UNIVERSITY OF SOUTHAMPTON

FACULTY OF MEDICINE

Clinical and Experimental Sciences

CHARACTERISATION OF THE HUMAN LUNG FIBROBLASTS ABILITY TO ACT AS AN ANTIGEN PRESENTING CELL FOR T HELPER CELLS OF THE IMMUNE SYSTEM

by

Andrew J Hutton

Thesis for the degree of Doctor of Philosophy

October 2015

ABSTRACT

T helper cells of the immune system are critical in mediating immune responses in the lung. Through the release of cytokines, T helper cells direct the wider immune response against a range of pathogens and are considered indispensible for effective immunity. T cell activation is governed by the interaction with antigen-presenting cells (APCs), which mediate antigen-specific T cell activation and thus control immune responses in areas such as the lung. While T helper cell activation by APCs such as dendritic cells is well established, the role of fibroblasts in T helper cell activation in the lung is poorly understood. Fibroblast antigen presentation was hypothesised to be a mechanism leading to activation of bacterial-specific lung T helper cells

Characterisation of T cell populations within the human distal lung was carried out. focusing upon examining the memory T cell populations. In addition, the cytokine production profile of T cells as a whole population and in response to specific lung bacterial antigen was examined.

Distal lung T cells were primarily CD4 T helper cells of an effector memory phenotype. IFNγ producing, Th1-type cells were the most abundant effector subset present in all T cell populations examined, while initial responses to the common lung bacteria non-typeable *haemophilus influenza* were more heterogeneous.

Human lung fibroblasts obtained from the distal lung were examined for expression of immune synapse molecules both *ex vivo* and *in vitro* using flow cytometry. The ability of lung fibroblasts to internalize environmental antigen was also investigated via flow cytometry and confocal microscopy. Using autologous lung fibroblasts and T helper cells, the ability of fibroblasts to present bacterial antigens non-typeable *haemophilus influenza* was examined. T helper activation was measured via the production of cytokines associated with the major T helper effector subsets

Lung fibroblasts expressed HLA-DR *ex vivo*, and were shown to upregulate HLA-DR and ICAM-1 by IFNγ treatment *in vitro,* but did not express CD80 or CD86. Fibroblasts were also able to internalize environmental antigen. Fibroblasts exposed to both IFNγ and a heat-killed form of non-typeable *haemophilus influenza* are shown to activate IFNγ and IL-17A producing T helper cells in an antigen dependent manner.

This study demonstrates that lung fibroblasts are able to function as an inducible APC and activate proinflammatory T helper cells. This previously unknown relationship between fibroblast and T cell may represent a key mechanism in lung immune responses to bacterial populations

Contents

List of tables

List of figures

ix

DECLARATION OF AUTHORSHIP

I, Andrew John Hutton

declare that the thesis entitled

"Characterisation of lung fibroblasts and their capacity to act as an antigen presenting cell for T helper cells of the immune system"

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

- this work was done wholly or mainly while in candidature for a research degree at this University;

- where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;

- where I have consulted the published work of others, this is always clearly attributed;

- where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;

- I have acknowledged all main sources of help;

- where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;

- none of this work has been published before submission,

-

- Signed: ……………………………………………………………………..

-

Date:…………………………………………………………………….

Acknowledgements

Definitions and Abbreviations

7-AAD	7-aminoactinomycin D
APC	Antigen presenting cell
APC-Cy7	Allophycocyanin-Cyanine7
APC	Allophycocyanin
AIRE	Autoimmune regulator
α-SMA	Alpha-smooth muscle actin
BSA	Bovine serum albumin
CIITA	Class II transactivator
CCR	C-C chemokine receptor
COPD	Chronic obstructive pulmonary disease
CD	Cluster of differentiation
CLIP	Class-II associated invariant chain peptide
CSE	Cigarette smoke exposure
CXCL	C-X-C motif ligand
DAPI	4', 6-diamidino-2-phenylindole
DAMP	Danger associated molecular pattern
DC	Dendritic cell
DMEM	Dulbecco modified eagles medium
DMSO	Dimethyl sulphoxide
DN T cell	Double negative ($CD4^-CD8^-$) T cell
DP T cell	Double positive ($CD4^+CD8^+$) T cell
ECM	Extracellular matrix
EpCAM	Epithelial cell adhesion molecule

FEV$_1$	Forced expiration volume in one second
FVC	Forced vital capacity
FITC	Fluorescein isothiocyanate
FoxP3	Forkhead box p3
GM-CSF	Granulocyte macrophage colony stimulating factor
GOLD	Global initiative for chronic obstructive pulmonary disease
HLA	Human leukocyte antigen
HS	Human serum
HSA	Human serum albumin
HLA	Human leukocyte antigen
ICAM-1	Intracellular adhesion molecule-1
ICOS	Inducible T cell co-stimulator
ICOSL	Inducible T cell co-stimulator ligand
IFNγ	Interferon gamma
IgG	Immunoglobulin G
Ii	Invariant chain
IL	Interleukin
iNOD	Inducible nitric oxide synthase
iTreg	Induced T regulatory cell
LAMP-1	Lysosomal-associated membrane protein 1
LFA-1	Lymphocyte function-associated antigen-1
LPS	Lipopolysaccharide
MAIT cell	Mucosal associated invariant T cell
MCP-1 (CCL2)	Monocyte chemotactic protein-1 (C-C motif ligand 2)

MFI	Mean fluorescent intensity
MIP-1α/β (CCL3/4)	Macrophage inflammatory protein- alpha/beta (C-C motif ligand3/4)
MoDC	Monocyte derived dendritic cell
MHC	Major histocompatibility complex
NCS	Neonatal calf serum
NK cell	Natural killer cell
NTHi	Nontypeable *Haemophilus influenzae*
nTreg	Natural T regulatory cell
PAMP	Pathogen associated molecular patters
PBMC	Peripheral blood mononuclear cell
PE- efluor 610	Phycoerythrin efluor 610
PE	Phycoerythrin
PE-Cy7	Phycoerythrin cyanin 7
PerCP-Cy5.5	Peridinin Chlorophyll-Cyanine5.5
PFA	Paraformaldehyde
PRR	Pattern recognition receptor
RPMI	Roswell park memorial institute
RSV	Respiratory syncytial virus
SDF-1	Stromal cell-derived factor 1
SLF	Synovial-like fibroblast
sMFI	Specific mean fluorescent intensity
TcR	T cell receptor
TGFβ	Transforming growth factor-beta
Th1, 2,17	T helper cell subtype 1,2,17

TLR	Toll-like receptor
TNF-α	Tumour necrosis factor- alpha
Treg	Regulatory T cell
TSLP	Thymic stromal lymphopoietin
U	Units
VLA-1	Very late antigen-1
V/V	Volume for volume
W/V	Weight for volume

1. INTRODUCTION

The human lung contains a vast array of cells from both the innate and adaptive arms of the immune system, as well as delicately arranged structural cells such as the lung epithelium, endothelium, and stromal cell populations (including fibroblasts). Together these cells form one of the most intricate organs of the body, which is designed to facilitate the gas exchange processes vital to life. The lung therefore must balance immune regulation (to prevent damage to the delicate architecture responsible for lung function), but at the same time possess the ability to launch robust immune responses to a wide array of pathogenic factors. Failure to respond robustly to infectious agents such as bacteria can result in pneumonia leading to systemic infection and morbidity.

Similar to the gut mucosa, the lung has its own bacterial population [1]. The majority of these bacteria are not normally pathogenic, but their growth and activity must be controlled by the immune system. Therefore, the lung must permit a switch from an immunosuppressive steady state to one that allows the development of a proinflammatory immune response against specific threats. The initial response involves the innate immune cells such as macrophages and dendritic cells (DCs) as well as the epithelial cells. Later in the response adaptive immune elements such as the T and B cell populations are involved in the clearance and subsequent development of memory responses to these threats. This complex interplay between different cell types creates a unique environment, involving the long-term maintenance of tissue-resident, antigen specific T cells. These T cells rely upon a number of lung resident cells to operate effectively. Using autologous cell model systems, the project aims to address whether lung fibroblasts – an archetypal structural cell- can contribute to lung immunity though presenting common lung bacterial antigens to T cells resulting in effector cytokine production.

1.1 An overview of immunity in the lung

The lung is a mucosal surface in close contact with the environment, resulting in considerable exposure to environmental antigens- the majority of which are not a threat to the host. To maintain lung integrity the body must ensure tolerance of the lung to environmental antigens to avoid inappropriate inflammation and immunopathology. However, the lung must also be able to quickly switch into action when a pathogenic insult occurs. The considerable number of bacteria present in the respiratory tract must also be controlled by the immune system [1]. Maintaining control over these bacterial populations

requires constant immune surveillance and responses over the course of a lifetime. The innate immune system is integral in this process of conditioning the lung to either a tolerogenic or proinflammatory nature.

The epithelial barrier provides the first line of innate defence, comprising epithelial cell-to-cell tight junctions, production of antimicrobial factors (e.g. complement proteins) and mucociliary clearance provided by the mechanical beating of ciliated lung epithelia. These epithelial surface immune elements provide a first line of defence against infection in the lung (reviewed in [2]. Mast cells present beneath the epithelium are a major innate immune cell population in the lung. These have the capability to respond in an antigen-specific manner through crosslinking of immunoglobulin class E (IgE) molecules bound on their surface via high affinity Fcε receptors. In doing so, this allows IgE-armed cells to respond to specific antigenic stimuli, and respond rapidly through the production of histamine and factors associated with a Th2 type response [3]. As such, they are associated primarily with allergic inflammation in the lung. [4]

There is also an array of innate immune cells resident within the lung alveolar and interstitial zones [5]. These cells include dendritic cells and macrophages, which make up the bodies main phagocytic sentinel cells, with primary responsibility for pathogen clearance through phagocytosis [6] and bactericidal factors (such as reactive oxygen species)[7]. These professional immune cells, along with epithelial cells, are potent sources of cytokines and chemokines [8]. Chemokines and cytokines produced by these cells go on to recruit neutrophils (major first responders of the innate system), blood monocytes and natural killer (NK) cells as well as adaptive immune cells all from the circulation. Dendritic cells are the body's professional antigen presenting cells (APCs), and are able to carry lung-based material to draining lymphatic nodes and present antigen to T cells of the adaptive immune system. Though this mechanism, DCs bridge the gap between innate and adaptive immunity. The careful balance between immunosuppression and inflammation is critical in maintaining lung function. In situations where chronic inflammation occurs, such as chronic obstructive pulmonary disease (COPD), the lungs can be irreparably damaged and lung function considerably impaired.

There are a wide variety of immune cells present in the human lung, however the T cells of the adaptive immune system are critical to the overall immune response to pathogenic threats. Through the production of distinct cytokines in response to antigen

stimulation, these cells are able to direct the wider innate and adaptive immune responses. T cell activity is related to both effective, long-term immunity as well as being linked to lung immunopathology. These cells are reliant upon interaction with the body's populations of APCs for activation and programming into distinct phenotypes.

1.2   T lymphocytes

T cells are a fundamental part of the adaptive immune system, responsible for the generation of highly specific responses and the development of memory-based immunity. Production of cytokines by the CD4 expressing T helper cells influences the wider immune response to pathogenic events and limit responses to self, preventing autoimmunity. CD8 expressing cytotoxic T cells are able to control viral infection and cancerous cell growth through causing direct destruction of the afflicted cell [9]. Both T cell types express a T cell receptor (TcR) that binds a specific antigen when presented within a major histocompatibility complex (MHC) molecule upon a cells surface. TcRs can be formed of alpha and beta subunits (which allow the T cell to bind protein antigen)[10] or gamma and delta subunits (which are thought to have specificity for phosphorylated antigen targets as opposed to peptide sequences)[11]. T cells undergo a number of developmental stages in the thymus, which results in populations of primarily αβ T cells that have been programmed into a CD4 or CD8 lineage. In addition, there are a number of important developmental checkpoints that remove highly self-reactive T cells, which would result in autoimmunity.

1.2.1   Thymocyte development in the Thymus

T cell bone marrow progenitors traffic to the thymus where they undergo a number of important developmental processes. Premature T cell progenitors –thymocytes- do not initially express a T cell receptor (which binds antigen and therefore determines the specificity of the T cell) or the CD4 or CD8 molecules. Double negative thymocytes enter the thymic cortex from the bone marrow and undergo gene rearrangement of the TcR beta, gamma and delta chains. In the majority of cases this process results in a functional beta chain rearrangement, which is then expressed on the cell surface in complex with an invariant pre-alpha TcR. Expression of this complex signals the cell to halt beta, gamma and delta chain gene rearrangement, and to express both CD4 and CD8 molecules (and are hence known as double positive- "DP"- thymocytes). This also triggers a round of cell proliferation, after which these DP thymocytes begin further gene rearrangement- this time of

the TcR alpha chain. The cell performs alpha chain rearrangement until a functional chain is produced which can complex with the TcR beta chain that has already been formed, and the resultant αβ TcR can bind either MHC class I or class II with low affinity[12]. It is the expression of the CD4 or CD8 co-receptors that allows the TcR to recognise antigen bound within a major histocompatibility (MHC) molecule. CD8 expression allows recognition of MHC class I bound antigen while CD4 recognises MHC class II bound antigen [13]

In the thymic environment, MHC class II and I molecules are expressed on specialized cortical epithelial cells, and appear to present a number of self-peptides to DP thymocytes. These specialized epithelial cells express autoimmune regulator (AIRE) genes, allowing them to produce and present a wide variety of self-antigens from a range of peripheral tissues to DP thymocytes. This presentation process can be seen as a delicate balance; DP thymocytes must have low-binding affinity for either MHC class I or II with bound antigen, otherwise they will be unable to effectively "see" MHC-peptide complexes [14]. The DP thymocytes unable to bind with low affinity die via neglect as they fail to receive survival signals through TcR:peptide MHC signalling. This process is termed positive selection. Of equal importance is the removal of thymocytes that bind AIRE expressed self-antigens with high affinity, as responding strongly would result in autoimmunity in the organ those antigens are present [15]. This process of removing high-reactive thymocytes is termed negative selection. If thymocytes respond with the correct level of affinity, they go on to commit to either CD4 or CD8 lineage. This lineage decision is thought to be determined by which MHC molecule the thymocyte interacts with upon the cortical epithelial cell [16] and completes the initial development process. These resultant T cells are referred to as "naïve" cells and are released into the periphery. These cells are distinguishable through expression of C-C chemokine receptor type 7 (CCR7), which permits entry into the bodys secondary lymphatic system [17]. CD45 isoform CD45RA is also preferentially expressed on naive T cells [17]. Therefore naïve T cells can be identified via CCR7 and CD45RA expression [17]. The process of gene rearrangement of alpha and beta subunits results in a T cell population with highly diverse TcRs and therefore an ability to bind huge numbers of different antigens. These naïve T cells traffic around the secondary lymph tissues, such as the draining lymph nodes, in order to encounter APCs trafficking antigen from peripheral tissue zones. The relationship between the T cell and the populations of antigen presenting cells is critical in enabling T cells to respond to antigen from peripheral tissues. Furthermore, T cell reliance upon antigen presentation in the context of MHC allows strict control over T cell activation

by the APC, ensuring proinflammatory responses are only generated if the APC has encountered appropriate warning signals in the periphery.

1.2.2   Naïve T cell activation in the secondary lymph system

Naïve T cells leave the thymus after development and begin a cycle of trafficking around the various secondary lymphoid tissues. These specialized organs bring together naïve T cells and professional APCs (such as dendritic cells and B cells) into a highly organised, compartmentalized structure. DCs stationed in peripheral tissues constantly sample the environment for antigen and upon receiving sufficient maturation signals, can migrate to local draining lymphatic tissues. Maturation signals involved in activating these APCs include the pathogen associated molecular patterns and/or danger associated molecular patterns (PAMPS/DAMPS respectively)[18]; for example, the Toll-like receptor (TLR) family of receptors respond to molecular patterns such as bacterial lipopolysaccharide (LPS) and unmethylated CpG sequences (figure 1)(reviewed in [19]. Upon receiving these maturation signals, dendritic cells will move from the peripheral tissue with their antigenic load to draining lymph tissue where they present tissue-based antigen to naïve T cells.
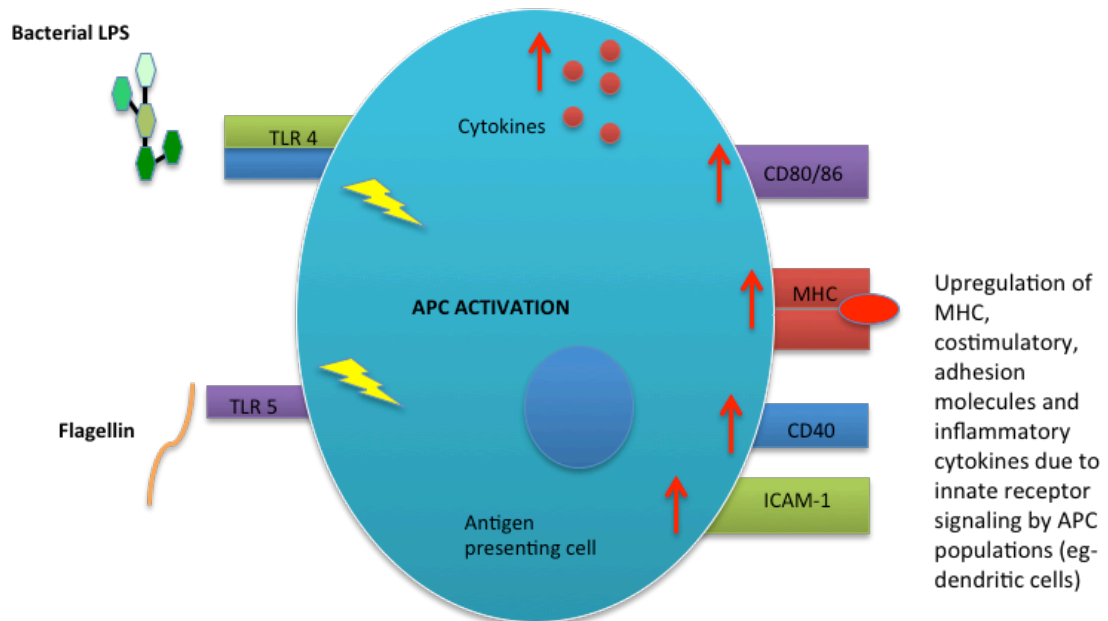
Figure 1-1- Antigen presenting cells receive activation signals through innate pattern recognition receptors (PRRs): Tissue resident professional antigen presenting cells such as dendritic cells can receive activation signals through receptors such as the toll like receptors (TLRs). For example, TLR4 binds bacterial LPS and TLR5 binds bacterial flagellin. Binding these trademark bacterial components acts as a warning of infection and signals the APC to activate. In response, APCs upregulate molecules vital for mediating activation of T cells and migrate from the tissue zone to draining lymphatics and into the lymph nodes

1.2.3   Antigen processing and presentation by APCs

Prior to presentation upon MHC molecules, potential sources of antigen must be processed within the APC. This relies on complex internal processes that ultimately lead to processed peptide antigen loading onto MHC molecules in internal vesicles and export to the cell surface for presentation

1.2.3.1   MHC class I and MHC Class II provoke different responses

As mentioned, T cells respond to peptide antigen via their T cell receptor. T helper cells bind MHC class II bound antigen by virtue of their CD4 expression, while CD8 expressing cytotoxic T cells respond to MHC class I presented antigens. Most cell types are able to process and present antigen upon MHC class I molecules. Antigens presented upon MHC class I are typically intracellular-based. Thus, MHC class I antigen presentation allows immunosurveillance of cells which may be cancerous or infected by an intracellular pathogen. MHC class II, in contrast, typically binds

7

environmentally acquired antigens, effectively allowing presentation of a broader variety of antigens present within the peripheral tissue. MHC class II presentation to T helper cells results in potent cytokine release that goes on to direct the wider response. In this role, T helper cells are critical to an effective immune response. The ability of fibroblasts to alert T helper cells to local pathogens, not just intracellular infections, is of central interest to this study.

1.2.3.2 The MHC Class II processing pathway

The MHC class II presentation pathway allows an APC to process and present antigenic material obtained via internalization of environmental materials (macropinocytosis [20], phagocytosis [21] or endocytosis [22]). Internalized material is typically moved through a series of internal vesicles, initially beginning in early endosomes and gradually moving to the progressively more acidic late endosomes and lysosomes. In these compartments, acid-pH optimum proteases- the cathepsins- degrade proteins into smaller fragments. The MHC class II molecules such as HLA-DR are themselves initially synthesised at the endoplasmic reticulum, and contain an invariant chain (Ii) within the antigen-binding pocket [17]. This Ii chain gives the MHC class II molecule stability and chaperones it to the endosomal compartments. As these compartments grow more acidic, the activity of cathepsin family proteases gradually degrades the Ii chain into a much smaller molecule- the Class II invariant chain peptide (CLIP)[23]. At this stage, both the MHC class II-CLIP complex and cathepsin-degraded antigenic material are present in the same internal vesicles. CLIP is finally removed by the enzymatic activity of the human leukocyte antigen-DM (HLA-DM) molecule, allowing antigens present in the vesicle to bind into the MHC class II pocket, forming a complete MHC class II:peptide complex (p;MHC). HLA-DM is suppressed by the action of the HLA-DO molecule, which is optimal at mildy acidic pH levels, thereby acting as a control mechanism to ensure peptides are only loaded onto MHC class II in late acidic vesicles [24]. Once loading is complete, the p:MHC class II is exported to the cell surface where it is able to present to T helper cells [25]. Professional APCs such as dendritic, macrophage and B cells are able to carry out MHC class II dependent processing and presentation of antigens constitutively. Non-professional APCs, typically non-haematopoietic cell populations that often require stimulation to activate MHC class II expression and internal processing, also exist within the human lung. These will be discussed in more depth in section 1.7.1. While

CD4 T helper cells respond to exogenously obtained antigen via this method, the process of autophagy allows presentation of cytosolic antigens upon MHC class II, allowing activation of T helper cells to antigens that would otherwise be excluded from the "classical" MHC class II pathway. Autophagy involves the sampling of cytosolic antigen through intracellular vesicle formation (an autophagosome) and its subsequent fusion into the lysosome, thus delivering internal cytosolic antigen into the class II pathway [26]. This process has been show to be relevant to education of T cells in the thymus to cytosolic-based antigens [27] as well as in macrophages during intracellular cytosolic infection [28]. This process allows CD4 T cells to respond to cytosolic antigens that otherwise may not be accessible via the exogenous class II pathway. The ability to process and present antigens in the context of MHC class II allows an APC to interact with antigen-specific CD4 T helper cells, potentially leading to T cell activation.

1.2.4   T cell activation

Naïve T cell activation is governed by its interaction with numerous molecules on the surface of APCs; termed the "immune synapse". APCs, which have been activated in peripheral zones (in response to PAMPS) and trafficked to draining lymphatic tissue, will upregulate molecules which allow the APC to activate naïve T cells specific for antigen it has encountered. The most important factor here is the MHC-antigen complex, which delivers the antigen-specific signal to the T cells (figure 1-2). This alone is not enough to activate T cells however. Presentation of antigen without adequate costimulation results in the T cell becoming unresponsive ("anergic") or to undergo apoptosis [29]. This level of control prevents immature APCs from activating T cells unless it has encountered PAMP signals. The co-stimulatory molecules CD80 and CD86 are upregulated upon mature professional APCs, which bind CD28 upon the interacting T cell and deliver a potent activation signal. This CD80/86 signalling through CD28 is absolutely essential for correct naïve T cell activation [30, 31]. Additional stimulatory molecules such as CD40 and cell-adhesion molecules such as intracellular adhesion molecule 1 (ICAM-1) also have roles in delivering activation signals and stabilizing the cell-to-cell interaction. Full stimulation results in T cell proliferation through autocrine action of interleukin 2 and upregulation of the IL-2 high affinity receptor (CD25) on T cells [32].
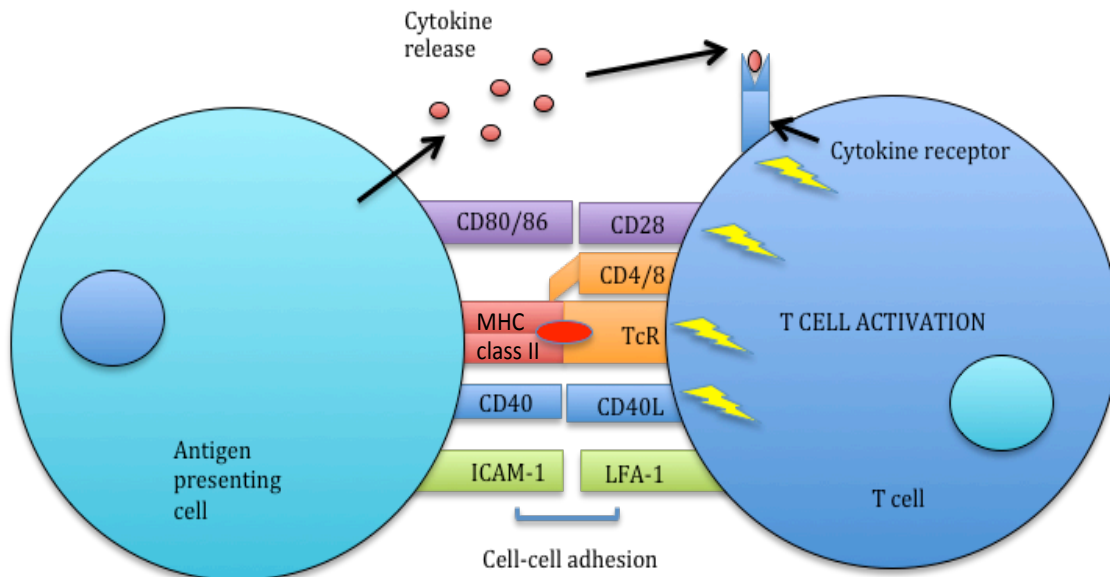
Figure 1-2- Activated APCs interact with and activate T cells: The interaction between T cell and antigen presenting cell has a number of cell-contact dependent mechanisms as well as soluble factor release. TcR interaction with the peptide:MHC molecule provides the main activating signal. The co-receptor of CD8 or CD4 will define whether the T cell is able to respond to MHC class I or II respectively. CD40:CD40L and CD80/86:CD28 provide costimulatory signals for activation and are vital in the priming of naïve T cells. Adhesive interaction between molecules such as ICAM-1 and LFA-1 stabilize the interaction between the two cells. Cytokines released by the APC as well as by the T cell at the time of activation provide further costimulation of the T cell as well as defining the T cells effector subtype (e.g.-Th1, Th2 etc.).

Soluble cytokine factors released by the APC will also affect the developmental outcome of T cell activation, dictating the nature of the response T cells will go on to promote. Once activated, T cells undergo clonal expansion, creating a pool of antigen specific clones ready to combat a particular pathogenic threat. The cells also undergo extensive phenotypic changes, going from an antigen-naïve state and into an antigen-experienced, memory T cell.

1.3   CD4 T cell effector subsets

As mentioned previously, conventional T cells fall into two main categories: The cytotoxic CD8+ T cell, and the CD4+ T helper cell. The CD4+ helper cells are a potent source of cytokines that activate and mobilize the wider immune response to threats, and they adopt different inflammatory and regulatory activity dependent upon the nature of the antigen insult. The differential production of helper cells can be split into what is known as "T

helper subsets". The development of these subsets initially occurs when naïve T cells interact with professional APCs. Typically, the production of conditioning cytokines by the APC will instruct interacting T cells to adopt one of the effector subsets (figure 1-3). APCs respond to pathogenic events via innate signalling mechanisms to preferentially promote a subset phenotype upon antigen-specific T cells. The primary subsets are the Th1, Th2, Th17 and T-regulatory (Treg) cells [33].
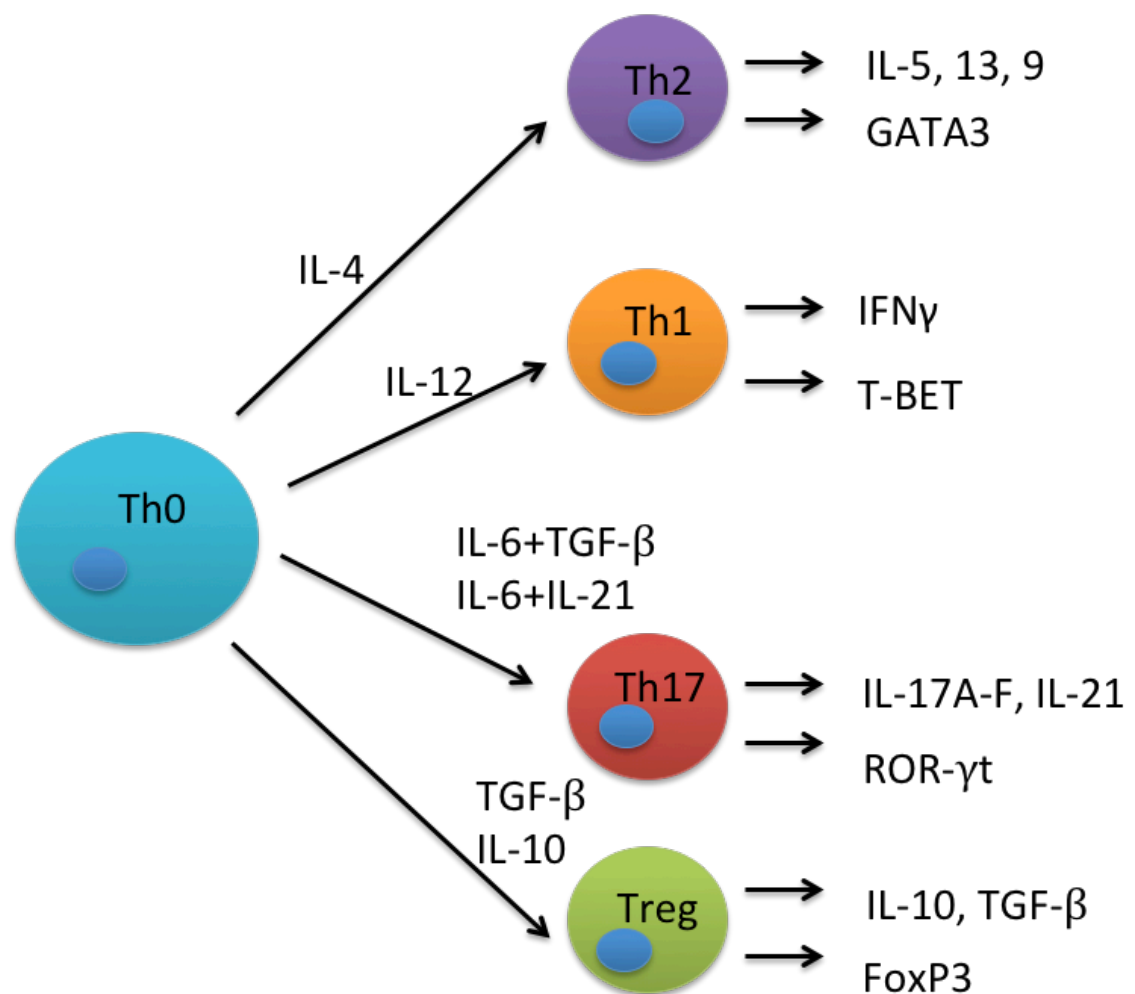
Figure 1-3- T helper cells are conditioned by local cytokines to adopt an effector subset: Once T cells are activated through interaction with activated APCs, cytokines released by the APC will drive the T helper cell to differentiate into an effector subset. These subsets are broadly defined by their cytokine release profile (upper arrow) and transcription factor expression (lower arrow). Shown above is a simplified diagram of the major T helper cell subsets, which are typically classed as Th1, Th2, Th17 and Treg phenotypes.

Th1 cells are promoted though APC production of IL-12 [34] and are classically associated with strong IFNγ production and the stimulation of anti-viral and bactericidal responses from other immune cells (primarily macrophages). IFNγ is considered the canonical antimicrobial cytokine, primarily affecting tissue-resident macrophage populations, driving antimicrobial actions such as the expression of inducible nitric oxide synthetase (iNOS) family enzymes which go on to produce reactive oxygen species and ultimately clear infection [35]. In addition, IFNγ is also able to amplify the adaptive response to an on-going infection through upregulation of the promoter class II transactivator (CIITA) and subsequent MHC class II expression on a wide number of cell types [36]. Th1 cells also promote isotype class switching by B cells (to the highly inflammatory IgG subclass of immunoglobulin- a hallmark of Th1 responses). Th2 on the other hand are promoted though IL-4 conditioning, and are associated with responses against extracellular pathogens [37]. This subset has been attributed with a central role in the pathogenesis of allergic disease such as asthma [38]. Th2 cells are defined by the release of IL-4, 5, 9 and 13, which cause eosinophilic inflammation, local fibrotic remodelling [39] and isotype class switching to the IgE class. Th2 development from naïve cells is promoted through IL-4 production by APCs [40]. Th17 cells are associated with IL-17 family cytokine release and exert their effects primarily upon nonhaematopoietic cells in mucosal surfaces, such as epithelial and fibroblast cells. Th17 cells condition the local area to promote the recruitment and activation of neutrophils from the circulation [41], and through this mechanism contribute to robust responses to bacterial and fungal [42] events. Due to the highly inflammatory nature of these cells, they have been implicated in a number of autoimmune conditions [43]. This subset of T cells is known to develop due to exposure of TGFβ in combination with IL-6, IL-21 or IL-1β, and seems to have reliance upon IL-23 in later stages in order to reach full functional competency (figure 1-3) (reviewed in [44]).

Regulatory T cells, on the other hand, serve to suppress inflammatory responses and restrict the activation of other immune cells. This function is crucial in limiting inflammation caused by immune responses as well as maintaining peripheral tolerance to self-antigen and to other environmental-based antigen such as food and commensal bacteria [45 2006]. Treg cells are broadly split into 2 categories: "natural" T regs and "induced" T regs (nTreg and iTreg, respectively). nTregs are thought to arise at the thymic development stage, where thymocytes with strong reactivity towards self antigen expressed by thymic stromal cells can drive the T cell to become regulatory. This will

effectively allow such T cells to moderate immune responses towards this particular self-ag, and enforce tolerance in the periphery [46]. In contrast, iTregs arise from conventional naïve T cells when exposed to cognate antigen under the correct circumstances. If activated in the presence of TGFβ, these cells take on a suppressive role (figure 1-3), and express molecules such as CTLA-4, GITR, and produce the potent suppressive cytokine IL-10 [45]. Both nTregs and iTregs upregulate the transcription factor FoxP3 to some degree, which is often used as an identifier of regulatory T cells, and both are able to suppress T cell proliferation and produce IL-10.

