenyi Biotech, Surrey, UK) was used. Tissue was prepared in the same manner but was added to pre-warmed unsupplemented RPMI alone. After digestion the solution was filtered through a 35 µm pore straining cap into 5 ml round-bottomed polypropylene FACS tubes (BD Biosciences) in preparation for FACS analysis.

Extracellular and intracellular cell marker staining and flow cytometry

T cells, macrophages and epithelial cells were identified using fluorescently-labelled antibodies (Figures 2.2-2.6) and a FACSAria (BD Biosciences) flow cytometer. Cells obtained from blood or tissue were suspended in MACS buffer (DPBS, 0.5% BSA and 0.5 M EDTA) containing 2 mg/ml human IgG (Sigma, Poole, UK). Appropriate isotype and fluorescence-matched control antibodies were added in a sample of the cells to aid gating of cell populations. These isotype control samples contained approximately 10% of the number of cells used for each sample. Cells were incubated on ice for 30 min in darkness after addition of antibodies. MACS buffer was added to the FACS tubes and these were centrifuged at 400 g, 4ºC for 5 min. Cell pellets were resuspended in 100 µl MACS buffer and a secondary stain was performed. Cells were again incubated on ice for 30 min in darkness after addition of antibodies. If only cell surface markers were analysed, MACS buffer was added to the FACS tubes and these were centrifuged at 400 g, 4ºC for 5 min before resuspension in MACS buffer for flow cytometry. If intracellular markers were analysed, cells were further washed in MACS buffer. The resulting cell pellets were resuspended in Cytofix/Cytoperm (BD Biosciences, Oxford, UK) and incubated on ice for 20 min in darkness. 1x Permwash was added to the FACS tubes and these were centrifuged at 400 g, 4ºC for 5 min before resuspending the cell pellet in 1x Permwash. Antibodies to identify intracellular markers were added to samples and these were incubated on ice for 30 min in darkness. 1x Permwash was added to the FACS tubes and these were centrifuged at 400 g, 4ºC for 5 min before resuspension in MACS buffer for flow cytometry. Data was

analysed using FACS Diva (v5.0.3, BD Biosciences, Oxford, UK) software. A complete list of antibodies used is shown in Table 2.5.

T cell staining

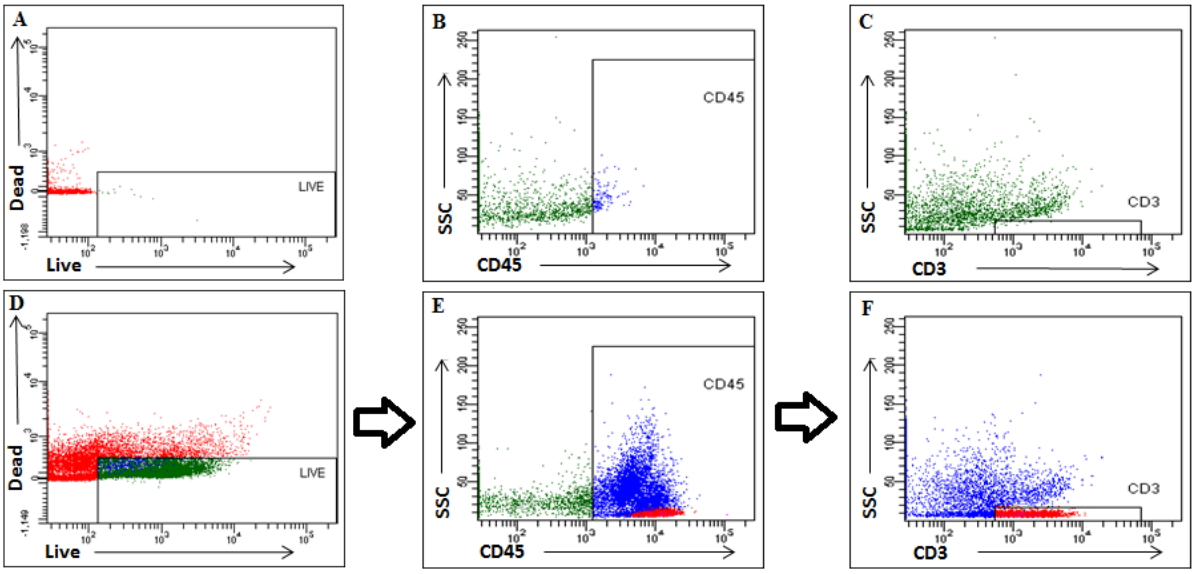

Figure 2.2. Flow cytometry gating strategy for T cells. (A) Unstained singlet population obtained from digested tissue. **(D)** Dead cells were excluded using LIVE/DEAD® Fixable Aqua Dead Cell Stain. **(B)** Live singlet population without CD45 antibody. **(E)** Live singlet CD45+ population. **(C)** Live CD45+ population with PE-Cy7 control antibody. **(F)** Live CD45+CD3+ T cell population.

Marker	Fluorochrome	Isotype	Clone	Quantity(µl/test)
CD3	PE-Cy7	Ms IgG1, κ	SK7	2.5
CD4	PerCP-Cy5.5	Ms IgG_1, κ	RPA-T4	5
CD8	APC-Cy7	Ms IgG1, κ	SK1	5
CD45	PE-CF594	Ms IgG_1, κ	HI30	2
CD279 (PD-1)	PE	Ms IgG_1, κ	MIH4	20
Granzyme B	FITC	Ms IgG_1, κ	GB11	20
IFNγ	FITC	Ms IgG2b, κ	25723.11	20
TIM-3*	APC	Ms IgG_1, κ	F38-2E2	5

Table 2.2. Mouse anti-human Antibodies used for T cell staining for flow cytometry analysis. Antibodies purchased from BD Biosciences (Oxford, UK) or eBiosciences (Hatfield, UK, marked with *).

The staining panel to detect the T cell population of PBMCs consisted of LIVE/DEAD® Fixable Aqua Dead Cell Stain (Invitrogen, Paisley, UK), anti-CD3 and anti-CD45 antibody (Figure 2.2). Addition of anti-CD4, anti-CD8 antibody allowed for identification of CD4+CD8- , CD4-CD8+ and CD4-CD8- (Double negative) T cells (Figure 2.3). Secondary staining of T cells included anti-CD279 (PD-1), and anti-TIM-3 antibody.

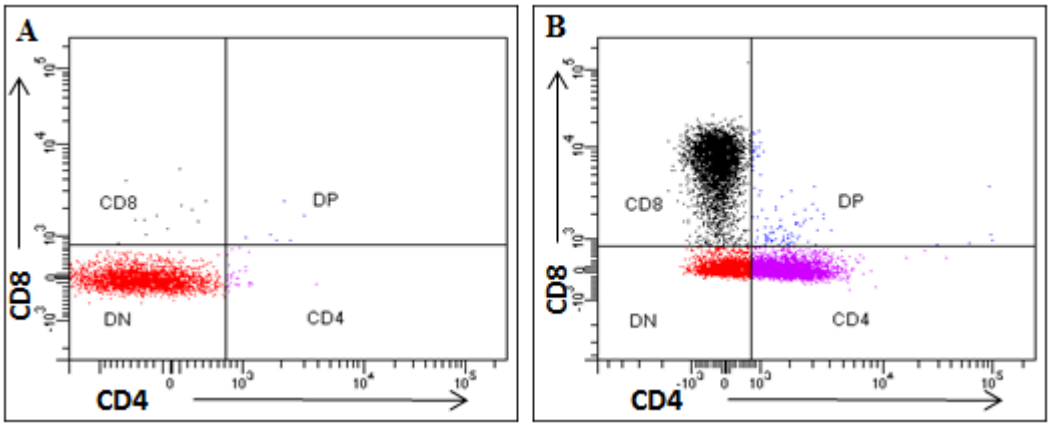

Figure 2.3. Flow cytometry gating strategy for CD4+ and CD8+ T cells. (A) Live singlet CD45+CD3+ T cells with PerCp-Cy5.5 and APC-Cy7 control antibody. **(B)** Gating of CD4+CD8- (purple), CD4-CD8+ (black) and DN T cells (red).

Memory T cell panel consisted of anti-CD3, anti-CD4, anti-CD8 and anti-CD45RA, and anti-CD197 (CCR7) antibody (de Bree et al., 2005; Purwar et al., 2011). Singlet cells were selected, and those expressing CD3+ were identified as T cells. T cells were divided by their expression of CD4+CD8- or CD4- CD8+. Expression of CD45RA and CCR7 were used to define memory phenotype (Figure 2.4). Secondary staining of the memory population consisted of anti-CD279 (PD-1), anti-TIM-3, anti-CD57 and anti-CD107a antibodies.

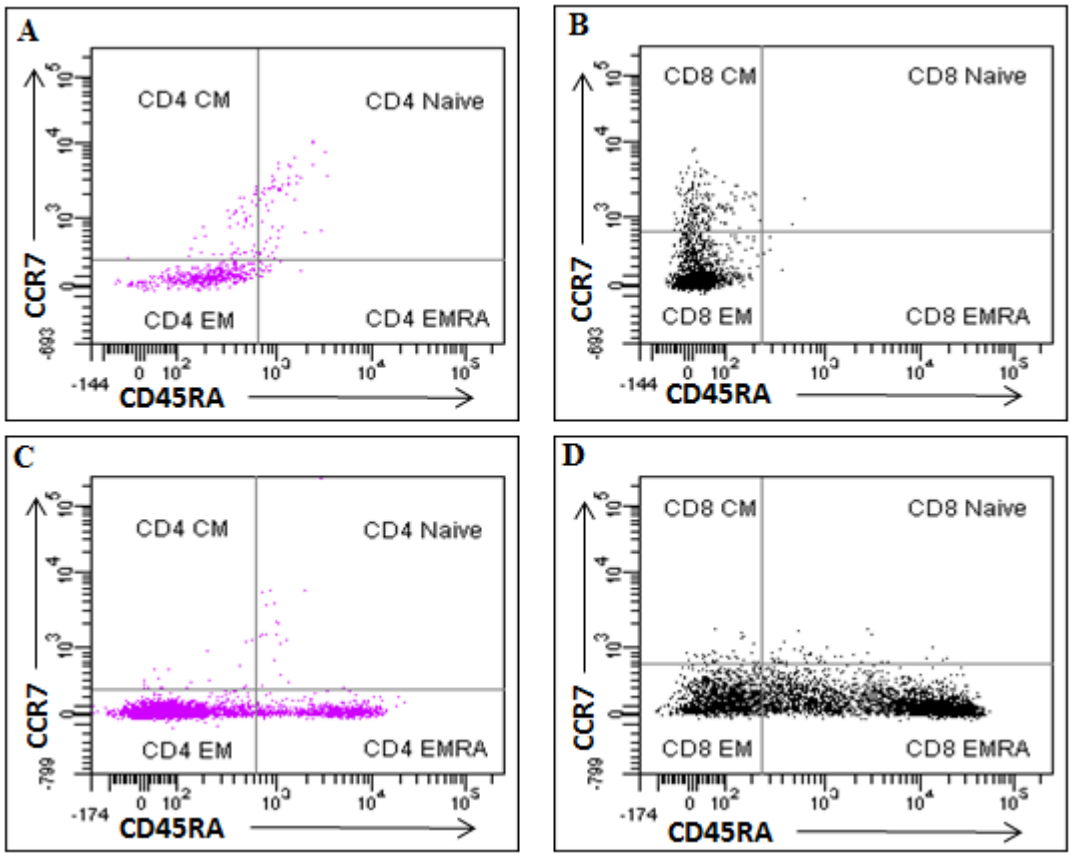

Figure 2.4. Flow cytometry gating strategy for memory CD4+ and CD8+ T cells. (A) Singlet CD3+CD4+ T cells with BV510 control antibody. **(B)** Singlet CD3+CD8+ T cells with BV510 control antibody. **(C)** Naïve CD4+ T cells were defined as CD3+CD4+CD45RA+CCR7+, Central memory cells were defined as CD3+CD4+CD45RA-CCR7+, Effector memory cells were defined as CD3+CD4+CD45RA-CCR7- and Effector memory CD45RA+ cells were defined as CD3+CD4+CD45RA+CCR7-. **(D)** Naïve CD8+ cells were defined as CD3+CD8+CD45RA+CCR7+, Central memory cells were defined as CD3+CD8+CD45RA-CCR7+, Effector memory cells were defined as CD3+CD8+CD45RA-CCR7- and Effector memory CD45RA+ cells were defined as CD3+CD8+CD45RA+CCR7-

Marker	Fluorochrome	Isotype	Clone	Quantity(µl/test)
CD3	PE-Cy7	Ms IgG1, κ	SK7	2.5
CD4	PerCP-Cy5.5	Ms IgG_1, κ	RPA-T4	5
CD8	APC-Cy7	Ms IgG1, κ	SK1	5
CD45RA	PE-CF594	Ms IgG_{2b}, κ	HI100	2
CD197 (CCR7)	BV510	Ms IgG2a	150503	5
CD57	FITC	Ms IgM, κ	NK-1	20
CD107a	BV421	Ms IgG1, κ	H4N3	5
CD279 (PD-1)	PE	Ms IgG_1, κ	MIH4	20
CD319 (NKG-2D)*	FITC	Ms IgG_1, κ	1D11	5
TIM-3	APC	Ms IgG_1, κ	F38-2E2	5

Table 2.3. Mouse anti-human antibodies used for Tmem staining for flow cytometry analysis. Antibodies purchased from BD Biosciences (Oxford, UK) or eBiosciences (Hatfield, UK, marked with *).Epithelial cell and macrophage staining

The epithelial cell and macrophage panel consisted of LIVE/DEAD® Fixable Aqua Dead Cell Stain, anti-CD45, anti-EpCAM-1, and anti-HLA-DR antibody (Figure 2.5) (Nicholas et al., 2013; Staples et al., 2012). Live singlet cells expressing CD45-EpCAM-1+ were defined as epithelial cells. Live singlet cells expressing CD45+HLA-DR+ were defined as macrophages. Secondary staining of epithelial cells and macrophages consisted of anti-CD274 (PD-L1), anti-CD80 and anti-CD86 antibodies. Intracellular staining for NP-1 (Influenza nucleoprotein) (3V Biosciences, Palo Alto, CA) was also performed in epithelial cells and macrophages.

Marker	Fluorochrome	Isotype	Clone	Quantity(µl/test)
CD45	PE-CF594	Ms IgG_1, κ	HI30	2
CD326 (EpCAM-1)	PerCP-Cy5.5	Ms IgG_1, λ	EBA-1	10
HLA-DR	APC-Cy7	Ms IgG_{2a}, κ	G46-6	5
CD274 (PD-L1)	PE	Ms IgG_1, κ	MIH1	20

Table 2.4. Mouse anti-human antibodies used for epithelial cell and macrophage staining for flow cytometry analysis. Antibodies purchased from BD Biosciences (Oxford, UK) or eBiosciences (Hatfield, UK, marked with *).

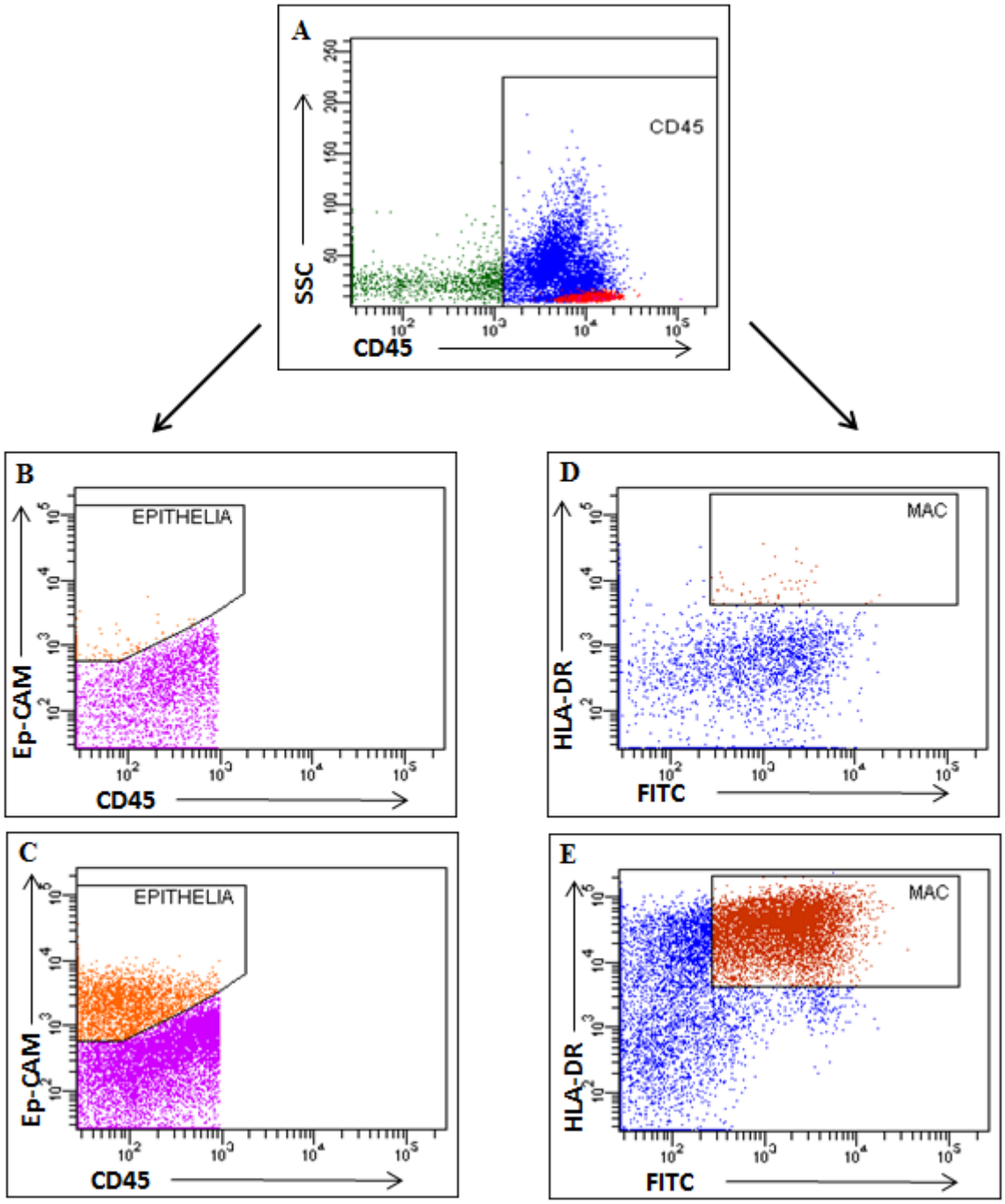

Figure 2.5. Flow cytometry gating strategy for epithelial cells and macrophages. **(A)** Live singlet cells were divided into CD45- or CD45+ populations. **(B)** Live CD45- population with PerCP-Cy5.5 control antibody. **(C)** Epithelial cells were defined as CD45-EpCAM-1+. **(D)** Live CD45+ population with APC-Cy7 control antibody. **(E)** Macrophage populations were defined as CD45+HLA-DR+.

Stimulation of peripheral T cells

T cells isolated from peripheral blood (as previously described) were cultured overnight in a flat-bottomed 48-well culture plate at 1 x 10^6 cells/ml in serum-free RPMI supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutmine (Gibco Life Sciences) and 250 ng/ml fungizone (Gibco Life Sciences, Paisley, UK) at 37ºC and 5% CO_2. Cells (suspended in serum-free RPMI) were removed from the wells of the culture plate and centrifuged at 400 g, 20ºC for 5 min. Cells were resuspended in fresh RPMI and counted before transfer to a flat-bottomed 48-well culture plate at 1 x 10^6 cells/ml. Cells were treated with 25 ng/ml phorbol 12-myristate 13-acetate (PMA) (Touraine et al., 1977) (Sigma, Poole, UK) and 100 ng/ml ionomycin (Sigma, Poole, UK) for 1 h, 2 h or 24 h, with control wells containing cells only. This was performed by direct pipetting of PMA and ionomycin into the wells of the 48-well plate. To measure granzyme B or IFNγ production, brefeldin A or monensin (both eBiosciences, Hatfield, UK) was added 6 h prior to harvest (18h after addition of PMA and ionomycin). For samples which were untimulated or stimulated at the 1h and 2h time points, brefeldin A or monensin were added 6 h prior to cell harvest. In these instances PMA and ionomycin were added after brefeldin A or monensin. Upon harvest, supernatant was removed and transferred to FACS tubes and centrifuged at 400 g, 4ºC for 5 min. Cells were resuspended in MACS buffer containing 2 mg/ml human IgG and staining was performed as previously described.

Infection of lung tissue with Influenza A Virus

1mm^3 samples of lung parenchymal tissue were prepared as previously described. 6-8 pieces of lung tissue were added to each well of a flat-bottomed 24-well culture plate containing RPMI. After resting overnight the media was replaced with serum-free RPMI supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutmine and 250 ng/ml fungizone. X31 Influenza A stock was provided by 3V Biosciences. Concentration of this stock was calculated by 3V Biosciences prior to transportation to the research group, and all virus concentrations are calculated from this data. 1 x 10^6 pfu/ml X31 Influenza A virus was added to designated wells containing 1mm^3 pieces of tissue (unless stated) by direct pipetting into the media in which tissue samples were submerged, with control non-infected wells and wells containing the same amount of UV-irradiated virus (UVX31). The culture plate was gently shaken by hand to disperse virus added to the samples. Tissue was incubated at 37ºC and 5% CO_2 for 2 h to allow for infection of cells residing in the tissue. Supernatant was removed and tissue was washed three times with unsupplemented RPMI in order to remove excess virus from the wells. Serum-free RPMI supplemented with 100 U/ml penicillin, 100µg/ml streptomycin, 2 mM L-glutmine and 250 ng/ml fungizone was added to the wells and tissue was incubated at 37ºC and 5% CO_2 for a further 22 h. Lung tissue was then digested and analysed by FACS as previously described.

Marker	Fluorochrome	Isotype	Clone	Quantity(µl/test)
CD3	PE-Cy7	Ms IgG1, κ	SK7	2.5
CD4	PerCP-Cy5.5	Ms IgG_1, κ	RPA-T4	5
CD8	APC-Cy7	Ms IgG1, κ	SK1	5
CD45	PE-CF594	Ms IgG_1, κ	HI30	2
CD45RA	PE-CF594	Ms IgG_{2b}, κ	HI100	2
CD57	FITC	Ms IgM, κ	NK-1	20
CD107a	BV421	Ms IgG1, κ	H4N3	5
CD197 (CCR7)	BV510	Ms IgG2a	150503	5
CD274 (PD-L1)	PE	Ms IgG_1, κ	MIH1	20
CD279 (PD-1)	PE	Ms IgG_1, κ	MIH4	20
CD319 (NKG-2D)*	FITC	Ms IgG_1, κ	1D11	5
CD326 (EpCAM-1)	PerCP-Cy5.5	Ms IgG_1, λ	EBA-1	10
Granzyme B	FITC	Ms IgG_1, κ	GB11	20
IFNγ	FITC	Ms IgG2b, κ	25723.11	20
HLA-DR	APC-Cy7	Ms IgG_{2a}, κ	G46-6	5
TIM-3*	APC	Ms IgG_1, κ	F38-2E2	5
APC Isotype control	APC	Ms IgG_1, κ	MOPC-21	20
APC-Cy7 Isotype control	APC-Cy7	Ms IgG_{2a}, κ	G155-178	2.5
BV421 Isotype control	BV421	Ms IgG_{2a}, κ	G155-178	5
BV510 Isotype control	BV510	Ms IgG_{2a}, κ	X40	5
FITC Isotype control	FITC	Ms IgG_1, κ	MOPC-21	20
FITC Isotype control	FITC	Ms IgM, κ	G155-228	20
PE Isotype control	PE	Ms IgG_1, κ	MOPC-21	20
PerCP-Cy5.5 Isotype control	PerCP-Cy5.5	Ms IgG_{2a}, κ	MOPC-173	2.5
PE-Cy7 Isotype control	PE-Cy7	Ms IgG_1, κ	MOPC-21	5

Table 2.5. Mouse anti-human antibodies used in flow cytometry analysis. Antibodies purchased from BD Biosciences (Oxford, UK) or eBiosciences (Hatfield, UK, marked with *).

RNA extraction from sorted cells

2.5 x 10^4 CD4 and CD8 T cells from tissue samples were sorted by flow cytometry into Stratagene lysis buffer (Agilent Technologies, Stockport, UK) supplemented with 0.7% 2-mercaptoethanol (Sigma, Poole, UK). Samples were stored at -80ºC until processing. RNA extraction was performed with an Agilent Nanoprep Kit (Agilent Technologies, Stockport, UK). Samples were diluted 1:1 with 70% ethanol solution before transfer into a spin cup fitted with a filter column. Spin cups were centrifuged at 12000 rpm, 12ºC for 1 min. Filtrate was reloaded into filter column and centrifuged at 12000 rpm, 12ºC for 1 min. This step was repeated once more. Filtrate was discarded and Low salt buffer (Agilent Technologies, Stockport, UK) was added to the spin cups. Samples were centrifuged at 12000 rpm, 12ºC for 1 min. Filtrate was discarded and spin cups were centrifuged at 12000 rpm, 12ºC for 2 min to allow membrane to dry. DNAse was diluted 1:5 and was added to the spin column. Samples were incubated at 37ºC for 15 min. High salt buffer (Agilent Technologies, Stockport, UK) was added and samples were then centrifuged at 12000 rpm, 12ºC for 1 min. Filtrate was discarded and Low salt buffer was added to the spin cups. Samples were centrifuged at 12000 rpm, 12ºC for 1 min and filtrate was discarded. Low salt buffer was again added to the column and samples were centrifuged at 13000 rpm, 12ºC for 1 min. Filtrate was discarded and spin cups were centrifuged at 12000 rpm, 12ºC for 2 min to allow membrane to dry. Elution buffer was added to the samples and incubated at room temperature for 30 min. Samples were centrifuged at 13000 rpm, 12ºC for 2 min, eluent was reloaded into the column and samples were again centrifuged at 13000 rpm, 12ºC for 2 min. This extracted RNA was stored at -80ºC.

cDNA synthesis of T cell RNA

Twenty nanograms RNA was reverse transcribed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Paisley, UK) and random hexamer primers. Reactions were prepared in RNase-free 0.2 ml tubes on ice and contained 1 x RT buffer, 4 mM dNTP mix, 1 x random primers, 50 U MultiScribe Reverse Transcriptase, 20 U RNase inhibitor made up to a 20 µl reaction volume with RNase/DNase-free water (all Applied Biosystems, Paisley, UK):

Tubes were gently vortexed and spun briefly in a microcentrifuge to eliminate air bubbles. Reverse transcription was performed using a DNA Engine Thermal Cycler (MJ Research) with the following conditions:

25 ºC 10 min (primer annealing)
37 ºC 120 min (first strand cDNA synthesis)
85 ºC 5 min (inactivation of reverse transcriptase)
Hold 4 ºC
The resulting cDNAs were stored at -20 ºC.

RT-PCR of DNA from sorted T cells

RT-PCR analysis of sorted CD4+ and CD8+ T cells from tissue was performed by Dr. C. Mirella Spalluto and Miss. Doriana Cellura

Quantitative PCR (qPCR) was performed using a 7900HT Fast Real-Time PCR System and predesigned Taqman Gene Expression Assays (Applied Biosystems, Paisley, UK). Reactions were prepared in duplicate in a 384 well PCR plate and contained 1 µl of cDNA generated as described above, 1 x Taqman Universal Master Mix II No AmpErase UNG, 1 x Taqman Gene Expression Assay buffer and made up to 5 µl reaction volume with RNase/DNase-free water (all from Applied Biosystems, Paisley, UK). RNase/DNase-free water was used as a negative control for these experiments, while no positive control was included.:

Reactions were performed using the following conditions:

95 ºC 10 min (polymerase activation step)
Followed by 40 cycles of:
95 ºC 15 sec (denaturing step)
60 ºC 1 min (annealing and extension step)

The following Taqman Gene Expression Assays were used:

CD27: Hs00386811_m1

CD57: Hs00218629_m1

CTLA-4: Hs03044418_m1

LAG-3: Hs00158563_m1

PDCD1: Hs01550088_m1

HAVCR2: Hs00958618_m1

(TIM-3)

β_2 M: Hs00984230_m1

Relative quantification of target gene expression was performed using the $\Delta\Delta C_T$ method. β_2 microglobulin (β_2 M) was used as the endogenous control.

ELISpot

ELISpot was performed using ELISpot for Human IFN-γ (MabTech, Sweden) and 0.45µm MultiScreen-IP Filter Plates (Millipore, Watford, UK) following manufacturer's instructions. Coating antibody (1-DIK) was diluted to 15µg/ml in sterile DPBS and was added to the wells of the plate before overnight incubation at 4ºC. MDMs were removed from 24-well culture plate using Cell dissociation solution, non-enzymatic (Sigma, Poole, UK) and transferred to sterile 1.5 ml eppendorfs. Cells were centrifuged at 400g, 4ºC for 5 min before resuspension in Serum-free (SF) RPMI (supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutmine and 250 ng/ml fungizone). MDMs were either not infected, or were treated with 2.5 x 10^4 pfu/ml X31 Influenza A H3N2 virus or UVX31. Infected cells were incubated at 37ºC for 2 h. Excess virus was removed by washing five times with basal media before resuspension with Reduced Serum (RS) RPMI (supplemented with 100 U/ml penicillin, 100µg/ml streptomycin, 2 mM L-glutmine and 250 ng/ml fungizone and 0.01% FCS). Monocyte-depleted PBMCs were defrosted at room temperature and suspended in RS RPMI. Excess antibody was removed from plate by washing five times with sterile DPBS before replacement with SF RPMI for 30 min at room temperature (RT). Media was removed and MDMs were added to the wells of the plate at a concentration of 5 x 10^4 cells/well. 1 x 10^5 monocyte-depleted PBMCs, 1 x 10^5 CD4+ T cells or 1 x 10^5 CD8+ T cells were added to MDM-containing wells and incubated at 37ºC for 22 h. MDM and T cells were also treated with 100 nM fluticasone propionate (Sigma, Poole, UK), 50 nM retinoic acid (Sigma, Poole, UK), 10 µg/ml PD-L1 blocking antibody or 10 µg/ml isotype control antibody (both

eBiosciences, Hatfield, UK) Cells were removed from the plate and wells were washed x5 with sterile DPBS+0.05% Tween20 (Sigma, Poole, UK). Detection antibody (7-B6-biotin) was diluted to 1 µg/ml in sterile DPBS+0.5% FCS and was added to the wells of the plate before 2 h incubation at RT. Excess antibody was removed from plate by washing x5 with sterile DPBS before replacement with Streptavidin-ALP (diluted 1:1000 in sterile DPBS+0.5% FCS) and was incubated for 1 h at RT. Excess antibody was removed from plate by washing five times with sterile DPBS before replacement with substrate solution (BCIP/NBT diluted 1:1:8 in sterile H2O). Plate was incubated at RT for 2-5 m until clear spots were visible. At this point wells were washed five times with H_2O and were allowed to dry at RT. Spot development was analysed using an AID EliSpot Reader (Germany) and AID EliSpot Software (Germany).

Statistics

Analysis of two groups was performed using either students t-test (parametric) or Wilcoxon signed rank test (non-parametric) according to sample variance within groups. Unpaired data was analysed using a Mann-Whitney test. For analysis of more than 2 groups, one-way ANOVA tests or Friedman tests were performed. GraphPad Prism (v6, GraphPad Software Inc., San Diego, USA) was used to perform statistical tests, Significance was determined by $p<0.05$ (*).

Chapter 3

Identifying Lung Resident Cells

Introduction

COPD is a heterogeneous condition involving a multitude of cell types and immune mechanisms. Epithelial cells, alveolar macrophages and T cells present in the lung may be involved in abnormal or deregulated responses in COPD, but would not be identified in the blood. It is therefore vital that the site of disease, the lung, is studied rather than potential secondary effects in the periphery. The analysis of lung parenchyma from human donors allows for the study of cells within the lung which may be implicated in the progression of COPD.

A pitfall encountered during research into tissue derived cells is that the preparation of these cells and tissues for analysis may alter the populations wishing to be studied. Flow cytometry is a valuable tool used to identify individual cells and cell populations, but this is only possible when a sample is in a single-cell suspension. Enzymatic digestion of tissue can result in the degradation and loss of the CD4 surface marker on T cells (Ford et al., 1996), and increased digestion times may increase cell yield but decrease marker expression. This chapter details the endeavours to yield CD4+ and CD8+ T cells, epithelial cells and macrophages from lung parenchymal tissue while preserving the characteristics of cells present in the lung.

Protocols used previously within our research group accounted for the potential loss of CD4 expression due to collagenase treatment, and T cells were defined as CD8+ or CD8- (Hinks, 2013). This CD8- population would include CD4+ T lymphocytes, but also innate-like cells such as NK T cells, MAIT cells and novel ILC populations (Moro et al., 2010; Tindemans et al., 2014). For this study, the aim was to optimise the digestion protocol to ensure both CD4+ and CD8+ T cells could be identified from human lung parenchyma. Digestion protocols would also require the yield of sufficient numbers of epithelial cells and macrophages. Initial experiments were performed using PBMCs to evaluate the effect of collagenase on T cells. Subsequent experiments were performed using human tissue to ensure adequate cell yield using the re-defined protocol.

Results and comments

Collagenase digestion duration does not affect cell viability or CD3 expression of T cells from blood

Initial collagenase experiments were performed with PBMCs rather than lung parenchyma in order to assess the direct effect of collagenase on surface marker expression and viability of the lymphocyte population. Any loss of viability or receptor expression in the PMBC model would be the direct result of collagenase treatment. Previous work performed by the group indicated that a 90 min collagenase digestion of lung parenchyma yielded good numbers of epithelial cells, macrophages and, importantly, T cells (Vijayanand et al., 2007). This was adopted as a "GOLD standard" by which to compared optimised protocol. Reduction of digestion time was one alteration to protocol which may preserve cell surface markers, but also may affect cell yield.

The duration of collagenase I treatment of PBMCs did not affect cell viability. At 15 min, 30 min, 60 min and 90 min digestion times, viability was unchanged with means ranging from 61.6% to 63.6% of the PBMC population (Figure 3.1). T cells from PBMCs were defined as singlet, live CD3+ cells (Figure 2.2.F). Using an identical protocol, the proportion of CD3+ T cells remained similar (ranging between 38.3% to 40.3% of live cells) at all incubation times (Figure 3. 2).

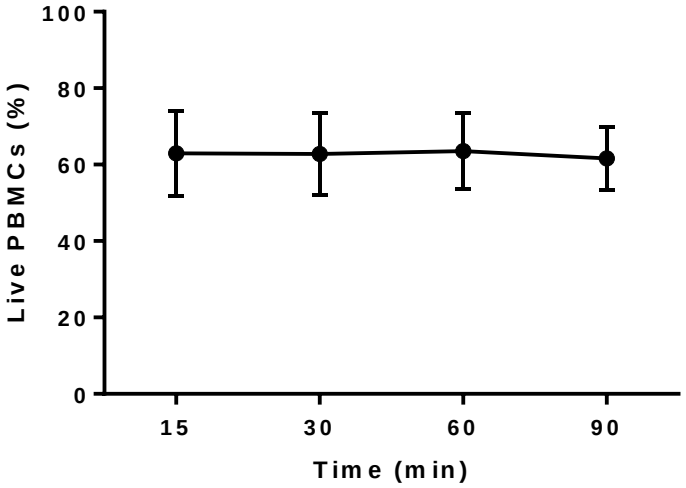

Figure 3.1. Proportion of live cells obtained from collagenase treatment of PBMCs. PBMCs were suspended in 1 mg/ml collagenase I solution and incubated at 37ºC with a magnetic stirrer. LIVE/DEAD® Fixable Aqua Dead Cell Stain was used to select live cells. n = 5. Mean and SEM shown. One-way ANOVA performed.

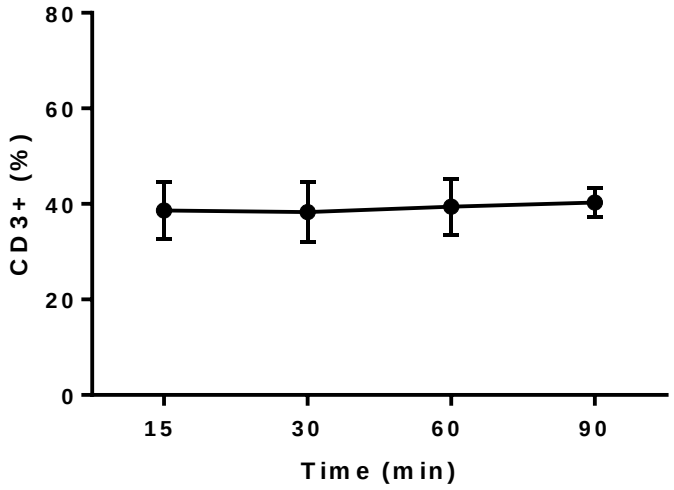

Figure 3.2. Proportion of live CD3+ cells obtained from collagenase treatment of PBMCs. PBMCs were suspended in 1 mg/ml collagenase I solution and incubated at 37ºC with a magnetic stirrer. CD3+ cells were gated on the live singlet cell population. n = 6. Mean and SEM shown. One-way ANOVA performed.

Collagenase digestion duration reduces CD4, but not CD8, expression by T cells from blood

With no loss of cell viability or the CD3 surface marker, CD4 and CD8 expression was measured using this protocol. A small but significant decrease in T cells expressing CD4 was recorded (mean decreasing from 48.8% to 43.6%, ANOVA p = 0.0127) with increasing duration of collagenase treatment (Figure 3.3). This is in agreement with Grange *et al*. (Grange et al., 2011) but not Abuzakouk *et al*. (Abuzakouk et al., 1996) who recorded no loss of CD4+ T cell detection after collagenase treatment. CD8 expression was unaffected by treatment duration (Figure 3.4) with means ranging from 33.3% to 34.6%. Double negative (DN) T cells are characterised by CD3+CD4-CD8- surface expression (Figure 2.3). Figure 3.5 shows a significant increase in the DN population from a mean of 15.9% to 21.2% as digestion time increases (ANOVA p = 0.0242). The increase in DN cells may be accounted for by CD4+ T cells losing this cell surface marker at the 60 min and 90 min time points. These CD4+ cells would be incorrectly identified as DN with longer digestions. Shorter digestion times were favoured in future work to preserve the CD4+ T cell population.

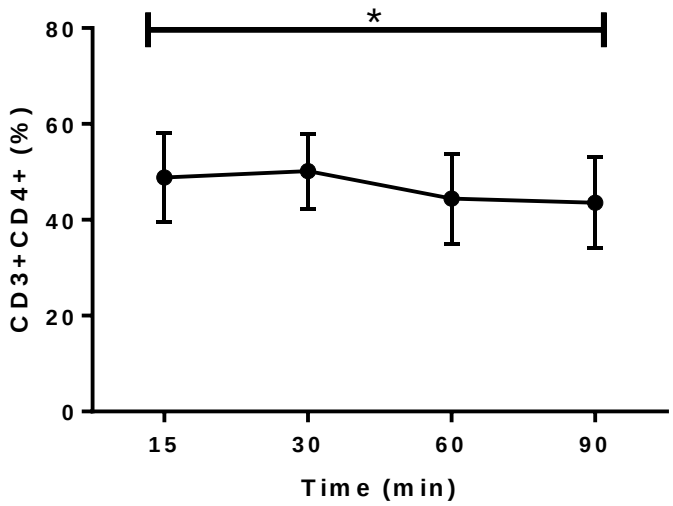

Figure 3.3. Proportion of live CD3+CD4+ cells obtained from collagenase treatment of PBMCs. PBMCs were suspended in 1 mg/ml collagenase I solution and incubated at 37ºC with a magnetic stirrer. CD4+ cells were gated on the live singlet CD3+CD4+CD8- population. n = 6. Mean and SEM shown. One-way ANOVA p = 0.0127

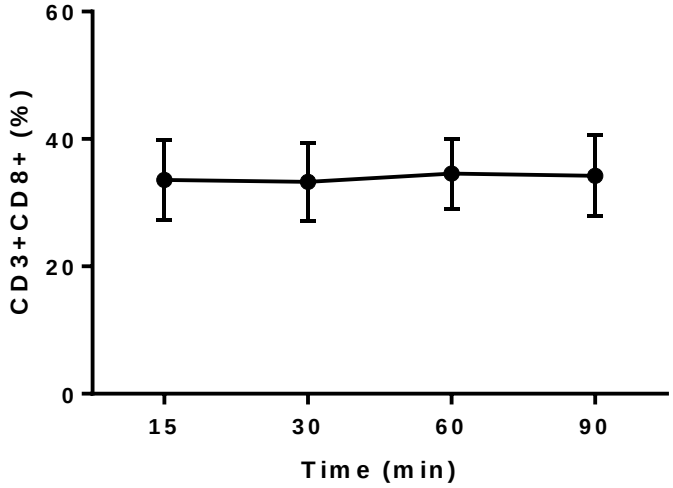

Figure 3.4. Proportion of live CD3+CD8+ cells obtained from collagenase treatment of PBMCs. PBMCs were suspended in 1 mg/ml collagenase I solution and incubated at 37ºC with a magnetic stirrer. CD8+ cells were gated on the live singlet CD3+CD4-CD8+ population. n = 6. Mean and SEM shown. One-way ANOVA performed

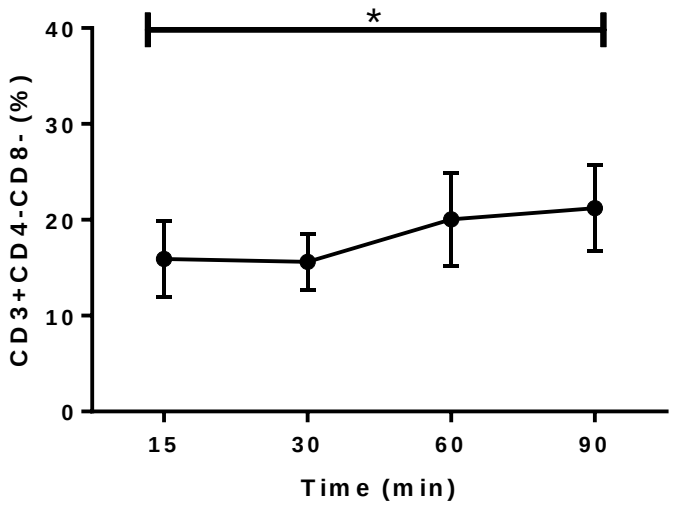

Figure 3.5. Proportion of live CD3+CD4-CD8- (DN) cells obtained from collagenase treatment of PBMCs. PBMCs were suspended in 1 mg/ml collagenase I solution and incubated at 37ºC with a magnetic stirrer. DN cells were gated on the live singlet CD3+CD4-CD8- population. n = 6. Mean and SEM shown. One-way ANOVA p = 0.0242.

Collagenase concentration does not affect cell viability or surface marker expression in PBMCs

The duration of 1 mg/ml collagenase treatment was shown to affect surface marker expression, but this may be lost at lower concentrations. Incubation time was 15 min for all experiments as this conserved CD4 to the greatest extent. As with previous experiments, cell viability and CD3 marker expression was unchanged with collagenase concentration. Increased collagenase concentration does not reduce CD4 expression (Figure 3.6) nor CD8 expression (Figure 3.7) during the 15 min treatment. DN populations also remained unchanged. Figure 3.6 represents values observed from 2 experiments. Values were not similar between experiments but the trends in response to increased collagenase treatment are similar. Therefore data from experiments were not combined and are shown individually.

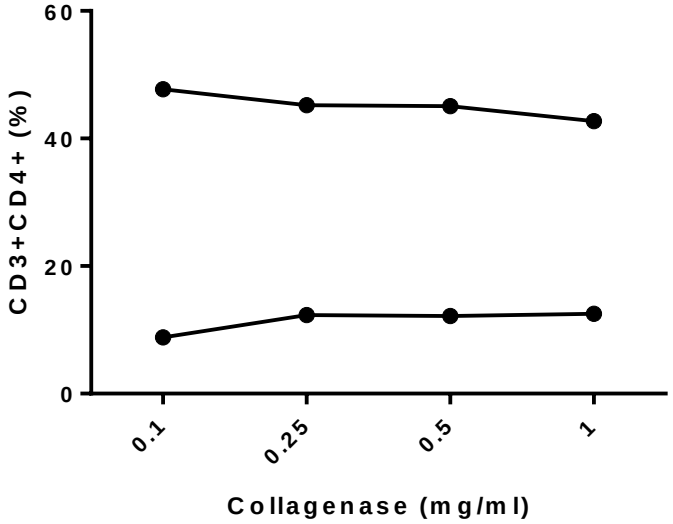

Figure 3.6. Proportion of live CD3+CD4+ cells obtained from collagenase treatment of PBMCs. PBMCs were suspended in collagenase I solution at the concentrations indicated and incubated at 37ºC for 15 min with a magnetic stirrer. CD4+ cells were gated on the live singlet CD3+CD8- population. n = 2.

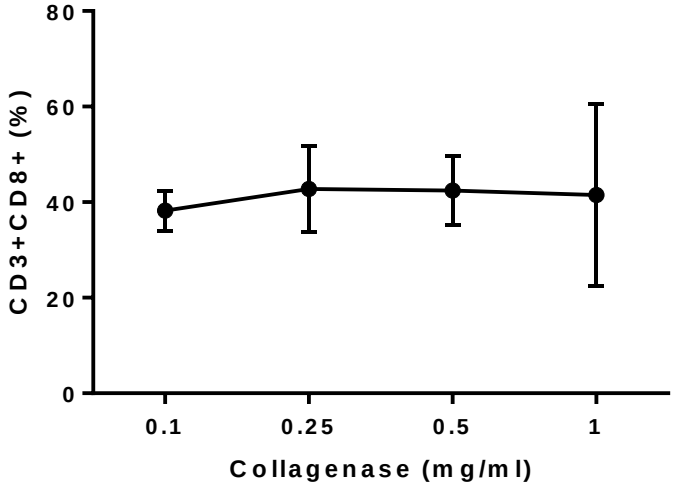

Figure 3.7. Proportion of live CD3+CD8+ cells obtained from collagenase treatment of PBMCs. PBMCs were suspended in collagenase I at the concentrations indicated solution and incubated at 37ºC for 15 min with a magnetic stirrer. CD8+ cells were gated on the live singlet CD3+CD4- population. n = 2. Mean and SD shown.

Non-enzymatic tissue disaggregation methods alone are not sufficient to yield high numbers of lung resident cells

As collagenase appeared to slightly reduce surface expression of CD4 and CD8 markers in PBMC, initial experiments using resected lung tissue investigated a non-enzymatic tissue disaggregation protocol utilising the GentleMACS Dissociator (Ruckwardt et al., 2010; Szymczak and Deepe, 2009). This method may preserve cell surface marker expression, but cell yield and cell viability were unknown prior to examination. Figure 3.8 shows the number of cells obtained from lung parenchyma using the GentleMACS Dissociator or by collagenase digestion. GentleMACS yielded a mean of 4,726 cells per mg of tissue. Although not statistically significant, this is lower than collagenase digestions of 30 min (mean=17,125), 60 min (mean=15,487) and 90 min (mean=12,083). As 3-4 times fewer cells were recovered from GentleMACS disaggregation than collagenase treatment, non-enzymatic digestion methods were not pursued for subsequent experiments.

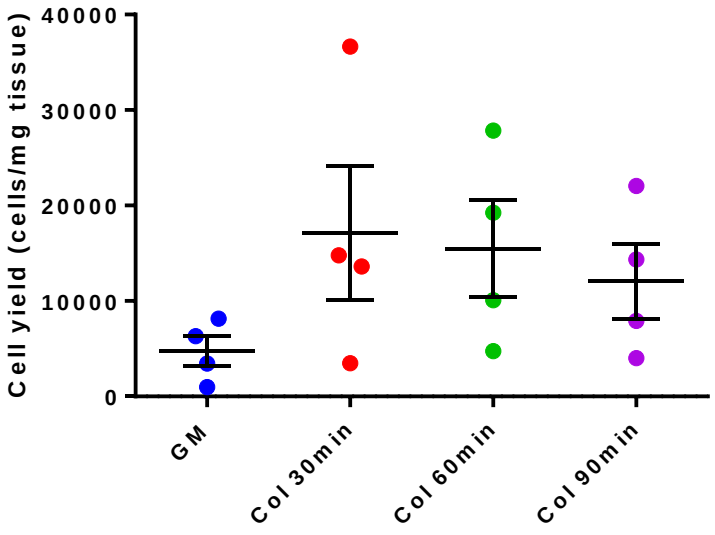

Figure 3.8. Numbers of cells obtained from GentleMACS dissociation or collagenase treatment of lung parenchymal tissue. 1 cm^3 sections of tissue were disaggregated using a GentleMACS Dissociator (GM) or digested with 0.5 mg/ml collagenase solution with a magnetic stirrer for 30 min, 60 min or 90 min. Cell yield is normalised to wet weight of tissue. n = 4. Mean and SEM shown.

Viability and T cell marker retention after collagenase digestion of lung parenchymal tissue

Analysis of cell surface marker expression on collagenase treated PBMCs allowed for the direct effects on T cells to be studied. As the key markers were relatively well preserved, similar experiments were performed using human lung tissue samples to ensure the protocol was sufficient to yield T cells from the complex cellular matrix of the tissue.

In agreement with Hagman *et al*. (Hagman et al., 2012), the duration of digestion did not affect the viability of cells yielded from the tissue (Figure 3.9). The proportion of live cells was between 8.6% to 11.2% of singlet cells, which was reduced from ~60% of PBMCs. Antibodies for the surface marker CD45 were included for tissue analysis, and the T cell population was defined as CD45+CD3+ cells. The proportion of CD45+CD3+ cells is unchanged (mean range 22.9% to 27%) with increased digestion time (Figure 3.10). A mean range of 22.9% to 27% CD45+ cells recovered from tissue were thus defined as T cells, compared to ~40% in blood.

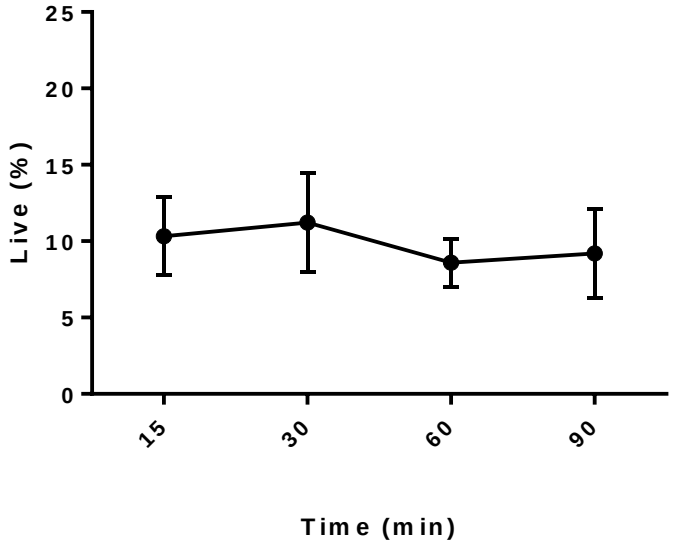

Figure 3.9. Proportion of live cells obtained from collagenase treatment of lung parenchymal tissue. 1 cm^3 sections of tissue were digested with 0.5 mg/ml collagenase solution with a magnetic stirrer for 15 min, 30 min, 60 min or 90 min. LIVE/DEAD® Fixable Aqua Dead Cell Stain was used to select live cells. n = 6. Mean and SEM shown. One-way ANOVA performed.

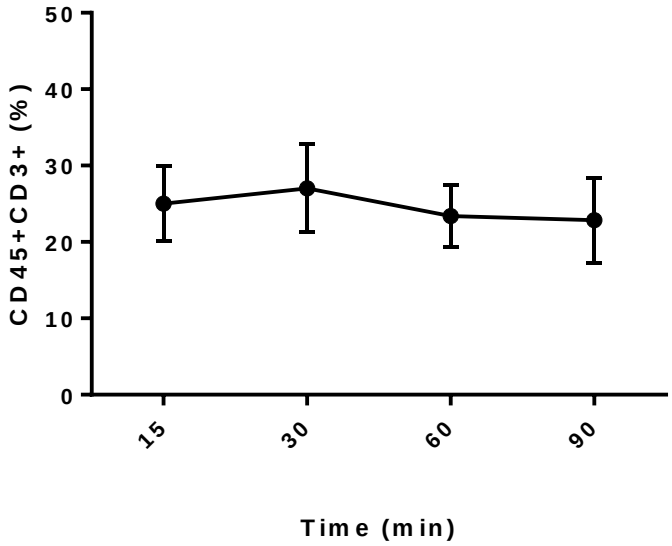

Figure 3.10. Proportion of live CD45+CD3+ cells obtained from collagenase treatment of lung parenchymal tissue. 1 cm^3 sections of tissue were digested with 0.5 mg/ml collagenase solution with a magnetic stirrer for 15 min, 30 min, 60 min or 90 min. T cells were defined as singlet live CD45+CD3+. n = 6. Mean and SEM shown. One-way ANOVA performed.

Increased collagenase digestion time trended towards a decreased CD4+ population from a mean of 17.7% to 11.2% (Figure 3.11), but this did not reach the statistical significance achieved by PBMC work (p = 0.38). CD8+ populations were conserved with increased time, accounting for 42.7% to 49.4% of T cells recovered from parenchyma (Figure 3.12). The population of DN T lymphocytes increased significantly (ANOVA p=0.02) from a mean of 31% to 45.7% (Figure 3.13). This increase of 14%-15% of the DN population may be due to the ~6% and ~7% loss seen in the CD4+ and CD8+ populations seen after 90 min digestion. The optimal conditions for further lung parenchymal digestion work were a collagenase concentration of 0.5 mg/ml and a digestion time of 15 min.

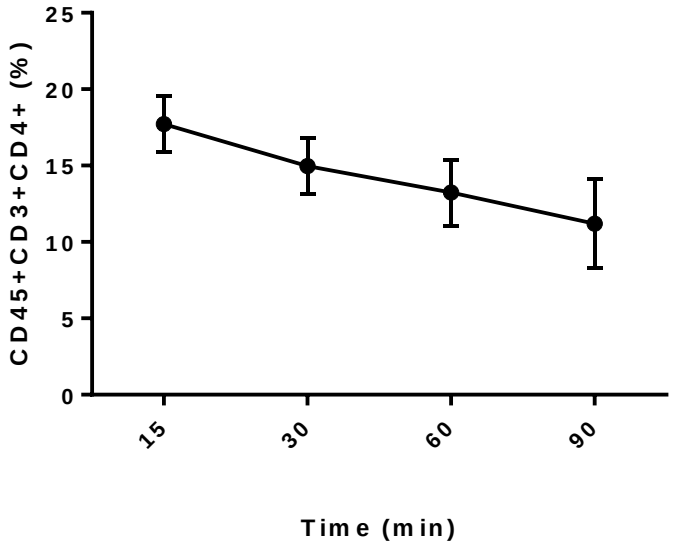

Figure 3.11. Proportion of live CD45+CD3+CD4+ cells obtained from collagenase treatment of lung parenchymal tissue. 1 cm^3 sections of tissue were digested with 0.5 mg/ml collagenase solution with a magnetic stirrer for 15 min, 30 min, 60 min or 90 min. CD4+ cells were gated on the live singlet CD45+CD3+CD4+CD8- population. n = 6. Mean and SEM shown. One-way ANOVA performed.

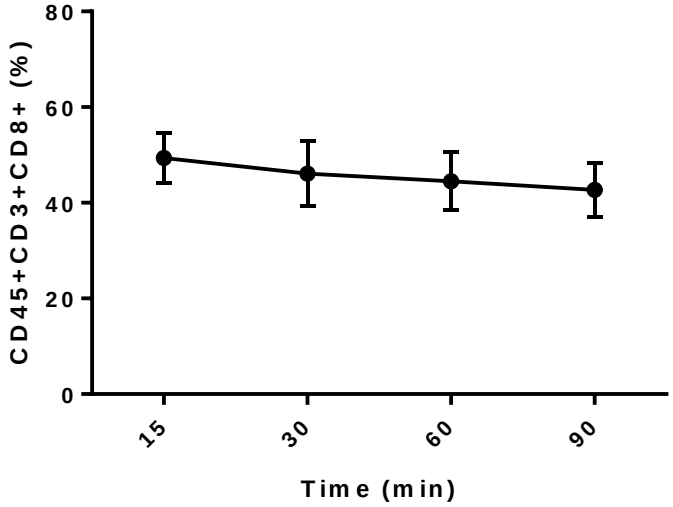

Figure 3.12. Proportion of live CD45+CD3+CD8+ cells obtained from collagenase treatment of lung parenchymal tissue. 1 cm^3 sections of tissue were digested with 0.5 mg/ml collagenase solution with a magnetic stirrer for 15 min, 30 min, 60 min or 90 min. CD8+ cells were gated on the live singlet CD45+CD3+CD4-CD8+ population. n = 6. Mean and SEM shown. One-way ANOVA performed

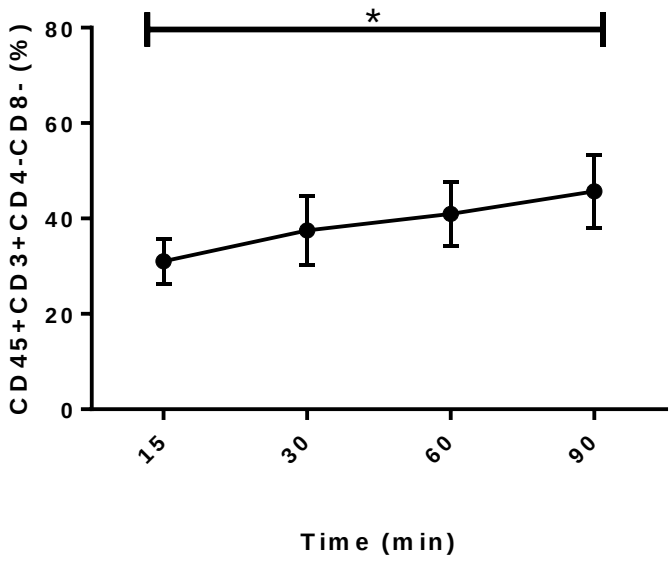

Figure 3.13. Proportion of live CD45+CD3+CD4-CD8- cells obtained from collagenase treatment of lung parenchymal tissue. 1cm^3 sections of tissue were digested with 0.5 mg/ml collagenase solution with a magnetic stirrer for 15 min, 30 min, 60 min or 90 min. DN cells were gated on the live singlet CD45+CD3+CD4-CD8- population. n = 6. Mean and SEM shown. One-way ANOVA p = 0.02.

Identification of memory T cell phenotypes in blood and tissue

T cells which have not been exposed to their specific antigen as designated by their TCR are naïve T cells. These T cells are most commonly found in the blood and lymph nodes and require co-stimulation from APCs to be activated. Naïve T cells can be defined by CD45RA+CCR7+ expression. After naïve T cell activation, T cells have the potential to differentiate into memory cells. Memory cells are long-lived and do not require co-stimulation in order to proliferate and perform other effector functions (Sallusto et al., 1999). Central Memory (T_{CM}) T cells migrate to, and are found in, lymphoid organs and tertiary lymphoid structures (Forster et al., 1999). This memory phenotype of T cells can be defined by CD45RA-CCR7+ expression. Upon recognition of antigen they can migrate to the site of infection to clear pathogens (Masopust et al., 2001). Effector Memory (T_{EM}) T cells can be defined by CD45RA-CCR7- expression. T_{EM} cells are potent cytokine producers and can promote T cell anti-viral responses through secretion of IFNγ and cytotoxic killing of infected cells (Sallusto et al., 1999). Memory T cells which are CD45RA+CCR7- appear to be terminally differentiated effector memory cells (Koch et al., 2008).

A key outcome for optimisation of the parenchyma digestion protocol was to allow for identification of T cells in the lung. Tissue was washed thoroughly with DPBS before resting and digestion in an attempt to rinse T cells present in blood vessels and capillaries out of the sample. To confirm that T cells from tissue were not derived from blood, the proportions of naïve and memory T cells from tissue and PBMCs was compared (Figure 3.14).