1.3.1   T cell plasticity between effector subsets

It is now recognised that there is subset plasticity between T helper cells. Traditionally, Th1 and Th2 cells are seen as terminally differentiated from one another and developmentally incompatible. This arose from studies demonstrating IL-12 and IFNγ (canonical Th1 development cytokines) directly oppose Th2 differentiation at a transcriptional level [47]. Likewise, Th2 IL-4 directly opposes the Th1 developmental pathway [40]. iTreg and Th17 cells are thought to have considerable plasticity with each other due to a similar developmental pathway and a reliance upon similar cytokines in their initial stimulation. Both subsets are reliant upon the action of TGFβ; iTregs will arise due to TGF-β alone, but if this cytokine is present in combination with inflammatory cytokines, the T cell is pushed into a Th17 phenotype. For example, a specific subtype of regulatory T cell, which expresses the IL-1β receptor, will change from a regulatory to a Th17 phenotype in the presence of IL-1β. Studies such as these indicate that memory T cells stationed in the peripheral tissues are subject to change depending upon local conditioning signals. These can come from dedicated immune cells, but also from the considerable number of structural type cells. Interaction between T cells and structural cells including epithelial, endothelial and fibroblast cells may have fundamental influences of T cell activity in these organs.

1.3.2   Mucosal associated invariant (MAIT) T cells

As well as conventional T cells, which express exquisite antigen specificity and as a population at large can respond to a huge variety of antigens, there also exist populations of T cells that have a greatly restricted antigen-specific response. This includes the mucosa-associated invariant (MAIT) T cells. MAIT cells are similar to conventional T cells in that

they are thymic in origin and express an αβ T cell receptor. However, these cells are defined by their expression of the Vα7.2 subunit of the TcR, which confers responsiveness to antigens bound through a unique antigen loading molecule- the MHC class I related molecule MR1 [48]. As a result, MAIT cell populations have much narrower antigen specificity, which appears to be focused on B vitamin metabolites produced by intracellular bacteria [49]. These cells are distributed primarily at the mucosal surfaces of the body but can also be found in the liver and the circulation. Critically, MAIT cells are able to respond rapidly to their cognate antigen, allowing an early response to threats in situations where there may be no established conventional T cell response [50]. These cells are therefore seen as a form of innate-like lymphocyte which is involved in bridging the gap between the initial insult in the mucosal zones and the generation of a full adaptive response. Their production of IFNγ, tumour necrosis factor-alpha (TNF-α) and IL-17A appear to be critical early on in the immune response to pathogenic insults [51]. MAIT cells also remain in the affected zone well into the late phase of the response, indicating they are not simply early responders that dissipate once the full adaptive response has been mobilized [52]. These studies identify such cell populations through their expression of the Vα7.2 TcR alpha chain and the surface marker CD161. These studies have focussed upon MAIT activity in mice, however there is currently very little understanding of MAIT cells in the human lung. Stationed in the mucosal surfaces of the body, MAIT cells may work in tandem with conventional T cells to fight infection in the lung environment. Through providing an early source of IFNγ, these cells may trigger amplification of antimicrobial responses by driving MHC class II expression on local cells, thereby facilitating activation of conventional T cells in the area. In peripheral areas like the lung, conventional T cells are predominantly of a "memory cell" phenotype.

1.4   Memory T cells

The activation of conventional T cells leads to the development of a large number of antigen-specific cells with different functions working to maintain long-term immunity. These cells differentiate into a number of possible subtypes. Some lose the ability to home to lymph nodes (through loss of the lymph node homing chemokine receptor CCR7) and upregulate homing molecules specific for peripheral tissues (such as CCR4 for lung homing [53]). This allows T cells to home to the zones where the antigenic insult is being experienced. These are the effector subtypes, capable of producing

potent cytokines and directing the wider immune response to pathogens in the tissue (particularly T helper cells). Many of these clonally expanded cells apoptose in the resolution phase of the response (with roughly 90% of clones dying) [54]. The activation of naïve T cells also provokes the generation of much longer-lived memory T cell populations. These long-lived cells are generated alongside the initial effector T cells and have distinct surface phenotypes relevant to their roles in maintaining long-term immunity. These cells can be stationed within the lymphatic system ("central memory" cells) or be circulating and residing in peripheral tissues ("effector memory" cells) and are responsible for any subsequent immune response after the initial antigen encounter [55]. All memory cells are differentiated from naïve cells by their expression of the CD45 isomer CD45RO (as opposed to naïve cell CD45RA)[56]. Central memory cells are further defined from effector memory cells by the presence of lymphatic homing molecules such as CCR7 and L-selectin [57]. These cells also seem to rely upon specific survival cytokines for homeostatic maintenance, such as interleukin 7 [58 2005]. It has been demonstrated that these memory cells (particularly central memory cells) have a striking ability to rapidly reactivate in response to antigen and produce a phenotypically diverse population of antigen-specific clones [59]. This behaviour is thought to contribute to the memory cells' ability to provide enhanced recall responses to antigen. Recently, a third memory cell phenotype has been described- the T resident memory population. These cells take up long-tem residency within a particular peripheral tissue and do not appear to re-enter the circulation. The presence of CD4 T resident memory cells has been described in the lung [60], where they rapidly respond to local infection and maintain long-term survival without requiring lymphatic recirculation. T resident memory cells appear to exhibit a distinct CD69 and CD103 phenotype, dependent upon the location in the periphery [61]. The development of these various memory cells originates from initial activation of a naïve cell, however it is not fully understood how the diverse and distinct activated subtypes are formed.

1.4.1 Memory T cell development

Three models have been proposed for the development of memory-type T cells. In the linear differentiation model, initially activated T cells are all effector cells, with central and effector memory populations developing out of this population at a later stage of the response [62] The bifurcating (or asymmetrical division) model describes initial polarization of the

dividing naïve T cell into daughter cells that have a pre-memory or pre-effector phenotype. These go on to divide further and form the central and effector memory populations, or the short-lived effector cells [63]. Alternatively, the progressive model places the development of the various post-activation memory phenotypes on a spectrum. Initially stem cell-like populations such as central memory populations form, and these serve as precursors for both memory and short lived effector populations as division continues [64]. There is evidence for all three theories and memory T cell genesis currently remains undefined. Interestingly, in the lung environment, there appears to be a role of T helper cell IFNγ production in order to develop optimal CD8 tissue resident memory populations [65], highlighting a potentially critical role for T helper cells in the development of this particular memory phenotype.

1.4.2   Differences in co-stimulatory requirements between naïve and memory T cells

An important difference between naïve and memory T cells is their requirement for co-stimulation signals. While naïve T cells are recognised as having an absolute requirement for signalling through CD28-CD80/86 interaction, it has been shown that memory T cell populations do not necessarily require this stimulation to reactivate [46, 66]. This would allow memory T cells to interact with APCs that do not express the CD80/86 molecules that are so critical for naïve T cell activation, and suggests memory cells are able to interact with a wider range of MHC class II expressing cells, not just professional APCs.

1.5   Control of immunity within the lung

The lung is a mucosal surface and therefore must be capable of dealing with numerous pathogens that enter the body via the respiratory tract, however the nature of T cell responses within the lung is still under scrutiny. Only recently has it been showed that even during homeostasis, the human lung contains up to 1×10^{10} T cells [67]. This large number of T cells is similar to the situation in human skin, and highlights that peripheral tissues are areas of major T cell residency, even in the steady state. The vast majority of human lung T cells exhibit a memory cell phenotype and bear conventional αβTcRs. Large numbers of both CD4 and CD8 lung T cells express CD103 and/or CD69+, which are involved in retaining cells in tissue zones and are indicative of a T resident memory phenotype [68]. Lung resident T cells also express vascular cell

adhesion molecule-1 (VLA-1). VLA-1 is proposed as a marker of lung residency, and in models of human influenza infection VLA-1+ T cells are the dominant source of IFNγ and respond rapidly during secondary challenge [67]. Interestingly, influenza challenge of lung based T cells compared to circulating blood and skin resident T cells reveals lung cells launch a much stronger response (14% of T cells proliferating compared to 1% and less than 1% respectively). These observations from human infection models provide strong evidence for T cells specific for lung pathogens lodging in the lung, rather than responses relying primarily upon recruitment of circulating effector cell populations [69]. Critically, it has been shown that T resident memory cells exist in distinct special niches within the lungs, in the submucosal regions directly beneath epithelial cells [53]. These resident memory clusters are situated directly adjacent to areas of previous antigenic insult, are potent sources of IFNγ and do not require replenishment from secondary lymphoid populations [60]. This last point suggests that resident T cells can be maintained in the lung microenvironment long-term, and rely upon local sources of stimulation for survival and activation.

1.5.1 Control of immunity by professional antigen presenting cell populations in the lung

It is clear there are significant populations of T cells residing in the lung mucosa, contributing to both steady state immunoregulation and to inflammatory responses to pathogens as required. T cells, by their nature, rely upon interaction with other cell populations to modulate their activation state and inflammatory profile. As previously mentioned, the professional APC populations represent the critical interacting cell during naïve T cell activation. However, once initial activation occurs and activated T cells leave the secondary lymphatic system and move to the lung periphery, they are able to interact with a wide number of lung cells that all work in concert to mediate immunity in this organ.

1.5.1.1 Lung dendritic cells as APCs

The lung contains a number of phenotypically distinct dendritic cell populations. Similar to other peripheral organs, DCs are important in transporting tissue antigen to

draining lymphatic tissue and priming T cell responses [70]. Distinct subsets of lung DCs appear to exist, with specialized functions relating to their interactions with T cells. CD103+ DCs are thought to be critical in the initial priming of CD8 T cells, due to an ability to "cross-present" exogenously obtained antigen into the MHC class I pathway [71]. While CD103+ DCs are required for initial priming, later in the response it appears that a CD11b+ DC subset is required to promote generation of central memory CD8 cells [72]. DCs also appear to have variable abilities to imprint effector subtypes upon T helper cells, albeit with conflicting results. Evidence using mouse modeling demonstrate that CD103+ DCs preferentially imprint a Th2 [73] or Th1 and Th17 [74] phenotype. Despite both studies using a similar mouse and antigen model, these differences may be context driven: exposing DCs to antigen *in vivo* appears to result in different T cell conditioning compared to *in vitro* antigen exposure. This demonstrates the importance of the local microenvironment in APC-T cell interaction and the subsequent nature of the T cell response.

Similar to most peripheral zones, lung DCs appear to be critical in initiating T cell responses by migrating to draining lymph nodes with peripheral tissue antigens and priming naïve T cells. However, lung DCs as a population are overshadowed by the much larger populations of macrophages [75]. These large macrophage populations are heavily involved in controlling both infection and immune responses in the lung

1.5.1.2 Lung macrophage populations as APCs

The professional APC compartment of the lung is dominated by large numbers of macrophage populations. These reside within the interstitium, alveolar and bronchial lung regions and are involved in immune regulation and the switch to inflammation, as required. Alveolar macrophages are the most abundant professional phagocyte in the lung [75]. Here, they are highly effective phagocytes, being able to quickly control bacterial colonization [6] while limiting inflammation. Mechanisms involved in maintaining this immunosuppressed steady state include low expression of immune synapse molecules MHC class II [7] and CD80 and CD86 [76], resulting in a poor ability to stimulate primary T cell responses. Furthermore, alveolar macrophages produce retinoic acid and TGFβ, which imprint a regulatory phenotype upon interacting T cells, further promoting immunosuppression [77]. These results have led to alveolar macrophages being seen as highly effective phagocytes with a relatively poor ability to

activate inflammatory T cell responses directly. However, alveolar macrophages are able to contribute to the switch from an anti- to pro inflammatory state, and their role in doing so is critical for combating pathogens [78]. A number of factors contribute to this change in macrophage behavior. Collectin family surfactant proteins A and D, produced by type II epithelial alveolar cells, suppress macrophage activity in the steady state through interacting with regulatory receptors via their globular heads. However, these same binding regions have affinity for PAMPs, and once bound, allow surfactant collagenous tail regions to bind activating receptors on macrophages and trigger both phagocytosis and proinflammatory activation [79]. Increased presence of PAMPs triggers TLR signaling and promotes further activation of macrophages, and also decreases sensitivity to immunoregulatory IL-10 [80]. This, along with the loss in anti-inflammatory surfactant activity and reduced IL-10 production by epithelial cells (as a result of infection and cell loss), tips the balance and results in a proinflammatory macrophage phenotype. TLR ligation under these circumstances results in a release of proinflammatory cytokines, triggering a wider immune response [8].

Both macrophage and DC populations in the lung are important in providing effective immunity and the promotion of anti or proinflammatory responses as required. However, as well as professional immune cells, there are large numbers of structural-type cells that contribute to the immune state of the lung and may also directly interact with tissue T cells. These include the epithelial and fibroblast cell populations.

1.6 The role of structural cells in lung immunology

The structural type cells of the lung include the epithelial, endothelial and fibroblasts populations. These cells have specialized roles in providing a physical barrier between the lumen and interstitial tissue zones as well as providing structural integrity to the organ as a whole. In addition, these cells have known immune capacity that contributes to immune responses. These cells may exert influence over tissue resident T cell populations. As previously discussed, initial activation of naïve T cells is reliant upon dendritic cells and occurs in the draining lymph tissues. However, once activated, these T cells traffic into the lung via the circulation. Upon lung entry, T cells are open to interact with a wide selection of lung cells, including both professional immune and structural cell types (figure 1-4)
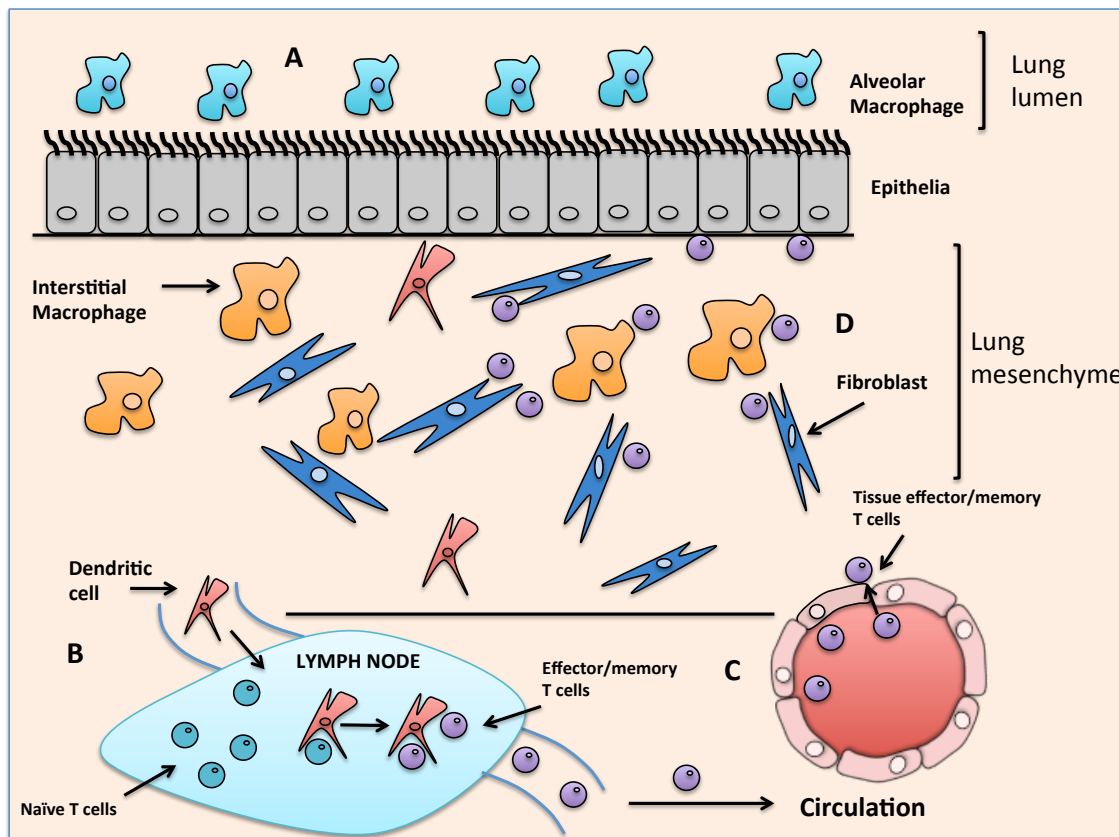
Figure 1-4: Memory T helper cells in the lung mesenchyme may interact with a variety of different cell types: Alveolar macrophages in the lung lumen are highly effective at clearing bacteria, but are not considered integral in activating T cells (**A**). This is carried out by dendritic cells (DCs), which take environmentally acquired antigen from the lung periphery and relocate to the lymph node via afferent draining lymphatics (**B**). Here, DCs are able to prime naïve T helper cells, which have specificity for lung antigens being presented via MHC class II on the DC cell surface. This triggers T cell activation, taking the T cell from a naïve to a memory or effector phenotype. These effector/memory cells then leave the lymph nodes via afferent lymphatics, enter circulation and traffic to the lung (**C**). After entering the lung environment, effector/memory T cells can interact with a wide number of cell types. These include interstitial macrophage populations as well as structural cell types, such as lung epithelial and fibroblast populations (**D**). Interaction with these various lung cells may affect T cell activation

1.6.1   Lung epithelial cells

Lung epithelial cells provide a physical and immunological barrier to environmental and pathogenic factors. Epithelial cells are involved in conditioning the lung into an anti-inflammatory state though a constitutive release of IL-10 [81], promoting

20

immunosuppression and formation of Treg cells [82]. Similar to alveolar macrophages, epithelial cells are able to switch to a proinflammatory phenotype under the correct circumstances. Epithelial cells are the primary cell type infected by a number of airborne pathogens, such as *Respiratory syncytial virus* (RSV) and *influenza virus*. Infection of the epithelia by these pathogens drives upregulation of TLRs (which are usually expressed at low levels), which in turn facilitate the release of proinflammatory mediators by making the cell responsive to PAMP signals [83, 84]. Responsiveness to TLR agonists is enhanced by exposure to cytokines TNFα and IFN γ produced by other lung cells. Ultimately, this leads to TLR-dependent release of proinflammatory cytokines such as IL-6 and IL-8 [85]. The epithelium is also a known source of IL-1α, a potent alarmin signal that can be released by damaged and dying epithelial cells, and proceeds to activate local fibroblast production of potent proinflammatory cytokines [86]. These fibroblast populations are located directly beneath the epithelial layers of the lung in the mesenchyme.

1.6.2   Lung fibroblast cells

Fibroblasts are bone marrow derived cells that inhabit the lung mesenchymal zones, where they are responsible for the production of extracellular matrix (ECM) components such as elastin, proteoglycans, fibronectin and the collagens. The ECM forms the structural framework and provides the means by which cells migrate through the organ. Fibroblasts themselves are characterised by an elongated, spindle-like cell body and are morphologically similar between different tissues within the body (figure 1-5).
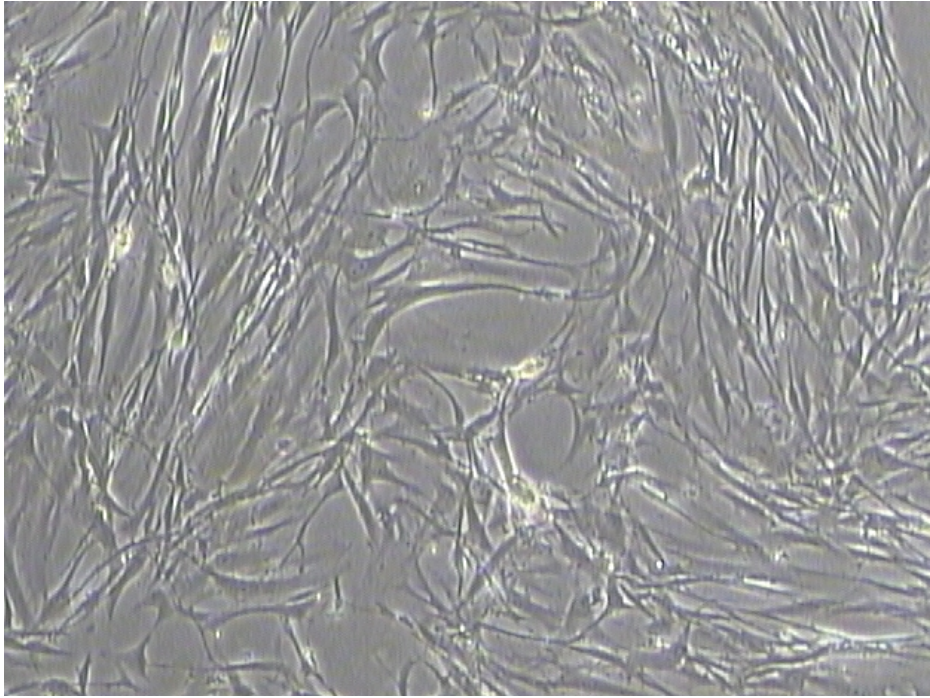
Figure 1-5- Human primary lung fibroblasts grown in culture: Human lung fibroblasts grown in culture are characterised by an elongated cell body with extending processes. Photograph kindly provided by Melanie Jannaway.

Fibroblasts are also capable of differentiating into myofibroblasts. In this situation, fibroblasts differentiate (typically due to TGFβ activation) and begin to express myosin and alpha-smooth muscle actin filaments, which allows them to exert contractile force and physically close wounds [87, 88]. Thus, fibroblasts and myofibroblasts have typically been considered a cell type involved in the production and maintenance of the scaffolding holding tissues together and in the wound repair process. Despite the ultrastructure of fibroblasts being incredibly similar between different tissue zones [89], there are major differences in the behaviour and response to inflammatory mediators by fibroblasts from different organs. This suggests that fibroblasts are not a universal cell type, but rather have a positional memory imprinted upon them that is defined by their location in the body. This locational conditioning affects the way in which these cells respond to specific mediators. As a result, fibroblasts from different tissues can exhibit starkly different levels of involvement in inflammatory reactions. Furthermore, it has been established that there are different phenotypes of fibroblasts within the same tissue. In the case of the lung, the proximal airway fibroblasts and the distal, parenchymal fibroblasts have notable differences in their ECM production [90] as well

as their cytokine response to inflammatory mediators [91]. This is just one example of fibroblast populations from the same organ exhibiting differing phenotypes. While it is well established that fibroblasts are heavily involved in matrix production and wound healing, understanding their level of involvement in immune responses is still a developing field.

1.6.3 Fibroblast immunological functions

Studies involving fibroblasts from a wide range of tissues have shown that fibroblasts from various locations have some involvement in immune responses. A common mechanism is the release of soluble factors such as the potent neutrophil chemotactic factor CXCL8 and macrophage chemotactic protein-1 (MCP-1), allowing a general ability to draw in innate responder cells [92]. While there is a generalized trend in the release of these particular factors between anatomically distinct fibroblasts, there are important divergent functions between fibroblasts from different sites. Fibroblasts can also be central to disease processes. Orbital fibroblasts are now understood to be implicated in the autoimmune conditions Graves ophthalmopathy and thyroid-associated ophthalmopathy. [93]. In these situations, disease-associated fibroblasts produce greater amounts of IL-6, IL-8 and MCP-1, all of which draw in innate responder cells. Ophthalmopathy-derived fibroblasts also can interact in a cell-to –cell contact manner via IFNγ-induced CD40 expression, allowing further IL-6 and IL8 production (Hwang et al., 2009). Synovial-like fibroblasts of the rheumatoid joints are another example of a fibroblast population exhibiting significant immune function. Here, synovial fibroblasts produce an array of inflammatory mediators that create a highly inflammatory microenvironment and recruit immune cells, including T helper cells (reviewed in [94]). These examples demonstrate disease-specific phenotypic changes in fibroblasts and also show how fibroblasts can exert significant influence on local organ-specific immunity.

Lung fibroblasts are subject to the same issues faced by other lung-resident cells; the need to maintain steady-state immunosuppression in the mucosa, but to switch to an inflammatory state during pathogenic insult. Fibroblasts are thought to contribute to the immunosuppressive steady-state atmosphere in the lung through suppression of T cell activation state. This mechanism appears to suppress recently activated T cell proliferation and production of TNF-α while maintaining immunoregulatory IL-10

release though a contact-dependent mechanism [95]. This immunosuppressive ability is in line with observations of lung resident APCs and epithelial cells promoting suppression of immune activity in the uninflamed lung. Interestingly, fibroblast suppression of T cell proliferation is impaired in patients with the inflammatory disorder chronic obstructive pulmonary disease, indicating fibroblasts respond to the local environment and have variable phenotypes with regard to immune capability [96]. While lung fibroblasts are immunosuppressive in the resting state, they are able to alter their function in response to local environmental cues. Exposure to IL-4 promotes the release of eotaxin-like compounds, allowing fibroblasts to contribute to type 2 immune reactions though eosinophil recruitment. IFNγ exposure also drives the production of CCL5, a potent T cell chemotactic factor [97]. Being located in the interstitium, fibroblasts are in a prime location to receive conditioning from the overlaying epithelial cell layers and studies have demonstrated lung fibroblasts exhibit strong responses to epithelia "alarmins" (factors released by dead or stressed cells). Lung fibroblasts respond to epithelial-derived IL-1α, exhibiting robust release of IL-6 and IL-8. Interestingly, the same response was not mirrored by macrophages, suggesting IL-1α release represents a specialized epithelial-fibroblast mechanism to recruit immune cells in cases of epithelial death [86].

From this growing body of work, it is clear fibroblasts are not immunologically inert, background cells to the immune response in the lung, but are capable of interacting with immune cells and amplifying inflammatory reactions. What is not fully established is the nature of T cell-fibroblast interaction, particularly the ability of fibroblasts to present antigen to T helper cells within the lung.

1.7   Lung structural cells as non-professional APCs

1.7.1   Lung epithelial cells as APCs

As well as providing a physical and chemical barrier to the lung surface, lung epithelial cells are also able to function as an APC. Constitutive expression of MHC class II [98, 99] suggests an ability to correctly process antigen and load it onto MHC class II molecules for presentation to T helper cells. Epithelial cells appear to use an antigen-processing pathway similar to that of professional APCs and this pathway is required for effective antigen presentation to T helper cells [100, 101]. Certain cathepsin family

proteins have been demonstrated to be critical in the ability of non-professional APCs like epithelial cells to produce MHC-antigen complexes [23], with variable reliance upon individual cathepsin proteases between different APCs. For example, cathepsins L and B are variably expressed in lung epithelia depending upon the surrounding cytokine milieu [102], and under inflammatory conditions the cathepsin-S molecule in particular appears to be dominant in antigen processing for both professional and a number of non-professional cells [23, 103]. The functional outcome of T cell interaction with these non-professional APCs has been poorly defined. Despite expression of MHC class II, human lung epithelial cells are unable to activate naïve T cells from blood. This is most likely due to a lack of CD80 and CD86 expression, resulting in an unresponsive ("anergic") state being imprinted upon T cells [104]. The feasibility of an epithelial cell- naïve T cell interaction is questionable, as the vast majority of T cells in the lung are of a memory phenotype while naïve cells should be restricted primarily to secondary lymphoid organs. Studies specifically investigating interaction with effector-type cells reveal that epithelial cells can in fact act as APCs and cause T cell activation. Effective antigen-dependent activation in this scenario was dependent upon epithelial expression of the CD80/86 related B-7 family molecule Inducible T-cell stimulator ligand (ICOSL) [105]. These studies suggest that memory and effector T cell populations are able to interact with non-professional APCs via other co-stimulatory molecules, and do not have an absolute requirement for CD80/86 stimulation as naïve T cells do.

While presentation of antigen via MHC class II to T helper cells is classically associated with cytokine release, recent developments in human influenza models have demonstrated a cytotoxic subset of T helper cells [99]. When stimulated with influenza peptides, a subset of T helper cells upregulate CD107a, indicating degranulation by the cell [106]. This process is shown to be sensitive to inhibition of the perforin/granzyme pathway used by cytotoxic cells to destroy virally infected cells [35]. Furthermore, CD4 T cells and not CD8 T cells correlated with protection in this model, suggesting a possible key role for these novel cells in responding to lung pathogens. Considering the fact influenza infects lung epithelial cells and drives upregulation of HLA-DR, these cytotoxic CD4 T cells most likely target epithelial cells via HLA-DR antigen presentation. These recent development demonstrate a previously unknown relationship between T helper cells and epithelial cells presenting antigen, and suggests T helper cell-structural cell interaction within the lung could be key to controlling infection.

25

The significant populations of T cells within the lung are reliant upon communication with local tissue-resident cells to operate effectively. While the roles of professional APCs and cell such as epithelial cells have been, and continue to be, characterized, the nature of T cell-fibroblast interaction in the lung is still largely unknown. In particular, the antigen-presentation function of fibroblasts in activating T helper cells has not been fully elucidated. This interaction is a major interest of the current study.

1.7.2 Fibroblasts as a potential structural APC

It has been demonstrated that a number of different cell types can upregulate MHC class II after exposure to IFNγ. Expression of MHC class II is a strong sign the cell is engaged in the processing and presentation of antigen and as a result, may be able to activate antigen-specific T helper cells. Based on this observation, attempts have been made to demonstrate the functional effects of fibroblast MHC class II expression upon T cell activation state. Dermal fibroblasts pre-conditioned with IFNγ are able to stimulate proliferation of T cells previously activated to candidate antigen, but were totally unable to prime naïve T cells to the same antigen [107]. The synovial-like fibroblast (SLF) has also been the subject of much interest, due to its central role in the pathogenesis of rheumatoid arthritis [94]. Using T cell hybridoma techniques, SLFs have been shown to present autoantigens via MHC class II and stimulate T cell division and production of IL-2 [108]. Studies using synovial joint-derived T cell lines driven against a candidate antigen further demonstrate FLS presentation though an ability to produce soluble IFNγ and again stimulate proliferation [109]. In all cases, fibroblasts require IFNγ in order to activate T cells. In cases where PBMCs have been used as a source of T cells, fibroblasts do not cause activation [110]. These studies demonstrate that fibroblasts have an inducible ability to stimulate T cells; particularly T cells inhabiting tissue zones and previously activated memory-type cells. While T cell proliferation appears to occur after fibroblast presentation, a comprehensive examination of cytokine production across the various T helper subsets has not been carried out. Examination of the antigen presenting capacity of lung fibroblast in particular is poorly defined. Considering the fact lung T cell populations appear to be primarily memory and resident-memory types [69], fibroblast antigen presentation may represent a key immune mechanism through presenting antigen to memory/resident

memory T helper cells in the lung environment. This may be of particular relevance in situations where infection breaches primary immune defences (in the form of alveolar macrophages and the overlying epithelium) and penetrates the mesenchyme zone.

1.8   Pathogenic events in the human lung

As a mucosal surface, the lung is regularly exposed to pathogenic and potentially pathogenic factors that must be dealt with by the immune system. These include both transient virus infections as well as bacterial populations that colonize the lungs in both healthy individuals and those with underlying lung pathologies. These colonizing bacteria are collectively referred to as the lung microbiome, a term originally coined to describe the vast numbers of bacteria resident at other mucosal surfaces [111]. While these colonizing bacteria are controlled in most individuals, under circumstances where immunity is compromised they are thought to contribute to aberrant lung inflammation. Studies examining lung viral infections have demonstrated how disrupting the delicate immune balance results in these bacteria taking on a central role in pathogenic processes.

1.8.1   Viral infection of the lung

Viruses such as *Influenza*, RSV and *Rhinovirus* are common lung pathogens that typically invade the epithelial cells of the respiratory tract. RSV invades primarily the upper respiratory tree and is a cause of morbidity and mortality in young children [112]. Rhinovirus again invades epithelial cells primarily in the upper respiratory tract in healthy individuals, although infection has been shown to spread to the lower tract [113]. Both RSV and rhinovirus infections are associated with a transient, mild illness and are usually effectively cleared in immunocompetent adults. In contrast, influenza infection can cause much more serious disease. Influenza can be broadly split into 2 main categories: seasonal and pandemic strains. Seasonal stains are typically dealt with more robustly by the immune system due to similarities between the current strain and the previously encountered seasonal strains [114]. Pandemic strains are much less well controlled due to their radically different antigenic profile (usually due to reassortment of immunodominant antigens between two different influenza strains)[115, 116]. Through infection of epithelial layers and considerable effects upon on the immune system, influenza is considered a major pathogenic viral lung infection. More recently,

the relationship between influenza infection and the local lung bacterial populations has become a major area of interest. Secondary bacterial pneumonia post viral infection appears to be a major cause of mortality in the context of influenza infections [117, 118]. This phenomena demonstrates how normally non-pathogenic bacteria can opportunistically cause disease due to immune dysregulation by a separate factor.

1.8.2   Bacteria within the lung

Whereas in the past the lung was considered a largely sterile environment beyond the nasopharyngeal area, it is now known that large numbers of bacteria inhabit the upper and lower respiratory tracts of the human lung [1]. Utilizing modern analysis techniques, it has been shown that an average of 2.2×10^3 bacteria are present per square cm of lung airway [119]. As these bacteria are present in healthy individuals, the immune system presumably maintains a level of control over these populations; ensuring bacterial numbers are kept in check while limiting inflammation to prevent immunopathology to a constant antigen stimulus. The role of these bacteria in lung biology is still a developing field, although recent advances highlight a critical role in both pneumonia events and chronic inflammatory conditions

1.8.2.1   Influenza infection leads to bacterial invasion of parenchymal tissue

While bacterial colonization appears to be "commensal" in healthy individuals, influenza infection drastically disrupts this balance. Pandemic influenza strains have been shown to lead to bacterial superinfection, leading to massive bacterial invasion of the lung parenchyme, increased neutrophil recruitment and morbidity [117] [17]. Studies examining virally induced bacterial pneumonia show infection with influenza or a bacterial load alone does not cause lung disease. However, administering a bacterial load to mice previously infected with influenza results in bacterial invasion beyond the epithelia into the mesenchyme [17]. This is accompanied by a drastic increase in neutrophil recruitment compared to influenza or bacteria exposed mice alone. Furthermore, these increased neutrophil populations exhibited impaired antimicrobial activity compared to single-infected equivalents [17], and the increased presence of these cells appears to drive pathology. Such observations suggest excessive neutrophil recruitment as a major factor in secondary bacterial mortality. Further studies demonstrate that influenza infection appears to drive massive alveolar macrophage

depletion (in some cases up to 90% depletion); after which secondary bacterial pneumonia occurs [120]. Taken together, these results show that influenza infection has a severe impact upon the lung, paving the way for bacterial invasion beyond its normal niche in the lung luminal areas. The loss of alveolar macrophages and the considerable epithelial cell death as a consequence of influenza infection impair frontline immunological barriers. This is followed by bacterial invasion into the mesenchyme and subsequent neutrophil recruitment. As fibroblasts reside in the mesenchyme, fibroblast responses to invading bacteria may be a key immune mechanism contributing to defence and/or immune pathology under these circumstances. As well as having a central role in secondary pneumonia, colonizing bacteria are now believed to contribute to pathogenic processes in chronic inflammatory conditions within the lung, such has COPD.