The proportion of blood naïve CD4+ T cells was approximately 45% of CD3+ cells, with central memory cells accounting for 29%. CD4+ effector memory cells accounted for 21% of this population, with 4.5% CD4+ T cells expressing and TEMRA phenotype. In blood naïve CD8+ T cells were the dominant phenotype, with 74.5% of cells expressing CD45RA and CCR7. TCM and TEMRA both accounted for 10% of CD8+ cells, with TEM representing approximately 5%.

In tissue (Figure 3.15), TEM population is dominant with a mean proportion of 91.3% with T_{EMRA} cells accounting for a mean of 7.2% of the CD4+ population. Naïve CD4+ T cells were detected at <1% in all tissue samples. A similar pattern was identified with CD8+ T cells. In tissue, the majority of CD8+ T cells are either TEM (mean = 52.8%) or TEMRA (mean = 42.5%) cells. As with CD4+ T cells, the naïve CD8+ population represented only a small proportion of cells identified in tissue.

CCR7 expression is used to identify naïve T cells and TCM cells. To ensure that the low expression of CCR7 in tissue was not due to collagenase cleavage of CCR7, PBMCs were incubated with or without collagenase. The mean sMFI for collagenase treated samples was 22, while non-treated samples expressed a mean sMFI of 37. Therefore CCR7 expression may be reduced in response to collagenase treatment, but it was not ablated to the extent that naïve and TCM populations would not be detectable.

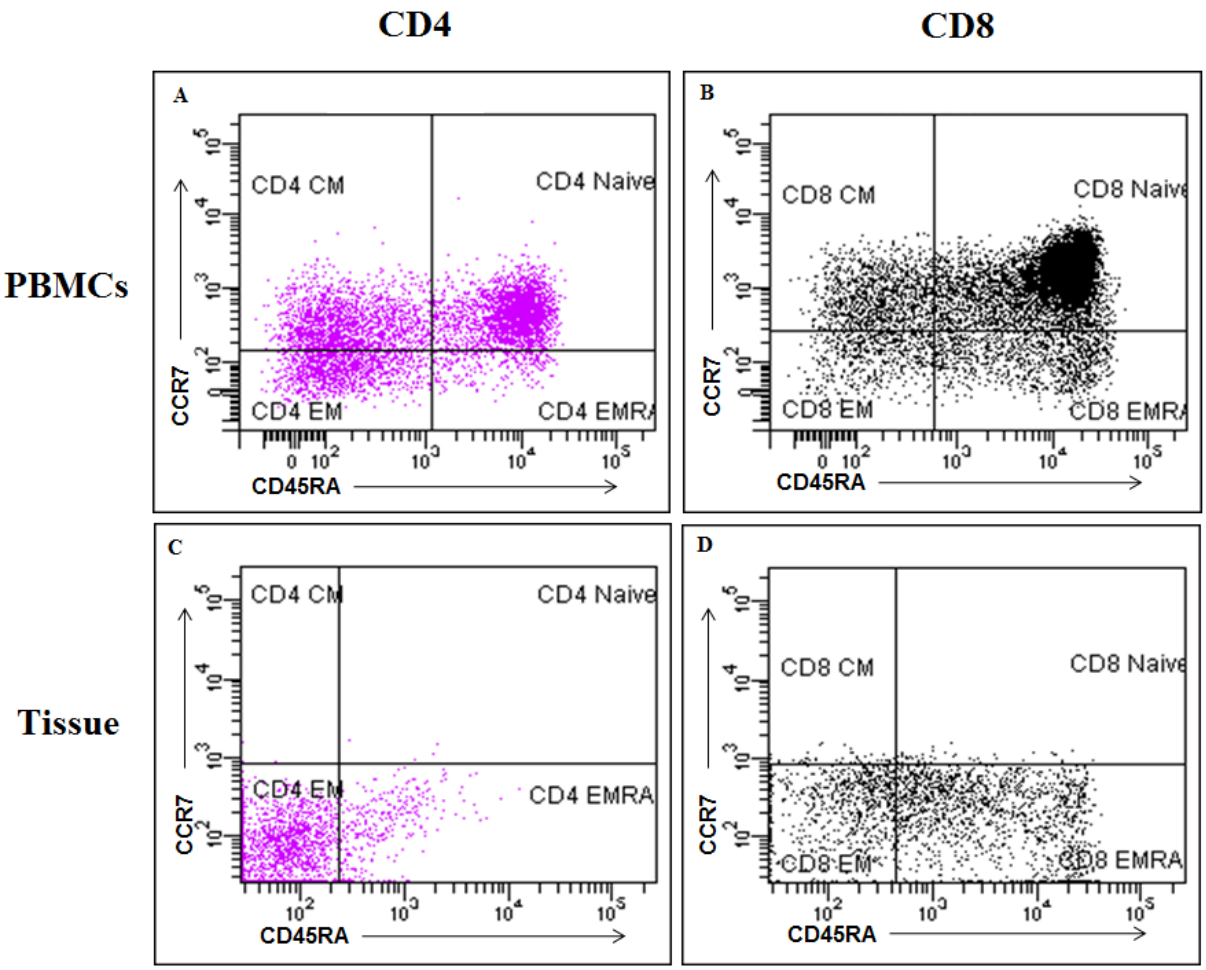

Figure 3.14. Naïve and Tmem cell populations in blood and tissue. PBMCs were isolated from blood using Ficoll-Paque density centrifugation. Tissue was prepared as previously described and digested in 0.5 mg/ml collagenase solution for 15 min. **(A+C)** T cell populations were gated on a singlet CD3+CD4+CD8- population or **(B+D)** a singlet CD3+CD4-CD8+ population. **(A)** CD4+ memory populations and **(B)** CD8+ populations in blood. **(C)** CD4+ memory populations and **(D)** CD8+ populations in lung parenchymal tissue.

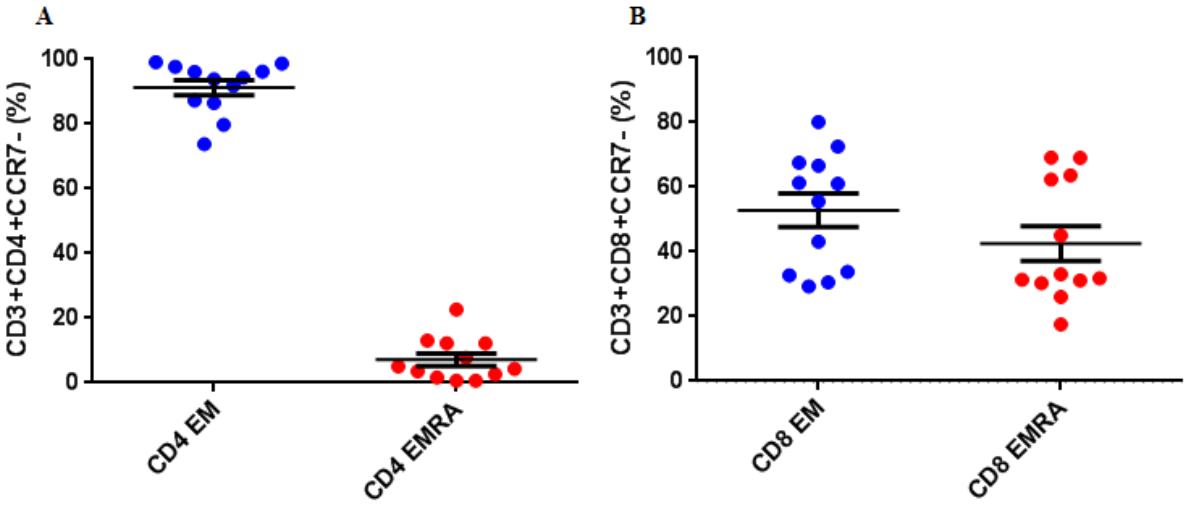

Figure 3.15. Memory T cell populations in human lung parenchyma. Lung tissue was digested with collagenase and cells analysed by flow cytometry. **(A)** Proportion of CD4+ **(B)** and CD8+ memory T cells are gated on the CD3+CCR7- population. n = 12. Mean and SEM shown. Mann-Whitney test was performed to compare control and COPD samples.

Optimisation of RT-PCR protocol

CD4+ and CD8+ T cells from lung parenchyma were gated and sorted for PCR analysis of genes associated with T cell exhaustion and senescence. In preparation for this work, the required number of cells to be sorted was evaluated. CD4+ and CD8+ T cells from tissue were sorted into Stratagene lysis buffer supplemented with 2-mercaptoethanol. RNA was extracted from T cells and cDNA was generated. The expression of the housekeeping genes GNBL1 and YWHAZ were measured by RT-PCR (Figure 3.16). Using 1 x 10^3 CD4+ or CD8+ T cells generated a Ct value of 33.1 and 33.9 respectively for GNBL1. Increasing the cell number used to 1 x 10^4 CD4+ or CD8+ T cells resulted in decreases Ct values (30.7 and 31.3 respectively). 2.5 x 10^4 CD4+ T cells recorded a Ct value of 27.6 and CD8+ T cells a Ct value of 29.3. The Ct value observed for 5 x 10^4 CD8+ T cells continued this pattern of decline with a value of 27.2. Use of 5 x 10^4 CD4+ T cells, however, recorded an increased Ct value of 29.6. Analysis of the YWHAZ gene followed a similar patter to GNBL1, but Ct values were higher at all cell concentrations. 1 x 10^3 CD4+ or CD8+ T cells generated a Ct value of 38.5 and 37.2 respectively for YWHAZ. Increasing cell numbers used to 1 x 10^4 CD4+ or CD8+ T cells resulted in decreases Ct values (34.4 and 34.7 respectively). Use of 2.5 x 10^4 CD4+ or CD8+ T cells recorded Ct values of 32.6 and 32. The lowest Ct values for this gene were recorded with 5 x 10^4 CD4+ or CD8+ T cells (31.9 and 30 respectively). The concentration of cells to be used in future experiments was 2.5 x 10^4 as this concentration recorded low Ct values for both housekeeping genes. Although the use of 5 x 10^4 CD8+ T cells recorded a lower Ct value, the increase seen in the CD4+ T cell sample compared to 2.5 x 10^4 cells was unfavourable.

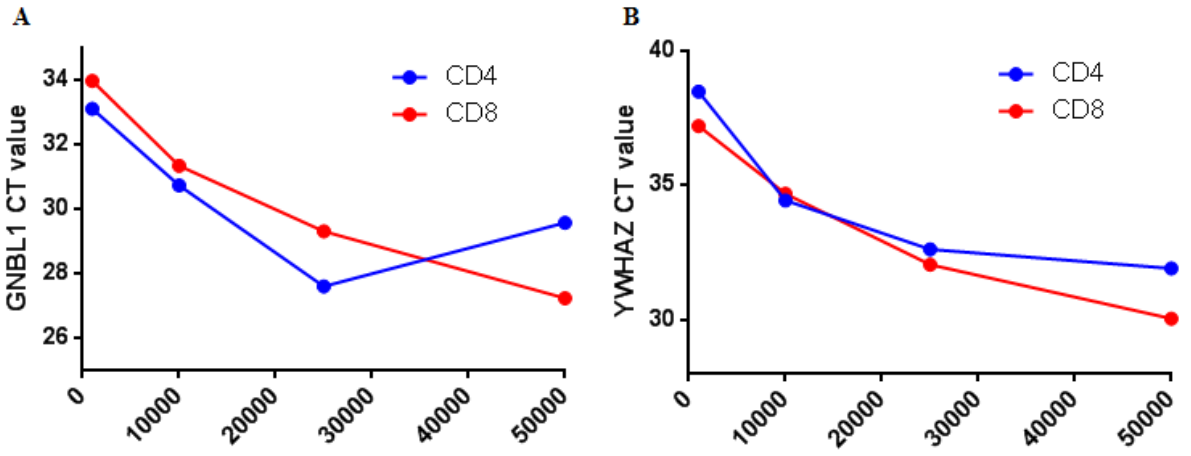

Figure 3.16. Housekeeping gene expression by lung CD4+ and CD8+ T cells. RT-PCR was performed with 1 x 10^3, 1 x 10^4, 2.5 x 10^4 or 5 x 10^4 CD4+ or CD8+ T cells. **(A)** GNBL1 or **(B)** YWHAZ gene expression. Expression represented as cycle threshold.

Conclusions

To avoid or limit potential loss of cell surface markers on tissue resident cell populations, several groups have investigated non-enzymatic tissue disaggregation methods. Initial attempts were time consuming, labour intensive or inefficient, requiring tissue aspiration (Ottesen et al., 1996), pressing (Singh, 1998) or scraping (Cornacchiari et al., 1995) to yield cells. As technology progresses so does the evolution of laboratory protocol. More recent studies have utilised automated tissue dissociation devices to rapidly relinquish cells from tissues. Novelli *et al.* (Novelli et al., 2000) performed tissue digestions using a Medimachine (DAKO – Agilent technologies). Decreased total cell number and cell viability was recorded compared to a methodology which included collagenase digestion. Grange *et al.* (Grange et al., 2011) also reported a decreased viability of cells obtained via Medimachine than collagenase alone. GentleMACS is a more modern technology employed for a similar purpose, but this too requires refinement to achieve yields gained by enzymatic digestion. These findings suggest that mechanical disaggregation alone does not allow for an optimal cell yield and also contribute to cell death.

Cell viability recorded in blood samples was significantly greater than in tissue samples. This may be in part due to the process in which tissue is obtained. Tissue samples are removed during surgery and stored in PBS before transportation to the laboratory. This can occur immediately, but as the priority in theatre is the patient, tissue transportation may be delayed. The length of time between lung parenchyma removal from the patient and arrival in the laboratory has not been studied by the group to determine differences in cell viability. Furthermore, while LIVE/DEAD staining was used in experiments performed in this chapter, degree of tissue necrosis or the "tissue quality" was not measured. Thus there may be unrecorded variation in tissue beyond the patient classification of control and COPD.

Incubation of PBMCs or human lung tissue in a collagenase solution for up to 90 min did not affect cell viability. Hagman *et al.* (Hagman et al., 2012) investigated the viability and yield of lymphocytes from adipose tissue after collagenase digestion. The group found that viability of cells was unchanged between 30 min and 120 min digestion times. Furthermore, CD3 expression was also unaffected by increased digestion time. Our data presented here in Chapter 3 agree with these findings, but in the context of lung parenchyma.

Analysis of the effects of collagenase in tissue is more complex than blood, as lung parenchyma is in part comprised of cells which are capable of producing proteases and other digestive enzymes. CD4 expression is partially lost in PBMCs digestions but only trends towards loss in tissue. This indicates that collagenase does have a direct effect on CD4 and does not require other enzymes, such as matrix metalloproteinases, to cleave the cell surface marker. Despite several studies reporting decreased detection of CD4+ lymphocytes as a result of enzymatic digestion (Van Damme et al., 2000), the mechanisms by which CD4 is lost is unknown. Our finding that CD4 expression is reduced in blood suggests either direct cleavage of CD4 or recycling of surface CD4 to intracellular vesicles. Concentrations of collagenase were similar for PBMC and lung tissue experiments. A greater variety of cell types and the inclusion of structural cells may limit the effect of collagenase in parenchyma compared to PBMCs alone. Loss of CD4 expression may have been enhanced in tissue if a greater concentration of collagenase was used.

The population of CD4+ T cells which lose CD4 expression are accounted for by an increased proportion of DN T cells. The DN T cell compartment is comprised of a wide variety of innate T cell subsets. MAIT cells, NK T cells and DN Treg cells have been identified in DN populations. These cells have been implicated in several pathogenic diseases, autoimmunity and transplantation (Juvet and Zhang, 2012; Prezzemolo et al., 2014; Wang et al., 2013). Misidentification of CD4+ T cell as DN cells may endow functions to these subsets that are associated with CD4+ counterparts. Enzymatic digestions must be performed with caution, with close examination of cell surface marker preservation. Experiments performed in this chapter finalised the tissue digestion protocol which would be performed in subsequent chapters.

The misidentification of cells derived from tissue with cells present in blood may prevent accurate quantification of T cell phenotypes at the site of interest. What is apparent is that the balance between memory and naïve T cells is altered between blood and tissue. The migration of naïve T cells into the lungs during infection has been identified in both the acute (Heidema et al., 2008) and chronic setting (Vezys et al., 2006). With the very low numbers of naïve T cells in tissue, the process of migration from the blood into the lung parenchyma may induce maturation and differentiation into effector cells. Furthermore, with a smaller fractional naïve T cell population in tissue compared to blood, it is likely that T cells within

the blood vessels of tissue samples only contributed a very small proportion of the total lymphocyte population.

Tmem cells are found in greater proportions in the lung than in blood (de Bree et al., 2007; de Bree et al., 2005; Purwar et al., 2011). Although this has been established in both murine models and humans, there is a poor consensus on their phenotype and function. This may largely be due to variable gating strategies when identifying Tmem cells. In an effort to fully characterise Tmem populations in the lungs, several combinations of receptors have been identified in the literature. These include CD45RO (Purwar et al., 2011), CD45RA (de Bree et al., 2005; Purwar et al., 2011), CCR7, CD127 (Vezys et al., 2006; Zhou and McElhaney, 2011), CD25 (Zhou and McElhaney, 2011), CD69 (Appay et al., 2008a; Kotturi et al., 2011; West et al., 2011) and CD28 (de Bree et al., 2005; Henson et al., 2011). Furthermore, other markers used to identify Tmem cells may be modulated by stimulation or exhaustion (Akbar and Henson, 2011; Duraiswamy et al., 2011; Jin et al., 2010; Punkosdy et al., 2011; Wang et al., 2011; Zhou and McElhaney, 2011), and thus compromise identification of T cells in models of viral infection. The expression of CD45RA and CCR7 was used to define naïve and Tmem cells in this study. Flow cytometry at facilities at Southampton University allowed for 9 fluorochromes to be used per sample. Expression of CD45RA and CCR7 could be used to identify 4 populations of T cells; naïve T cells, TCM, TEM and TEMRA. As only two flurochromes were used to define the Tmem populations, remaining fluorochromes could be used to identify T cell functional markers, such as PD-1, TIM-3, CD107a and CD57. Expression of functional markers could be measured on the naïve and memory T lymphocyte populations to further define their phenotype.

TEM and TEMRA populations may be dominant in lung parenchyma due to their ability to respond rapidly to the recognition of pathogens (Sallusto et al., 1999). T memory cells do not require stimulation by antigen presenting cells to perform effector functions, and may limit infection until T cells are recruited from the periphery (Ely et al., 2003; Heidema et al., 2008). Furthermore, there is evidence that naïve T cell may not express appropriate adhesion molecules to remain in tissue in the steady-state (Cerwenka et al., 1999; Galkina et al., 2005). Sorting of CD4+ and CD8+ T cells from lung tissue yields highly purified populations which can be further investigated. RT-PCR can be performed using sorted cell populations which allows for measurement of gene expression. This is especially useful if the gene of interest does not regulate the expression of a cell surface marker which could be identified by flow

cytometry. CD4+ and CD8+ T cells were sorted in adequate numbers to yield sufficient RNA. This RNA in turn was used to perform RT-PCR with the GNBL1 and YWHAZ housekeeping genes, identifying their expression in CD4+ and CD8+ T lymphocyte populations. Optimisation of cell numbers required for RT-PCR was required for Chapter 4, in which expression of genes associated with senescence and exhaustion were quantified in T cell populations.

Work performed in Chapter 3 identified the effects of collagenase on cell viability, CD3, CD4, CD8 and CCR7 expression. Optimisation of tissue digestion has generated a robust model for investigating lung resident T cell populations from explanted tissue.

Chapter 4

Lung Resident Inflammatory Cell Populations in Health and COPD

Introduction

Chapter 3 detailed the development of the protocol used to identify cells from human lung parenchyma. This protocol allowed for conservation of the CD4+ and CD8+ T cell markers, and also the identification of macrophages. Tmem populations were also identified in both blood and lung parenchyma.

Differences in inflammatory cell populations both in lung parenchyma and the periphery can indicate an imbalanced immune response. Skewing of inflammatory cell populations could indicate a role for cells in either a protective or pathological role in disease. Recurrent respiratory infections in COPD patients (Hurst et al., 2010) may result in a greater anti-viral CD8+ T cell population in their lungs compared to controls who are either have competent barrier functions preventing infection, or functioning immune responses to clear infection during the acute phase.

Characterisation of T cells human cancers and murine models of chronic infection have revealed that T cell exhaustion occurs at the tumour site (Ahmadzadeh et al., 2009) or site of infection (Aubert et al., 2011; Barber et al., 2006). Therefore the importance of characterising T cell phenotypes in lung parenchyma is crucial in the understanding of lymphocyte functions in COPD. As COPD individuals are more susceptible to viral infections, T cells may become exhausted due to chronic antigen exposure. This exhaustion may be responsible for more severe symptoms in COPD patients during viral infection (Mallia et al., 2011).

T lymphocytes from tissue were identified and cell surface expression of PD-1 and TIM-3 were measured by flow cytometry. CD4+ and CD8+ T cells were sorted by FACS and further phenotypic markers were analysed at a gene expression level using RT-PCR (Table 4.1). Identification of T cell phenotype requires several markers to distinguish between activation, exhaustion or protective roles of T cells. T lymphocytes were also quantified and phenotyped by their cell surface expression of PD-1 and CD57, revealing potential functional characteristics of T_{EM} and T_{EMRA} cells.

Marker/Gene	Function
CD27	Loss of expression indicates T cells are fully differentiated. Negative expression suggests cytotoxic function.
CD57	Marker of T cell senescence
CD152 (CTLA-4)	Inhibitory receptor inducing T cell inhibition
CD223 (LAG-3)	Marker of T cell exhaustion
CD279 PD-1 (*PDCD1*)	Marker of T cell exhaustion. Also expressed on activated T cells
TIM-3 (*HAVCR2*)	Marker of T cell exhaustion

Table 4.1. Genes analysed by RT-PCR and their function.

Results and Comment

Patient Cohort

Blood and lung parenchyma analysed in Chapter 4 was obtained from the patient cohort described in Chapter 2. Tissue from the entire cohort was not used in these experiments due to limited mass of tissue obtained after surgery. Table 4.2 details patient data used in this chapter. Numbers of control (n=20) and COPD (n=24) individuals was similar, and groups were age matched. Proportions of males and females were similar between groups. Pack years appeared to be greater in COPD patients than controls (28.50 ± 27.60 vs. 46.49 ± 30.69) but this was not statistically significant (p = 0.1). FEV_1% was significantly lower in COPD than controls (95.28 ± 16.99 vs. 76.21 ± 15.73). Thus the control and COPD individuals analysed in Chapter 4 are similar to the entire cohort.

	Control	COPD	p Value
n	20	24	-
Age	65.05 (60.25 - 74.75)	67.04 (59.75 - 74)	0.9
Gender M/F	9 / 11	12 / 12	-
Smoker (Never/Ex/Current)	5 / 11 / 4	1 / 16 / 7	-
Pack Years	28.50 (0.875 – 55.25)	46.49 (29 – 56.25)	0.1
Cancer Diagnosis (Yes/Unknown)	13 / 7	14 / 10	
FEV_1%	95.28 (80.18 – 111.8)	76.21 (64.56 – 88.88)	0.001
FEV_1/FVC%	0.764 (0.727 – 0.807)	0.597 (0.524 – 0.668)	< 0.0001

Table 4.2. Patient phenotypes of individuals used in cell proportion work. Individuals were defined as COPD by spirometry if $FEV_1/FVC < 0.7$. Mean and IQR shown. Unpaired t test or Mann-Whitney test performed.

Peripheral T cell populations are unchanged in COPD

The identification of T lymphocytes had been optimised for blood and lung parenchyma in Chapter 3. Optimisation allowed for T cell populations in blood and tissue to be compared between control and COPD patients. CD4+ (Figure 4.1A) and CD8+ (Figure 4.1B) T cell populations in blood were measured from control and COPD patients. The proportion of CD4+ T cells was unchanged in disease (p = 0.6). A mean of 65% T cells were CD4+ in

controls compared to 59.2% in COPD patients. CD8+ cells comprised 24.4% of T cells in controls and 31.9% in COPD patients but this was not statistically significant (p = 0.6).

	Control	COPD	p Value
n	7	5	-
Age	67.86 (63 - 73)	68 (60 – 75.5)	0.98
Gender M/F	4 / 3	0 / 5	-
Smoker (Never/Ex/Current)	1 / 4 / 2	0 / 4 / 1	-
Pack Years	24.43 (0 – 40)	71.9 (30 – 118.5)	0.034
Cancer Diagnosis (Yes/Unknown)	4 / 3	2 / 3	-
FEV_1%	92.59 (75 – 112.7)	64.64 (53.65 – 74.45)	0.02
FEV_1/FVC%	0.742 (0.71 – 0.782)	0.577 (0.462 – 0.679)	0.004

Table 4.3. Patient phenotypes of individuals used in PBMC experiments. Individuals were defined as COPD by spirometry if FEV_1/FVC <0.7. Mean and IQR shown. Unpaired t test performed.

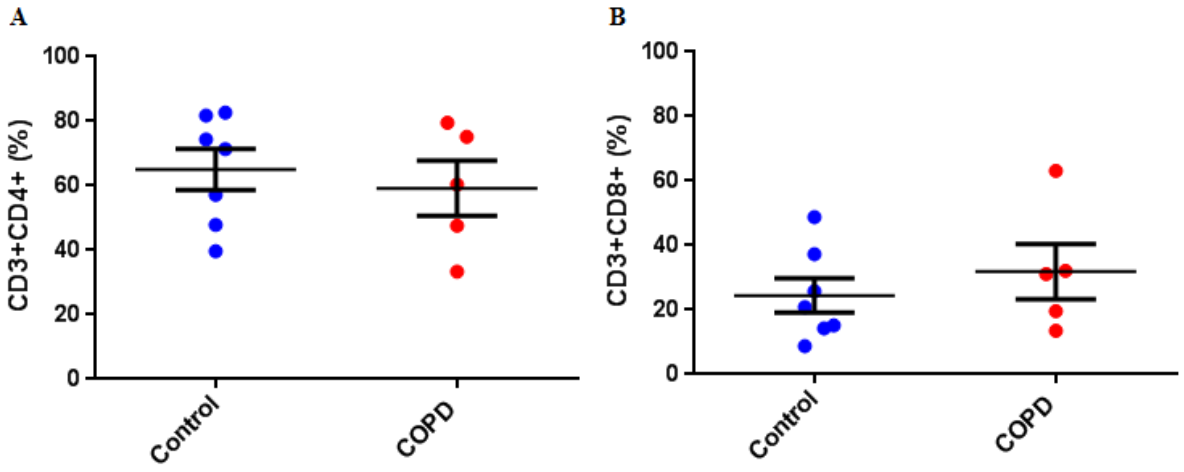

Figure 4.1. T cell populations in blood of control and COPD patients. PBMCs were separated from blood using Ficoll-Paque density centrifugation and cells were analysed by flow cytometry. T lymphocytes were defined as live singlet CD3+ cells. **(A)** Proportion of CD4+ **(B)** and CD8+ T cells are gated on the live CD3+ population. Control n = 7, COPD n = 5. Mean and SEM shown. Unpaired students t test was performed.

PD-1 is in not expressed by T cells in the periphery but is upregulated upon stimulation

Although T cell proportions in blood were unchanged between health and disease, the potential for T lymphocytes to express an exhausted phenotype in COPD had not yet been established. PD-1 expression in blood was not seen in LCMV murine models of chronic infection, but had been detected in humans with HIV (Peretz et al., 2012; Trautmann et al., 2006). A recent study by Kalathil *et al.* (Kalathil et al., 2014) detected PD-1 expression in a population of blood CD4+CD127+ T cells, although there was no evidence of functional exhaustion. T cells from human blood were analysed for their expression of PD-1. Samples taken from control and COPD patients recorded undetectable or very low proportions of T cells expressing PD-1 (Figure 4.2).

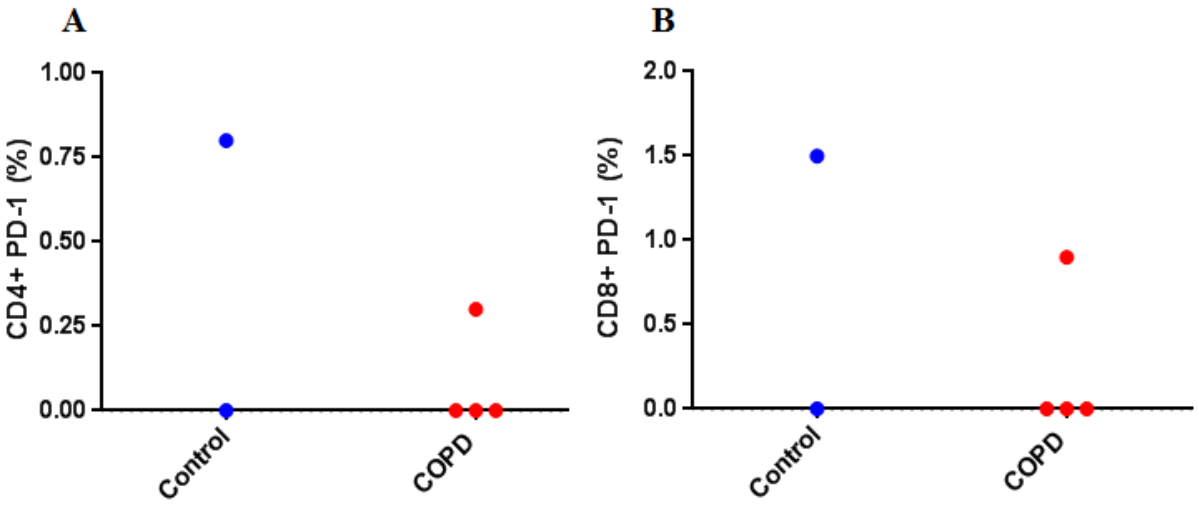

Figure 4.2. Proportion of peripheral T cells expressing PD-1. PBMCs were separated from blood using Ficoll-Paque density centrifugation and cells were analysed by flow cytometry. T lymphocytes were defined as live singlet CD3+ cells. **(A)** Proportion of CD4+ **(B)** and CD8+ T cells are gated on the live CD3+ population. Control n = 2, COPD = 4. Mean and SEM shown.

A cohort of healthy individuals was recruited to observe T cell responses to stimulation. To investigate the regulation of PD-1, T cells were stimulated with PMA and ionomycin to activate T cells and induce PD-1 upregulation (Figure 4.4). Untreated CD4+ and CD8+ T cells expressed low levels of PD-1 at all recorded time points during the assay. At 24 h CD4+ ($p = 0.006$) and CD8+ ($p = 0.03$) T cells significantly upregulated PD-1 compared to unstimulated controls. Thus PD-1 expression can be induced in human T cells and could be detected by flow cytometry.

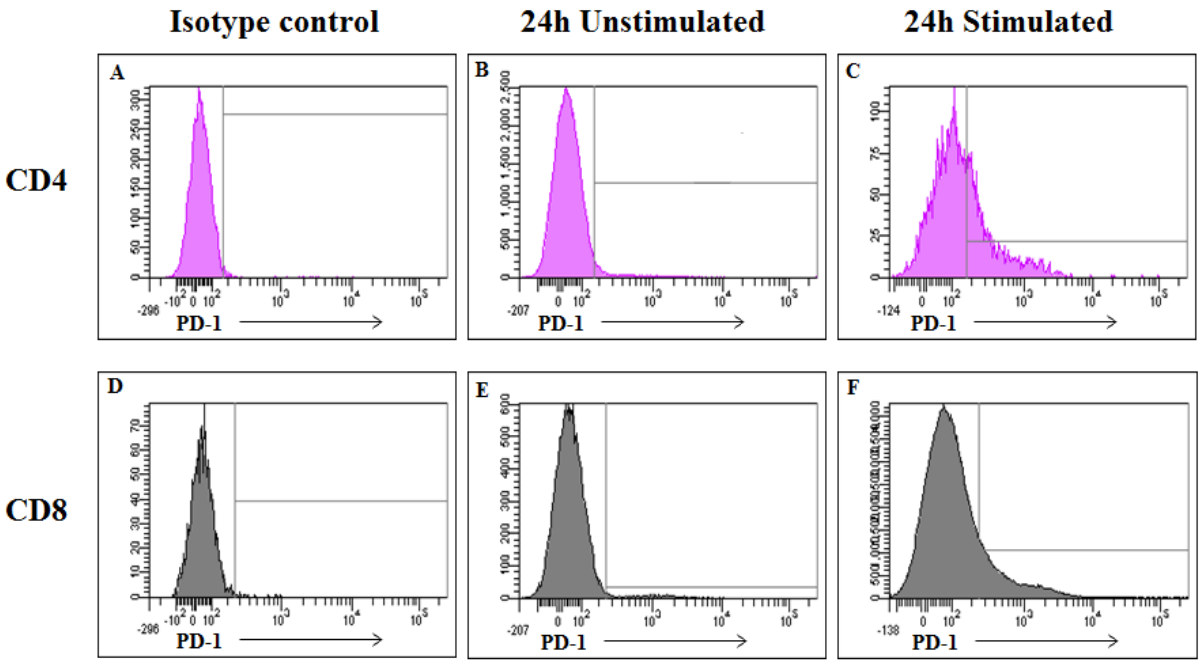

Figure 4.3. Representative plot of PD-1 expression by T cells stimulated by PMA and ionomycin. PBMCs were cultured at a concentration of 1 x 10^6 cells/ml in RPMI and treated with PMA (25ng/ml) and ionomycin (100ng/ml) for 24h. PD-1 expression was measured by flow cytometry after incubation with fluorescently-labelled antibody. **(A)** CD4+ T cells **(D)** and CD8+ T cells stained with isotype control antibody. **(B)** PD-1 expression by unstimulated CD4 T cells **(E)** and CD8 T cells. **(C)** PD-1 expression by stimulated CD4 T cells **(F)** and CD8 T cells. Plots representative of 3 experiments.

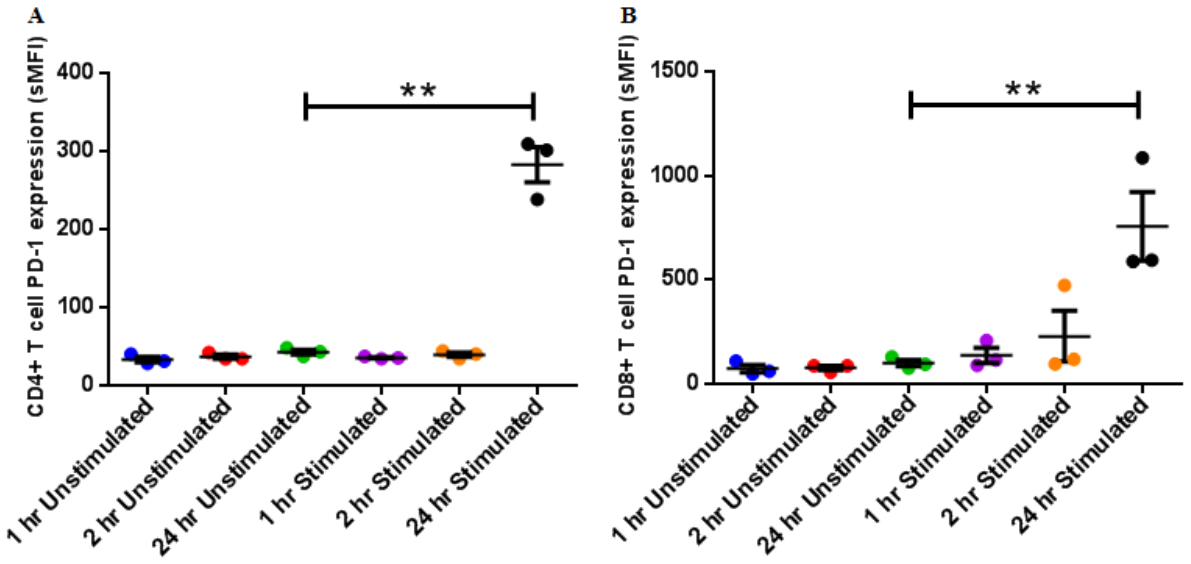

Figure 4.4. PD-1 expression is upregulated on stimulated T cells from blood. PBMCs were cultured at a concentration of 1 x 10^6 cells/ml in RPMI and treated with PMA (25ng/ml) and ionomycin (100ng/ml) for 1hr, 2h or 24h. PD-1 expression was measured by flow cytometry after incubation with fluorescently-labelled antibody. T lymphocytes were defined as singlet CD3+ cells. **(A)** The expression levels on individual cell (sMFI) was measured for CD4+ **(B)** and CD8+ cells. n = 3. Mean and SEM shown. Paired students t test used to determine p value between 2 groups. (*) represents $p < 0.05$, (**) represents $p < 0.01$.

Stimulation of T cells isolated from blood generated functional immune responses (Figure 4.6 and Figure 4.8). Intracellular staining of CD8+ T cells showed an increased granzyme B production after 24 h stimulation ($p = 0.028$) compared to 1 h. This is an expected result due to the cytotoxic functions of CD8+ T lymphocytes. The granzyme B content of CD4+ T cells was also increased by 24 h stimulation ($p = 0.028$). The cytotoxic capabilities of CD4+ T cells is less well understood than CD8+ counterparts, but there is a growing literature to suggest cytotoxic CD4+ cells are able to kill virally infected cells (Bratke et al., 2009; Fang et al., 2012). Granzyme B expression is greater in CD8+ (mean sMFI = 296.3) than CD4+ (mean sMFI = 44.3) ($p = 0.008$). This suggests that although CD4+ T cells may produce granzyme B, CD8+ T cells may be the dominant cytotoxic phenotype.

The production of IFNγ by stimulated CD4+ and CD8+ T cells was also measured using flow cytometry (Figure 4.8). The expression of IFNγ by CD4+ T cells was not detected in stimulated or unstimulated samples. CD8+ IFNγ expression appeared to be upregulated at 24 h, but this was not significantly different to expression at 1 h ($p = 0.112$). The IFNγ values seen in unstimulated CD8+ T cell samples fluctuated between time points. At 1 h, unstimulated CD8+ T cells expressed a mean sMFI of 1, which is in practical terms is undetectable. At 2 h the mean sMFI was 47.33, and at 24 h it had returned to near-undetectable mean sMFI of 6. Therefore while stimulation of CD8+ T cells induced IFNγ production as expected, CD4+ T cell responses were not observed. Table 4.4 illustrates the responses of CD4+ and CD8+ peripheral T cells to PMA and ionomycin stimulation. The poor quantification of IFNγ production by T cells is likely due to a failure of the experiments rather than the inability of T cells to produce IFNγ. This prompted the decision to use ELISpot to detect IFNγ production rather than flow cytometry (Chapter 6).

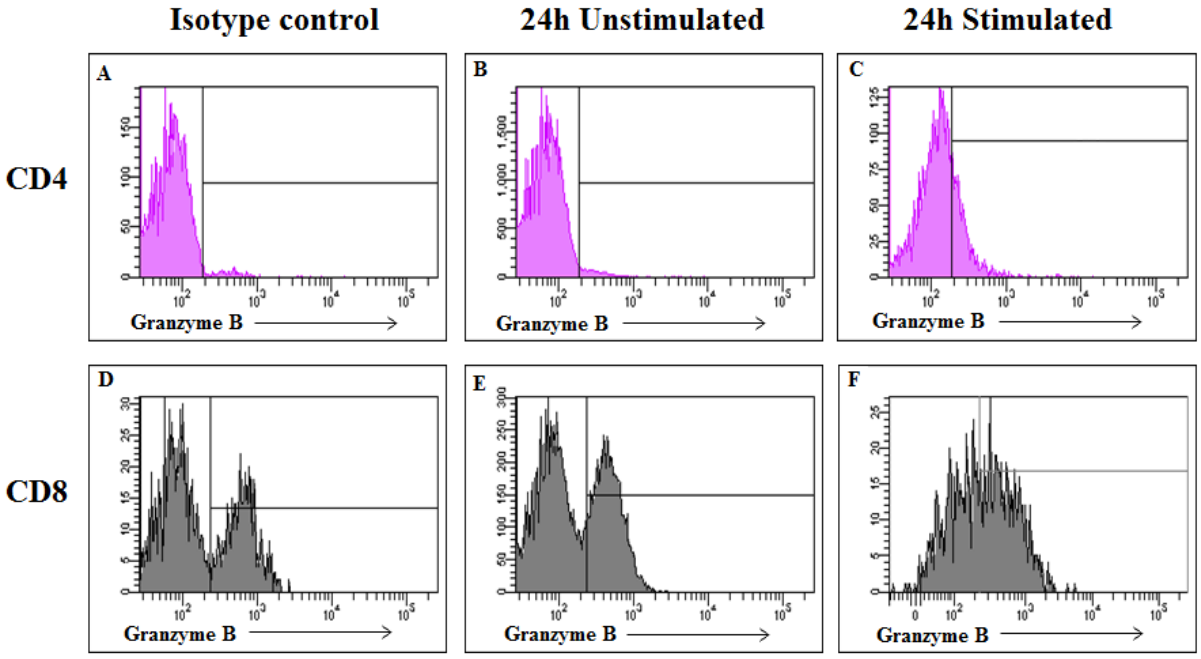

Figure 4.5. Representative plot of Granzyme B expression by T cells stimulated by PMA and ionomycin. PBMCs were cultured at a concentration of 1 x 10^6 cells/ml in RPMI and treated with PMA (25ng/ml) and ionomycin (100ng/ml) for 24h. Granzyme-B expression was measured by flow cytometry after incubation with fluorescently-labelled antibody. **(A)** CD4+ T cells **(D)** and CD8+ T cells stained with isotype control antibody. **(B)** Granzyme B expression by unstimulated CD4 T cells **(E)** and CD8 T cells. **(C)** Granzyme B expression by stimulated CD4 T cells **(F)** and CD8 T cells. Plots representative of 3 experiments.

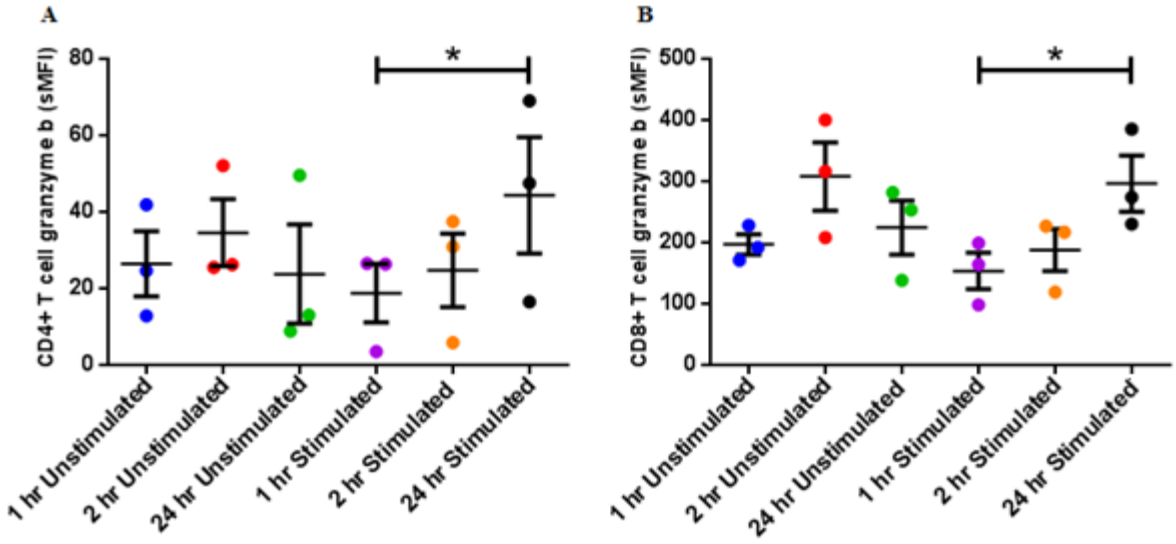

Figure 4.6. Granzyme B production by stimulated T cells from blood. PBMCs were prepared to a concentration of 1x10^6 cells/ml in RPMI and treated with PMA (25ng/ml) and ionomycin (100ng/ml) for 1hr, 2h or 24h. Granzyme b expression was measured by flow cytometry after incubation with fluorescently-labelled antibody. T lymphocytes were defined as singlet CD3+ cells. **(A)** The expression levels on individual cell (sMFI) was measured for CD4+ **(B)** and CD8+ cells. n = 3. Mean and SEM shown. Friedman test p = 0.028 for CD4+ and CD8+ cells. (*) represents $p < 0.05$.

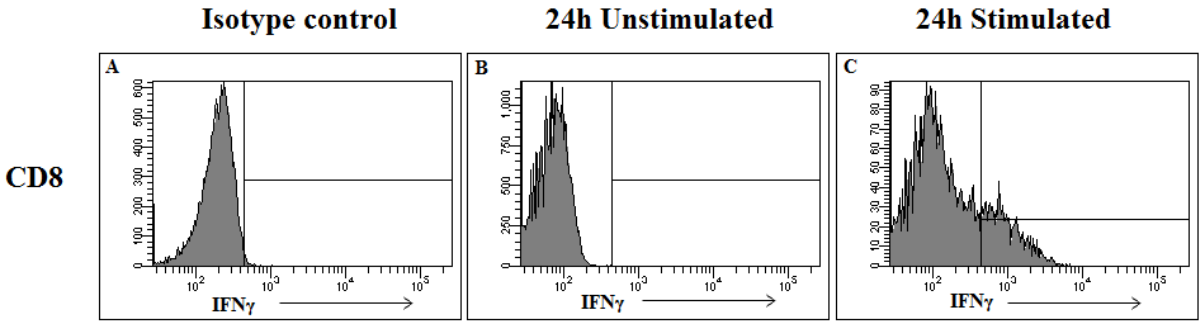

Figure 4.7. Representative plot of IFNγ expression by T cells stimulated by PMA and ionomycin. PBMCs were cultured at a concentration of 1 x 10^6 cells/ml in RPMI and treated with PMA (25ng/ml) and ionomycin (100ng/ml) for 24h. IFNγ expression was measured by flow cytometry after incubation with fluorescently-labelled antibody. **(A)** CD8+ T cells stained with isotype control antibody. **(B)** IFNγ expression by unstimulated CD8 T cells. **(C)** IFNγ expression by stimulated and CD8 T cells. As results from these experiments were inconsistent, plots are representative of one experiment.

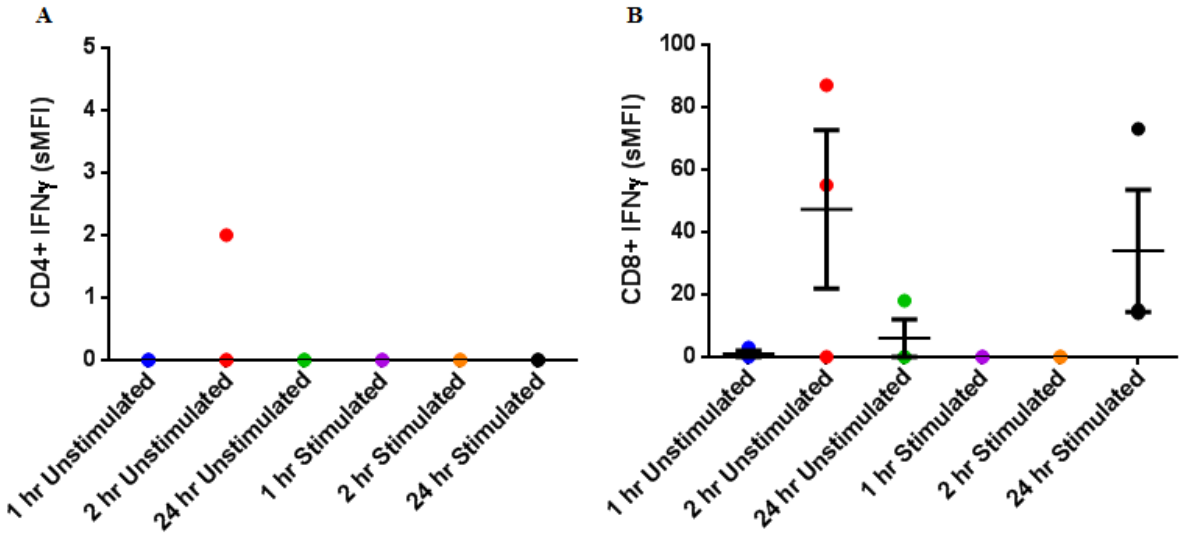

Figure 4.8. IFNγ production by stimulated T cells from blood. PBMCs were prepared to a concentration of 1x10^6 cells/ml in RPMI and treated with PMA (25ng/ml) and ionomycin (100ng/ml) for 1hr, 2h or 24h. Granzyme b expression was measured by flow cytometry after incubation with fluorescently-labelled antibody. T lymphocytes were defined as singlet CD3+ cells. **(A)** The expression levels on individual cell (sMFI) was measured for CD4+ **(B)** and CD8+ cells. n = 3. **(A)** Median values shown. **(B)** Mean and SEM shown. n=3. One way ANOVA and paired students t test performed.

	PD-1	Granzyme B	IFNγ
CD4 T cells	Increased	Increased	Not detected
CD8 T cells	Increased	Increased	Likely increased

Table 4.4. CD4 and CD8 T cell response to stimulation.

Tissue resident T cell populations are altered in COPD

Previously, Polosukhin *et al.* (Polosukhin et al., 2011) found increased numbers of CD4+ and CD8+ T cells in the lungs of COPD individuals compared to controls, and these numbers increased as defects in epithelial cell structure increased. Absolute T cell numbers were not analysed due to tissue yield variations, but altered cell proportions may also indicate differences between healthy and diseased lung. A small study characterising inflammatory cells from human bronchial biopsies observed that increased CD8+ T lymphocyte numbers correlated with a decreased FEV_1 value (O'Shaughnessy et al., 1997). A similar study by Saetta *et al*. (Saetta et al., 1998) identified increased CD8+ T cell proportions of lymphocytes in the COPD lungs compared to controls, as measured by immunohistochemistry. Using the tissue digestion protocol described in Chapter 3 (Figure 4.9), the proportion of CD4+ T cells was lower (p =0.016) in COPD (mean = 39.3%) than controls (mean = 47.3%). Conversely, the proportion of CD8+ T cells was greater (p=0.004) in COPD (mean = 42.7%) than controls (31.2%). Therefore in agreement with previous studies (O'Shaughnessy et al., 1997; Saetta et al., 1998), there does seem to be an increased proportion of CD8+ T cells in COPD lungs validating our FACS methodology and implicating CD8+ T cells in the inflammation and pathology associated with disease.

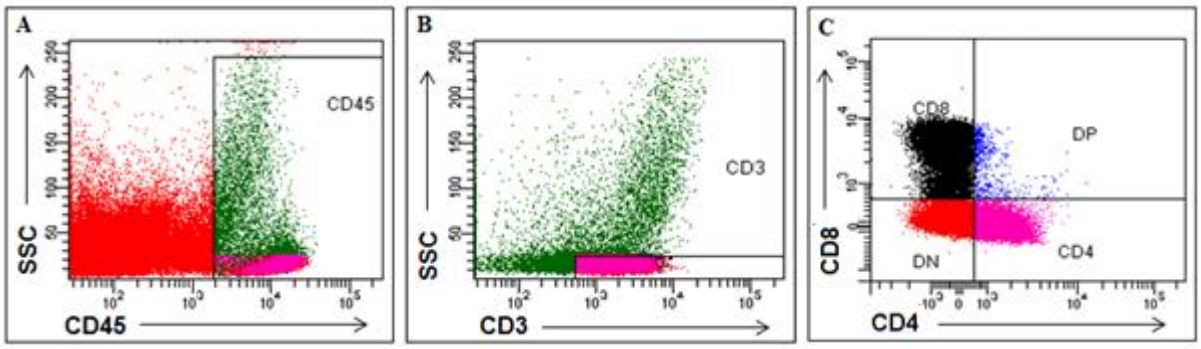

Figure 4.9. Gating of CD4+ and CD8+ T cells from lung parenchyma. Lung tissue was digested with collagenase and cells analysed by flow cytometry. **(A)** CD45+ cells were selected from the live singlet population. **(B)** T cells were defined as CD45+CD3+. **(C)** T cells were divided into CD4+CD8-, CD4-CD8+, double negative or double positive populations.

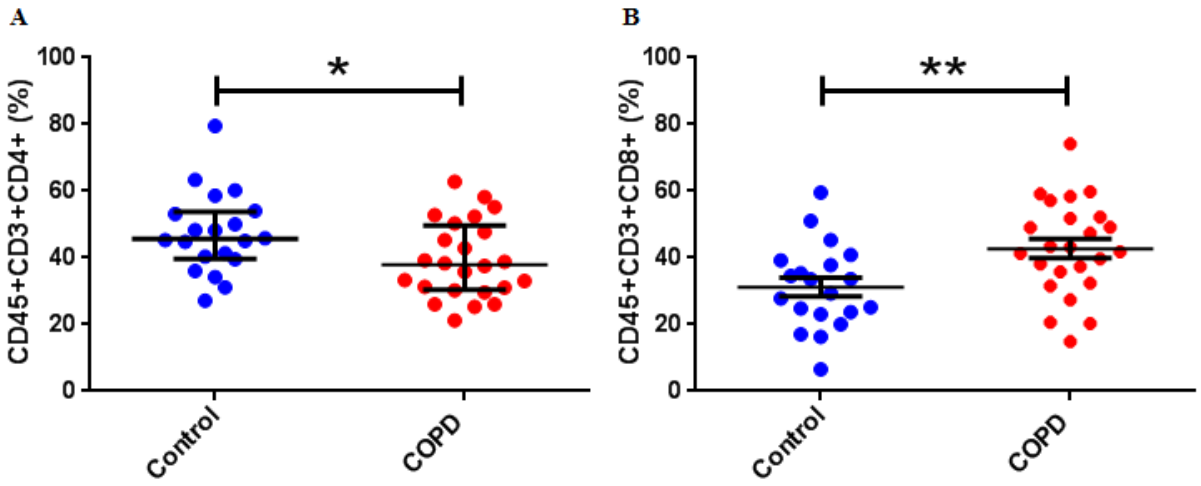

Figure 4.10. T cell populations in lung parenchyma of control and COPD patients. Lung tissue was digested with collagenase and lymphocytes analysed by flow cytometry. **(A)** Proportion of CD4+ **(B)** and CD8+ T cells are gated on the live CD45+CD3+ population. Control n = 20, COPD n = 24. Mean and SEM shown. Unpaired students t test was performed. (*) represents $p < 0.05$, (**) represents $p < 0.01$. Patient phenotype data for this experiment is shown in Figure 4.2.

PD-1 expression by lung resident T cells in controls and COPD

As described above, the proportions of T cells expressing PD-1 in blood was very low or undetectable (Figure.4.3). Murine models have identified CD8+ T cell populations expressing PD-1 in lungs and spleen of chronically infected mice (Barber et al., 2006; Jin et al., 2010). Therefore PD-1 expression may occur in the diseased organ rather than the periphery. To investigate this, PD-1 expression by T cells in the lung was quantified in control and COPD patients (Figure 4.12). The mean proportion of CD4+ T cells from controls that expressed PD-1 was 1.68%, compared to a mean of 4.73% in COPD tissue ($p = 0.1316$). A mean of 4.39% of CD8+ T cells from control lung tissue expressed PD-1, while a mean of 12.14% expressed PD-1 in COPD tissue ($p = 0.0778$). There is therefore a trend for a greater proportion of CD4+ and CD8+ T cells to express PD-1 in COPD lungs compared to control tissue.

	Control	COPD	p Value
n	9	10	-
Age	67.78 (56.5 - 77)	70.44 (66.5 – 76.5)	0.55
Gender M/F	4 / 5	5 / 5	-
Smoker (Never/Ex/Current)	2 / 6 / 1	0 / 7 / 3	-
Pack Years	27.08 (0 – 48)	39.28 (27.05 – 53.13)	0.33
Cancer Diagnosis (Yes/Unknown)	5 / 4	5 / 5	-
FEV_1%	101.6 (92.5 – 115)	75 (58 – 84.5)	0.003
FEV_1/FVC%	0.782 (0.765 – 0.798)	0.573 (0.508 – 0.635)	< 0.0001

Table 4.5. Patient phenotypes of individuals used in T cell PD-1 experiments. Individuals were defined as COPD by spirometry if FEV_1/FVC <0.7. Mean and IQR shown. Unpaired t test performed.

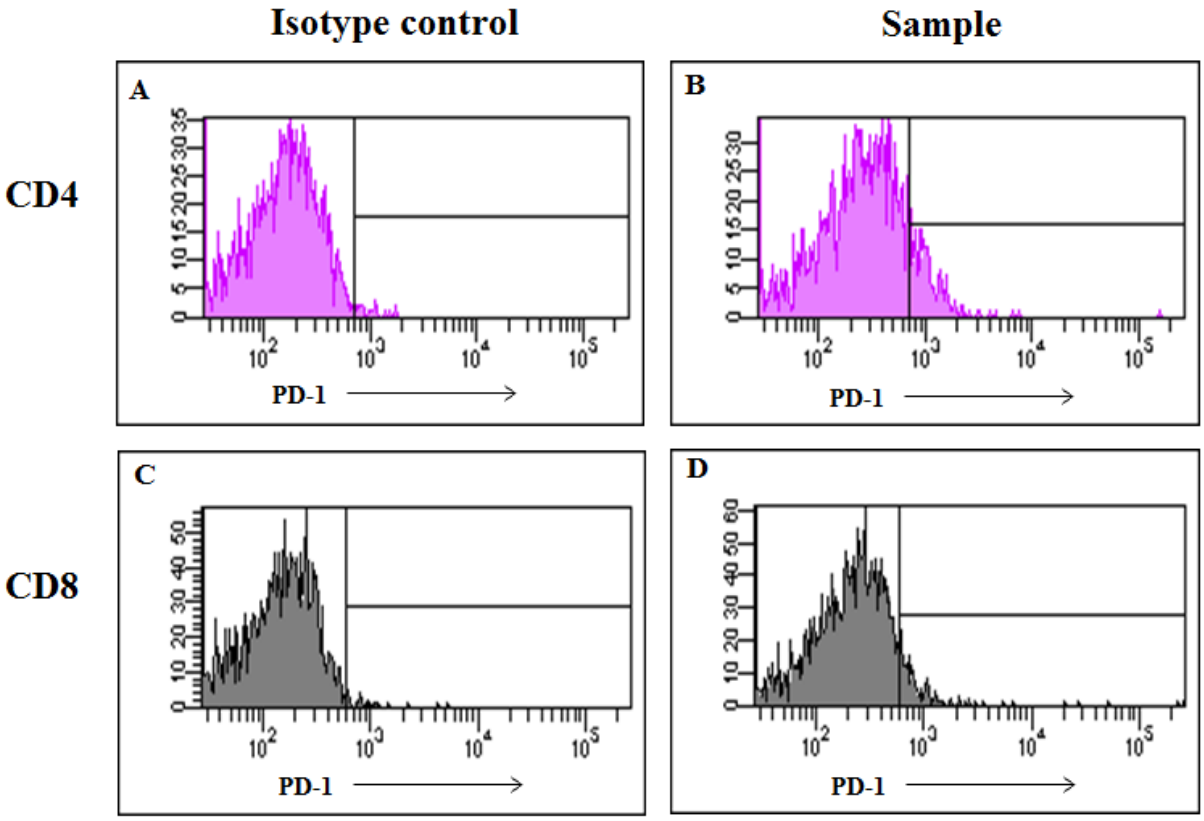

Figure 4.11. Representative plot of PD-1 expression by lung T cells. Lung tissue was digested with collagenase and cells analysed by flow cytometry. T cells are gated on the live CD45+CD3+ population. **(A)** CD4+ T cells **(C)** and CD8+ T cells stained with isotype control antibody. **(B)** PD-1 expression by lung CD4 T cells **(D)** and lung CD8 T cells. Plots are representative of 19 experiments.

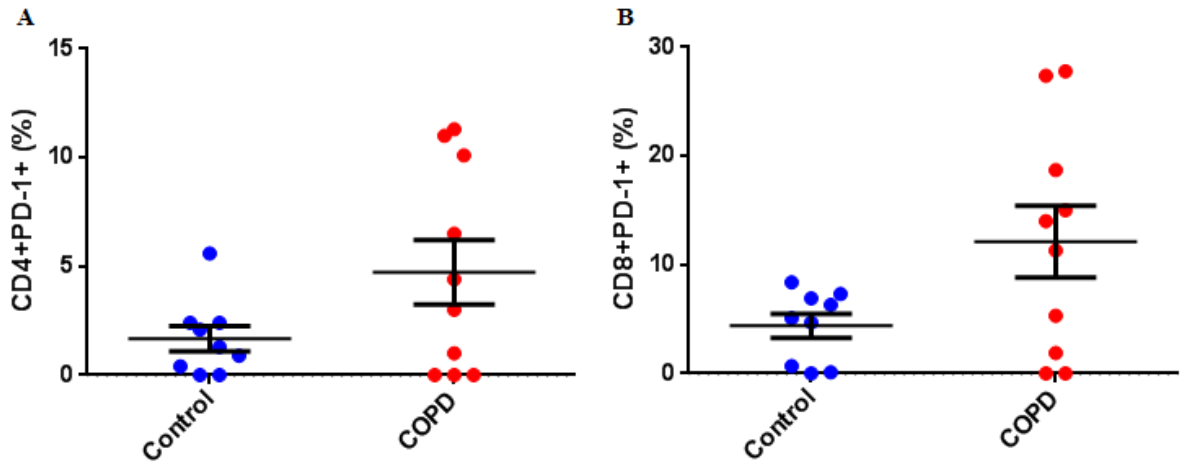

Figure 4.12. Intrinsic PD-1 expression by CD4 and CD8 T cells in controls and COPD. Lung tissue was digested with collagenase and cells analysed by flow cytometry. T cells are gated on the live CD45+CD3+ population. **(A)** Proportion of CD4+ **(B)** and CD8+ T cells expressing surface PD-1. Control n = 9, COPD n = 10. Mean and SEM shown. Mann-Whitney test was performed.

Gene Expression of Sorted CD4 and CD8 T cells in control and COPD tissue

The co-expression of PD-1 and TIM-3 has been used to identify functionally exhausted T cells in murine models (Jin et al., 2010). In Figure 4.12, populations of CD4+ and CD8+ T lymphocytes which expressed PD-1 had been identified in human lung parenchyma of control and COPD patients. In contrast, TIM-3 expressing T cells were not identified in blood or tissue from either controls or COPD individuals. To ensure that lack of TIM-3 detection was due to a lack of TIM-3 expression, RT-PCR experiments were performed using sorted CD4+ and CD8+ T cells from lung parenchyma (Figure 4.9). *PCDC1* (the gene encoding PD-1) was also analysed by RT-PCR to allow comparison of gene expression with surface marker expression on T cells (Figure 4.14). Gene expression of LAG-3, CTLA-4, CD27 and CD57 was also measured, with their functions described in Table 4.1 RT-PCR was performed by Dr. Spalluto and Miss. Cellura. Patient phenotype data for these experiments can is shown in Table 4.6.

	Control	COPD	p Value
n	6	6	-
Age	68.67 (63 – 72.25)	66 (57.25 – 75.25)	0.57
Gender M/F	3 / 3	3 / 3	-
Smoker (Never/Ex/Current)	3 / 2 / 1	0 / 4 / 2	-
Pack Years	31 (0 – 63.75)	55 (30.75 – 85.5)	0.3
Cancer Diagnosis (Yes/Unknown)	4 / 2	3 / 3	-
FEV_1%	95.74 (82.11 – 113.3)	75 (73.22 – 90.14)	0.082
FEV_1/FVC%	0.77 (0.701 – 0.831)	0.627 (0.595 – 0.676)	< 0.0001

Table 4.6. Patient phenotypes of individuals used in RT-PCR experiments. Individuals were defined as COPD by spirometry if FEV_1/FVC <0.7. Mean and IQR shown. Unpaired t test performed.

TIM-3 was not expressed by most CD4+ or CD8+ T cell samples from control or COPD lung parenchyma. One individual in the COPD group showed upregulated TIM-3 gene expression compared to housekeeping gene in CD4+ and CD8+ T cells, but this may be an outlier due to low Ct values recorded in other COPD individuals. As T lymphocyte TIM-3 expression was not detected by flow cytometry or RT-PCR, it may not play an inhibitory role in stable COPD.

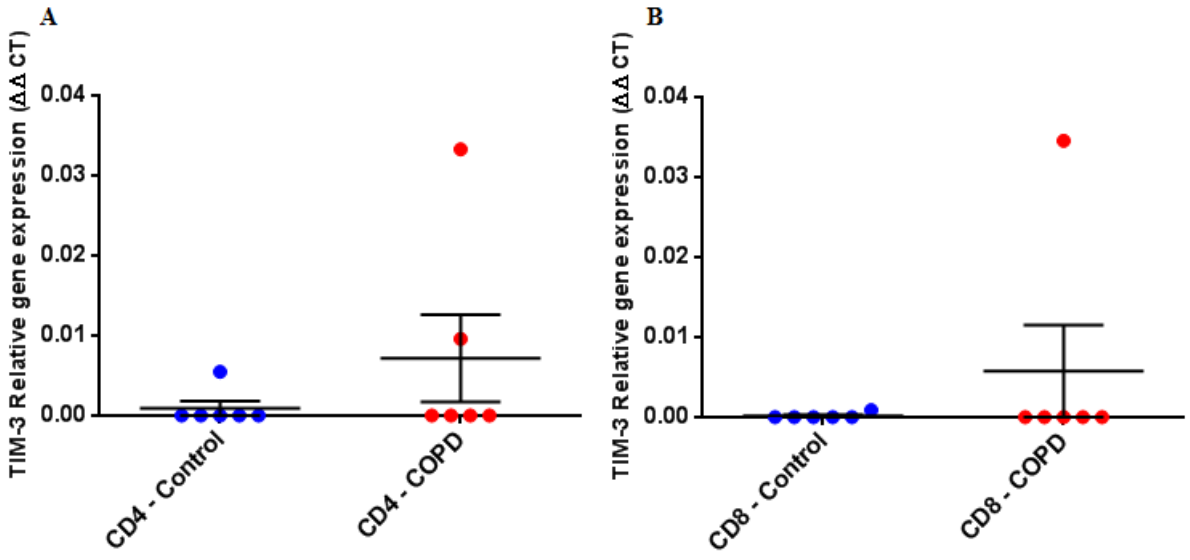

Figure 4.13. RT-PCR gene expression of TIM-3. T cells were gated on the live CD45+CD3+ population. **(A)** RT-PCR was performed with 2.5 x 10^4 CD4+ **(B)** or CD8+ T cells sorted from control or COPD lung parenchyma. ΔΔCt value calculated using B_2M housekeeping gene expression. n = 6. Mann-Whitney test performed.

In addition to TIM-3, PD-1 gene expression was also quantified in sorted CD4+ and CD8+ T lymphocytes from lung. Expression of PD-1 was detected in most samples. There was no difference in PD-1 expression by CD4+ T cells between control and COPD samples (p = 0.28). There was a trend for CD8+ T cell PD-1 expression to be lower in COPD individuals than controls (p = 0.1135). This is contradictory to flow cytometry data analysed in Figure 4.12. 50% of CD8+ T cells samples from the COPD group did not express detectable PD-1 measured by RT-PCR. Only 20% of COPD samples did not express detectable PD-1 as measured by flow cytometry. Therefore this trend for decreased PD-1 gene expression may be due to low sample numbers, which may have been overcome in flow cytometry data.

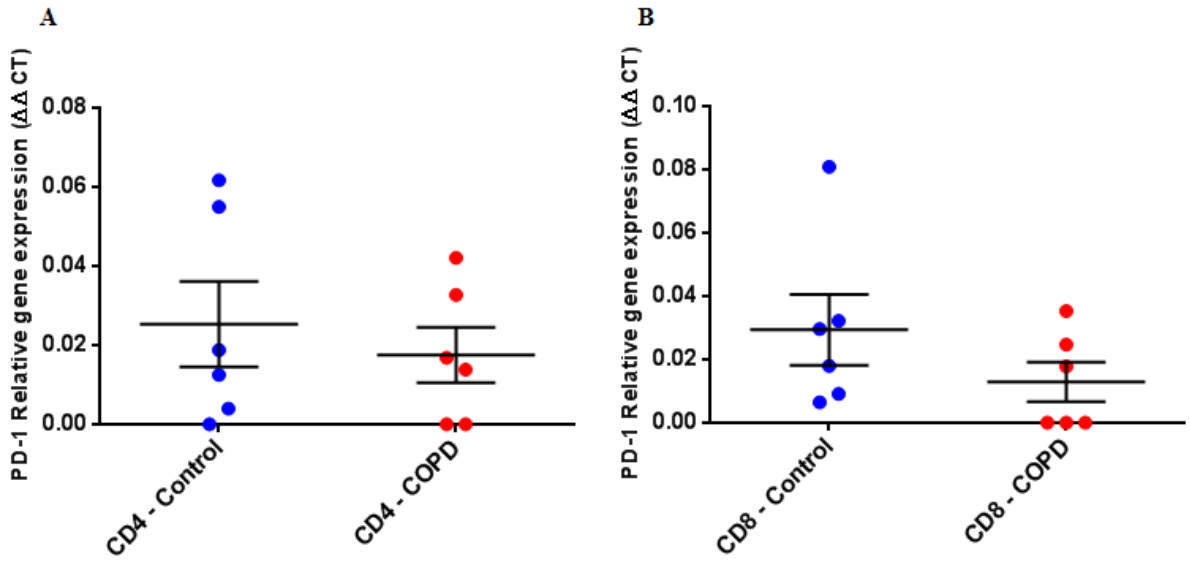

Figure 4.14. RT-PCR gene expression of PD-1. T cells are gated on the live CD45+CD3+ population. **(A)** RT-PCR was performed with 2.5 x 10^4 CD4+ **(B)** or CD8+ T cells sorted from control or COPD lung parenchyma. ΔΔCt value calculated using B_2M housekeeping gene expression. n=6. Unpaired students t test performed.

LAG-3 expression is associated with T cell exhaustion as T cells expressing LAG-3 may also express PD-1 and TIM-3 (Richter et al., 2010; Wherry et al., 2007). LAG-3 expression was detected in the majority of CD4+ T cell samples (Figure 4.15A), but there was no difference in expression between controls and COPD (p = 0.369). LAG-3 was also expressed by CD8+ T cell samples (Figure 4.15B). No differences in expression were recorded between control and COPD individuals (p = 0.398).

CTLA-4 is an inhibitory receptor expressed by T cells. CTLA-4 is not associated with T cell exhaustion (Yokosuka et al., 2012), and blocking of CTLA-4 in murine models of chronic infection does not restore exhausted T cell function (Barber et al., 2006). T cell inhibition by

CTLA-4 is therefore likely to be independent of the PD-1:PD-L1 signalling pathway, and may implicate that alternative inhibitory mechanisms are present in disease. CTLA-4 expression was detected in CD4+ and CD8+ T lymphocytes. There was no difference in expression between controls and COPD samples for CD4+ T cells ($p = 0.497$). There is a trend for greater CTLA-4 expression in controls than in COPD samples of CD8+ T cells ($p = 0.0688$). This reduced expression of CTLA-4 suggests that potential T cell inhibition in COPD is CTLA-4 independent, or that COPD T cells have fewer mechanisms of regulation than in controls.

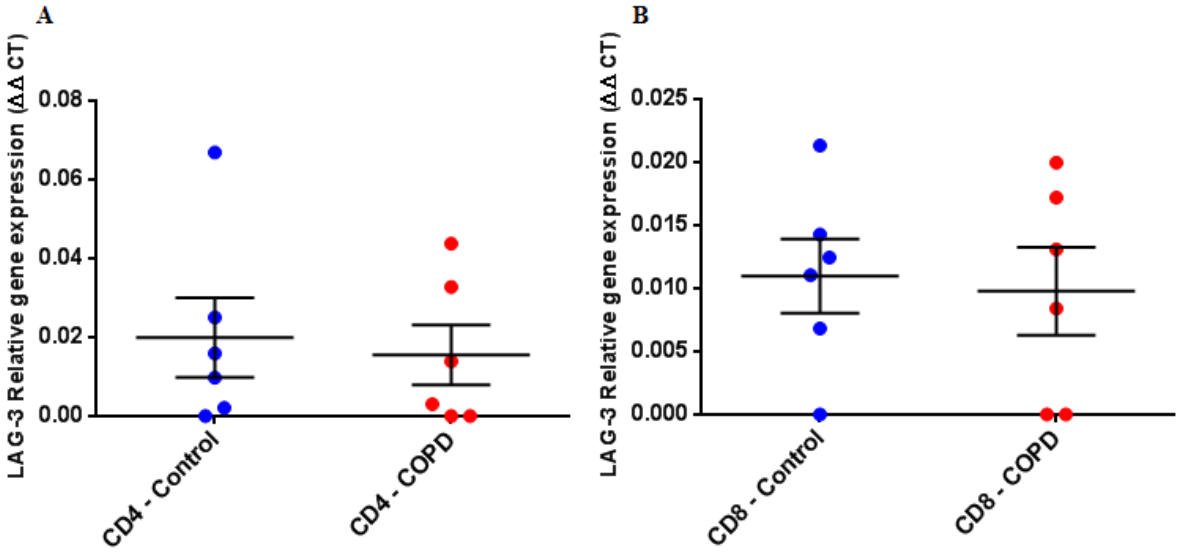

Figure 4.15. RT-PCR gene expression of LAG-3. T cells are gated on the live CD45+CD3+ population. **(A)** RT-PCR was performed with 2.5 x 10^4 CD4+ **(B)** or CD8+ T cells sorted from control or COPD lung parenchyma. ΔΔCt value calculated using B_2M housekeeping gene expression. n = 6. Unpaired students t test performed

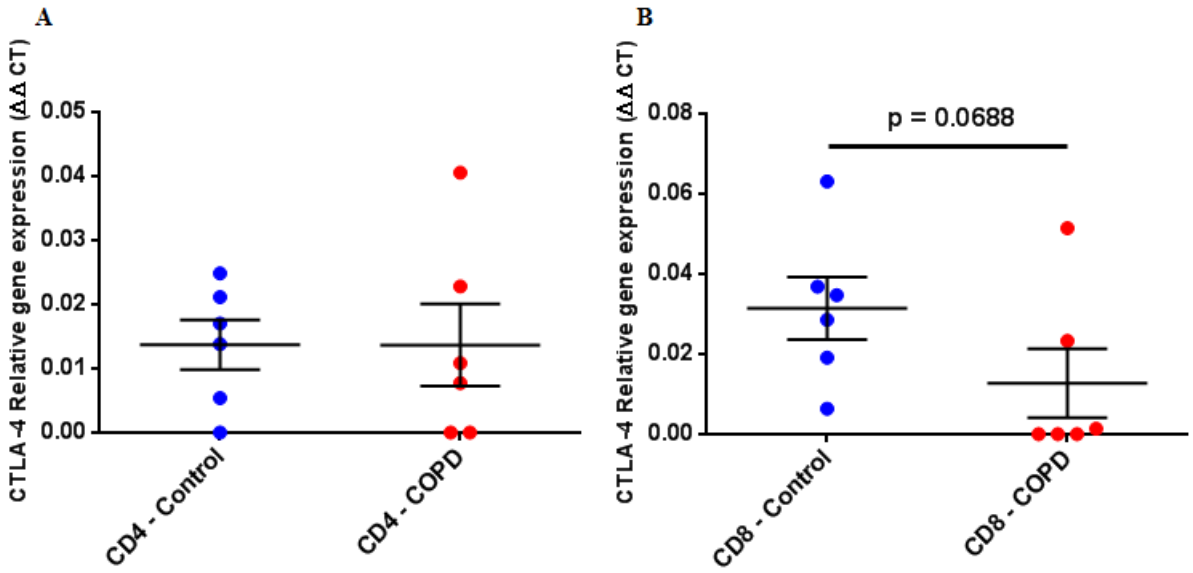

Figure 4.16. RT-PCR gene expression of CTLA-4. T cells are gated on the live CD45+CD3+ population. **(A)** RT-PCR was performed with 2.5 x 10^4 CD4+ **(B)** or CD8+ T cells sorted from control or COPD lung parenchyma. ΔΔCt value calculated using B_2M housekeeping gene expression. n = 6. Unpaired students t test performed.

CD27 is a marker of T cell differentiation. It is not expressed on naïve T cells, but is upregulated upon antigen encounter. Long-lived memory cells which have undergone multiple proliferations lose CD27 expression, and these are termed terminally differentiated (Akbar and Henson, 2011; Koch et al., 2008; Sauce et al., 2007). CD4+ T cell expression of CD27 was unchanged between control and COPD samples (p = 0.495). CD8+ T cell expression of CD27 appeared greater for COPD samples compared to controls, but this was not significant (p = 0.153).

CD57 is a marker of T cell senescence (Dolfi et al., 2013). In a similar manner to CD27 it is associated with cells with a poor proliferative capacity (Wagar et al., 2011). CD57 is also associated with age and reduced telomere length (Appay et al., 2008b). CD57 is expressed by most CD4+ T cell samples, but there is no difference between controls and disease (p = 0.252). There is also no difference in CD57 expression by CD8+ T cells between controls and COPD (p = 0.194). CD8+ T cell expression appears to be divided in COPD samples, with two individuals expressing higher ΔΔCt values than four other COPD patients (Figure 4.18B). In contrast, control samples have only a small degree of difference for CD57 expression by CD8+ T lymphocytes. Greater sample numbers are required to fully elucidate the relationship between COPD and CD57 expression.

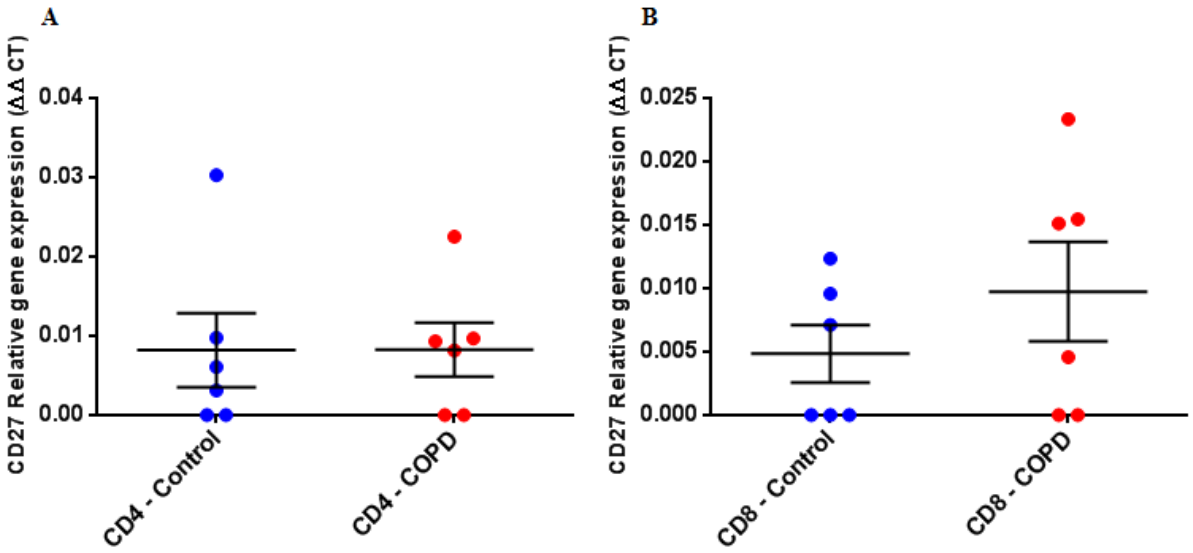

Figure 4.17. RT-PCR gene expression of CD27. T cells are gated on the live CD45+CD3+ population. **(A)** RT-PCR was performed with 2.5 x 10^4 CD4+ **(B)** or CD8+ T cells sorted from control or COPD lung parenchyma. ΔΔCt value calculated using B_2M housekeeping gene expression. n=6. Unpaired students t test performed.

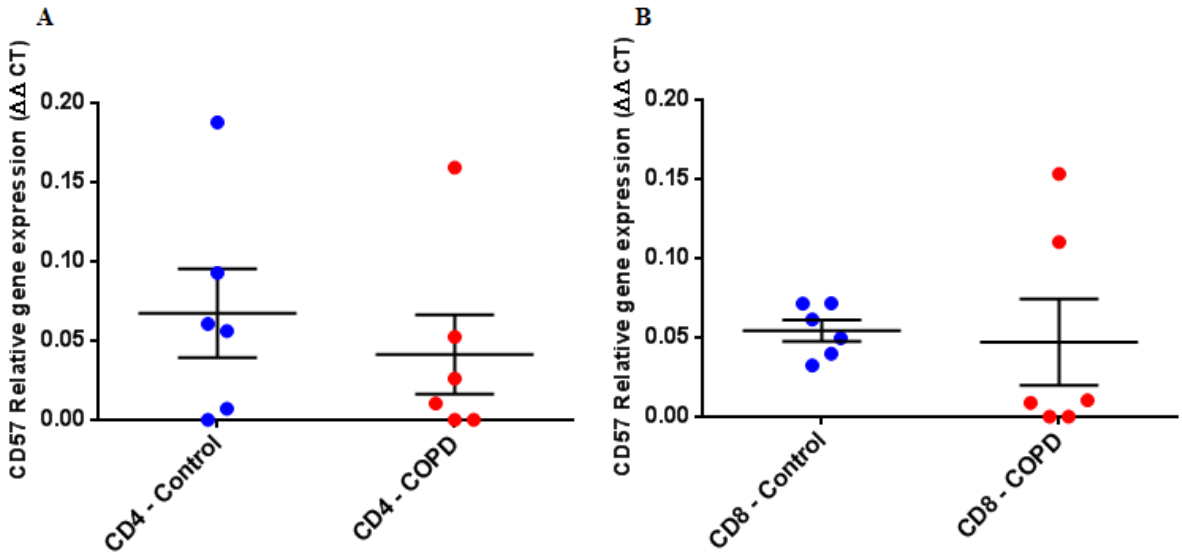

Figure 4.18. RT-PCR gene expression of CD57. T cells are gated on the live CD45+CD3+ population. **(A)** RT-PCR was performed with 2.5 x 10^4 CD4+ **(B)** or CD8+ T cells sorted from control or COPD lung parenchyma. ΔΔCt value calculated using B_2M housekeeping gene expression. n=6. Unpaired students t test or Mann-Whitey test performed.

T memory population in COPD

The increased proportion of CD8+ T cells in COPD lung had previously been reported, and was confirmed by Figure 4.10. To expand upon this work, the proportion of TEM and TEMRA lymphocytes was compared between control and COPD patients (Figure 4.19). Tmem cells are antigen-experienced T cells which can elicit potent anti-viral responses without co-stimulation from APCs. Increased proportions of TEM or TEMRA would suggest increased requirement of these cells in the tissue. Conversely, decreased proportions of Tmem cells may indicate impaired immune responses in the lung parenchyma, which may explain increased frequency and severity of respiratory infections in COPD. The gating strategy for T mem cells is different from that of CD4+ and CD8+ T cells shown in Figure 4.10. Tmem cells are gated as singlet CD3+ lymphocytes, then by expression of CD4+ or CD8+. Further division were identified by the expression of CCD7 and CD45RA.

There was no significant difference between either CD8+ TEM or TEMRA proportions in control or COPD groups. Intriguingly, individuals in the control group appeared to be separated into two further groups. 3 patients had a low proportion of TEM with a corresponding high proportion of TEMRA cells. The remaining 4 patients had a high proportion of TEM and low proportion of TEMRA cells. COPD patients did not group in this manner.

	Control	COPD	p Value
n	7	5	-
Age	64.86 (52 - 76)	66.8 (58.5 – 74)	0.75
Gender M/F	3 / 4	2 / 3	-
Smoker (Never/Ex/Current)	1 / 5 / 1	0 / 3 / 2	-
Pack Years	12.25 (0.375 – 34.38)	30.65 (14.44 – 40)	0.26
Cancer Diagnosis (Yes/Unknown)	2 / 5	3 / 2	-
FEV_1%	98.71 (92 – 112)	75.4 (71.5 – 78.5)	0.001
FEV_1/FVC%	0.763 (0.752 – 0.8)	0.607 (0.555 – 0.659)	0.0025

Table 4.7. Patient phenotypes of individuals used in Tmem experiments. Individuals were defined as COPD by spirometry if $FEV_1/FVC < 0.7$. Mean and IQR shown. Unpaired t test performed.

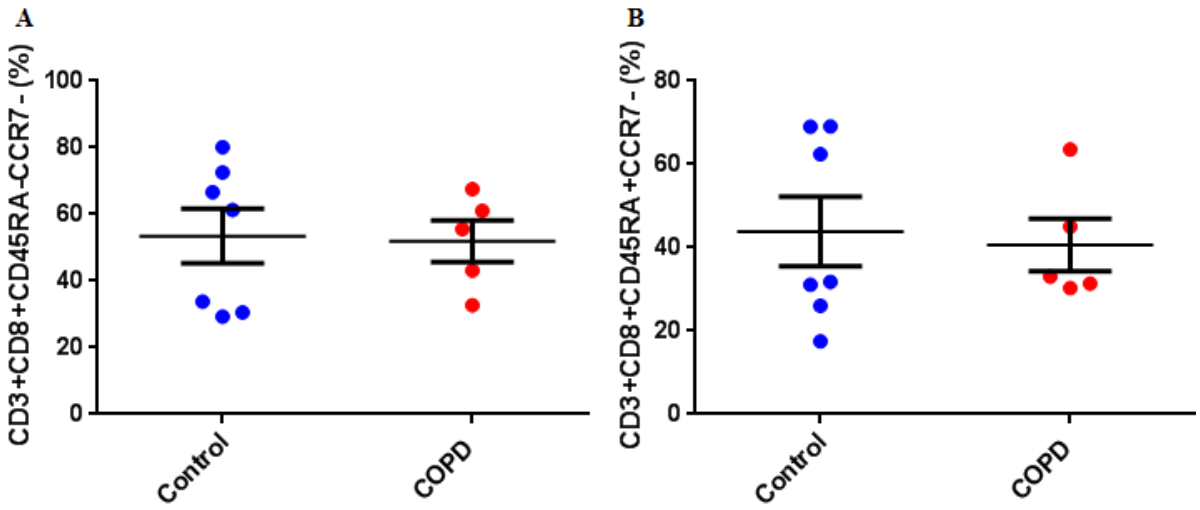

Figure 4.19. CD8+ Memory T cell populations in control and COPD lung. Lung tissue was digested with collagenase and cells analysed by flow cytometry. Memory T cells were gates as singlet CD3+ lymphocytes. T cells were divided by their expression of CD4 or CD8 before gating of memory phenotype by expression of CD45RA and CCR7 **(A)** Proportion of CD8+ T_{EM} (CD45RA-CCR7-) **(B)** and CD8+ T_{EMRA} (CD45RA+CCR7-) cells in control and COPD lung parenchyma. Control n = 7, COPD n = 5. Mean and SEM shown. Mann-Whitney test was performed.

PD-1 and CD57 expression by Tmem cells in controls and COPD

Memory T cell populations account for the majority of T lymphocytes found in lung parenchyma and are likely to have previously encountered respiratory viral infection, further work was performed to investigate phenotypic differences between Tmem subsets. Expression of PD-1 by CD8+ T_{EM} cells was not significantly different between control and COPD samples ($p = 0.227$). CD8+ T_{EMRA} cells also expressed PD-1, but expression was unchanged between control and COPD patients ($p = 0.3617$). CD8+ T_{EM} cells expressed similar levels of CD57 between controls and COPD individuals ($p = 0.423$). Although it appeared that CD57 was expressed at a lower level by CD8+ T_{EMRA} cells in COPD, this was not a significant difference ($p = 0.133$).

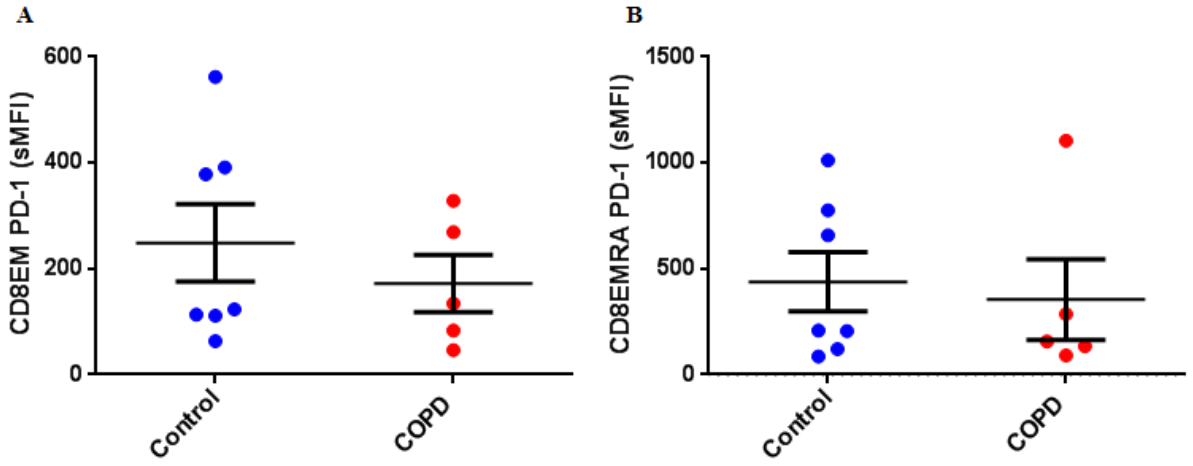

Figure 4.20 Intrinsic PD-1 expression by CD8EM and CD8EMRA T cells in controls and COPD. Lung tissue was digested with collagenase and cells analysed by flow cytometry. Memory T cells were gates as singlet CD3+ lymphocytes. T cells were divided by their expression of CD4 or CD8 before gating of memory phenotype by expression of CD45RA and CCR7 **(A)** Proportion of CD8+ T_{EM} (CD45RA-CCR7-) **(B)** and CD8+ T_{EMRA} (CD45RA+CCR7-) cells in control and COPD lung parenchyma.. Control n = 7, COPD n = 5. Mean and SEM shown. Unpaired students t test or Wilcoxon signed-rank test was performed.

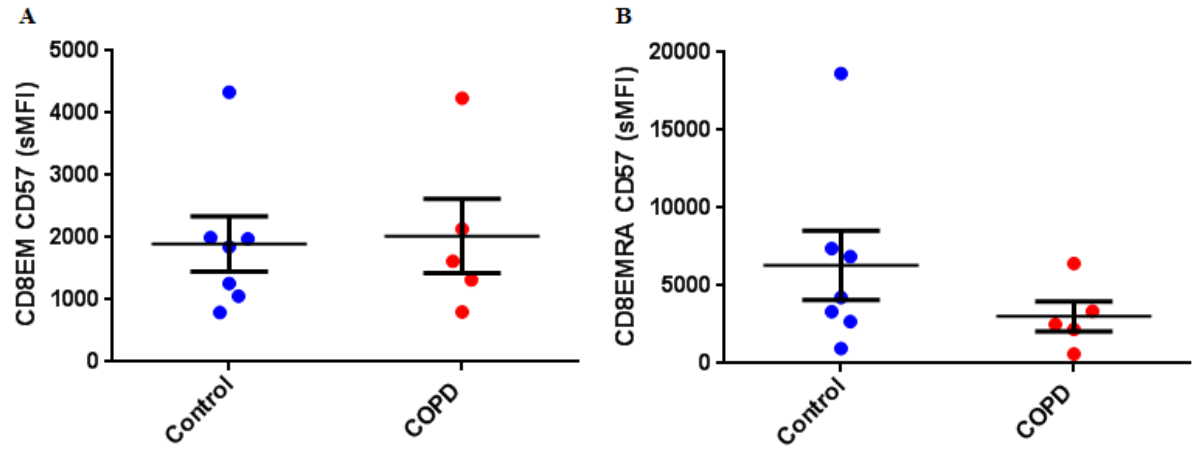

Figure 4.21. Intrinsic CD57 expression by CD8EM and CD8EMRA T cells in controls and COPD. Lung tissue was digested with collagenase and cells analysed by flow cytometry. Memory T cells were gates as singlet CD3+ lymphocytes. T cells were divided by their expression of CD4 or CD8 before gating of memory phenotype by expression of CD45RA and CCR7 **(A)** Proportion of CD8+ T_{EM} (CD45RA-CCR7-) **(B)** and CD8+ T_{EMRA} (CD45RA+CCR7-) cells in control and COPD lung parenchyma. Control n = 7, COPD n = 5. Mean and SEM shown. Unpaired students t test or Wilcoxon signed-rank test was performed.

No differences of PD-1 or CD57 expression by Tmem cells were seen between control and COPD samples (Figure 4.20 and 4.21). Functional marker expression was then compared between CD8+ TEM and TEMRA populations to characterise phenotypic differences between

populations (Figure 4.22). CD8+ TEMRA cells appeared to express greater levels of PD-1 compared to TEM cells in both controls (p = 0.185) and COPD (p = 0.228), but neither increase was statistically significant. Significant differences were observed between TEM and TEMRA expression of CD57. TEMRA cells expressed CD57 to a significantly greater level than TEM in control samples (p = 0.0156). Increased CD57 expression by TEMRA cells was seen as a trend in COPD samples (p = 0.0625). This expression of CD57 by CD8+ TEMRA cells suggests that these are terminally-differentiated cytotoxic cells, while TEM cells may have greater proliferative potential.

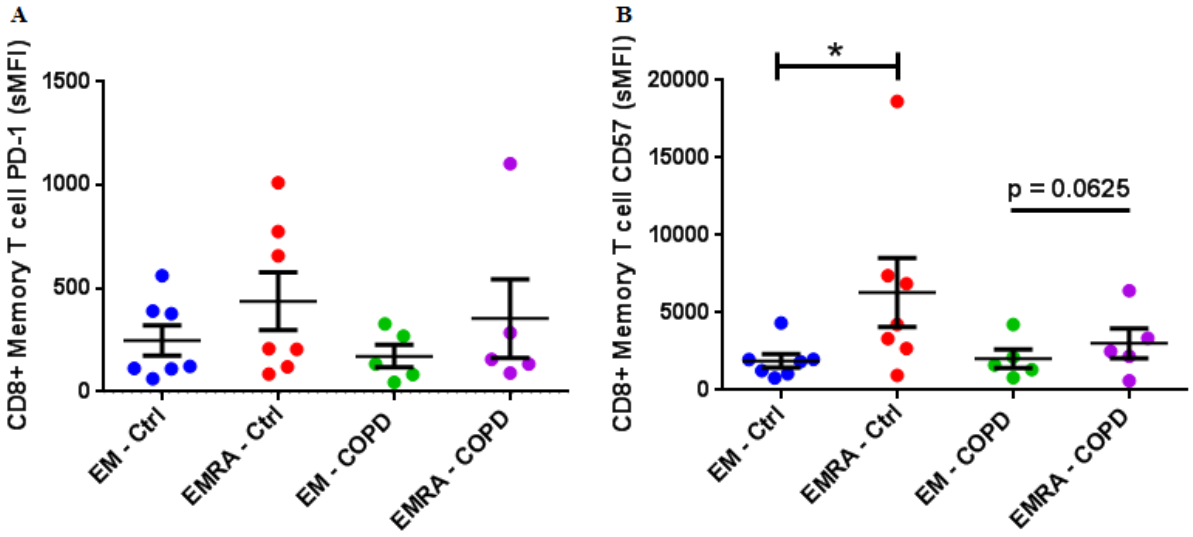

Figure 4.22. Comparison of PD-1 and CD57 expression by CD8EM and CD8EMRA T cells. Lung tissue was digested with collagenase and cells analysed by flow cytometry. Memory T cells were gates as singlet CD3+ lymphocytes. T cells were divided by their expression of CD4 or CD8 before gating of memory phenotype by expression of CD45RA and CCR7. **(A)** CD8+ TEM and TEMRA cells were identified and expression of PD-1 **(B)** and CD57 was measured in controls and COPD tissue. Control n = 7, COPD n = 5. Mean and SEM shown. Wilcoxon signed-rank test was performed. (*) represents $p < 0.05$

Macrophage proportions are unchanged in COPD

T cells require interaction with antigen presenting cells in order to be activated and perform their effector functions. In tissue, APCs can interact with T cells in the lung to promote or inhibit cytokine or cytotoxic immune responses. Macrophages have also been identified as being recruited to diseased lungs. Finkelstein *et al.* (Finkelstein et al., 1995) displayed a relationship between lung parenchymal T cell and macrophage numbers in smokers, but patients were not categorised by their lung function. Investigations into the role of macrophages in COPD has largely been performed using samples from BAL (Wen et al., 2010) or induced-sputum (Domagala-Kulawik et al., 2003). Macrophages were identified in

the lungs as being CD45+HLA-DR+ but there was no statistical difference in the proportions of these cells between control (mean = 16.2%) and COPD individuals (mean = 16.8%) (p = 0.86, Figure 4.23).

	Control	COPD	p Value
n	16	12	-
Age	63.94 (52.5 – 74.75)	65.58 (59 – 73)	0.7
Gender M/F	6 / 6	7 / 5	-
Smoker (Never/Ex/Current)	3 / 9 / 4	0 / 9 / 3	-
Pack Years	23.29 (0.875 – 51.25)	51.34 (26 – 50)	0.025
Cancer Diagnosis (Yes/Unknown)	11 / 5	7 / 5	-
FEV_1%	96 (82.35 – 111.4)	78.92 (68.5 – 92.95)	0.015
FEV_1/FVC%	0.767 (0.729 – 0.812)	0.61 (0.542 – 0.668)	< 0.0001

Table 4.8. Patient phenotypes of individuals used in macrophage experiments. Individuals were defined as COPD by spirometry if FEV_1/FVC <0.7. Mean and IQR shown. Unpaired t test performed.

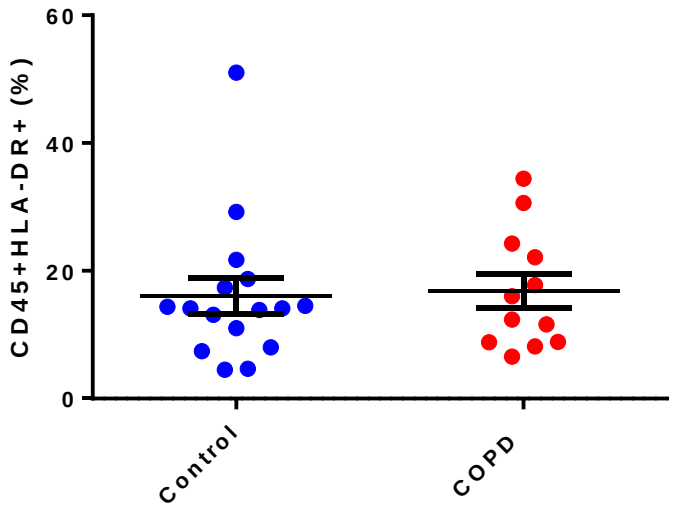

Figure 4.23. Macrophage proportions in lung parenchymal tissue. Lung tissue was digested with collagenase and cells analysed by flow cytometry. Macrophages were defined as CD45+HLA-DR+ cells. Control n = 16, COPD n = 12. Mean and SEM shown. Unpaired students t test was performed.

Conclusions

Quantification of T cell populations was expanded to investigate lymphocyte proportions in COPD, as changes in T lymphocyte composition in COPD may infer a protective or pathogenic role in disease. Initial phenotyping of T lymphocytes was performed in peripheral blood. Peripheral CD4+ and CD8+ T lymphocyte populations were identified and compared between controls and disease. Blood lymphocytes expressed low levels of PD-1, but this was upregulated in response to stimulation. Granzyme B was produced by CD4+ and CD8+ T cells in response to stimulation, but IFNγ responses as measured by flow cytometry were less conclusive.

Identification of the lymphocyte population in control and COPD lung parenchyma was performed utilising the optimised digestion protocol from Chapter 3. The proportion of CD8+ T cells was elevated in COPD lung compared to controls, and PD-1 expression by CD4+ and CD8+ T cells appeared to be upregulated in disease. The majority of CD4+ T cells in the lung were T_{EM} cells, while CD8+ T cells were mainly T_{EM} or T_{EMRA} phenotypes.

Blood and lung tissue were obtained from controls and COPD patients prior to undergoing surgery. COPD individuals in this cohort where classified as either GOLD I or GOLD II according to their lung function. Significant differences and trends between health and disease identified in this chapter and in later chapters may be enhanced or made clearer with the inclusion of COPD patients with GOLD III or GOLD IV diagnosis. Study of these individuals is currently limited in Southampton as few surgeries are performed on individuals with the later stages of COPD. Blood work, BAL and bronchial biopsies could be obtained from GOLD III and GOLD IV individuals in future studies, allowing for comparisons to be made between disease severities.

The exhaustion marker TIM-3 was not detected on the surface of T lymphocytes by flow cytometry, and RT-PCR experiments confirmed that it was not expressed by CD4+ or CD8+ T cells in lung tissue. RT-PCR was also performed to measure gene expression of PD-1, LAG-3, CTLA-4, CD27 and CD57. RT-PCR data suggested that CTLA-4 is downregulated in CD8+ T cells from COPD tissues.

The proportion of macrophages found in lung tissue was unchanged between health and COPD. With evidence confirming their protective role in respiratory viral infections (Kim et al., 2008; Schneider et al., 2014), the ability to mediate immune functions via anti-viral cytokine secretion (Kohlmeier et al., 2010; Kumagai et al., 2007) may be of greater importance than their proportion in tissue. DCs are understood to perform similar immune-regulatory functions to macrophages. Previous work performed by the group investigated the prevalence of DCs in human lung parenchyma. Using a similar gating strategy that was utilised for identification of macrophages, a lineage marker was also used (Staples et al., 2012). Data from these experiments identified approximately 1% of the CD45+EpCam-1-HLA-DR+ population as being DCs. As Macrophages accounted for almost 20% of CD45+EpCam-1-HLA-DR+ cells, macrophages are more dominant drivers of immune responses in the lung than DCs.

The proportions of CD4+ and CD8+ T cells in the periphery were unchanged between controls and COPD patients. This does not agree with the finding by Koch *et al.*, who identified an increase in peripheral CD4+ proportions (Koch et al., 2007). Koch *et al.* divided their patient cohort into non-smokers, smokers and smokers with COPD. Such a distinction was not made in our study, with the majority of controls and COPD patients being either ex-smokers or current smokers. The greater CD4+ proportion of T cells in blood in this previous study may be accounted for by the migration of CD8+ T cells into the parenchyma of COPD patients as described herein and by others (Figure 4.10) (Ely et al., 2003). Such a change in T cell proportions has been identified by Heidema *et al*. (Heidema et al., 2008) who observed a decrease in RSV-specific CD8+ lymphocytes in blood during acute RSV infection. The CD4:CD8 ratio then returned to pre-infection levels after viral clearance. Therefore the frequency of CD4+ and CD8+ T cells appears to be dynamic, with proportions skewed depending on smoking status, COPD diagnosis and viral infection.

In blood the majority of T cells were CD4+ (Control mean = 65%, COPD mean = 59.2%) compared to 47.3% and 39.3% in tissue. CD8+ cells accounted for 24.4% of T lymphocytes in control and 31.9% in COPD blood, compared to 31.2% and 42.7% respectively in tissue. Different cell compositions in blood and lung may reflect different immune requirements for host protection (Heidema et al., 2008; Teijaro et al., 2011; Teijaro et al., 2010). Furthermore changes in immune cell numbers in blood may indicate systemic disease while cell composition in the lung may indicate site specific disease responses. T cell proportions in

blood were determined using a total of 7 control patients and 5 COPD individuals. This is a relatively small sample size compared to lung parenchymal work, in which 20 control and 24 COPD patients were characterised. Therefore T cell proportions calculated from blood samples may not be as accurate as those analysed from tissue.

The increased proportion of CD8+ T cells in the lung parenchyma of COPD patients may be due to their previous encounter with viral antigen, rather than a naïve population in blood. As CD8+ T cells can elicit potent anti-viral responses (Mueller et al., 2010; Topham et al., 1997; Yap et al., 1978), the raised proportions of CD8+ T lymphocytes in COPD lungs may indicate increased frequency or recurring infection in these patients (Benfield et al., 2008; Sethi et al., 2002). Thus mechanism of anti-viral defence may involve peripheral anti-viral CD8+ T cell recruitment to the lungs, but also memory cells may be retained in tissues to ensure a swift and specific immune response to pathogens. Knudson *et al.* (Knudson et al., 2014) investigated the localisation of T cells in a murine model of viral infection. Infecting mice with RSV or Influenza A virus saw recruitment of CD8+ T cells to the lungs, with viral-specific T cells residing in the lungs for 30 days. Furthermore, the majority of IFNγ-secreting CD8+ cells were found in lung tissues rather than in the periphery.

The increased CD8+ population in tissue may account for the significant decrease in CD4+ T cells rather than their absolute number. Anti-viral T cell responses are associated with CD8+ dominant responses, but CD4+ T cells are also required. Teijaro *et al.* (Teijaro et al., 2011; Teijaro et al., 2010) have highlighted the importance of a competent CD4+ memory T cell response in a murine model of influenza infection. Mice subjected to a lethal influenza challenge were rescued by transfer of lung resident CD4+ memory T cells, an effect which was significantly greater than transfer of naïve or spleen memory CD4+ T cells. Furthermore Wilkinson *et al.* (Wilkinson et al., 2012) recently demonstrated that in humans a cytotoxic CD4+ population responds to influenza challenge. Intriguingly, individuals in which influenza-specific CD4+ Tmem cells were detected prior to infection had reduced symptoms and better disease outcomes upon infection. Therefore while inflammatory cell proportions can indicate the role of certain cell phenotypes in disease, it is the functions of these cells which confers protection.

Significant differences were seen for CD4+ and CD8+ T cells populations between health and disease, but proportions of each subset overlapped between groups. This is not

uncommon when analysing data from humans due to genetic and environmental variability between individuals (Martin et al., 2014; Ye et al., 2014). Patients were age, gender and pack years matched, but these are only a few key factors accounted for. Patients were excluded from analysis if they had a significant co-morbidity which may affect results such as idiopathic pulmonary fibrosis (Papiris et al., 2005), bronchiectasis (Gaga et al., 1998) or asbestosis (Kumagai-Takei et al., 2013). The patients were diagnosed with cancer, and often surgery performed would remove solid tumours. It was expected that in these instances the lung tissue would have a greater proportion of CD8+ T cells as part of the immune response to the tumours than healthy lungs (Fridman et al., 2012). The significant increase in CD8+ T cells in COPD lungs is therefore greater than a control group which itself may have elevated CD8+ T cell numbers.

PD-1-expressing lymphocyte populations were identified in lung parenchyma, with a trend towards greater expression in COPD individuals. PD-1 expression was expressed at very low levels in the periphery, but was significantly upregulated by CD4+ and CD8+ T cells in response to stimulation. The inducible nature of PD-1 in response to activation was first identified by Agata *et al.* (Agata et al., 1996) and this previous work agrees with the findings presented above. PD-1 is a marker of T cell exhaustion, but it is also expressed by activated T cells which appear to be fully functional (Wu et al., 2014). PD-1 is upregulated by T lymphocytes in acute models of LCMV, but these are resolved before T cells display an exhausted phenotype (Barber et al., 2006). Thus in the absence of TIM-3 and without any functional evidence of exhaustion it would appear that, in the context of COPD, PD-1 is more a marker of T cell activation. Thus the dynamics of PD-1 expression by T lymphocytes is required.

TIM-3 was not expressed on the cell surface of CD4+ or CD8+ T cells derived from lung. Previously, Gao *et al.* (Gao et al., 2012) identified TIM-3+ CD4+ and CD8+ T cells in the parenchyma of patients with lung cancer using flow cytometry. Undetectable levels of TIM-3 were expressed in the periphery, while approximately 23% of CD4+ and 28% of CD8+ lymphocytes expressed TIM-3 at the tumour site. Goa *et al.* also analysed T cell populations from tissue distal to the tumour site, similar to work shown in Figure 4.10. Data from this study identified 11.5% of CD4+ and 8% CD8+ T cells were TIM-3 positive. Results from our study do not agree with these findings, as TIM-3 was not detected on any of the T cell subsets

derived from tissue. Moreover there was no expression of TIM-3 at the mRNA level (Figure 4.13) further suggesting no TIM-3 expression in these samples.

The T cell exhaustion phenotype has been characterised as PD-1+TIM-3+ T cells. Key to the exhaustion mechanism is inhibition of antigen-specific T lymphocytes due to repeated presentation of antigen. By only looking at the overall T cell populations from lung parenchyma we have not dissected the nature of T cell specificity. Identification of T cell exhaustion may require presentation of antigens which may be chronically expressed in COPD. Candidates include antigen from RSV (Wilkinson et al., 2006a), nontypeable haemophilus influenzae (NTHi) (Garmendia et al., 2014; Lugade et al., 2014) or potential self-antigens (Lee et al., 2007).

CTLA-4 gene expression trends towards down-regulation in CD8+ T cells of COPD patients. Current literature is unclear as to the implications of CTLA-4 expression in COPD, but investigations into CTLA-4 polymorphisms appear to be the focus of several studies. Zhu *et al.* (Zhu et al., 2009) reported CTLA-4 polymorphisms associated with disease, but a separate study by Deng *et al.* (Deng et al., 2013) did not identify a relationship. CTLA-4 has also been reported as being co-expressed with PD-1 on CD8+ T cells (Duraiswamy et al., 2011), but CTLA-4 expression has not been implicated in T cell exhaustion directly. It is therefore unclear as to whether down-regulation of CTLA-4 by CD8+ T cells would limit their ability to be regulated by APCs and Treg cells.

Low levels of LAG-3 gene expression were detected in CD4+ and CD8+ lymphocytes in lung parenchyma. In a murine model of influenza A infection, Strutt *et al.* (Strutt et al., 2012) identified LAG-3 expression on secondary responder lymphocytes, while primary responses did not express LAG-3. Furthermore, CD8+ T cells upregulate LAG-3 in a model of LCMV chronic infection (West et al., 2011). Thus, LAG-3 may not be differentially expressed in the steady state between controls and COPD. LAG-3 expression is poorly defined in humans, and as is yet to be identified as a human marker of T cell exhaustion. As LAG-3 was identified in the steady state in both controls and disease, its expression in humans requires further examination.

CD27 gene expression by CD4+ lymphocytes is unchanged between controls and disease, but CD8+ T cells from COPD parenchyma may express CD27 to a greater level than controls. Tmem cells which do not express surface CD27 are highly cytotoxic cells (Hamann et al., 1997), and smokers have a greater proportion of CD8+ T cells which are CD27- (Koch et al., 2007). Furthermore, influenza-specific CD8+CD27+ cells do not produce IFNγ to the same level as CD27- counterparts (He et al., 2003). Previous literature, combined with our data, suggest that CD8+ T cells from COPD lung parenchyma may have impaired anti-viral responses compared to controls.

T cell senescence is a natural phenomenon associated with aging (Akbar and Fletcher, 2005; Effros, 2004). Analysis of CD57 was therefore performed with caution, as symptoms and diagnosis of COPD are mostly seen in the elderly population. COPD samples used in this chapter are age-matched with control samples, and thus a "baseline" level of senescence can be identified. CD57 expression was unchanged in lymphocytes, but CD8+ T cells from COPD sample appeared to diverge into $CD57^{lo}$ and $CD57^{hi}$ populations. CD57-expressing lymphocytes have a poorly proliferative, terminally differentiated phenotype (Focosi et al., 2010), but these cells also display potent cytotoxic responses (Brenchley et al., 2003; Chattopadhyay et al., 2009). In the context of COPD, Olloqueli *et al*. (Olloquequi et al., 2012) sampled the small airways and lung parenchyma of patients undergoing airway resection or lung transplantation. Olloqueli *et al.* observed that CD57 expression on lymphocytes significantly correlated with severe COPD. Combining our RT-PCR data yields potentially conflicting results. With a population of poorly cytotoxic CD27+ T cells, but also a population of CD57+ highly cytotoxic lymphocytes in the lungs of COPD patients. These results, however, may also emphasise the complex nature of T cell functions in the lung, with a combination of anti-viral response and regulatory inhibition occurring in a simultaneous manner. Furthermore, if T cells are expressing an activated phenotype in stable COPD, further investigation is required to identify what is the source of T cell activation. The implications of marker expression are shown in Table 4.9.

Marker	Stable	Activated	Senescent	Exhausted	COPD
PD-1	Low	High	Unknown	High	*Potentially elevated*
TIM-3	Undetected	Undetected	Undetected	High	*Undetected*
LAG-3	*Low expression*	Undetected	Undetected	High	*Low expression*
CTLA-4	*Low expression*	Low/High	High	Unknown	*Downregulated*
CD27	*Variable*	Upregulated	Low	Unknown	*Upregulated by CD8+ only*
CD57	*Variable*	Low (High on T_{EMRA} cells)	High	Unknown	*Potentially downregulated*

Table 4.9. Functional markers and their expression during different activation states. Table adapted from findings by (Agata et al., 1996; Barber et al., 2006; Duraiswamy et al., 2011; Focosi et al., 2010; Hamann et al., 1997; He et al., 2003; Jin et al., 2010; Koch et al., 2007; Olloquequi et al., 2012; Strutt et al., 2012; West et al., 2011; Wu et al., 2014). Expression status in italics represents findings from work performed in this thesis.

A limitation to the RT-PCR was again small sample numbers analysed. CD4+ and CD8+ T cells from 6 control and 6 COPD tissue samples were used for RT-PCR. There are two key reasons as to why sample numbers were low. Firstly, tissue did not always yield the required 2.5 x 10^4 CD4+ and CD8+ T cells. In samples yielding insufficient cell numbers, data generated by flow cytometry could still be analysed. Secondly, tissue that was used for the influenza infection model (Chapter 5), required cell fixation, making it unsuitable for RT-PCR. On occasion there was not a sufficient mass of tissue to perform experiments with both fixed and unfixed tissue-resident cells. This work, however, has allowed the appraisal of including RT-PCR work in future studies.

Work in Chapter 4 implicates CD8+ T cells in COPD by their increased proportion in lung. T cells residing in tissue appear to have elevated PD-1 expression, but this alone does not indicate an exhausted phenotype. RT-PCR data revealed that lymphocyte populations in lung parenchyma may differentially express functional markers such as CTLA-4, CD27 and CD57, and as such may not have a conserved phenotype in COPD. This was reiterated with Tmem population characterisation, which saw significant differences in CD57 expression between CD8+ T_{EM} and T_{EMRA} cells. With populations of cells identified and analysed in the steady state, work involving viral challenge of human lung ex planted tissue could be performed to analyse anti-viral inflammatory cell responses.

Chapter 5

Experimental Study of PD-1 and PD-L1 expression In Lung Parenchyma

Introduction

Influenza A virus is a global concern, and one which is clinically relevant in COPD disease outcomes. Influenza is a seasonal virus (Jenkins et al., 2012) which can cause an acute infection in healthy individuals, but have fatal outcomes in the immunocompromised. Exacerbations are life-threatening events in COPD, and a study by Gerke *et al.* (Gerke et al., 2013) identified a correlation between hospitalisation due to COPD exacerbation and hospital admission due to influenza infection. Furthermore Papi *et al.* (Papi et al., 2006) identified respiratory viral infections, including influenza, as potential contributors to COPD exacerbations by measuring patient sputum. Influenza can infect the lower airways of the lung (Dakhama et al., 1999), and thus could be expected to infect epithelial cells and macrophages of lung parenchyma *in vivo*. Therefore an inability to control influenza infection by COPD patients may induce disease exacerbation and unchecked viral persistence. This chapter focuses on T lymphocyte responses to tissue infection by X31 influenza virus, and whether T cell anti-viral functions are impaired in COPD.

The archetypal role of T cell immunity is to protect against viral infection. Studies to date in animal models have identified the important role of exhaustion signalling in choreographing appropriate anti-viral immune responses (Freeman et al., 2000). Dysregulation of this axis has been shown to lead to immune pathology when underactive (Erickson et al., 2012) or chronic infection when persistent signalling occurs (Barber et al., 2006; Trautmann et al., 2006; Velu et al., 2009). Lung derived cells are the key population of T cells in protection against acute viral infection, however it is not possible to obtain lung samples from patients experiencing acute viral infections. This limitation has led to the development of an *ex-vivo* model of acute viral infection of lung tissue. The model is described and its utility in determining dynamics of exhaustion signalling in this context is described herein.

Experimental infection of lung tissue by X31 influenza A virus was optimised to ensure a significant proportion of epithelial cells and alveolar macrophages were infected. The dynamic expression of the PD-1 ligand, PD-L1 was measured on epithelial cells and macrophages in response to infection to determine their regulatory potential in this model.
Expression of PD-1 and TIM-3 was measured on lymphocytes by flow cytometry to identify T cell exhaustion in response to virus. CD57 and CD107a expression was measured on CD4+ and CD8+ T cells to assess their potential to upregulate an anti-viral immune response. T cell

functional markers were also measured on TEM and TEMRA cells to identify phenotypic differences between lung memory populations.

Patient cohort

Patient data for influenza infection experiments was similar to that of the entire cohort and that of Chapter 4. Numbers of control (n = 11) and COPD (n = 12) individuals was similar, and groups were age matched (Table 5.1). Proportions of males and females were similar between groups. Pack years appeared to be greater in COPD patients than controls (27.5 vs. 39.38) but this was not statistically significant (p = 0.29). FEV_1% was significantly lower in COPD than controls (102.4 vs. 74.45).

	Control	COPD	p Value
N	11	12	-
Age	69.73 (61 – 78)	68.17 (63 – 74.75)	0.69
Gender M/F	5 / 6	5 / 7	-
Smoker (Never/Ex/Current)	3 / 7 / 1	0 / 8 / 4	-
Pack Years	27.5 (0.75 – 53.25)	39.38 (31.25 – 50)	0.29
Cancer Diagnosis (Yes/Unknown)	6 / 5	6 / 6	
FEV_1%	102.4 (93.5 – 113)	74.45 (70 – 80)	0.0001
FEV_1/FVC%	0.773 (0.752 – 0.796)	0.58 (0515 – 0.642)	< 0.0001

Table 5.1. Patient phenotypes of control and COPD patients from which parenchymal tissue was infected with X31 influenza virus. Individuals were defined as COPD by spirometry if $FEV_1/FVC < 0.7$. Mean and IQR shown.

Influenza A infection of human parenchymal tissue

In order to infect epithelial cells and lung resident macrophages without killing cells, titration experiments were performed to calculate optimal virus dose (Figure 5.2). Infection rates were determined by intracellular expression of the influenza protein Nucleoprotein-1 (NP-1), which is only expressed during viral replication within the host cell (Figure 5.1). Increased virus concentrations added to samples resulted in increased infection of epithelial cells (p = 0.004) and macrophages (p = 0.008). At the highest concentration used, a mean of 16.33% of epithelial cells and 26.58% of macrophages were infected. No NP-1 expression was identified in non-infected samples. Previous work by the group had shown that T cells were not infected

by X31 in this explant infection model (Nicholas et al., 2013). A concentration of 1 x 10^6 pfu/ml X31 Influenza A virus was used for subsequent experiments.