1.8.3   Chronic obstructive pulmonary disease (COPD)

COPD is a chronic inflammatory condition characterised by irreversible destruction of the alveolar regions (emphysema) and fibrotic remodelling of the larger airways (chronic bronchitis). Cigarette smoke exposure (CSE) is the primary environmental driving force in COPD, and it is associated with increased numbers of inflammatory cells within the lung [5]. Particular emphasis has been placed on CD8 cytotoxic T cells, which appear in greater proportions in COPD patients and correlate with declining lung function [121]. T helper cell numbers are also increased in COPD patients, but the nature of these cells in the COPD is still not completely understood. The microbiota of the lung also appears to change in individuals with COPD. Recent work examining these changes suggests colonizing bacterial populations contribute to the COPD disease process in the lung.

1.8.4   COPD and the relationship with lung bacteria

COPD is defined by distinct changes in the lung microbiome [1], with bacteria such as Non-typeable *Haemophilus influenza* (NTHi) being postulated as a main risk factor in driving disease exacerbations [122]. NTHi is a small uncapsulated cocbacillus, recognised to be relatively harmless in most individuals [123], able to exist in lung in both extracellular biofilm [124] and intracellular states [125]. Immunity to NTHi appears to be impaired in the COPD lung. NTHi clearance by alveolar macrophages is

impaired in COPD individuals [126], possibly due to the actions of CSE which has been shown to cause apoptosis [127] and impairment of alveolar macrophage immunological functions [128]. Mice models further demonstrate that CSE causes a significant reduction in both IFNγ production and protective antibody generation to NTHi. Conversely, CSE increased neutrophilic inflammation associated IL-17 production [129], suggesting promotion of neutrophil responses to bacteria, similar to what is found in secondary bacterial models [17]. Recent work has demonstrated that lung T cell responses to NTHi are also altered in patients with COPD compared to smoking "healthy" equivalents. T helper and cytotoxic T cell cytokine responses to NTHi skew significantly towards a Th2 and Th17 response in individuals with COPD [130]. Skewing towards a Th2 response suggests an inappropriate response to NTHi in COPD subjects as Th1 cells are considered key in antimicrobial immunity [131]. These studies collectively indicate that the COPD lung has an impaired ability to control bacteria such as NTHi, and seems to promote a Th17, neutrophilic inflammatory profile. However, despite the fact NTHi is a leading cause of exacerbation, the exact means by which NTHi contribute to exacerbations is not understood. Similar to influenza-induced pneumonia, impairment of "primary" immune defences through an external factor (such as chronic CSE) may result in a loss of control over lung resident NTHi. As this control is lost, infection of epithelial cells can occur [125] and bacterial populations may invade the underlying mesenchyme. The role of fibroblasts in contributing to immune responses in such situations is poorly understood, particularly with regard to communication with tissue resident T cell populations.

1.8.5 SUMMARY

The human lung contains a huge number of T cells that are involved in responding to bacteria. These adaptive immune cells rely upon interaction with tissue resident APCs in order to receive antigen-specific activation signals. Through this interaction, T cells are able to direct the wider immune response via potent cytokine release. The ability of lung fibroblasts to present bacterial antigens is currently unknown, but may represent a mechanism contributing to responses against bacterial invasion of the mesenchyme. This would be particularly relevant in situations where epithelial and macrophage defences are impaired, such as is seen in pneumonia and chronic inflammatory conditions.

1.9   AIMS AND HYPOTHESIS

The hypothesis of this study is that human lung fibroblasts will act as an inducible APC and will present bacterial antigens to memory T helper cells, resulting in T cell cytokine production. Project aims are as follows

1- Characterise the nature of T cell populations present in the human distal lung, including an examination of the memory T cell populations as well as more novel cell types involved in microbial immunity and particularly in the early release of IFNγ

2- To examine if there are differences in these T cell populations between subjects with no obstructive lung disease and those with COPD.

3- Characterise the expression of the critical antigen presenting MHC class II molecule upon human lung fibroblasts from *ex vivo* lung samples.

4- Characterise the ability of lung fibroblasts to upregulate immune synapse molecules MHC class II, ICAM-1, CD80 and CD86 in response to common lung inflammatory stimulus *in vitro*

5- Examine the ability of lung fibroblasts to internalize exogenous antigens into a MHC class II processing pathway.

6- Develop NTHi-specific T helper cell lines from human lung samples for use in co-culture assays.

7- Co-culture NTHi-specific T helper cell lines with either autologous dendritic cells or lung fibroblasts previously exposed to NTHi antigens.

2. MATERIALS AND METHODS

2.1 Media and buffers

"**Pharm lyse" lysis buffer x10** (BD biosciences,Cambridge, UK). This solution contained a concentrated ammonium chloride based buffered solution and was diluted in sterile distilled water to a x1 working concentration solution.

"Perm wash" permeabilization buffer (BD biosciences,Cambridge, UK). This comes as a x10 concentrated solution containing foetal bovine serum and saponin. Solution was diluted in sterile distilled water to a x1 working concentration solution prior to use.

FoxP3 Fix/Perm Kit (eBioscience, Hatfield, UK). Fixation/permeabilization concentrate (containing paraformaldehyde) is diluted 1:4 with provided fix/perm dilutent solution and used for fixation of cells. This is followed by treatment with a permabilization buffer (provided as x10 solution, diluted in distilled sterile water to a x1 working solution)

Cell stimulation cocktail (eBioscience, Hatfield, UK) is provided as a x500 solution containing phorbol 12-myristate 13-acetate (PMA) and ionomycin, and was diluted to a working concentration of x1 through dilution into 10% HS fully supplemented RPMI (see "RPMI-1640" below)

Annexin V binding buffer (eBioscience, Hatfield, UK) is supplied as a x10 concentrate. This was diluted in sterile distilled water to a x1 working concentration.

Hanks balances salt solution (HBSS) with Ca^{2+}/Mg^{2+} (GE healthcare lifesciences, Buckinghamshire, UK)

Roswell Park Memorial Institute-1640 media (RPMI, Life Technologies, New York, USA**)** was supplemented with 1% penicillin/streptomycin (V/V), 1% sodium pyruvate (V/V), 1% glutamine (V/V), 1% non-essential amino acids (V/V) and either 10% neonatal calf serum (Life technologies, Paisley, UK) or 10% human serum (V/V) (Sigma-Aldrich, Gillingham, UK).

Dulbellco Modified Eagles Medium (**DMEM**, GE healthcare, Buckinghamshire, UK). This was supplemented with 1% penicillin/streptomycin (V/V), 1% sodium pyruvate (V/V), 1% glutamine (V/V), 1% non-essential amino acids (V/V) and 10% neonatal calf serum (V/V)(all Life technologies, Paisley, UK). For serum starvations steps as part of an experimental procedure, neonatal calf serum was omitted from the preparation

Triton-X 100 (Sigma-Aldrich, Gillingham, UK) was diluted into PBS solution to a final concentration of 0.1% (V/V)

Paraformaldehype (4%) (Sigma-Aldrich, Gillingham, UK) was prepared from powdered stock and reconstituted in PBS solution heated to 60°c and with sodium hydroxide added to allow the power to form a solution. Once fully dissolved pH was balanced using hydrochloric acid to a pH of 7.3. The final working concentration of PFA in solution was 4% (W/V).

FACS buffer contained PBS supplemented with 0.01% sodium azide (W/V) and 0.5% bovine serum albumin (W/V).

2.2   Patient lung resection samples

Patients undergoing resection surgery for suspected lung cancer or bullous repair surgery at Southampton General Hospital were approached for this study. Patient consent and participation was approved of by the "NRES Committee South Central" (Ethics number: 08/H0502/32). A study code number unrelated to the subjects name identified data collected from the resulting lung samples. Participant clinical details (age, gender, FEV_1, FEV_1 percent predicted, FVC, smoking history, pack years and years ex) were gathered in order to relate experimental data to clinical details. All distal lung tissue samples used in this study were taken from the marginal, non-cancerous zones of resection tissue and were classified as healthy non-cancerous by a pathologist.

2.3   Isolation of T cell populations for initial phenotyping

The isolation of lymphocytes for initial CD4 and CD8 memory T cell and MAIT cell characterization was carried out on populations isolated through mechanical and enzymatic disruption of tissue fragments. In these cases, mechanically and enzymatically processed (type 1 collagenase, 1mg/ml, for 30 mins at 37°C- Sigma-Aldrich, Gillingham, UK) samples were filtered through a 70μm nylon cell strainer and treated with pharm lyse solution (both BD biosciences) for up to five minutes to remove erythrocytes and washed twice in PBS (centrifuged at 400g for 5mins). Lymphocyte populations were then cultured overnight at 37°C in RPMI 1640 (Life technologies, New York, USA) media supplemented with 1% penicillin/streptomycin, 1% sodium pyruvate, 1% glutamine, 1% non-essential amino acids and 10% human serum

(Henceforth referred to as 10% HS RPMI). In addition, these cell populations were exposed to pro-survival Interleukin-2 (50U) overnight before being stained for cell surface markers for flow cytometry analysis of T cell memory and MAIT cell populations.

2.4   Non-enzymatic isolation of cells for NTHi-specific T cell responses and T cell lines

For the isolation of distal lung white blood cell cultures for initial heat killed NTHi T cell responder frequency and NTHi-specific line generation, lung fragments were cut into 2-3mm fragments and rested overnight in RPMI 1640 (Life technologies, New York, USA) media supplemented with 1% penicillin/streptomycin, 1% sodium pyruvate, 1% glutamine, 1% non-essential amino acids and 10% neonatal calf serum (all from Life technologies, Paisley, UK and henceforth referred to as 10% NCS RPMI) in order to allow cells to egress from tissue. Cells were not exposed to enzymatic treatment in order to prevent cell death and possible activation of T cell populations. After overnight egression from the tissue fragments to the culture media, cells were separated via lymphoprep density separation to isolate mononuclear cells and washed twice in PBS. Cells were suspended in PBS and layered on top of an equal measure of lymphoprep. This was then centrifuged at 490g for 30 minutes and washed twice in PBS to remove residual lymphoprep . Resultant cell populations were then used for either measuring the initial T cell response to heat killed NTHi by flow cytometry or for long-term culture to generate NTHi-responder enriched T cell populations (see diagram). For the generation of NTHi specific lines, cells obtained after the lymphoprep stage were exposed to 1500 heat killed NTHi bacteria and cultured for 5 days. After 5 days, cells were given a cytokine boost of 50 units IL-2 and 100 units IL-7. This method is designed to firstly activate only NTHi-specific T cells over the initial 5 days, and then promote proliferation of these responsive clones through the introduction of the growth cytokines IL-2 and 7. To verify that this method promoted the expansion of T helper cells, populations were taken after the initial 10 days and screened for the expression of proliferation-associated marker Ki-67 via flow cytometry. Cells were then maintained through feeding every 3 days with RMPI 10% supplemented with IL-2 (50U) and IL-7 (100U). Cell lines were used within 5 weeks of initial isolation. (See figure 2-1)
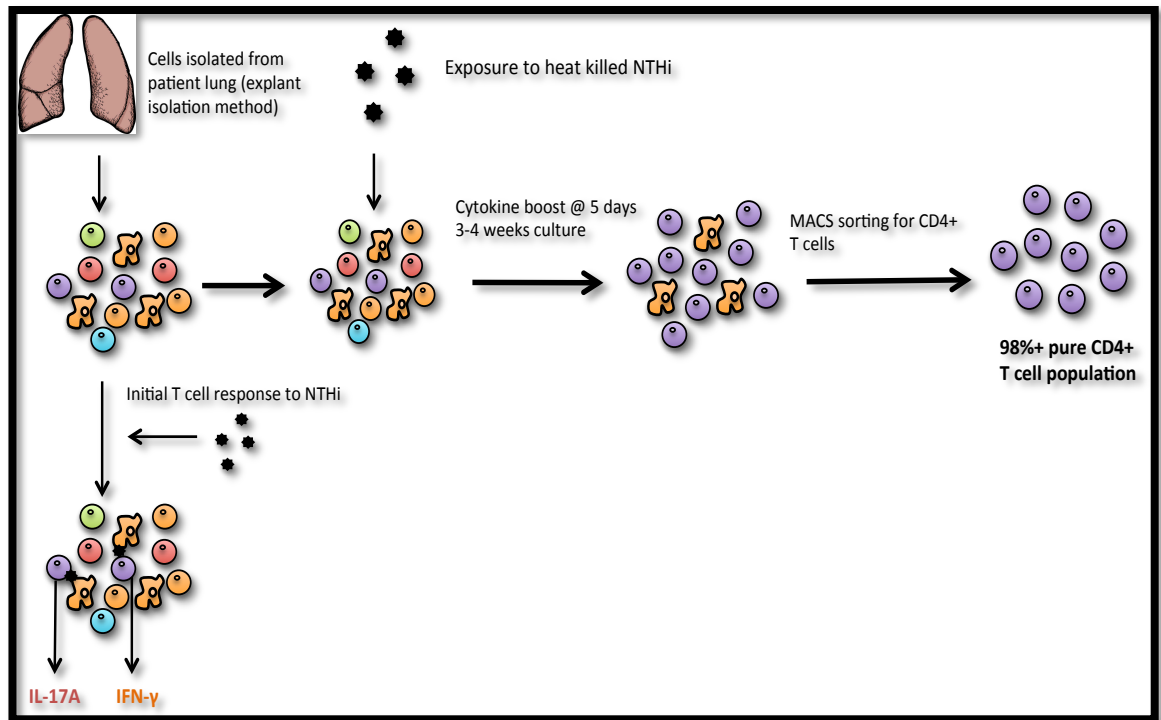
Figure 2-1 Outlining the procedure of isolating human lung immune cell populations, screening for initial T cell reactivity to NTHi and the development of a NTHi-specific T cell line: Human lung immune cell populations were isolated via egression from finely cut lung tissue fragments. Subsequent recovered cells were separated through a lymphoprep density gradient to isolate mononuclear cells. This results in a cell population including alveolar macrophages and lymphocytes. This initial population was used to firstly screen for the initial T cell response to heat killed NTHi via intracellular cytokine release. This represents the hosts' initial T cell responder frequency to NTHi in the lung. Cells recovered in this way were also used to generate an NTHi-responder enriched population. This was done by exposure of egression-isolated cells to heat-killed NTHi and long-term culture to produce a large number of NTHi specific clones. These cells were then used to test the ability of matched fibroblasts to present NTHi antigen and reactivate these clones

2.5   Isolation of human lung fibroblasts for *ex vivo* analysis

For the *ex vivo* analysis of different lung cell population (fibroblasts, epithelial cells and alveolar macrophages) expression of HLA-DR, lung fragments were enzymatically digested with type 1 collagenase (1mg/ml- Sigma-Aldrich, Gillingham, UK) for 30 minutes at 37°C. Once complete, digests were filtered through a 70µm nylon cell strainer (VWR international, Lutterworth, UK) and washed with PBS. Resultant enzymatically liberated cell populations were immediately stained with lineage markers and for expression of HLA-DR before analysis via flow cytometry.

2.6   Isolation of human lung fibroblasts for *in vitro* analysis

For further analysis of human lung fibroblast expression of immune synapse molecules, *in vitro* methods were used. Explanted tissue samples were cut into 2-4mm fragments and cultured in 6-well plastic plates with DMEM (PAA, Cambridge, UK) supplemented with 1% penicillin/streptomycin, 1% sodium pyruvate, 1% glutamine, 1% non-essential amino acids and 10% neonatal calf serum (Henceforth referred to as "DMEM 10% NCS"). This allowed for adherent fibroblast populations to migrate out of the tissue and populate the well surface. These were initially cultured in plastic 6-well plates and consequently passaged into T-25 and T-75 flasks (Sigma-Aldrich, Gillingham, UK) to accommodate greater cell growth and numbers.

Fibroblasts were grown to confluence in T75 cell culture flasks. Once confluent, cells were removed via treatment with tryple express and re-plated into welled plates for exposure to stimulants or for co-culture. For flow cytometry analysis of surface molecules, cells were seeded directly into plastic multi-well culture plates. Populations of fibroblasts were analysed for immune synapse molecules at baseline or after exposure to increasing levels of IFNγ (Peprotech, London, UK), IL-1α (Miltenyi biotechnology, Surrey, UK) or heat killed NTHi (Pfizer, New York, USA) for 48 hours. All cells were cultured in serum-free DMEM during this 48h incubation period. For flow cytometry, adherent fibroblasts were removed from plastic culture wells via accutase (eBioscience, Hatfield, UK) treatment to prevent the cleavage of cell surface epitopes. Cells were then transferred into FACS tubes in preparation for FACS staining and analysis

For confocal microscopy, fibroblasts were seeded upon sterilized glass cover slips precoated with 1% BSA and then placed within 6 well plates for incubation. Again, fibroblasts were cultured in serum-free DMEM while undergoing exposure to proinflammatory stimulation for 48h.

2.7 Generation of monocyte-derived dendritic cells from patient blood

Autologous matched professional APC populations in the form of monocyte-derived dendritic cells (moDCs) were required for comparative analysis of antigen-presenting function against lung fibroblasts. This required the isolation of peripheral blood mononuclear cells (PBMCs) from whole blood and subsequent freezing in order to store these progenitors until both matched fibroblast and T cell populations reached sufficient numbers for experimental procedures (figure 2-2). Patient PBMCs were isolated from whole blood via lymphoprep density gradient separation. Briefly, blood was diluted 1:1 with PBS before being layered upon an equal measure of lymphoprep (Stemcell technologies, Cambridge, UK) and centrifuged for 30 minutes at 490g. The resultant buffy coat was washed twice in PBS before the now isolated PBMCs were suspended in a pre-chilled freezing solution of 10% dimethyl sulfoxide (DMSO, Sigma-Aldrich, Gillingham, UK) and 90% human serum (Sigma-Aldrich, Gillingham, UK). Cells were frozen at a density of 10 million cells per ml, and stored at -80°C until required for co-culture. When required, cells were defrosted by rapid thawing of vial in a pre-warmed water bath set to 40°C. Once the majority of solution was defrosted, 1ml of fully supplemented RPMI+10% NCS was added to each vial. The resultant 2ml total volume in each well was then further diluted into 8ml RPMI+10% NCS and centrifuged to recover cells. Cells were immediately resuspended in 80ul sorting buffer (PBS containing 0.2M EDTA and 0.5% BSA) with 10ul of Miltenyi CD14 magnetically labelled beads. This solution was mixed well before being incubated at 4°C for 15 minutes. Cells were then washed with sorting buffer before being passed through a magnetic column to separate out CD14+ monocytes (moDC progenitor cells). The resultant monocyte populations were cultured at a cell density of 500,000 cells/ml in fully supplemented RPMI+10% NCS containing growth factors interleukin-4 (IL-4, Miltenyi biotechnology, Surrey, UK) at 250U/ml and granulocyte-macrophage colony-stimulating factor (GM-CSF, Miltenyi biotechnology, Surrey, UK) at 500U/ml. Cells were cultured in this manner for 5 days, with feeding on the third. By day five cells were ready for exposure to factors prior to co-culture.
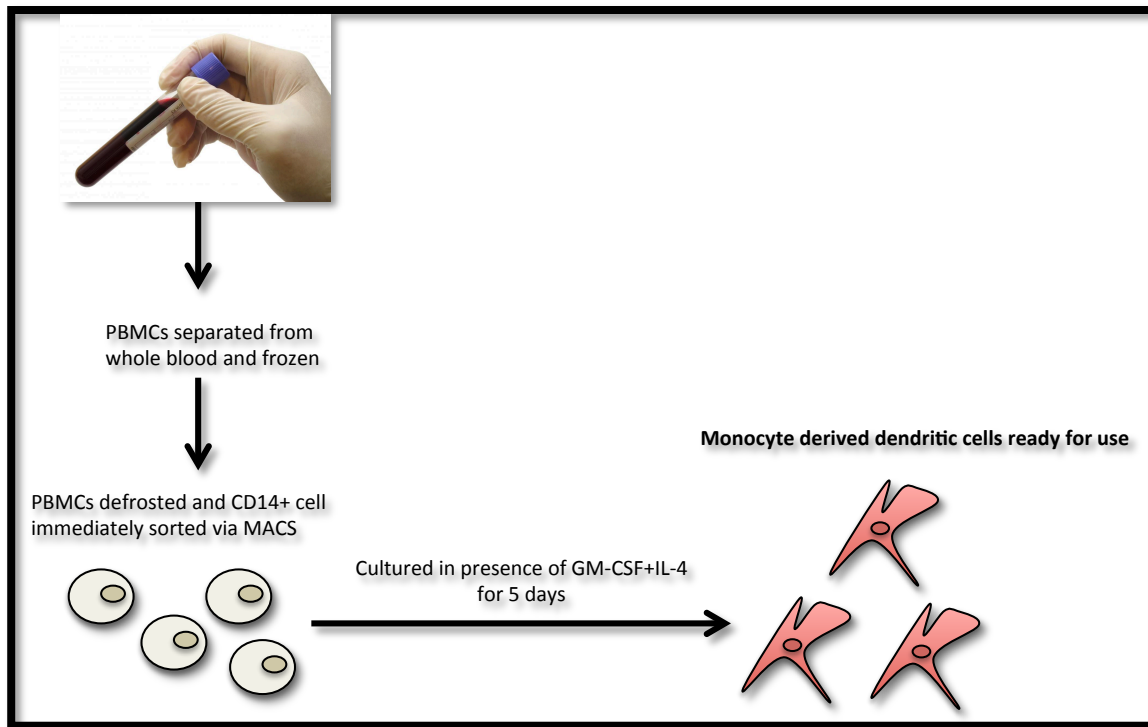
Figure 2-2- Isolation of patient PBMCs and generation of monocyte-derived dendritic cells from CD14+ progenitors: Subject whole blood samples were passed through lymphoprep density gradient separation to isolate PBMCS. These cells were immediately frozen in 10% DMSO+90% human serum and stored at -80°C. Once patient-matched fibroblast and NTHi-specific T cell populations were approaching adequate numbers for experimental use, PBMCs were defrosted and immediately magnetically sorted on the basis of CD14 expression to isolate monocytes. These cells were then cultured in the presence of the growth factors GM-CSF and IL-4 for 5 days in order to promote the generation of moDCs. After 5 days, cells were ready to receive conditioning in preparation for co-culture with autologous T helper cells.

2.8 FLOW CYTOMETRY OF HUMAN LUNG T CELLS

2.8.1 Initial characterization of human lung T cells by flow cytometry.

Lung cells were isolated via previously described enzymatic methods, and resultant populations allowed to rest in culture for 24 hours (see cell isolation section). Cells were then analysed via flow cytometry. Cells were identified as cytotoxic CD8+, T helper CD4+, mucosal associated invariant T cells (MAIT cells) and memory populations through fluorescently-labelled antibody staining (see table 2-1). Cells were suspended in FACS buffer (PBS+0.05% sodium azide+0.5% BSA) containing 10% human serum for 20 minutes on ice to block Fc receptors and prevent nonspecific binding of antibodies. Cells were then stained with either fluorescently labelled antibody for specific cell-surface epitopes, or for corresponding isotype controls for these antibodies. This would allow for effective gating on positively stained cell populations. Cells were then incubated on ice for 30 minutes in darkness to allow antibody staining. Cells were then washed twice in FACS buffer via centrifugation at 400g for 5 mins. Cells were then resuspended in 300-400μl FACS buffer for flow cytometry analysis.

Figure 2-3 is representative of the gating strategy used for analyzing the CD4 and CD8 T cells fractions from enzymatically digested lung samples. Firstly, cells were identified as lymphocytes based upon size and granularity (figure 2-3 A) before dead cells exclusion through uptake of dead cell markers annexin V and/or 7-AAD (figure 2-3 B). Live lymphocytes were then screened for CD3 expression (figure2-3 C), and this population further scrutinized for CD4 and CD8 expression (figure 2-3 D). In addition to examining CD3+ T cell populations, it was also possible to analyse CD3- CD8+ (possible natural killer cell) populations using this staining panel. For the analysis of these populations, cells were identified via size and granularity and dead cells excluded as stated previously, and the resultant CD3⁻ fraction of these cells examined for the expression of CD8 (see figure 2-4).

ANTIBODY TARGET	CONJUGATED FLUOROPHORE	ISOTYPE	CLONE	QUANTITY (µl/test)
CD3	FITC	IgG2a, kappa	HIT3a	2
CD4	APC	IgG1, kappa	SK-3	2
CD8	PE	IgG1, kappa	RPA-T8	2
Vα7.2*	APC-Cy7	IgG1, kappa	3C10	2
CD161	PerCP Cy5.5	IgG1	HP-3G10	2
CCR7	APC-eFluor780	IgG2a, kappa	3D12	2
CD45RO	PerCP-eFluor710	IgG2a, kappa	UCHL1	2
Ki-67	FITC	IgG1, kappa	20Raj1	2

Table 2-1- Antibodies used in flow cytometry cell surface analysis of T cell CD4 and CD8 memory and MAIT cell populations as well as cell proliferation in response to NTHi : The antibodies listed above were used for the analysis of CD4 T helper and CD8 cytotoxic cell populations within human lung tissue samples. This included identifying memory cell populations and MAIT cell populations within each subset. Antibodies marked with * were procured from biolegend (London, UK). All others were procured from eBioscience (Hatfield,UK)
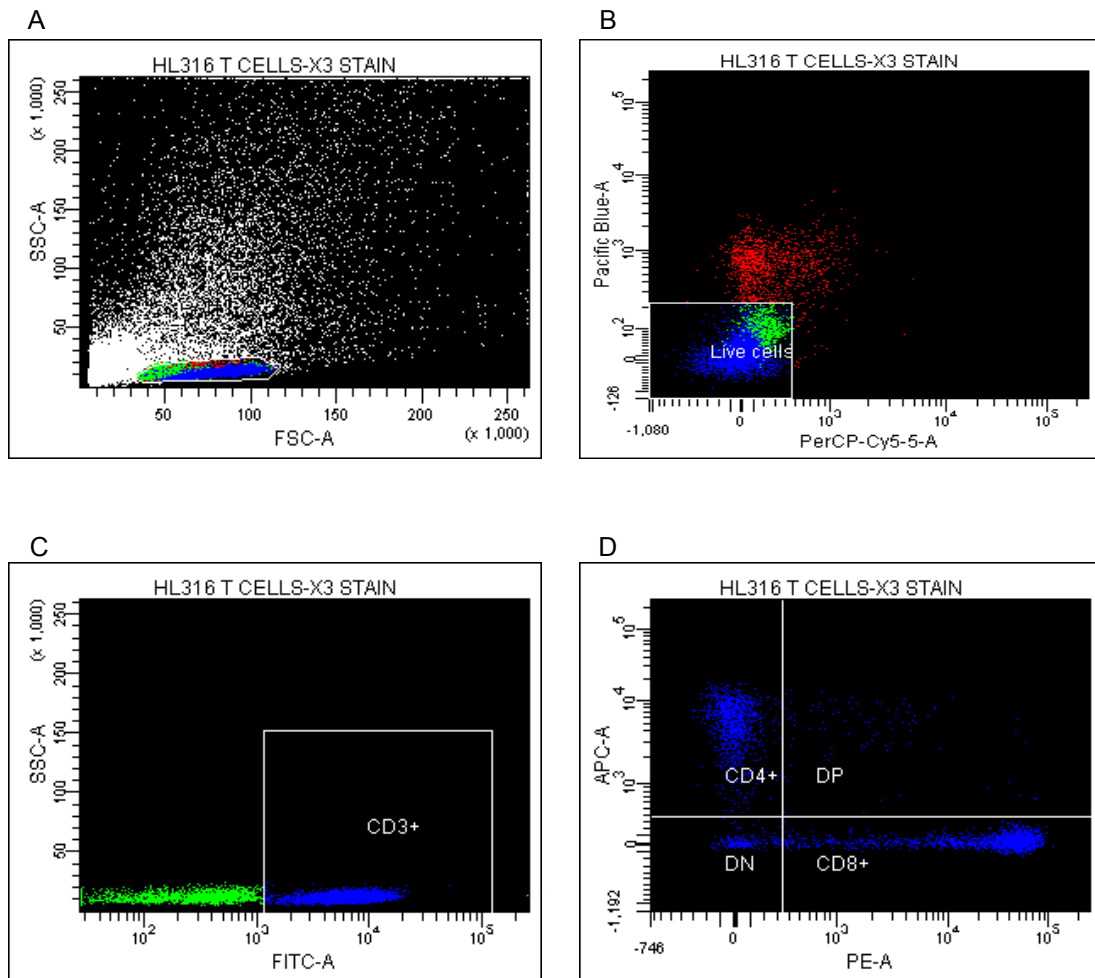
Figure 2-3- Flow cytometry gating strategy for T cell analysis: Lymphocytes were isolated from lung tissue via enzymatic digestion and rested overnight prior to analysis. Lymphocytes were gated through their forward scatter and side scatter profile (A). Dead cells were then excluded from further analysis by giving a positive signal for Annexin V and/or 7-AAD (B). Live lymphocytes that were CD3 positive (C) were then finally analysed for CD4 and CD8 expression (D)
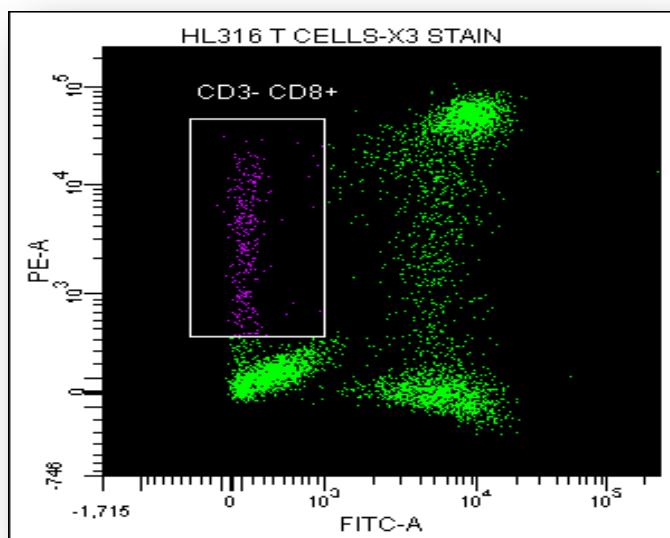
Figure 2-4 Flow cytometry gating strategy for CD3⁻ CD8⁺ cell analysis: Cells Isolated via tissue enzymatic digestion were analysed for the presence of CD3-CD8+ cells. Cells were identified through size and granularity before live dead exclusion as stated in figure 2-3. Cells were then selected on being CD3- and then analysed for the expression of CD8. Representative staining of CD3- CD8+ populations is shown above.

A second panel was also used to analyse the proportion of MAIT cells in patient enzymatically digested lung samples. Representative gating for the identification of MAIT cells is shown in figure 2-5. Cells were again firstly identified through size and granularity (figure 2-5A), and this population screened to exclude dead cells through Annexin V (figure 2-5B) positive staining. Resultant live lymphocytes were then stained for CD3 expression (figure 2-5C), and this population further split via CD4 and CD8 expression into CD3+CD4+ and CD3+CD8+ populations, as well as the CD4CD8 double positive and double negative populations (figure 2-5D). The resultant four CD3+ populations (CD4+, CD8+, DN and DP) were then screened for expression of MAIT cell markers CD161 and the Vα7.2 T cell receptor (figure 2-5E). Expression of both of these markers would strongly suggest that the cell is a MAIT cell..
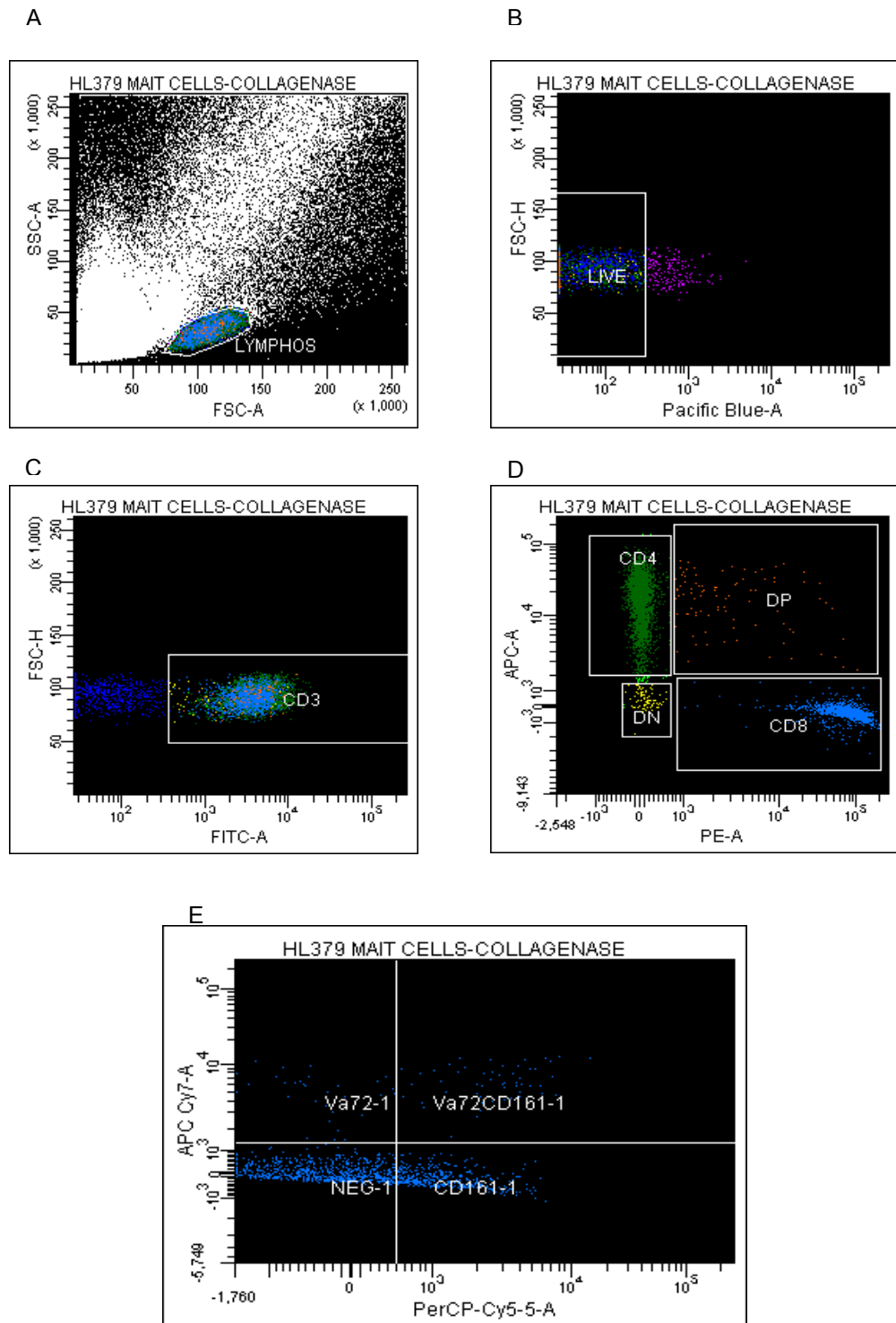
Figure 2-5-Flow cytometry gating strategy for MAIT cell analysis: Cells isolated via collagenase digestion of lung tissue were screened for the presence of MAIT cells via flow cytometry. Lymphocytes are initially identified through their forward scatter and side scatter profile (A). Dead cells are then excluded from further analysis by giving a positive signal for Annexin V (B). Live lymphocytes that are CD3 positive (C) are then categorised via their expression of CD4 and CD8 expression (D). Resultant populations are then analysed for their expression of CD161 and Vα7.2 TcR (E).