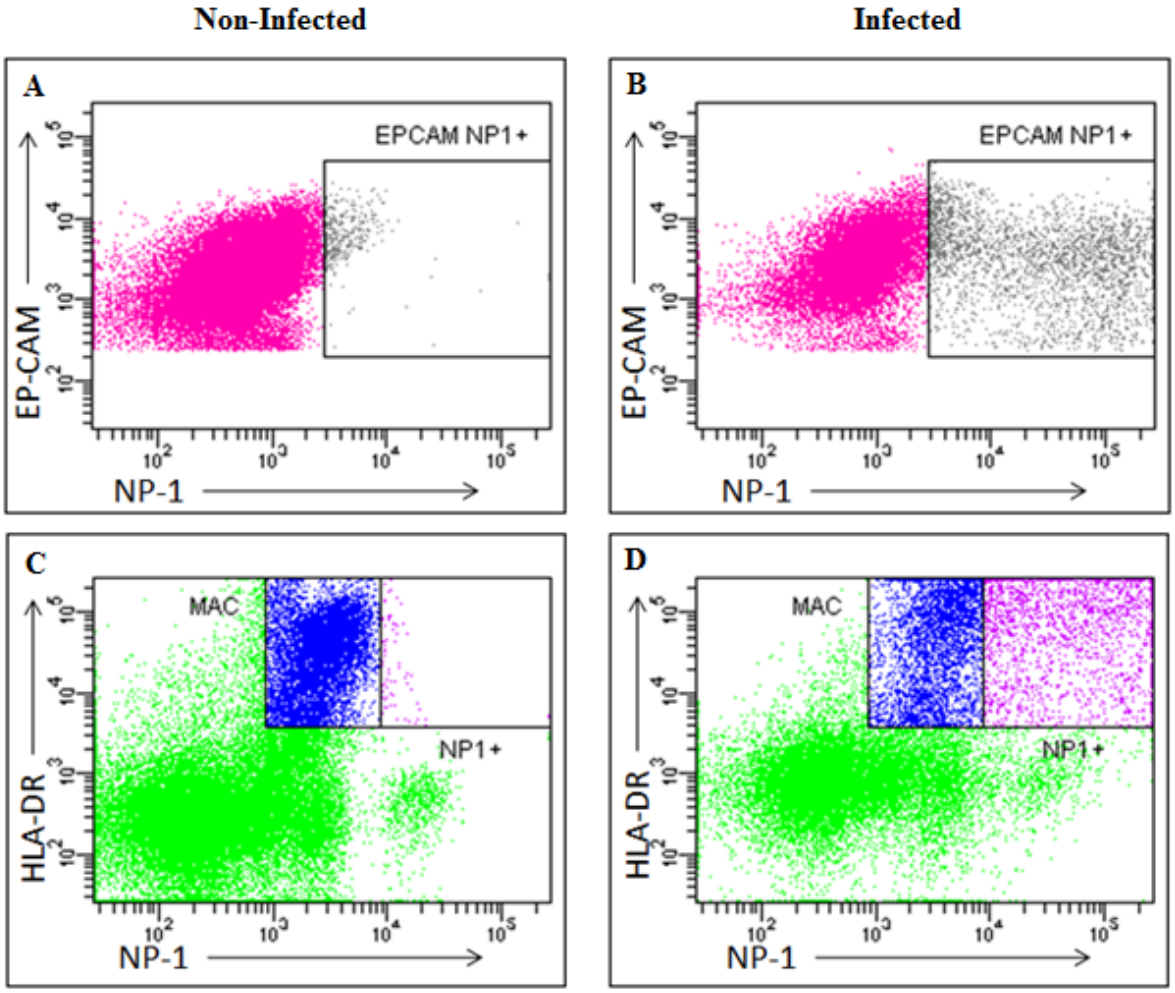

Figure 5.1. NP-1 expression in non-infected and infected epithelial cells and macrophages. 1cm^3 sections of tissue were treated with 1x10^6 pfu/ml live X31 virus (X31) or non-infected (NI) for 2 h before a 22 h incubation. **(A)** Proportions of uninfected **(B)** and infected epithelial cells **(C)** and uninfected **(D)** and infected macrophages expressing NP-1. Infected epithelial cells are represented as grey dots, infected macrophages are represented as purple dots.

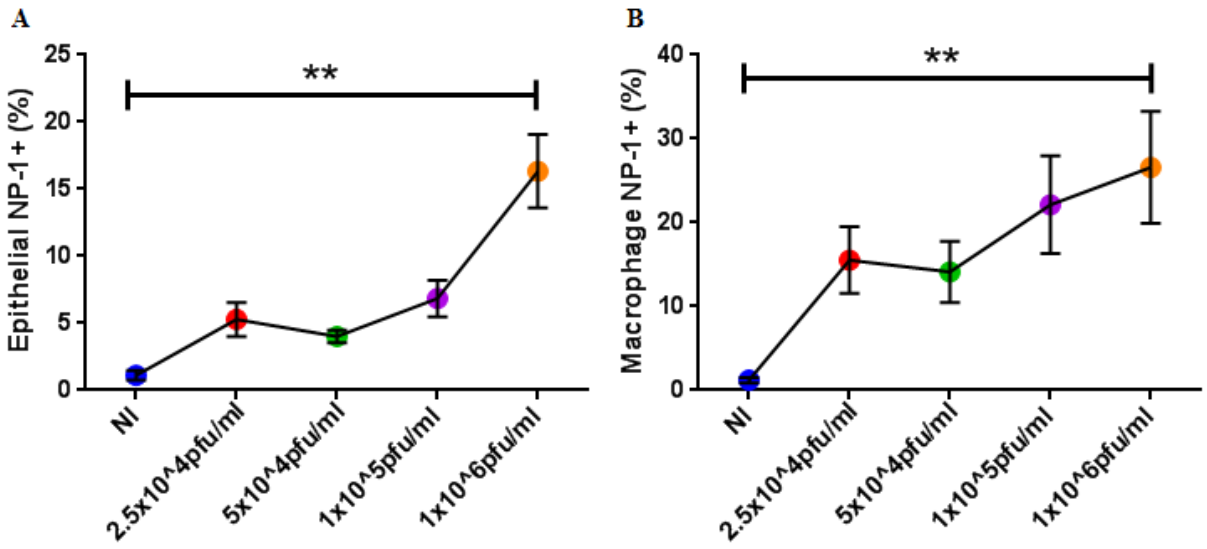

Figure 5.2. Treatment of parenchymal tissue with increasing X31 influenza A virus concentrations results in increased epithelial cell and macrophage infection. 1 cm^3 sections of tissue were infected 2.5 x 10^4 pfu/ml, 5 x 10^4 pfu/ml, 1 x 10^5 pfu/ml or 1 x 10^6 pfu/ml X31 Influenza A virus stock for 2h followed by a 22 h incubation. No virus was added to the non-infected (NI) samples. Tissue was collagenase digested as previously described **(A)** epithelial cells **(B)** and macrophages were analysed for infection by flow cytometry using FITC-labelled anti-NP-1antibody. n = 6. Mean and SEM shown. One-way ANOVA p = 0.004 and p = 0.009 respectively.

Observational studies have reported that COPD patients are more susceptible to common viral infections (Hurst et al., 2005), and that viral infection results in more severe symptoms (Mallia et al., 2011) than control patients. The susceptibility of parenchymal tissue to infection was measured in control and COPD samples (Figure 5.3). UV-treated X31 influenza virus (UVX31) was included in the assays to control for innate immune responses. This was no longer able to replicate, but could potentially be recognised by PAMPs on innate immune cells and epithelial cells. Thus mechanisms driven by viral infection could be compared to reactions generated by a non-infectious agent. The ability of X31 influenza A model had previously been shown to infect epithelial cells and macrophages in human bronchial and parenchymal tissues (Nicholas et al., 2013). Endothelium, fibroblasts, B cells and T cells were not infected by X31. Therefore infection of epithelial cells and macrophages was quantified. Epithelial cells and macrophages from UVX31 treated tissue did not express NP-1. A mean of 10.38% of epithelial cells from control patients expressed NP-1 (Figure 5.3A) compared to 9.13% form COPD samples (p = 0.7). There were no significant differences (p = 0.7) between proportion of macrophages from control (mean = 18.12%) or COPD (mean = 16.52%) tissue infected with X31 virus (Figure 5.3B). Thus it appears that susceptibility to X31 infection was unchanged between controls and disease samples.

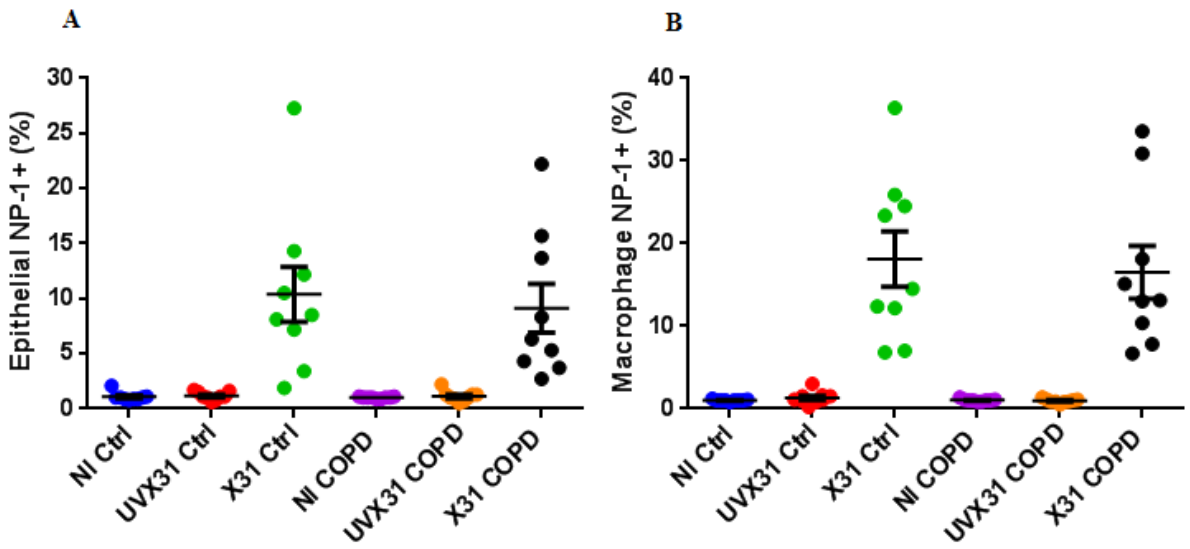

Figure 5.3. Epithelial cell and macrophage infection by X31 Influenza A virus and UV-irradiated control in human lung tissue. 1cm^3 sections of tissue were treated with UVX31, 1x10^6 pfu/ml live X31 virus (X31) or non-infected (NI) for 2 h before a 22 h incubation. **(A)** Proportions of epithelial cells **(B)** and macrophages expressing NP-1 was quantified by flow cytometry. n=9 for controls and COPD. Mean and SEM shown. Unpaired two-tailed t test was performed.

PD-1 is upregulated during Influenza A infection

As there was no difference in the proportion of infected cells between health and COPD, these data suggested that the mechanisms leading to COPD exacerbations may arise as a failure to adequately control the immune response. PD-1 and PD-L1 expression were therefore measured to investigate differential immune responses to epithelial cell and macrophage infection. PD-1 expressing T cells have been obtained from the lung, spleen and liver of murine models of acute and chronic viral infection (Aubert et al., 2011; Jin et al., 2010), but these findings had not been replicated in humans. Figure 5.5 shows that during X31 infection PD-1 was upregulated on CD4+ and CD8+ T cells in both control and COPD parenchymal samples. CD4+ PD-1 expression in controls increased from a NI mean sMFI of 250.8 to 350.4 in X31 samples ($p = 0.02$). NI CD4+ T cells from COPD patients express a mean sMFI PD-1 of 219.4 which increased to 288.8 with X31 treatment ($p = 0.003$). A similar result was seen for CD8+ T cells (Figure 5.5B). Infection upregulated PD-1 expression in controls (mean sMFI = 177.1 to 240.9, $p = 0.014$) and COPD samples (mean sMFI = 217.2 to 263, $p = 0.049$). CD4+ expression of PD-1 in NI samples is not significantly different between the control group and COPD ($p = 0.2142$). There is also no difference seen in CD8+ T cell expression in these samples ($p = 0.4638$). PD-1 is upregulated during X31

infection, but the level of expression in CD4+ (p = 0.1815) and CD8+ (p = 0.4189) T cells in unchanged between control and COPD samples.

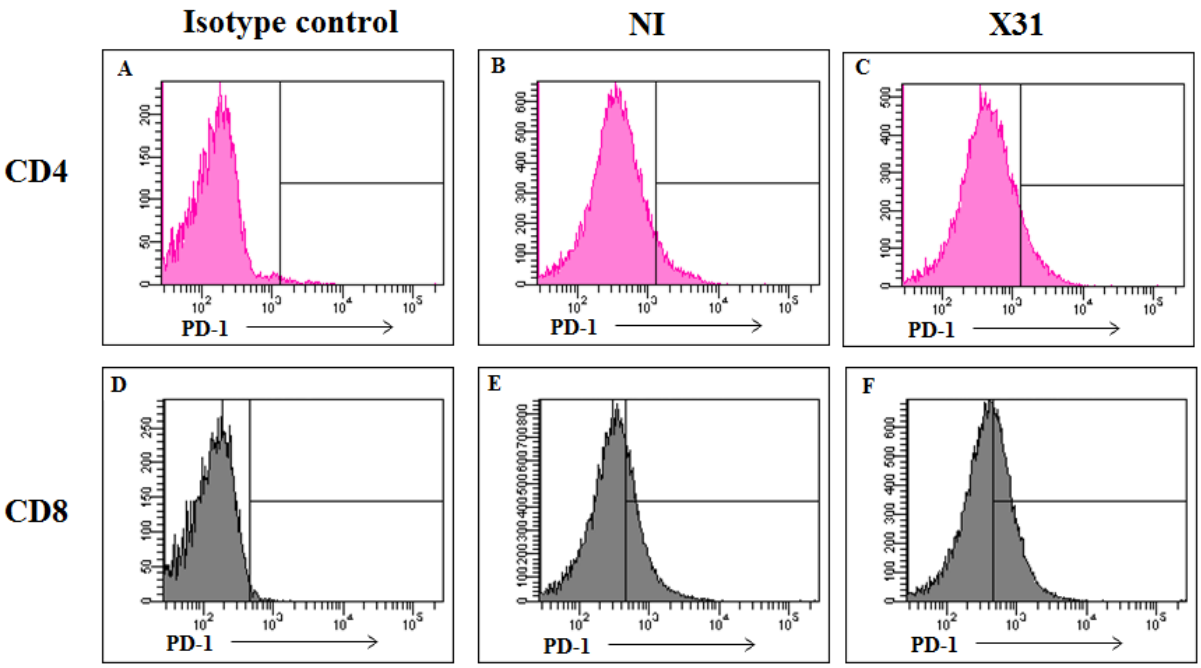

Figure 5.4. Representative plot of PD-1 expression by T cells after X31 Influenza infection. 1cm^3 sections of tissue were treated with UVX31, X31 or NI for 2 h before a 22 h incubation. PD-1 expression was measured by flow cytometry after incubation with fluorescently-labelled antibody. **(A)** CD4+ T cells **(D)** and CD8+ T cells stained with isotype control antibody. **(B)** PD-1 expression by NI CD4 T cells **(E)** and CD8 T cells. **(C)** PD-1 expression by X31-treated CD4 T cells **(F)** and CD8 T cells. Plots representative of 20 experiments.

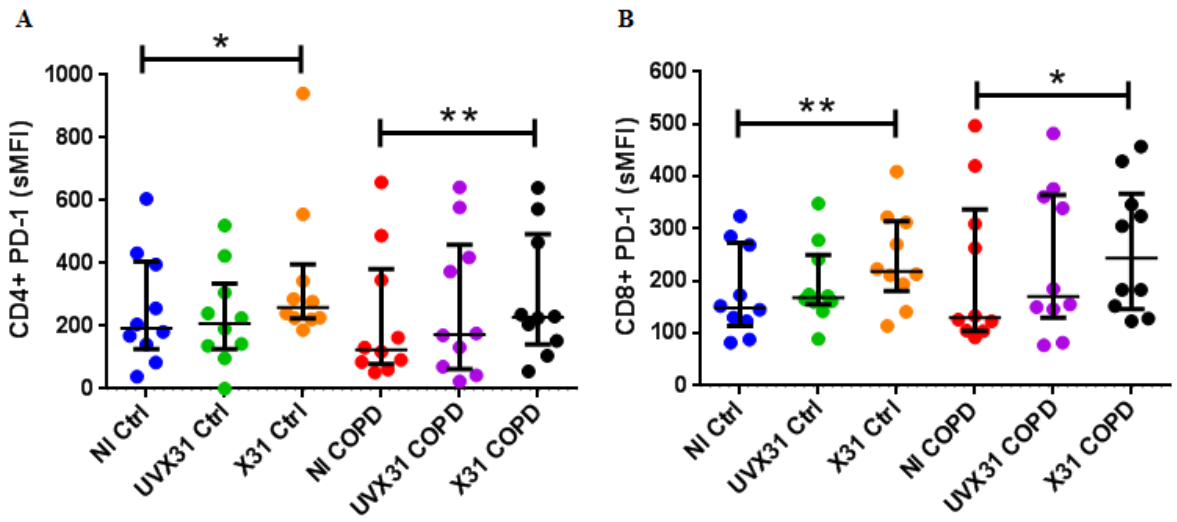

Figure 5.5. PD-1 expression by T cells during X31 infection of lung parenchyma. 1cm^3 sections of tissue were treated with UVX31, X31 or NI for 2 h before a 22 h incubation. **(A)** Proportions of CD4+ **(B)** and CD8+ T cells expressing PD-1 were quantified by flow cytometry. n = 10 for controls and COPD. Mean and SEM shown. Wilcoxon signed rank test performed when comparing within disease groups. Mann-Whitney test performed when comparing control group with COPD group. (*) represents $p < 0.05$, (**) represents $p < 0.01$.

Memory T cell populations have been identified in the lung as discussed in Chapter 4. As these cells account for the majority of T cells found in lung parenchyma and are likely to carry memory for past respiratory viral infections, further work was performed to investigate phenotypic differences between Tmem subsets (Figure 5.6). PD-1 was upregulated by CD8+ TEM cells from control ($p = 0.0234$) and COPD ($p = 0.0313$) samples in response to X31 infection. Expression of PD-1 prior to infection was not significantly different between control and COPD samples ($p = 0.2266$), nor was there a difference between infected samples ($p = 0.1926$). CD8EMRA cells from control samples also upregulated PD-1 in response to infection ($p = 0.0078$), but this was not seen in the COPD samples ($p = 0.3125$). As with CD8EM results, there are no significant differences between PD-1 expression in control and COPD samples in either non-infected ($p = 0.3617$) or X31-treated samples ($p = 0.1654$).

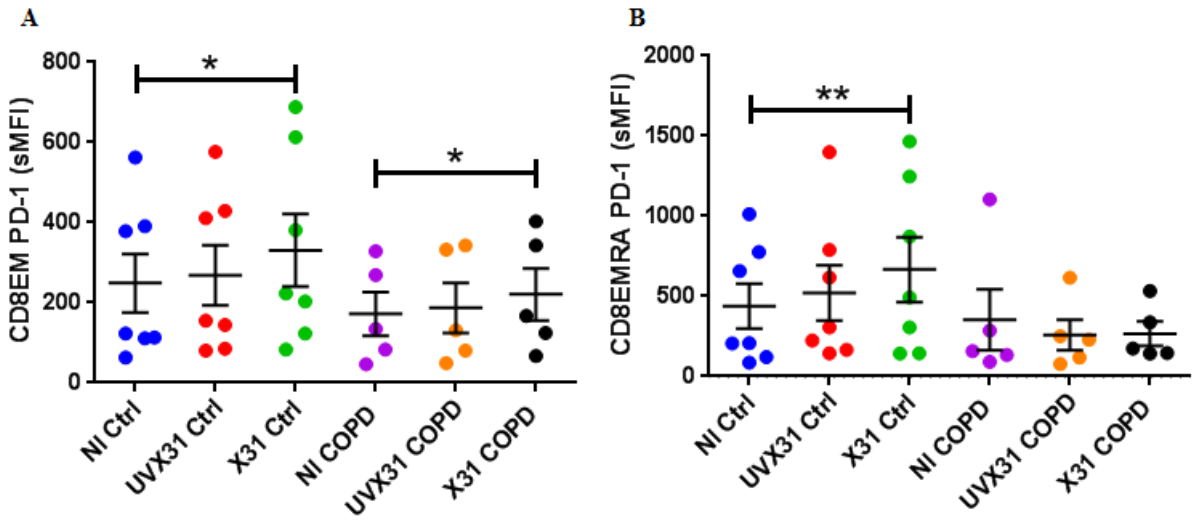

Figure 5.6. PD-1 expression by Tmem cells during X31 infection of lung parenchyma. 1cm^3 sections of tissue were treated with UVX31, X31 or NI for 2 h before a 22 h incubation. **(A)** Proportions of CD8+CD45RA-CCR7- **(B)** and CD8+CD45RA+CCR7- T cells expressing PD-1 was quantified by flow cytometry. Control n = 7, COPD n = 5. Mean and SEM shown. Unpaired t test or Wilcoxon signed rank test performed when comparing within disease groups. Mann-Whitney test performed when comparing control group with COPD group. (*) represents $p < 0.05$, (**) represents $p < 0.01$.

CD107a, but not TIM-3 or CD57 are upregulated by Influenza A infection

Expression of PD-1 alone is not sufficient to classify T cells as having an exhausted phenotype. Co-expression of TIM-3 with PD-1 has previously been used to delineate between activated and exhausted cells (Jin et al., 2010; West et al., 2011). Figure 5.8 shows combined data from control and COPD samples. For CD4+ and CD8+ T cells, TIM-3 is not expressed in non-infected lung parenchyma. Unlike PD-1 expression, TIM-3 is also not upregulated in response to X31 infection.

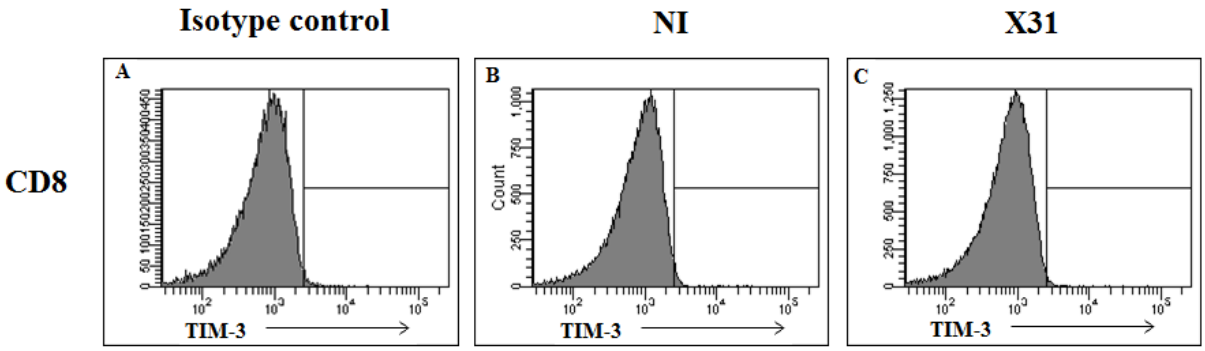

Figure 5.7. Representative plot of TIM-3 expression by T cells after X31 Influenza infection. 1cm^3 sections of tissue were treated with UVX31, X31 or NI for 2 h before a 22 h incubation. TIM-3 expression was measured by flow cytometry after incubation with fluorescently-labelled antibody. **(A)** CD8+ T cells stained with isotype control antibody. **(B)** TIM-3 expression by NI CD8 T cells. **(C)** TIM-3 expression by X31-treated CD8 T cells. Plots representative of 6 experiments.

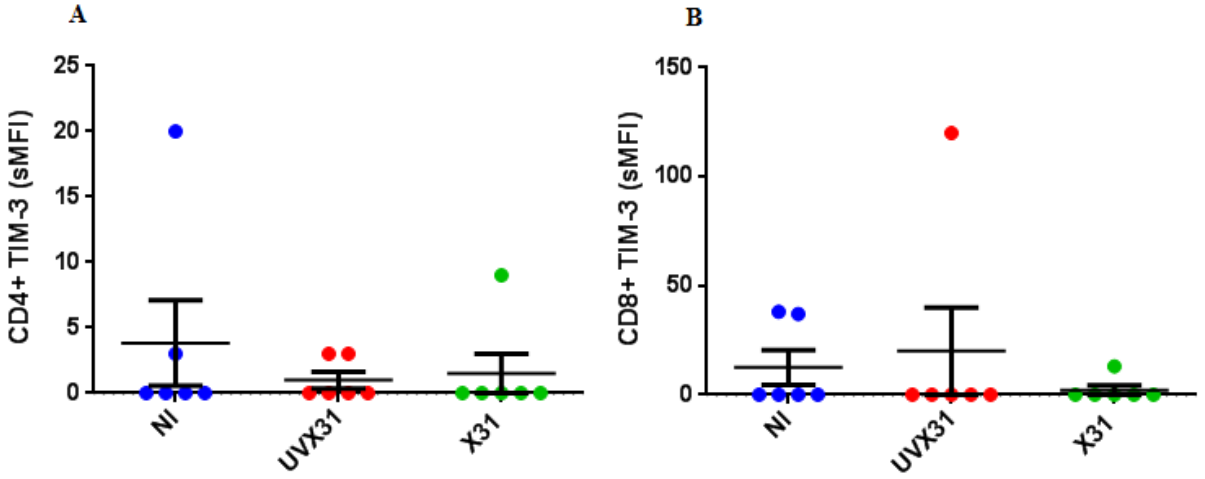

Figure 5.8. TIM-3 expression by T cells during X31 infection of lung parenchyma. 1cm^3 sections of tissue were treated with UVX31, X31 or NI for 2 h before a 22 h incubation. **(A)** Proportions of CD4+ **(B)** and CD8+ T cells expressing TIM-3 were quantified by flow cytometry. n = 6. Mean and SEM shown. Unpaired t test or Wilcoxon signed rank test performed when comparing within disease groups.

CD57 is associated with fully differentiated cells and cells (Brenchley et al., 2003; Papagno et al., 2004) which have potent anti-viral effector functions (Brenchley et al., 2003; Chattopadhyay et al., 2009; Ibegbu et al., 2005). CD4+ T cells from control tissue and COPD tissue recorded a wide range of CD57 expression values (NI sMFI 265 to 3945 and 422 to 2204 respectively), but CD57 was not upregulated in response to X31 (Figure 5.10A). CD8+ T cells expressed CD57 in non-infected tissues of controls and COPD samples, but also did not upregulate CD57 in response to X31.

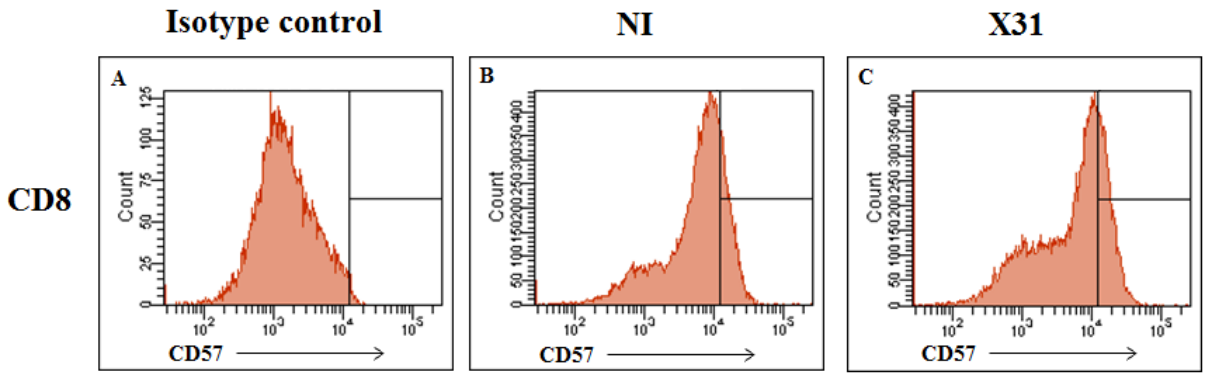

Figure 5.9. Representative plot of CD57 expression by T cells after X31 Influenza infection. 1cm^3 sections of tissue were treated with UVX31, X31 or NI for 2 h before a 22 h incubation. CD57 expression was measured by flow cytometry after incubation with fluorescently-labelled antibody. **(A)** CD8+ T cells stained with isotype control antibody. **(B)** CD57 expression by NI CD8 T cells. **(C)** CD57 expression by X31-treated CD8 T cells. Plots representative of 12 experiments.

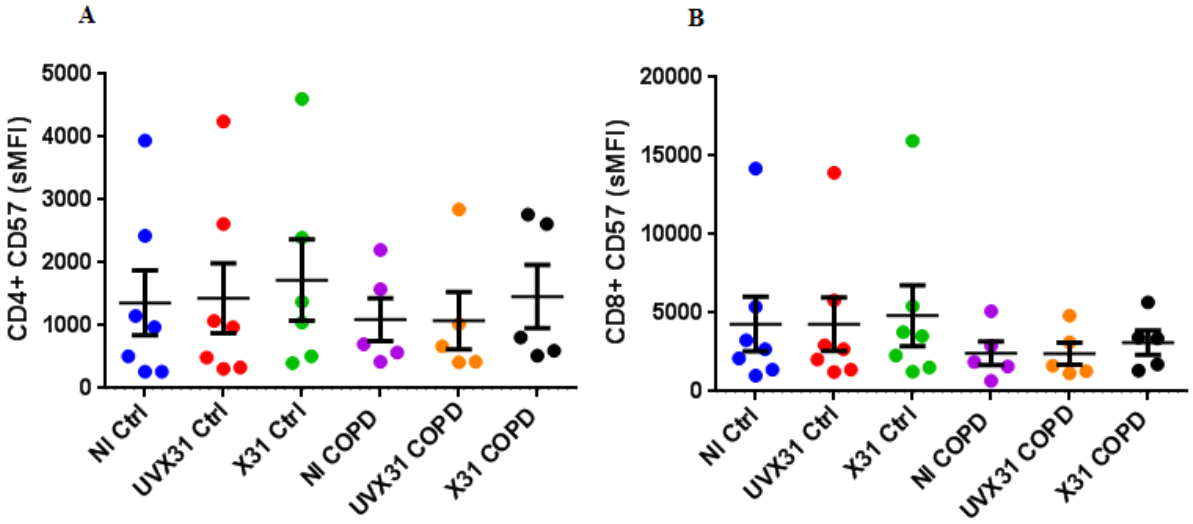

Figure 5.10 CD57 expression by T cells during X31 infection of lung parenchyma. 1cm^3 sections of tissue were treated with UVX31, X31 or NI for 2 h before a 22 h incubation. **(A)** Proportions of CD4+ **(B)** and CD8+ T cells expressing CD57 were quantified by flow cytometry. Control n = 7, COPD n = 5. Mean and SEM shown. Unpaired t test or Wilcoxon signed rank test performed when comparing within disease groups. Mann-Whitney test performed when comparing control group with COPD group.

Figure 5.11 illustrates the modulation of CD57 expression on Tmem cells in response to X31 treatment. CD57 expression is slightly but significantly upregulated by CD8+ TEM cells in control ($p = 0.0391$) and COPD tissues ($p = 0.0313$) in response to infection. As with PD-1 expression, there were no differences in CD57 expression between control and COPD groups at baseline ($p = 0.4234$) or during X31 infection ($p = 0.271$). TEMRA cells, however did not upregulate CD57 in response to infection (Figure 5.11B). There was no significant increase in

CD57 expression in control samples (p = 0.1094), but a trend (p = 0.0625) for increased CD57 was observed in infected COPD tissues. Again there were no differences in CD57 expression between control and COPD groups at baseline (p = 0.2659) or during X31 infection (p = 0.345).

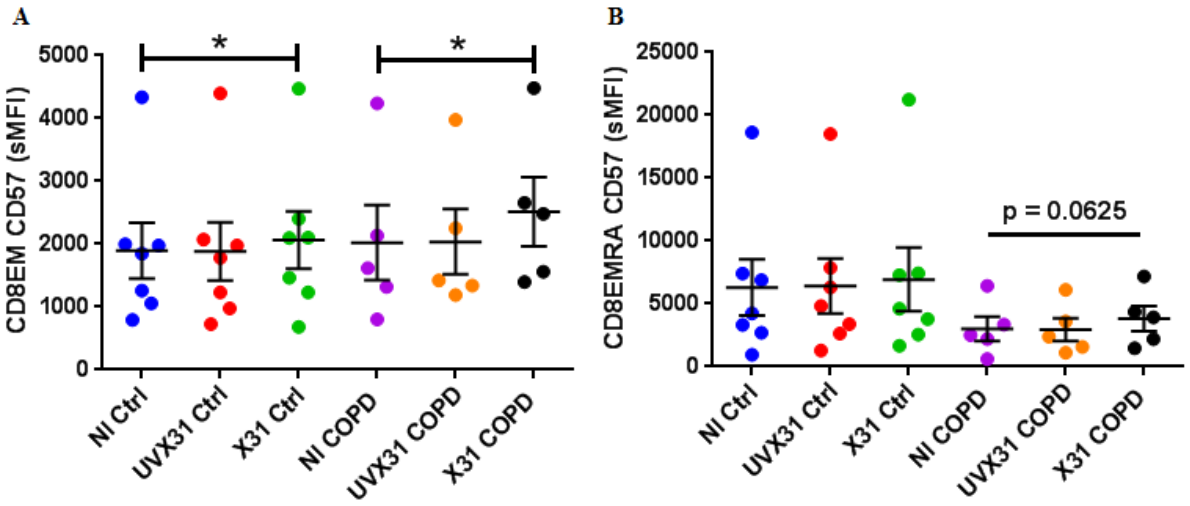

Figure 5.11. CD57 expression by Tmem cells during X31 infection of lung parenchyma. 1cm^3 sections of tissue were treated with UVX31, X31 or NI for 2 h before a 22 h incubation. **(A)** Proportions of CD8+CD45RA-CCR7- **(B)** and CD8+CD45RA+CCR7- T cells expressing CD57 was quantified by flow cytometry. Control n = 7, COPD n = 5. Mean and SEM shown. Unpaired t test or Wilcoxon signed rank test performed when comparing within disease groups. Mann-Whitney test performed when comparing control group with COPD group. (*) represents $p < 0.05$.

A key immunological mechanism to control viral infection is the ability for T cells and NK cells to kill infected cells via the release of perforin and granzyme. Cell degranulation can be identified by the expression of CD107a on the surface of cytotoxic cells (Figure 5.13). CD4+ T cells from control samples significantly upregulated CD107a in response to infection (p = 0.03). There was a trend for a similar result with cells from COPD samples, but this did not achieve significance (p = 0.12). This is possibly due to a small sample size and one patient recording undetectable levels of CD107a for all conditions. CD8+ T cells also upregulated CD107a in control explants (p = 0.03) but not in COPD samples.

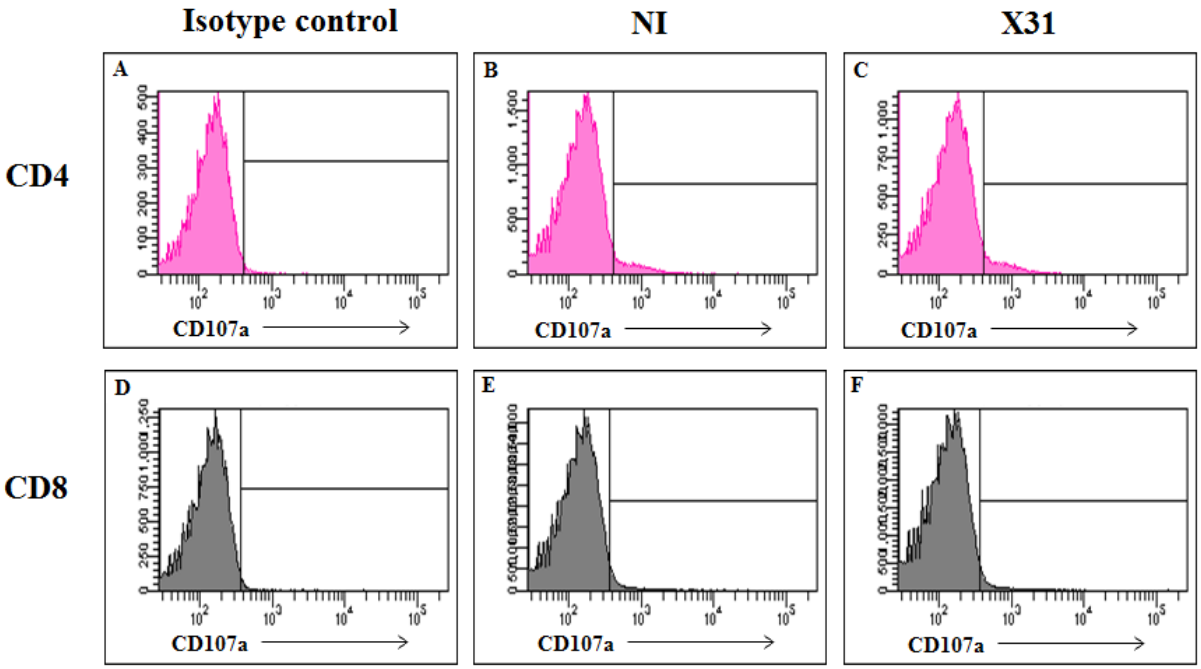

Figure 5.12. Representative plot of CD107a expression by T cells after X31 Influenza infection. 1cm^3 sections of tissue were treated with UVX31, X31 or NI for 2 h before a 22 h incubation. CD107a expression was measured by flow cytometry after incubation with fluorescently-labelled antibody. **(A)** CD4+ T cells **(D)** and CD8+ T cells stained with isotype control antibody. **(B)** CD107a expression by NI CD4 T cells **(E)** and CD8 T cells. **(C)** CD107a expression by X31-treated CD4 T cells **(F)** and CD8 T cells. Plots representative of 10 experiments.

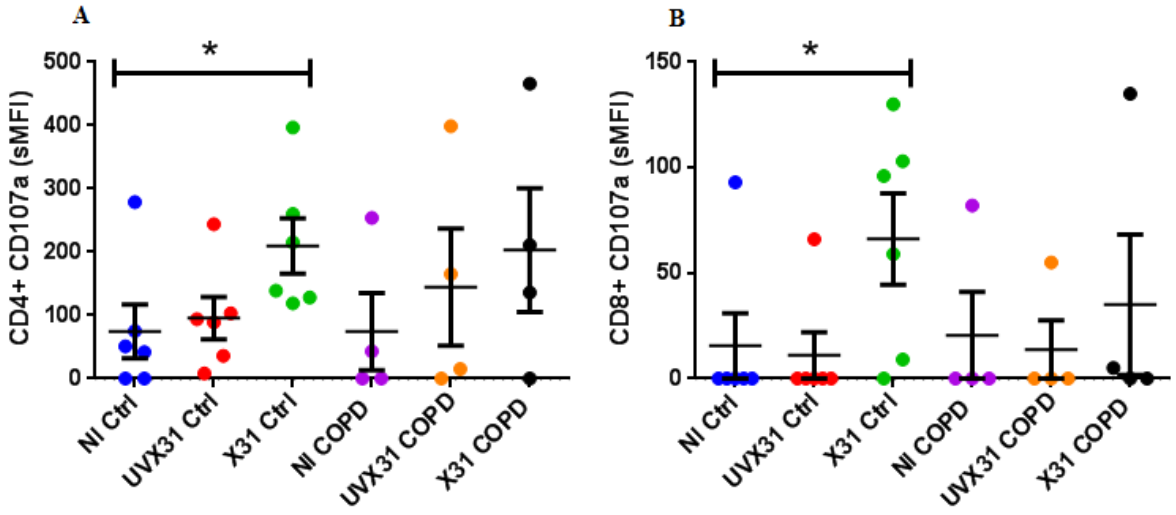

Figure 5.13. CD107a expression by T cells during X31 infection of lung parenchyma. 1cm^3 sections of tissue were treated with UVX31, X31 or NI for 2 h before a 22 h incubation. **(A)** Proportions of CD4+ **(B)** and CD8+ T cells expressing CD107a were quantified by flow cytometry. Control n = 6, COPD n = 4. Mean and SEM shown. Unpaired t test or Wilcoxon signed rank test performed when comparing within disease groups. Mann-Whitney test performed when comparing control group with COPD group. (*) represents $p < 0.05$.

Analysis of Tmem populations also found disease-related differences in CD107a upregulation in response to infection (Figure 5.14). As expected, TEM (p = 0.0112) and TEMRA (p = 0.0346) cells from control patients upregulated CD107a in response to X31 influenza A infection. Neither TEM (p = 0.125) nor TEMRA (p = 0.1995) cells from COPD samples unregulated CD107a during acute infection. This distinction in responses between disease states was emphasised by a trend suggesting that CD107a expression in TEM cells from infected control tissue was greater than in COPD infected samples (p = 0.0559). The expression of CD107a by TEMRA cells in infected tissue from controls was not significantly higher than that of TEMRA cells in COPD (P = 0.36). This is likely due to samples from control and COPD groups which did not express detectable CD107a.

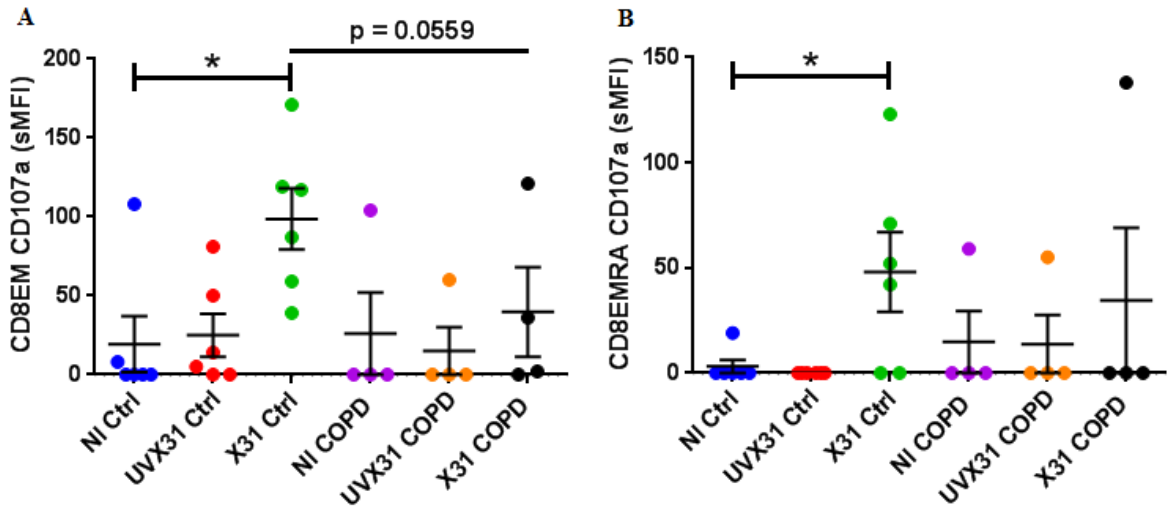

Figure 5.14. CD107a expression by Tmem cells during X31 infection of lung parenchyma. 1cm^3 sections of tissue were treated with UVX31, X31 or NI for 2 h before a 22 h incubation. **(A)** Proportions of CD8+CD45RA-CCR7- **(B)** and CD8+CD45RA+CCR7- T cells expressing PD-1 was quantified by flow cytometry. Control n = 6, COPD n = 4. Mean and SEM shown. Unpaired t test or Wilcoxon signed rank test performed when comparing within disease groups. Mann-Whitney test performed when comparing control group with COPD group. (*) represents $p < 0.05$.

PD-L1 is upregulated by macrophages but not epithelial cells in response to X31 infection

Exhausted T cells lose their effector functions due to ligation of PD-1 by PD-L1. Thus PD-L1 was measured on epithelial cells and macrophages (Figure 5.16) to elucidate whether this corresponded to viral infection and T cell upregulation of PD-1. The results indicated that epithelial cells express PD-L1 in human parenchyma, but its expression is lower (NI pooled controls and COPD sMFI = 142) than in macrophages (NI pooled sMFI = 442.47) and is not

regulated by acute X31 infection. Macrophages, however, upregulate PD-L1 in response to infection in control samples (p = 0.016) and COPD samples (p = 0.049). The expression of PD-L1 by macrophages in NI samples is unchanged between controls and COPD samples (p = 0.0824). Although this p value could be described as a trend for increased expression PD-L1 in healthy tissue, the majority of macrophages in both groups recorded undetectable levels of the ligand. There was a similar trend for lower expression of PD-L1 in COPD observed when comparing infected samples (p = 0.0926). Intriguingly, only one sample in the control group did not express PD-L1 in response to infection, while macrophages in 4 of 9 COPD samples fail to upregulate the ligand.

	Control	COPD	p Value
n	9	9	-
Age	71.11 (64 – 79.5)	66.44 (57 – 76.5)	0.36
Gender M/F	4 / 5	3 / 6	-
Smoker (Never/Ex/Current)	3 / 5 / 1	0 / 4 / 5	-
Pack Years	23.21 (0 – 45)	54.75 (39.38 – 75.25)	0.031
Cancer Diagnosis (Yes/Unknown)	6 / 3	5 / 4	-
FEV_1%	102.5 (95 – 115)	72.25 (60 – 79.25)	0.0007
FEV_1/FVC%	0.78 (0.756 – 0.798)	0.62 (0.602 – 0.659)	< 0.0001

Table 5.2. Patient phenotypes of individuals used in epithelial cell and macrophage experiments. Individuals were defined as COPD by spirometry if FEV_1/FVC <0.7. Mean and IQR shown. Unpaired t test performed.

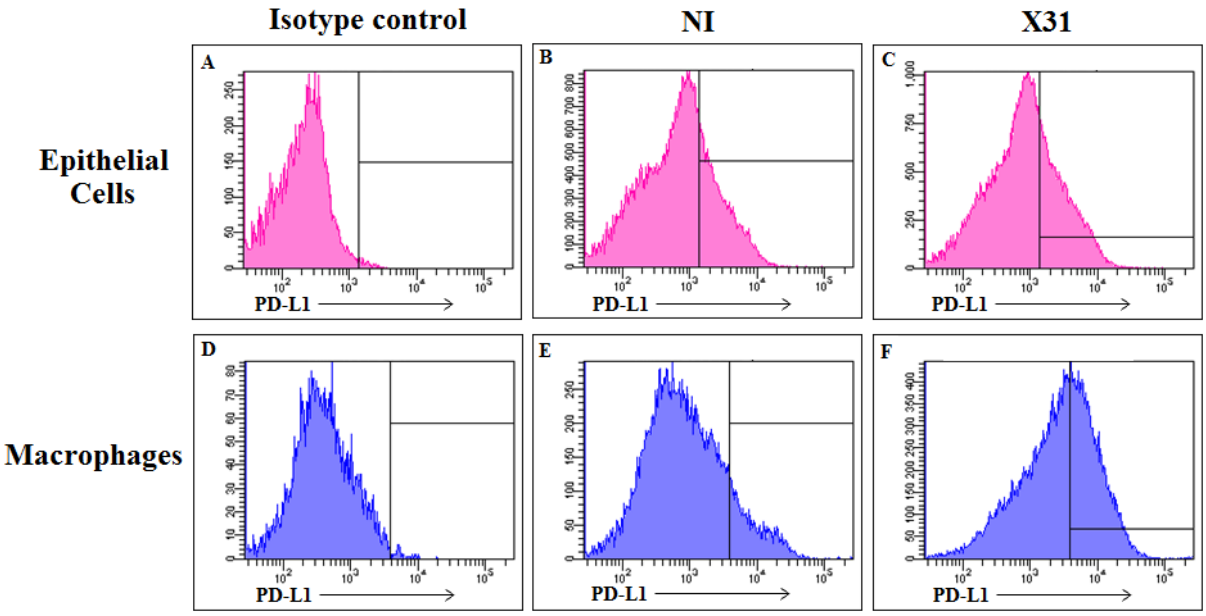

Figure 5.15. Representative plot of PD-L1 expression by epithelial cells and macrophages after X31 Influenza infection. 1cm^3 sections of tissue were treated with UVX31, X31 or NI for 2 h before a 22 h incubation. PD-L1 expression was measured by flow cytometry after incubation with fluorescently-labelled antibody. **(A)** Epithelial cells **(D)** and macrophages stained with isotype control antibody. **(B)** PD-L1 expression by NI epithelial cells **(E)** and macrophages. **(C)** PD-L1 expression by X31-treated epithelial cells **(F)** and macrophages. Plots representative of 18 experiments.

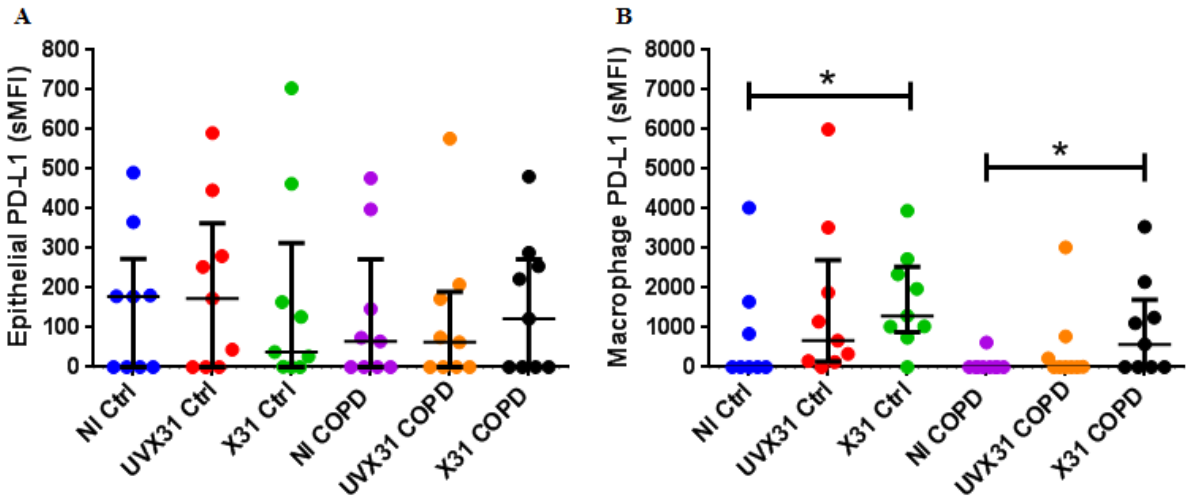

Figure 5.16. PD-L1 expression by epithelial cells and macrophages during X31 infection of lung parenchyma. 1cm3 sections of tissue were treated with UVX31, X31 or NI for 2 h before a 22 h incubation. **(A)** Proportions of epithelial **(B)** and macrophages expressing PD-L1 was quantified by flow cytometry. n = 9 for controls and COPD. Mean and SEM shown. Unpaired t test or Wilcoxon signed rank test performed when comparing within disease groups. Mann-Whitney test performed when comparing control group with COPD group. (*) represents $p < 0.05$

Conclusion

Lung tissue was infected to observe changes in functional markers on T cells, epithelial cells and macrophages in response to X31 influenza A virus. This challenge model was utilised to attempt to characterise dysfunctional T cells responses to influenza, and identify potential T cell exhaustion. CD4+ T cells from control and COPD tissue appeared to responds to infection in a functional manner, while CD8+ T cells in COPD showed inhibited cytotoxic capabilities. Further investigation of the CD8+ lymphocyte population identified TEMRA cells as being dysfunctional in response to influenza virus. This dysfunction did not appear to be regulated by the exhaustion signalling pathway. Macrophage from control and COPD samples upregulated PD-L1 in response to infection, but this was not reproduced by epithelial cells.

Increased concentration of X31 virus used to challenge parenchymal tissue increased the proportion of epithelial cell and macrophage infection. As shown previously (Nicholas et al., 2013), only epithelial cells and macrophages had detectable NP-1 expression and thus were targeted for infection by influenza virus. Infection of these cells is likely to be representative of infection *in vivo*. Sheng *et al.* (Sheng et al., 2011) performed autopsies and immunohistochemical analysis on lungs from individuals who died from infection with the 1918 pandemic influenza H1N1 virus. A key finding of their work was that viral RNA was detected in epithelial cells and macrophages, but no other cell types. As epithelial cells provide a physicochemical barrier in the lungs and macrophages are involved in tissue surveillance, these cells may be the first to encounter virus and thus may be targeted for infection by influenza.

There were no differences in the proportion of epithelial cells and macrophages which expressed NP-1 between control and COPD groups. Previous literature suggests that individuals with COPD are more susceptible to infection than healthy individuals (Hurst et al., 2005; Inghammar et al., 2014). Our work had limitations which potentially could have led to no differences in susceptibility between controls and COPD. The COPD patient cohort consisted of patients classified as GOLD stage I or stage II as these patients were suitable for surgical lung resection. Viral infection is a trigger of disease exacerbations (Seemungal et al., 2001; Wilkinson et al., 2006b), and exacerbation frequency correlates with COPD severity (Hurst et al., 2010). Therefore as GOLD stage III and IV patients were not recruited, the most

susceptible individuals with COPD may not have been investigated. Analysis of NP-1 expression is an accurate method of identifying infected epithelial cells and macrophages. The ability of influenza virus to successfully shed has been measured by Dr. Nicholas using similar methodology, but determining re-infection has proved challenging and this was not measured in my study. Therefore while control and COPD tissue was infected equally, potential differences in viral shedding or viral persistence is unknown in this model. The concentration of virus used to infected samples was selected as it infected the greatest proportion of epithelial cells and macrophages. This level of infection may be the plateau of infection before excessive cell death is induced. Therefore challenge of lung parenchyma with lower concentrations may reveal different susceptibilities to infection between control and COPD groups. Influenza is only one virus for which COPD individuals may be more susceptible. RSV (Mallia et al., 2011), rhinovirus (George et al., 2014; Wilkinson et al., 2006a) and adenovirus (McManus et al., 2007) have all been implicated in COPD exacerbations, and may infect COPD patients to a greater extent than healthy individuals. Data for the degree of X31 infection was generated from 9 control and 9 COPD samples. These patients may be representative of their respective groups, but they may be relative outliers which skew results and prevent accurate conclusions from being made.

As epithelial cells can function as antigen presenting cells (Papi et al., 2000), it would stand to reason that they may be able to modulate and potentially inhibit immune responses. The expression of PD-L1 by epithelium in the tumour microenvironment indicates poor prognosis in several solid cancers (Hino et al., 2010; Nakanishi et al., 2007). In recent years this has progressed from mouse tumour cell lines (Iwai et al., 2002) to murine models and now has clinical applications (Thompson et al., 2004; Topalian et al., 2012). It appears that although epithelial expression of PD-L1 does not attenuate T cell responses in the acute model of X31 infection, these cells may play a role in chronic infection.

Upregulation of PD-L1 in response to viral infection has been shown in human cell lines, yet PD-L1 was inconsistently expressed and was not modulated by X31 infection in our analysis of the human lung epithelium. Utilising flow cytometric techniques, Stanciu *et al.* (Stanciu et al., 2006) reported the upregulation of PD-L1 by H292, BEAS-2B and A549 epithelial cell lines in response to 24 h RSV infection. Further work by the group of Sebastian Johnston with BEAS-2B showed that RSV-induced upregulation of PD-L1 was associated with T cell inhibition (Telcian et al., 2011). Discrepancies between PD-1 expression may be due to

differential PD-L1 responses between epithelial cells in bronchial cell lines and epithelial cells in primary parenchyma. A secondary finding in a study by Erickson *et al.* (Erickson et al., 2012) was the requirement of PD-L1 to induce inhibition of T cell responses to influenza infection. This group performed RT-PCR analysis on human bronchial epithelial cells lines and lung tissue from influenza-infected mice. This suggests that PD-L1 expression may be upregulated after 24 h at the gene expression level, but may require more time for that to be translated into cell surface expression.

Using the explant model of acute Influenza A infection it was demonstrated that PD-1 was upregulated by CD4+ and CD8+ T cells in both control and COPD tissues (Figure 5.5). This is insufficient evidence to suggest that these cells display an exhausted phenotype, but it does indicate T cell activation in response to X31 treatment. Models of LCMV infection have established the dynamics of PD-1 expression in response to acute and chronic viral infection in mice. Responses to acute and chronic strains of LCMV are similar until approximately 7-8 d post-infection (Barber et al., 2006; Wherry et al., 2007; Youngblood et al., 2011). Erickson *et al.* (Erickson et al., 2012) investigated T cell responses in the acute setting using a murine model of RSV and Influenza infection. PD-1 was upregulated as with LCMV models, with virus-specific cells showing the greatest levels of expression. McNally *et al.* (McNally et al., 2013) also recorded PD-1 expression by CD4+ and CD8+ T cells in response to acute Influenza infection in a murine model. As our experiments were harvested at 24 h, conclusions regarding chronic infection cannot be made. However, acute infection in the human *ex vivo* model appears to yield similar results to those of the previously reported *in vivo* murine model (McNally et al., 2013).

Work performed in Chapter 4 indicated that there was a trend for an increased proportion of CD4+ T cells to express PD-1 in the lungs of stable COPD patients compared to controls. Comparison of non-infected samples in this chapter did not reveal a similar trend in the measurement of PD-1 sMFI on CD4+ T cells. This may be due to sampling differences as tissue analysed for PD-1 expression in Chapter 4 was not used for infection work performed in Chapter 5. Furthermore analysis of the proportion of PD-1 expressing cells and the expression of PD-1 on a per cell basis (sMFI) may not be interchangeable for the PD-1 marker. Analysis of antigen-specific modulation of PD-1 expression is required, as this may be lost when analysing the total CD4+ or CD8+ T cell population.

PD-1 upregulation by CD8+ T cells in models of chronic infection is expected due to their well characterised anti-viral (Bivas-Benita et al., 2013; Cox et al., 2013) and anti-tumour properties (van der Bruggen et al., 1991; Wolfel et al., 1995). Whether CD4+ T cells can exhibit an exhausted phenotype, and whether their progressive loss of function is similar to CD8+ T cells, is currently unknown. In the explant model PD-1 was upregulated by both CD4+ and CD8+ T cells in response to X31 infection. It has been established that CD4+ T helper cells can promote CD8+ T cell responses during viral infections (Cardin et al., 1996; Matloubian et al., 1994). Therefore T cell exhaustion in CD4+ T cells may prevent promotion of CD8+ responses, even if CD8+ T cells were fully functional.

As it appears that PD-1 and PD-L1 were upregulated during infection, it was perhaps surprising to find no expression of TIM-3 by cells from the lung parenchyma. Jin *et al.* (Jin et al., 2010) addressed the importance of TIM-3 expression in a murine model of LCMV. While T cells expressing PD-1 had displayed inhibited effector functions, cells co-expressing PD-1 and TIM-3 were most affected. Intriguingly blocking TIM-3 did not restore T cell function which was shown by PD-L1 blocking antibodies. As T cells exhaustion is a progressive loss of function, TIM-3 may only be expressed at the least functional "end-stage" of exhaustion. What can be concluded from the lung TIM-3 data is that there is no evidence of TIM-3 expression prior to viral challenge, and TIM-3 does not appear to contribute to T cell dysfunction in COPD. Furthermore the acute explant model may not be sufficient to identify T cells at their most exhausted state.

The release of granzyme B and perforin is utilised by CD8+ T cells to induce apoptosis of virally-infected cells (Peters et al., 1991). Intercellular staining of these proteins was not performed due to variable levels of Granzyme B detection in non-stimulated samples (Chapter 4), but CD107a can be used as a surrogate marker for T cell cytotoxic degranulation (Peters et al., 1991). CD107a is a lysosomal membrane protein which is revealed on the surface of degranulated cells, implicating CD107a-expressing cells in secretion of perforin and granzyme B. Antibodies used to identify CD107a expression were added to cell samples after X31 infection protocol and 22h incubation had been completed. Expression of CD107a is dynamic during T cell stimulation, and may be intracellularily recycled from the cell surface. Thus the levels of CD107a detected on T cells is representative of the end stage of the infection which may differ from those taken at earlier time points. CD107a was upregulated by CD4+ and CD8+ T cells in controls but not COPD samples in response to

influenza infection. An inability to produce cytotoxic proteins in response to viral infection infers a susceptibility to infection and potentially a failure to clear the pathogen. Loss of cytotoxic function is a characteristic of T cell exhaustion, thus further emphasising that upregulation of PD-1 alone may not indicate T cell exhaustion, but in combination with impaired CD107a responses it can suggest impaired T cell function. Cytotoxic responses are predominantly associated with CD8 T cell and NK cells, but in this model CD107a was also upregulated by CD4 T cells. This finding adds to the growing literature of cytotoxic CD4+ T cells in viral infection (Fang et al., 2012; Jellison et al., 2005), including influenza (Wilkinson et al., 2012). It is unclear as to whether cytotoxic CD4+ T cells are a unique subset of T cells, or whether their killing ability is induced during an impaired CD8+ response.