In addition to the analysis of basic CD4 and CD8 populations and MAIT cell populations within the lung, the proportion of memory type T cells was also examined (figure 2-6). These cells were examined from both enzymatically digested and non-enzymatic tissue egression isolation methods. As in previous gating strategies, cells were first identified via size and granularity (figure 2-6A) before exclusion of dead cells via annexin V staining (figure 2-6B) and gating upon the CD3+ cell fraction (figure 2-6C). From this CD3+ pool, CD4 and CD8 cells were identified (figure 2-6D). It was then possible to further analyse these populations for the expression of the memory cell marker CD45RO and the chemokine receptor CCR7 (figure 2-6E).
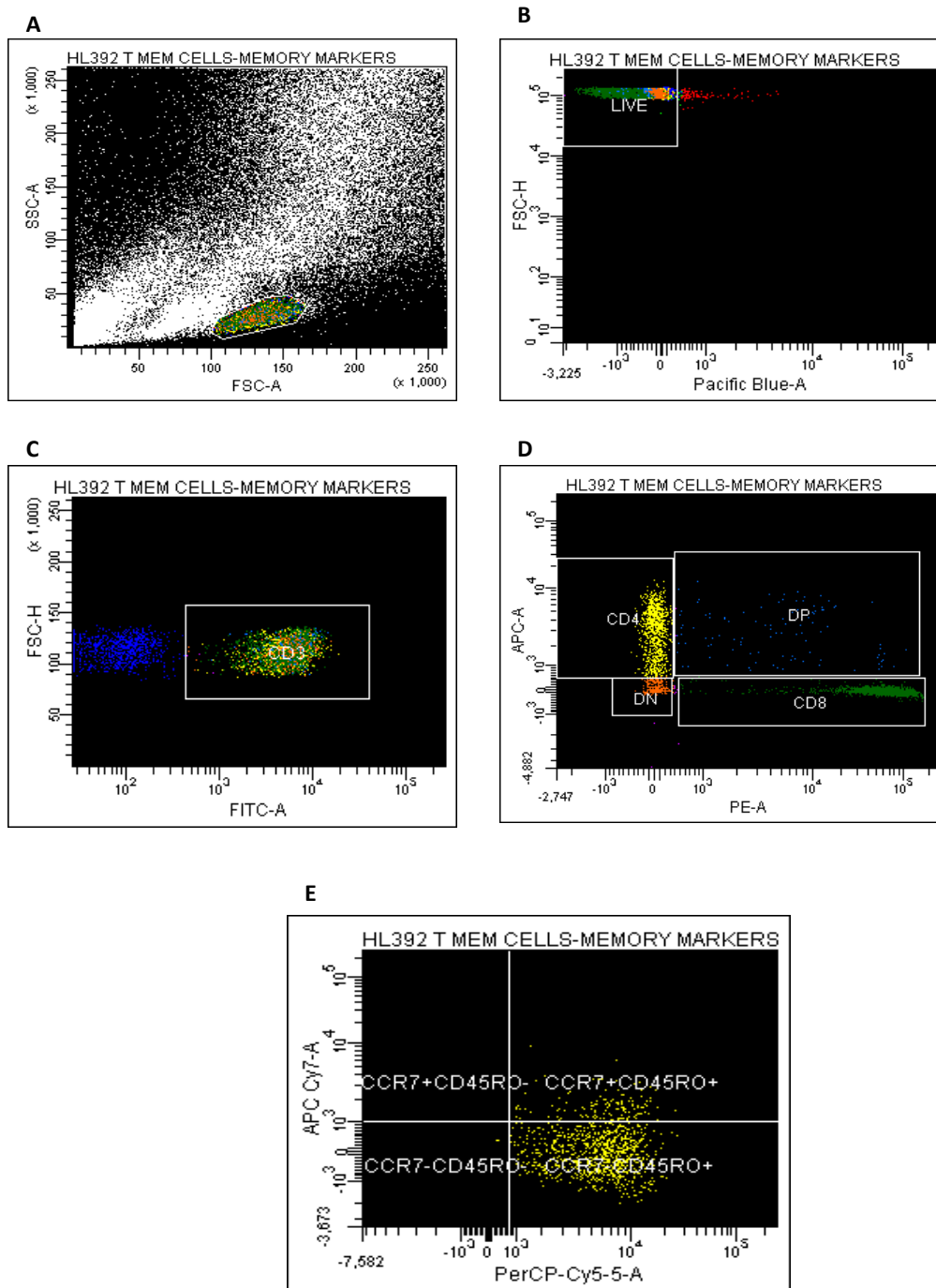
Figure 2-6- Flow cytometry gating strategy for memory cell population analysis: Cells isolated via collagenase digestion of lung tissue were analysed for memory T cell populations. Cells were first identified via size and granularity (A) before dead cell exclusion (B). CD3 expressing cells were then selected (C) and examined for CD4 and CD8 expression (D). From these resultant CD3+ populations, cells were split into categories based upon the expression of CD4 and/or CD8 and these populations further analysed for the expression of CCR7 and CD45RO. Figure 2-6E shows representative staining of CD3+CD4+ cell expression of CD45RO and CCR7.

2.8.2 Intracellular staining of T cell populations for proliferation marker Ki-67

T cell populations exposed to heat-killed NTHi for 5 days and boosted with growth cytokines IL-2 and IL-7 for a further five days were screened for the expression of the intracellular proliferation-associated marker Ki-67. Cells were stained for T cell lineage markers CD4 (helper) and CD8 (cytotoxic) before being screened for Ki-67 (see table X for antibodies). Cells were first stained for surface markers before being washed (as outlined previously). Cells were then fixed for 20 minutes at 4°C in Fox P3 fixation fluid (eBioscience, Hatfield, UK). After fixation, cells were washed twice in Fox P3 perm wash before being incubation with anti-Ki-67 antibody for 30 minutes on ice. Once staining was complete, cells were washed twice in Fox P3 perm wash before being resuspended in FACS buffer in preparation for analysis

2.8.3 Intracellular cytokine staining by flow cytometry of T cell populations

The following staining procedure was used for both the analysis of initial NTHi-responder T cell populations and the reactivation of the NTHi T cell line after co-culture with autologous fibroblasts. Cells were initially stained for cell-surface markers of T cell lineage (CD3, 4 and 8) as previously outlined, but with the addition of a fixable live/dead stain-efluor 450 (eBioscience, Hatfield, UK). This was added at a dilution of 1/1000 and incubated with cells for 30 minutes on ice and protected from light. After live dead viability staining, cells were washed twice in PBS. Once surface and viability staining was completed, cells were fixed through incubation with 4% paraformaldehyde (4% PFA) for 20 minutes at 4°C in darkness. Cells were then washed with FACS buffer before being incubated in saponin-based permeabilization buffer (BD biosciences, Cambridge, UK) for 20mins at 4°C in darkness. Cells were then centrifuged at 400g for 5 minutes and resuspended in permeabilization buffer to a final volume of 100μl. Cells were then ready for intracellular staining. Cells were stained with anti-human IFN-γ and IL-13 ("panel 1"), or with anti-human IL-10 and IL-17A ("panel 2") intracellular cytokine antibodies and incubated for 30 minutes at room temperature and protected from light (see table 2-2 for full antibody details). After staining, cells were washed twice in permeabilization buffer before being resuspended in FACS buffer ready for analysis.

Representative gating strategy for the intracellular cytokine staining of T cells is shown in figure 2-7. Cells were first differentiated as live or dead (figure 2-7A) before the identification of lymphocytes as based on size and granularity (figure 2-7B). Cells were then gated for doublet discrimination before identifying the T cell population through CD3 expression (figure 2-7C). CD3+ events were then analysed for CD4 or CD8 expression. The CD3+CD4+ T helper and CD3+CD8+ T cell populations were then analysed for the production of IFNγ and IL-13 (staining panel 1) or IL-10 and IL-17A (staining panel 2). Representative staining for antibody panel 2 (IL-17A and IL-10) of T helper cells (figure 2-7F) and cytotoxic T cells (figure 2-7G) in response to heat-killed NTHi stimulation are shown. The results for both cytokine production by initial NTHi responders and PMA+ionomycin activation are presented as the percentage of CD4+ cells positive for one cytokine minus the percentage of unstimulated CD4 cells positive for that particular cytokine.

ANTIBODY TARGET	CONJUGATED FLUOROPHORE	ISOTYPE	CLONE	QUANTITY (µl/test)
CD3	PE- eFluor 610	IgG1, kappa	UCHT1	2
CD4	APC	IgG1, kappa	SK-3	2
CD8	PerCP Cy5.5	IgG1, kappa	RPA-T8	2
Interferon-ɣ	PE	IgG1, kappa	4S.B3	2.5
Interleukin-13	FITC	IgG1	PVM13-1	2.5
Interleukin-17A	PE	IgG1, kappa	eBio64CAP17	2.5
Interleukin-10	PE-Cy7	IgG1, kappa	JES3-9D7	2.5

Table 2-2- Antibodies used in the analysis of T cell populations intracellular cytokine production and associated fluorophores: The antibody panel listed above was used to identify T cell populations via cell surface markers and to examine the intracellular production of cytokines associated with T cell effector subsets. All antibodies purchased from eBioscience (Hatfield, UK)
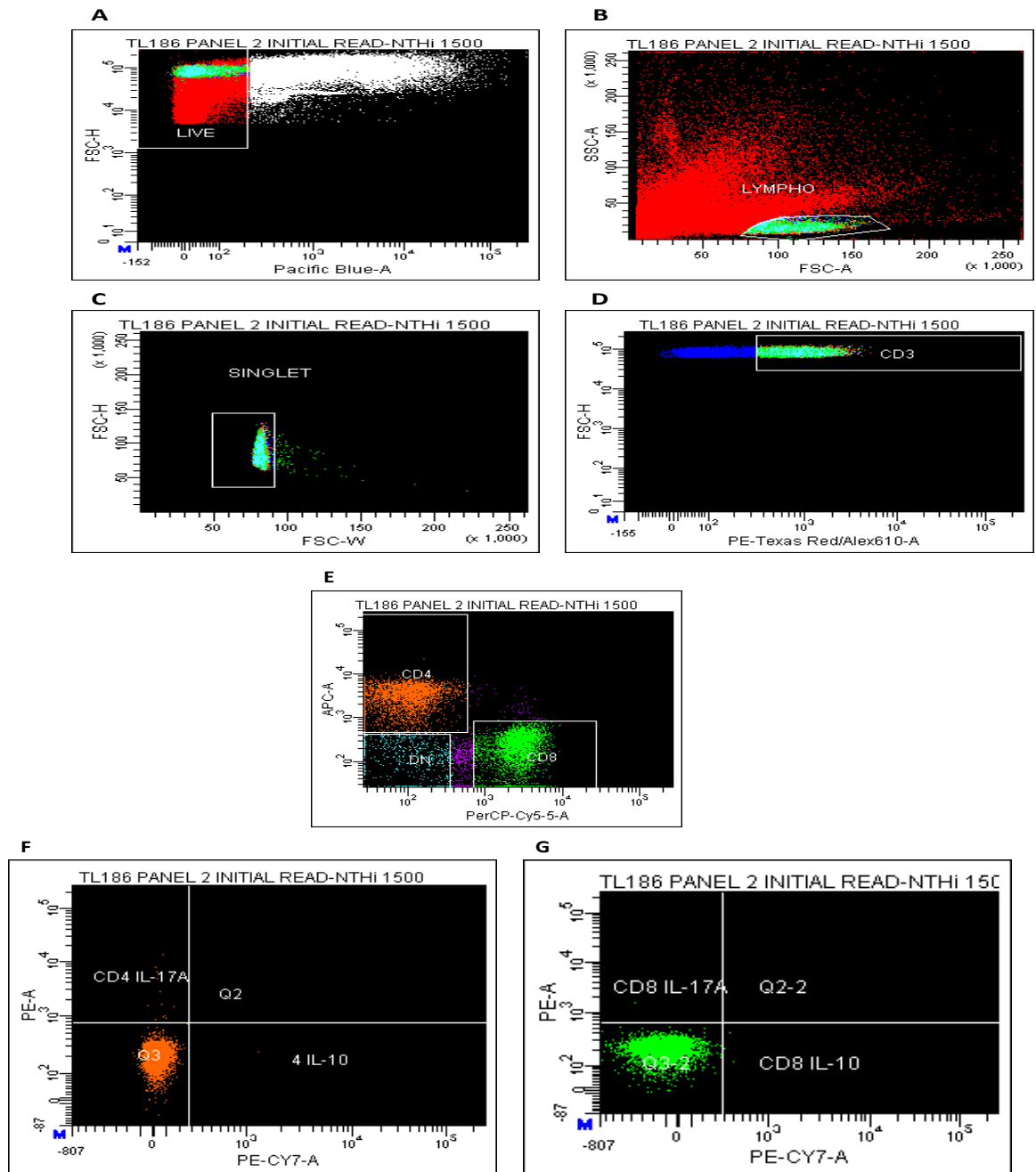
Figure 2-7- Flow cytometry gating strategy for the analysis of NTHi responsive T cells in initial patient lung cell isolates: Lung cells isolated via egression from lung tissue fragments were used for intracellular cytokine analysis of T cell responses to NTHi. T Cells were first identified as live or dead (A) before lymphocytes were gated upon through size and granularity (B). Singlet cells were selected via forward scatter width and height. Cells were then screened for CD3 expression (C), and CD3$^+$ cells then analysed for CD4 and CD8 expression (D). CD4$^+$ and CD8$^+$ cells were identified, and each T cell population examined for the intracellular production of cytokines. The figure above shows the production of cytokines IL-17A and IL-10, which are detailed as "panel 2". CD4$^+$ T helper cell cytokine production (F) and CD8$^+$ cytotoxic T cell production (G) of these two cytokines are shown. A separate panel ("panel 1") was used for the examination of IFN-γ and IL-13 producing T cells. This substituted IL-17A-PE and IL-10-PE Cy7 for IFN-γ PE and IL-10 FITC.

51

2.9 Flow cytometry of human lung fibroblasts

2.9.1 The *Ex vivo* analysis of lung populations for HLA-DR expression by flow cytometry

Lung cell populations were isolated via previously mentioned collagenase digestion (1mg/ml for 30 minutes at 37°C). Resultant cell populations were then immediately stained for lineage markers associated with macrophages, epithelial cells and fibroblasts as well as for HLA-DR expression (see table 2-3). Cells isolated from collagenase digestions were blocked in 10% human serum-FACS buffer for 20 minutes on ice. Cells were then stained with relevant antibodies for 30 minutes on ice and protected from light. Cells were washed twice in FACS buffer before being incubated in fixation buffer (FoxP3 fixation buffer, eBioscience, Hatfield, UK) for 30 minutes at 4°C protected from light. Cells were then washed twice in permeabilization buffer (eBioscience, Hatfield, UK), resuspended in 100μl of permeabilization buffer, and then stained with antibodies for intracellular antigens for 30 minutes at 4°C in darkness. After this staining incubation, cells were washed twice with permabilization buffer and resuspended in FACS buffer in preparation for analysis by flow cytometry. The gating strategy used for analysis is outlined in figure 2-8. Cells were initially gated via their forward scatter area and width profile (figure 2-8A). This population was then gated as either CD45+ or CD45- (figure 2-8B). The CD45+ fraction was then further analysed and a FITC autofluorescent, HLA-DR^{hi} expressing population identified as macrophages (figure 2-8D). The CD45- fraction analysed for the expression of EpCAM and CD90, with CD90+EpCAM- cells being classed as fibroblasts (figure 2-8C). The CD90+ EpCAM- population was then analysed for the expression of **α**-SMA in order to gate out myofibroblast populations. These macrophages, epithelial cell and fibroblast cells were then analysed for the expression of HLA-DR (figures 2-8 E, F and G respectively). Corresponding isotype control antibodies were used to assess the level of nonspecific and background signal of HLA-DR. This was deducted from the final analysis in order to give a specific mean fluorescent intensity (sMFI) of HLA-DR staining that accurately depicts the level of expression on the cell populations.

ANTIBODY TARGET	CONJUGATED FLUOROPHORE	Isotype	Clone	Quantity (µl/test)
CD45*	NCF 594	IgG1, kappa	HI30	5
EpCAM*	PerCP-Cy5.5	IgG1, lambda	EBA-1	5
CD90	APC	IgG1, kappa	eBio5E10	5
α-SMA◆	FITC	IgG2a, kappa	1A4	5
HLA-DR	APC-eFluor 780	IgG2b, kappa	LN3	5

Table 2-3-Antibodies used for the analysis of *ex vivo* expression of HLA-DR within human lung cell populations: Antibodies used for the identification of human lung macrophages, epithelial and fibroblast cell populations from collagenase-digested human lung tissue samples and subsequent expression of HLA-DR on each lung cell subset. Antibodies purchased from BD bioscience (Oxford, UK, marked with *), Abcam (Cambridge, UK, marked with w)
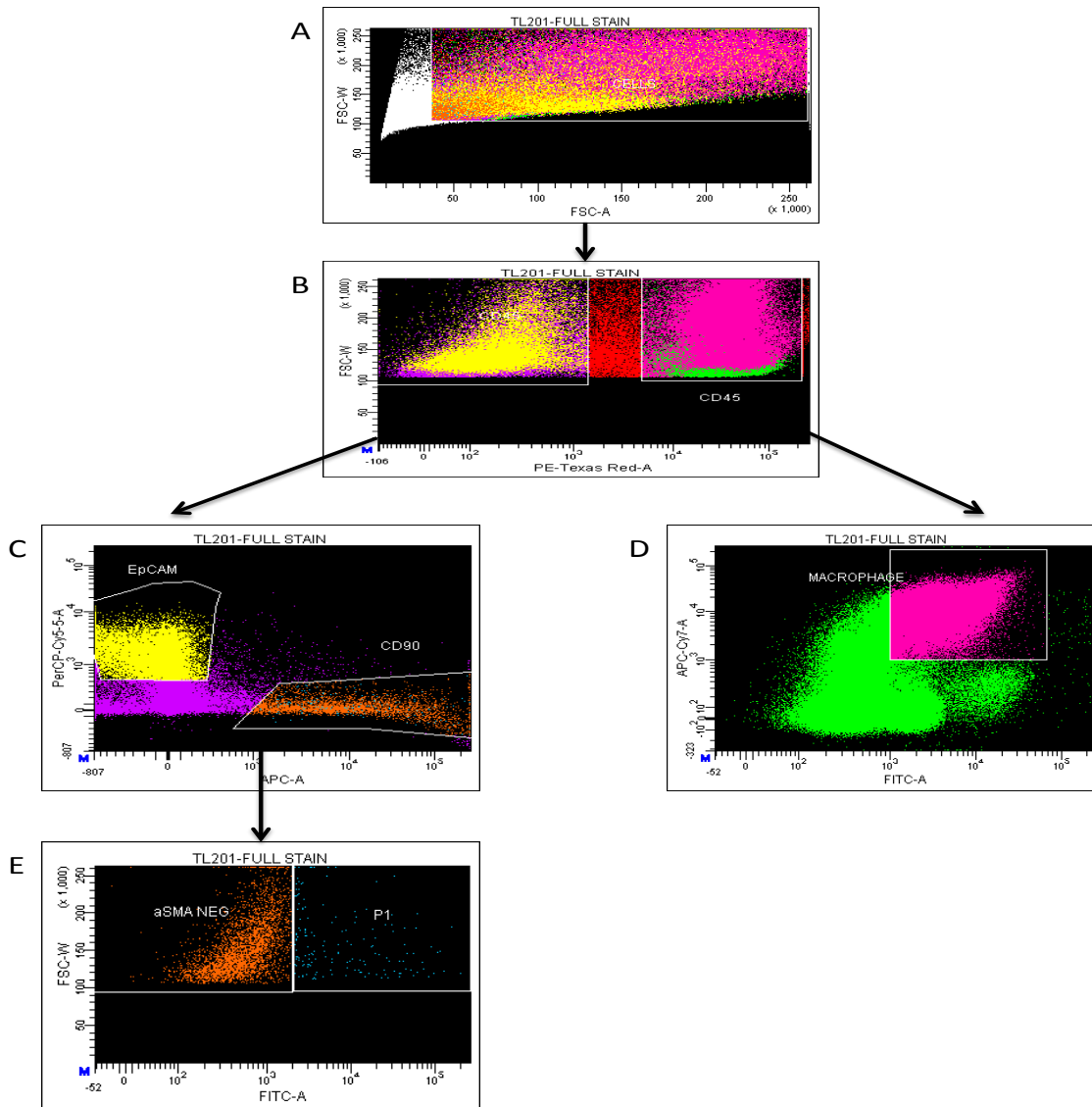
Figure 2-8- Gating strategy for the identification of alveolar macrophages, epithelial and fibroblasts cell populations and their expression of HLA-DR: Lung samples were immediately enzymatically disaggregated via collagenase treatment, and the resultant cell populations stained for lineage markers and HLA-DR expression. After initial forward scatter identification of whole cell populations (A), cells were screened for CD45 expression. The CD45$^+$ population was then further analysed for FITC autofluorescence and HLA-DR expression, with macrophages being identified as having high ("Hi") expression on both parameters (B). The CD45$^-$ population was further analysed for expression of either EpCAM or CD90 (C). The EpCAM population represents epithelial cells. The CD90$^+$ fraction was further analysed for **α**-SMA expression, with the high expressing fraction excluded from analysis as these represent myofibroblasts (E). Macrophages, fibroblasts and epithelial cells identified via this gating strategy were then screened for HLA-DR expression.

2.9.2   Analysis of *In vitro* cultured fibroblast lineage markers

Fibroblasts were obtained through outgrowth from lung tissue fragments, as previously mentioned. This resulted in a monoculture population of adherent cells bearing distinct fibroblast morphology. Representative cultures of these cells were screened for lineage markers (see table 2-4 for antibody details) in order to positively identify fibroblasts (CD45- EpCAM- with CD90 expression and low, if any, αSMA expression) as well as identifying contaminating epithelial cells (CD45- EpCAM+ cells) and leukocytes (CD45+ cells). Myofibroblasts were artificially generated from these adherent cell populations through 48h exposure to 9ng/ml TGFβ (peprotech, London, UK). Myofibroblasts are possible contaminant cells that may grow out alongside fibroblast populations and bear similar morphology, but can be differentiated via their higher expression of αSMA. Therefore myofibroblasts were generated in order to provide a reference against which cells could be classed as fibroblasts or myofibroblasts from patient cultured populations. Cells were removed from plastic culture wells after outgrowth with accutase enzymatic treatment to prevent cleavage of surface epitopes and stained for lineage markers (see table 2-4). Expression of these markers was then analysed via flow cytometry. Representative flow cytometry gating strategy for this analysis is shown in figure 2-9. Cells were intitally identified via forward and side scatter profile (figure 2-9A), before gating upon CD45- and CD45+ populations (figure 2-9B). The CD45- fraction was then screened for the expression of EpCAM and CD90 (figure 2-9C). Finally, the CD45-EpCAM-CD90+ fraction was analysed for αSMA (figure 2-9D).

ANTIBODY TARGET	CONJUGATED FLUOROPHORE	Isotype	Clone	Quantity (μl/test)
CD45*	NCF 594	IgG1, kappa	HI30	2
EpCAM*	PerCP-Cy5.5	IgG1, lambda	EBA-1	2
CD90	APC	IgG1, kappa	eBio5E10	2
α-SMA♦	FITC	IgG2a, kappa	1A4	2

Table 2-4- Antibodies used for the analysis of *in vitro* cultured fibroblasts for lineage markers and possible contaminating cell populations: Antibodies used for the identification of human lung fibroblast cell populations as well as for possible leukocyte and epithelial cells which may contaminate grown cultures obtained from primary tissue samples. Antibodies purchased from BD bioscience (Oxford, UK, marked with *), Abcam (Cambridge, UK, marked with +)
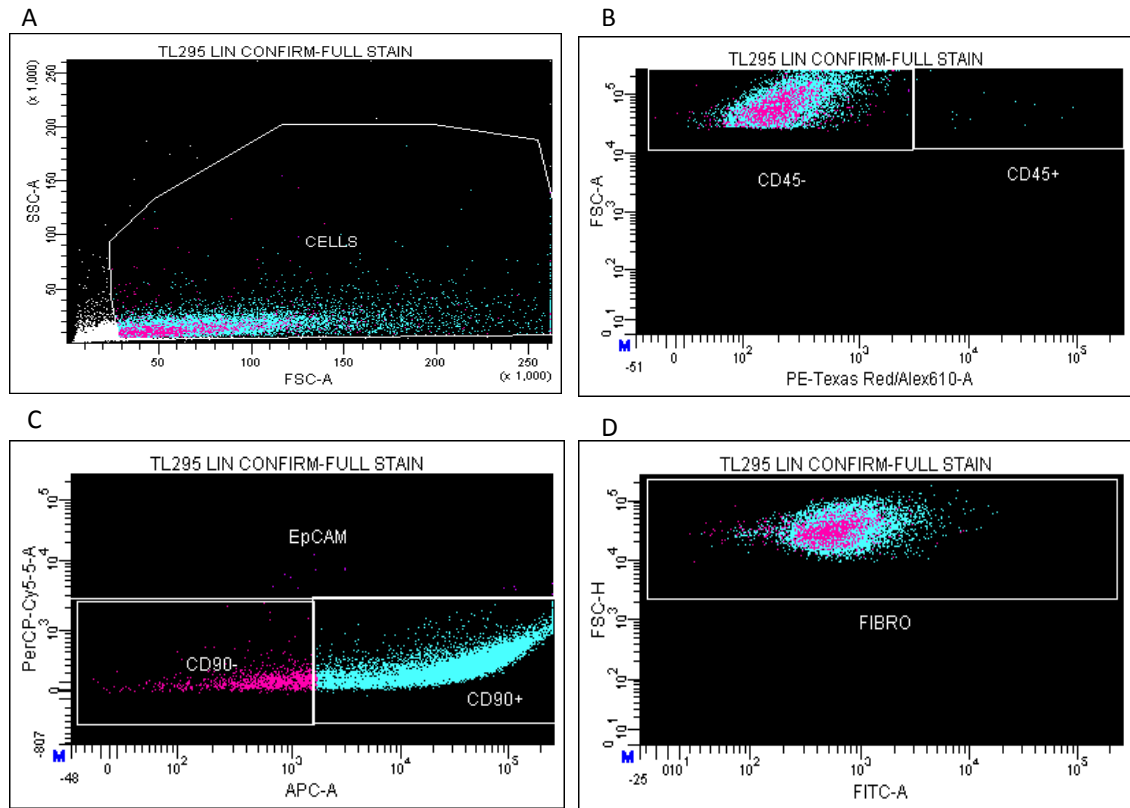
Figure 2-9- Gating strategy for the analysis of *in vitro* fibroblasts and possible contaminating leukocytes, epithelial and myofibroblast cells: Adherent cell populations grown from lung tissue fragments over a 3-4 week culture period were analysed for lineage markers of fibroblasts, epithelial cells and leukocytes. Cells were first identified via size and granularity (A) before screening for CD45 expression (B). CD45$^-$ cells were then further analysed for EpCAM and CD90 expression (C). The CD45$^-$CD90$^+$ fraction was then further analysed for the expression of α-SMA (D)

2.9.3   Analysis of *in vitro* cultured fibroblasts expression of immune synapse molecules

Human lung fibroblasts grown *in vitro* from lung fragments were screened for the expression of immune synapse molecules HLA-DR, ICAM-1, CD80 and CD86 (see table 2-5 for antibody details) both at baseline and after exposure to various proinflammatory stimuli (see figure 2-10). Fibroblasts were removed from culture wells via tryple express enzymatic treatment (Gibco Life technologies, Paisley, UK) and stained for cell surface molecules. Cells were analysed on a FACS Aria 1 (BD Biosciences) with a 2.0 neutral density filter to reduce the apparent size of the cells via forward and side scatter for effective cell gating. The gating strategy used for the cultured fibroblast expression of immune synapse molecules is outlined in figure 2-11.
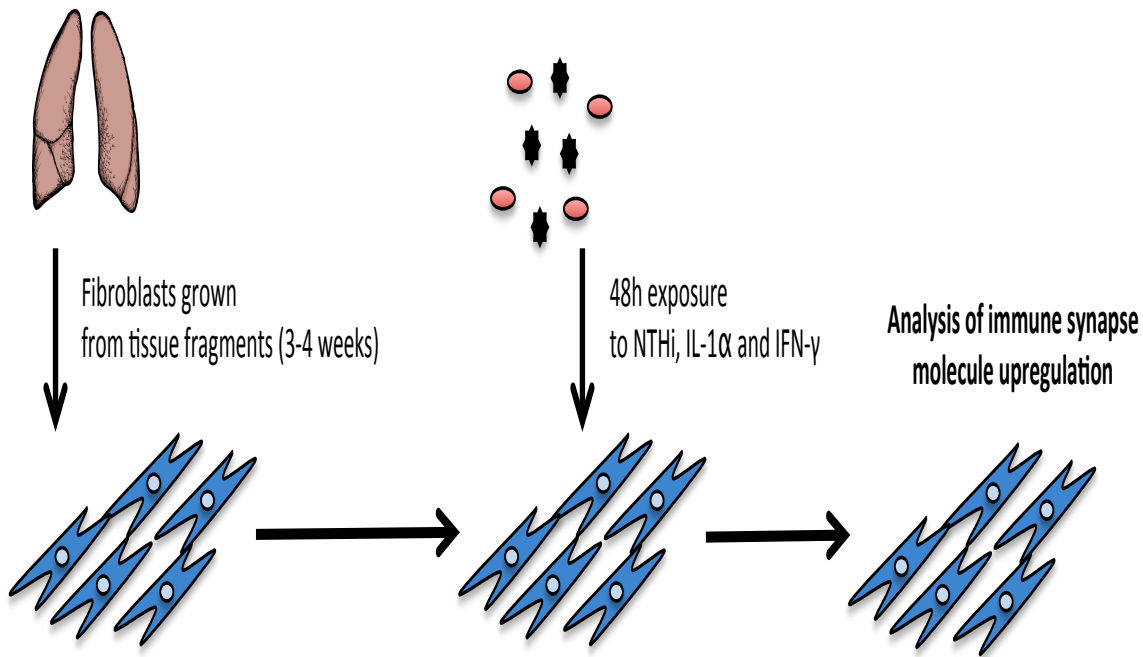
Figure 2-10- Analysis of *in vitro* human lung fibroblasts for the expression of immune synapse molecules in response to 48h exposure to proinflammatory stimuli: Human lung fibroblasts were obtained through outgrowth from lung tissue fragments over the course of a 3-4 week period. Resultant adherent fibroblast monocultures were then exposed to proinflammatory signals IFNγ, IL-1α or heat killed NTHi. After 48h exposure, the level of expression of immune synapse molecules HLA-DR, ICAM-1, CD80, CD86 or CD40 was measured via flow cytometry. The baseline level of expression as well as any upregulation in response to increasing amounts of proinflammatory signal was measured.

Antibody Target	Conjugated Fluorophore	Isotype	Clone	Quantity (µl/test)
HLA-DR, -DP, -DQ ❖	PE	Mouse IgG1	TDR31.1	1.4
ICAM-1	APC	IgG1, kappa	HA58	2
CD80	FITC	IgG1, kappa	2D10.4	2
CD86	PE	IgG2b, kappa	IT2.2	2
CD40	PerCP eFluor 710	IgG1, kappa	5C3	2

Table 2-5- Antibodies used to analyse *in vitro* human lung fibroblasts for molecules of the immune synapse: Antibodies used to analyse the expression of immune synapse molecules upon human lung fibroblasts. Antibodies were purchased from eBioscience (Hatfield, UK) and Ancell (Bayport, USA, marked with **v**)
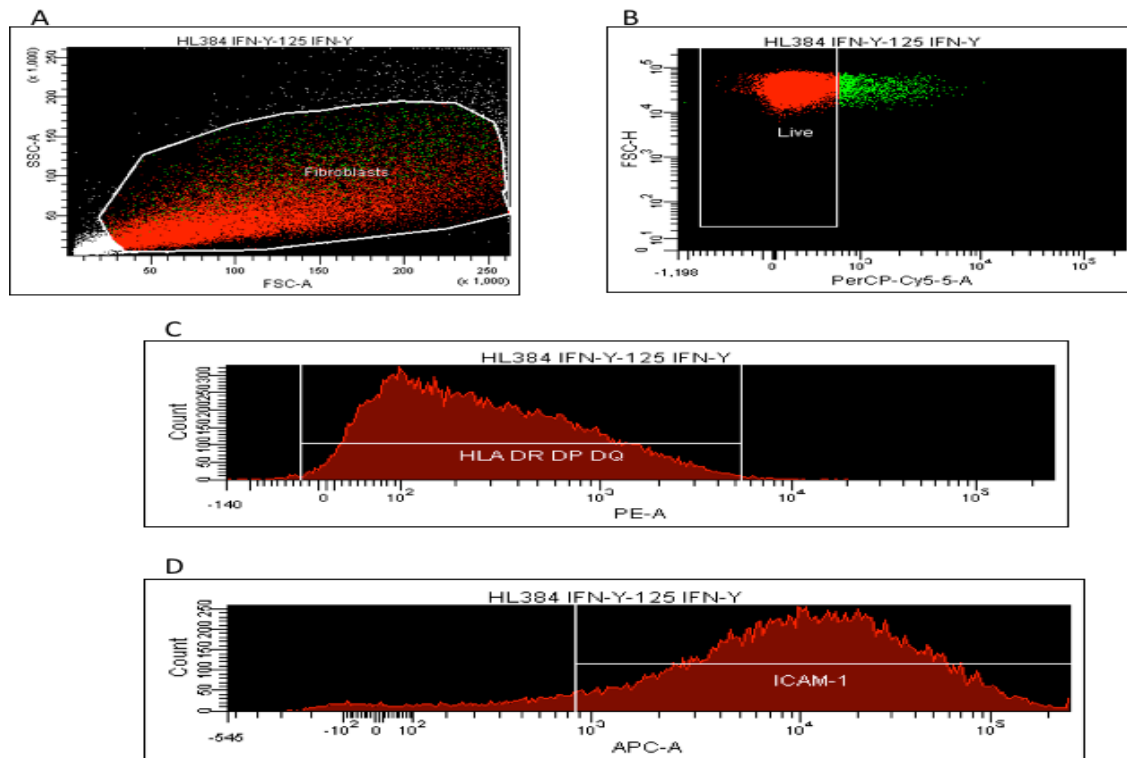
Figure 2-11- Flow cytomety gating strategy for the analysis of *in vitro* fibroblast immune synapse molecules: Fibroblast cell populations were obtained from outgrowth from lung tissue fragments before exposure to proinflammatory mediators and subsequent analysis of immune synapse molecules via flow cytometry. Cells were first identified as fibroblasts though size and granularity (A). Dead cells were then excluded from further analysis through 7-AAD staining (B). The levels of expression of HLA-DR, -DP, -DQ (C) and ICAM-1 (D) were then analysed. Similar gating strategy was used for analysing the expression of CD80 and CD86.

Antibody Target	Fluorophore	Isotype	Clone	Quantity (µg/ml)
α-SMA*	FITC	IgG2a	1A4	4
Anti-Cathepsin S	N/A (Primary)	IgG	Polyclonal	0.2
Anti goat IgG (secondary)	Alexafluor-647	IgG	Polyclonal	0.4

Table 2-6- Antibodies involved in the analysis of fibroblasts via immunofluorescent imaging: For the analysis of fibroblasts through either inverted immunofluorescent or confocal microscopy, the above antibodies were used. The fluorophore of each antibody used is also detailed   All antibodies obtained from Abcam, unless marked with *, which are obtained from Sigma-Aldrich

2.10 Gating strategy for the phenotyping of monocyte-derived dendritic ells (moDCs)

MoDCs were generated from PBMCs progenitors through conditioning with IL-4 and GM-CSF (see section 2.7 for full protocol). After 7 days of conditioning, cells were analysed for lineage markers to verify moDC phenotype (see table 2-7). Cells were screened for the expression of CD11c, CD123 and HLA-DR. Expression of HLA-DR and CD11c and the absence of CD123 would indicate that cells are conventional myeloid dendritic cells. Gating strategy for the identification of these moDCs is outlined in figure 2-12.

Antibody target	Fluorophore	Isotype	Clone	Quantity (ul/test)
CD11c	APC	1gG2b	S-HLC-3	5
CD123	PE	IgG1, kappa	9F5	20
HLA-DR*	APC-eFluor 780	IgG2b, kappa	LN3	5

Table 2-7 Antibodies involved in confirmation of moDC lineage: monocytes were cultured in the presence of IL-4+GM-CSF to drive the differentiation of moDCs. To confirm the lineage of differentiated cells, the above antibody panel was used. All antibodies were procured from BD biosciences unless marked with *, which are procured from eBiosciences.
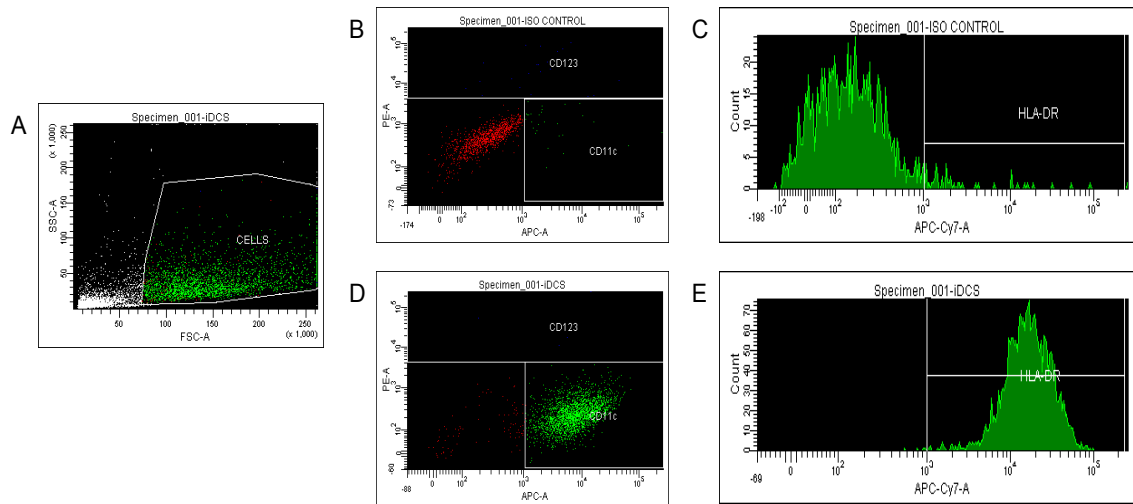
Figure 2-12 - Gating strategy for the phenotype confirmation of IL-4+GM-CSF moDCs from patient blood: In order to confirm that IL-4 and GM-CSF conditioned CD14+ monocytes expressed a conventional moDC phenotype, samples were screened for the expression of CD11c, CD123 and HLA-DR. Firstly, cells were identified though forward and side scatter (A). Cells were then analysed for the expression of CD11c and CD123 (D). Corresponding isotpye control staining for CD123 and CD11c is also shown (B). CD11c+ CD123- moDCs were then analysed for HLA-DR expression (E). Corresponding isotype control for HLA-DR staining is also shown (C). Figure representative of 3 experiments.

2.11 CO-CULTURE OF AUTOLOGOUS PROFESSIONAL OR NON-PROFESSIONAL (FIBROBLAST) ANTIGEN-PRESENTING CELLS WITH A NTHi-SPECIFIC T CELL LINE

In order to measure the ability of human lung fibroblasts to cause T cell activation in an antigen-dependent manner, a co-culture system was designed (figure 2-14). This made use of patient lung-derived or PBMC-derived T cell populations that were enriched to provide a large pool of NTHi responder cells. These NTHi-enriched populations were then placed in co-culture with either autologous lung fibroblasts or moDCs. Tissue T cells were obtained via the aforementioned tissue egression model (see section 2.4). Resultant immune cell populations were exposed to heat-killed NTHi (1500 units) for 5 days before receiving a cytokine boost of 50 units/ml recombinant human Iinterleukin-2 (IL-2, Miltenyi biotechnology, Surrey, UK) and 100U/ml interleukin-7 (IL-7, Miltenyi biotechnology, Surrey, UK). Cultures were then allowed up to 4 weeks to allow proliferation of NTHi-specific T helper cell clones from the T cell population. The aim of this procedure was to provide a large number of T helper cells with a known antigen

specificity that could be exploited in order to test the ability of lung fibroblasts to present NTHi antigens and reactivate these T cells. Fibroblasts were grown out from tissue fragments as previously mentioned for *in vitro* analysis assays (see section 2.6). Once adequate numbers of both cell types were obtained, fibroblasts were seeded into a flat-bottomed 96-well plate at a density of 25,000/well. After being allowed to adhere overnight, cells were then exposed to either serum free DMEM alone, or supplemented with IFNγ (500 units), NTHi (1500 heat-killed units) or IFNγ (500 units)+NTHi (1500 heat killed units). Fibroblasts were incubated with conditioning factors for 48h. MoDCs were treated in the same manner; cells were seeded out at 25,000/well and exposed to activation factors for 48h in serum-free media. After incubation, cells were washed extensively with media to remove conditioning factors. APC cultures at this point were ready for co-culture with the autologous NTHi-specific T cells.

24 hours prior to co-culture, NTHi-exposed T cell cultures were sorted using miltenyi magnetic activated cell sorting (MACS), based upon CD4 positive selection. Briefly, NTHi exposed lung cell cultures were washed in MACS Buffer (PBS containing 0.2M EDTA and 0.5% BSA) and resuspended in 60μl MACS buffer post-wash. Cells were then given 40μl Miltenyi CD4 magnetic microbeads, mixed with the cell suspension, and incubated for 15 minutes at 4°C. Cells were then washed and resuspended in 1ml MACS buffer. At this point, cells were separated on the basis of CD4 expression using Miltenyi MACS cell separation magnets through a separation column. Magnetically captured CD4 expressing cells were then recovered by removing the column from the magnetic field and flushing with 5ml of MACS buffer. This ensures resultant populations are only CD4+ T helper cells (and removes unwanted contaminating cells such as B cells, CD8+ T cells and NK cells which may have an effect on the culture). Cells isolated in this manner are 98%+ T helper cells (figure 2-13)
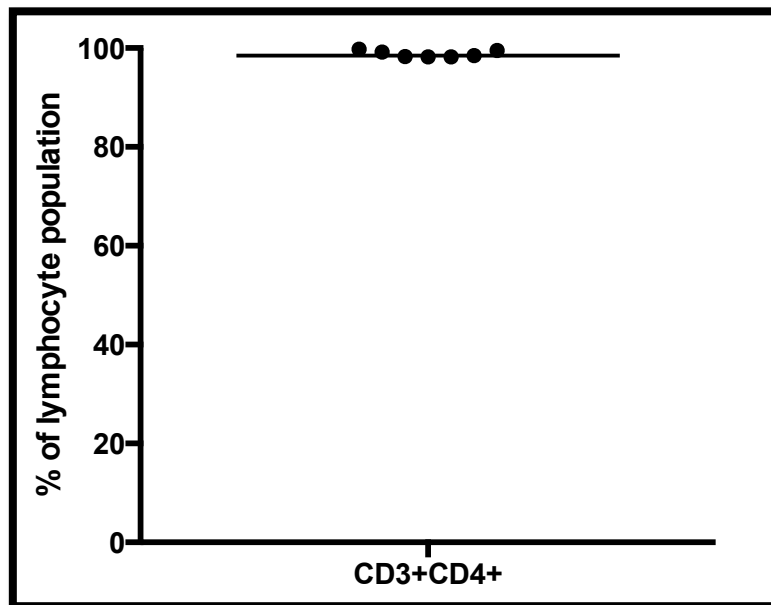
Figure 2-13- MACS sorting of CD4+ cells yields high-purity T helper cell populations: Lung leukocyte cultures exposed to NTHi and cultured over a 4 week period were sorted via MACS for CD4 expression. This was to provide a purified population of T helper cells. Verification of cell purify was carried out via flow cytometry. MACS sorting based upon CD4 expression resulted in a population of CD3+CD4+ T helper cells. Purity was consistently above 98% in all samples. N=7

Sorted cells were then rested overnight in cytokine-free 10% human serum RPMI. Cytokine-free conditions at this stage should prevent a baseline activation state of the sorted CD4+ T helper populations. After overnight resting, cells are ready for co-culture alongside previously activated fibroblasts or moDCs (see figure 2-14).

T cells were added to previously described fibroblasts or moDCs in wells of a 96-well plate at a density of 100,000 CD4$^+$ T helper cells to 25,000 APC (4:1 ratio). In certain cultures, HLA-DR blocking antibodies were added at 10μg/ml. Corresponding isotype controls of equivalent protein concentration were also setup to verify any observed effect of HLA-DR blockade was not an artefact of the assay setup. Where APCs were initially exposed to NTHi, another dose of NTHi was administered at the point of T cell co-culture. As well as co-cultures, T cell monocultures were setup-including T cells exposed to 1500 NTHi, PMA+ionomycin (40.5**μM** and 670μM respectively) or no stimulation. Cells were cultured for 1 hour before the addition of brefeldin A (3μg/ml) to allow the intracellular build-up of cytokine within T cells for

analysis. Cultures were then incubated overnight before T cells removed and stained for T cell lineage markers and the production of cytokines.
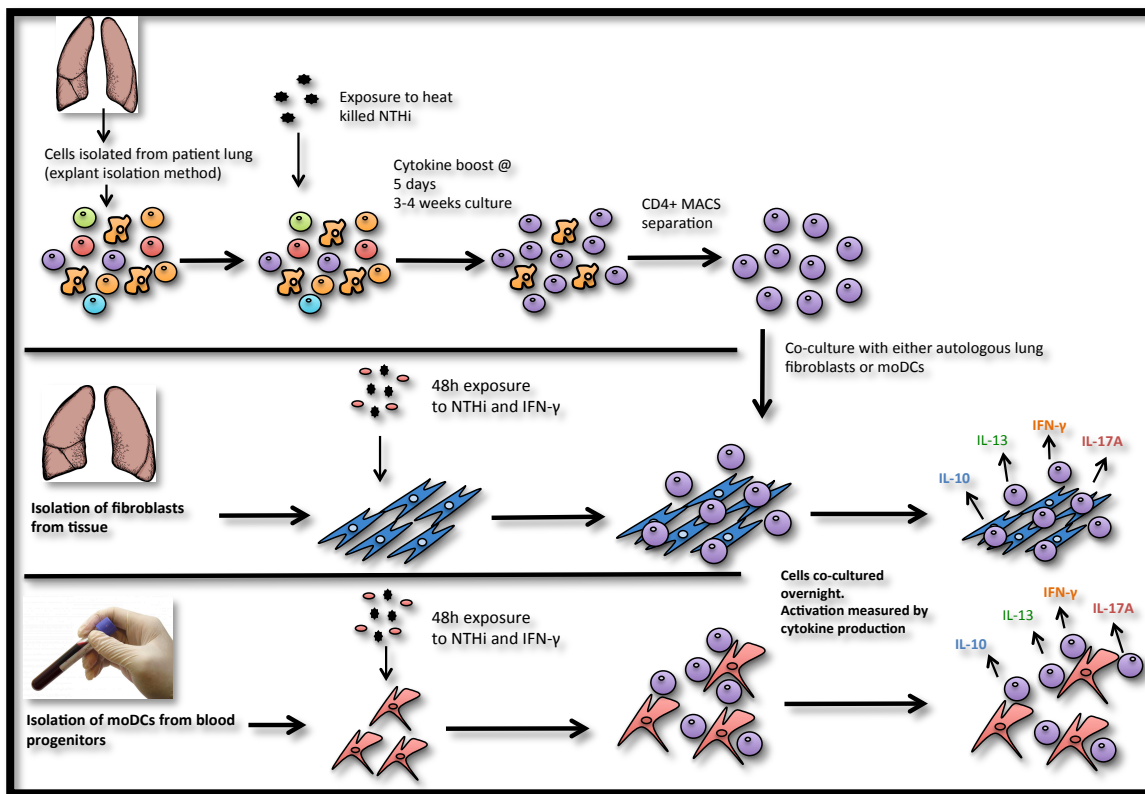
Figure 2-14- Isolation and culture of autologous human lung fibroblasts or moDCs and CD4^{+} T helper populations for co-culture in order to examine the ability of fibroblasts to act as an antigen-presenting cell: Both lung fibroblast and lung T cells were isolated via the aforementioned *in vitro* fibroblast and T cell egression methods. Briefly, fibroblasts were outgrown from lung fragments over a 3-4 week period until sufficient numbers were attained in culture. Patient-matched moDCs were obtained from blood based CD14+ progenitor cells conditioned with IL-4+GM-CSF (see section 2.7). Concurrently, patient-matched lung derived immune cells were isolated and subsequently exposed to heat killed NTHi in order to drive the generation of a NTHi-responder enriched T cell line. This provided a pool of T helper cells with a known Ag-specificity that can be used to test the ability of matched lung fibroblasts to present NTHi-Ag and reactivate this line. In cases where patient blood samples were available, it was possible to pair NTHi-responder enriched lines with matched moDCs. This represents activation of T helper cells by a professional, bonafide APC population. T helper cell activation after co-culture was measured by flow cytometry intracellular cytokine staining.

Statistics

Non-parametric data analyses were used in this study. For the analysis of paired datasets, a Wilcoxon test was used. For the analysis of unpaired dataset, a Matt Whitney U test was used. Where indicated, * represents $P>0.05$, ** $P>0.01$, *** $P>0.001$, **** $P>0.0001$

Chapter 2: Materials and Methods

3. CHARACTERIZATION OF T CELL POPULATIONS WITHIN THE HUMAN DISTAL LUNG

Chapter 3

3.1 Introduction

Substantial numbers of T cells reside within the human lungs. These cells help maintain steady-state tolerance but also mediate proinflammatory responses to pathogens. Many of these peripheral tissue T cells are thought to be of a memory-phenotype [69], and are able to release effector cytokines that are critical in directing immune responses in the lung. Of particular importance are the T helper cells, which are able to launch differential cytokine responses that go on to dictate the nature of the inflammatory response by the wider immune system and are critical to effective host immunity [40, 132, 133]. In addition to conventional CD4+ and CD8+ populations, there are a number of more specialized T cell populations, such as the MAIT cells. These cells are recognised to be a potent source of proinflammatory cytokines in mucosal zones and have a restricted antigen specificity that is currently thought to be directed against bacterially generated B vitamin precursors.

This study uses human lung T cells derived from distal lung samples. The majority of lung samples were obtained from subjects undergoing resection surgery for lung cancer (along with a small number of younger bullectomy subjects, receiving surgery for non-cancerous pneumothorax). Although only non-cancerous tissue is used in this study, a number of subjects have underlying pathology in the form of COPD. This condition is known to have altered T cell activity compared to healthy equivalents [121]. Therefore the obstructive lung status of subjects is examined in relation to the various immune cell populations found within the lung. In this chapter, lung T cells are examined, scrutinizing T memory and MAIT cells in particular. The cytokine production profile of conventional T cell is also explored, allowing identification of effector T cell subsets. Firstly, cell isolation techniques are compared. A collagenase digestion technique is used for examining all lung cell populations within distal lung samples, while non-enzymatic techniques were used for isolation of T cells for long-term culture and functional assays.

3.2   Results

3.2.1   The effect of collagenase treatment on T cell receptor expression

Collagenase treatment is widely used to liberate lymphocytes from lung tissue samples [134, 135]. While useful for increasing cell yield, it has been known to cleave cell surface epitopes [136, 137]. Any such cleavage has the potential to alter the ratio of T cell populations observed via flow cytometry. To assess the effect of type 1 collagenase exposure (1mg/ml for half an hour at 37°C) upon receptor expression, patient lung preparations were treated with lysis buffer alone or collagenase followed by red cell lysis buffer and the resultant populations analysed after a resting period of 24 hours in RPMI 10% HS. Cleavage of CD4, CD8 (figure 3-1), CD45RO and CCR7 (figure 3-2) was measured.
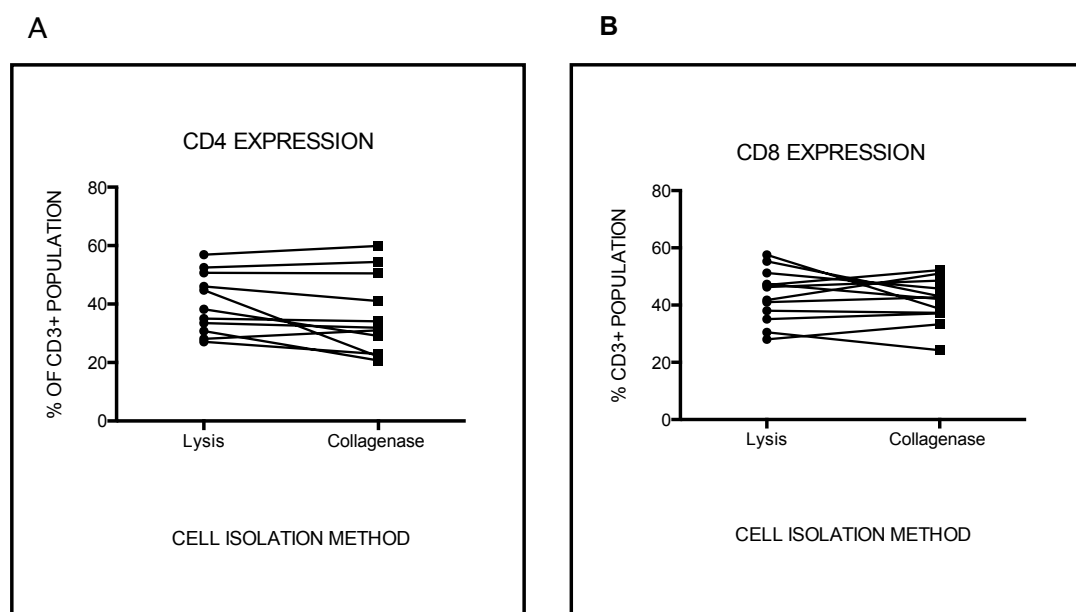
Figure 3-1- The effect of collagenase treatment on CD4 and CD8 surface expression: Lung tissue samples were either mechanically disrupted and enzymatically digested via collagenase exposure (1mg/ml for half an hour at 37°C) followed by red cell lysis, or mechanically disrupted without collagenase treatment and red cell lysis performed. Both lysis alone and type I collagenase with lysis buffer were compared to measure cleavage of T cell surface receptors CD4 (A) and CD8 (B). Neither receptor is significantly cleaved by collagenase treatment (Wilcoxon signed-rank test) N=11
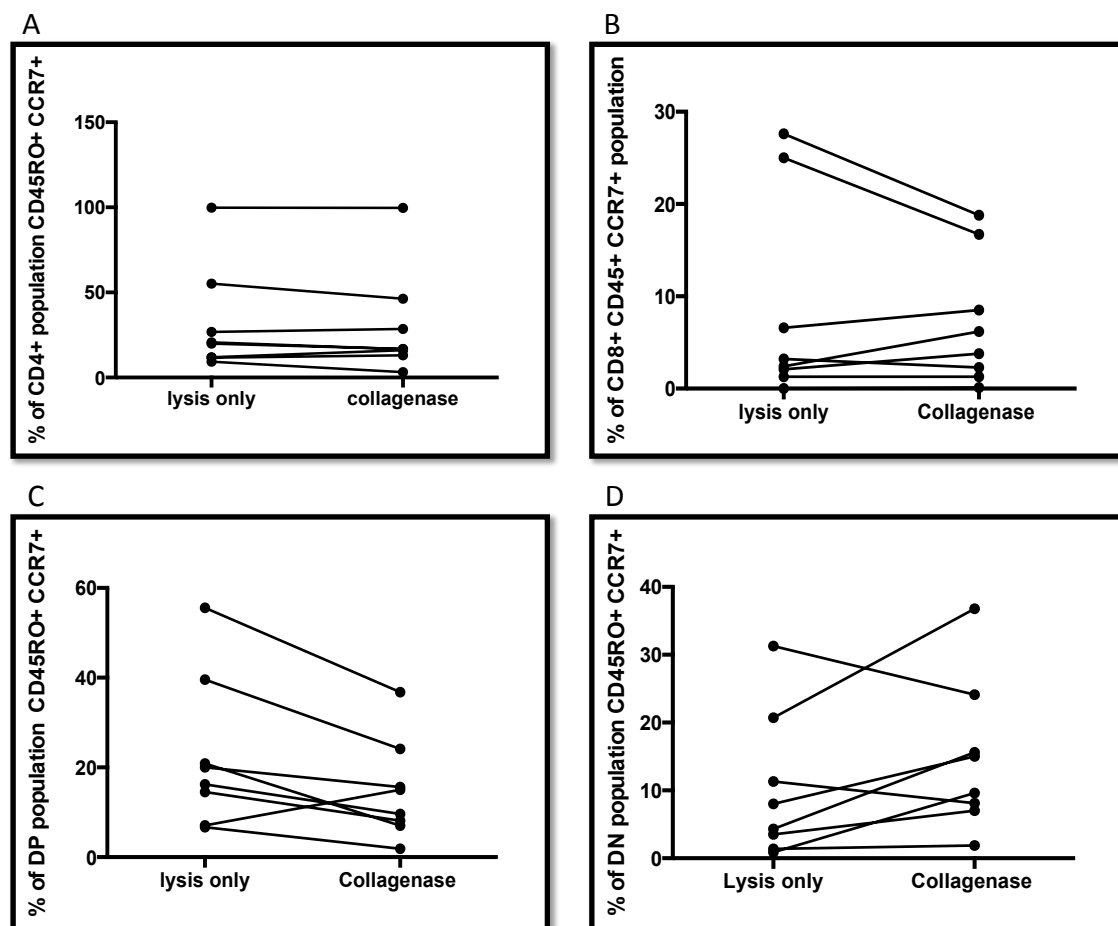
Figure 3-2- The effect of collagenase treatment upon expression of memory T cell markers CD45RO and CCR7 (central memory T cells): Subject matched lung tissue samples were either mechanically disrupted and enzymatically digested via collagenase exposure (1mg/ml for half an hour at 37°C) followed by red cell lysis, or mechanically disrupted without enzyme treatment followed by red cell lysis. Both enzymatic and non enzymatic methods were compared to measure cleavage of memory cell markers CD45RO and CCR7 across CD4+ (A), CD8+ (B), CD4+CD8+ double positive (DP) (C) and CD4-CD8- double negative (DN) (D) T cell populations. Differences between the two isolation methods were non significant. Statistical analysis carried out via Wilcoxon signed-rank test. N=8

The use of collagenase treatment to digest lung tissue does not seem to have a major effect upon the expression of any of the surface markers examined. DP T cell populations exhibit a small level of decreased memory receptor expression when treated with collagenase (figure 3-2C), however this apparent decrease did not reach statistical significance through using a two-tailed Wilcoxon's paired signed-rank test. A two-tailed test was used for this analysis, as the effect of collagenase may not necessarily result in a decrease in this cell population. For example, collagenase treatment may release greater numbers of these cells from lung tissue than lysis isolation alone. Therefore, an unbiased two-tailed test was opted for. Furthermore, considering this

phenomena does not occur upon CD4 and CD8 cells (figure 3-2A and B respectively), it seems unlikely that cleavage accounts for this apparent decrease.

3.2.1.1   Isolation of T cell populations with collagenase versus tissue explant methods

For functional analysis of T cells and the generation of NTHi-specific T cell lines (primarily for use in co-cultures- see chapter 5), a tissue explant isolation technique was employed. This involved cutting tissue into small 2-3mm fragments and allowing the migration of cells from the tissue without enzymatic treatment or red cell lysis to avoid cell death or inducing T cell activation. This isolation method used density gradient separation to purify mononuclear cells only (See section 2.4 for full protocol). It was important to ensure that cells isolated non-enzymatically did not result in T memory cells being "trapped" within tissue, and an increased in contamination from circulatory naïve T cells occurring.

Comparing the proportions of memory and naïve T cell populations reveals there is very little difference between the two techniques for T helper cell isolation. The T helper CD45RO+ effector memory cells are stable between both techniques (figure 3-3A), while there appears to be only minor differences in the CD8 effector memory population (Figure 3-3D). Very small, if any, naïve T cells were present in both subsets (Figure 3-3B and E). The central memory CD4+ populations appear to be largely unchanged (figure 3-3C) between both isolation methods. There appears to be minor changes in CD8+ central memory cell proportions between the two isolation methods (figure 3-3F), although there is no clear trend. These results indicate that the explant isolation method does not cause a noticeable drop in effector or memory T helper cells, which are of central interest to this study (particularly CD4 memory cell populations). This suggests non-enzymatic isolation methods are suitable for procuring T helper cell populations. Furthermore, this method does not seem to result in an increase in contaminant naïve T cells from the circulation.
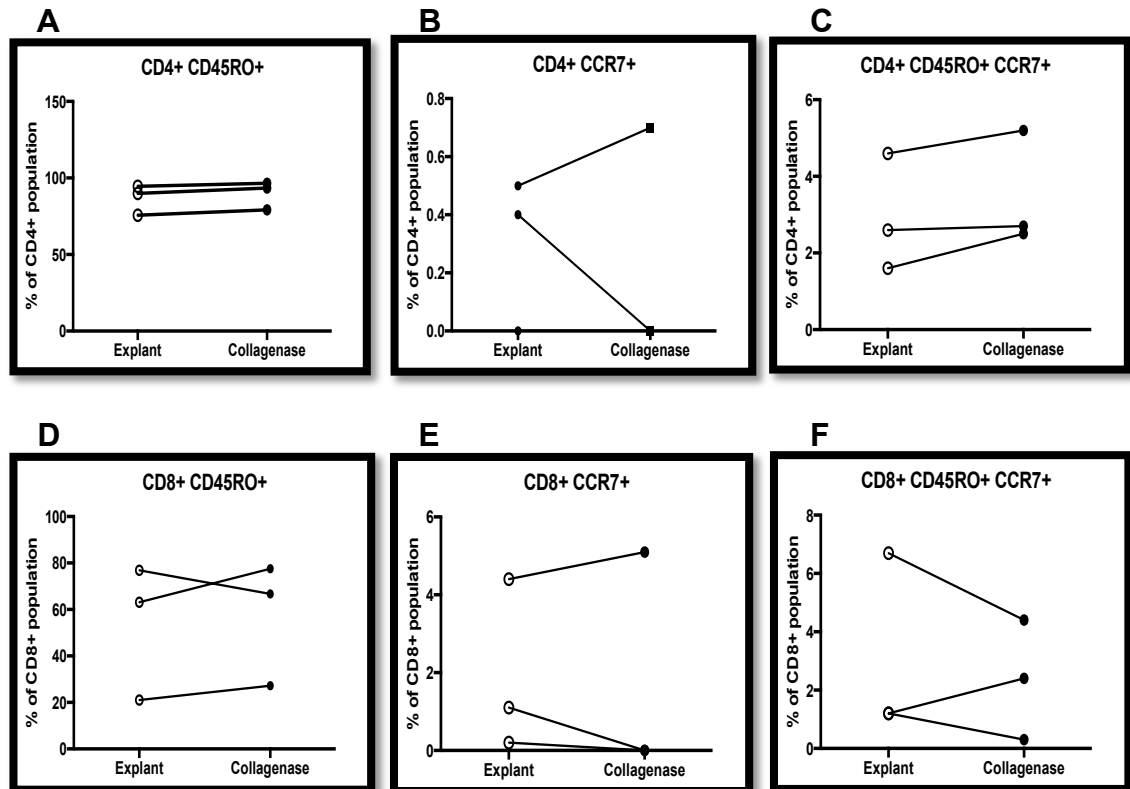
Figure 3-3- Comparison of explant and collagenase digest methods of T cell isolation from lung tissue for differences in memory cell profiles: Subject matched human lung samples were either enzymatically digested to release lung cells, or cells were isolated after egression from explanted tissue fragments. Both methods were compared for proportions of memory T cells via flow cytometry. CD3+CD4+ T helper and CD3+CD8+ cytotoxic T cells were analysed for the expression of CD45RO alone (memory/effector phenotype, panels A, D), CCR7 alone (naïve cell phenotype, panels B, E) and CD45RO+ CCR7+ (central memory phenotype, panels C, F). N=3

3.2.2 CD4 and CD8 T cell populations in the human lung

Using flow cytometry, the proportions of CD4, CD8, CD4CD8 double positive (DP) and CD4CD8 double negative (DN) T cells were examined. Analysis of collagenase digested lung samples demonstrate CD4 T helper cells as the largest lung T cell population, followed by a large CD8 cytotoxic T cell fraction and smaller populations of DP and DN T cells (figure 3-4).
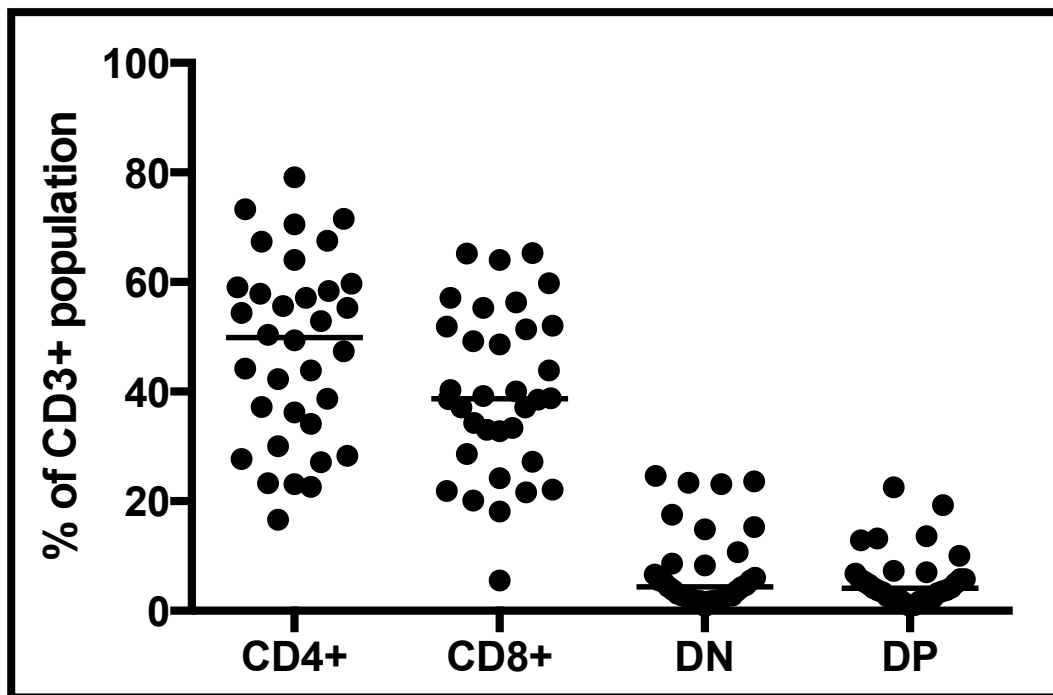
Figure 3-4- The proportions of T cells present within the human distal lung: CD3+ T cells were isolated from patient lung samples through enzymatic disruption and red cell lysis of tissue. Resultant cell preparations were analysed for the proportion of T cells expressing CD4 and CD8 via flow cytometry. The total percentages of CD3+ cells that are CD4+, CD8+, CD4-CD8- (DN) or CD4+CD8+ (DP) are shown above. Median values represented on graph. N=34