It was intended that investigation of the Tmem population in the explant model would identify anti-viral responses by long-lived T cells which have already encountered respiratory pathogens. As COPD individuals may suffer recurrent or prolonged infections, it was the Tmem compartment which would be most likely to express dysfunctional characteristics in response to influenza challenge. Two distinct functional populations were identified in the CD8+ T cell subset, TEM and TEMRA cells. The role of these cells was elucidated by differential expression of CD57, CD107a and PD-1 (illustrated in Table 5.3). The lack of CD107a upregulation combined with upregulated CD57 by TEMRA cells in COPD tissue would suggest an anergic phenotype of this cell population (Table 4.9). The failure to upregulate PD-1 implies that this inhibition or dysfunction is independent of the classical T cell exhausted phenotype. Therefore there may be regulatory mechanisms in the COPD lung which inhibit the cytotoxic capabilities of TEMRA cells, but this has not been identified in our work. It appears that the terminally differentiated TEMRA population in COPD show signs of senescence and inhibited functionality. It is yet to be elucidated as to whether these cells fully display an exhausted or anergic phenotype, or to the extent they can protect the host in an *in vivo* context.

	PD-1	CD57	CD107a
TEM Control	Upregulated	Upregulated	Upregulated
TEM COPD	Upregulated	Upregulated	Trend towards upregulation
TEMRA Control	Upregulated	Unchanged	Upregulated
TEMRA COPD	Unchanged	Trend for upregulation	Unchanged

Table 5.3. Expression of key cell markers by memory CD8 T cells in response to X31 infection of tissue.

In summary, work performed in Chapter 5 identified epithelial cells and macrophages as targets for viral infection in the human lung explant. Macrophages upregulate PD-L1 in response to infection, although there is a trend for reduced expression of PD-L1 in COPD tissue. Epithelial cells do not appear to modulate PD-L1 expression in response to infection, but this may be due to the relatively short incubation time (24 h). Therefore macrophages may be the key regulatory cell during acute influenza infection. CD4+ and CD8+ T cells from tissue upregulated PD-1 in response to infection but did not express TIM-3 suggesting these cells do not express an exhausted phenotype. CD107a expression, however, was upregulated by CD4+ T cells in control and COPD, but only in controls was CD107a upregulated by CD8+ T cells. This implies CD8+ T cells from COPD lung tissue have functionally impaired cytotoxic capabilities. CD8+ TEM and TEMRA appeared to have different functional profiles through characterisation of CD107a and CD57 expression. This work addressed inflammatory cell responses to influenza infection of lung parenchyma. Further work to explore mechanisms of T cell-macrophage interactions can be investigated using an *in vitro* model of infection using blood-derived cells, in which cell surface marker and IFNγ responses can be measured in response to X31 challenge.

Chapter 6

Analysis of Control of Human Functional T cell Responses During Experimental Influenza Infection

Introduction

Chapter 5 detailed the responses of T cells and macrophages to influenza infection in the lung. A key finding of this work was the upregulation of CD107a by CD8+ T cells during acute infection, demonstrating a functional anti-viral immune response in healthy controls, but this was lacking in COPD patients.

To extend our study to investigate the role of T cells in response to acute viral infection, assays to measure T cell cytokine production were utilised. ELISpot is a validated methodology to identify and quantify the frequency of cytokine-producing cells. This assay utilises monoclonal antibodies to identify cells producing a pre-determined cytokine (such as IFNγ). Cytokine producing cells are identified by coloured spots developing upon a membrane which are then counted. If the number of cells present in the well is known, the number of spots formed can be represented as number of spot forming colonies (SFCs)/1 x 10^6 cells. Furthermore ELISpot is a flexible system in which supplements can be added to wells to measure the promotion or inhibition of cytokine production.

A limitation of the lung parenchyma infection model is the small yield of cells compared to blood models and culled murine models of infection. Work discussed in Chapter 2 emphasised that cell marker preservation was of paramount importance in this study, even if this resulted in potential lower cell yields. Each well of an ELISpot requires a minimum recommended number of 5 x 10^4 effector cells. Due to the number of variables wishing to be tested, it was not practical to continue using cells derived from lung tissue. The mass of tissue required for such investigations would exceed what was obtained from surgery and factoring in the cost of antibodies required, time to process tissue and sort cells using FACS necessitated another source of cells to be sought.

Work was therefore continued and expanded to understand control of T cell responses to viral infection that would help inform interpretation of our earlier data by utilising an *in vitro* model of infection of monocyte-derived macrophages (MDM) and autologous T cells derived from blood. Blood was obtained from healthy volunteers, and thus comparisons between T cell responses between health and COPD were not performed. In this chapter, IFNγ production by T cells was measured by ELISpot in response to co-culture with Influenza A-infected MDMs. IFNγ responses were also measured when cells were treated with fluticasone

propionate, retinoic acid or αPD-L1 blocking antibody. T cells were also analysed for expression of PD-1, TIM-3, CD107a and NKG2D in response to these treatments.

MDMs are infected by X31 Influenza A virus

The differentiation of blood monocytes to MDMs has previously been validated as a model of lung macrophages (Tudhope et al., 2008; Young et al., 1990) and has been utilised by the research group (Staples et al., 2012). Dr. Staples previously constructed a protocol for the infection of monocytes by influenza A virus to give similar levels of infection to that observed in tissue explants (K.J. Staples et al., 2014) (Figure 5.3).

To ensure that MDMs were infected, MDMs were exposed to X31 Influenza A virus for 2 h followed by washing. These cells were then cultured for a further 22 h before analysis by flow cytometry. Figure 6.1. shows the infection of MDMs by X31 influenza using a similar gating strategy to tissue macrophages. Approximately 55% of MDMs were infected using this protocol. PD-L1 expression was also measured on these infected MDMs (Figure 6.2). NI samples expressed a sMFI of 507, and this is upregulated to a sMFI of 1883 upon infection. Thus, like macrophages in lung parenchyma, MDMs are infected by X31 influenza A and respond to this infection by upregulating PD-L1 surface expression.

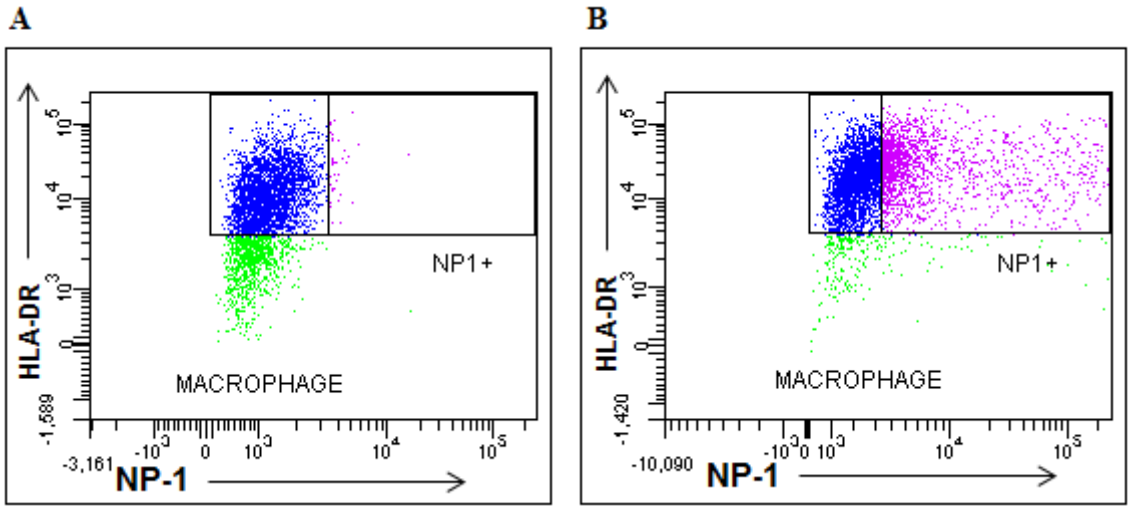

Figure 6.1. MDM infection by X31 Influenza A Virus. MDMs were treated with X31 or NI for 2 h before washing. MDMs were gated on the singlet cell population expressing CD45+ and HLA-DR+. Expression of NP-1 was used to determine MDM infection by X31. **(A)** Non-infected sample and **(B)** X31-treated sample. Blue dots represent uninfected MDMs, pink dots represent infected MDMs. Data representative of 2 experiments.

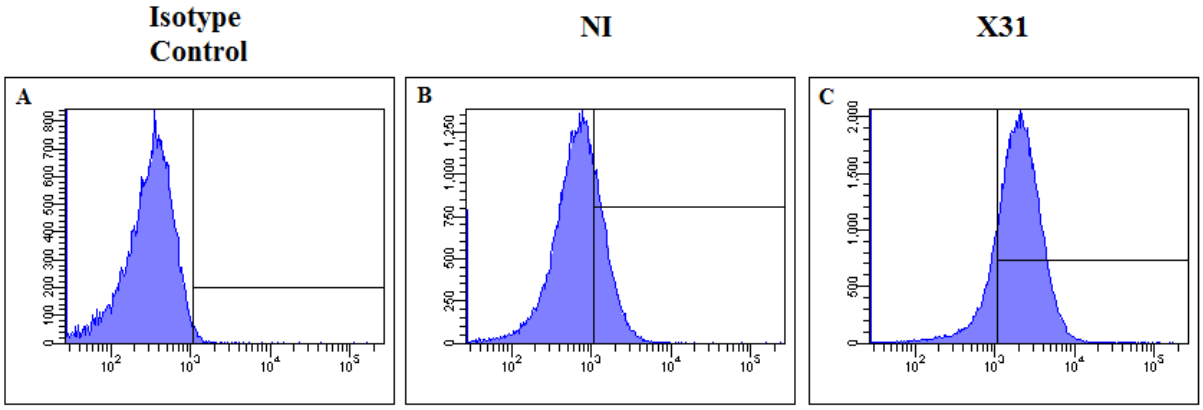

Figure 6.2. MDMs upregulate PD-L1 in response to X31 Influenza A infection. MDMs were treated with X31 or NI for 2 h before washing. **(A)** Isotype control antibody was added to measure background fluorescence. **(B)** Non-infected **(C)** or X31-treated MDMs were labelled with PE-labelled PD-L1 antibody.

Interaction between Influenza A infected MDMs and T cell induces IFNγ production

Experiments had been previously performed with PBMCs to quantify IFNγ production by intra-cellular staining (Chapter 3). There was difficulty in optimisation of this protocol, with undetectable levels of IFNγ expression recorded in CD4+ T cells. Therefore an ELISpot technique was employed to assess T cell IFNγ production.

In order to observe virus-specific IFNγ production rather than T cell activation due to HLA-mismatch, autologous lymphocytes were incubated with MDMs were infected by X31, in ELISpot assays. MDMs were incubated with X31 virus for two hours before washing and addition to an ELISpot plate prepared for detection of IFNγ. Autologous T cells were added to the ELISpot wells and cells were cultured for 18 h. The ELISpot protocol was then performed as described in Chapter 2 (Figure 2.1). For initial experiments 5 x 10^4 MDMs and 2.5 x 10^5 T cells were used per well to give a 1:5 Target:Effector ratio as recommended by the manufacturer as responder frequency was unknown and possibly low.

Figure 6.3 shows the number of SFCs detected by ELISpot in response to MDM and T cell co-culture after treatment of MDMs with UV-irradiated virus or live X31 virus. The number of T cells producing IFNγ is significantly greater in wells containing X31-infected MDMs (mean SFCs = 804.6) than non-infected MDMs (mean SFCs = 90, $p = 0.0086$). Live X31 also induces greater IFNγ production than UVX31 ($p = 0.0182$).

To ensure that infected MDMs were required for T cell secretion of IFNγ, experiments were performed in which monocyte-depleted PBMCs (lymphocytes) were treated with X31 in a similar manner to MDMs. IFNγ production under these conditions is shown in Figure 6.4 MDMs do not produce IFNγ in response to infection. Similarly T cells alone produce very low levels of IFNγ (mean sMFI = 80.56), and this is significantly lower than wells containing infected MDMs and autologous lymphocytes (mean sMFI = 788.3, p = 0.0368). Therefore live virus alone is not sufficient to induce IFNγ responses by T cells in the absence of MDMs. Work previously performed by the group indicated that MDMs are preferably infected by X31, and T cells do not appear to be targets of infection (Staples et al., 2009). Furthermore it appears that antigen presentation by MDMs is required for optimal T cell responses. The lymphocyte population comprises of T cells, but also B cells which can function as APCs (Avalos and Ploegh, 2014). In this model, however, it appears that no cell in the lymphocyte population could present antigen as there was no IFNγ release without the co-stimulation provided by MDMs Previous work by the group has shown than B cells are not infected by X31 influenza virus in a similar model, and this may be a reason for poor T cell stimulatory responses.

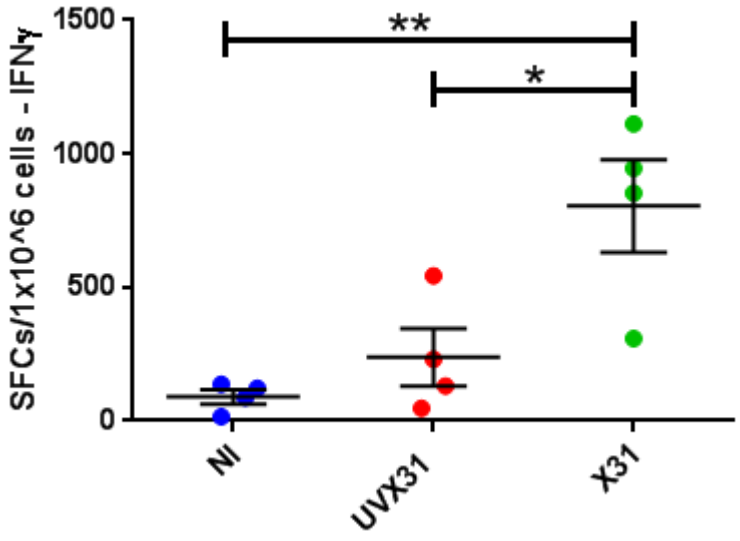

Figure 6.3. IFNγ production by T cells in response to infected MDMs. MDMs were treated with UVX31, X31 or NI for 2 h before washing. 5 x 10^4 MDMs were added to wells of an ELISPOT plate and were co-cultured with 2.5 x 10^5 lymphocytes. The ELISPOT plate was incubated at 37ºC for 18 h before analysis. n = 4. Mean and SEM shown. Paired students t test was performed. $p < 0.05$ (*), $p < 0.01$ (**).

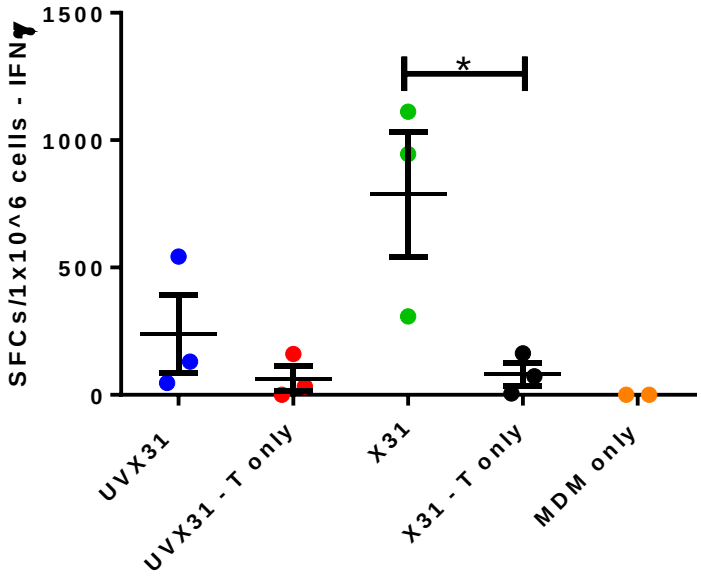

Figure 6.4. IFNγ production by T cells requires MDMs. MDMs or lymphocytes were treated with UVX31 or X31 for 2 h before washing. 5 x 10^4 MDMs were added to wells of an ELISPOT plate and were co-cultured with 2.5 x 10^5 lymphocytes. Wells not containing MDMs (T only), and wells containing MDMs only were also measured for their ability to produce IFNγ. The ELISPOT plate was incubated at 37ºC for 18 h before analysis. n = 3. Mean and SEM shown. Paired students t test was performed. $p < 0.05$ (*).

Initial ELISpot work was performed using 2.5 x 10^5 lymphocytes/well, but subsequent experiments recorded values which were beyond the limit of ELISpot detection (Figure 6.5). as the software was unable to give reproducible counts above 350 spots per well. If wells exceeded this number of spots, colonies overlapped and did not allow for accurate assignment and counting by the software. As IFNγ production is routinely reported as spot forming colonies (SFCs)/1x10^6 cells, the number of spots in each well was multiplied by 4 to give a final value in these preliminary experiments. Figure 6.5 B shows results from 3 experiments in which X31 infection recorded excessive values (these were represented as 1400 SFCs/1 x 10^6 cells). The numbers of lymphocytes was reduced to 1 x 10^5 cells/well, with the Target:Effector ratio reduced to 1:2 (Figure 6.6). As fewer cells were present within each well, the maximum SFC detection limit was increased from 1400 to 3500 SFC/1 x 10^6 cells (spots counted in wells were multiplied by 10 rather than 4). X31 infection of MDMs resulted in increased IFNγ production by T cells ($p = < 0.0001$) as seen in Figure 6.6. From a total of 11 experiments, of which samples were performed in triplicate, only 1 well exceeded the detection limit (recorded as 3500 SFC/1 x 10^6 cells). Therefore the concentration of 5 x 10^4 MDMs and 1 x 10^5 lymphocytes/T cells was used for all further experiments.

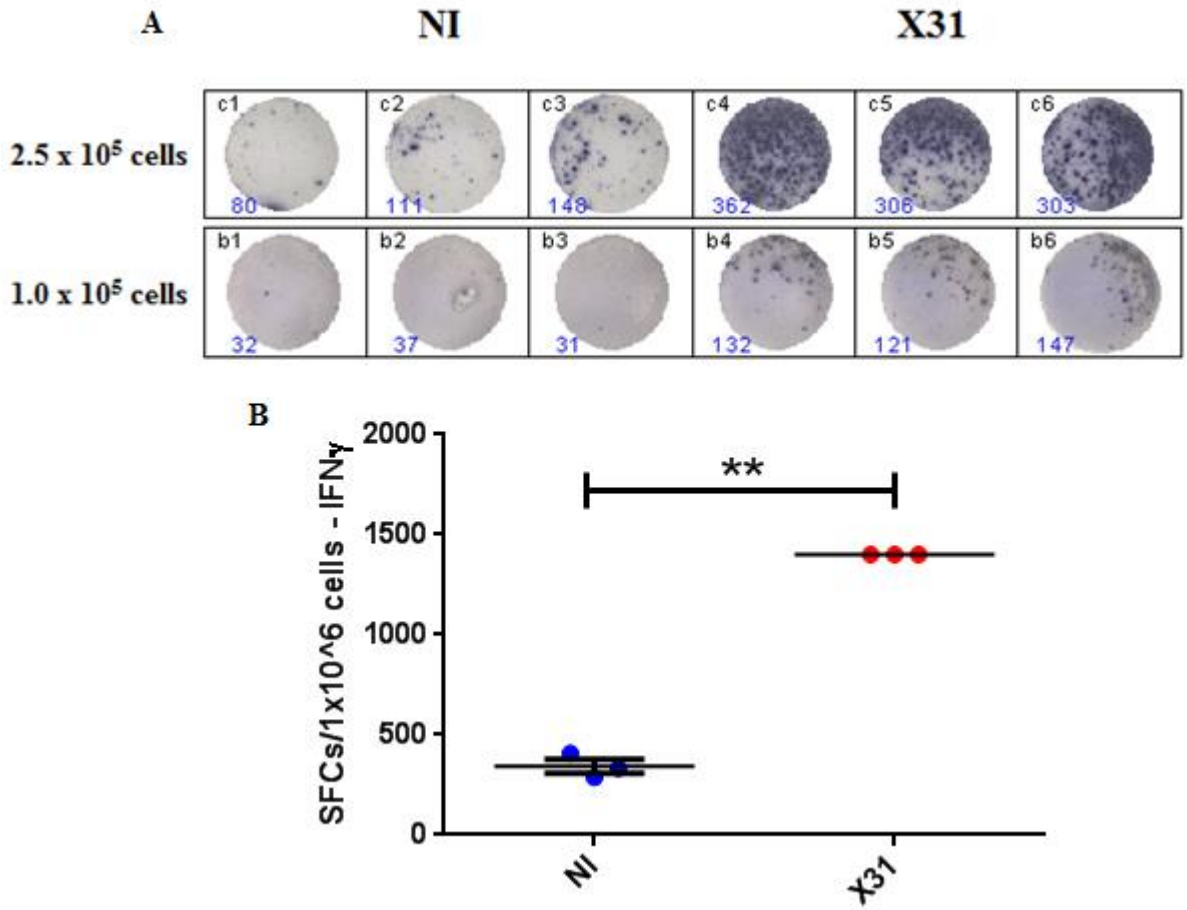

Figure 6.5. IFNγ production by T cells exceeded detection limit in response to infected MDMs. MDMs were treated with X31 or NI for 2 h before washing. 5 x 10^4 MDMs were added to wells of an ELISPOT plate and were co-cultured with 2.5 x 10^5 lymphocytes. The ELISPOT plate was incubated at 37ºC for 18 h before analysis. **(A)** Well of ELISpot plate under saturated and unsaturated conditions. Picture is representative of 3 experiments. **(B)** Number of SFCs/1 x 10^6 lymphocytes co-cultured with NI or X31-treated MDMs. Mean and SEM shown. n = 3. Paired students t test was performed. p = 0.0008 (**).

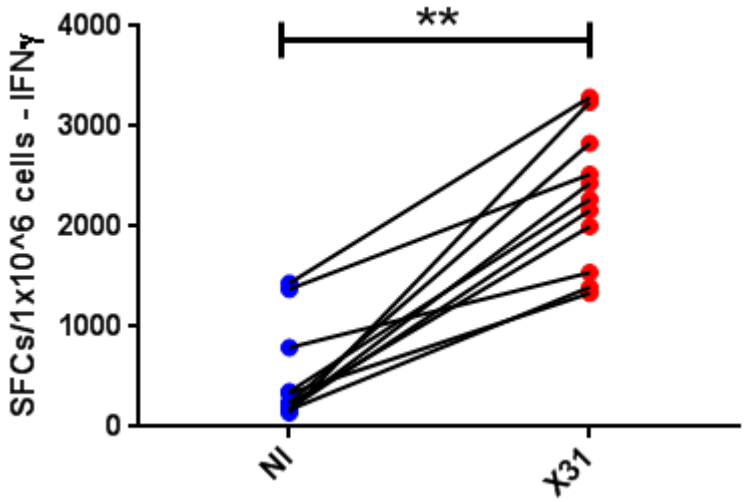

Figure 6.6 IFNγ production was upregulated by T cells in response to infected MDMs. MDMs were treated with X31 or NI for 2 h before washing. 5 x 10^4 MDMs were added to wells of an ELISPOT plate and were co-cultured with 1 x 10^5 lymphocytes. The ELISPOT plate was incubated at 37ºC for 18 h before analysis. n = 11. Mean and SEM shown. Paired students t test was performed. $p < 0.01$ (**).

IFNγ is produced by CD4 and CD8 T cells

IFNγ was produced by lymphocytes in the presence of infected MDMs, but these cells consist of a mixed T cell population (i.e. CD4+ and CD8+ cells), NK cells and other innate lymphoid cells which may also produce IFNγ. Therefore the lymphocyte population was purified to yield CD4+ and CD8+ T cells. 1 x 10^5 CD4 +or CD8+ T cells were added to 5 x 10^4 MDMs in a 1:2 Target:Effector ratio. Figure 6.7 shows that both CD4+ T cells ($p = 0.0114$) and CD8+ T cells ($p = 0.001$) significantly upregulate IFNγ production in response to X31-infected MDMs compared to NI MDMs. CD8+ T cells (mean sMFI = 1555) appear to be the dominant T cell subset for IFNγ production compared to CD4+ T cells (mean sMFI = 899.3, $p = 0.0037$).

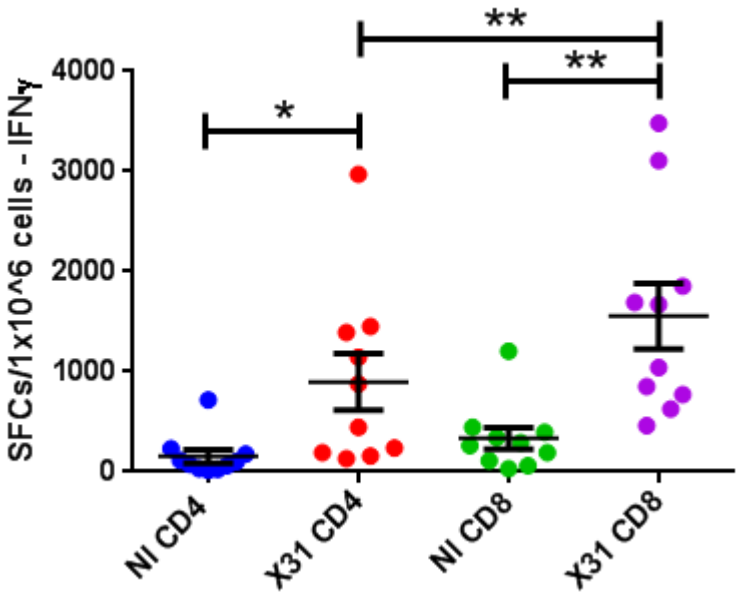

Figure 6.7 IFNγ production was upregulated by CD4 and CD8 T cells in response to infected MDMs. MDMs were treated with X31 or NI for 2 h before washing. 5 x 10^4 MDMs were added to wells of an ELISPOT plate and were co-cultured with 1 x 10^5 CD4+ or CD8+ T cells. The ELISPOT plate was incubated at 37ºC for 18 h before analysis. n = 10. Mean and SEM shown. Paired students t test was performed. $p < 0.05$ (*), $p < 0.01$ (**).

The production of IFNγ as detected by ELISpot was combined with flow cytometic analysis of PD-1 expression using the *in vitro* model (Figure 6.8). MDMs were separated after 2 h infection. These cells were either used in the previously described ELISpot experiment, or they were co-cultured with autologous T cells for 18 h before labelling with antibody and analysed by flow cytometry. Figure 6.9 illustrates PD-1 expression by T cells co-cultured with non-infected or X31 infected MDMs. Only one CD4+ sample showed increased PD-1 expression in response X31, with the remaining 3 samples recording unchanged expression (Figure 6.10A). All CD8+ samples showed increased PD-1 expression upon co-culture with X31 infected MDMs (Figure 6.10B), but this did not achieve statistical significance ($p = 0.119$).

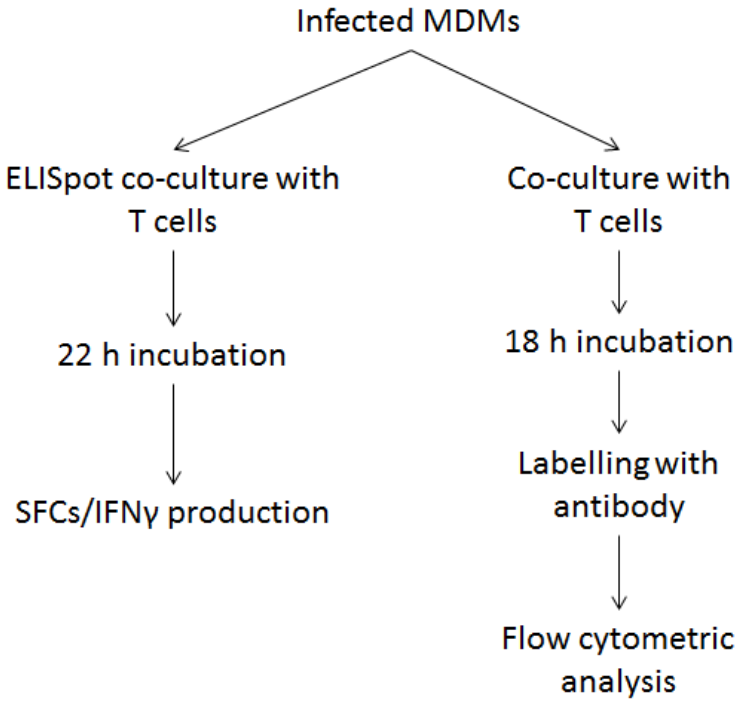

Figure 6.8. Combination of ELISpot and flow cytometry. MDMs were infected as previously described. A proportion of these cells were used in ELISpot experiments to quantify IFNγ responses by T cells. Remaining MDMs were used in flow cytometic experiments in which key cell surface markers on T cells were analysed.

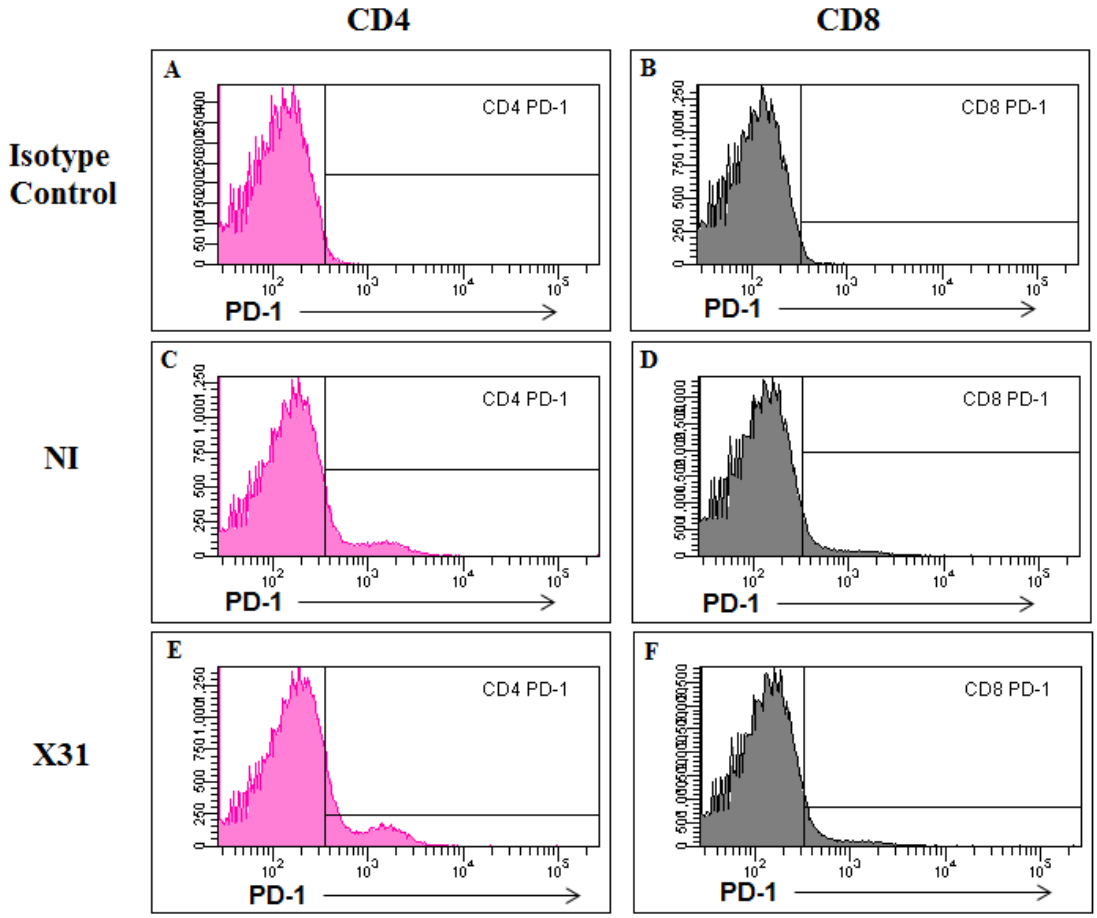

Figure 6.9 PD-1 expression by T cells co-cultured with MDMs. After co-culture with MDMs, PD-1 expression by T cells was measured. **(A)** Isotype control was added to measure background fluorescence for CD4 and **(B)** CD8 T cells. **(C)** PD-1 expression by CD4 **(D)** and CD8 T cells cultured with non-infected MDMs. **(E)** PD-1 expression by CD4 **(F)** and CD8 T cells cultured with X31 infected MDMs. Data representative of 4 experiments.

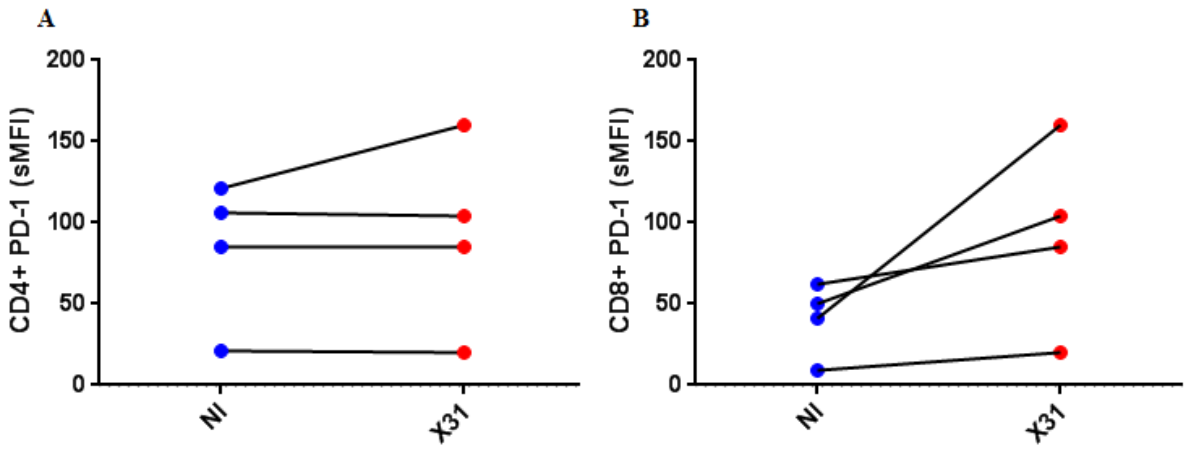

Figure 6.10 PD-1 expression by T cells in response to infected MDMs. MDMs were treated with X31 or NI for 2 h before washing. 5 x 10^4 MDMs were co-cultured with 1 x 10^5 lymphocytes at 37ºC for 18 h. **(A)** sMFI of CD4+ **(B)** and CD8+ T cells expressing PD-1 was quantified by flow cytometry. n = 4. Paired students t test was performed.

The expression of CD107a was also measured on T cells after co-culture with MDMs. This expression is illustrated in Figure 6.11. CD107a appears to be upregulated by CD4+ (Figure 6.12A) and to a greater extent CD8+ T cells (Figure 6.12B) in response to X31-infected MDMs. Upregulation is not significant, possibly due to low sample numbers and one sample which did not express any detectable CD107a in either cell phenotype. It is currently unknown if the lack of CD107a expression was reflective of the patient phenotype or whether there was an error in sample processing.

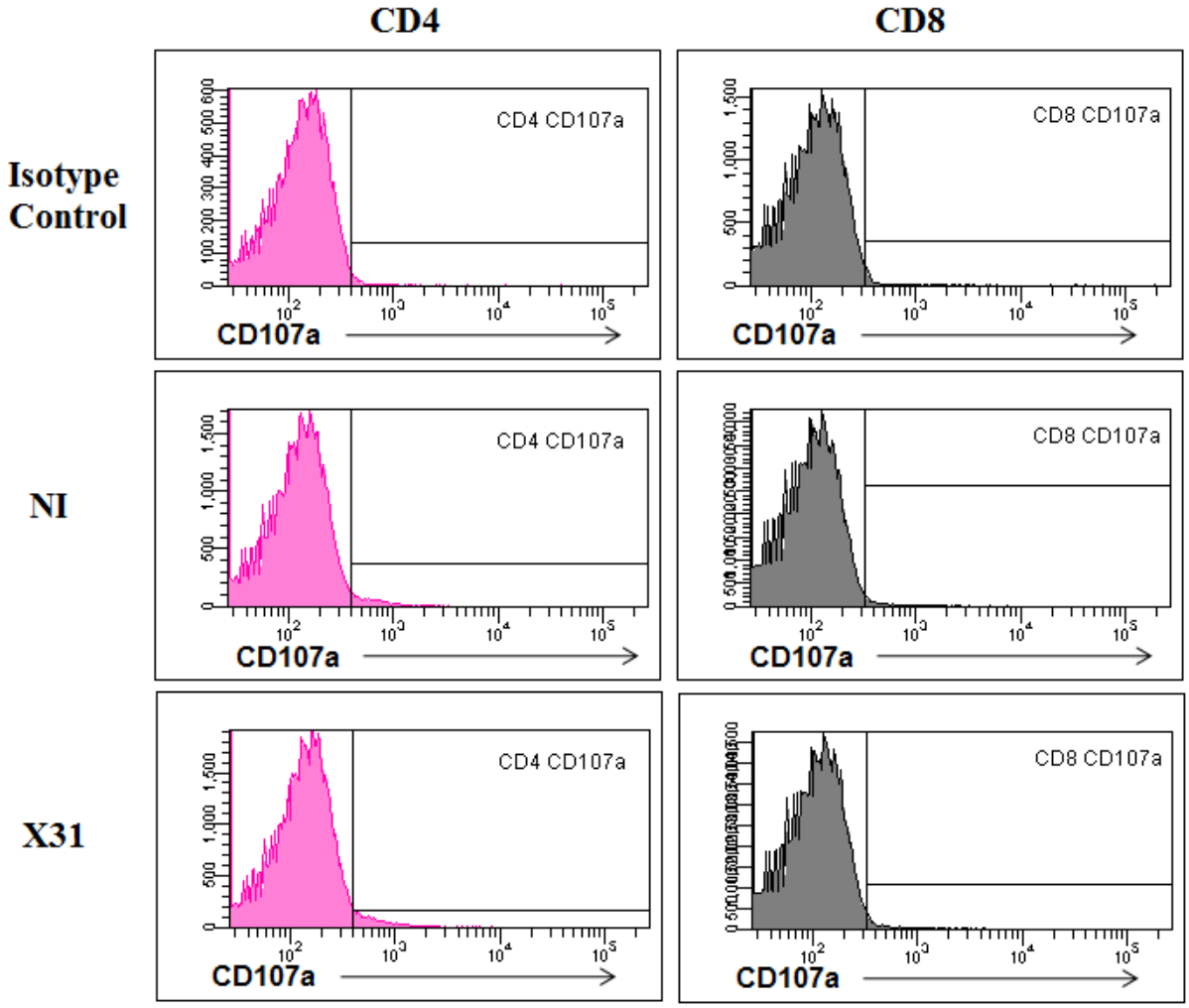

Figure 6.11 CD107a expression by T cells co-cultured with MDMs. After co-culture with MDMs, CD107a expression by T cells was measured. **(A)** Isotype control was added to measure background fluorescence for CD4 and **(B)** CD8 T cells. **(C)** CD107a expression by CD4 **(D)** and CD8 T cells cultured with non-infected MDMs. **(E)** CD107a expression by CD4 **(F)** and CD8 T cells cultured with X31 infected MDMs. Data representative of 3 experiments.

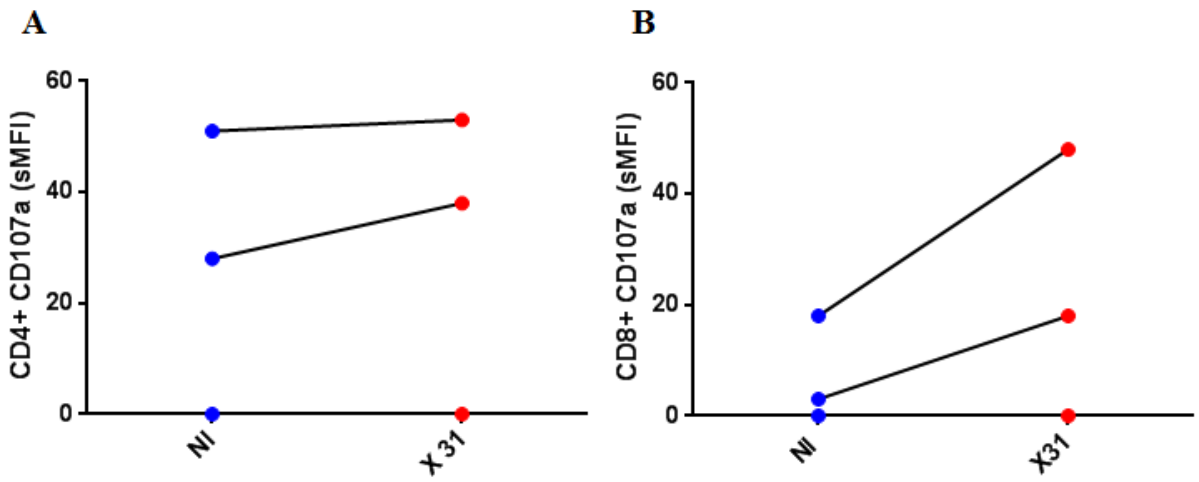

Figure 6.12. CD107a expression by T cells in response to infected MDMs. MDMs were treated with X31 or NI for 2 h before washing. 5 x 10^4 MDMs were co-cultured with 1 x 10^5 lymphocytes at 37ºC for 18 h. **(A)** sMFI of CD4+ **(B)** and CD8+ T cells expressing CD107a was quantified by flow cytometry. n = 3.

As with work in lung parenchyma (Chapter 5), the expression of TIM-3 was analysed by flow cytometry. TIM-3 was also not expressed at a detectable level in this blood model of acute influenza infection (Figure 6.13). In addition to PD-1, CD107a and TIM-3, NKG2D expression was measured (Figure 6.14). NKG2D is a receptor expressed in some innate-like cells and T cells which can detect virally-infected cells or tumour cells (Lanier, 2008). Identification of NKG2D expression within the T cell population would indicate that IFNγ production was due to the cytokine environment or TLR ligation (bystander activation) (Sckisel et al., 2014) rather than TCR-dependant T cell activation. In these experiments, however, NKG2D was not detected and thus production of IFNγ was associated with CD4+ and CD8+ T cells TCR-dependant responses.

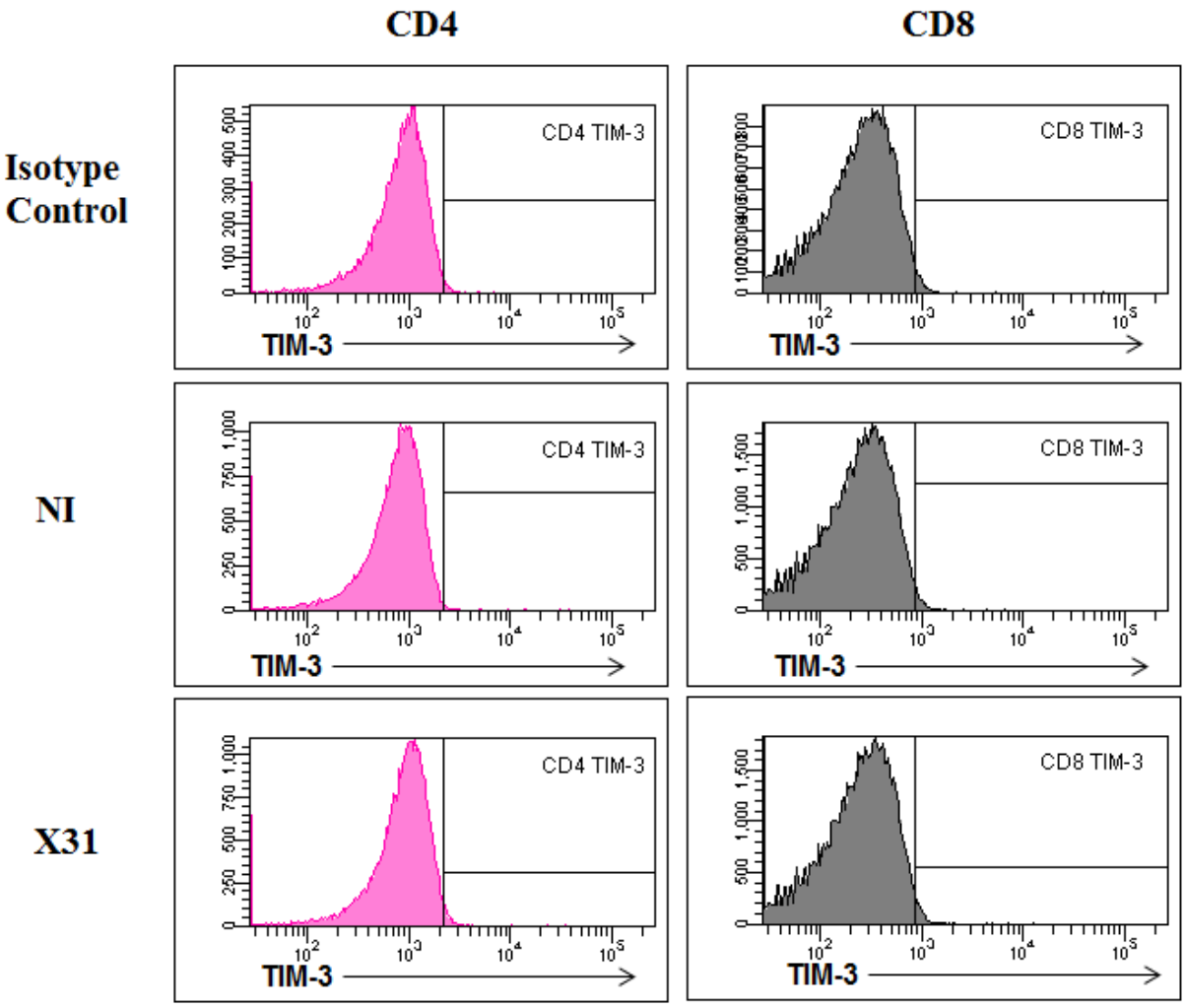

Figure 6.13 TIM-3 expression by T cells co-cultured with MDMs. After co-culture with MDMs, TIM-3 expression by T cells was measured. **(A)** Isotype control was added to measure background fluorescence for CD4+ and **(B)** CD8+ T cells. **(C)** TIM-3 expression by CD4+ **(D)** and CD8+ T cells cultured with non-infected MDMs. **(E)** TIM-3 expression by CD4+ **(F)** and CD8+ T cells cultured with X31 infected MDMs. Data representative of 3 experiments.

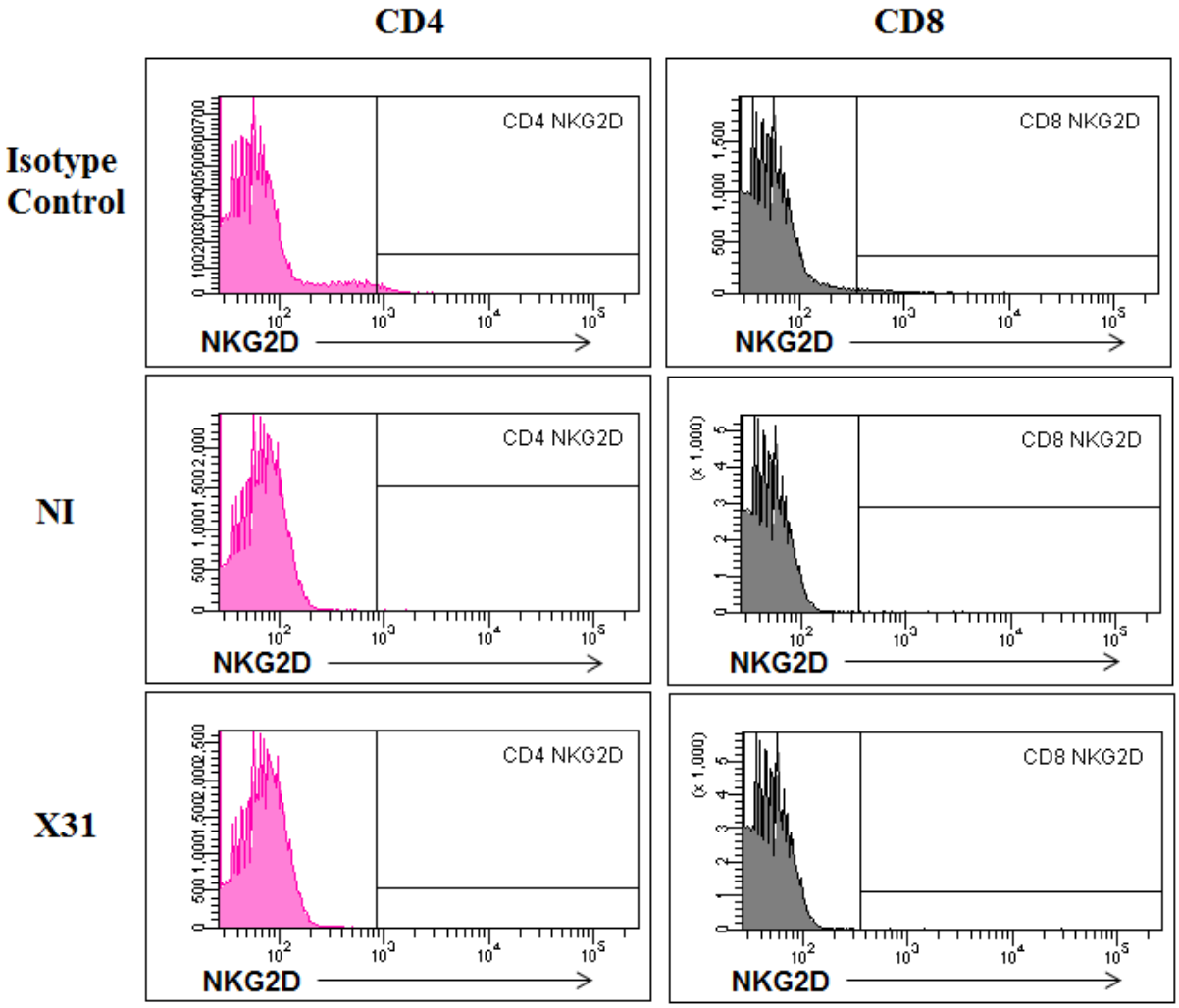

Figure 6.14 NKG2D expression by T cells co-cultured with MDMs. After co-culture with MDMs, NKG2D expression by T cells was measured. **(A)** Isotype control was added to measure background fluorescence for CD4 **(B)** and CD8+ T cells. **(C)** NKG2D expression by CD4+ **(D)** and CD8+ T cells cultured with non-infected MDMs. **(E)** NKG2D expression by CD4+ **(F)** and CD8+ T cells cultured with X31 infected MDMs. Data representative of 3 experiments.

Fluticasone inhibits T cell IFNγ production

Corticosteroids are commonly used in the treatment of COPD despite the inability of these drugs to slow disease progression (Yang et al., 2012), to improve mortality rate (Calverley et al., 2007), and patient populations who are not responsive to treatment. Corticosteroids can reduce inflammation in the lung and may be beneficial during disease exacerbations (Woods et al., 2014), but this may also inhibit the immune response to respiratory pathogens. In order to ascertain whether the phenotypes we were observing in the COPD lung were an epiphenomenon caused by steroid use, the effects of the steroid fluticasone propionate on T cell responses to MDM infection were analysed.

ELISpot experiments were performed using the lymphocyte population in which fluticasone propionate was used as a steroid treatment MDM post-infection (Figure 6.15). In a similar result to Figure 6.6, X31 infected samples recorded more spot forming colonies than non-infected samples ($p = 0.0107$). Fluticasone-treated samples infected with X31 also upregulated T cell IFNγ production compared to non-infected samples ($p = 0.0178$). In infected samples, treatment with fluticasone (mean sMFI = 761.7 SFC) significantly reduced the number of spot forming colonies seen compared to non-treated cells (mean sMFI = 2448, $p = 0.0105$). To ensure that fluticasone was inhibiting IFNγ but not killing cells, experiments were performed to measure cell viability and MDM infection levels in fluticasone-treated samples (Table 6.1). Facilities for flow cytometry prohibited the use of live virally-infected cells, requiring cells to be fixed for analysis. Thus viability was only measured in uninfected samples. Treatment of MDMs and lymphocytes slightly reduced cell viability from 71.4% to 65.3%. The proportion of infected MDMs was also slightly reduced from 36.8% to 33.4% with fluticasone treatment. Although cell viability and infection of MDMs may be lowered in fluticasone-treated samples, it is unlikely to be sufficient to account for the significant decrease in IFNγ production seen in Figure 6.15. Representative ELISpot wells can be seen in Figure 6.15.

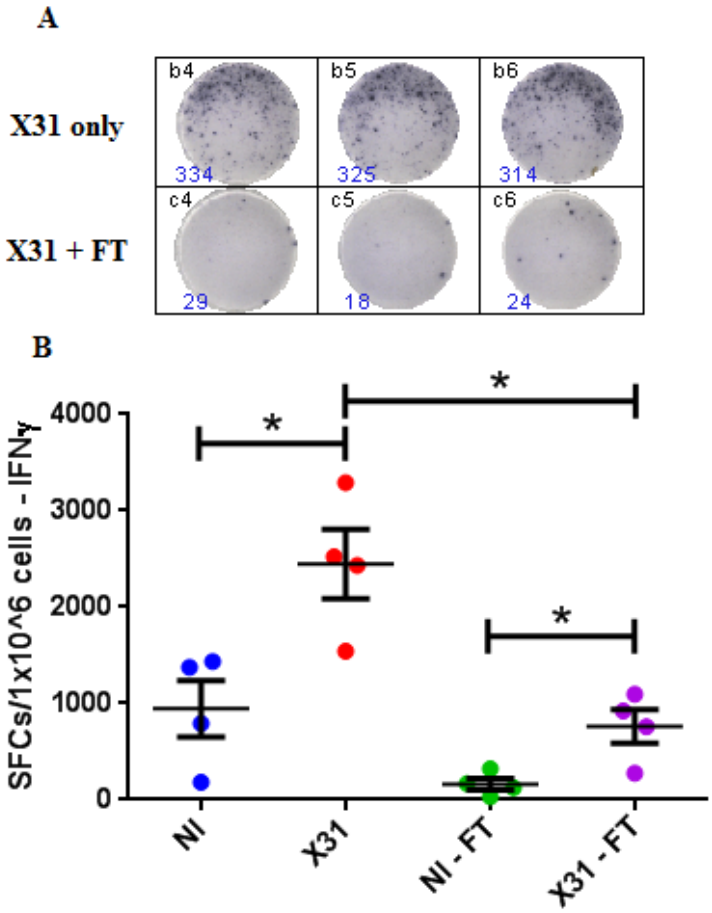

Figure 6.15 Fluticasone inhibits IFNγ production by T cells. MDMs were treated with X31 or NI for 2 h before washing. 5 x 10^4 MDMs were co-cultured with 1 x 10^5 lymphocytes with or without 100 nM fluticasone propionate (FT) at 37ºC for 18 h. **(A)** Wells of ELISpot plate with or without fluticasone. Picture is representative of 4 experiments. **(B)** Number of SFCs/1 x 10^6 lymphocytes co-cultured with NI or X31-treated MDMs. n=4. Mean and SEM shown. Paired students t tests were performed. $p < 0.05$ (*).

	NI	NI + FT	X31	X31 + FT	P value
Viability (%)	60.9 (12.2)	52.88 (11.79)	-	-	0.125
Infection (%)	-	-	31.58 (14.84)	28.26 (16.89)	0.188

Table 6.1 Fluticasone effects on cell viability and MDM infection. MDMs were culture and co-cultured with lymphocytes as previously described. Viability and infection of cells was measured by flow cytometry. Mean and SD shown. Viability n=4, Infection n=5. Wilcoxon signed rank test performed.

CD4+ and CD8+ T cells were again isolated from lymphocytes and co-cultured with infected MDMs and fluticasone (Figures 6.16). There were no differences in baseline IFNγ expression for either CD4+ (Figure 6.16A) or CD8+ T cells (Figure 6.16B), despite one sample

recording greater IFNγ production in samples without fluticasone treatment. Comparing infected samples, there was a trend (p = 0.0631) for CD4+ T cell IFNγ production to be inhibited with fluticasone treatment (Figure 6.16C). CD8+ T cell IFNγ production was significantly decreased (p = 0.0117) in response to fluticasone treatment (Figure 6.16D). Both CD4+ and CD8+ T cell IFNγ production is attenuated with fluticasone treatment, but CD8+ T cells appear to be more susceptible to inhibition by corticosteroids.

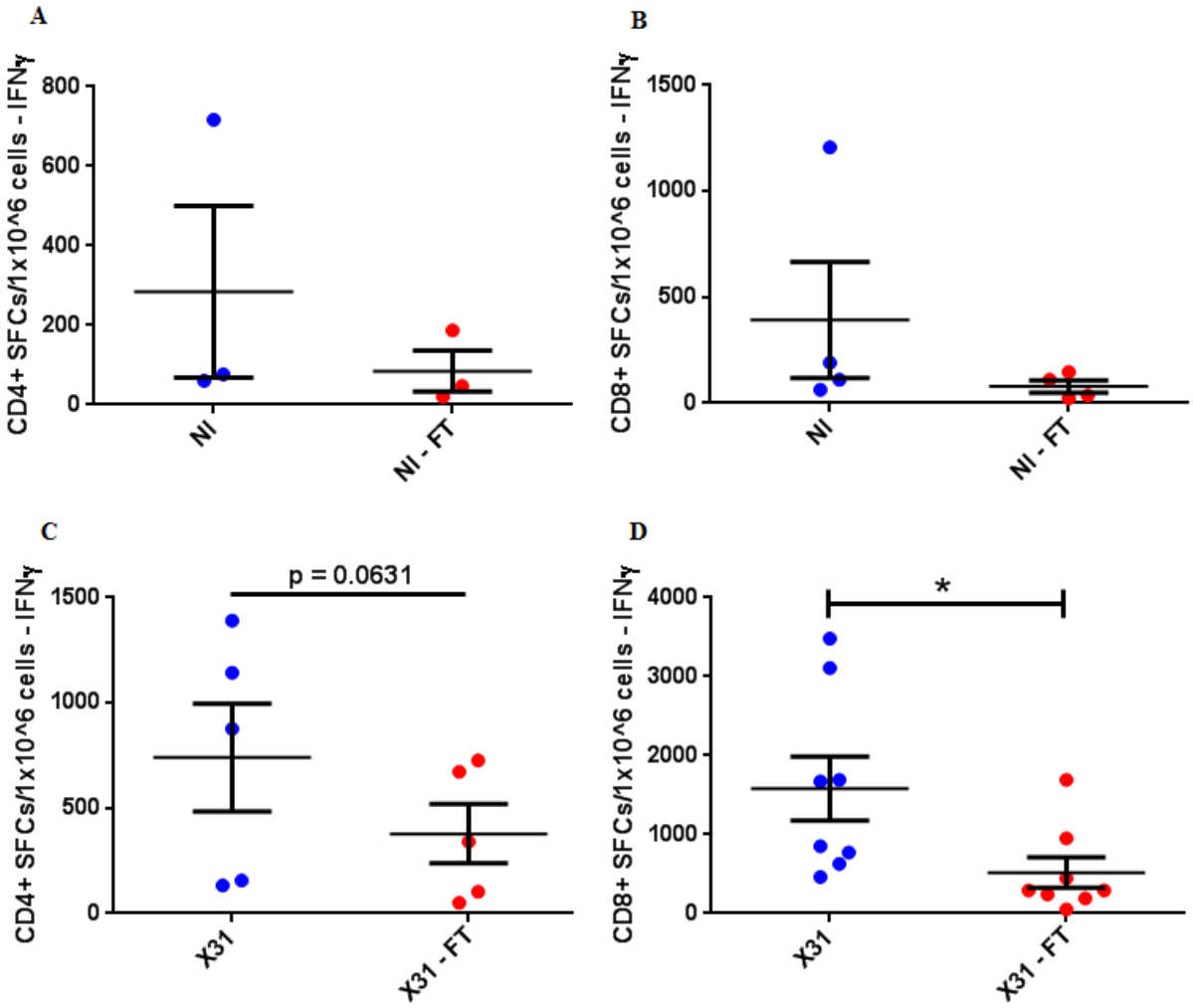

Figure 6.16 IFNγ production by CD4 and CD8 T cells treated with fluticasone. MDMs were treated with X31 **(C-D)** or NI **(A-B)** for 2 h before washing. 5 x 10^4 MDMs were co-cultured with **(A+C)** 1 x 10^5 CD4+ **(B+D)** or CD8+ T cells and **(C-D)** 100 nM fluticasone propionate at 37ºC for 18 h. **(A)** n = 3, **(B)** n = 4., **(C)** n = 5, **(D)** n=8 Mean and SEM shown. Paired students t tests were performed. $p < 0.05$ (*).