3.2.3   T memory cell populations in the human lung

Flow cytomery was used to further examine T cell populations for the expression of effector memory (CD45RO+CCR7-), central memory (CD45RO+CCR7+) and naïve (CD45RO-CCR7-) phenotypes. Memory cells were screened for across CD4 helper (Figure 3-5B), CD8 cytotoxic (Figure 3-5C), DN (Figure 3-5D) and DP T cell (Figure 3-5E) populations. Across all T cell categories, CD45RO+CCR7- effector type cells were dominant, with a small population of central memory CD45RO+CCR7+ also apparent. CD45RO-CCR7- naïve T cells were rarely, if at all, present within distal lung samples.
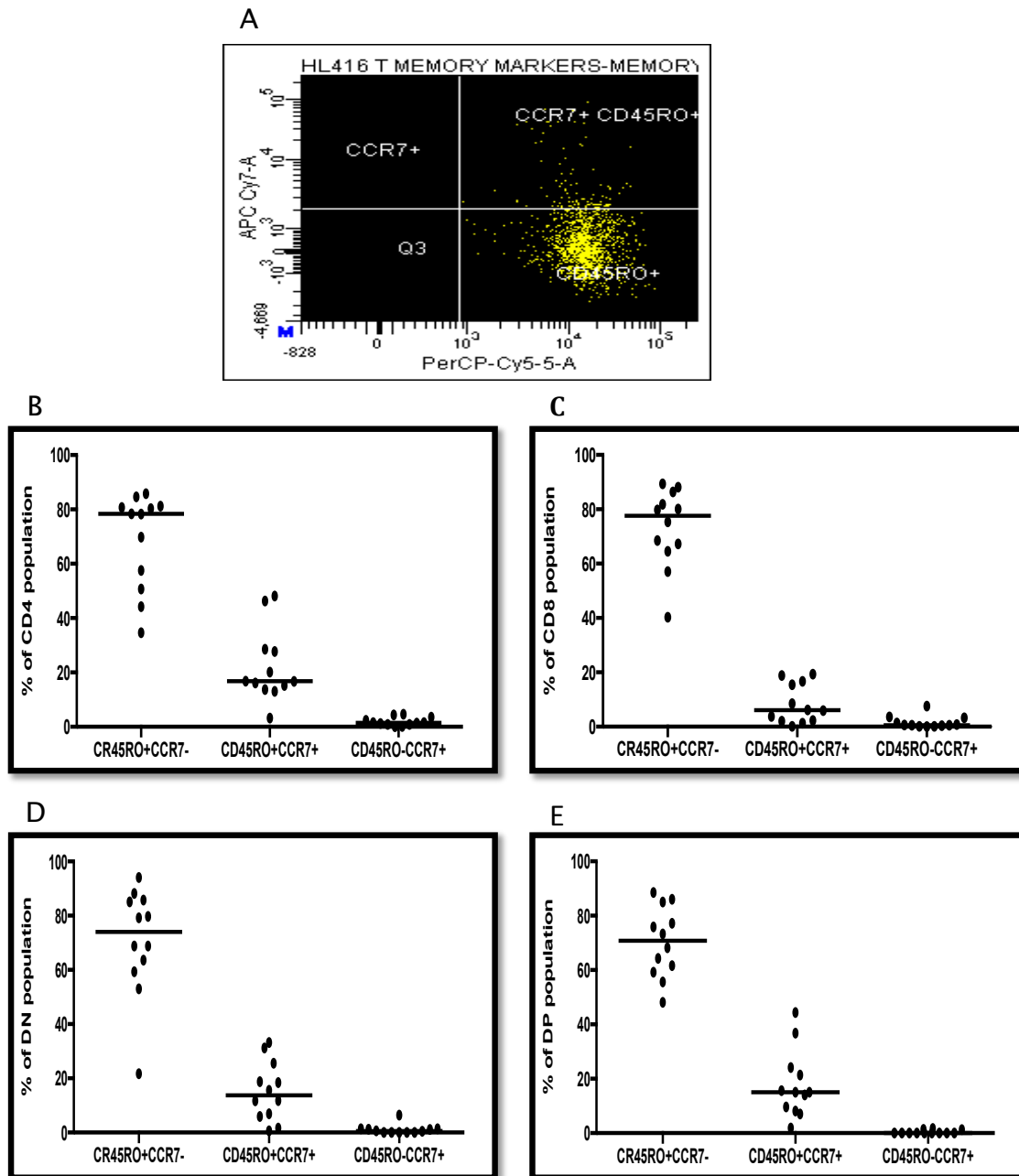
Figure 3-5- Memory cell marker expression upon CD4+, CD8+, DN and DP T cell populations: CD3+ T cell populations were isolated from human lung samples via enzymatic disruption and red cell lysis. Resultant lung T cells were examined for the expression of T cell memory markers. Different memory T cell populations and naive T cells were identified through the expression of CD45RO and CCR7 via flow cytometry (A). T effector memory (CD45RO+ CCR7-), T central memory (CD45RO+ CCR7+) and Naïve phenotype (CD45RO-CCR7-) populations for each of the CD4+ (B), CD8+ (C), DN (D) and DP (E) populations are shown. Median values are shown. N= 12

3.2.4   T cell effector subsets in human distal lung

T cell populations isolated from tissue via non-enzymatic explant methods (see section 2.4) were used for the functional analysis of lung T cell populations, particularly the production of cytokines relating to different T effector subsets (IFNγ for Th1, IL-17A for Th17, IL-10 for Treg and IL-13 for Th2). Lung cells were exposed to PMA+ionomycin to activate cytokine production within T cells, which was then measured by flow cytometry intracellular cytokine staining. Cytokine production by CD4 T helper, CD8 cytotoxic and CD4-CD8- DN T cells was examined (figure 3-6).
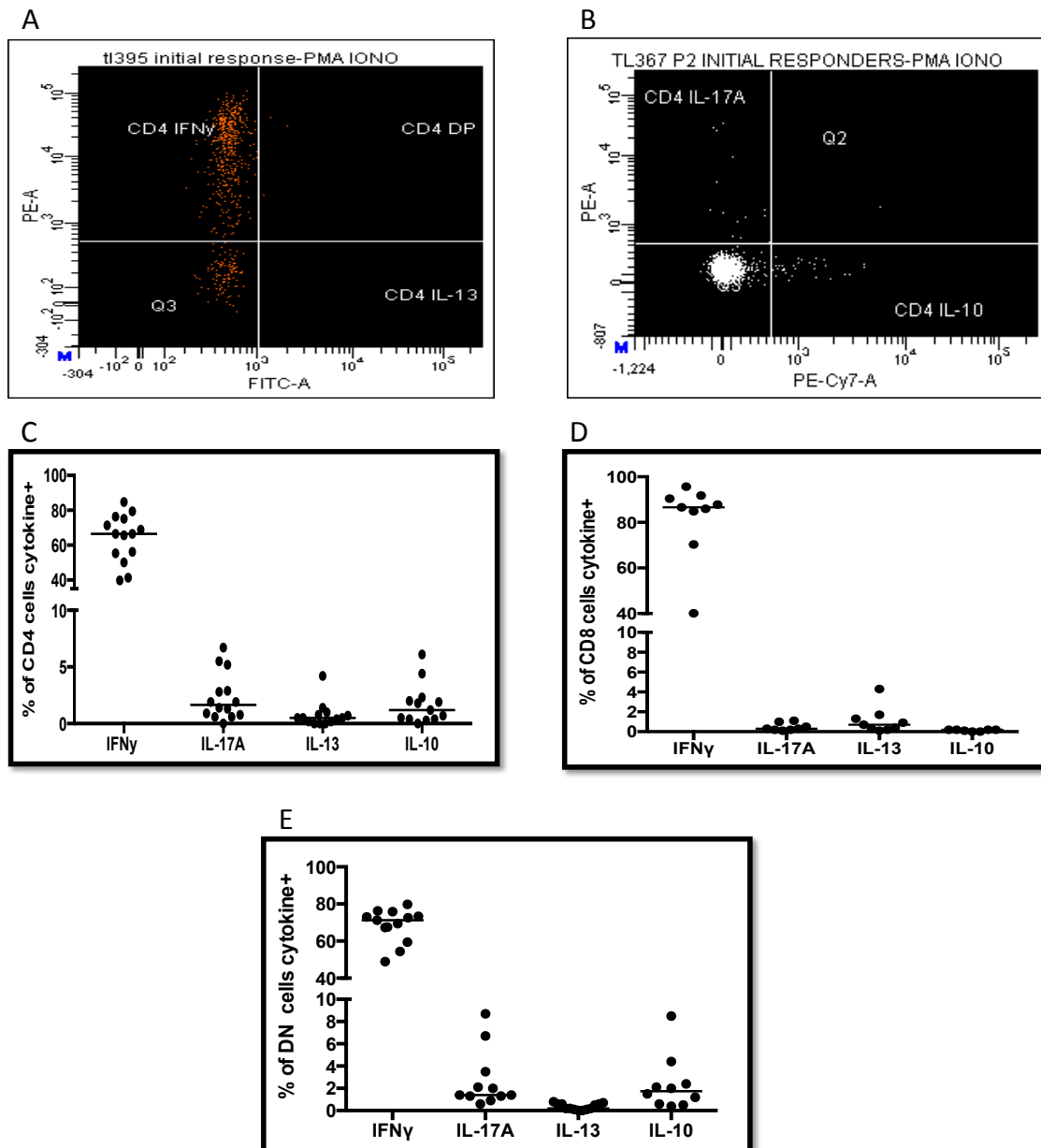
Figure 3-6- Production of cytokines by lung T cell populations after PMA+Ionomycin stimulation: Human lung T cells were isolated via explant migration and exposed to PMA+ionomycin to stimulate cytokine production. Cells were screened for the production of IFNγ and IL-13 on one panel (A) and IL-17A and IL-10 on a separate panel (B). Production of cytokines is shown across CD4 helper (A), CD8 cytotoxic (B) and DN (C) T cell populations. Results are depicted as the percentage of each T cell population positive for one cytokine, corrected for the percentage of unstimulated cells producing that particular cytokine.

In all three T cell populations, IFNγ producing cells were the dominant subset. Both CD4+ and DN T cells also had smaller IL-17A and IL-10 producing populations, while CD8+ cells had little to none. In all subsets there were very few IL-13 producing cells.

While CD4 T helper cells and CD8 cytotoxic cells have well defined role in immunity, the role of DN populations is less well defined, although it is though these cells contain significant numbers of the invariant MAIT cell populations.

3.3 Mucosal-associated invariant T (MAIT) cells in the lung

3.3.1 Effect of collagenase on MAIT-associated markers

In addition to the analysis of CD4 and CD8 populations, the presence of MAIT cell populations within the human distal lung was examined. These cells are thought to exist as a subset of the CD4+, CD8+ and DN T cell fractions [48]. Again, the effect of collagenase treatment upon MAIT-associated markers CD161 and Vα7.2 was examined (figure 3-7). Expression of these molecules appears to be largely unaffected by collagenase treatment across CD4, CD8 and DN T cell populations.
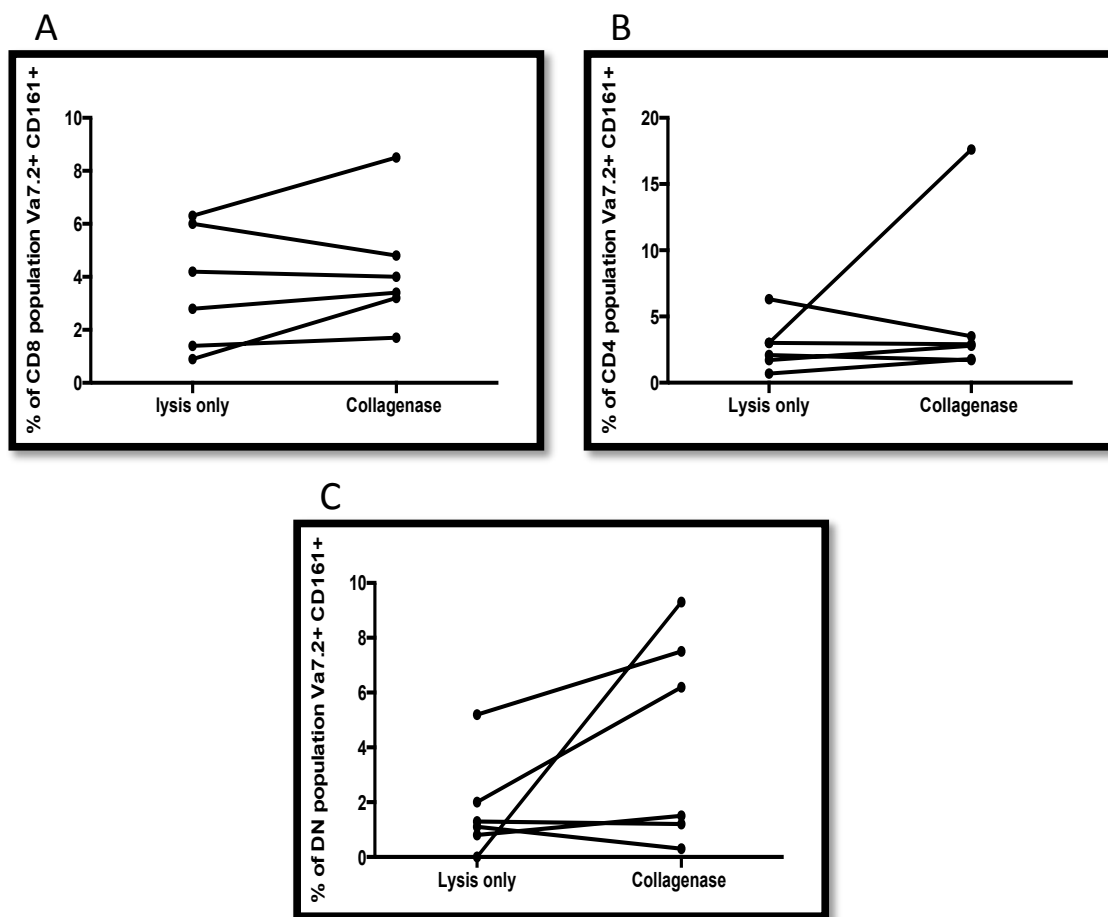
Figure 3-7- The effect of collagenase treatment on MAIT cell surface markers CD161 and Vα7.2: Lung tissue T cell populations were isolated through either enzymatic digestion followed by red cell lysis, or red cell lysis alone. Both isolation methods were compared to measure cleavage of MAIT cell surface receptors CD161 and Vα7.2 TcR via flow cytometry. MAIT cells were identified across CD4+ (A), CD8+ (B) and DN (C) populations. No significant differences between both conditions for either population were observed (Wilcoxon signed-rank) N=6

3.3.2   MAIT cells within human distal lung

CD3+ T cells were split into CD4, CD8 and DN expressing populations before being analysed for expression of the invariant TcR Vα7.2 chain and the surface molecule CD161- expression of both identifying a cell as a MAIT cell (figure 3-8). MAIT cells appear to be predominantly CD8+ cells, with a generally smaller proportion being DN (figure 3-8C). The majority of subjects did not have many CD4+ T cells expressing the CD161 and Vα7.2 MAIT lineage markers.
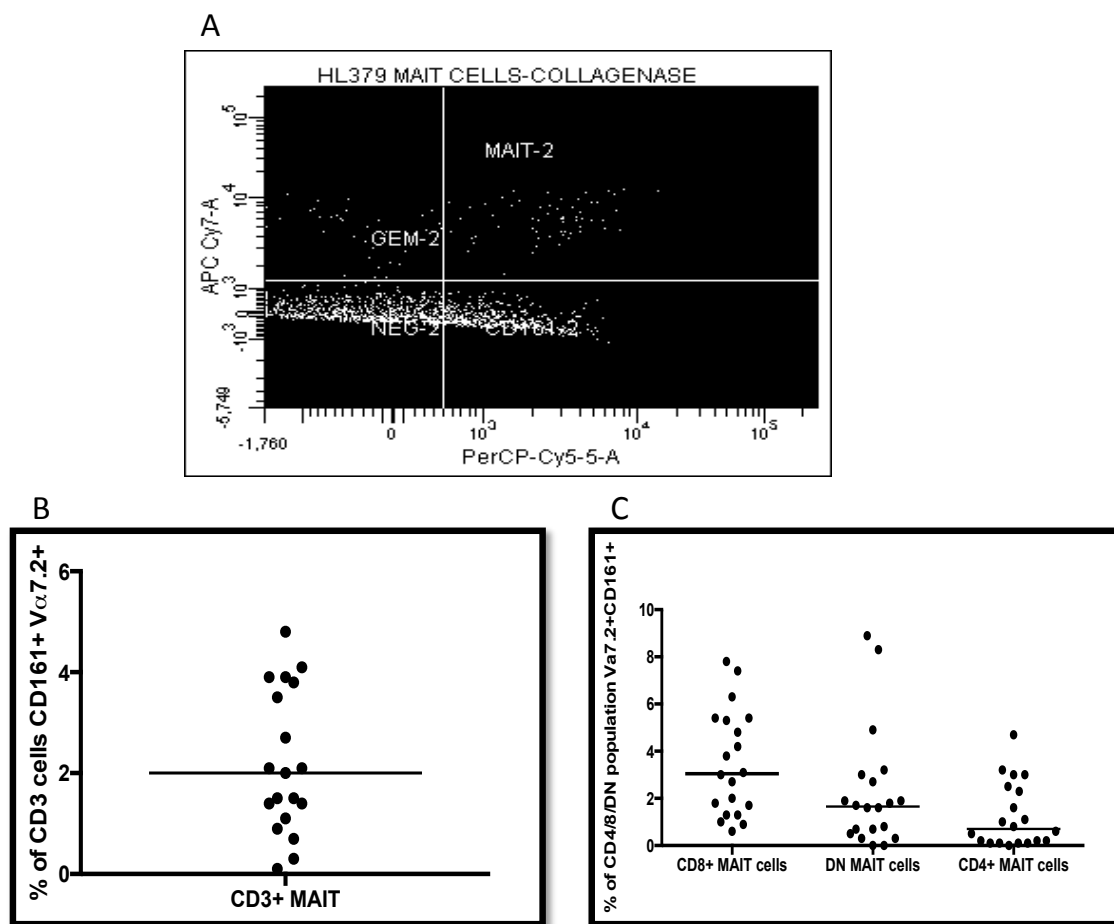
Figure 3-8- MAIT cell populations across all patient groups: Lung cell populations were isolated from distal lung samples through enzymatic disruption before red cell lysis. Resultant lung cell populations were analysed via flow cytometry for the presence of MAIT cells. MAIT cells are identified by their expression of both CD161 and Vα7.2 (A). The percentage of these cells that make up the general T cell population (as identified by CD3 expression) are shown in B. MAIT cell populations within individual T cell subsets CD3+CD4+, CD3+CD8+ and CD3+CD4-CD8- (DN) are shown above (C). Median values are shown. N=20

3.3.3   CD3-CD8+ cell populations within the lung

Staining of cells using CD3, 4 and 8 also allowed the identification of a CD3-CD8+ leukocyte cell fraction from collagenase digested tissue preperations (See section 2.8.1 for gating strategy). This population most likely consists of natural killer (NK) cells [138],[139]. Flow cytometry analysis demonstrates there are large proportions of these cells present within distal lung samples (figure3-9).

A

B
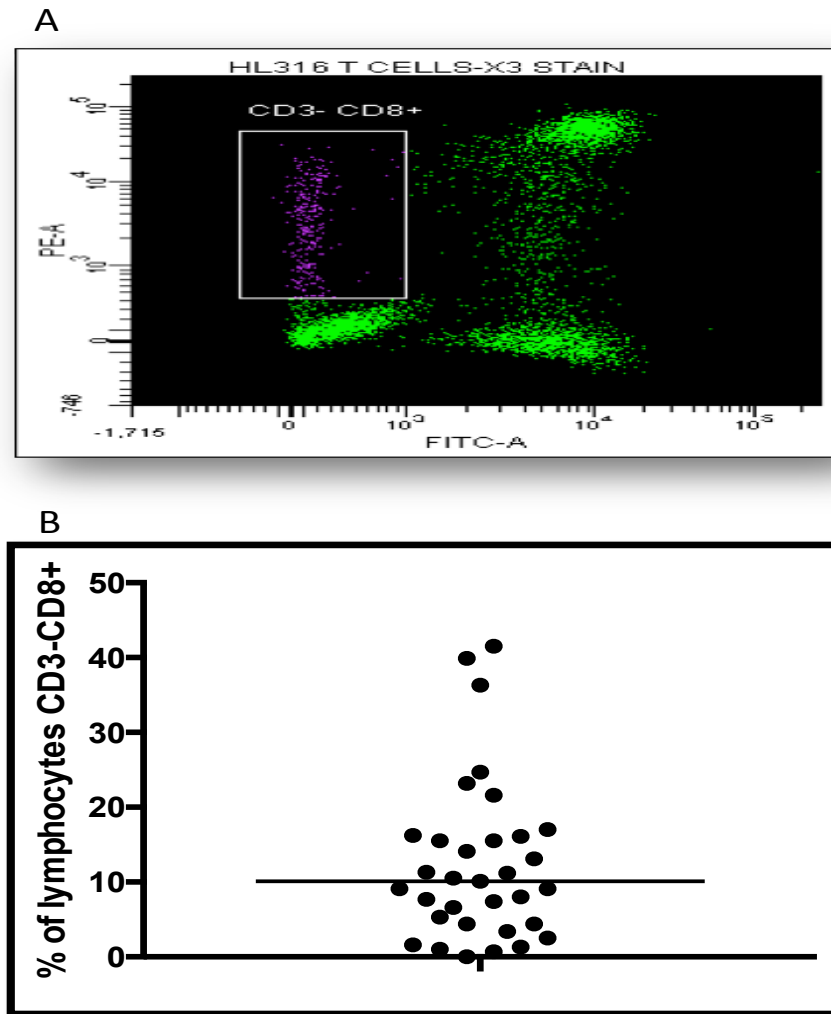
Figure 3-9 The presence of CD3-CD8+ cells within the human distal lung: Human distal lung samples were collagenase digested and stained for CD3, CD4 and CD8. As well as allowing identification of CD3+ T cell populations, CD3-CD8+ could also be identified (A). Results above show the CD3-CD8+ cell fraction as a percentage of the lymphocyte gate, as defined by cell size and granularity (see section 2.8.1 for gating strategy). N=33

3.3.4   CD3-CD8+ Cells can be isolated via egression from explanted tissue:

In order to examine cytokine production by CD3-CD8+ cells, a non-enzymatic, tissue-egression method was used to isolate cells for functional analysis (see section 2-4). A comparison of collagenase digested vs tissue egression was again performed to ensure the tissue egression model did not result in CD3-CD8+ cell trapping within tissue. CD3-CD8+ cells, as a proportion of the total lymphocyte-gated population, were compared between the isolation methods. Explant isolation appears to yield a slightly higher

proportion of CD3-CD8+ cells compared to the collagenase isolation method (figure 3-10).
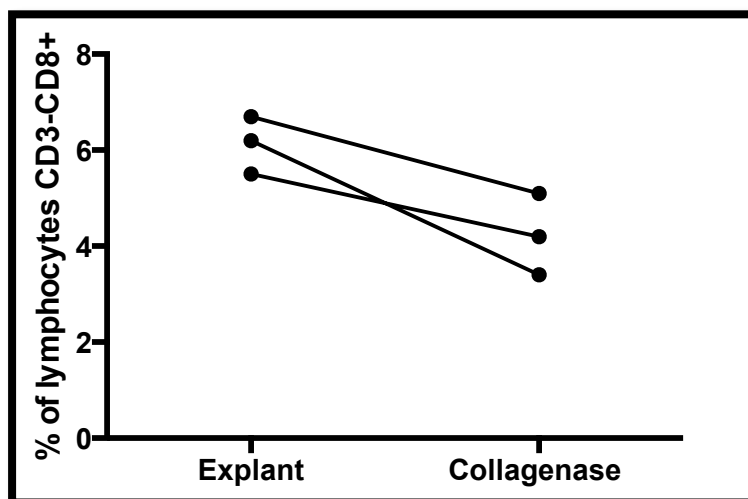
Figure 3-10- Comparison of explant and collagenase digested methods for the isolation of CD3-CD8+ cells from distal lung tissue: Lung cells were isolated from distal lung samples through either collagenase digestion or migration from tissue explants. Resultant cells were analysed for a CD3-CD8+ cell surface profile via flow cytometry. The proportion of CD3-CD8+ cells obtained via each isolation method was then compared. N=3.

3.3.5   CD3-CD8+ cells produce IFNγ

NK cells are attributed with production of the cytokine IFNγ [140]. Therefore, the production of IFNγ by CD3-CD8+ cells after PMA+ionomycin stimulation was examined via flow cytometry. Similar to cytokine production of T cell subsets, explant isolated cell populations were used. Intracellular flow cytometry demonstrates CD3-CD8+ cells produce large amounts of IFNγ (figure 3-11)

A

B
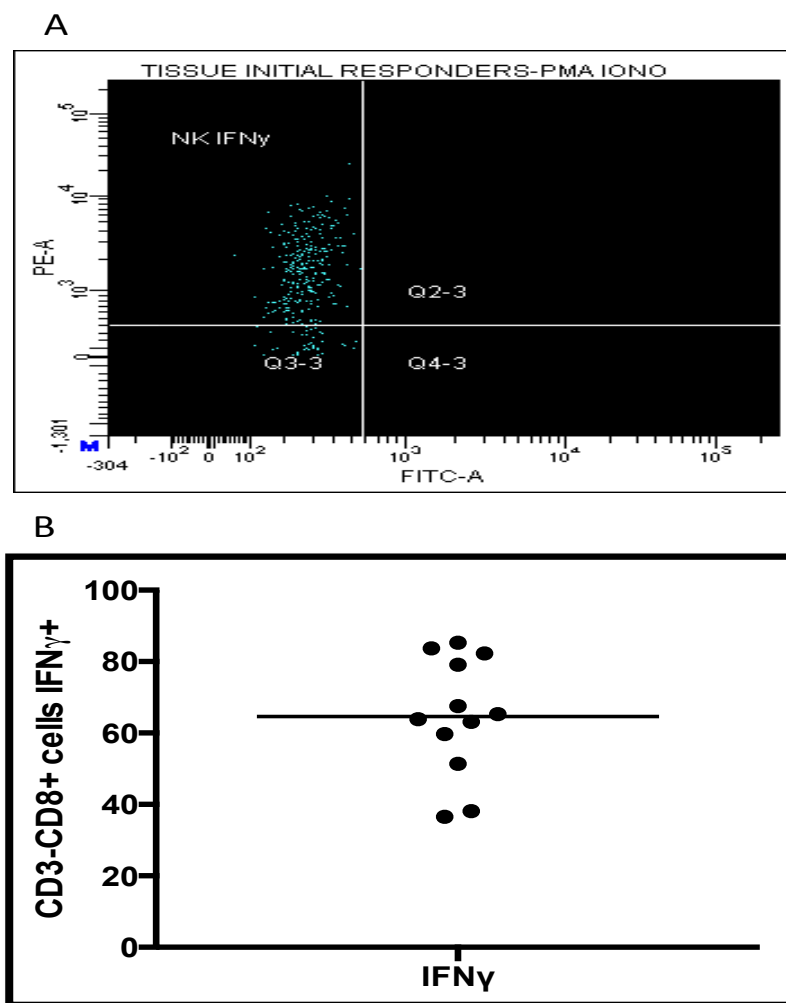
Figure 3-11- The production of IFNγ by CD3-CD8+ lung cells after PMA+ionomycin stimulation: Human lung cells were isolated via migration from distal tissue explants and activated via PMA+ionomycin stimulation to provoke cytokine production. The production of IFNγ by CD3-CD8+ cells was then measured by intracellular flow cytometry (A). Results are shown as the percentage of CD3-CD8+ cells positive for IFNγ corrected for the percentage of unstimulated cells producing IFNγ (B). N=12.

A cell surface profile of CD3-CD8+ along with robust IFNγ production would strongly suggest these cells are lung NK cells.

These results demonstrate that lung T cell populations predominantly consist of memory-phentype cells capable of producing IFNγ with very little, if any, naïve cells present. MAIT cell populations are also present, along with a CD3-CD8+IFNy+ cell type that most likely represents NK cells. It is important to understand how subject characteristics such as underlying disease state affect T cell populations, and therefore may affect later assays that make uses of lung-derived T cell populations.

3.3.6 CD4 and CD8 T cell populations in COPD and non-COPD lung

It has been shown that there are increased proportions of CD8 T cells within the lungs of COPD subjects [121]. As a number of subjects in this study have COPD as defined by spirometry, we compared the proportions of CD4 and CD8 expressing T cells based on disease status. Lung tissue was obtained from subjects undergoing resection or bullus repair surgery at Southampton general hospital. The subjects that make up the cohort for the analysis of CD4 and CD8 proportions are shown in table 3-1.

	Young Bullectomy	No COPD	COPD
Age	24.2± 4.9	65.6 ± 3.8	66.9±2.6
Gender	6M	6M 8F	8M 6F
FEV1/FVC	N/A	0.76±0.02	0.60±0.02
FEV1 % predicted	N/A	96.8%±3.5	75.6%±4
Smoking history	1 Current 1 Ex 3 Never 1 Unknown	3 Current 9 Ex 1 Never 1 Unknown	4 Current 9 Ex 1 Unknown
Pack years	N/A	30.4±6.47	40.9±5.4
Years ex	N/A	12.6±3.8	12.3±8.9

Table 3-1- Subject cohort for the analysis of CD4 and CD8 T cell populations: Human lung tissue from subjects undergoing resection was obtained following written consent along with clinical subject information where available. Subjects were classed as either young bullectomy or resection patients with or without obstructive lung disease (COPD) as measured by FEV1/FVC.

The proportions of CD3+ T cells that are CD4+ T helper cells, CD8+ cytotoxic T cells, DP or DN were examined via flow cytometry (Figure 3-12). Cell populations are shown between COPD, non-COPD and young bullectomy subject groups. There appeared to be a non-significant increase in T helper cells in non-COPD subjects (figure 3-12A), and a non-significant increase in the DN proportion in bullectomy subjects

(figure 3-13C). In the case of bullectomy subjects, the sample population consisted of only 6 subjects and contained considerable variation between subjects.
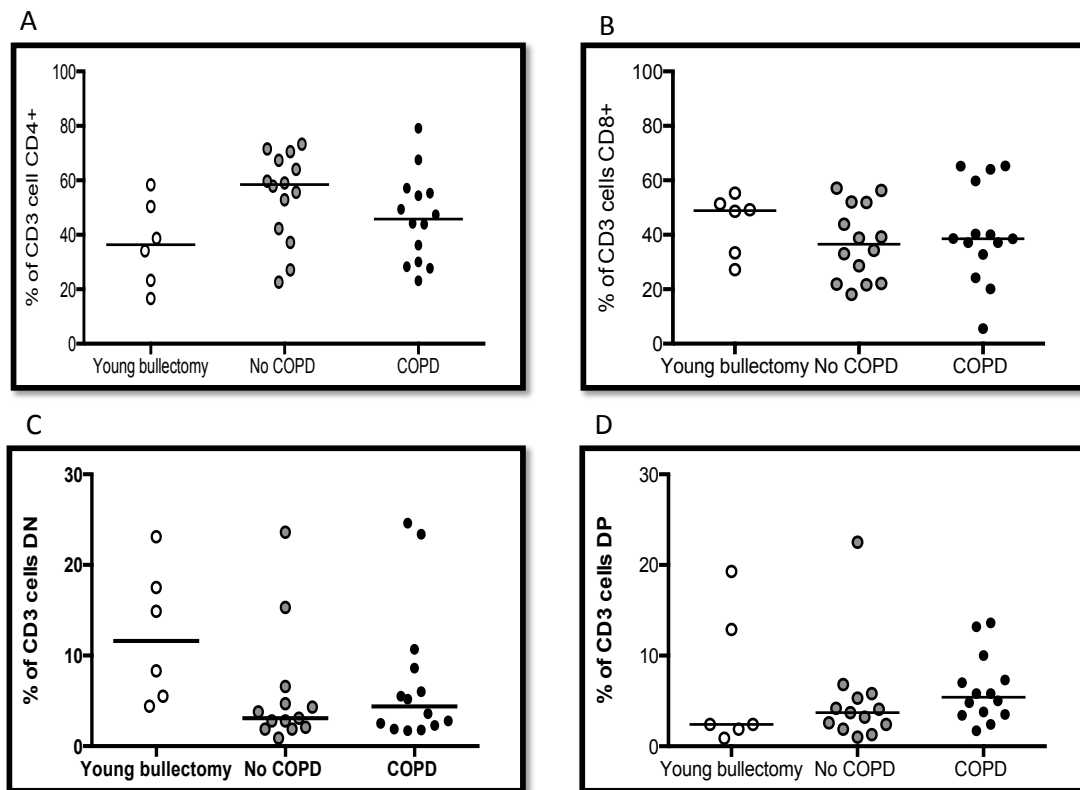
Figure 3-12- T cell populations between patient groups: T cells were isolated from patient lung tissue through enzymatic and mechanical disruption before undergoing red cell lysis. Resultant populations were analysed for the proportion of CD4 and CD8 T cell populations via flow cytometry. Total proportions of CD3+ cells that are CD4+(A), CD8+(B), CD4-CD8-double negative (C) or CD4+CD8+ double positive (D) between different patient groups are shown above. Proportions are representative of the percentage of cells that come from the CD3+ (therefore T cells) population. Median values represented on graphs.