Measurement of PD-1 in CD4+ and CD8+ cells (Figure 6.17) did not reveal differences in expression during fluticasone treatment, suggesting corticosteroids do not have a direct role in promotion of T cell exhaustion. CD4+ T cells did not appear to have altered CD107a expression in response to fluticasone treatment (Figure 6.18A). Expression of CD107a on fluticasone-treated CD8+ T cells followed a similar pattern to untreated samples (Figure

6.18B). Although appearing to upregulate CD107a, CD8+ T cells that were fluticasone and X31-treated did not achieve statistical significance when compared to untreated samples (p = 0.0938). As with data shown in Figure 6.12, the inability to detect CD107a expression on one sample may conceal patterns of CD107a expression in response to either influenza virus or fluticasone.

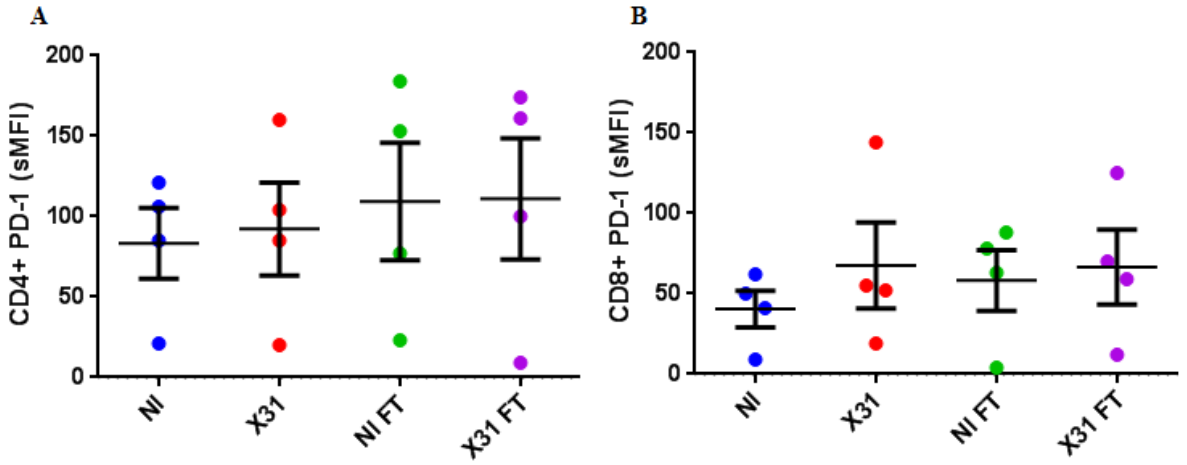

Figure 6.17 PD-1 expression by CD4 and CD8 T cells treated with fluticasone. MDMs were treated with X31 or NI for 2 h before washing. 5 x 10^4 MDMs were co-cultured with **(A)** 1 x 10^5 CD4+ **(B)** or CD8+ T cells with or without 100 nM fluticasone propionate at 37ºC for 18 h. **(A)** sMFI of CD4+ **(B)** and CD8+ T cells expressing PD-1 was quantified by flow cytometry. n=4. Mean and SEM shown. Paired students t tests were performed.

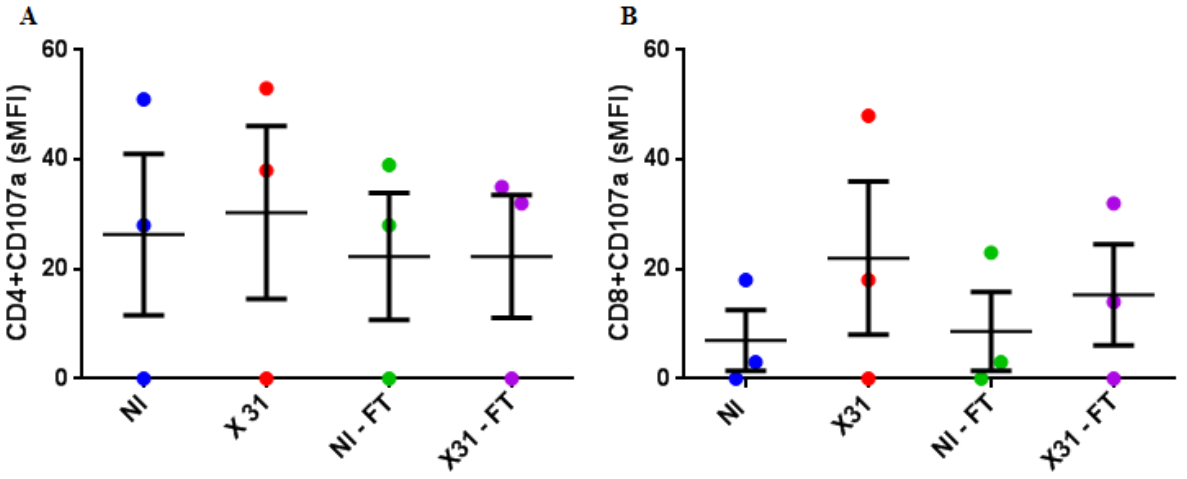

Figure 6.18. CD107a expression by CD4 and CD8 T cells treated with fluticasone. MDMs were treated with X31 or NI for 2 h before washing. 5 x 10^4 MDMs were co-cultured with **(A)** 1 x 10^5 CD4+ **(B)** or CD8+ T cells with or without 100 nM fluticasone propionate at 37ºC for 18 h. **(A)** sMFI of CD4+ **(B)** and CD8+ T cells expressing CD107a was quantified by flow cytometry. n=3. Mean and SEM shown. Paired students t tests were performed.

Retinoic acid attenuates T cell IFNγ production

COPD patients may be characterised with nutritional deficiencies which contribute to the progression of disease. Hall *et al* . (Hall et al., 2011a) have shown that in Vitamin A deficient mice, T cell IFNγ responses are significantly reduced. During *T. gondii* infection, retinoic acid treatment of mice recovered T cell potential to produce IFNγ and significantly increase parasite clearance. Conversely, retinoic acid appears to play a role in the generation and maintenance of inducible Treg cells (iTreg) which can maintain tolerance to non-harmful antigens at mucosal sites (Benson et al., 2007; Curotto de Lafaille et al., 2008; Hadis et al., 2011).

Experiments were performed to elucidate the ability of retinoic acid to promote or inhibit T cell function in the context of influenza infection (representative wells shown in Figure 6.19A). Figure 6.19B shows that addition of retinoic acid inhibits upregulation of IFNγ production in response to X31 treatment ($p = 0.1622$). When compared to X31 infection without RA (Figure 6.19.C), IFNγ producing T cells are significantly fewer in RA treated samples (mean sMFI= 1116, $p = 0.0173$) than X31 alone (mean sMFI = 2370).

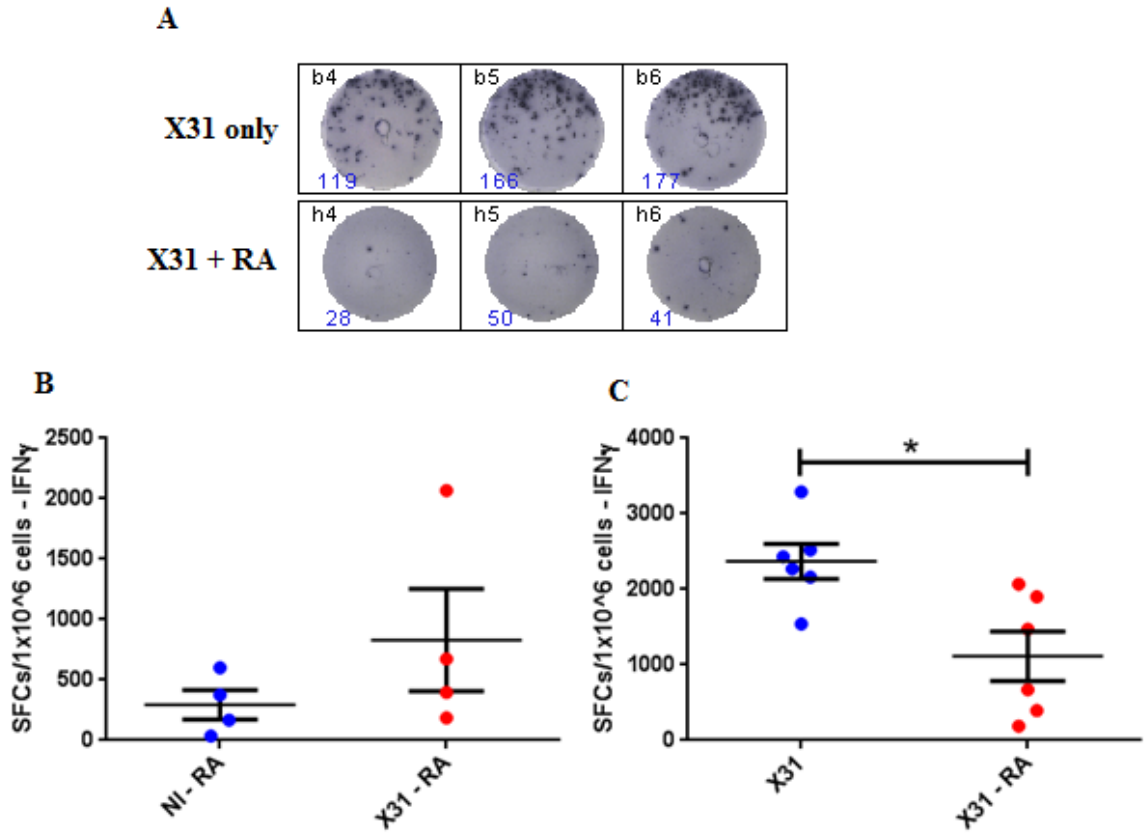

Figure 6.19. Retinoic acid inhibits IFNγ production by T cells. MDMs were treated with X31 or NI for 2 h before washing. 5 x 10^4 MDMs were co-cultured with 1 x 10^5 lymphocytes with or without 50 nM retinoic acid (RA) at 37ºC for 18 h. **(A)** Wells of ELISpot plate with or without 50 nM retinoic acid. Picture is representative of 6 experiments. **(B)** Number of SFCs/1 x 10^6 lymphocytes co-cultured with NI or X31-treated MDMs with or without 50 nM retinoic acid. n=4. **(C)** Number of SFCs/1 x 10^6 lymphocytes co-cultured with X31-treated MDMs with or without 50 nM retinoic acid. n = 6. Mean and SEM shown. Paired students t tests were performed. $p < 0.05$ (*).

The lymphocyte population was again separated to yield CD4+ and CD8+ T cell populations (Figure 6.20). There was no difference at baseline levels of IFNγ production by CD4+ T cells in response to retinoic acid treatment. Differences in means may be accounted for by one sample in which relatively a high number of IFNγ-producing cells were identified. Frequency of IFNγ-producing CD8+ T cells was similar between non-infected samples and retinoic acid treated samples. When comparing retinoic treated and non-treated infected samples (Figure 6.20), CD4+ T cells appeared to have reduced IFNγ production compare to non-treated counterparts but significance was not achieved ($p = 0.1373$). CD8+ T cells from retinoic acid treated samples expressed a mean SFC of 1049, while non-treated infected samples recorded a SFC mean of 1247. Therefore retinoic acid treatment of CD4 T+ cells resulted in a 40% loss of SFCs, while this loss was 16% in CD8+ T cells. Therefore retinoic acid may play a more significant role in CD4+ T cell IFNγ production compared to CD8+ T cells,

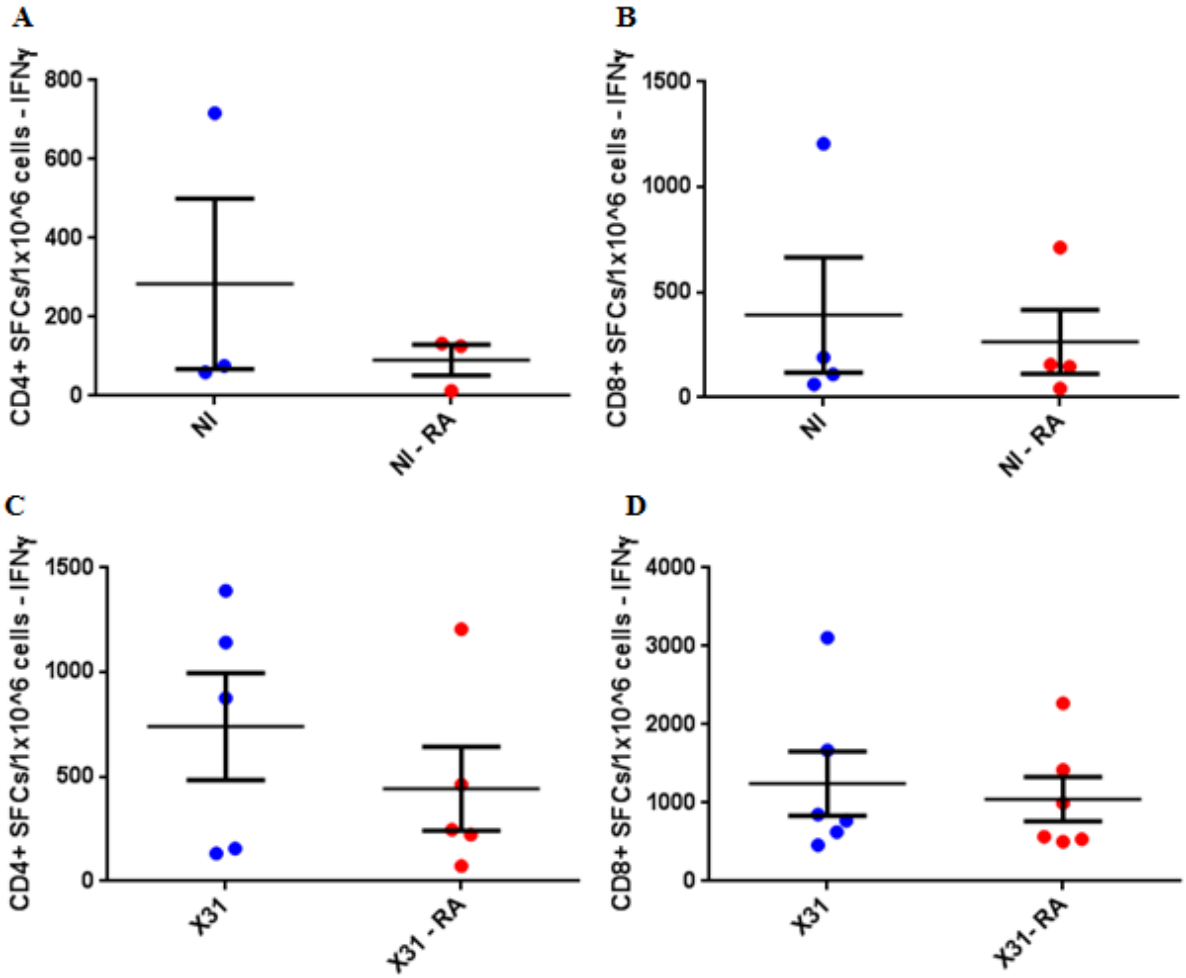

Figure 6.20. IFNγ production by CD4 and CD8 T cells treated with retinoic acid. MDMs were treated with X31 or NI for 2 h before washing. 5 x 10^4 MDMs were co-cultured with **(A)** 1 x 10^5 CD4+, **(B)** CD8+ T cells with or without 50 nM retinoic acid at 37ºC for 18 h. **(C)** X31-treated MDMs with 1 x 10^5 CD4+ or **(D)** CD8+ T cells supplemented with 50 nM retinoic acid. **(A)** n=3, **(B)** n=4. **(C)** n=5. **(D)** n=6. Mean and SEM shown. Paired students t tests were performed.

PD-1 expression by retinoic acid treated cells was unchanged in response to X31 infection of MDMs (Figure 6.21). PD-1 expression for two samples was increased in response to retinoic acid treatment independently of X31 infection. Figure 6.21 illustrates the upregulation of PD-1 expression by retinoic acid treatment alone. Thus retinoic acid may play a role in PD-1 expression, but the mechanism is currently unknown. Retinoic acid treatment also appeared to upregulate CD107a expression independently of X31 infection (Figure 6.23). Retinoic acid-treated CD4+ T cells did not significantly increase CD107a expression in non-infected samples ($p = 0.1647$) nor X31-treated samples ($p = 0.1563$). CD8+ T cells also did not achieve significance in response to retinoic acid (NI $p = 0.1597$, X31 $p = 0.1406$).

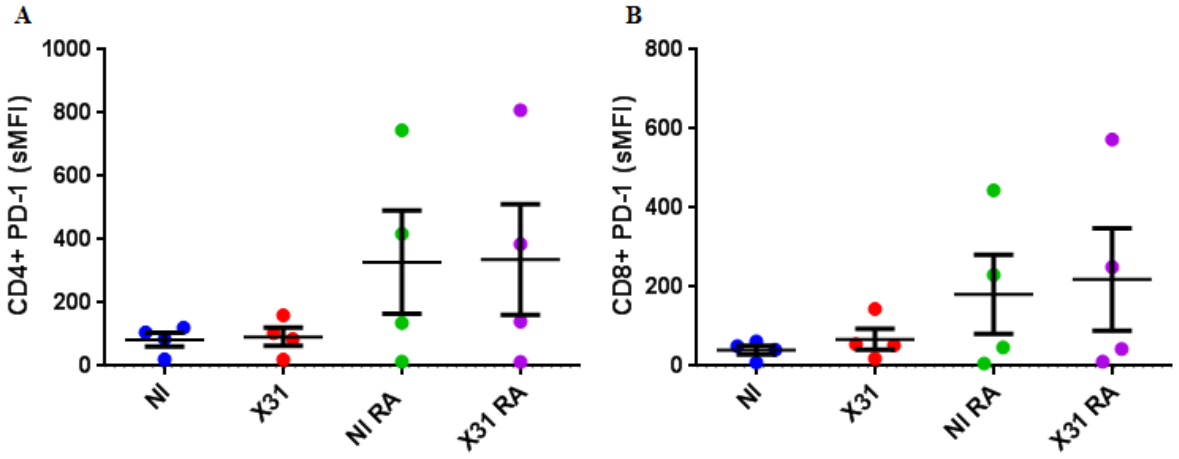

Figure 6.21 PD-1 expression by CD4 and CD8 T cells treated with retinoic acid. MDMs were treated with X31 or NI for 2 h before washing. 5 x 10^4 MDMs were co-cultured with **(A)** 1 x 10^5 CD4+ **(B)** or CD8+ T cells with and without 50 nM retinoic acid at 37ºC for 18 h. **(A)** sMFI of CD4+ **(B)** and CD8+ T cells expressing PD-1 was quantified by flow cytometry. n=4. Mean and SEM shown. Paired students t tests were performed.

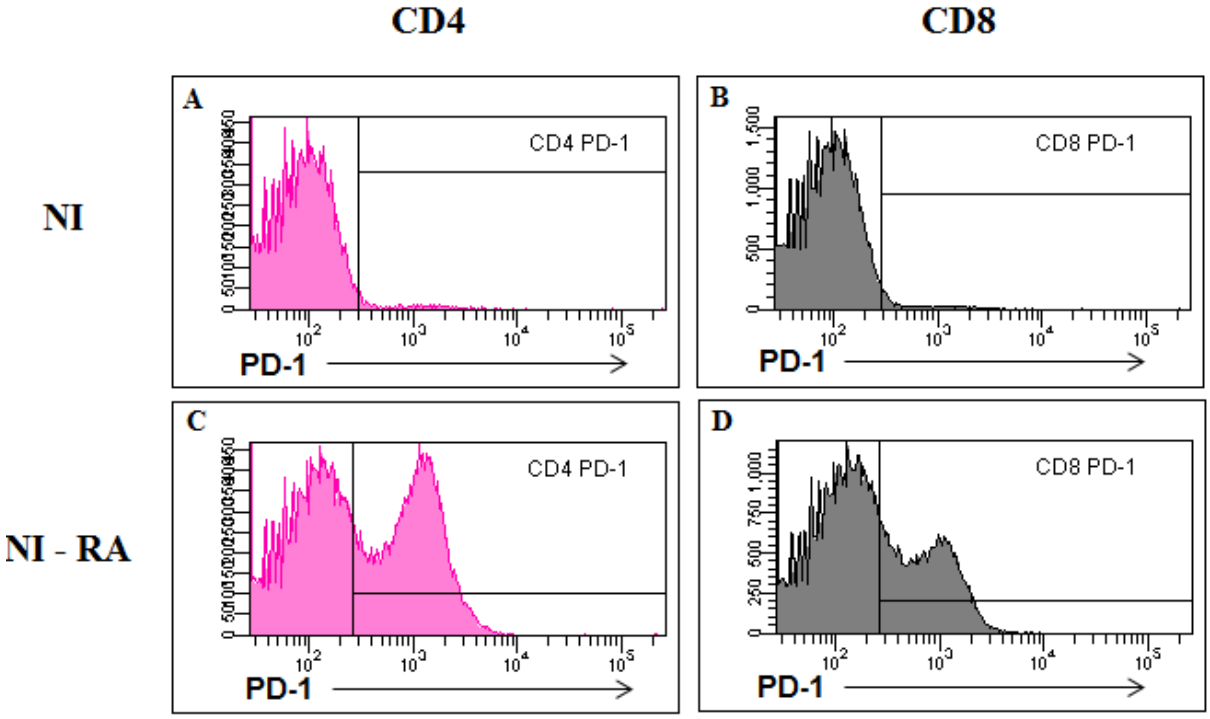

Figure 6.22. PD-1 expression by T cells co-cultured with MDMs. After co-culture with MDMs, PD-1 expression by T cells was measured. **(A)** PD-1 expression by CD4 **(B)** and CD8+ T cells cultured with non-infected MDMs. **(C)** PD-1 expression by CD4 **(D)** and CD8+ T cells cultured with non-infected MDMs and 50 nM retinoic acid.

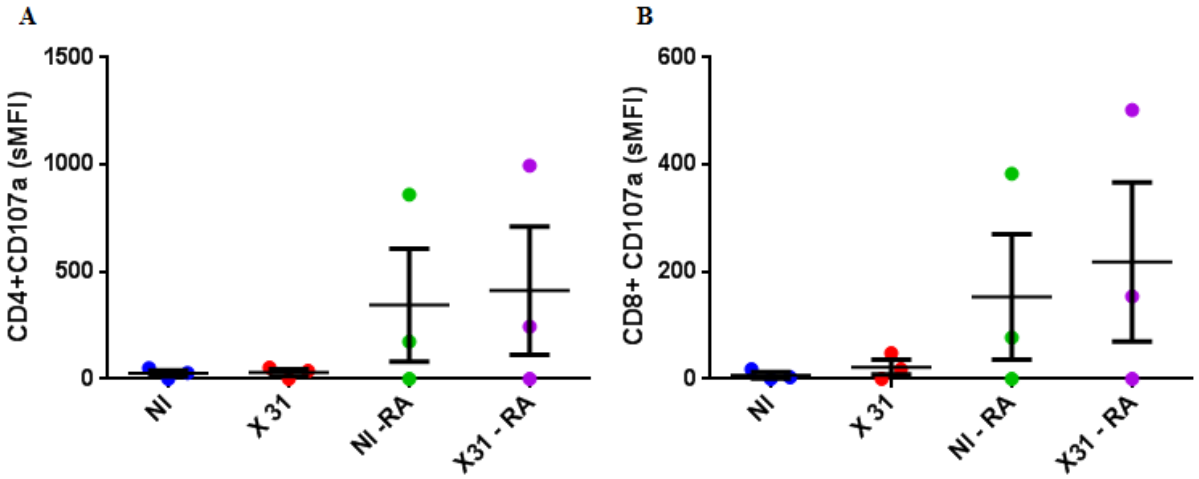

Figure 6.23. CD107a expression by CD4 and CD8 T cells treated with retinoic acid. MDMs were treated with X31 or NI for 2 h before washing. 5 x 10^4 MDMs were co-cultured with **(A)** 1 x 10^5 CD4+ **(B)** or CD8+ T cells with and without 50 nM retinoic acid at 37ºC for 18 h. **(A)** sMFI of CD4+ **(B)** and CD8+ T cells expressing CD107a was quantified by flow cytometry. n=3. Mean and SEM shown. Paired students t tests were performed.

Effects of PD-L1 blocking antibody on T cell IFNγ production

Blocking of PD-1:PD-L1 interactions has been shown to reverse T cell exhaustion in models of chronic viral infection (Barber et al., 2006). MDMs and lymphocytes were supplemented with anti-PD-L1 blocking antibody (αPD-L1) and the number of SFCs was measured (Figure 6.24). X31 infection combined with isotype antibody recorded a SFC mean of 2072, while αPD-L1 treatment recorded a SFC mean of 1966. These values were both lower than X31 infection alone in which the SFC mean was 2448. As there was no apparent effect of blocking PD-L1 for the entire lymphocyte population, experiments were performed with purified CD4+ and CD8+ T cells (Figure 6.25). Treatment with either isotype control antibody ($p = 0.0908$) or αPD-L1 ($p = 0.091$) trended towards decreased IFNγ production compared to X31 treatment alone, but this did not achieve significance. Treatment with αPD-L1 antibody did not significantly increase IFNγ production by CD4+ T cells compared to isotype antibody ($p = 0.1142$). There was a trend, however, for CD8+ T cells supplemented with treated αPD-L1 to record a greater IFNγ response than with isotype control antibody supplementation ($p = 0.0881$).

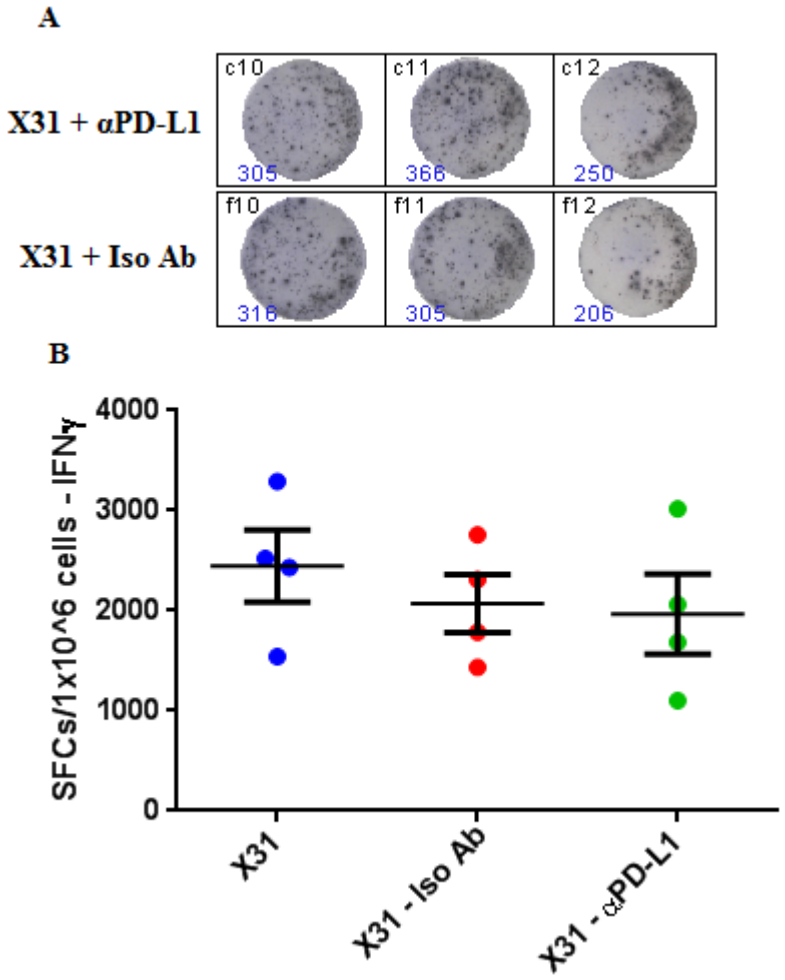

Figure 6.24. IFNγ production by T cells in response to blocking PD-L1. MDMs were treated with X31 or NI for 2 h before washing. 5 x 10^4 MDMs were co-cultured with 1 x 10^5 lymphocytes with isotype control antibody or αPD-L1 blocking antibody at 37ºC for 18 h. **(A)** Wells of ELISpot plate with isotype control antibody or αPD-L1 blocking antibody. Picture is representative of 4 experiments. **(B)** Number of SFCs/1 x 10^6 lymphocytes co-cultured with X31-treated MDMs with isotype control antibody or αPD-L1 blocking antibody. n = 4. Mean and SEM shown. Paired students t tests were performed.

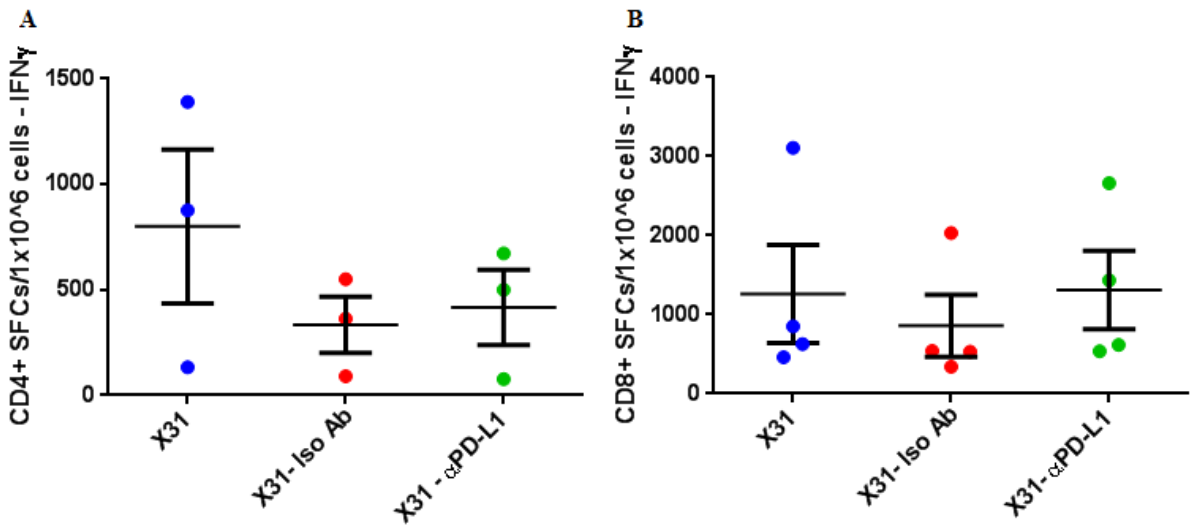

Figure 6.25. IFNγ production by CD4 and CD8 T cells is not increased in response to blocking PD-L1. MDMs were treated with X31 for 2 h before washing. **(A)** 5 x 10^4 MDMs were co-cultured with 1 x 10^5 CD4+ or **(B)** CD8+ T cells with isotype control antibody or αPD-L1 blocking antibody at 37ºC for 18 h. **(A)** n = 3, **(B)** n = 4. Mean and SEM shown. Paired students t tests were performed.

Analysis of PD-1 expression on CD4+ T cells suggested that PD-1 was not upregulated on T cells in response to αPD-L1 or isotype control antibody. The three samples analysed, however, recorded a wide range in PD-1 expression (X31 αPD-L1 sMFI range 19-185 – Figure 6.26) which may mask treatment effects. CD8 T cells expressed a lower variation of surface PD-1, but again neither isotype antibody nor αPD-L1 treatment appeared to induce IFNγ production. CD107a expression was unchanged on CD4+ T cells in response to control antibody or blocking of PD-L1 (Figure 6.27). Again one sample had no detectable CD107a expression under any treatment which could cloud analysis. Intriguingly, one sample upregulated CD107a when treated with αPD-L1 antibody (sMFI = 48) compared to isotype control (sMFI = 17). Due to small sample numbers it cannot be hypothesised as to whether blocking PD-L1 has had an effect on CD107a expression, or whether this sample is merely an outlier.

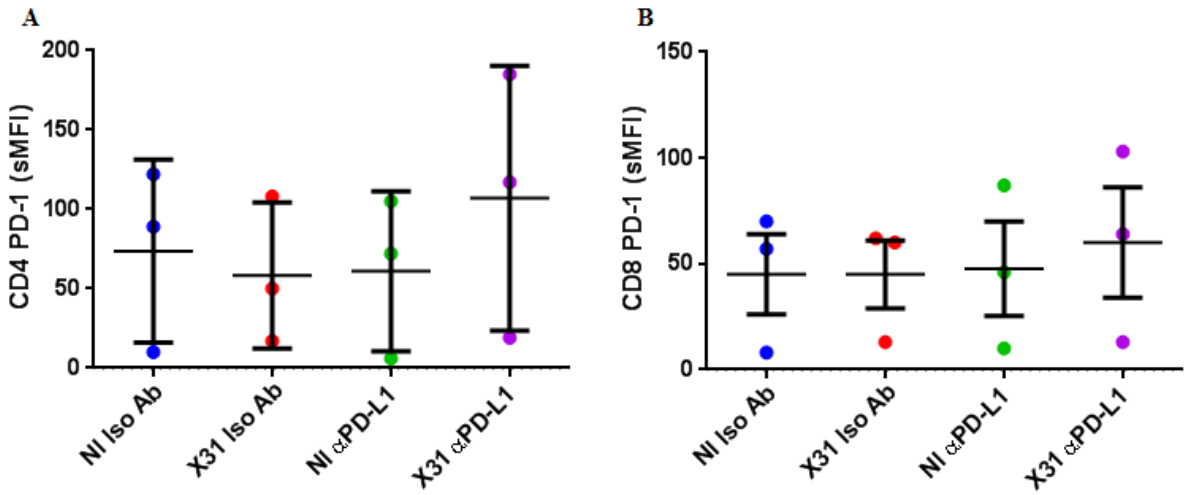

Figure 6.26 PD-1 expression by CD4 and CD8 T cells treated with αPD-L1 blocking antibody. MDMs were treated with X31 or NI for 2 h before washing. 5 x 10^4 MDMs were co-cultured with **(A)** 1 x 10^5 CD4+ **(B)** or CD8+ T cells and and Isotype control antibody or αPD-L1 antibody at 37ºC for 18 h. **(A)** sMFI of CD4+ **(B)** and CD8+ T cells expressing PD-1 was quantified by flow cytometry. **(A)** n = 3, **(B)** n = 4. Mean and SEM shown. Paired students t tests were performed.

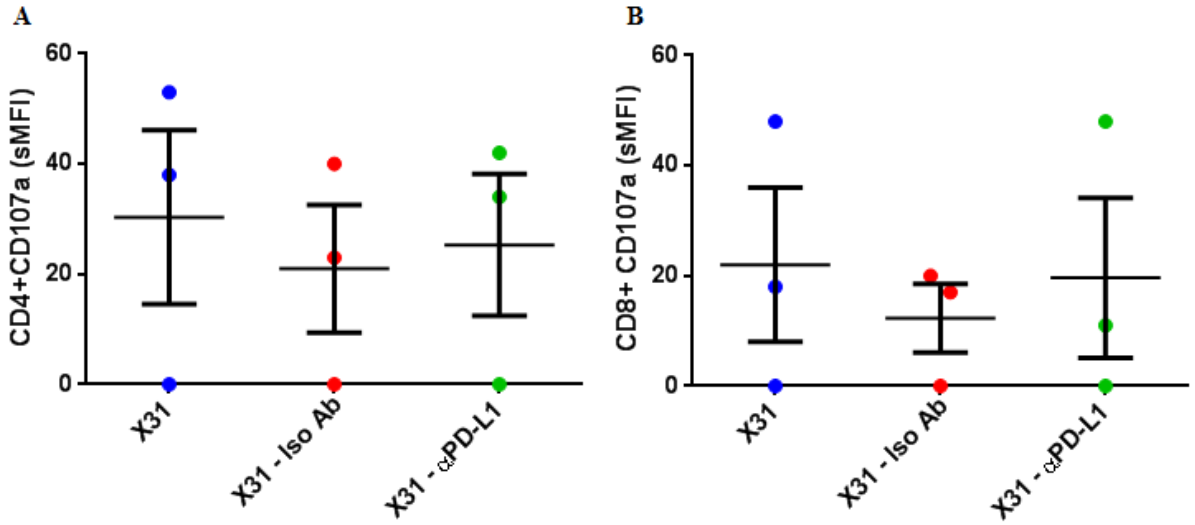

Figure 6.27 CD107a expression by CD4 and CD8 T cells treated with αPD-L1 blocking antibody. MDMs were treated with X31 or NI for 2 h before washing. 5 x 10^4 MDMs were co-cultured with **(A)** 1 x 10^5 CD4+ **(B)** or CD8+ T cells with and without 10µg/ml isotype control antibody or αPD-L1 blocking antibody at 37ºC for 18 h. **(A)** sMFI of CD4+ **(B)** and CD8+ T cells expressing CD107a was quantified by flow cytometry. n = 3. Mean and SEM shown. Paired students t tests were performed.

Conclusions

Loss of IFNγ production is characteristic of the T cell exhaustion phenotype (Wherry et al., 2003; Yi et al., 2010). Inhibited T cell response may occur in response to corticosteroids, the most commonly administered treatment to limit inflammation in COPD lungs. Supplementation with retinoic acid in this model allowed for elucidation on its regulatory properties, which may inhibit cell responses to influenza. Furthermore, blocking of PD-L1 on infected MDMs was postulated to increase T cell IFNγ responses during infection. Therefore therapeutics could be developed in order to prevent PD-1:PD-L1 signalling and enhance anti-viral T cell responses in COPD patients.

CD4+ and CD8+ T cells upregulated IFNγ production in response to influenza-infected MDMs. CD4+ and CD8+ lymphocytes appeared to upregulate CD107a, suggesting cytotoxic capabilities. CD4+ T cells did not upregulate PD-1, but there was a trend for PD-1 upregulation by CD8+ T cells in response to infected MDMs. Fluticasone inhibited IFNγ production by T cells but did not appear to modulate PD-1 or CD107a expression. Retinoic acid supplementation decreased IFNγ production, but upregulated PD-1 and CD107a on T cells. α PD-L1 blocking antibody had no effect on IFNγ production by CD4+ T cells, but it may increase IFNγ response by CD8+ T cells. Treatment effects are summarised in Table 6.2

Macrophages display many immune-regulatory functions and plasticity depending on their environment (Stout et al., 2005). A key function in viral infection is the ability to internalise, process and present viral antigen to T cells (Hume, 2008; McCormack et al., 1993). In the ELISpot experiments, MDMs were required in order for T cells to secrete IFNγ, with virus alone insufficient to generate a response. Therefore the ability of MDMs to present viral antigen was identified as a key mechanism for IFNγ production. Previously the group, in collaboration with Prof. McMichael and colleagues, had shown the ability of Tmem cells to upregulate IFNγ production in response to APCs loaded with influenza peptide pools (Wilkinson et al., 2012). McMichael and colleagues identified the Tmem population as IFNγ producers, while work in Chapter 6 did not phenotype T cells as naïve or memory cells. If the naïve population is not responsive in this model, measurement of PD-1 and CD107a would have a diluted effect and potential upregulation by Tmem cells may be lost in the total lymphocyte population. Thus, categorising Tmem responses may identify a more focused response which is not seen in the total T cell population.

While MDMs were shown to process and present antigen to T cells, B cells from blood did not in this model. B cells are a component of the lymphocyte population which was treated with X31 Influenza A virus. X31-treated lymphocytes did not produce IFNγ in response to this viral challenge. B cells have been established as APCs (Avalos and Ploegh, 2014), but this may be limited to lymph node follicles and secondary/tertiary lymphoid organs (Batista and Harwood, 2009). Whitmire *et al.* (Whitmire et al., 2009) studied the effect of B cell depletion on T cells in a murine model of virus infection. It was observed that primary responses were not inhibited by B cell depletion, but these mice responded poorly to secondary infection. Therefore B cells may have a greater role in generating T cell memory responses rather than inducing upregulation of protective IFNγ during an acute viral infection. In response to MDM infection by X31 Influenza A, the mean number of SFCs produced by the lymphocyte population was 2275 SFCs/1 x 10^6 cells. Using this value the responder frequency was calculated as 0.2275% (range = 0..133-0.329%). Using the same calculation, CD4+ responder frequency was 0.089% and CD8+ responder frequency was 0.155%. Heidema *et al*. (Heidema et al., 2008) utilised virus-specific tetramer staining to quantify and locate RSV and Influenza specific T cells during acute infection. Tetramer staining of CD8+ T cells revealed that 0.1-0.83% of CD8+ T cells recognised a single RSV epitope. A responder frequency of 0.2275% generated by the ELISpot experiments is thus within this range. As NKG2D, a marker for bystander T cell activation (Chu et al., 2013), it was not expressed by T cells in a virus-free environment, nor was it upregulated in response to infection. These data suggest that IFNγ production in the MDM infection model is likely due to activated TCR-dependant influenza-specific CD4+ and CD8+ T cells. Walsh *et al.* (Walsh et al., 2008) determined that peak NKG2D expression by bystander CD8+ T cells was achieved at day 7 in a murine model of virus-induced encephalitis. Analysis of NKG2D expression in our study was measured at 24 h, and may be premature to determine bystander activation.

PD-1 upregulation by CD4+ and CD8+ T cells in response to X31 influenza A was recorded in the lung tissue but only by CD8+ T cells in the *in vitro* blood model. Furthermore, macrophages in lung tissue and MDMs in the blood model both upregulated PD-L1 in response to infection. This may be explained by the greater naïve T cell proportions in blood than in tissue. Naïve T cells which are not antigen experienced may not upregulate PD-1 within 24 h of first antigen exposure, while this process may be accelerated for Tmem cells. Using a murine model of chronic infection, Vezys *et al.* (Vezys et al., 2006) hypothesised

that infiltrating naïve T cells to the site of infection generated a diverse T cell pool in conjunction with poorly-proliferative Tmem cells. These naive cells provided anti-viral responses which were not seen in the Tmem cells. Therefore in the blood model of influenza infection, CD4+ T cells may not upregulate PD-1 until a later time point, beyond the acute phase of infection.

The role of PD-1:PD-L1 interactions is well detailed in models of chronic infection, but is less clear in an acute setting. Barber *et al*. (Barber et al., 2006) described upregulation of CD8+ T cell effector function when PD-L1 was blocked during chronic LCMV infection, but no change was seen during acute LCMV infection. Rowe *et al*. (Rowe et al., 2008) described a decreased proliferative and protective ability of CD8 T cells in a model of *Listeria monocytogenes* infection in response to PD-L1 blocking, while Channappanavar *et al.* (Channappanavar et al., 2012) describe enhanced CD8+ T cell responses to primary and secondary HSV infection. Findings from Chapter 6 suggest that αPD-L1 blocking antibody may enhance CD8+ IFNγ responses and therefore do not concur with the original finding of Barber *et al.* in which blocking of PD-L1 in an acute setting does not enhance or inhibit IFNγ production.

CD4+ T cells and CD8+ T cells from healthy individuals upregulated CD107a in the tissue infection model, but the expression of CD107a expression was unclear in the blood model. CD107a appeared to be upregulated in response to X31, especially for the CD8+ T cell population, but small sample numbers were a limitation for analysis. Increased sample numbers would determine whether the sample expressing no detectable CD107a was representative of T cell cytotoxic responses in this model. The ELISpot technique is a measurement of IFNγ-producing cells, and does not identify T cell cytotoxicity. In this study CD107a was used as a surrogate marker for degranulation, but this may not be as accurate as a functional cytotoxic assay such as a Cr^{51}-release assay which measures target-cell killing (Wilkinson et al., 2012). From results in Chapter 6 there are potentially conflicting hypothesis generated. In this model the production of IFNγ by T cells may be the dominant effector response, with little or no MDM cell-killing. Alternatively, T cells may kill infected MDMs using other mechanisms such as Fas:FasL interactions. A desired outcome of this study was to compare T cell function in the lung infection model with the blood infection model. While there are similarities in PD-1 and CD107a expression between the two models, more samples are required for the lung and blood systems to be directly comparable.

Delmas *et al.* (Delmas et al., 2007) observed that virally-infected APCs may not be subject to cell killing. Experiments by Delmas *et al*. showed that cell killing preferentially favoured peptide-pulsed cells, rather than virally-infected cells. In order to test this finding by Delmas *et al*., a Low-density lipoprotein (LDL) assay could be included in future work. This experiment assesses the level of cell killing from culture supernatants. Supernatants from ELISpot wells containing infected MDMs with lymphocytes could be compared to wells containing non-infected MDMs and lymphocytes. This work could be expanded to assess killing in response to fluticasone, retinoic acid and αPD-L1 blocking antibody.

As previously discussed, the expression of TIM-3 may only occur at the end stages of T cell exhaustion. As the data herein are derived from an acute infection of MDMs and their interactions with naïve T cells, it was unlikely that either TIM-3 expression or upregulation would be observed in this model. TIM-3 expressing T cells have been identified in the blood of HIV patients (Jones et al., 2008; Sakhdari et al., 2012). This is likely due to the chronic nature of the disease and also the key viral targets of infection are CD4+ T cells themselves.

Data from ELISpot experiments illustrated that treatment of MDMs and lymphocytes with fluticasone inhibits T cell production of IFNγ. Fluticasone treatment did not appear to affect MDM viability or the proportion of infected cells (Table 6.1). Other functions, such as antigen presentation and cytokine rerelease, were not measured and therefore may play factor in decrease IFNγ production in fluticasone-treated samples. Therefore inhibition may be induced due to macrophage, T cell or dual fluticasone-induced mechanisms. Although there was no effect on CD107a expression on T cells, loss of IFNγ production alone may prevent the ability of T cells to elicit an anti-viral response. COPD patients in the lung parenchyma infection cohort had been receiving corticosteroid treatment prior to surgery. Therefore a potential confounding factor when comparing control and COPD patients in the tissue model may be the use of steroids. As fluticasone had no effect on PD-1 or CD107a expression, these data suggest patient administration of steroids may also have no effect in the tissue model. The use of corticosteroids as a treatment for COPD patients, especially in later stages of disease, is intended to reduce inflammation in the lungs. If this treatment, however, impairs the ability of T cells to confer protection against viruses, these patients may be susceptible to recurrent, more severe or prolonged viral infections. Thus, further studies to assess the effects of steroids on cytotoxic T cell activity are warranted both in the MDM model and also in the lung explant model.

Retinoic acid treatment appeared to inhibit IFNγ production by lymphocytes, but CD8+ T cells alone did not appear to be inhibited. Nolting *et al.* (Nolting et al., 2009) and Hall *et al.* (Hall et al., 2011b) have described the role of retinoic acid and its receptors in the differentiation of naïve CD4+ T cells into inducible T regulatory cells (iTreg). This alone could explain the inhibition of IFNγ production in samples containing both CD4 T cells and retinoic acid, but the upregulation of PD-1 independently of infection is also characteristic of iTreg cells. A recent publication by Penaloza-MacMaster *et al*. (Penaloza-MacMaster et al., 2014) elegantly details the dynamics of Treg function in the context of a murine LCMV model of acute and chronic infection. In their investigation, Tregs constitutively expressed PD-1 prior to infection. Thus PD-1 expression may be upregulated due to expansion of the Treg population independently of X31 infection. Alternatively, concentrations of retinoic acid used may have a toxic effect on the T cells. No experiments were performed to assess potential T cell death or apoptosis in response to retinoic acid treatment and therefore cannot be rejected as a mechanism for altering T cell surface marker expression.

Treatment	IFNγ	PD-1	CD107a
X31	Increased	Increased by CD8 T cells	Increased
Fluticasone	Inhibits	No effect	No effect
Retinoic Acid	Inhibits	Increase	Increased
αPD-L1 blocking antibdy	Increased by CD8 T cells	No effect	No effect

Table 6.2. Treatment effects of T cell outputs from the MDM infection co-culture model.

Work in Chapter 6 demonstrates the importance of antigen presenting cells for the generation of anti-viral responses by T lymphocytes. Secretion of IFNγ by T cells appears to correlate with increased PD-1 and CD107a expression but small sample numbers may mask these processes. When combined with data from Chapter 5, PD-1 expression appears to be a marker of T cell activation and does not confer an exhausted or inhibited phenotype in the acute infection setting. Treg expansion may explain inhibited IFNγ production and increased PD-1 expression in response to retinoic acid treatment.

Chapter 7

Discussion

Conclusions

The aim of this project was to improve our understanding of key mechanism which contribute to the development and progression of COPD. By characterising T cell populations in the blood and lungs of COPD patients and controls. I aimed to identify the contribution of exhaustion and loss of T cell function to the clinical phenotype. My intention was to elucidate the paradox of COPD individuals with increased CD8+ T cell numbers in the lungs who poorly controlled viral respiratory infections. In this final chapter I will summarise results from this thesis and discuss their limitations. I will then discuss how this work could be expanded.

Defining COPD in the cohort

COPD is not a singular condition in itself, it is a combination of chronic bronchitis, emphysema and small airways disease, and abnormal inflammatory responses culminating in a progressive loss of lung function. In this study individuals were defined as COPD by spirometry if their FEV_1/FVC ratio was < 0.7. COPD patients were either GOLD I or GOLD II severity and these were combined to form the "COPD" group. Individuals from GOLD III and GOLD IV were unavailable for recruitment as they were not suitable for surgery. Since disease exacerbation frequency was previously shown to correlate with COPD severity (Hurst et al., 2010), the inclusion of more severe COPD patients may have revealed more prominent T cell dysfunction in these individuals. Further attempts to characterise disease were made but this was rarely fruitful and often bed-side notes were the only form of data available to the research group. The inability to phenotype patients in a detailed manner was due to two main factors. Firstly, patients recruited to the study mostly lived outside Southampton, and on occasion they did not live in the Hampshire area. This made access to patient lung function data and possible CT scans challenging. Secondly patients were recruited from individuals scheduled for lung re-section surgery to remove lung tumours on an *ad hoc* basis dependent upon when surgery was scheduled and if patients consented to tissue donation. Therefore patients were identified by their availability and willingness to participate rather than COPD characteristics.

The evidence of CD8+ T cells in COPD pathology

Two key papers have observed that an increased proportion of CD8+ T cells are present in human bronchial (O'Shaughnessy et al., 1997) and subpleural parenchyma (Saetta et al., 1998) tissues of COPD lung. These papers were the results of very small sample numbers. O'Shaughnessy *et al.* recruited 5 control and 11 COPD individuals, while Saetta *et al.* recruited 7 controls and 9 COPD patients. CD8+ cell numbers were also calculated by immunohistochemistry and manual counting of T lymphocytes. In comparison, work in this thesis measured CD4+ and CD8+ T cell proportions in 20 control and 24 COPD individuals. Furthermore high-throughput flow cytometry analysis allowed much greater numbers of cells to be analysed per-sample than with immunohistochemistry. My data indicating that greater proportions of CD8+ T cells are present in human lung parenchyma of COPD individuals is therefore a valuable addition to current literature confirming and expanding these previous studies. However, the significance of this shift in CD4:CD8 ratio in the COPD lung remains to be elucidated. In this thesis I have looked at markers of T cell activation and exhaustion in order to identify dysfunction in T lymphocytes which may contribute to severity or pathogenesis of COPD.

Altered T cell proportions are seen in parenchyma but not blood

In contrast to the data derived from lung tissue, the proportions of CD4+ and CD8+ cells were unchanged in blood from control and COPD patients in a small sample of the cohort. Also, while blood contained a mixed proportion of naïve and memory T cell subsets, the majority of T lymphocytes in tissue were either TEM or TEMRA cells. As the T cell composition of blood is different to tissues, the study of explanted lung parenchyma could reveal novel mechanisms of protection or dysregulation. The majority of published human COPD research investigates samples from blood (Grundy et al., 2013; Koch et al., 2007), sputum (Wang et al., 2013; Wen et al., 2010), BAL (Grundy et al., 2013; Wen et al., 2010) and bronchial biopsies (Hattotuwa et al., 2002; Wen et al., 2010). A complete characterisation of disease, however, cannot be complete without study of the small airway tissues, a key site for the disease mechanisms of COPD.

T cells express PD-1 but no other markers of exhaustion in stable COPD

The expression of an exhausted T cell phenotype has been identified in murine models of chronic infection (Barber et al., 2006; Fourcade et al., 2010), in human cancer (Ahmadzadeh et al., 2009) and HIV (Peretz et al., 2012; Trautmann et al., 2012). PD-1 expression appeared to be expressed on a greater proportion of CD4+ and CD8+ T cells in COPD tissue compared to controls using flow cytometric analysis but this was not significant and was not replicated in RT-PCR work. Furthermore, TIM-3 expression was undetectable by flow cytometry and RT-PCR. Contrastingly, LAG-3 expression was detected by RT-PCR, but there was no difference in expression between T lymphocytes from control or COPD tissue. Thus poor control of viral infection in COPD does not appear to be due to functionally exhausted T cells. Instead, trends towards elevated expression of CD27 and downregulated expression of CD57 in CD8+ T cells by RT-PCR suggests activation rather than exhaustion in stable COPD. Therefore, T cells may be activated in the stable lungs of COPD patients, but the origin of this activation is unknown. Expansion of sample numbers and inclusion of LAG-3, CD27 and CTLA-4 into flow cytometry work may allow more defined activation status of lung T cells. However, baseline expression of markers may not provide a full picture of cellular function and responses to viral infection. I therefore utilised a model of influenza infection in order to investigate the how lung T cells respond to a respiratory viral challenge.

CD8+CD45RA+CCR7- TEMRA cells express impaired responses to influenza infection in COPD

Work investigating T cell responses to influenza A infection of lung parenchyma identified functional impairment in the Tmem population. T memory cells are characterised by their prior exposure to antigen, thus it was these cells which may be most likely to be exhausted. CD8+ TEMRA cells from COPD tissue did not upregulate PD-1 in response to tissue infection, unlike CD8+ TEM and CD4+ T cells. CD8+ TEMRA also did not upregulate CD107a in COPD tissues, unlike their counterparts in control tissue. CD8+ TEMRA from COPD patients therefore may have impaired cytotoxic capabilities and may be unable to kill virally-infected cells during infection of COPD patients. At the time of investigation there was no literature found which addressed the role of CD8+ T_{EMRA} cells in COPD. Shen *et al.* (Shen et al., 2010) correlated expression of PD-1 on TEMRA cells in human blood with higher viral loads of HCV. T_{EMRA} cells may be inhibited in response to chronic viral infection, with no upregulation of CD107a in response to tissue infection. PD-1 was not upregulated by CD8+ T_{EMRA} in the

tissue infection model, but this may reflect a lack of activation rather than an absence of exhaustion. Furthermore the upregulation of CD107a by CD4+ T cells may indicate an attempt by the host to compensate an impaired anti-viral response by CD8+ T_{EMRA} cells. Investigation is required into the role of CD8+ T_{EMRA} cells both in COPD and in the context of respiratory viral infection, not least the antigen specificity of these cells. PD-1 expression and associated exhaustion is associated with chronic viral infection and there are reports of chronic RSV infection in a frequently exacerbating COPD cohort (Wilkinson et al., 2006a). Screening all of the explant tissue obtained for chronic viral infection was beyond the scope of the present study, but given the variation in T cell responses derived from COPD tissue it would be interesting in future studies to correlate the T cell phenotype observed with the presence of chronic viral infection.

Corticosteroids inhibit T cell IFNγ expression but do not modulate PD-1 or CD107a

Corticosteroids are the main therapy used to limit lung inflammation during COPD exacerbations (Barnes, 2011; Woods et al., 2014), but there is a recognised concern that corticosteroids may have little benefit in stable COPD (Calverley et al., 2007; Suissa and Barnes, 2009; Yang et al., 2012). In Chapter 4, a trend for an increased proportion of T cells expressing PD1 was observed in COPD patients. In order to exclude this upregulation being an effect of inhaled corticosteroid use in COPD, in chapter 6 the effect of administration of fluticasone on MDM-T cell co-cultures was investigated. Whilst fluticasone inhibited T cell production of IFNγ, this steroid did not modulate PD-1 or CD107a responses. Furthermore, PD-1 upregulation in response to COPD tissue infection was upregulated by CD4+ and CD8+ T cell populations in response to viral infection. As these patients used corticosteroids prior to surgery, it appears PD-1 expression is independent of steroid treatment. Corticosteroids do dampen IFNγ-mediated T cell responses, but its mechanism of inhibition does not appear to work through enhancement of T cell exhaustion. Corticosteroids, however, remain a confounding factor for research comparing samples from control and COPD patients.

Retinoic acid mediates PD-1 expression independently of infection

Retinoic acid has been shown to promote CD4+ T cell differentiation into a regulatory phenotype (Hall et al., 2011a; Nolting et al., 2009). Retinoic acid also promotes Treg migration to mucosal sites, implicating a role in tolerance (Hall et al., 2011b). Current literature suggests that Treg cells constitutively express PD-1 (Penaloza-MacMaster et al.,

2014), and this may explain PD-1 expression levels in retinoic acid treated samples in Chapter 6. Treg populations were not measured in the lung parenchymal tissues, nor was the nutritional status of patients known. Therefore more work is required to elucidate the role of retinoic acid, Treg cells and abnormal inflammation in COPD lungs.

Effects of blocking PD-1:PD-L1 interactions in acute influenza infection.

The low number of samples analysed prevented firm conclusions and disease implications to be made, however my data suggests blocking PD-L1 does not increase IFNγ production by CD4+ T cells during acute influenza infection. Blocking of PD-1:PD-L1 has been shown to restore exhausted T cell functions in chronically infected mice (Barber et al., 2006), and also may have clinical applications in human cancers (Lipson et al., 2013; Topalian et al., 2012). Barber *et al.* did not find an upregulation of T cell responses in a murine model of acute LCMV, but data from chapter 6 identified a trend for CD8+ T cells to increase IFNγ production in response to X31 to a greater level than control antibody. This is certainly a point of interest revealed by the MDM infection model. X31 infection of tissue could be supplemented with αPD-L1 blocking antibodies to investigate IFNγ responses. ELISpot would not be a suitable measure for IFNγ production in this model, but supernatant analysis by ELISA may reveal different levels of IFNγ production by tissue T cells.

Macrophages express PD-L1 in response to viral infection

Previous investigation into PD-L1 regulation of responses to respiratory viruses has focussed on expression by epithelial cells (Erickson et al., 2012; Stanciu et al., 2006; Telcian et al., 2011). Viral infection of control and COPD tissue did not modulate PD-L1 expression by epithelial cells, although it was constitutively expressed, albeit at a very low level. In contrast, macrophages from these tissues did significantly upregulate PD-L1, although macrophages from almost half of the COPD samples did not express PD-L1. Therefore the COPD group may be phenotypically divided by the expression of PD-L1. If αPD-L1 blocking antibody were to be considered in treatment of COPD, there may be a population of COPD individuals who may not be responsive to treatment.

The hypothesis that T cell exhaustion contributed to COPD was not confirmed, although further work is required to identify virus-specific T cells. PD-1 was expressed at low levels on lung T cells and was upregulated in response to influenza challenge. PD-L1 was constitutively expressed on epithelial cells but was only upregulated by macrophages. αPD-L1 blocking antibody promoted limited IFNγ responses in CD8+ T cells in response to MDM infection. Corticosteroids inhibit T cell function independently of the exhaustion pathway, while retinoic acid may promote Treg differentiation.

Future work

As previously discussed, sample numbers are low in many experiments. This is especially true for surface expression analysis of PD-1, CD57 and CD107a in the explant model of X31 infection. Expansion of the number of lung samples challenged with influenza *ex vivo* would help eradicate the skewing of results by potential outliers and allow confirmation or rejection of current trends. Furthermore, due to the heterogeneity of COPD, more detailed patient data is required, both in terms of lung function, but also characterisation of potential emphysema chronic bronchitis and air trapping. Targeted recruitment of COPD patients for study would allow for a highly phenotyped cohort to be studied, and this may provide more consistent results than was seen in this thesis. Access to full lung function data, including Transfer Factor of the lung for carbon monoxide (TL_{CO} – measurement of air trapping), Diffusing Capacity divided by the alveolar volume (DLCO/VA - pulmonary gas exchange measurement) and K_{CO} (diffusing capacity of the lung) could be assessed, while CT scanning of patients would also aid the identification of emphysema. Due to the current nature of patient recruitment, this is not currently available at Southampton in respect of explanted lung. Bronchial biopsies obtained via bronchoscopy could be performed to yield tissue from these sites, but transbronchial biopsies are not routinely performed for safety reasons and thus disease mechanisms restricted to the small airways could not be analysed.

To further understand the relationship between CD8+ T cells in the lungs and viral infection, PCR analysis could be performed using lung parenchyma to detect viral or bacterial RNA/DNA which would suggest current or latent infection of the individual. As T cell exhaustion is associated with chronic infection, identification of pathogen genetic material would imply pathogen antigen availability. Furthermore if virus was detected in tissue samples, this would focus lymphocyte investigation on T cells specific for that pathogen.

Tetramer staining for anti-influenza T cells was originally proposed before initiation of this study. This was not fulfilled due to the nature of patient recruitment as HLA expression of patients was unknown. If patient details were known in advance of surgery, HLA-typing could be performed, Tetramers could not be HLA-matched and anti-viral T cells could be identified. T cell exhaustion has been shown to be an antigen-specific response to chronic infection (Barber et al., 2006; Wherry et al., 2007). Thus the current protocol examining the entire CD4+ and CD8+ population, and the whole Tmem populations, may not be focussed

enough to identify exhaustion. The blood infection model revealed a responder frequency rate of 0.2275% of lymphocytes. Even if this population is increased in tissue (the site of influenza infection), the exhaustion signal may be lost amongst non-specific T cells. Expression exhaustion and functional markers would be analysed to characterise these cells in COPD tissue, and identify whether influenza-specific exhaustion contributes to disease severity.

In addition, the role of CD8+ TEMRA cells is poorly understood in respiratory disease. This thesis evaluated response of several T cell phenotypes simultaneously in response to X31 challenge. As CD8+ TEMRA cells appear to be functionally impaired, further analysis with greater focus is required.

As discussed in chapter 6, CD107a is a surrogate marker for T cell degranulation. The MDM infection model could be expanded to include Cr^{51} release assays which would determine specific T cell killing. This would be especially relevant in the context of CD8+ TEMRA cell which appear to have impaired upregulation of CD107a, and whose cytotoxic potential may be inhibited.

Supernatants from lung parenchyma infection experiments have been collected and stored at -80ºC. It was the intention of this study to analyse supernatants for cytokine production, including IL-6, IL-8, IFNγ and IL-10. Multi-parameter Luminex experiments were not performed due to time restrictions, but cytokine profiles can be compared between controls and COPD samples in the future. Inflammatory cytokine environments may activate T cells in an antigen-independent manner (Sckisel et al., 2014). While viral-specific T cells may be exhausted and allow for impaired viral clearance, bystander activation of T cells may result in tissue damage by poorly regulated T cell cytotoxic function.

Summary

This thesis has detailed results from a number of different techniques to further understand the role of tissue resident lung T cells in COPD pathogenesis and the responses of these cells to viral challenge. Optimisation of both blood and tissue models of infection has revealed new T cell subtypes of interest which may be associated with dysfunctional response to infection. The importance of macrophages as mediators of T cell function via PD-L1 expression is a novel finding in the context of COPD, and this work provides a platform for further work to be performed in the lung parenchymal model. T cell exhaustion was not identified in stable state nor in response to influenza infection. T cell dysfunction, however, is likely to be heterogeneous across patients and disease phenotypes. The ability to analyse tissue cells in a flexible challenge model offers real opportunities to develop new therapeutic interventions which may improve outcomes in this important disease.

References

Abdelaziz, M.M., J.L. Devalia, O.A. Khair, M. Calderon, R.J. Sapsford, and R.J. Davies. 1995. The effect of conditioned medium from cultured human bronchial epithelial cells on eosinophil and neutrophil chemotaxis and adherence in vitro. *Am J Respir Cell Mol Biol* 13:728-737.

Abuzakouk, M., C. Feighery, and C. O'Farrelly. 1996. Collagenase and Dispase enzymes disrupt lymphocyte surface molecules. *Journal of immunological methods* 194:211-216.

Agata, Y., A. Kawasaki, H. Nishimura, Y. Ishida, T. Tsubata, H. Yagita, and T. Honjo. 1996. Expression of the PD-1 antigen on the surface of stimulated mouse T and B lymphocytes. *Int Immunol* 8:765-772.

Agostini, C., M. Facco, M. Chilosi, and G. Semenzato. 2001. Alveolar macrophage-T cell interactions during Th1-type sarcoid inflammation. *Microscopy research and technique* 53:278-287.

Agusti, A., W. MacNee, K. Donaldson, and M. Cosio. 2003. Hypothesis: does COPD have an autoimmune component. *Thorax* 58:832-834.

Ahmadzadeh, M., L.A. Johnson, B. Heemskerk, J.R. Wunderlich, M.E. Dudley, D.E. White, and S.A. Rosenberg. 2009. Tumor antigen-specific CD8 T cells infiltrating the tumor express high levels of PD-1 and are functionally impaired. *Blood* 114:1537-1544.

Akbar, A.N., and J.M. Fletcher. 2005. Memory T cell homeostasis and senescence during aging. *Current opinion in immunology* 17:480-485.

Akbar, A.N., and S.M. Henson. 2011. Are senescence and exhaustion intertwined or unrelated processes that compromise immunity? *Nat Rev Immunol* 11:289-295.

Akbar, A.N., and M. Vukmanovic-Stejic. 2007. Telomerase in T lymphocytes: use it and lose it? *J Immunol* 178:6689-6694.

Allie, S.R., W. Zhang, C.Y. Tsai, R.J. Noelle, and E.J. Usherwood. 2013. Critical role for all-trans retinoic acid for optimal effector and effector memory CD8 T cell differentiation. *J Immunol* 190:2178-2187.

Andersson, F., S. Borg, S.A. Jansson, A.C. Jonsson, A. Ericsson, C. Prutz, E. Ronmark, and B. Lundback. 2002. The costs of exacerbations in chronic obstructive pulmonary disease (COPD). *Respir Med* 96:700-708.

Appay, V., D.C. Douek, and D.A. Price. 2008a. CD8+ T cell efficacy in vaccination and disease. *Nature medicine* 14:623-628.

Appay, V., R.A. van Lier, F. Sallusto, and M. Roederer. 2008b. Phenotype and function of human T lymphocyte subsets: consensus and issues. *Cytometry. Part A : the journal of the International Society for Analytical Cytology* 73:975-983.

Araya, J., S. Cambier, J.A. Markovics, P. Wolters, D. Jablons, A. Hill, W. Finkbeiner, K. Jones, V.C. Broaddus, D. Sheppard, A. Barzcak, Y. Xiao, D.J. Erle, and S.L. Nishimura. 2007. Squamous metaplasia amplifies pathologic epithelial-mesenchymal interactions in COPD patients. *J Clin Invest* 117:3551-3562.

Artigas, M.S., D.W. Loth, L.V. Wain, S.A. Gharib, M. Obeidat, W. Tang, G. Zhai, J.H. Zhao, A.V. Smith, J.E. Huffman, E. Albrecht, C.M. Jackson, D.M. Evans, G. Cadby, M. Fornage, A. Manichaikul, L.M. Lopez, T. Johnson, M.C. Aldrich, T. Aspelund, I. Barroso, H. Campbell, P.A. Cassano, D.J. Couper, G. Eiriksdottir, N. Franceschini, M. Garcia, C. Gieger, G.K. Gislason, I. Grkovic, C.J. Hammond, D.B. Hancock, T.B. Harris, A. Ramasamy, S.R. Heckbert, M. Heliovaara, G. Homuth, P.G. Hysi, A.L. James, S. Jankovic, B.R. Joubert, S. Karrasch, N. Klopp, B. Koch, S.B. Kritchevsky,

L.J. Launer, Y. Liu, L.R. Loehr, K. Lohman, R.J. Loos, T. Lumley, K.A. Al Balushi, W.Q. Ang, R.G. Barr, J. Beilby, J.D. Blakey, M. Boban, V. Boraska, J. Brisman, J.R. Britton, G.G. Brusselle, C. Cooper, I. Curjuric, S. Dahgam, I.J. Deary, S. Ebrahim, M. Eijgelsheim, C. Francks, D. Gaysina, R. Granell, X. Gu, J.L. Hankinson, R. Hardy, S.E. Harris, J. Henderson, A. Henry, A.D. Hingorani, A. Hofman, P.G. Holt, J. Hui, M.L. Hunter, M. Imboden, K.A. Jameson, S.M. Kerr, I. Kolcic, F. Kronenberg, J.Z. Liu, J. Marchini, T. McKeever, A.D. Morris, A.C. Olin, D.J. Porteous, D.S. Postma, S.S. Rich, S.M. Ring, F. Rivadeneira, T. Rochat, A.A. Sayer, I. Sayers, P.D. Sly, G.D. Smith, A. Sood, J.M. Starr, A.G. Uitterlinden, J.M. Vonk, S.G. Wannamethee, P.H. Whincup, C. Wijmenga, O.D. Williams, A. Wong, M. Mangino, K.D. Marciante, W.L. McArdle, B. Meibohm, A.C. Morrison, K.E. North, E. Omenaas, L.J. Palmer, K.H. Pietilainen, I. Pin, O. Pola Sbreve Ek, A. Pouta, B.M. Psaty, A.L. Hartikainen, T. Rantanen, S. Ripatti, J.I. Rotter, I. Rudan, A.R. Rudnicka, H. Schulz, S.Y. Shin, T.D. Spector, I. Surakka, V. Vitart, H. Volzke, N.J. Wareham, N.M. Warrington, H.E. Wichmann, S.H. Wild, J.B. Wilk, M. Wjst, A.F. Wright, L. Zgaga, T. Zemunik, C.E. Pennell, F. Nyberg, D. Kuh, J.W. Holloway, H.M. Boezen, D.A. Lawlor, R.W. Morris, N. Probst-Hensch, J. Kaprio, J.F. Wilson, C. Hayward, M. Kahonen, J. Heinrich, A.W. Musk, D.L. Jarvis, S. Glaser, M.R. Jarvelin, B.H. Ch Stricker, P. Elliott, G.T. O'Connor, D.P. Strachan, S.J. London, I.P. Hall, V. Gudnason, and M.D. Tobin. 2011. Genome-wide association and large-scale follow up identifies 16 new loci influencing lung function. *Nature genetics*

Aubert, R.D., A.O. Kamphorst, S. Sarkar, V. Vezys, S.J. Ha, D.L. Barber, L. Ye, A.H. Sharpe, G.J. Freeman, and R. Ahmed. 2011. Antigen-specific CD4 T-cell help rescues exhausted CD8 T cells during chronic viral infection. *Proceedings of the National Academy of Sciences*

Avalos, A.M., and H.L. Ploegh. 2014. Early BCR Events and Antigen Capture, Processing, and Loading on MHC Class II on B Cells. *Frontiers in immunology* 5:92.

Badham, C. 1814. An essay on bronchitis: with a supplement containing remarks on simple pulmonary abscess. Callow, London.

Baitsch, L., P. Baumgaertner, E. Devevre, S.K. Raghav, A. Legat, L. Barba, S. Wieckowski, H. Bouzourene, B. Deplancke, P. Romero, N. Rufer, and D.E. Speiser. 2011. Exhaustion of tumor-specific CD8(+) T cells in metastases from melanoma patients. *J Clin Invest* 121:2350-2360.

Barber, D.L., E.J. Wherry, D. Masopust, B. Zhu, J.P. Allison, A.H. Sharpe, G.J. Freeman, and R. Ahmed. 2006. Restoring function in exhausted CD8 T cells during chronic viral infection. *Nature* 439:682-687.

Barnes, N.C., Y.S. Qiu, I.D. Pavord, D. Parker, P.A. Davis, J. Zhu, M. Johnson, N.C. Thomson, P.K. Jeffery, and S.C.O.S. Group. 2006. Antiinflammatory effects of salmeterol/fluticasone propionate in chronic obstructive lung disease. *Am J Respir Crit Care Med* 173:736-743.

Barnes, P.J. 2011. Glucocorticosteroids: current and future directions. *British Journal of Pharmacology* 163:29-43.

Barnes, P.J. 2013. New anti-inflammatory targets for chronic obstructive pulmonary disease. *Nature reviews. Drug discovery* 12:543-559.

Batista, F.D., and N.E. Harwood. 2009. The who, how and where of antigen presentation to B cells. *Nat Rev Immunol* 9:15-27.

Behera, D., S.K. Jindal, and H.S. Malhotra. 1994. Ventilatory function in nonsmoking rural Indian women using different cooking fuels. *Respiration* 61:89-92.

Belkaid, Y., C.A. Piccirillo, S. Mendez, E.M. Shevach, and D.L. Sacks. 2002. CD4+CD25+ regulatory T cells control Leishmania major persistence and immunity. *Nature* 420:502-507.

Belz, G.T., D. Wodarz, G. Diaz, M.A. Nowak, and P.C. Doherty. 2002. Compromised influenza virus-specific CD8(+)-T-cell memory in CD4(+)-T-cell-deficient mice. *J Virol* 76:12388-12393.

Benfield, T., P. Lange, and J. Vestbo. 2008. COPD stage and risk of hospitalization for infectious disease. *Chest* 134:46-53.

Benson, M.J., K. Pino-Lagos, M. Rosemblatt, and R.J. Noelle. 2007. All-trans retinoic acid mediates enhanced T reg cell growth, differentiation, and gut homing in the face of high levels of co-stimulation. *J Exp Med* 204:1765-1774.

Bettini, M., and D.A. Vignali. 2009. Regulatory T cells and inhibitory cytokines in autoimmunity. *Current opinion in immunology* 21:612-618.

Bhowmik, A., T.A. Seemungal, R.J. Sapsford, and J.A. Wedzicha. 2000. Relation of sputum inflammatory markers to symptoms and lung function changes in COPD exacerbations. *Thorax* 55:114-120.

Biagioli, M.C., P. Kaul, I. Singh, and R.B. Turner. 1999. The role of oxidative stress in rhinovirus induced elaboration of IL-8 by respiratory epithelial cells. *Free radical biology & medicine* 26:454-462.

Biernacki, W.A., S.A. Kharitonov, and P.J. Barnes. 2003. Increased leukotriene B4 and 8-isoprostane in exhaled breath condensate of patients with exacerbations of COPD. *Thorax* 58:294-298.

Biesalski, H.K., and D. Nohr. 2003. Importance of vitamin-A for lung function and development. *Molecular aspects of medicine* 24:431-440.

Bivas-Benita, M., G.O. Gillard, L. Bar, K.A. White, R.J. Webby, A.H. Hovav, and N.L. Letvin. 2013. Airway CD8(+) T cells induced by pulmonary DNA immunization mediate protective anti-viral immunity. *Mucosal Immunol* 6:156-166.

Boman, C., B. Forsberg, and T. Sandstrom. 2006. Shedding new light on wood smoke: a risk factor for respiratory health. *Eur Respir J* 27:446-447.

Bommart, S., G. Marin, A. Bourdin, N. Molinari, F. Klein, M. Hayot, I. Vachier, P. Chanez, J. Mercier, and H. Vernhet-Kovacsik. 2014. Relationship between CT air trapping criteria and lung function in small airway impairment quantification. *BMC pulmonary medicine* 14:29.

Borchers, M.T., M.P. Carty, and G.D. Leikauf. 1999. Regulation of human airway mucins by acrolein and inflammatory mediators. *Am J Physiol* 276:L549-555.

Bouvier, N.M., and P. Palese. 2008. The biology of influenza viruses. *Vaccine* 26 Suppl 4:D49-53.

Brahmer, J.R., S.S. Tykodi, L.Q. Chow, W.J. Hwu, S.L. Topalian, P. Hwu, C.G. Drake, L.H. Camacho, J. Kauh, K. Odunsi, H.C. Pitot, O. Hamid, S. Bhatia, R. Martins, K. Eaton, S. Chen, T.M. Salay, S. Alaparthy, J.F. Grosso, A.J. Korman, S.M. Parker, S. Agrawal, S.M. Goldberg, D.M. Pardoll, A. Gupta, and J.M. Wigginton. 2012. Safety and Activity of Anti-PD-L1 Antibody in Patients with Advanced Cancer. *The New England journal of medicine*

Bratke, K., H. Goettsching, M. Kuepper, S. Geyer, W. Luttmann, and J.C. Virchow. 2009. Interleukin-4 suppresses the cytotoxic potential of in vitro generated, adaptive regulatory CD4 T cells by down-regulation of granzyme B. *Immunology* 127:338-344.

Brenchley, J.M., N.J. Karandikar, M.R. Betts, D.R. Ambrozak, B.J. Hill, L.E. Crotty, J.P. Casazza, J. Kuruppu, S.A. Migueles, M. Connors, M. Roederer, D.C. Douek, and R.A. Koup. 2003. Expression of CD57 defines replicative senescence and antigen-induced apoptotic death of CD8+ T cells. *Blood* 101:2711-2720.

Brincks, E.L., A.D. Roberts, T. Cookenham, S. Sell, J.E. Kohlmeier, M.A. Blackman, and D.L. Woodland. 2013. Antigen-Specific Memory Regulatory CD4+Foxp3+ T Cells Control Memory Responses to Influenza Virus Infection. *J Immunol* 190:3438-3446.

Brown, D.M., A.M. Dilzer, D.L. Meents, and S.L. Swain. 2006. CD4 T cell-mediated protection from lethal influenza: perforin and antibody-mediated mechanisms give a one-two punch. *J Immunol* 177:2888-2898.

Brown, D.M., S. Lee, L. Garcia-Hernandez Mde, and S.L. Swain. 2012. Multifunctional CD4 cells expressing gamma interferon and perforin mediate protection against lethal influenza virus infection. *J Virol* 86:6792-6803.

Brown, J.A., D.M. Dorfman, F.R. Ma, E.L. Sullivan, O. Munoz, C.R. Wood, E.A. Greenfield, and G.J. Freeman. 2003. Blockade of programmed death-1 ligands on dendritic cells enhances T cell activation and cytokine production. *J Immunol* 170:1257-1266.

Bucks, C.M., J.A. Norton, A.C. Boesteanu, Y.M. Mueller, and P.D. Katsikis. 2009. Chronic antigen stimulation alone is sufficient to drive CD8+ T cell exhaustion. *J Immunol* 182:6697-6708.

Buist, A.S., W.M. Vollmer, and M.A. McBurnie. 2008. Worldwide burden of COPD in high- and low-income countries. Part I. The burden of obstructive lung disease (BOLD) initiative. *The international journal of tuberculosis and lung disease : the official journal of the International Union against Tuberculosis and Lung Disease* 12:703-708.

Burrows, B., C.M. Fletcher, B.E. Heard, N.L. Jones, and J.S. Wootliff. 1966. The emphysematous and bronchial types of chronic airways obstruction. A clinicopathological study of patients in London and Chicago. *Lancet* 1:830-835.

Calverley, P.M., J.A. Anderson, B. Celli, G.T. Ferguson, C. Jenkins, P.W. Jones, J.C. Yates, J. Vestbo, and T. investigators. 2007. Salmeterol and fluticasone propionate and survival in chronic obstructive pulmonary disease. *The New England journal of medicine* 356:775-789.

Cardin, R.D., J.W. Brooks, S.R. Sarawar, and P.C. Doherty. 1996. Progressive loss of CD8+ T cell-mediated control of a gamma-herpesvirus in the absence of CD4+ T cells. *J Exp Med* 184:863-871.

Celli, B.R., R.J. Halbert, R.J. Nordyke, and B. Schau. 2005. Airway obstruction in never smokers: results from the Third National Health and Nutrition Examination Survey. *The American journal of medicine* 118:1364-1372.

Cerveri, I., A.G. Corsico, S. Accordini, R. Niniano, E. Ansaldo, J.M. Anto, N. Kunzli, C. Janson, J. Sunyer, D. Jarvis, C. Svanes, T. Gislason, J. Heinrich, J.P. Schouten, M. Wjst, P. Burney, and R. de Marco. 2008. Underestimation of airflow obstruction among young adults using FEV1/FVC <70% as a fixed cut-off: a longitudinal evaluation of clinical and functional outcomes. *Thorax* 63:1040-1045.

Cerwenka, A., T.M. Morgan, A.G. Harmsen, and R.W. Dutton. 1999. Migration kinetics and final destination of type 1 and type 2 CD8 effector cells predict protection against pulmonary virus infection. *J Exp Med* 189:423-434.

Chana, K.K., P.S. Fenwick, A.G. Nicholson, P.J. Barnes, and L.E. Donnelly. 2014. Identification of a distinct glucocorticosteroid-insensitive pulmonary macrophage phenotype in patients with chronic obstructive pulmonary disease. *The Journal of allergy and clinical immunology* 133:207-216 e201-211.

Chang, J., and T.J. Braciale. 2002. Respiratory syncytial virus infection suppresses lung CD8+ T-cell effector activity and peripheral CD8+ T-cell memory in the respiratory tract. *Nature medicine* 8:54-60.

Channappanavar, R., B.S. Twardy, and S. Suvas. 2012. Blocking of PDL-1 interaction enhances primary and secondary CD8 T cell response to herpes simplex virus-1 infection. *PLoS One* 7:e39757.

Chapman, K.R., D.M. Mannino, J.B. Soriano, P.A. Vermeire, A.S. Buist, M.J. Thun, C. Connell, A. Jemal, T.A. Lee, M. Miravitlles, S. Aldington, and R. Beasley. 2006. Epidemiology and costs of chronic obstructive pulmonary disease. *Eur Respir J* 27:188-207.

Chattopadhyay, P.K., M.R. Betts, D.A. Price, E. Gostick, H. Horton, M. Roederer, and S.C. De Rosa. 2009. The cytolytic enzymes granyzme A, granzyme B, and perforin: expression patterns, cell distribution, and their relationship to cell maturity and bright CD57 expression. *Journal of leukocyte biology* 85:88-97.

Chemnitz, J.M., R.V. Parry, K.E. Nichols, C.H. June, and J.L. Riley. 2004. SHP-1 and SHP-2 associate with immunoreceptor tyrosine-based switch motif of programmed death 1 upon primary human T cell stimulation, but only receptor ligation prevents T cell activation. *J Immunol* 173:945-954.

Chen, J., Z. Chen, T. Narasaraju, N. Jin, and L. Liu. 2004. Isolation of highly pure alveolar epithelial type I and type II cells from rat lungs. *Laboratory investigation; a journal of technical methods and pathology* 84:727-735.

Cheng, X., H. Dai, N. Wan, Y. Moore, R. Vankayalapati, and Z. Dai. 2009. Interaction of programmed death-1 and programmed death-1 ligand-1 contributes to testicular immune privilege. *Transplantation* 87:1778-1786.

Cheng, X., V. Veverka, A. Radhakrishnan, L.C. Waters, F.W. Muskett, S.H. Morgan, J. Huo, C. Yu, E.J. Evans, A.J. Leslie, M. Griffiths, C. Stubberfield, R. Griffin, A.J. Henry, A. Jansson, J.E. Ladbury, S. Ikemizu, M.D. Carr, and S.J. Davis. 2013. Structure and interactions of the human programmed cell death 1 receptor. *The Journal of biological chemistry* 288:11771-11785.

Chizzolini, C., R. Chicheportiche, D. Burger, and J.M. Dayer. 1997. Human Th1 cells preferentially induce interleukin (IL)-1beta while Th2 cells induce IL-1 receptor antagonist production upon cell/cell contact with monocytes. *Eur J Immunol* 27:171-177.

Chu, T., A.J. Tyznik, S. Roepke, A.M. Berkley, A. Woodward-Davis, L. Pattacini, M.J. Bevan, D. Zehn, and M. Prlic. 2013. Bystander-activated memory CD8 T cells control early pathogen load in an innate-like, NKG2D-dependent manner. *Cell reports* 3:701-708.

Churg, A., H. Tai, T. Coulthard, R. Wang, and J.L. Wright. 2006. Cigarette smoke drives small airway remodeling by induction of growth factors in the airway wall. *Am J Respir Crit Care Med* 174:1327-1334.

CIBA. 1959. Terminology, definitions, and classification of chronic pulmonary emphysema and related conditions. *Thorax* 286–299.

Cielen, N., K. Maes, and G. Gayan-Ramirez. 2014. Musculoskeletal disorders in chronic obstructive pulmonary disease. *BioMed research international* 2014:965764.

Connors, A.F., Jr., N.V. Dawson, C. Thomas, F.E. Harrell, Jr., N. Desbiens, W.J. Fulkerson, P. Kussin, P. Bellamy, L. Goldman, and W.A. Knaus. 1996. Outcomes following acute exacerbation of severe chronic obstructive lung disease. The SUPPORT investigators (Study to Understand Prognoses and Preferences for Outcomes and Risks of Treatments). *Am J Respir Crit Care Med* 154:959-967.

Cornacchiari, A., P.G. Grigolato, F. Facchetti, M.L. Morassi, M. Cadei, F. Alpi, S. Battocchio, and E. Chirico. 1995. Usefulness of the scraping method for DNA flow cytometry in breast tumors. *Cytometry* 19:263-266.

Cox, M.A., S.M. Kahan, and A.J. Zajac. 2013. Anti-viral CD8 T cells and the cytokines that they love. *Virology* 435:157-169.

Cruz-Guilloty, F., A.M. Saeed, S. Duffort, M. Cano, K.B. Ebrahimi, A. Ballmick, Y. Tan, H. Wang, J.M. Laird, R.G. Salomon, J.T. Handa, and V.L. Perez. 2014. T cells and macrophages responding to oxidative damage cooperate in pathogenesis of a mouse model of age-related macular degeneration. *PLoS One* 9:e88201.

Culpitt, S.V., D.F. Rogers, P. Shah, C. De Matos, R.E. Russell, L.E. Donnelly, and P.J. Barnes. 2003. Impaired inhibition by dexamethasone of cytokine release by alveolar macrophages from patients with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 167:24-31.

Curotto de Lafaille, M.A., N. Kutchukhidze, S. Shen, Y. Ding, H. Yee, and J.J. Lafaille. 2008. Adaptive Foxp3+ regulatory T cell-dependent and -independent control of allergic inflammation. *Immunity* 29:114-126.

d'Hennezel, E., E. Yurchenko, E. Sgouroudis, V. Hay, and C.A. Piccirillo. 2011. Single-cell analysis of the human T regulatory population uncovers functional heterogeneity and instability within FOXP3+ cells. *J Immunol* 186:6788-6797.

Dakhama, A., R.G. Hegele, G. Laflamme, E. Israel-Assayag, and Y. Cormier. 1999. Common respiratory viruses in lower airways of patients with acute hypersensitivity pneumonitis. *Am J Respir Crit Care Med* 159:1316-1322.

Dalrymple, H., B.P. Barna, A. Malur, A.G. Malur, M.S. Kavuru, and M.J. Thomassen. 2013. Alveolar macrophages of GM-CSF knockout mice exhibit mixed M1 and M2 phenotypes. *BMC immunology* 14:41.

de Boer, W.I., A. van Schadewijk, J.K. Sont, H.S. Sharma, J. Stolk, P.S. Hiemstra, and J.H. van Krieken. 1998. Transforming growth factor beta1 and recruitment of macrophages and mast cells in airways in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 158:1951-1957.

de Bree, G.J., H. Daniels, M. Schilfgaarde, H.M. Jansen, T.A. Out, R.A. van Lier, and R.E. Jonkers. 2007. Characterization of CD4+ memory T cell responses directed against common respiratory pathogens in peripheral blood and lung. *The Journal of infectious diseases* 195:1718-1725.

de Bree, G.J., E.M. van Leeuwen, T.A. Out, H.M. Jansen, R.E. Jonkers, and R.A. van Lier. 2005. Selective accumulation of differentiated CD8+ T cells specific for respiratory viruses in the human lung. *J Exp Med* 202:1433-1442.

Delmas, S., P. Brousset, D. Clement, E. Le Roy, and J.L. Davignon. 2007. Anti-IE1 CD4+ T-cell clones kill peptide-pulsed, but not human cytomegalovirus-infected, target cells. *The Journal of general virology* 88:2441-2449.

Deng, L., H. Zhou, J. Yang, J. Xiao, B. Wang, L. Wang, X. Ou, and Y. Feng. 2013. CTLA-4 gene polymorphisms and susceptibility to chronic obstructive pulmonary disease. *International journal of clinical and experimental pathology* 6:2548-2553.

Denning, T.L., Y.C. Wang, S.R. Patel, I.R. Williams, and B. Pulendran. 2007. Lamina propria macrophages and dendritic cells differentially induce regulatory and interleukin 17-producing T cell responses. *Nat Immunol* 8:1086-1094.

Di Stefano, A., A. Capelli, M. Lusuardi, P. Balbo, C. Vecchio, P. Maestrelli, C.E. Mapp, L.M. Fabbri, C.F. Donner, and M. Saetta. 1998. Severity of airflow limitation is associated with severity of airway inflammation in smokers. *Am J Respir Crit Care Med* 158:1277-1285.

Dolfi, D.V., K.D. Mansfield, A.M. Polley, S.A. Doyle, G.J. Freeman, H. Pircher, K.E. Schmader, and E.J. Wherry. 2013. Increased T-bet is associated with senescence of influenza virus-specific CD8 T cells in aged humans. *Journal of leukocyte biology* 93:825-836.

Domagala-Kulawik, J., M. Maskey-Warzechowska, I. Kraszewska, and R. Chazan. 2003. The cellular composition and macrophage phenotype in induced sputum in smokers and ex-smokers with COPD. *Chest* 123:1054-1059.

Donaldson, G.C., T. Seemungal, D.J. Jeffries, and J.A. Wedzicha. 1999. Effect of temperature on lung function and symptoms in chronic obstructive pulmonary disease. *Eur Respir J* 13:844-849.

Donaldson, G.C., T.A. Seemungal, A. Bhowmik, and J.A. Wedzicha. 2002. Relationship between exacerbation frequency and lung function decline in chronic obstructive pulmonary disease. *Thorax* 57:847-852.

Duan, M., W.C. Li, R. Vlahos, M.J. Maxwell, G.P. Anderson, and M.L. Hibbs. 2012. Distinct Macrophage Subpopulations Characterize Acute Infection and Chronic Inflammatory Lung Disease. *J Immunol*

Dullaers, M., R. De Bruyne, F. Ramadani, H.J. Gould, P. Gevaert, and B.N. Lambrecht. 2012. The who, where, and when of IgE in allergic airway disease. *The Journal of allergy and clinical immunology* 129:635-645.

Dunnill, M.S., G.R. Massarella, and J.A. Anderson. 1969. A comparison of the quantitative anatomy of the bronchi in normal subjects, in status asthmaticus, in chronic bronchitis, and in emphysema. *Thorax* 24:176-179.

Duraiswamy, J., C.C. Ibegbu, D. Masopust, J.D. Miller, K. Araki, G.H. Doho, P. Tata, S. Gupta, M.J. Zilliox, H.I. Nakaya, B. Pulendran, W.N. Haining, G.J. Freeman, and R. Ahmed. 2011. Phenotype, function, and gene expression profiles of programmed death-1(hi) CD8 T cells in healthy human adults. *J Immunol* 186:4200-4212.

Dustin, M.L. 2008. T-cell activation through immunological synapses and kinapses. *Immunological reviews* 221:77-89.

Effros, R.B. 2004. Replicative senescence of CD8 T cells: effect on human ageing. *Experimental gerontology* 39:517-524.

Eisner, M.D., J. Balmes, P.P. Katz, L. Trupin, E.H. Yelin, and P.D. Blanc. 2005. Lifetime environmental tobacco smoke exposure and the risk of chronic obstructive pulmonary disease. *Environmental health : a global access science source* 4:7.

Ekici, A., M. Ekici, E. Kurtipek, A. Akin, M. Arslan, T. Kara, Z. Apaydin, and S. Demir. 2005. Obstructive airway diseases in women exposed to biomass smoke. *Environmental research* 99:93-98.

Ely, K.H., L.S. Cauley, A.D. Roberts, J.W. Brennan, T. Cookenham, and D.L. Woodland. 2003. Nonspecific recruitment of memory CD8+ T cells to the lung airways during respiratory virus infections. *J Immunol* 170:1423-1429.

Ely, L.K., S.R. Burrows, A.W. Purcell, J. Rossjohn, and J. McCluskey. 2008. T-cells behaving badly: structural insights into alloreactivity and autoimmunity. *Current opinion in immunology* 20:575-580.

Eppert, B.L., B.W. Wortham, J.L. Flury, and M.T. Borchers. 2013. Functional characterization of T cell populations in a mouse model of chronic obstructive pulmonary disease. *J Immunol* 190:1331-1340.

Epstein, S.L., W.P. Kong, J.A. Misplon, C.Y. Lo, T.M. Tumpey, L. Xu, and G.J. Nabel. 2005. Protection against multiple influenza A subtypes by vaccination with highly conserved nucleoprotein. *Vaccine* 23:5404-5410.

Erickson, J.J., P. Gilchuk, A.K. Hastings, S.J. Tollefson, M. Johnson, M.B. Downing, K.L. Boyd, J.E. Johnson, A.S. Kim, S. Joyce, and J.V. Williams. 2012. Viral acute lower respiratory infections impair CD8+ T cells through PD-1. *J Clin Invest*

Erwig, L.P., and P.M. Henson. 2008. Clearance of apoptotic cells by phagocytes. *Cell death and differentiation* 15:243-250.

Evans, H.G., N.J. Gullick, S. Kelly, C. Pitzalis, G.M. Lord, B.W. Kirkham, and L.S. Taams. 2009. In vivo activated monocytes from the site of inflammation in humans specifically promote Th17 responses. *Proc Natl Acad Sci U S A* 106:6232-6237.

Fadok, V.A., D.L. Bratton, A. Konowal, P.W. Freed, J.Y. Westcott, and P.M. Henson. 1998. Macrophages that have ingested apoptotic cells in vitro inhibit proinflammatory cytokine production through autocrine/paracrine mechanisms involving TGF-beta, PGE2, and PAF. *J Clin Invest* 101:890-898.

Fang, M., N.A. Siciliano, A.R. Hersperger, F. Roscoe, A. Hu, X. Ma, A.R. Shamsedeen, L.C. Eisenlohr, and L.J. Sigal. 2012. Perforin-dependent CD4+ T-cell cytotoxicity contributes to control a murine poxvirus infection. *Proc Natl Acad Sci U S A*

Feenstra, T.L., M.L. van Genugten, R.T. Hoogenveen, E.F. Wouters, and M.P. Rutten-van Molken. 2001. The impact of aging and smoking on the future burden of chronic obstructive pulmonary disease: a model analysis in the Netherlands. *Am J Respir Crit Care Med* 164:590-596.

Finkelstein, R., R.S. Fraser, H. Ghezzo, and M.G. Cosio. 1995. Alveolar inflammation and its relation to emphysema in smokers. *Am J Respir Crit Care Med* 152:1666-1672.

Fletcher, C., and R. Peto. 1977. The natural history of chronic airflow obstruction. *British medical journal* 1:1645-1648.

Focosi, D., M. Bestagno, O. Burrone, and M. Petrini. 2010. CD57+ T lymphocytes and functional immune deficiency. *Journal of leukocyte biology* 87:107-116.

Fontenot, J.D., M.A. Gavin, and A.Y. Rudensky. 2003. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *Nat Immunol* 4:330-336.

Ford, A.L., E. Foulcher, A.L. Goodsall, and J.D. Sedgwick. 1996. Tissue digestion with dispase substantially reduces lymphocyte and macrophage cell-surface antigen expression. *Journal of immunological methods* 194:71-75.

Forster, R., A. Schubel, D. Breitfeld, E. Kremmer, I. Renner-Muller, E. Wolf, and M. Lipp. 1999. CCR7 coordinates the primary immune response by establishing functional microenvironments in secondary lymphoid organs. *Cell* 99:23-33.

Fourcade, J., Z. Sun, M. Benallaoua, P. Guillaume, I.F. Luescher, C. Sander, J.M. Kirkwood, V. Kuchroo, and H.M. Zarour. 2010. Upregulation of Tim-3 and PD-1 expression is associated with tumor antigen-specific CD8+ T cell dysfunction in melanoma patients. *J Exp Med* 207:2175-2186.

Freeman, G.J., A.J. Long, Y. Iwai, K. Bourque, T. Chernova, H. Nishimura, L.J. Fitz, N. Malenkovich, T. Okazaki, M.C. Byrne, H.F. Horton, L. Fouser, L. Carter, V. Ling, M.R. Bowman, B.M. Carreno, M. Collins, C.R. Wood, and T. Honjo. 2000. Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation. *J Exp Med* 192:1027-1034.

Fridman, W.H., F. Pages, C. Sautes-Fridman, and J. Galon. 2012. The immune contexture in human tumours: impact on clinical outcome. *Nature reviews. Cancer* 12:298-306.

Fujimoto, K., M. Yasuo, K. Urushibata, M. Hanaoka, T. Koizumi, and K. Kubo. 2005. Airway inflammation during stable and acutely exacerbated chronic obstructive pulmonary disease. *Eur Respir J* 25:640-646.

Fulton, R.B., D.K. Meyerholz, and S.M. Varga. 2010. Foxp3+ CD4 regulatory T cells limit pulmonary immunopathology by modulating the CD8 T cell response during respiratory syncytial virus infection. *J Immunol* 185:2382-2392.

Gaajetaan, G.R., T.H. Geelen, J.H. Vernooy, M.A. Dentener, N.L. Reynaert, G.G. Rohde, E.V. Beuken, G.E. Grauls, C.A. Bruggeman, and F.R. Stassen. 2013. Interferon-beta induces a long-lasting antiviral state in human respiratory epithelial cells. *The Journal of infection* 66:163-169.

Gaensler, E.A. 1950. Air velocity index; a numerical expression of the functionally effective portion of ventilation. *American review of tuberculosis* 62:17-28.

Gaga, M., A.M. Bentley, M. Humbert, J. Barkans, F. O'Brien, C.G. Wathen, A.B. Kay, and S.R. Durham. 1998. Increases in CD4+ T lymphocytes, macrophages, neutrophils and interleukin 8 positive cells in the airways of patients with bronchiectasis. *Thorax* 53:685-691.

Galkina, E., J. Thatte, V. Dabak, M.B. Williams, K. Ley, and T.J. Braciale. 2005. Preferential migration of effector CD8+ T cells into the interstitium of the normal lung. *J Clin Invest* 115:3473-3483.

Ganesan, S., A.T. Comstock, and U.S. Sajjan. 2013. Barrier function of airway tract epithelium. *Tissue barriers* 1:e24997.

Gao, X., Y. Zhu, G. Li, H. Huang, G. Zhang, F. Wang, J. Sun, Q. Yang, X. Zhang, and B. Lu. 2012. TIM-3 expression characterizes regulatory T cells in tumor tissues and is associated with lung cancer progression. *PLoS One* 7:e30676.

Garmendia, J., C. Viadas, L. Calatayud, J.C. Mell, P. Marti-Lliteras, B. Euba, E. Llobet, C. Gil, J.A. Bengoechea, R.J. Redfield, and J. Linares. 2014. Characterization of Nontypable Haemophilus influenzae Isolates Recovered from Adult Patients with Underlying Chronic Lung Disease Reveals Genotypic and Phenotypic Traits Associated with Persistent Infection. *PLoS One* 9:e97020.

Gascoigne, N.R., and E. Palmer. 2011. Signaling in thymic selection. *Current opinion in immunology* 23:207-212.

Gazzinelli, R.T., M. Wysocka, S. Hieny, T. Scharton-Kersten, A. Cheever, R. Kuhn, W. Muller, G. Trinchieri, and A. Sher. 1996. In the absence of endogenous IL-10, mice acutely infected with Toxoplasma gondii succumb to a lethal immune response dependent on CD4+ T cells and accompanied by overproduction of IL-12, IFN-gamma and TNF-alpha. *J Immunol* 157:798-805.

Geissmann, F., S. Jung, and D.R. Littman. 2003. Blood monocytes consist of two principal subsets with distinct migratory properties. *Immunity* 19:71-82.

George, S.N., D.S. Garcha, A.J. Mackay, A.R. Patel, R. Singh, R.J. Sapsford, G.C. Donaldson, and J.A. Wedzicha. 2014. Human rhinovirus infection during naturally occurring COPD exacerbations. *Eur Respir J*

Gerke, A.K., F. Tang, M. Yang, E.D. Foster, J.E. Cavanaugh, and P.M. Polgreen. 2013. Predicting chronic obstructive pulmonary disease hospitalizations based on concurrent influenza activity. *COPD* 10:573-580.

Ghiotto, M., L. Gauthier, N. Serriari, S. Pastor, A. Truneh, J.A. Nunes, and D. Olive. 2010. PD-L1 and PD-L2 differ in their molecular mechanisms of interaction with PD-1. *Int Immunol* 22:651-660.

Gielen, V., S.L. Johnston, and M.R. Edwards. 2010. Azithromycin induces anti-viral responses in bronchial epithelial cells. *Eur Respir J* 36:646-654.

GOLD. 2014. Global Strategy for the Diagnosis, Management and Prevention of COPD.

Golden-Mason, L., B. Palmer, J. Klarquist, J.A. Mengshol, N. Castelblanco, and H.R. Rosen. 2007. Upregulation of PD-1 expression on circulating and intrahepatic hepatitis C virus-specific CD8+ T cells associated with reversible immune dysfunction. *J Virol* 81:9249-9258.

Golden-Mason, L., B.E. Palmer, N. Kassam, L. Townshend-Bulson, S. Livingston, B.J. McMahon, N. Castelblanco, V. Kuchroo, D.R. Gretch, and H.R. Rosen. 2009. Negative immune regulator Tim-3 is overexpressed on T cells in hepatitis C virus infection and its blockade rescues dysfunctional CD4+ and CD8+ T cells. *J Virol* 83:9122-9130.

Grange, C., J. Letourneau, M.A. Forget, J. Godin-Ethier, J. Martin, M. Liberman, M. Latour, H. Widmer, J.B. Lattouf, C.A. Piccirillo, J.F. Cailhier, and R. Lapointe. 2011. Phenotypic characterization and functional analysis of human tumor immune infiltration after mechanical and enzymatic disaggregation. *Journal of immunological methods* 372:119-126.

Grube, D. 2004. Constants and variables in immunohistochemistry. *Archives of histology and cytology* 67:115-134.

Grundy, S., J. Plumb, S. Lea, M. Kaur, D. Ray, and D. Singh. 2013. Down regulation of T cell receptor expression in COPD pulmonary CD8 cells. *PLoS One* 8:e71629.

Guilliams, M., I. De Kleer, S. Henri, S. Post, L. Vanhoutte, S. De Prijck, K. Deswarte, B. Malissen, H. Hammad, and B.N. Lambrecht. 2013. Alveolar macrophages develop from fetal monocytes that differentiate into long-lived cells in the first week of life via GM-CSF. *J Exp Med* 210:1977-1992.

Hackett, T.L., F. Shaheen, A. Johnson, S. Wadsworth, D.V. Pechkovsky, D.B. Jacoby, A. Kicic, S.M. Stick, and D.A. Knight. 2008. Characterization of side population cells from human airway epithelium. *Stem Cells* 26:2576-2585.

Hadis, U., B. Wahl, O. Schulz, M. Hardtke-Wolenski, A. Schippers, N. Wagner, W. Muller, T. Sparwasser, R. Forster, and O. Pabst. 2011. Intestinal tolerance requires gut homing and expansion of FoxP3+ regulatory T cells in the lamina propria. *Immunity* 34:237-246.

Haeberle, H.A., R. Takizawa, A. Casola, A.R. Brasier, H.J. Dieterich, N. Van Rooijen, Z. Gatalica, and R.P. Garofalo. 2002. Respiratory syncytial virus-induced activation of nuclear factor-kappaB in the lung involves alveolar macrophages and toll-like receptor 4-dependent pathways. *The Journal of infectious diseases* 186:1199-1206.

Hagman, D.K., J.N. Kuzma, I. Larson, K.E. Foster-Schubert, L.Y. Kuan, A. Cignarella, E. Geamanu, K.W. Makar, J.R. Gottlieb, and M. Kratz. 2012. Characterizing and quantifying leukocyte populations in human adipose tissue: impact of enzymatic tissue processing. *Journal of immunological methods* 386:50-59.

Hagstad, S., A. Bjerg, L. Ekerljung, H. Backman, A. Lindberg, E. Ronmark, and B. Lundback. 2014. Passive smoking exposure is associated with increased Risk of COPD in never smokers. *Chest* 145:1298-1304.

Halbert, R.J., J.L. Natoli, A. Gano, E. Badamgarav, A.S. Buist, and D.M. Mannino. 2006. Global burden of COPD: systematic review and meta-analysis. *Eur Respir J* 28:523-532.

Hall, J.A., J.L. Cannons, J.R. Grainger, L.M. Dos Santos, T.W. Hand, S. Naik, E.A. Wohlfert, D.B. Chou, G. Oldenhove, M. Robinson, M.E. Grigg, R. Kastenmayer, P.L. Schwartzberg, and Y. Belkaid. 2011a. Essential role for retinoic acid in the promotion of CD4(+) T cell effector responses via retinoic acid receptor alpha. *Immunity* 34:435-447.

Hall, J.A., J.R. Grainger, S.P. Spencer, and Y. Belkaid. 2011b. The role of retinoic acid in tolerance and immunity. *Immunity* 35:13-22.

Halpern, M.T., R.H. Stanford, and R. Borker. 2003. The burden of COPD in the U.S.A.: results from the Confronting COPD survey. *Respir Med* 97 Suppl C:S81-89.

Hamann, D., P.A. Baars, M.H. Rep, B. Hooibrink, S.R. Kerkhof-Garde, M.R. Klein, and R.A. van Lier. 1997. Phenotypic and functional separation of memory and effector human CD8+ T cells. *J Exp Med* 186:1407-1418.

Han, M.K. 2011. Update in chronic obstructive pulmonary disease in 2010. *Am J Respir Crit Care Med* 183:1311-1315.

Hang do, T.T., J.Y. Song, M.Y. Kim, J.W. Park, and Y.K. Shin. 2011. Involvement of NF-kappaB in changes of IFN-gamma-induced CIITA/MHC-II and iNOS expression by influenza virus in macrophages. *Molecular immunology* 48:1253-1262.

Hardie, J.A., A.S. Buist, W.M. Vollmer, I. Ellingsen, P.S. Bakke, and O. Morkve. 2002. Risk of over-diagnosis of COPD in asymptomatic elderly never-smokers. *Eur Respir J* 20:1117-1122.

Hattotuwa, K.L., M.J. Gizycki, T.W. Ansari, P.K. Jeffery, and N.C. Barnes. 2002. The effects of inhaled fluticasone on airway inflammation in chronic obstructive pulmonary disease: a double-blind, placebo-controlled biopsy study. *Am J Respir Crit Care Med* 165:1592-1596.

He, X.S., K. Mahmood, H.T. Maecker, T.H. Holmes, G.W. Kemble, A.M. Arvin, and H.B. Greenberg. 2003. Analysis of the frequencies and of the memory T cell phenotypes of human CD8+ T cells specific for influenza A viruses. *The Journal of infectious diseases* 187:1075-1084.

Heidema, J., J.W. Rossen, M.V. Lukens, M.S. Ketel, E. Scheltens, M.E. Kranendonk, W.W. van Maren, A.M. van Loon, H.G. Otten, J.L. Kimpen, and G.M. van Bleek. 2008. Dynamics of human respiratory virus-specific CD8+ T cell responses in blood and airways during episodes of common cold. *J Immunol* 181:5551-5559.

Henrickson, S.E., and U.H. von Andrian. 2007. Single-cell dynamics of T-cell priming. *Current opinion in immunology* 19:249-258.

Henson, S.M., R. Macaulay, O. Franzese, and A.N. Akbar. 2011. Reversal of Functional Defects in highly differentiated young and old CD8 T Cells by PDL Blockade. *Immunology*

Heo, J.S., J.S. Park, E.J. Lee, T.H. Kim, A.S. Jang, S.W. Park, J.N. Kim, Y.G. Kim, S.T. Uh, J.S. Choi, J.O. Na, Y.H. Kim, S.H. Jeong, Y.B. Kim, S.R. Kim, and C.S. Park. 2013. Retinoic acid receptor alpha: One of plasma biomarkers associated with exacerbation of chronic obstructive pulmonary disease. *COPD*

Hilleman, D.E., N. Dewan, M. Malesker, and M. Friedman. 2000. Pharmacoeconomic evaluation of COPD. *Chest* 118:1278-1285.

Hinks, T.S.C. 2013. The Role Of T Cell Subsets In The Airways In Asthma. In Clinical and Experiment Sciences. University of Southampton, Southampton.

Hino, R., K. Kabashima, Y. Kato, H. Yagi, M. Nakamura, T. Honjo, T. Okazaki, and Y. Tokura. 2010. Tumor cell expression of programmed cell death-1 ligand 1 is a prognostic factor for malignant melanoma. *Cancer* 116:1757-1766.

Hnizdo, E., P.A. Sullivan, K.M. Bang, and G. Wagner. 2004. Airflow obstruction attributable to work in industry and occupation among U.S. race/ethnic groups: a study of NHANES III data. *American journal of industrial medicine* 46:126-135.

Hogg, J.C., F. Chu, S. Utokaparch, R. Woods, W.M. Elliott, L. Buzatu, R.M. Cherniack, R.M. Rogers, F.C. Sciurba, H.O. Coxson, and P.D. Pare. 2004. The nature of small-airway obstruction in chronic obstructive pulmonary disease. *The New England journal of medicine* 350:2645-2653.

Hogg, J.C., and W. Timens. 2009. The pathology of chronic obstructive pulmonary disease. *Annual review of pathology* 4:435-459.

Hogg, J.C., J.L. Wright, B.R. Wiggs, H.O. Coxson, A. Opazo Saez, and P.D. Pare. 1994. Lung structure and function in cigarette smokers. *Thorax* 49:473-478.

Holt, P.G., B.W. Robinson, M. Reid, U.R. Kees, A. Warton, V.H. Dawson, A. Rose, M. Schon-Hegrad, and J.M. Papadimitriou. 1986. Extraction of immune and inflammatory cells from human lung parenchyma: evaluation of an enzymatic digestion procedure. *Clin Exp Immunol* 66:188-200.

Hume, D.A. 2008. Macrophages as APC and the dendritic cell myth. *J Immunol* 181:5829-5835.

Hurst, J.R., G.C. Donaldson, W.R. Perera, T.M. Wilkinson, J.A. Bilello, G.W. Hagan, R.S. Vessey, and J.A. Wedzicha. 2006. Use of plasma biomarkers at exacerbation of chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 174:867-874.

Hurst, J.R., G.C. Donaldson, T.M. Wilkinson, W.R. Perera, and J.A. Wedzicha. 2005. Epidemiological relationships between the common cold and exacerbation frequency in COPD. *Eur Respir J* 26:846-852.

Hurst, J.R., J. Vestbo, A. Anzueto, N. Locantore, H. Mullerova, R. Tal-Singer, B. Miller, D.A. Lomas, A. Agusti, W. Macnee, P. Calverley, S. Rennard, E.F. Wouters, and J.A. Wedzicha. 2010. Susceptibility to exacerbation in chronic obstructive pulmonary disease. *The New England journal of medicine* 363:1128-1138.

Hutchinson, J. 1846. On the capacity of the lungs, and on the respiratory functions, with a view of establishing a precise and easy method of detecting disease by the spirometer. *Medico-chirurgical transactions* 29:137-252.

Ibegbu, C.C., Y.X. Xu, W. Harris, D. Maggio, J.D. Miller, and A.P. Kourtis. 2005. Expression of killer cell lectin-like receptor G1 on antigen-specific human CD8+ T lymphocytes during active, latent, and resolved infection and its relation with CD57. *J Immunol* 174:6088-6094.

Inghammar, M., G. Engstrom, B. Ljungberg, C.G. Lofdahl, A. Roth, and A. Egesten. 2014. Increased incidence of invasive bacterial disease in chronic obstructive pulmonary disease compared to the general population--a population based cohort study. *BMC infectious diseases* 14:163.

Ishida, M., Y. Iwai, Y. Tanaka, T. Okazaki, G.J. Freeman, N. Minato, and T. Honjo. 2002. Differential expression of PD-L1 and PD-L2, ligands for an inhibitory receptor PD-1, in the cells of lymphohematopoietic tissues. *Immunology letters* 84:57-62.

Ishida, Y., Y. Agata, K. Shibahara, and T. Honjo. 1992. Induced expression of PD-1, a novel member of the immunoglobulin gene superfamily, upon programmed cell death. *The EMBO journal* 11:3887-3895.

Ishiwata, K., N. Watanabe, M. Guo, K. Tomihara, M.J. Brumlik, H. Yagita, D. Pardoll, L. Chen, and T. Shin. 2010. Costimulator B7-DC attenuates strong Th2 responses induced by Nippostrongylus brasiliensis. *J Immunol* 184:2086-2094.

Iwai, Y., M. Ishida, Y. Tanaka, T. Okazaki, T. Honjo, and N. Minato. 2002. Involvement of PD-L1 on tumor cells in the escape from host immune system and tumor immunotherapy by PD-L1 blockade. *Proc Natl Acad Sci U S A* 99:12293-12297.

Iwata, M., A. Hirakiyama, Y. Eshima, H. Kagechika, C. Kato, and S.Y. Song. 2004. Retinoic acid imprints gut-homing specificity on T cells. *Immunity* 21:527-538.

Janeway, C.A., Jr., and R. Medzhitov. 2002. Innate immune recognition. *Annual review of immunology* 20:197-216.

Jellison, E.R., S.K. Kim, and R.M. Welsh. 2005. Cutting edge: MHC class II-restricted killing in vivo during viral infection. *J Immunol* 174:614-618.

Jenkins, C.R., B. Celli, J.A. Anderson, G.T. Ferguson, P.W. Jones, J. Vestbo, J.C. Yates, and P.M.A. Calverley. 2012. Seasonality and determinants of moderate and severe COPD exacerbations in the TORCH study. *European Respiratory Journal* 39:38-45.

Jin, H.T., A.C. Anderson, W.G. Tan, E.E. West, S.J. Ha, K. Araki, G.J. Freeman, V.K. Kuchroo, and R. Ahmed. 2010. Cooperation of Tim-3 and PD-1 in CD8 T-cell exhaustion during chronic viral infection. *Proc Natl Acad Sci U S A* 107:14733-14738.

Johannessen, A., T.D. Skorge, M. Bottai, T.B. Grydeland, R.M. Nilsen, H. Coxson, A. Dirksen, E. Omenaas, A. Gulsvik, and P. Bakke. 2013. Mortality by Level of Emphysema and Airway Wall Thickness. *Am J Respir Crit Care Med*

Jones, R.B., L.C. Ndhlovu, J.D. Barbour, P.M. Sheth, A.R. Jha, B.R. Long, J.C. Wong, M. Satkunarajah, M. Schweneker, J.M. Chapman, G. Gyenes, B. Vali, M.D. Hyrcza, F.Y. Yue, C. Kovacs, A. Sassi, M. Loutfy, R. Halpenny, D. Persad, G. Spotts, F.M. Hecht, T.W. Chun, J.M. McCune, R. Kaul, J.M. Rini, D.F. Nixon, and M.A. Ostrowski. 2008. Tim-3 expression defines a novel population of dysfunctional T cells with highly elevated frequencies in progressive HIV-1 infection. *J Exp Med* 205:2763-2779.

Juvet, S.C., and L. Zhang. 2012. Double negative regulatory T cells in transplantation and autoimmunity: recent progress and future directions. *Journal of molecular cell biology* 4:48-58.

K.J. Staples, C. M. Spalluto, R. T. McKendry, and T.M.A. Wilkinson. 2014. Influenza infection of human lung macrophages increases PDL1 expression via autologous IFNβ. In European Respiratory Society International Conference. Munich, Germany.

Kalathil, S.G., A.A. Lugade, V. Pradhan, A. Miller, G.I. Parameswaran, S. Sethi, and Y. Thanavala. 2014. T-regulatory cells and programmed death 1(+) T cells contribute to effector T-cell dysfunction in patients with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 190:40-50.

Karakurt, P., and A. Unsal. 2013. Fatigue, anxiety and depression levels, activities of daily living of patients with chronic obstructive pulmonary disease. *International journal of nursing practice* 19:221-231.

Kaul, P., M.C. Biagioli, I. Singh, and R.B. Turner. 2000. Rhinovirus-induced oxidative stress and interleukin-8 elaboration involves p47-phox but is independent of attachment to intercellular adhesion molecule-1 and viral replication. *The Journal of infectious diseases* 181:1885-1890.

Kaur, M., L.J. Smyth, P. Cadden, S. Grundy, D. Ray, J. Plumb, and D. Singh. 2012. T lymphocyte insensitivity to corticosteroids in chronic obstructive pulmonary disease. *Respir Res* 13:20.

Keatings, V.M., P.D. Collins, D.M. Scott, and P.J. Barnes. 1996. Differences in interleukin-8 and tumor necrosis factor-alpha in induced sputum from patients with chronic obstructive pulmonary disease or asthma. *Am J Respir Crit Care Med* 153:530-534.

Keir, M.E., S.C. Liang, I. Guleria, Y.E. Latchman, A. Qipo, L.A. Albacker, M. Koulmanda, G.J. Freeman, M.H. Sayegh, and A.H. Sharpe. 2006. Tissue expression of PD-L1 mediates peripheral T cell tolerance. *J Exp Med* 203:883-895.

Kim, H.M., Y.W. Lee, K.J. Lee, H.S. Kim, S.W. Cho, N. van Rooijen, Y. Guan, and S.H. Seo. 2008. Alveolar macrophages are indispensable for controlling influenza viruses in lungs of pigs. *J Virol* 82:4265-4274.

Knowles, M.R., and R.C. Boucher. 2002. Mucus clearance as a primary innate defense mechanism for mammalian airways. *J Clin Invest* 109:571-577.

Knudson, C.J., K.A. Weiss, S.M. Hartwig, and S.M. Varga. 2014. The pulmonary localization of virus-specific T lymphocytes is governed by the tissue tropism of infection. *J Virol* 88:9010-9016.

Koch, A., M. Gaczkowski, G. Sturton, P. Staib, T. Schinkothe, E. Klein, A. Rubbert, K. Bacon, K. Wassermann, and E. Erdmann. 2007. Modification of surface antigens in blood CD8+ T-lymphocytes in COPD: effects of smoking. *Eur Respir J* 29:42-50.

Koch, S., A. Larbi, E. Derhovanessian, D. Ozcelik, E. Naumova, and G. Pawelec. 2008. Multiparameter flow cytometric analysis of CD4 and CD8 T cell subsets in young and old people. *Immunity & ageing : I & A* 5:6.

Kohansal, R., P. Martinez-Camblor, A. Agusti, A.S. Buist, D.M. Mannino, and J.B. Soriano. 2009. The natural history of chronic airflow obstruction revisited: an analysis of the Framingham offspring cohort. *Am J Respir Crit Care Med* 180:3-10.

Kohlmeier, J.E., T. Cookenham, A.D. Roberts, S.C. Miller, and D.L. Woodland. 2010. Type I interferons regulate cytolytic activity of memory CD8(+) T cells in the lung airways during respiratory virus challenge. *Immunity* 33:96-105.

Kotturi, M.F., J.A. Swann, B. Peters, C.L. Arlehamn, J. Sidney, R.V. Kolla, E.A. James, R.S. Akondy, R. Ahmed, W.W. Kwok, M.J. Buchmeier, and A. Sette. 2011. Human CD8 and CD4 T cell memory to lymphocytic choriomeningitis virus infection. *J Virol* 85:11770-11780.

Kronenberg, M., and Y. Kinjo. 2009. Innate-like recognition of microbes by invariant natural killer T cells. *Current opinion in immunology* 21:391-396.

Kumagai-Takei, N., Y. Nishimura, M. Maeda, H. Hayashi, H. Matsuzaki, S. Lee, J. Hiratsuka, and T. Otsuki. 2013. Effect of asbestos exposure on differentiation of cytotoxic T lymphocytes in mixed lymphocyte reaction of human peripheral blood mononuclear cells. *Am J Respir Cell Mol Biol* 49:28-36.

Kumagai, Y., O. Takeuchi, H. Kato, H. Kumar, K. Matsui, E. Morii, K. Aozasa, T. Kawai, and S. Akira. 2007. Alveolar macrophages are the primary interferon-alpha producer in pulmonary infection with RNA viruses. *Immunity* 27:240-252.

Laënnec, R. 1821. A treatise on mediate auscultation and on diseases of the lungs and heart. Bailliere, London.

Lamprecht, B., L. Schirnhofer, B. Kaiser, M. Studnicka, and A.S. Buist. 2007. Farming and the prevalence of non-reversible airways obstruction: results from a population-based study. *American journal of industrial medicine* 50:421-426.