3.3.7 CD3-CD8+ cell proportions between COPD and non-COPD lung

The proportion of CD3-CD8+ cells present in collagenase digested lung preparations from each subject disease category was also examined. There appeared to be no significant difference between bullectomy, non-COPD and COPD subjects (figure 3-13).
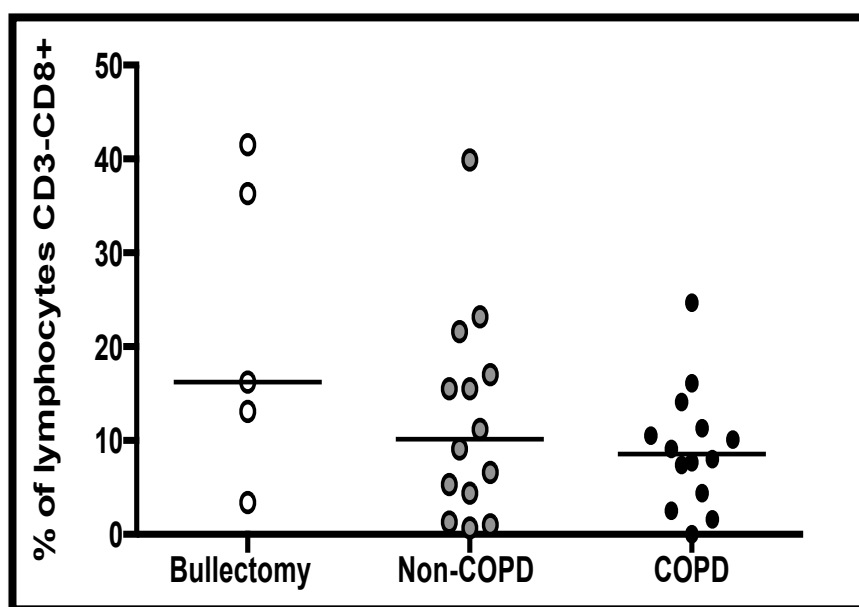
Figure 3-13- CD3-CD8+ populations between patient groups: Lung cell populations were isolated via enzymatic disruption followed by red cell lysis of lung tissue samples. Resultant populations were analysed via flow cytometry for the expression of CD8 upon CD3- leukocytes. Results are shown as the percentage of CD3- cells within the leukocyte gate positive of CD8. The proportions of CD3-CD8+ cells are shown across all three patient categories. Median values represented on graph.

3.3.8   Memory T cell proportions in normal and COPD lung

In addition to examining the basic CD4 and CD8 expressing T cell proportions between COPD and non-COPD subjects, memory cell populations were also compared. Table 3-2 outlines the details of the subjects used in this analysis.

	Young Bullectomy	No COPD	COPD
Age	23.5±6.4	64.5 ±4.2	70±1
Gender	2M	6M, 6F	4M, 4F
FEV1/FVC	N/A	0.8	0.6
FEV1% predicted	N/A	99.7±7.7	85.8±20.4
Smoking history	2 current	5 Ex, 5 Current, 2 Unknown	6 Ex, 2 Current
Pack years	N/A	38.1±3.3	39.5±3.3
Years ex	N/A	18.3	16±3.9

Table 3-2- **Subject cohort for the analysis of memory T cell populations between subject disease categories:** Human lung tissue from subjects undergoing resection was obtained following written consent along with clinical subject information where available. Subjects were classed as either young bullectomy or resection patients with or without obstructive lung disease (COPD) as measured by FEV1/FVC.

Lung cells isolated via collagenase digestion of distal lung tissue were screened for T cell lineage via flow cytometry. Resultant CD4, CD8, DP and DN T cell populations were then analysed for the expression of a CD45RO+CCR7- effector memory phenotype (figure 3-14). In all T cell categories (CD4, CD8, DP and DN), there were no noticeable differences in the effector memory proportions between different subject conditions.
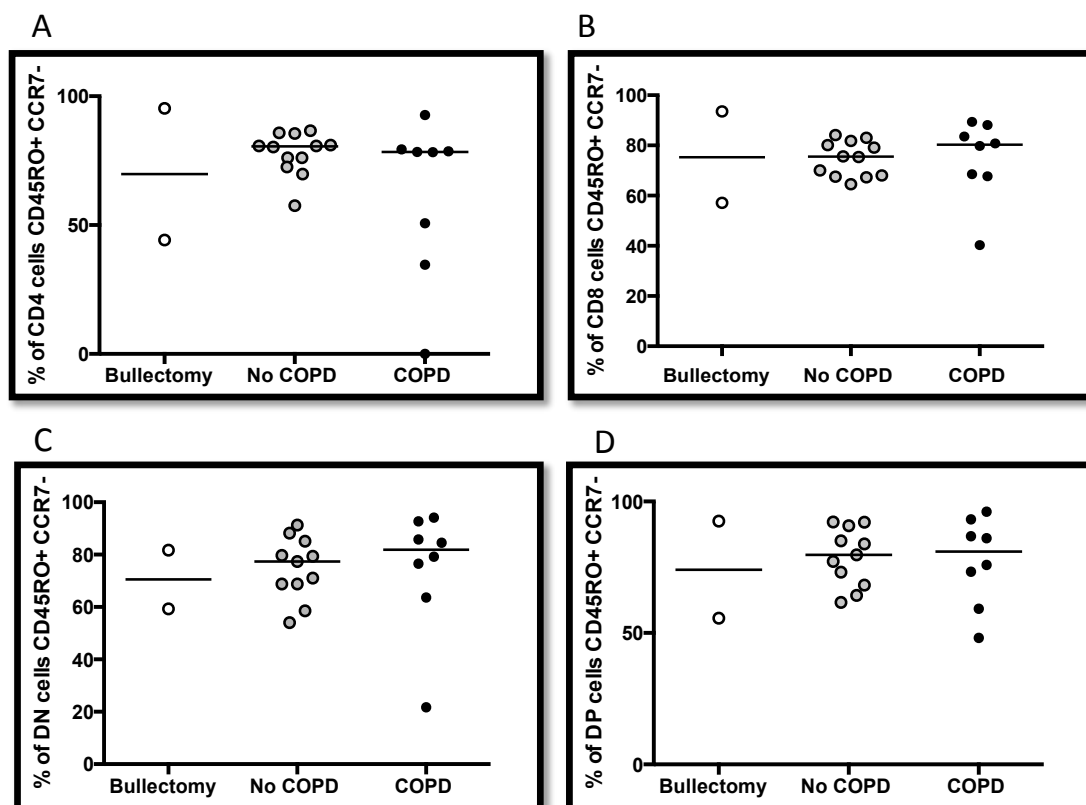
Figure 3-14- CD45RO+CCR7- Effector memory T cell proportions between COPD and non-COPD subjects: Lung cell populations were isolated from distal lung samples via enzymatic disruption and red cell lysis. Resultant cells were examined for an effector memory T cell profile (CD45RO+CCR7-). Effector memory populations of each of the CD4+ (A), CD8+ (B), DN (C) and DP (D) T cell populations are shown. In each case, the proportion of effector memory cells is compared between bullectomy, COPD and non-COPD subjects. Median values are shown.

CD45RO+CCR7+ central memory T cells were also screened for across CD4, CD8 DN and DP T cell populations (figure 3-15). Central memory proportions were similar across all T cell subsets and between subject disease categories.
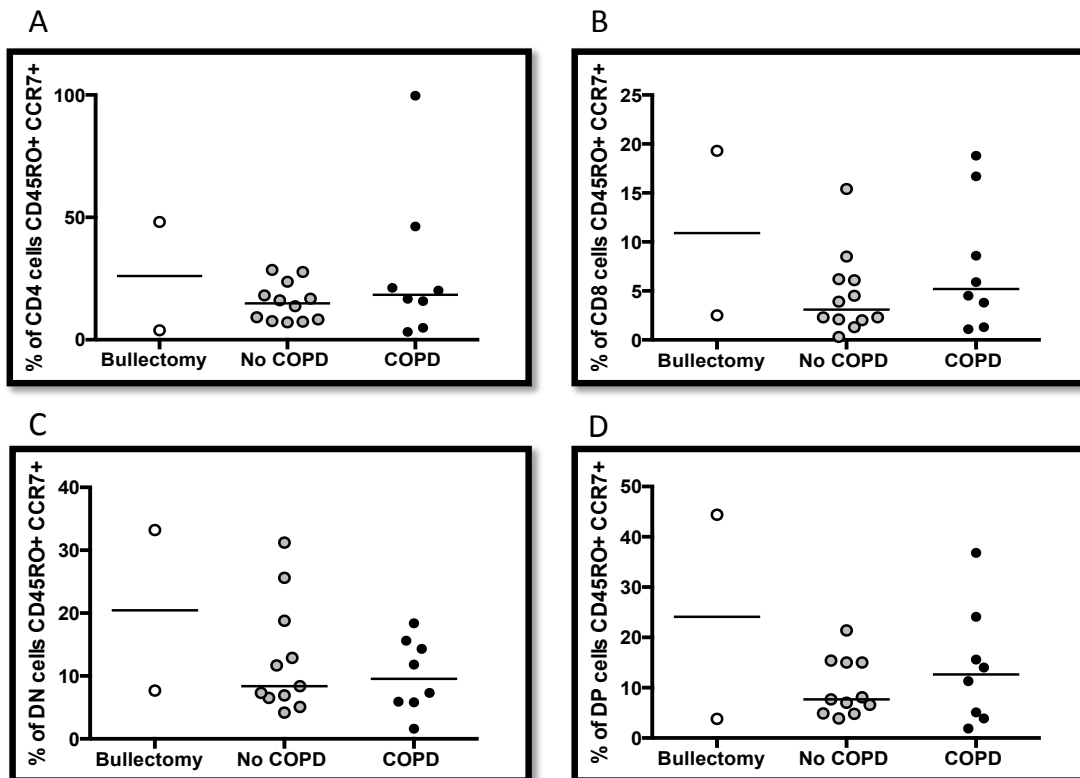
Figure 3-15- CD45RO+CCR7+ Central memory T cell proportions between COPD and non-COPD subjects: Lung cell populations were isolated from distal lung samples via enzymatic disruption and red cell lysis. Resultant cells were examined for a central memory T cell profile (CD45RO+CCR7+). T central memory populations of each of the CD4+ (A), CD8+ (B), DN (C) and DP (D) T cell populations are shown. In each case, the proportion of central memory cells is compared between bullectomy, COPD and non-COPD subjects. Median values are shown. N= 12

3.3.9   MAIT cells populations in the COPD and non-COPD lung

In addition to examining basic T cell, memory cell and CD3-CD8+ cells populations between subject groups, it was also possible to examine differences in MAIT cell populations. The details of the subjects used in this analysis are outlined in table 3-3.

	Young bullectomy	Non COPD	COPD
Age	19	64±2.9	72.3±1.3
Gender	M	3 M, 7F	4M, 4F
FEV1/FVC	N/A	0.7	0.6
FEV1 % predicted	N/A	100±3.1	86.8±3.4
Smoking history	Current	5 Ex, 4 Current, I Unknown	4 Ex, 4 Current
Pack years	-	34.5±11	37.5±4.9
Years ex	N/A	26.3±4.7	14.7±3.9

Table 3-3- Subject cohort for the analysis of MAIT cell populations between subject disease categories: Human lung tissue from subjects undergoing resection was obtained following written consent along with clinical subject information where available. Subjects were classed as either young bullectomy or resection patients with or without obstructive lung disease (COPD) as measured by FEV1/FVC.

Distal lung samples were collagenase digested and stained for CD3 as a marker of T cell lineage and for MAIT cell markers CD161 and Vα7.2 Total MAIT cell proportions were then compared between subject disease categories (figure 3-16). Flow cytometry analysis shows the total proportion of MAIT cells within the entire CD3+ T cell population are not significantly different between subject categories, although a non-significant trend for decreased MAIT cells in COPD was apparent.
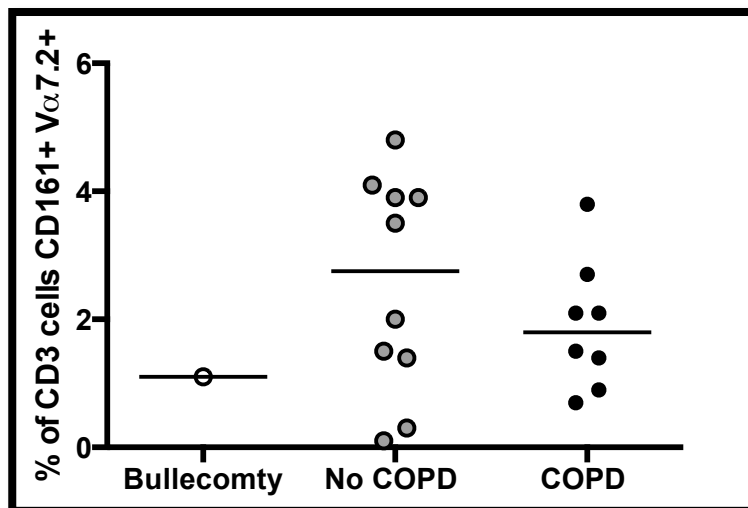
Figure 3-16- Total CD3+ MAIT cell proportions between COPD and non-COPD subjects:
Lung cell populations were isolated from distal lung samples via enzymatic disruption and red
cell lysis. Resultant cells were examined for T cell lineage markers and MAIT cell markers
CD161 and Vα7.2 via flow cytometry. MAIT cell proportions within the total CD3+ T cell
fraction are shown. Results are shown as the percentage CD3+ cells positive for both CD161
and Vα7.2. The proportion of MAIT cells is compared between bullectomy, COPD and non-
COPD subjects. Median values are shown. N= 19

The proportion of MAIT cells in each of the CD4, CD8, and DN T cell subsets was then
compared between subject disease categories, again showing no significant differences
(figure 3-17)
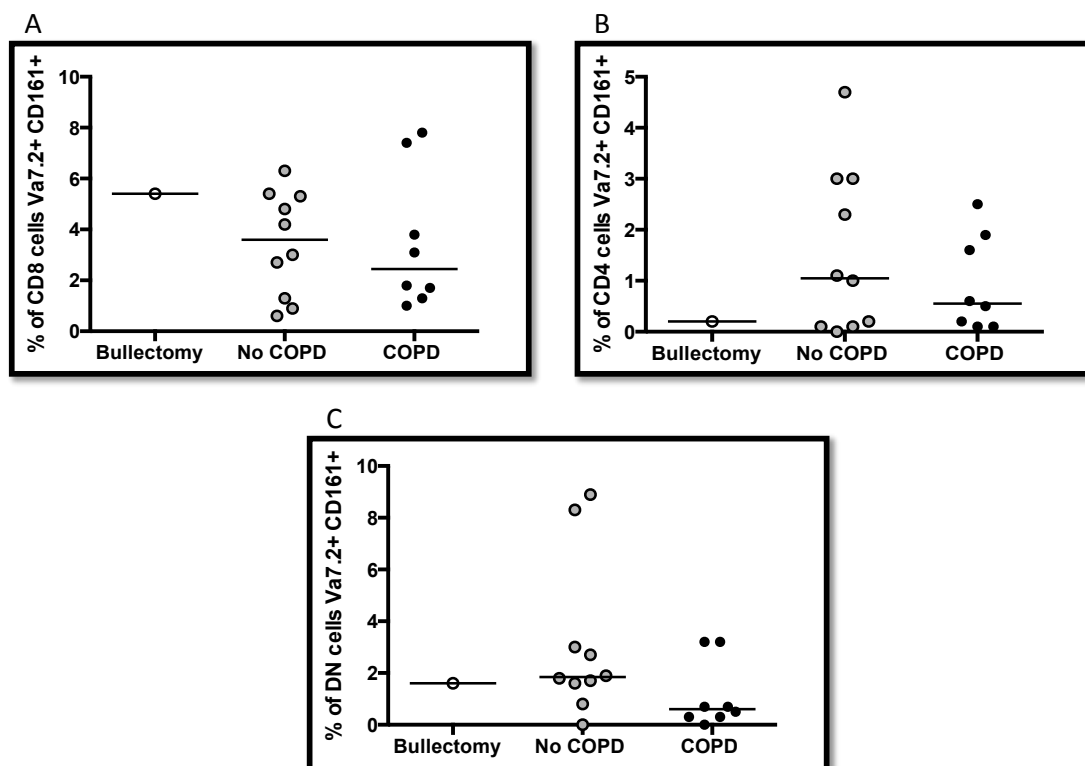
Figure 3-17- CD4, CD8 and DN MAIT cell proportions between COPD and non-COPD subjects: Lung cell populations were isolated from distal lung samples via enzymatic disruption and red cell lysis. Resultant cells were examined for T cell lineage markers and MAIT cell markers CD161 and Vα7.2 via flow cytometry. MAIT cell proportions within each of the CD4+ (A), CD8+ (B) and DN (C) T cell populations are shown. Results are shown as the percentage of each T cell population positive for both CD161 and Vα7.2. In each case, the proportion of MAIT cells is compared between bullectomy, COPD and non-COPD subjects. Median values are shown. N= 19

Overall, comparison of the various T cell populations as well as CD3-CD8+ cells within human distal lung does not highlight any differences between subjects based upon obstructive lung disease state in this study.

3.4   DISCUSSION

Using flow cytometry we aimed to characterise the T cell populations within the human distal lung, primarily examining the proportion of CD4, CD8 and double positive/negative populations. We demonstrate that the majority of distal lung T cells are CD4 T helpers, followed closely by CD8 cytotoxic T cells and smaller proportions of DN and DP cells. This is in line with previous work showing T helper cells as the dominant T cell population present within the human lung [69]. Furthermore, the CD45RO+CCR7- effector memory phenotype was prevalent in all T cell subsets, along with smaller numbers of CD45RO+CC7+ central memory cells. Almost no CD45RO-CCR7+ naïve T cells were present in distal lung samples. This lack of naïve cells indicates minimal contamination from circulatory blood and confirms that naive cells are rare within the peripheral lung environment [69]. As well as bearing a memory cell phenotype, the vast majority of T cells exhibit an IFNγ producing, Th1-phenotype. Smaller populations of IL-17A and IL-10 producing cells were also apparent, particularly in the T helper cell fraction. In addition to conventional T cells, mucosal associated invariant T (MAIT) cells were also present within distal lung samples along with populations of CD3-CD8+ cells; most likely representing the innate lymphocyte NK cells. These CD3-CD8+ cells are also demonstrated to be a source of IFNγ, further indicating the likelihood of these being NK cells [141].

Ultimately, lung derived T cell populations are used to examine lung fibroblast antigen presentation capabilities. It is therefore important to establish how underlying disease states may affect T cells within the lung as this may impact upon later experiments.   Tissue was only used from resected marginal zones that were classed as non-cancerous by a pathologist prior to use in this study. Beyond this, the most notable underlying lung pathology is COPD. COPD is known to have an altered T cell profile compared to healthy equivalents. In particular, CD8 cytotoxic T cells have been attributed as a major factor in COPD due to their increased proportions and association with declining lung function [121]. In the current study, both CD4 and CD8 cells are found in equal proportions in both COPD and non-COPD patient lung samples. This discrepancy may be explained by a number of factors. The study by Saetta focused upon subjects with chronic bronchitis, a particular endotype of COPD. Our current study classifies patients as COPD or non-COPD via lung spirometry tests alone-which

will not make distinctions between chronic bronchitis and other endotypes of obstructive lung disease (such as emphysema). Furthermore, we quantify T cell populations through flow cytometry analysis, whereas the previous study used immunohistochemical microscopy and manual cell counting to analyse T cell populations. These different analysis techniques may cause differences in the end results, therefore experimental design may also contribute towards the discrepancy in results in relation to T cell populations within the COPD lung.

Studies examining differences in COPD and non-COPD subject T helper memory cells report an apparent decrease in the effector memory subset in COPD [142]. While we do not find such a trend, the study by Freeman et al quantify memory cells via expression of CD62L and CD27. While expression of CD62L and CD27 can identify a memory-cell phenotype, it is notably different from CCR7 and CD45RO expression used in our study to identify memory cells. CD27 expression categorises memory cell populations into distinct subsets with different activation requirements and responsiveness to viral pathogens [143, 144]. T cells responding to insults early in the response appear to express CD27, and this is progressively lost as the response proceeds on (Appay et al). These CD27- cells are then associated with strong antigen-recall responses and a higher activation state compared to CD27 expressing equivalents (Schiott et al). These discrepancies in classifying memory T cells may account for the difference in results with the current study as these memory cell phenotypes represent discrete cell populations to some degree.

Through staining for expression of the CD45 isoform CD45RO and the lymphatic homing receptor CCR7, it is possible to identify effector memory (CD45RO+CCR7-) and central memory (CD45RO+CCR7+) T cells. These cells differ in their ability to circulate through peripheral zones (effector memory) and within the secondary lymphatics (central memory) [57]. Our results indicate that the majority of T cells across the CD4 and CD8 categorized populations are CD45RO+ CCR7-effector memory cells. Similar lung studies examining T cell populations have also uncovered large proportions of CD45RO+ cells, ranging from 75% to 97% of the T cell population dependent upon the isolation method used [69]. Other peripheral zones such as the skin share this trait, with the vast majority of T cells bearing a memory [145]. These studies also indicate a significant proportion of this CD45RO population are tissue resident memory cells, and important class of non-circulatory, long lived cell which inhabit

peripheral zones and provide frontline defence against pathogens [69]. Undoubtedly this CD45RO fraction in our study will contain a proportion of these resident memory cells, and further screening for resident memory associated markers CD103, CD69 and VLA-1 may have demonstrated the presence of these populations.

We also report no statistically significant differences in the MAIT cell and CD3-CD8+ cell fractions between COPD and non-COPD patients. While the nature of these cells is understood in terms of effector function and activation, their role and potential dysfunction in COPD are still under investigation. NK cells have been shown to be increased in the blood of COPD subjects [146], however no differences in parenchymal lung NK numbers between COPD and non COPD lung have been demonstrated previously [147]. Further investigations are required to identify the role and potential dysfunction of these cells in lung diseases such as COPD.

The vast majority of T cells isolated from the lung exhibit a Th1 phenotype, as shown by the production of IFNγ (figure 3-6). Th1 responses are considered to be protective against microbial and viral threats [146], and many of these cells will be specific for lung pathogens such as *influenza* [69] and bacteria such as NTHi [130]. IL-17A producing Th17 cells are also present, albeit in much smaller proportions. Again this T helper subset is known to be involved in the response to *influenza* [148] and bacteria [130] within the lung. The predominance of these cell types most likely represents the requirement to have large numbers of inflammatory cells stationed in the lung to deal with infectious agents. Analysis of T helper subsets also shows a small population of IL-10 producing cells (figure 3-6). The production of IL-10 is associated with anti-inflammatory, regulatory function. IL-10 is able to promote tolerance to self-antigens through immunosuppressive activity upon other T cells [149] as well as being implicated in tumour pathogenesis through similar immunosuppressive pathways [150]. IL-10 production was initially associated with Th2 cells [151]. However, more recent developments have led to the classification of a phenotypically distinct "Treg" cell, which produces primarily IL-10 and has a specialized role in suppressing the responses of other T cells [152]. However, work in human studies has shown that Th17 [153] and Th1 [154] subsets are also capable of producing IL-10 along with their respective effector cytokines in what is considered a self-limiting control of inflammatory capacity. Therefore, although we identify IL-10 producing T cells, it is difficult to conclusively classify these as true Treg populations.

In addition to screening for IL-10, IL-17A and IFNγ, the presence of IL-13 producing T cells was investigated (figure 3-6). Very few IL-13 producing T cells were identified via PMA+ionomycin stimulation. IL-13 production is associated with the Th2 subset [155], and drives mucus hypersecretion, fibrosis and promotion of an allergic-type response [156]. IL-13 is therefore associated with the pathogenic processes involved in asthma [157]. IL-13 has also been postulated to be involved in the COPD pathogenic process. Mouse modelling highlights a role for IL-13 driven emphysema via MMP production, demonstrating a possible mechanism by which IL-13 producing T cells may drive COPD pathogenesis [158]. With such low numbers in the cohort of subjects examined in this study, it is difficult to extrapolate if IL-13 production is related to a disease state in the individuals examined. Further analysis focusing upon clearly defined COPD and non COPD subjects without confounding conditions such as asthma may prove beneficial in delineating the role of IL-13 in COPD.

While cytokine analysis is a powerful tool to examine T cell subsets, the increasingly complex cytokine profiles of T cell subsets make it difficult to definitively identify effector subsets. Utilizing flow cytometry to examine the transcription factor is another approach that would allow identification of T cell subsets. T cell effector subsets can be identified by expression of distinct transcription factors, which are upregulated in response to cytokines produced by APCs interacting with the T cell [159, 160]. For example, Expression of transcription factor T-bet is associated with Th1 [159] cells, while GATA3 is associated with Th2 cells [161]. Using both cytokine production profiles and the transcription factor expression profile of T cells would allow a more robust analysis of the nature of T cell effector populations the distal lung.

The isolation of T cells for initial screening was done though enzymatic digestion of lung tissue to liberate as many cells as possible. However, for the isolation of T cells for use in co-culture models with fibroblasts and intracellular cytokine analysis, a non-enzymatic model was used. Although we show no cleavage of markers such as CD4 due to collagenase in our system, cleavage of other markers not screened for that may impact T cell activation cannot be ruled out. Therefore collagenase was avoided to prevent unintended effects upon these T cells being used for long-term culture. Comparisons between the enzymatic and non-enzymatic explant method demonstrate 2 important points; there is no apparent increase in naïve T cells in the explant method,

indicating that allowing cell migration from tissue doesn't increase the proportions of blood-based T cells in culture. Secondly, the T helper memory-type populations are largely unaltered between the two methods, indicating memory T cells are still obtained without collagenase digestion. This is critical as later experiments focus upon lung T helper-fibroblast interaction, and contaminant blood cells may affect the outcome of these experiments. CD8 memory cell populations did change slightly between enzymatic and explant methods, particularly in the CD45RO+CCR7+ fraction. However, as this method of cell isolation is focused upon T helper cells rather than cytotoxic T cells, this finding should not impair the ability of the explant isolation method for this study.

In addition to the presence of single positive cells, our study also reports the presence of smaller, yet distinct populations of double negative (CD3+CD4-CD8-) and double positive (CD3+CD4+CD8+) T cells. While smaller than the single positive cell fractions, these populations have been implicated in a number of disease situations. Double positive cells appear to be involved in the response to chronic viral infections through both their increased presence in the peripheral area of insult and their release of potent inflammatory mediators [162]. Whilst we have not investigated the function of the DP populations, previous work indicates that these DP cells arise through chronic inflammation in the lung and have the capability to contribute to the inflammatory reaction [162]. The presence of these cells therefore suggests that a proportion of subjects have underlying lung inflammation.

The double negative T cells have been linked to rapid and robust responses to antigen stimulation. In particular, it has been shown that DN T cells are one of the largest sources of IL-17 in the autoimmune condition systemic lupus erythematosus [163]. Lung DN populations have also been associated with a strong response to the respiratory infection *Francisella Tularensis.* Subsequent investigations have demonstrated that the DN cells responding to this form of pulmonary infection are the newly described MAIT cell population [52].

These MAIT cells have been described as CD3+ DN, CD4 or CD8 T cells expressing CD161 and the Vα 7.2 chain TcR [48]. These MAIT cells respond to vitamin B metabolites bound and presented by MHC-related protein 1 (MR1) upon APCs. We demonstrate that human distal lung samples do contain MAIT cell populations,

primarily in the CD8 fraction but also considerable numbers in the DN fraction. CD4 MAIT cells were also present in a smaller number of subjects. These cells may nave a role producing cytokines rapidly in response to bacterial infection. The early release of IFNγ by MAIT cells in particular is though to be critical in conditioning the local area to drive optimal Th1 responses [164]. Production of IFNγ by MAIT cells in this way may condition local fibroblasts and allow their contribution to T cell immunity in the area.

As well as being able to examine the T cell CD3+ populations, it was also possible to analyse the proportion of CD3-CD8+ cells. These cells most likely represent the natural killer cell population, which is known to express CD8 [139]. Figure 14 shows that both COPD and non-COPD subjects have similar proportions of NK cells, with the lower cohort size bullectomy category also showing a wide spread of variability in the proportion of these cells present. Therefore NK cells appear to be well represented in the lung regardless of underlying disease processes. NK cells are cytotoxic, being able to directly kill infected and cancerous cells [165], and their presence in resection subjects may have been explained by underlying responses to cancer. However the presence of CD3-CD8+ cells in young bullectomies indicate these cells are present regardless of cancer and also that age discrepancies do not seem to impact their presence in the lung.

More recently, NK cells have also been shown to respond to bacterial infection indirectly via signals from professional APC populations [166], releasing cytokines such as IFNγ in the early-phase of an immune response. Similar to previously mentioned MAIT cells, early release of IFNγ may be important in conditioning the local environment, facilitating recruitment and activation of other immune cells and also affecting local stromal cells such as fibroblasts. We show that a considerable proportion of CD3-CD8+ cells are capable of producing IFNγ, further indicating that a portion of these cells are most likely NK cells. Identifying cells merely as CD3-CD8+ can result in the identification of other non-T cell, CD8-expressing cells, such as dendritic cells [167], although flow cytometry gating upon lymphocytes based upon size and granularity should exclude these cells. Inclusion of a more specific NK marker, such as CD56, would confirm these as NK cells [168]. Therefore while this population is most likely an NK population, we cannot confirm NK lineage with absolute certainty.

Our analysis highlights that there are large populations of CD4 T helper cells present in the human lung, both in healthy individuals and those with obstructive lung disease. Our analysis corroborates finding by others that the vast majority of T cells within the lung are memory-type cells and naïve T cells are not a prominent feature in the parenchymal lung [69]. Presumably a number of these memory cells are stationed to control lung-specific insults including influenza virus and microbes such as NTHi. We also demonstrate the presence of MAIT cells and CD3-CD8+ cells, a portion of which are most likely NK cells. Both these cell types rapidly produce IFNγ upon activation in the periphery, and may represent a critical source of stimulation for local fibroblasts and driving expression of HLA-DR upon these cells, thereby facilitating fibroblast-T helper cell interaction. Furthermore, nonezymatic isolation techniques are shown to result in unaltered ratios of memory T helper cells from tissue samples. Therefore, non-enzymatic isolation can be used to provide a source of lung memory T helper cells for fibroblast-T cell co-culture assays.

4. PHENOTYPIC ANALYSIS OF HUMAN LUNG FIBROBLAST EXPRESSION OF IMMUNE SYNAPSE MOLECULES AND INTERNALIZATION OF EXOGENOUS ANTIGEN

4.1   Introduction

Lung fibroblasts are positioned in the mesenchymal zone of the lung, directly beneath the epithelial cells and alveolar macrophage populations. In this location, fibroblasts are ideally positioned to receive both local inflammatory signals, such as IL-1α from damaged overlying epithelia [86] IFNγ produced by local immune cell populations. In addition, fibroblasts may come into contact with microbial factors during bacterial invasion into the mesenchyme [17, 118]. The relationship between lung fibroblast and lung resident memory T helper cells is poorly defined, particularly in regard to the fibroblasts ability to present antigen to these immune cells in the local environment. In order to present antigen to a T helper cell, the cell in question must fulfil a number of criteria- of central importance being the expression of immune synapse molecules, as well as an ability to sample their environment for antigenic material. These characteristics allow APCs to internalize antigens from the local area and present them in complex with MHC Class II molecules, which in humans are the human leukocyte antigen (HLA) class II molecules. Of these HLA molecules, HLA-DR is one of the primary HLA class II molecule. The aim of this chapter is to examine the expression of vital immune synapse molecules on human lung fibroblasts both *ex vivo* and *in vitro*. In addition, the ability of *in vitro* cultured fibroblasts to internalize environmental material is also investigated. This includes examining the expression of an internal vesicle marker associated with the MHC class II pathway. The expression of critical immune synapse molecules and an ability to sample environmental antigen would strongly suggest that human lung fibroblasts have the capability to antigen-present to T helper cells.

4.2 RESULTS

4.2.1 Human lung fibroblast, epithelial cell and macrophage proportions within human distal lung

Fibroblasts and epithelial cells are structural cells that make up part of the CD45- cell population, while macrophages are one of the most abundant CD45+ leukocytes present in the lung. Through flow cytometry, it was possible to assess the general proportions of these cell types present within human distal lung samples. The percentage of macrophages (CD45+FITC-hi HLA-DR-hi), epithelial cells (CD45-EpCAM+CD90-) and fibroblasts (CD45-EpCAM-CD90+) as a percentage of total lung cells was assessed (figure 4-1). Macrophages were the most abundant cell type present in this analysis, while in the structural cell compartment epithelial cells were more abundant than fibroblasts.
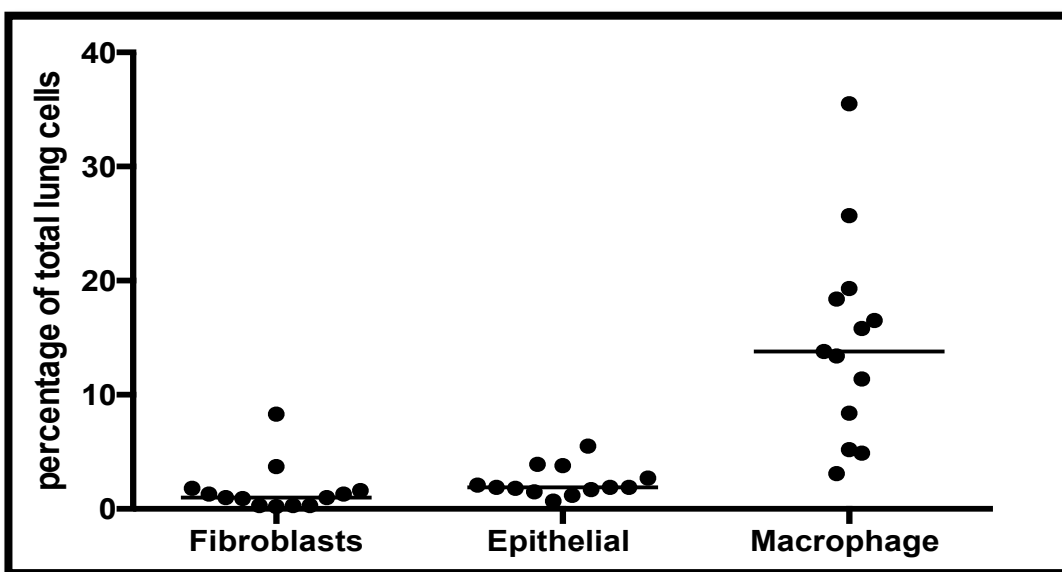
Figure 4-1- Proportions of epithelial, fibroblast and macrophage cells in human distal lung samples: Human distal lung samples were enzymatically digested to release cell populations. Cells were then stained for lineage markers to identify epithelial, macrophage and fibroblast populations. Shown above is the percentage of fibroblast and epithelial cells that make up the CD45- cell fraction. Macrophages are shown as a percentage of the total CD45+ cell fraction.