Landsman, L., and S. Jung. 2007. Lung macrophages serve as obligatory intermediate between blood monocytes and alveolar macrophages. *J Immunol* 179:3488-3494.

Lanier, L.L. 2008. Up on the tightrope: natural killer cell activation and inhibition. *Nat Immunol* 9:495-502.

Laskin, D.L., B. Weinberger, and J.D. Laskin. 2001. Functional heterogeneity in liver and lung macrophages. *Journal of leukocyte biology* 70:163-170.

Latchman, Y., C.R. Wood, T. Chernova, D. Chaudhary, M. Borde, I. Chernova, Y. Iwai, A.J. Long, J.A. Brown, R. Nunes, E.A. Greenfield, K. Bourque, V.A. Boussiotis, L.L. Carter, B.M. Carreno, N. Malenkovich, H. Nishimura, T. Okazaki, T. Honjo, A.H. Sharpe, and G.J. Freeman. 2001. PD-L2 is a second ligand for PD-1 and inhibits T cell activation. *Nat Immunol* 2:261-268.

Laurell, C.B., and A. Eriksson. 1963. The electrophoretic alpha 1-globulin pattern of serum in alpha 1-antitrypsin deficiency. *Scandinavian journal of clinical and laboratory investigation* 15:132–140.

Lee, S.H., S. Goswami, A. Grudo, L.Z. Song, V. Bandi, S. Goodnight-White, L. Green, J. Hacken-Bitar, J. Huh, F. Bakaeen, H.O. Coxson, S. Cogswell, C. Storness-Bliss, D.B. Corry, and F. Kheradmand. 2007. Antielastin autoimmunity in tobacco smoking-induced emphysema. *Nature medicine* 13:567-569.

Leigh, J.P., P.S. Romano, M.B. Schenker, and K. Kreiss. 2002. Costs of occupational COPD and asthma. *Chest* 121:264-272.

Li, D., R. Guabiraba, A.G. Besnard, M. Komai-Koma, M.S. Jabir, L. Zhang, G.J. Graham, M. Kurowska-Stolarska, F.Y. Liew, C. McSharry, and D. Xu. 2014. IL-33 promotes ST2-dependent lung fibrosis by the induction of alternatively activated macrophages and innate lymphoid cells in mice. *The Journal of allergy and clinical immunology*

Liang, S.C., Y.E. Latchman, J.E. Buhlmann, M.F. Tomczak, B.H. Horwitz, G.J. Freeman, and A.H. Sharpe. 2003. Regulation of PD-1, PD-L1, and PD-L2 expression during normal and autoimmune responses. *Eur J Immunol* 33:2706-2716.

Lieberman, J., B. Winter, and A. Sastre. 1986. Alpha 1-antitrypsin Pi-types in 965 COPD patients. *Chest* 89:370-373.

Lipson, E.J., W.H. Sharfman, C.G. Drake, I. Wollner, J.M. Taube, R.A. Anders, H. Xu, S. Yao, A. Pons, L. Chen, D.M. Pardoll, J.R. Brahmer, and S.L. Topalian. 2013. Durable cancer regression off-treatment and effective reinduction therapy with an anti-PD-1 antibody. *Clinical cancer research : an official journal of the American Association for Cancer Research* 19:462-468.

Lugade, A.A., P.N. Bogner, T.H. Thatcher, P.J. Sime, R.P. Phipps, and Y. Thanavala. 2014. Cigarette Smoke Exposure Exacerbates Lung Inflammation and Compromises Immunity to Bacterial Infection. *J Immunol*

Lutz, M.B., and C. Kurts. 2009. Induction of peripheral CD4+ T-cell tolerance and CD8+ T-cell cross-tolerance by dendritic cells. *Eur J Immunol* 39:2325-2330.

Mahajan, D., Y. Wang, X. Qin, Y. Wang, G. Zheng, Y.M. Wang, S.I. Alexander, and D.C. Harris. 2006. CD4+CD25+ regulatory T cells protect against injury in an innate murine model of chronic kidney disease. *Journal of the American Society of Nephrology : JASN* 17:2731-2741.

Maino, V.C., and L.J. Picker. 1998. Identification of functional subsets by flow cytometry: intracellular detection of cytokine expression. *Cytometry* 34:207-215.

Mallia, P., J. Footitt, R. Sotero, A. Jepson, M. Contoli, M.B. Trujillo-Torralbo, T. Kebadze, J. Aniscenko, G. Oleszkiewicz, K. Gray, S.D. Message, K. Ito, P.J. Barnes, I.M. Adcock, A. Papi, L.A. Stanciu, S.L. Elkin, O.M. Kon, M. Johnson, and S.L. Johnston. 2012. Rhinovirus infection induces degradation of antimicrobial peptides and secondary bacterial infection in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 186:1117-1124.

Mallia, P., S.D. Message, V. Gielen, M. Contoli, K. Gray, T. Kebadze, J. Aniscenko, V. Laza-Stanca, M.R. Edwards, L. Slater, A. Papi, L.A. Stanciu, O.M. Kon, M. Johnson, and S.L. Johnston. 2011. Experimental rhinovirus infection as a human model of chronic obstructive pulmonary disease exacerbation. *Am J Respir Crit Care Med* 183:734-742.

Man, W.D., P. Kemp, J. Moxham, and M.I. Polkey. 2009. Skeletal muscle dysfunction in COPD: clinical and laboratory observations. *Clin Sci (Lond)* 117:251-264.

Manicassamy, S., R. Ravindran, J. Deng, H. Oluoch, T.L. Denning, S.P. Kasturi, K.M. Rosenthal, B.D. Evavold, and B. Pulendran. 2009. Toll-like receptor 2-dependent induction of vitamin A-metabolizing enzymes in dendritic cells promotes T regulatory responses and inhibits autoimmunity. *Nature medicine* 15:401-409.

Mantovani, B., M. Rabinovitch, and V. Nussenzweig. 1972. Phagocytosis of immune complexes by macrophages. Different roles of the macrophage receptor sites for complement (C3) and for immunoglobulin (IgG). *J Exp Med* 135:780-792.

Marin, A., J. Garcia-Aymerich, J. Sauleda, J. Belda, L. Millares, M. Garcia-Nunez, I. Serra, M. Benet, A. Agusti, J.M. Anto, E. Monso, and G. On Behalf Of The Pac-Copd Study. 2012. Effect of Bronchial Colonisation on Airway and Systemic Inflammation in Stable COPD. *COPD*

Martin, A.R., H.A. Costa, T. Lappalainen, B.M. Henn, J.M. Kidd, M.C. Yee, F. Grubert, H.M. Cann, M. Snyder, S.B. Montgomery, and C.D. Bustamante. 2014. Transcriptome sequencing from diverse human populations reveals differentiated regulatory architecture. *PLoS genetics* 10:e1004549.

Masopust, D., V. Vezys, A.L. Marzo, and L. Lefrancois. 2001. Preferential localization of effector memory cells in nonlymphoid tissue. *Science* 291:2413-2417.

Masopust, D., V. Vezys, E.J. Wherry, and R. Ahmed. 2007. A brief history of CD8 T cells. *Eur J Immunol* 37 Suppl 1:S103-110.

Mathers, C.D., and D. Loncar. 2006. Projections of global mortality and burden of disease from 2002 to 2030. *PLoS Med* 3:e442.

Matloubian, M., R.J. Concepcion, and R. Ahmed. 1994. CD4+ T cells are required to sustain CD8+ cytotoxic T-cell responses during chronic viral infection. *J Virol* 68:8056-8063.

Maus, U.A., S. Janzen, G. Wall, M. Srivastava, T.S. Blackwell, J.W. Christman, W. Seeger, T. Welte, and J. Lohmeyer. 2006. Resident alveolar macrophages are replaced by recruited monocytes in response to endotoxin-induced lung inflammation. *Am J Respir Cell Mol Biol* 35:227-235.

McCormack, J.M., D. Askew, and W.S. Walker. 1993. Alloantigen presentation by individual clones of mouse splenic macrophages. Selective expression of IL-1 alpha in response to CD8+ T cell-derived IFN-gamma defines the alloantigen-presenting phenotype. *J Immunol* 151:5218-5227.

McManus, T.E., A.M. Marley, N. Baxter, S.N. Christie, J.S. Elborn, L.G. Heaney, P.V. Coyle, and J.C. Kidney. 2007. Acute and latent adenovirus in COPD. *Respir Med* 101:2084-2090.

McNally, B., F. Ye, M. Willette, and E. Flano. 2013. Local Blockade of Epithelial PDL-1 in the Airways Enhances T Cell Function and Viral Clearance during Influenza Virus Infection. *J Virol* 87:12916-12924.

Mehta, A.J., D. Miedinger, D. Keidel, R. Bettschart, A. Bircher, P.O. Bridevaux, I. Curjuric, H. Kromhout, T. Rochat, T. Rothe, E.W. Russi, T. Schikowski, C. Schindler, J. Schwartz, A. Turk, R. Vermeulen, N. Probst-Hensch, and N. Kuenzli. 2012. Occupational Exposure to Dusts, Gases and Fumes and Incidence of COPD in SAPALDIA. *Am J Respir Crit Care Med*

Mercer, P.F., J.K. Shute, A. Bhowmik, G.C. Donaldson, J.A. Wedzicha, and J.A. Warner. 2005. MMP-9, TIMP-1 and inflammatory cells in sputum from COPD patients during exacerbation. *Respir Res* 6:151.

Miller, M.R., J. Hankinson, V. Brusasco, F. Burgos, R. Casaburi, A. Coates, R. Crapo, P. Enright, C.P. van der Grinten, P. Gustafsson, R. Jensen, D.C. Johnson, N. MacIntyre, R. McKay, D. Navajas, O.F. Pedersen, R. Pellegrino, G. Viegi, J. Wanger, and A.E.T. Force. 2005. Standardisation of spirometry. *Eur Respir J* 26:319-338.

Mohamed Hoesein, F.A., B. de Hoop, P. Zanen, H. Gietema, C.L. Kruitwagen, B. van Ginneken, I. Isgum, C. Mol, R.J. van Klaveren, A.E. Dijkstra, H.J. Groen, H.M. Boezen, D.S. Postma, M. Prokop, and J.W. Lammers. 2011. CT-quantified emphysema in male heavy smokers: association with lung function decline. *Thorax* 66:782-787.

Moro, K., T. Yamada, M. Tanabe, T. Takeuchi, T. Ikawa, H. Kawamoto, J. Furusawa, M. Ohtani, H. Fujii, and S. Koyasu. 2010. Innate production of T(H)2 cytokines by adipose tissue-associated c-Kit(+)Sca-1(+) lymphoid cells. *Nature* 463:540-544.

Morris, G.P., and P.M. Allen. 2012. How the TCR balances sensitivity and specificity for the recognition of self and pathogens. *Nat Immunol* 13:121-128.

Mosser, D.M., and J.P. Edwards. 2008. Exploring the full spectrum of macrophage activation. *Nat Rev Immunol* 8:958-969.

Mueller, S.N., W.A. Langley, E. Carnero, A. Garcia-Sastre, and R. Ahmed. 2010. Immunization with live attenuated influenza viruses that express altered NS1 proteins results in potent and protective memory CD8+ T-cell responses. *J Virol* 84:1847-1855.

Mueller, S.N., M. Matloubian, D.M. Clemens, A.H. Sharpe, G.J. Freeman, S. Gangappa, C.P. Larsen, and R. Ahmed. 2007. Viral targeting of fibroblastic reticular cells contributes to immunosuppression and persistence during chronic infection. *Proc Natl Acad Sci U S A* 104:15430-15435.

Mulder, D.J., A. Pooni, N. Mak, D.J. Hurlbut, S. Basta, and C.J. Justinich. 2011. Antigen presentation and MHC class II expression by human esophageal epithelial cells: role in eosinophilic esophagitis. *The American journal of pathology* 178:744-753.

Nakanishi, J., Y. Wada, K. Matsumoto, M. Azuma, K. Kikuchi, and S. Ueda. 2007. Overexpression of B7-H1 (PD-L1) significantly associates with tumor grade and postoperative prognosis in human urothelial cancers. *Cancer immunology, immunotherapy : CII* 56:1173-1182.

Nicholas, B.L., K.J. Staples, S. Moese, J. Ward, P. Dennison, T. Havelock, T.S.C. Hinks, K. Amer, E. Woo, M. Chamberlain, N. Singh, M. North, S. Pink, T.M.A. Wilkinson, and R. Djukanović. 2013. A human lung explant model for the study of anti-viral drugs. In European Respiratory Society International Conference. Barcelona, Spain.

Nishimura, H., M. Nose, H. Hiai, N. Minato, and T. Honjo. 1999. Development of lupus-like autoimmune diseases by disruption of the PD-1 gene encoding an ITIM motif-carrying immunoreceptor. *Immunity* 11:141-151.

Nishimura, H., T. Okazaki, Y. Tanaka, K. Nakatani, M. Hara, A. Matsumori, S. Sasayama, A. Mizoguchi, H. Hiai, N. Minato, and T. Honjo. 2001. Autoimmune dilated cardiomyopathy in PD-1 receptor-deficient mice. *Science* 291:319-322.

Nishimura, K., T. Izumi, M. Tsukino, and T. Oga. 2002. Dyspnea is a better predictor of 5-year survival than airway obstruction in patients with COPD. *Chest* 121:1434-1440.

Nolting, J., C. Daniel, S. Reuter, C. Stuelten, P. Li, H. Sucov, B.G. Kim, J.J. Letterio, K. Kretschmer, H.J. Kim, and H. von Boehmer. 2009. Retinoic acid can enhance conversion of naive into regulatory T cells independently of secreted cytokines. *J Exp Med* 206:2131-2139.

Novelli, M., P. Savoia, I. Cambieri, R. Ponti, A. Comessatti, F. Lisa, and M.G. Bernengo. 2000. Collagenase digestion and mechanical disaggregation as a method to extract and immunophenotype tumour lymphocytes in cutaneous T-cell lymphomas. *Clinical and experimental dermatology* 25:423-431.

O'Neil, S.E., B. Lundback, and J. Lotvall. 2011. Proteomics in asthma and COPD phenotypes and endotypes for biomarker discovery and improved understanding of disease entities. *Journal of proteomics* 75:192-201.

O'Shaughnessy, T.C., T.W. Ansari, N.C. Barnes, and P.K. Jeffery. 1997. Inflammation in bronchial biopsies of subjects with chronic bronchitis: inverse relationship of CD8+ T lymphocytes with FEV1. *Am J Respir Crit Care Med* 155:852-857.

Okazaki, T., and T. Honjo. 2006. The PD-1-PD-L pathway in immunological tolerance. *Trends in immunology* 27:195-201.

Olloquequi, J., J. Garcia-Valero, E. Rodriguez, M.A. Montero, J. Ferrer, and J.F. Montes. 2012. Lung CD57+ cell density is increased in very severe COPD. *Histology and histopathology* 27:39-47.

Orozco-Levi, M., J. Garcia-Aymerich, J. Villar, A. Ramirez-Sarmiento, J.M. Anto, and J. Gea. 2006. Wood smoke exposure and risk of chronic obstructive pulmonary disease. *Eur Respir J* 27:542-546.

Ott, P.A., F.S. Hodi, and C. Robert. 2013. CTLA-4 and PD-1/PD-L1 blockade: new immunotherapeutic modalities with durable clinical benefit in melanoma patients. *Clinical cancer research : an official journal of the American Association for Cancer Research* 19:5300-5309.

Ottesen, G.L., I.J. Christensen, J.K. Larsen, B. Hansen, and J.A. Andersen. 1996. Tissue disaggregation for flow cytometric DNA analysis: comparison of fine-needle aspiration and an automated mechanical procedure. *Cytometry* 26:65-68.

Owen, C.A. 2005. Proteinases and oxidants as targets in the treatment of chronic obstructive pulmonary disease. *Proc Am Thorac Soc* 2:373-385; discussion 394-375.

Packard, T.A., Q.Z. Li, G.P. Cosgrove, R.P. Bowler, and J.C. Cambier. 2013. COPD is associated with production of autoantibodies to a broad spectrum of self-antigens, correlative with disease phenotype. *Immunologic research* 55:48-57.

Papagno, L., C.A. Spina, A. Marchant, M. Salio, N. Rufer, S. Little, T. Dong, G. Chesney, A. Waters, P. Easterbrook, P.R. Dunbar, D. Shepherd, V. Cerundolo, V. Emery, P. Griffiths, C. Conlon, A.J. McMichael, D.D. Richman, S.L. Rowland-Jones, and V. Appay. 2004. Immune activation and CD8+ T-cell differentiation towards senescence in HIV-1 infection. *PLoS biology* 2:E20.

Papi, A., C.M. Bellettato, F. Braccioni, M. Romagnoli, P. Casolari, G. Caramori, L.M. Fabbri, and S.L. Johnston. 2006. Infections and airway inflammation in chronic obstructive pulmonary disease severe exacerbations. *Am J Respir Crit Care Med* 173:1114-1121.

Papi, A., L.A. Stanciu, N.G. Papadopoulos, L.M. Teran, S.T. Holgate, and S.L. Johnston. 2000. Rhinovirus infection induces major histocompatibility complex class I and costimulatory molecule upregulation on respiratory epithelial cells. *The Journal of infectious diseases* 181:1780-1784.

Papiris, S.A., A. Kollintza, P. Kitsanta, G. Kapotsis, M. Karatza, J. Milic-Emili, C. Roussos, and Z. Daniil. 2005. Relationship of BAL and lung tissue CD4+ and CD8+ T lymphocytes, and their ratio in idiopathic pulmonary fibrosis. *Chest* 128:2971-2977.

Parker, C.M., N. Voduc, S.D. Aaron, K.A. Webb, and D.E. O'Donnell. 2005. Physiological changes during symptom recovery from moderate exacerbations of COPD. *Eur Respir J* 26:420-428.

Parry, R.V., J.M. Chemnitz, K.A. Frauwirth, A.R. Lanfranco, I. Braunstein, S.V. Kobayashi, P.S. Linsley, C.B. Thompson, and J.L. Riley. 2005. CTLA-4 and PD-1 receptors inhibit T-cell activation by distinct mechanisms. *Molecular and cellular biology* 25:9543-9553.

Penaloza-MacMaster, P., A.O. Kamphorst, A. Wieland, K. Araki, S.S. Iyer, E.E. West, L. O'Mara, S. Yang, B.T. Konieczny, A.H. Sharpe, G.J. Freeman, A.Y. Rudensky, and R. Ahmed. 2014. Interplay between regulatory T cells and PD-1 in modulating T cell exhaustion and viral control during chronic LCMV infection. *J Exp Med* 211:1905-1918.

Peretz, Y., Z. He, Y. Shi, B. Yassine-Diab, J.P. Goulet, R. Bordi, A. Filali-Mouhim, J.B. Loubert, M. El-Far, F.P. Dupuy, M.R. Boulassel, C. Tremblay, J.P. Routy, N. Bernard, R. Balderas, E.K. Haddad, and R.P. Sekaly. 2012. CD160 and PD-1 co-expression on HIV-specific CD8 T cells defines a subset with advanced dysfunction. *PLoS pathogens* 8:e1002840.

Perez-Padilla, R., J. Regalado, S. Vedal, P. Pare, R. Chapela, R. Sansores, and M. Selman. 1996. Exposure to biomass smoke and chronic airway disease in Mexican women. A case-control study. *Am J Respir Crit Care Med* 154:701-706.

Pesci, A., M. Majori, A. Cuomo, N. Borciani, S. Bertacco, G. Cacciani, and M. Gabrielli. 1998. Neutrophils infiltrating bronchial epithelium in chronic obstructive pulmonary disease. *Respir Med* 92:863-870.

Peters, P.J., J. Borst, V. Oorschot, M. Fukuda, O. Krahenbuhl, J. Tschopp, J.W. Slot, and H.J. Geuze. 1991. Cytotoxic T lymphocyte granules are secretory lysosomes, containing both perforin and granzymes. *J Exp Med* 173:1099-1109.

Peters, W., H.M. Scott, H.F. Chambers, J.L. Flynn, I.F. Charo, and J.D. Ernst. 2001. Chemokine receptor 2 serves an early and essential role in resistance to Mycobacterium tuberculosis. *Proc Natl Acad Sci U S A* 98:7958-7963.

Pilgaard, L., P. Lund, J.G. Rasmussen, T. Fink, and V. Zachar. 2008. Comparative analysis of highly defined proteases for the isolation of adipose tissue-derived stem cells. *Regenerative medicine* 3:705-715.

Pino-Lagos, K., Y. Guo, C. Brown, M.P. Alexander, R. Elgueta, K.A. Bennett, V. De Vries, E. Nowak, R. Blomhoff, S. Sockanathan, R.A. Chandraratna, E. Dmitrovsky, and R.J.

Noelle. 2011. A retinoic acid-dependent checkpoint in the development of CD4+ T cell-mediated immunity. *J Exp Med* 208:1767-1775.

Plumb, J., L. Robinson, S. Lea, A. Banyard, J. Blaikley, D. Ray, A. Bizzi, G. Volpi, F. Facchinetti, and D. Singh. 2013. Evaluation of glucocorticoid receptor function in COPD lung macrophages using beclomethasone-17-monopropionate. *PLoS One* 8:e64257.

Polosukhin, V.V. 2001. Ultrastructural of the bronchial epithelium in chronic inflammation. *Ultrastructural pathology* 25:119-128.

Polosukhin, V.V., J.M. Cates, W.E. Lawson, R. Zaynagetdinov, A.P. Milstone, P.P. Massion, S. Ocak, L.B. Ware, J.W. Lee, R.P. Bowler, A.V. Kononov, S.H. Randell, and T.S. Blackwell. 2011. Bronchial secretory immunoglobulin a deficiency correlates with airway inflammation and progression of chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 184:317-327.

Prezzemolo, T., G. Guggino, M.P. La Manna, D. Di Liberto, F. Dieli, and N. Caccamo. 2014. Functional Signatures of Human CD4 and CD8 T Cell Responses to Mycobacterium tuberculosis. *Frontiers in immunology* 5:180.

Punkosdy, G.A., M. Blain, D.D. Glass, M.M. Lozano, L. O'Mara, J.P. Dudley, R. Ahmed, and E.M. Shevach. 2011. Regulatory T-cell expansion during chronic viral infection is dependent on endogenous retroviral superantigens. *Proc Natl Acad Sci U S A* 108:3677-3682.

Purwar, R., J. Campbell, G. Murphy, W.G. Richards, R.A. Clark, and T.S. Kupper. 2011. Resident memory T cells (T(RM)) are abundant in human lung: diversity, function, and antigen specificity. *PLoS One* 6:e16245.

Qiu, Y., J. Zhu, V. Bandi, R.L. Atmar, K. Hattotuwa, K.K. Guntupalli, and P.K. Jeffery. 2003. Biopsy neutrophilia, neutrophil chemokine and receptor gene expression in severe exacerbations of chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 168:968-975.

Rai, K.R., A. Sawitsky, E.P. Cronkite, A.D. Chanana, R.N. Levy, and B.S. Pasternack. 1975. Clinical staging of chronic lymphocytic leukemia. *Blood* 46:219-234.

Ralainirina, N., A. Poli, T. Michel, L. Poos, E. Andres, F. Hentges, and J. Zimmer. 2007. Control of NK cell functions by CD4+CD25+ regulatory T cells. *Journal of leukocyte biology* 81:144-153.

Rhodes, A., B. Jasani, A.J. Balaton, and K.D. Miller. 2000. Immunohistochemical demonstration of oestrogen and progesterone receptors: correlation of standards achieved on in house tumours with that achieved on external quality assessment material in over 150 laboratories from 26 countries. *Journal of clinical pathology* 53:292-301.

Richter, K., P. Agnellini, and A. Oxenius. 2010. On the role of the inhibitory receptor LAG-3 in acute and chronic LCMV infection. *Int Immunol* 22:13-23.

Riley, J.L., and C.H. June. 2005. The CD28 family: a T-cell rheostat for therapeutic control of T-cell activation. *Blood* 105:13-21.

Rohde, G., A. Wiethege, I. Borg, M. Kauth, T.T. Bauer, A. Gillissen, A. Bufe, and G. Schultze-Werninghaus. 2003. Respiratory viruses in exacerbations of chronic obstructive pulmonary disease requiring hospitalisation: a case-control study. *Thorax* 58:37-42.

Rowe, J.H., T.M. Johanns, J.M. Ertelt, and S.S. Way. 2008. PDL-1 blockade impedes T cell expansion and protective immunity primed by attenuated Listeria monocytogenes. *J Immunol* 180:7553-7557.

Ruckwardt, T.J., C. Luongo, A.M. Malloy, J. Liu, M. Chen, P.L. Collins, and B.S. Graham. 2010. Responses against a subdominant CD8+ T cell epitope protect against immunopathology caused by a dominant epitope. *J Immunol* 185:4673-4680.

Rutten, F.H., K.G. Moons, M.J. Cramer, D.E. Grobbee, N.P. Zuithoff, J.W. Lammers, and A.W. Hoes. 2005. Recognising heart failure in elderly patients with stable chronic obstructive pulmonary disease in primary care: cross sectional diagnostic study. *BMJ* 331:1379.

Saetta, M., A. Di Stefano, G. Turato, F.M. Facchini, L. Corbino, C.E. Mapp, P. Maestrelli, A. Ciaccia, and L.M. Fabbri. 1998. CD8+ T-lymphocytes in peripheral airways of smokers with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 157:822-826.

Saito, J., A.J. Mackay, C. Rossios, D. Gibeon, P. Macedo, R. Sinharay, P.K. Bhavsar, J.A. Wedzicha, and K.F. Chung. 2014. Sputum-to-serum hydrogen sulfide ratio in COPD. *Thorax* 69:903-909.

Sakaguchi, S., N. Sakaguchi, M. Asano, M. Itoh, and M. Toda. 1995. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J Immunol* 155:1151-1164.

Sakhdari, A., S. Mujib, B. Vali, F.Y. Yue, S. MacParland, K. Clayton, R.B. Jones, J. Liu, E.Y. Lee, E. Benko, C. Kovacs, J. Gommerman, R. Kaul, and M.A. Ostrowski. 2012. Tim-3 negatively regulates cytotoxicity in exhausted CD8+ T cells in HIV infection. *PLoS One* 7:e40146.

Sakuishi, K., L. Apetoh, J.M. Sullivan, B.R. Blazar, V.K. Kuchroo, and A.C. Anderson. 2010. Targeting Tim-3 and PD-1 pathways to reverse T cell exhaustion and restore anti-tumor immunity. *J Exp Med* 207:2187-2194.

Salazar, L.M., and A.M. Herrera. 2011. Fibrotic response of tissue remodeling in COPD. *Lung* 189:101-109.

Sallusto, F., J. Geginat, and A. Lanzavecchia. 2004. Central memory and effector memory T cell subsets: function, generation, and maintenance. *Annual review of immunology* 22:745-763.

Sallusto, F., D. Lenig, R. Forster, M. Lipp, and A. Lanzavecchia. 1999. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature* 401:708-712.

Sauce, D., J.R. Almeida, M. Larsen, L. Haro, B. Autran, G.J. Freeman, and V. Appay. 2007. PD-1 expression on human CD8 T cells depends on both state of differentiation and activation status. *AIDS* 21:2005-2013.

Schneider, C., S.P. Nobs, A.K. Heer, M. Kurrer, G. Klinke, N. van Rooijen, J. Vogel, and M. Kopf. 2014. Alveolar macrophages are essential for protection from respiratory failure and associated morbidity following influenza virus infection. *PLoS pathogens* 10:e1004053.

Schneider, D., S. Ganesan, A.T. Comstock, C.A. Meldrum, R. Mahidhara, A.M. Goldsmith, J.L. Curtis, F.J. Martinez, M.B. Hershenson, and U. Sajjan. 2010. Increased cytokine response of rhinovirus-infected airway epithelial cells in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 182:332-340.

Schroeder, J.D., A.S. McKenzie, J.A. Zach, C.G. Wilson, D. Curran-Everett, D.S. Stinson, J.D. Newell, Jr., and D.A. Lynch. 2013. Relationships between airflow obstruction and quantitative CT measurements of emphysema, air trapping, and airways in subjects with and without chronic obstructive pulmonary disease. *AJR. American journal of roentgenology* 201:W460-470.

Schwartz, A.G., and J.C. Ruckdeschel. 2006. Familial lung cancer: genetic susceptibility and relationship to chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 173:16-22.

Sckisel, G.D., J.K. Tietze, A.E. Zamora, H.H. Hsiao, S.O. Priest, D.E. Wilkins, L.L. Lanier, B.R. Blazar, N. Baumgarth, and W.J. Murphy. 2014. Influenza infection results in local expansion of memory CD8(+) T cells with antigen non-specific phenotype and function. *Clin Exp Immunol* 175:79-91.

Seamark, D.A., S.D. Blake, C.J. Seamark, and D.M. Halpin. 2004. Living with severe chronic obstructive pulmonary disease (COPD): perceptions of patients and their carers. An interpretative phenomenological analysis. *Palliative medicine* 18:619-625.

Seemungal, T., R. Harper-Owen, A. Bhowmik, I. Moric, G. Sanderson, S. Message, P. Maccallum, T.W. Meade, D.J. Jeffries, S.L. Johnston, and J.A. Wedzicha. 2001. Respiratory viruses, symptoms, and inflammatory markers in acute exacerbations and stable chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 164:1618-1623.

Sethi, S., N. Evans, B.J. Grant, and T.F. Murphy. 2002. New strains of bacteria and exacerbations of chronic obstructive pulmonary disease. *The New England journal of medicine* 347:465-471.

Sethi, S., R. Sethi, K. Eschberger, P. Lobbins, X. Cai, B.J. Grant, and T.F. Murphy. 2007. Airway bacterial concentrations and exacerbations of chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 176:356-361.

Shafiani, S., G. Tucker-Heard, A. Kariyone, K. Takatsu, and K.B. Urdahl. 2010. Pathogen-specific regulatory T cells delay the arrival of effector T cells in the lung during early tuberculosis. *J Exp Med* 207:1409-1420.

Shapiro, S.D., E.J. Campbell, D.K. Kobayashi, and H.G. Welgus. 1991. Dexamethasone selectively modulates basal and lipopolysaccharide-induced metalloproteinase and tissue inhibitor of metalloproteinase production by human alveolar macrophages. *J Immunol* 146:2724-2729.

Sharpe, A.H., E.J. Wherry, R. Ahmed, and G.J. Freeman. 2007. The function of programmed cell death 1 and its ligands in regulating autoimmunity and infection. *Nat Immunol* 8:239-245.

Shen, T., J.J. Zheng, C.H. Xu, J. Liu, W.D. Zhang, F.M. Lu, and H. Zhuang. 2010. PD-1 expression on peripheral CD8+TEM/TEMRA subsets closely correlated with HCV viral load in chronic hepatitis C patients. *Virol J* 7:

Sheng, Z.M., D.S. Chertow, X. Ambroggio, S. McCall, R.M. Przygodzki, R.E. Cunningham, O.A. Maximova, J.C. Kash, D.M. Morens, and J.K. Taubenberger. 2011. Autopsy series of 68 cases dying before and during the 1918 influenza pandemic peak. *Proc Natl Acad Sci U S A* 108:16416-16421.

Sheppard, K.A., L.J. Fitz, J.M. Lee, C. Benander, J.A. George, J. Wooters, Y. Qiu, J.M. Jussif, L.L. Carter, C.R. Wood, and D. Chaudhary. 2004. PD-1 inhibits T-cell receptor induced phosphorylation of the ZAP70/CD3zeta signalosome and downstream signaling to PKCtheta. *FEBS letters* 574:37-41.

Shinohara, T., M. Taniwaki, Y. Ishida, M. Kawaichi, and T. Honjo. 1994. Structure and chromosomal localization of the human PD-1 gene (PDCD1). *Genomics* 23:704-706.

Short, K.R., E.J. Grant, M. Vissers, P.C. Reading, D.A. Diavatopoulos, and K. Kedzierska. 2013. A Novel Method Linking Antigen Presentation by Human Monocyte-Derived Macrophages to CD8(+) T Cell Polyfunctionality. *Frontiers in immunology* 4:389.

Sidney, S., M. Sorel, C.P. Quesenberry, Jr., C. DeLuise, S. Lanes, and M.D. Eisner. 2005. COPD and incident cardiovascular disease hospitalizations and mortality: Kaiser Permanente Medical Care Program. *Chest* 128:2068-2075.

Siegel, R., J. Eskdale, and G. Gallagher. 2011. Regulation of IFN-lambda1 Promoter Activity (IFN-lambda1/IL-29) in Human Airway Epithelial Cells. *J Immunol*

Sin, D.D., T. Stafinski, Y.C. Ng, N.R. Bell, and P. Jacobs. 2002. The impact of chronic obstructive pulmonary disease on work loss in the United States. *Am J Respir Crit Care Med* 165:704-707.

Singh, N.P. 1998. A rapid method for the preparation of single-cell suspensions from solid tissues. *Cytometry* 31:229-232.

Sirois, J., G. Menard, A.S. Moses, and E.Y. Bissonnette. 2000. Importance of histamine in the cytokine network in the lung through H2 and H3 receptors: stimulation of IL-10 production. *J Immunol* 164:2964-2970.

Soler, N., A. Torres, S. Ewig, J. Gonzalez, R. Celis, M. El-Ebiary, C. Hernandez, and R. Rodriguez-Roisin. 1998. Bronchial microbial patterns in severe exacerbations of chronic obstructive pulmonary disease (COPD) requiring mechanical ventilation. *Am J Respir Crit Care Med* 157:1498-1505.

Sood, A., H. Petersen, C.M. Blanchette, P. Meek, M.A. Picchi, S.A. Belinsky, and Y. Tesfaigzi. 2010. Wood smoke exposure and gene promoter methylation are associated with increased risk for COPD in smokers. *Am J Respir Crit Care Med* 182:1098-1104.

Soriano, J.B., D.D. Sin, X. Zhang, P.G. Camp, J.A. Anderson, N.R. Anthonisen, A.S. Buist, P.S. Burge, P.M. Calverley, J.E. Connett, S. Petersson, D.S. Postma, W. Szafranski, and J. Vestbo. 2007. A pooled analysis of FEV1 decline in COPD patients randomized to inhaled corticosteroids or placebo. *Chest* 131:682-689.

Sperr, W.R., H.C. Bankl, G. Mundigler, G. Klappacher, K. Grossschmidt, H. Agis, P. Simon, P. Laufer, M. Imhof, T. Radaszkiewicz, D. Glogar, K. Lechner, and P. Valent. 1994. The human cardiac mast cell: localization, isolation, phenotype, and functional characterization. *Blood* 84:3876-3884.

Stahl, E., A. Lindberg, S.A. Jansson, E. Ronmark, K. Svensson, F. Andersson, C.G. Lofdahl, and B. Lundback. 2005. Health-related quality of life is related to COPD disease severity. *Health and quality of life outcomes* 3:56.

Stanciu, L.A., C.M. Bellettato, V. Laza-Stanca, A.J. Coyle, A. Papi, and S.L. Johnston. 2006. Expression of programmed death-1 ligand (PD-L) 1, PD-L2, B7-H3, and inducible costimulator ligand on human respiratory tract epithelial cells and regulation by respiratory syncytial virus and type 1 and 2 cytokines. *The Journal of infectious diseases* 193:404-412.

Stanescu, D., A. Sanna, C. Veriter, S. Kostianev, P.G. Calcagni, L.M. Fabbri, and P. Maestrelli. 1996. Airways obstruction, chronic expectoration, and rapid decline of FEV1 in smokers are associated with increased levels of sputum neutrophils. *Thorax* 51:267-271.

Staples, K.J., C.W. Bragg, B. Nicholas, T.M.A. Wilkinson, and R. Djukanovic. 2009. Influenza infection of monocyte-macrophages is insensitive to inhibition by NH4Cl. In European Respiratory Society International Conference. Vienna.

Staples, K.J., T.S. Hinks, J.A. Ward, V. Gunn, C. Smith, and R. Djukanovic. 2012. Phenotypic characterization of lung macrophages in asthmatic patients: overexpression of CCL17. *The Journal of allergy and clinical immunology* 130:1404-1412 e1407.

Starr, T.K., S.C. Jameson, and K.A. Hogquist. 2003. Positive and negative selection of T cells. *Annual review of immunology* 21:139-176.

Stewart, C.C. 1992. Clinical applications of flow cytometry. Immunologic methods for measuring cell membrane and cytoplasmic antigens. *Cancer* 69:1543-1552.

Stone, J.D., A.S. Chervin, and D.M. Kranz. 2009. T-cell receptor binding affinities and kinetics: impact on T-cell activity and specificity. *Immunology* 126:165-176.

Stout, R.D., C. Jiang, B. Matta, I. Tietzel, S.K. Watkins, and J. Suttles. 2005. Macrophages sequentially change their functional phenotype in response to changes in microenvironmental influences. *J Immunol* 175:342-349.

Stringer, K.A., M. Tobias, H.C. O'Neill, and C.C. Franklin. 2007. Cigarette smoke extract-induced suppression of caspase-3-like activity impairs human neutrophil phagocytosis. *Am J Physiol Lung Cell Mol Physiol* 292:L1572-1579.

Strutt, T.M., K.K. McKinstry, Y. Kuang, L.M. Bradley, and S.L. Swain. 2012. Memory CD4+ T-cell-mediated protection depends on secondary effectors that are distinct from and superior to primary effectors. *Proc Natl Acad Sci U S A* 109:E2551-2560.

Sugita, S. 2009. Role of ocular pigment epithelial cells in immune privilege. *Archivum immunologiae et therapiae experimentalis* 57:263-268.

Suissa, S., and P.J. Barnes. 2009. Inhaled corticosteroids in COPD: the case against. *Eur Respir J* 34:13-16.

Sullivan, S.D., S.D. Ramsey, and T.A. Lee. 2000. The economic burden of COPD. *Chest* 117:5S-9S.

Szymczak, W.A., and G.S. Deepe, Jr. 2009. The CCL7-CCL2-CCR2 axis regulates IL-4 production in lungs and fungal immunity. *J Immunol* 183:1964-1974.

Taams, L.S., J.M. van Amelsfort, M.M. Tiemessen, K.M. Jacobs, E.C. de Jong, A.N. Akbar, J.W. Bijlsma, and F.P. Lafeber. 2005. Modulation of monocyte/macrophage function by human CD4+CD25+ regulatory T cells. *Human immunology* 66:222-230.

Taraseviciene-Stewart, L., and N.F. Voelkel. 2008. Molecular pathogenesis of emphysema. *J Clin Invest* 118:394-402.

Taylor, C.R., and R.M. Levenson. 2006. Quantification of immunohistochemistry--issues concerning methods, utility and semiquantitative assessment II. *Histopathology* 49:411-424.

Teijaro, J.R., D. Turner, Q. Pham, E.J. Wherry, L. Lefrancois, and D.L. Farber. 2011. Cutting Edge: Tissue-Retentive Lung Memory CD4 T Cells Mediate Optimal Protection to Respiratory Virus Infection. *J Immunol*

Teijaro, J.R., D. Verhoeven, C.A. Page, D. Turner, and D.L. Farber. 2010. Memory CD4 T cells direct protective responses to influenza virus in the lungs through helper-independent mechanisms. *J Virol* 84:9217-9226.

Telcian, A.G., V. Laza-Stanca, M.R. Edwards, J.A. Harker, H. Wang, N.W. Bartlett, P. Mallia, M.T. Zdrenghea, T. Kebadze, A.J. Coyle, P.J. Openshaw, L.A. Stanciu, and S.L. Johnston. 2011. RSV-induced bronchial epithelial cell PD-L1 expression inhibits CD8+ T cell nonspecific antiviral activity. *The Journal of infectious diseases* 203:85-94.

Telenga, E.D., R.F. Hoffmann, t.K. Ruben, S.J. Hoonhorst, B.W. Willemse, A.J. van Oosterhout, I.H. Heijink, M. van den Berge, L. Jorge, P. Sandra, D.S. Postma, K. Sandra, and N.H. ten Hacken. 2014. Untargeted lipidomic analysis in chronic obstructive pulmonary disease. Uncovering sphingolipids. *Am J Respir Crit Care Med* 190:155-164.

Thompson, R.H., M.D. Gillett, J.C. Cheville, C.M. Lohse, H. Dong, W.S. Webster, K.G. Krejci, J.R. Lobo, S. Sengupta, L. Chen, H. Zincke, M.L. Blute, S.E. Strome, B.C. Leibovich, and E.D. Kwon. 2004. Costimulatory B7-H1 in renal cell carcinoma patients: Indicator of tumor aggressiveness and potential therapeutic target. *Proc Natl Acad Sci U S A* 101:17174-17179.

Thornton Snider, J., J.A. Romley, K.S. Wong, J. Zhang, M. Eber, and D.P. Goldman. 2012. The Disability burden of COPD. *COPD* 9:513-521.

Tindemans, I., N. Serafini, J.P. Di Santo, and R.W. Hendriks. 2014. GATA-3 Function in Innate and Adaptive Immunity. *Immunity* 41:191-206.

Topalian, S.L., F.S. Hodi, J.R. Brahmer, S.N. Gettinger, D.C. Smith, D.F. McDermott, J.D. Powderly, R.D. Carvajal, J.A. Sosman, M.B. Atkins, P.D. Leming, D.R. Spigel, S.J. Antonia, L. Horn, C.G. Drake, D.M. Pardoll, L. Chen, W.H. Sharfman, R.A. Anders, J.M. Taube, T.L. McMiller, H. Xu, A.J. Korman, M. Jure-Kunkel, S. Agrawal, D. McDonald, G.D. Kollia, A. Gupta, J.M. Wigginton, and M. Sznol. 2012. Safety, Activity, and Immune Correlates of Anti-PD-1 Antibody in Cancer. *The New England journal of medicine*

Topham, D.J., R.A. Tripp, and P.C. Doherty. 1997. CD8+ T cells clear influenza virus by perforin or Fas-dependent processes. *J Immunol* 159:5197-5200.

Touraine, J.L., J.W. Hadden, F. Touraine, E.M. Hadden, R. Estensen, and R.A. Good. 1977. Phorbol myristate acetate: a mitogen selective for a T-lymphocyte subpopulation. *J Exp Med* 145:460-465.

Trautmann, L., L. Janbazian, N. Chomont, E.A. Said, S. Gimmig, B. Bessette, M.R. Boulassel, E. Delwart, H. Sepulveda, R.S. Balderas, J.P. Routy, E.K. Haddad, and R.P. Sekaly. 2006. Upregulation of PD-1 expression on HIV-specific CD8+ T cells leads to reversible immune dysfunction. *Nature medicine* 12:1198-1202.

Trautmann, L., F.M. Mbitikon-Kobo, J.P. Goulet, Y. Peretz, Y. Shi, J. Van Grevenynghe, F.A. Procopio, M.R. Boulassel, J.P. Routy, N. Chomont, E.K. Haddad, and R.P. Sekaly. 2012. Profound metabolic, functional, and cytolytic differences characterize HIV-specific CD8 T cells in primary and chronic HIV infection. *Blood* 120:3466-3477.

Trupin, L., G. Earnest, M. San Pedro, J.R. Balmes, M.D. Eisner, E. Yelin, P.P. Katz, and P.D. Blanc. 2003. The occupational burden of chronic obstructive pulmonary disease. *Eur Respir J* 22:462-469.

Tudhope, S.J., T.K. Finney-Hayward, A.G. Nicholson, R.J. Mayer, M.S. Barnette, P.J. Barnes, and L.E. Donnelly. 2008. Different mitogen-activated protein kinase-dependent cytokine responses in cells of the monocyte lineage. *The Journal of pharmacology and experimental therapeutics* 324:306-312.

Ulvestad, B., B. Bakke, W. Eduard, J. Kongerud, and M.B. Lund. 2001. Cumulative exposure to dust causes accelerated decline in lung function in tunnel workers. *Occupational and environmental medicine* 58:663-669.

Vallbracht, S., H. Unsold, and S. Ehl. 2006. Functional impairment of cytotoxic T cells in the lung airways following respiratory virus infections. *Eur J Immunol* 36:1434-1442.

Van Damme, N., D. Baeten, M. De Vos, P. Demetter, D. Elewaut, H. Mielants, G. Verbruggen, C. Cuvelier, E.M. Veys, and F. De Keyser. 2000. Chemical agents and enzymes used for the extraction of gut lymphocytes influence flow cytometric detection of T cell surface markers. *Journal of immunological methods* 236:27-35.

van den Berge, M., K. Steiling, W. Timens, P.S. Hiemstra, P.J. Sterk, I.H. Heijink, G. Liu, Y.O. Alekseyev, M.E. Lenburg, A. Spira, and D.S. Postma. 2014. Airway gene expression in COPD is dynamic with inhaled corticosteroid treatment and reflects biological pathways associated with disease activity. *Thorax* 69:14-23.

van den Boom, G., M.P. Rutten-van Molken, P.R. Tirimanna, C.P. van Schayck, H. Folgering, and C. van Weel. 1998. Association between health-related quality of life and consultation for respiratory symptoms: results from the DIMCA programme. *Eur Respir J* 11:67-72.

van der Bruggen, P., C. Traversari, P. Chomez, C. Lurquin, E. De Plaen, B. Van den Eynde, A. Knuth, and T. Boon. 1991. A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma. *Science* 254:1643-1647.

Varol, C., L. Landsman, D.K. Fogg, L. Greenshtein, B. Gildor, R. Margalit, V. Kalchenko, F. Geissmann, and S. Jung. 2007. Monocytes give rise to mucosal, but not splenic, conventional dendritic cells. *J Exp Med* 204:171-180.

Velu, V., K. Titanji, B. Zhu, S. Husain, A. Pladevega, L. Lai, T.H. Vanderford, L. Chennareddi, G. Silvestri, G.J. Freeman, R. Ahmed, and R.R. Amara. 2009. Enhancing SIV-specific immunity in vivo by PD-1 blockade. *Nature* 458:206-210.

Vestbo, J., and J.C. Hogg. 2006. Convergence of the epidemiology and pathology of COPD. *Thorax* 61:86-88.

Vezys, V., D. Masopust, C.C. Kemball, D.L. Barber, L.A. O'Mara, C.P. Larsen, T.C. Pearson, R. Ahmed, and A.E. Lukacher. 2006. Continuous recruitment of naive T cells contributes to heterogeneity of antiviral CD8 T cells during persistent infection. *J Exp Med* 203:2263-2269.

Vijayanand, P., G. Seumois, C. Pickard, R.M. Powell, G. Angco, D. Sammut, S.D. Gadola, P.S. Friedmann, and R. Djukanovic. 2007. Invariant natural killer T cells in asthma and chronic obstructive pulmonary disease. *The New England journal of medicine* 356:1410-1422.

Wagar, L.E., B. Gentleman, H. Pircher, J.E. McElhaney, and T.H. Watts. 2011. Influenza-specific T cells from older people are enriched in the late effector subset and their presence inversely correlates with vaccine response. *PLoS One* 6:e23698.

Walker, M.R., B.D. Carson, G.T. Nepom, S.F. Ziegler, and J.H. Buckner. 2005. De novo generation of antigen-specific CD4+CD25+ regulatory T cells from human CD4+CD25- cells. *Proc Natl Acad Sci U S A* 102:4103-4108.

Walsh, K.B., L.L. Lanier, and T.E. Lane. 2008. NKG2D receptor signaling enhances cytolytic activity by virus-specific CD8+ T cells: evidence for a protective role in virus-induced encephalitis. *J Virol* 82:3031-3044.

Walters, J.A., D.J. Tan, C.J. White, P.G. Gibson, R. Wood-Baker, and E.H. Walters. 2014. Systemic corticosteroids for acute exacerbations of chronic obstructive pulmonary disease. *Cochrane Database Syst Rev* 9:CD001288.

Wang, C., P. Hillsamer, and C.H. Kim. 2011. Phenotype, effector function, and tissue localization of PD-1-expressing human follicular helper T cell subsets. *BMC immunology* 12:53.

Wang, J., R.A. Urbanowicz, P.J. Tighe, I. Todd, J.M. Corne, and L.C. Fairclough. 2013. Differential activation of killer cells in the circulation and the lung: a study of current smoking status and chronic obstructive pulmonary disease (COPD). *PLoS One* 8:e58556.

Wang, L., N.F. Lovejoy, and D.L. Faustman. 2012. Persistence of prolonged C-peptide production in type 1 diabetes as measured with an ultrasensitive C-peptide assay. *Diabetes care* 35:465-470.

Wang, P.S., J. Avorn, M.A. Brookhart, H. Mogun, S. Schneeweiss, M.A. Fischer, and R.J. Glynn. 2005. Effects of noncardiovascular comorbidities on antihypertensive use in elderly hypertensives. *Hypertension* 46:273-279.

Ward, M.M., H.S. Javitz, W.M. Smith, and A. Bakst. 2000. Direct medical cost of chronic obstructive pulmonary disease in the U.S.A. *Respir Med* 94:1123-1129.

Welsh, R.M., and L.K. Selin. 2002. No one is naive: the significance of heterologous T-cell immunity. *Nat Rev Immunol* 2:417-426.

Wen, Y., D.W. Reid, D. Zhang, C. Ward, R. Wood-Baker, and E.H. Walters. 2010. Assessment of airway inflammation using sputum, BAL, and endobronchial biopsies in current and ex-smokers with established COPD. *International journal of chronic obstructive pulmonary disease* 5:327-334.

West, E.E., B. Youngblood, W.G. Tan, H.T. Jin, K. Araki, G. Alexe, B.T. Konieczny, S. Calpe, G.J. Freeman, C. Terhorst, W.N. Haining, and R. Ahmed. 2011. Tight regulation of memory CD8(+) T cells limits their effectiveness during sustained high viral load. *Immunity* 35:285-298.

Wherry, E.J. 2011. T cell exhaustion. *Nat Immunol* 12:492-499.

Wherry, E.J., J.N. Blattman, K. Murali-Krishna, R. van der Most, and R. Ahmed. 2003. Viral persistence alters CD8 T-cell immunodominance and tissue distribution and results in distinct stages of functional impairment. *J Virol* 77:4911-4927.

Wherry, E.J., S.J. Ha, S.M. Kaech, W.N. Haining, S. Sarkar, V. Kalia, S. Subramaniam, J.N. Blattman, D.L. Barber, and R. Ahmed. 2007. Molecular signature of CD8+ T cell exhaustion during chronic viral infection. *Immunity* 27:670-684.

Whitmire, J.K., M.S. Asano, S.M. Kaech, S. Sarkar, L.G. Hannum, M.J. Shlomchik, and R. Ahmed. 2009. Requirement of B cells for generating CD4+ T cell memory. *J Immunol* 182:1868-1876.

Wilkinson, T.M., G.C. Donaldson, S.L. Johnston, P.J. Openshaw, and J.A. Wedzicha. 2006a. Respiratory syncytial virus, airway inflammation, and FEV1 decline in patients with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 173:871-876.

Wilkinson, T.M., J.R. Hurst, W.R. Perera, M. Wilks, G.C. Donaldson, and J.A. Wedzicha. 2006b. Effect of interactions between lower airway bacterial and rhinoviral infection in exacerbations of COPD. *Chest* 129:317-324.

Wilkinson, T.M., C.K. Li, C.S. Chui, A.K. Huang, M. Perkins, J.C. Liebner, R. Lambkin-Williams, A. Gilbert, J. Oxford, B. Nicholas, K.J. Staples, T. Dong, D.C. Douek, A.J. McMichael, and X.N. Xu. 2012. Preexisting influenza-specific CD4(+) T cells correlate with disease protection against influenza challenge in humans. *Nature medicine*

Williams, S.K., S. McKenney, and B.E. Jarrell. 1995. Collagenase lot selection and purification for adipose tissue digestion. *Cell transplantation* 4:281-289.

Wilson, L., E.B. Devine, and K. So. 2000. Direct medical costs of chronic obstructive pulmonary disease: chronic bronchitis and emphysema. *Respir Med* 94:204-213.

Wilson, R. 1998. The role of infection in COPD. *Chest* 113:242S-248S.

Wohlfert, E., and Y. Belkaid. 2008. Role of endogenous and induced regulatory T cells during infections. *Journal of clinical immunology* 28:707-715.

Wolfel, T., M. Hauer, J. Schneider, M. Serrano, C. Wolfel, E. Klehmann-Hieb, E. De Plaen, T. Hankeln, K.H. Meyer zum Buschenfelde, and D. Beach. 1995. A p16INK4a-insensitive CDK4 mutant targeted by cytolytic T lymphocytes in a human melanoma. *Science* 269:1281-1284.

Woods, J.A., J.S. Wheeler, C.K. Finch, and N.A. Pinner. 2014. Corticosteroids in the treatment of acute exacerbations of chronic obstructive pulmonary disease. *International journal of chronic obstructive pulmonary disease* 9:421-430.

Wu, X., H. Zhang, Q. Xing, J. Cui, J. Li, Y. Li, Y. Tan, and S. Wang. 2014. PD-1(+) CD8(+) T cells are exhausted in tumours and functional in draining lymph nodes of colorectal cancer patients. *British journal of cancer* 111:1391-1399.

Yamane, H., and W.E. Paul. 2012. Cytokines of the gamma(c) family control CD4+ T cell differentiation and function. *Nat Immunol* 13:1037-1044.

Yang, I.A., M.S. Clarke, E.H. Sim, and K.M. Fong. 2012. Inhaled corticosteroids for stable chronic obstructive pulmonary disease. *Cochrane Database Syst Rev* 7:CD002991.

Yao, H., and I. Rahman. 2011. Current concepts on oxidative/carbonyl stress, inflammation and epigenetics in pathogenesis of chronic obstructive pulmonary disease. *Toxicology and applied pharmacology* 254:72-85.

Yap, K.L., G.L. Ada, and I.F. McKenzie. 1978. Transfer of specific cytotoxic T lymphocytes protects mice inoculated with influenza virus. *Nature* 273:238-239.

Ye, C.J., T. Feng, H.K. Kwon, T. Raj, M.T. Wilson, N. Asinovski, C. McCabe, M.H. Lee, I. Frohlich, H.I. Paik, N. Zaitlen, N. Hacohen, B. Stranger, P. De Jager, D. Mathis, A. Regev, and C. Benoist. 2014. Intersection of population variation and autoimmunity genetics in human T cell activation. *Science* 345:1254665.

Yi, J.S., M.A. Cox, and A.J. Zajac. 2010. T-cell exhaustion: characteristics, causes and conversion. *Immunology* 129:474-481.

Yin, L., J. Scott-Browne, J.W. Kappler, L. Gapin, and P. Marrack. 2012. T cells and their eons-old obsession with MHC. *Immunological reviews* 250:49-60.

Yohannes, A.M., and G.S. Alexopoulos. 2014. Depression and anxiety in patients with COPD. *European respiratory review : an official journal of the European Respiratory Society* 23:345-349.

Yokosuka, T., and T. Saito. 2009. Dynamic regulation of T-cell costimulation through TCR-CD28 microclusters. *Immunological reviews* 229:27-40.

Yokosuka, T., M. Takamatsu, W. Kobayashi-Imanishi, A. Hashimoto-Tane, M. Azuma, and T. Saito. 2012. Programmed cell death 1 forms negative costimulatory microclusters that directly inhibit T cell receptor signaling by recruiting phosphatase SHP2. *Journal of Experimental Medicine*

Yoo, J.S., H. Kato, and T. Fujita. 2014. Sensing viral invasion by RIG-I like receptors. *Current opinion in microbiology* 20C:131-138.

Young, D.A., L.D. Lowe, and S.C. Clark. 1990. Comparison of the effects of IL-3, granulocyte-macrophage colony-stimulating factor, and macrophage colony-stimulating factor in supporting monocyte differentiation in culture. Analysis of macrophage antibody-dependent cellular cytotoxicity. *J Immunol* 145:607-615.

Young, R.P., R.J. Hopkins, T. Christmas, P.N. Black, P. Metcalf, and G.D. Gamble. 2009. COPD prevalence is increased in lung cancer, independent of age, sex and smoking history. *Eur Respir J* 34:380-386.

Young, R.P., R.J. Hopkins, B.A. Hay, M.J. Epton, P.N. Black, and G.D. Gamble. 2008. Lung cancer gene associated with COPD: triple whammy or possible confounding effect? *Eur Respir J* 32:1158-1164.

Youngblood, B., K.J. Oestreich, S.J. Ha, J. Duraiswamy, R.S. Akondy, E.E. West, Z. Wei, P. Lu, J.W. Austin, J.L. Riley, J.M. Boss, and R. Ahmed. 2011. Chronic Virus Infection Enforces Demethylation of the Locus that Encodes PD-1 in Antigen-Specific CD8(+) T Cells. *Immunity* 35:400-412.

Youngnak, P., Y. Kozono, H. Kozono, H. Iwai, N. Otsuki, H. Jin, K. Omura, H. Yagita, D.M. Pardoll, L. Chen, and M. Azuma. 2003. Differential binding properties of B7-H1 and B7-DC to programmed death-1. *Biochemical and biophysical research communications* 307:672-677.

Zajac, A.J., J.N. Blattman, K. Murali-Krishna, D.J. Sourdive, M. Suresh, J.D. Altman, and R. Ahmed. 1998. Viral immune evasion due to persistence of activated T cells without effector function. *J Exp Med* 188:2205-2213.

Zandvoort, A., D.S. Postma, M.R. Jonker, J.A. Noordhoek, J.T. Vos, and W. Timens. 2008. Smad gene expression in pulmonary fibroblasts: indications for defective ECM repair in COPD. *Respir Res* 9:83.

Zhang, X., J.C. Schwartz, X. Guo, S. Bhatia, E. Cao, M. Lorenz, M. Cammer, L. Chen, Z.Y. Zhang, M.A. Edidin, S.G. Nathenson, and S.C. Almo. 2004. Structural and functional analysis of the costimulatory receptor programmed death-1. *Immunity* 20:337-347.

Zhao, J., J. Zhao, C. Fett, K. Trandem, E. Fleming, and S. Perlman. 2011. IFN-gamma- and IL-10-expressing virus epitope-specific Foxp3(+) T reg cells in the central nervous system during encephalomyelitis. *J Exp Med* 208:1571-1577.

Zhen, Y., J. Zheng, and Y. Zhao. 2008. Regulatory CD4+CD25+ T cells and macrophages: communication between two regulators of effector T cells. *Inflammation research : official journal of the European Histamine Research Society ... [et al.]* 57:564-570.

Zhou, S., R. Ou, L. Huang, and D. Moskophidis. 2002. Critical role for perforin-, Fas/FasL-, and TNFR1-mediated cytotoxic pathways in down-regulation of antigen-specific T cells during persistent viral infection. *J Virol* 76:829-840.

Zhou, X., and J.E. McElhaney. 2011. Age-related changes in memory and effector T cells responding to influenza A/H3N2 and pandemic A/H1N1 strains in humans. *Vaccine* 29:2169-2177.

Zhu, A., D. Ge, J. Zhang, Y. Teng, C. Yuan, M. Huang, I.M. Adcock, P.J. Barnes, and X. Yao. 2014. Sputum myeloperoxidase in chronic obstructive pulmonary disease. *European journal of medical research* 19:12.

Zhu, G., A. Agusti, A. Gulsvik, P. Bakke, H. Coxson, D.A. Lomas, E.K. Silverman, S.G. Pillai, and I. investigators. 2009. CTLA4 gene polymorphisms are associated with chronic bronchitis. *Eur Respir J* 34:598-604.

Zhu, J., Y.S. Qiu, S. Majumdar, E. Gamble, D. Matin, G. Turato, L.M. Fabbri, N. Barnes, M. Saetta, and P.K. Jeffery. 2001. Exacerbations of Bronchitis: bronchial eosinophilia and gene expression for interleukin-4, interleukin-5, and eosinophil chemoattractants. *Am J Respir Crit Care Med* 164:109-116.

Zhu, J., H. Yamane, and W.E. Paul. 2010. Differentiation of effector CD4 T cell populations (*). *Annual review of immunology* 28:445-489.

Zizzo, G., and P.L. Cohen. 2013. IL-17 stimulates differentiation of human anti-inflammatory macrophages and phagocytosis of apoptotic neutrophils in response to IL-10 and glucocorticoids. *J Immunol* 190:5237-5246.