4.2.2   Human lung fibroblast, epithelial cell and macrophage expression of HLA-DR within human distal lung

MHC class II is the most critical molecule for mediating antigen-specific activation of T helper cells. It is responsible for binding and presenting peptide antigen to the corresponding T cell receptor; therefore its expression would be fundamental for fibroblast APC function. The human MHC Class II molecule HLA-DR is expressed upon macrophage and epithelial cell [169] populations, both of which have known APC function.   Therefore screening fibroblasts, macrophages and epithelial cells simultaneously for HLA-DR would allow a comparison of HLA-DR expression on fibroblasts to that of these established APCs. This was done though screening of *ex vivo* distal lung populations (full protocol in section 2.5) via flow cytometry (figure 4-2).
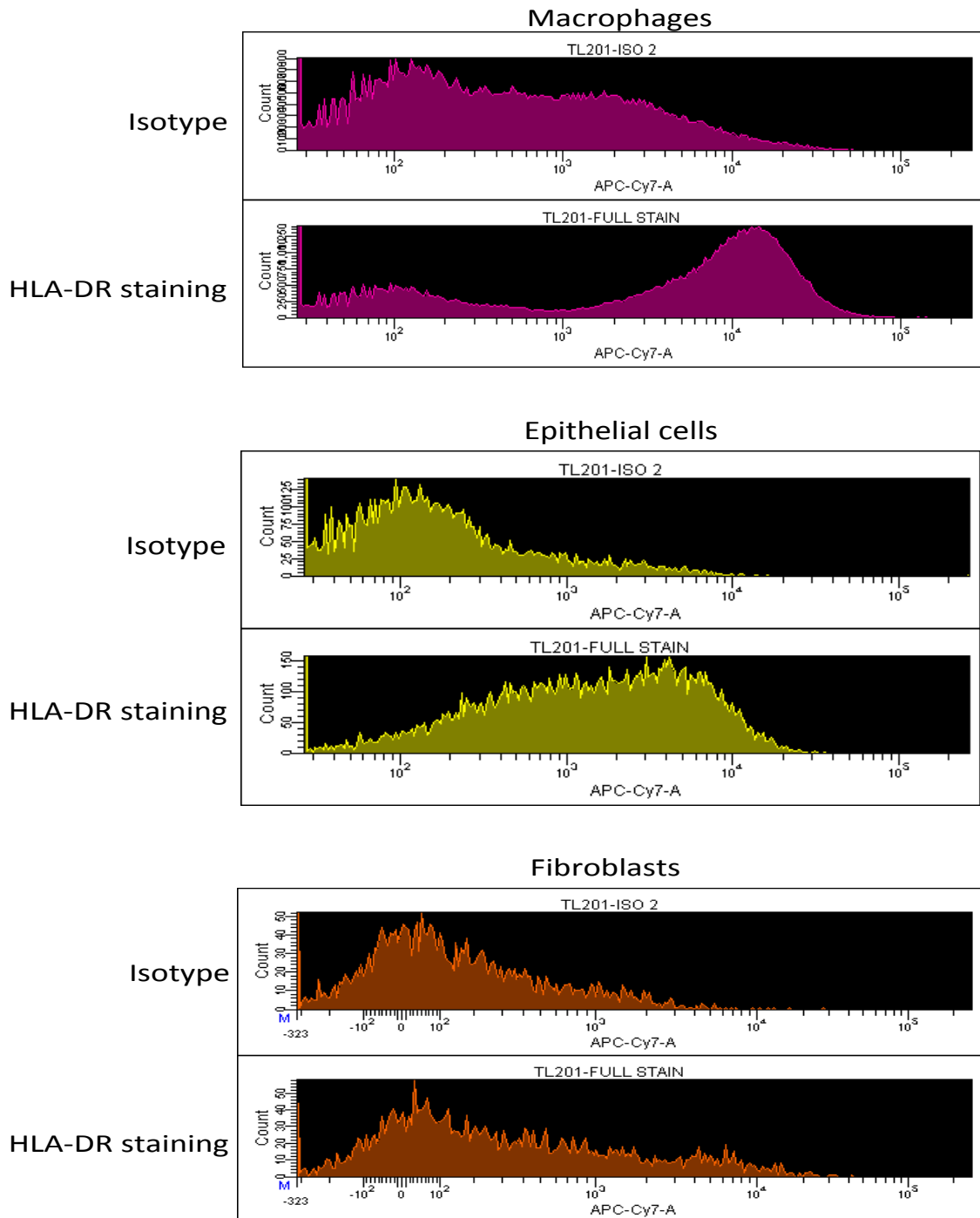
Figure 4-2- representative flow cytometry histograms of HLA-DR expression by macrophages, epithelial and fibroblast cells: Distal lung samples were collagenase digested to release cells and stained for lineage markers. Once identified as epithelial (CD45- EpCAM+ CD90-) or fibroblast (CD45- EpCAM- CD90+ αSMA lo) cells, populations were then screened for the expression of HLA-DR. Shown above are representative isotype control samples for nonspecific binding (upper panels) and the HLA-DR expression by each population (lower panels).

Examination of HLA-DR expression upon these three lung cell populations demonstrates that macrophages exhibit the highest expression, with epithelial cells also having notable levels and fibroblasts having the lowest-yet still detectable- levels (figure 4-3). This shows that human lung fibroblasts express HLA-DR within the human lung environment, and highlights its expression relative to cells with known HLA-DR dependent antigen-presentation capabilities.
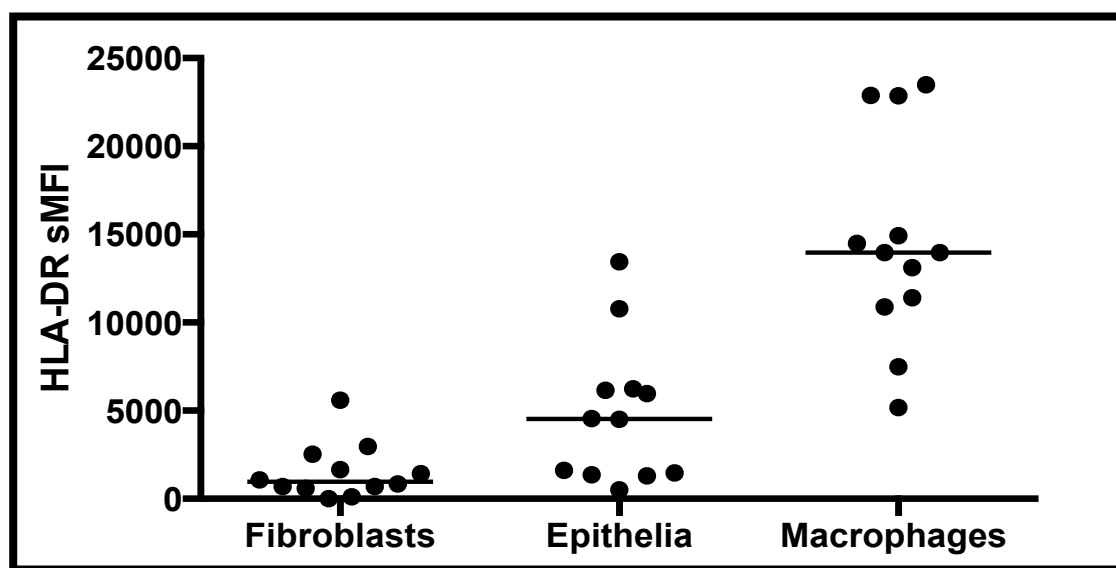
Figure 4-3- *Ex vivo* **expression of HLA-DR on human lung cell populations:** Distal lung tissue samples were collagenase digested (1mg/ml, 30 mins) to release cells, and cell populations stained for lineage markers. Cells were classified as fibroblasts (CD45- EpCAM- CD90+), epithelial cells (CD45- EpCAM+ CD90-) or macrophages (CD45+ HLA-DR-Hi). Each population was then screened for the expression of HLA-DR. Results above show the specific mean fluorescence intensity (sMFI) for the expression of HLA-DR of each lung cell population. Bars represent median values. N=12

4.2.3 Verification of fibroblast lineage from tissue growth and influence of culture conditions on *in vitro* grown fibroblast

For *in vitro* examination of fibroblast populations, cells were obtained via growth of adherent cells from human distal lung explants. While this is a recognised method for obtaining fibroblasts [170], a phenotypic analysis of cells isolated this way was carried out to ensure resultant outgrown cells exhibit a fibroblast phenotype. Of particular concern would be the presence of epithelial or leukocyte populations, both of which are known to express immune synapse molecules and affect T cell activation. Adherent cell

populations were therefore stained for lineage markers of fibroblasts, epithelial cells and leukocytes and analysed via flow cytometry (representative gating shown in figure 4-4).
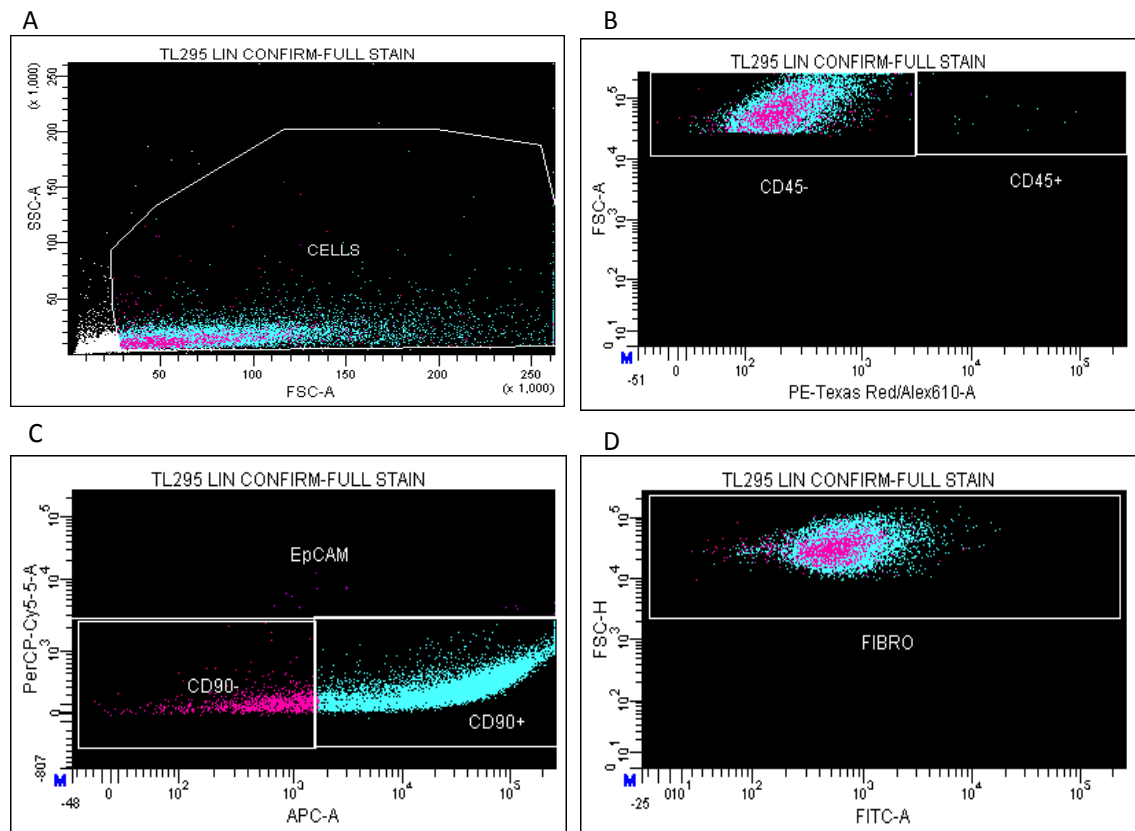
Figure 4-4- Representative flow cytometry plots of primary *in vitro* fibroblast and TGFβ stimulated myofibroblast expression of lineage markers and αSMA: Subject matched *in vitro* cultured fibroblast and myofibroblasts derived from exposure of fibroblasts to TGFβ were stained for CD45, EpCAM and CD90. The CD45-EpCAM-CD90+ fraction was then analysed for αSMA expression. Cells were first gated upon based on size and granularity (A) before being screened for CD45 expression (B). The CD45- cell fraction was then examined for EpCAM and CD90 expression. The CD90+ fraction was then further examined for αSMA expression (D).

Results demonstrate that the vast majority of adherent cells cultures obtained from tissue outgrowth contain CD45-EpCAM-CD90+ cells (figure 4-5). This verification step indicates there are no leukocyte or epithelial cell populations present, and most cells bear a CD45- EpCAM- fibroblast lineage phenotype. This analysis indicates outgrowth from tissue yields fibroblast populations.
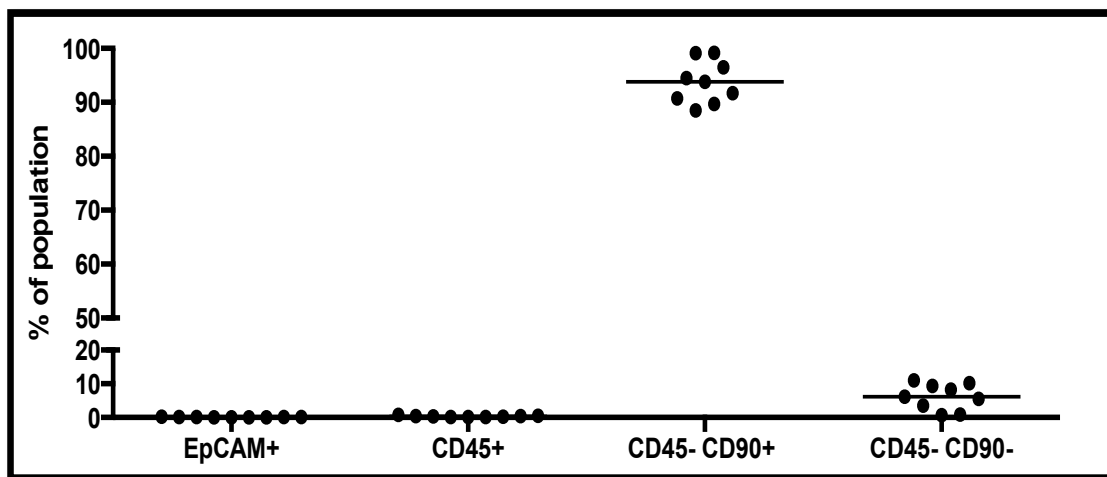
Figure 4-5- Phenotypic characterization of cultured human lung fibroblasts: Adherent cells grown from distal lung tissue fragments were analysed for expression of pan-leukocyte marker CD45, epithelial cell marker EpCAM and the fibroblast-associated marker CD90. Cells were analysed for expression of these markers via flow cytometry. Results are given as the percent of population expressing each of the various profiles: CD45 expressing cells are read from the total cell population, while EpCAM and CD90 populations are read from the CD45- fraction and represented as a percent of this population. Bars represent median values. N=9

Fibroblasts and myofibroblasts are closely related cell types that exist in the interstitium, with myofibroblasts differentiating from fibroblasts under certain circumstances. Culture conditions have been shown to drive fibroblasts into a myofibroblast phenotype from adherent outgrown cell populations. Therefore, staining for the myofibroblast associated marker alpha-smooth muscle actin (αSMA) [171] was carried out to verify the phenotype of adherent outgrown cells. This procedure involved exposing cultures of adherent cells to TGFβ in order to drive cells into a myofibroblast phenotype, serving as a positive control for the expression of αSMA [39]. Figure 4-6 shows representative flow cytometry plots of patient-matched fibroblast and myofibroblast αSMA expression.
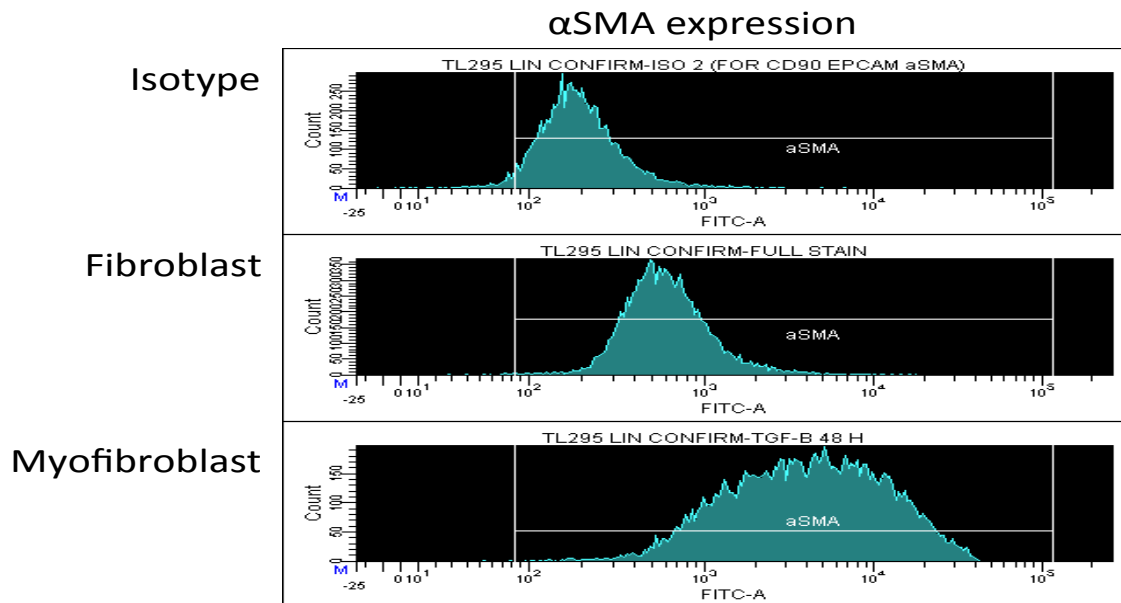
Figure 4-6- Representative flow cytometry histogram plots of primary fibroblast and TGFβ stimulated myofibroblast αSMA expression: Cultured fibroblasts and TGFβ conditioned myofibroblasts were identified as being CD45- EpCAM- CD90+ (see figure X) This population was then examined for the expression of αSMA. Shown above are the representative histograms of the isotype controls (for nonspecific antibody binding),,fibroblast and myofibroblast expression of αSMA.

Figure 4-7 shows the whole sample cohort expression of αSMA between both the EpCAM-CD45-CD90+ populations (with the TGFβ-exposed populations being classed as myofibroblasts and unexposed adherent cells being classed as fibroblasts). The CD45- EpCAM- CD90+ fibroblasts that grow from explanted tissue over the 4 week culture period appear to have lower expression of αSMA compared to TGFβ derived myofibroblasts.
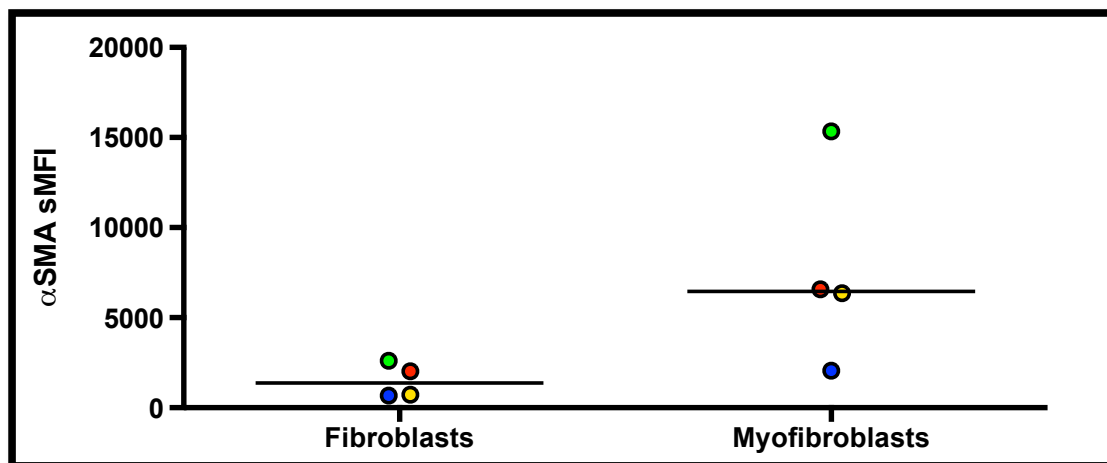
Figure 4-7- The expression of α-SMA within cultured fibroblast and TGFβ: Adherent cell populations obtained from growth from tissue fragments were classed as a fibroblast-like population based up a CD45- EpCAM- CD90+ cell surface profile. This cell fraction was then examined for the expression of α-SMA. Sample populations of outgrown cells were exposed to TGFβ for 48 hours to drive myofibroblasts development and provide a reference population for known α-SMA expression. Bars represent median values. N= 4

Inverted fluorescent microscopy was also used to examine αSMA expression between cultured fibroblasts and TGFβ driven myofibroblasts. TGFβ exposed myofibroblasts develop distinct αSMA fibres (figure 4-8- bottom), while fibroblasts failed to do so (figure 4-8- top). Taken together, these results shows that culture techniques used in this study result in fibroblast populations and do not promote differentiation into myofibroblasts.
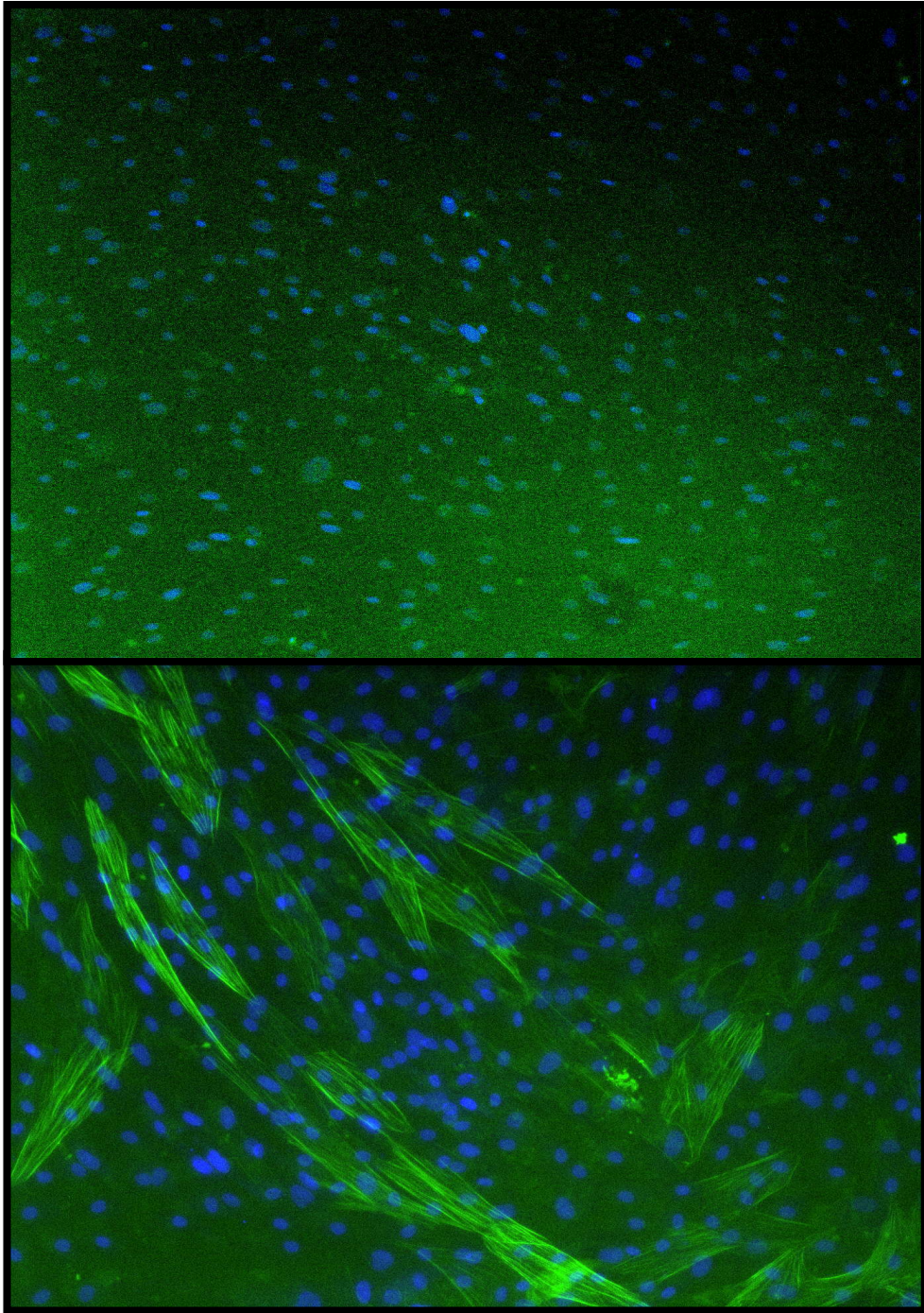
Figure 4-8- Comparison of adherent cell cultures to TGF-β - driven myofibroblast cultures: Adherent cells grown from human lung explants were either maintained in culture under normal conditions (upper panel) or treated with 9ng/ml TGFβ for 48 hours. Cells were stained with anti α-SMA antibody (green) and counterstained with Hoechst nuclear stain (blue). The upper panel shows no visible α-SMA fiber formation, while distinct fiber expression by myofibroblasts is common (lower panel). Patient (study number "HL310") fibroblasts (upper) and myofibroblasts (lower) are shown. Hoerchst nuclear dye = blue, anti α-SMA= green

4.2.4 The expression of immune synapse molecules by cultured fibroblasts *in vitro* in response to common lung inflammatory factors

Fibroblasts are able to respond to a number of inflammatory stimuli by production of soluble factors and expression of surface molecules. Of particular interest for this study is the fibroblasts upregulation of immune synapse molecules in response to common lung cytokines IFNγ and IL-1α. IL-1α is produced by stressed and dying lung epithelium and has been shown to act as a potent alarmin signal for fibroblasts. Activation of fibroblasts via IL-1α provokes release of cytokine and chemokine factors [86]. IFNγ is produced by a number of lung resident cells, particularly T cells and NK cells, and is considered the canonical cytokine for driving MHC class II expression on both immune and non-immune cells [36]. In addition to examining the response to cytokines, the response to a heat-killed form of the common lung microbe nontypeable *haemophilus influenzae* (NTHi) is examined. This bacteria is also used as a source of antigen in later experiments, therefore it is important to establish the effect it has on fibroblast activation state. We examined the effect these factors have on lung fibroblasts expression of the MHC class II molecule HLA-DR (and in some cases, -DP and DQ), which are critical in binding and presenting antigen to CD4 T helper cells [25]. Expression of the co-stimulatory molecules CD80 and CD86 is examined, as these are known to be expressed by professional APCs and provide further activation signals to T cells {Bromley et al., 2001}. The expression of ICAM-1 is also investigated. This molecule facilitates T cell activation though stabilizing cell-to-cell interaction between the APC and T cell, and thus is considered an important in effective activation of T cells [172]. The expression of these molecules was measured at baseline levels and after 48 hours of stimulation. All analysis was done via flow cytometry and results are expressed as either the raw mean fluorescent intensity (MFI) or the specific MFI (sMFI).

4.2.4.1   Upregulation of MHC Class II molecules upon human lung fibroblasts by interferon gamma, interleukin 1 alpha and heat killed *Haemophilus Influenzae*

Fibroblasts do not express notable levels of MHC class II molecule HLA-DR, -DP, -DQ at baseline after 3-4 weeks of culture outside of the human body (figure 4-9). However expression can be driven by exposure to increasing levels of IFNγ (ranging from 125 to 1000 units/ml)

Figure 4-9- Dose-response increase in expression of HLA-DR,-DP, -DQ in response to IFN-γ exposure: Fibroblasts obtained through growth from tissue explants were analysed for the expression of the human MHC class II molecules HLA-DR, -DP, -DQ in the resting state (baseline expression) or in response to increasing levels of the proinflammatory mediator IFN-γ. Cells were treated with increasing levels of IFN-γ from 125U/ml up to a maximum of 1000U/ml. Fibroblast cultures were incubated for 48 hours to allow upregulation and expression measured via flow cytometry. The specific mean fluorescence intensity (sMFI) of HLA-DR,DP,DQ expression for each patient is shown. Bars represent median values. Statistical analysis was done through a Wilcoxon paired test **** $P<0.0001$. N=18.

In contrast to IFNγ, exposure of fibroblasts to IL-1α does not cause any upregulation of MHC Class II (figure 4-10), even at the highest dose of stimulation used in these experiments (500u/ml).
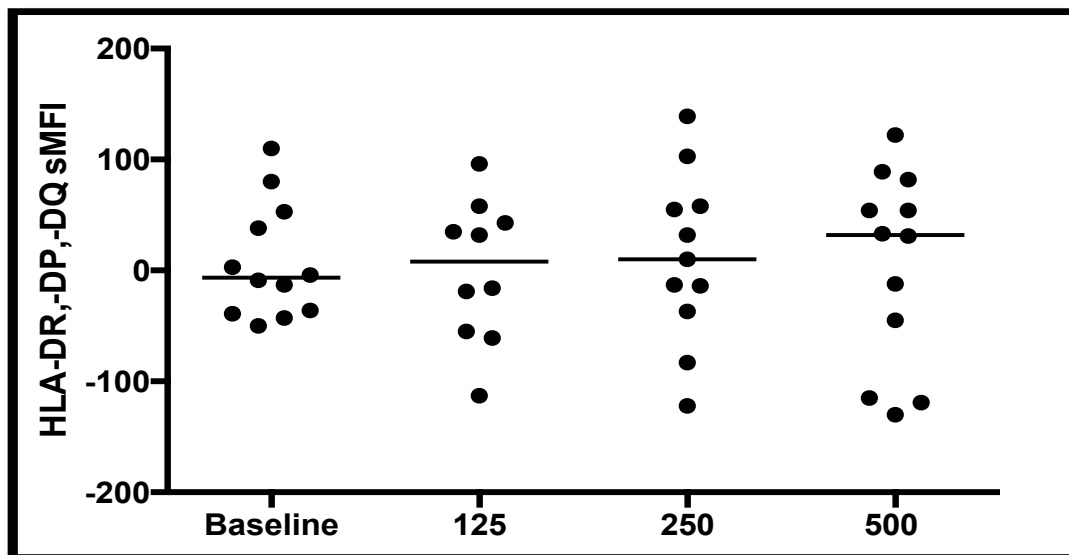
Figure 4-10- Dose-response increase in expression of HLA-DR, -DP, -DQ in response to IL-1α exposure: Fibroblasts obtained through growth from tissue explants were analysed for the expression of the human MHC class II molecules HLA-DR, -DP, -DQ in the resting state (baseline expression) or in response to increasing levels of the inflammatory cytokine IL-1 α. Cells were treated with increasing levels of IL-1α from 125U/ml to a maximum of 500U/ml. Fibroblasts were incubated in the presence of cytokine for 48 hours to allow upregulation and expression before analysis via flow cytometry. The specific mean fluorescence intensity (sMFI) value for the expression of HLA-DR, DP, DQ is shown. Bars represent median values. N=12.

As both IL-1α and IFNγ were administered in equal strengths (i.e.- both were measured in units of activity and administered at 125, 250 and 500U/ml), a unit-for-unit comparison of their ability to drive MHC class II upregulation could be carried out. This was performed on a smaller cohort of subject-matched fibroblasts that were concurrently exposed to either cytokine and analysed together. Figure 4-11 highlights the contrasting ability of these cytokines ability to drive MHC class II expression, with IFNγ driving greater upregulation than IL-1α.
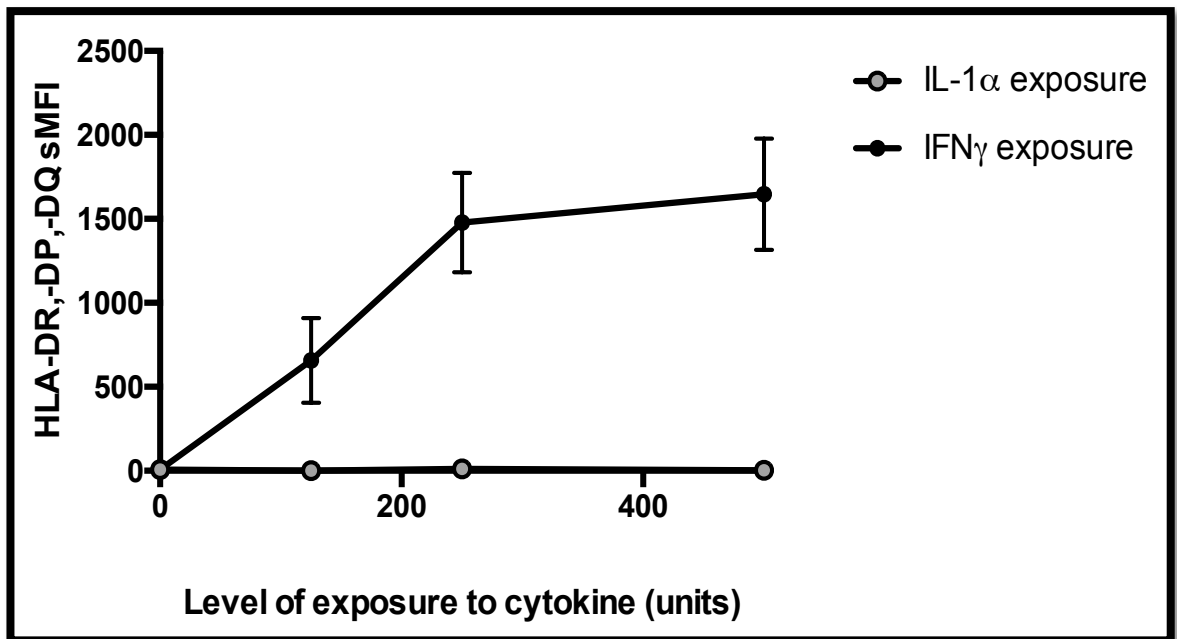
Figure 4-11: Comparison of the ability of IFN-γ and IL-1α to drive upregulation of HLA-DR, -DP, -DQ: The comparative ability of IFN-γ (closed circles) and IL-1α (open circles) on the upregulation of MHC class II molecules was examined. Fibroblasts were cultured with equivalent levels of either cytokine for 48 hours before molecule expression was measured via flow cytometry. The ability of both cytokines to increase expression is shown as the specific mean fluorescent intensity (sMFI) value. Bars represent the SEM value. N=12

The effect of heat-killed NTHi exposure upon fibroblast expression of HLA-DR was also carried out. Exposure to 1500 or 15,000 heat killed bacteria failed to drive any upregulation above baseline levels (Figure 4-12). Patient matched fibroblasts were concurrently stimulated with IFNγ (500U/ml) to serve as a positive control. Taken together, these results indicate that IFNγ is the only tested inflammatory stimulus capable of driving the upregulation of MHC class II on the surface of human lung fibroblasts.
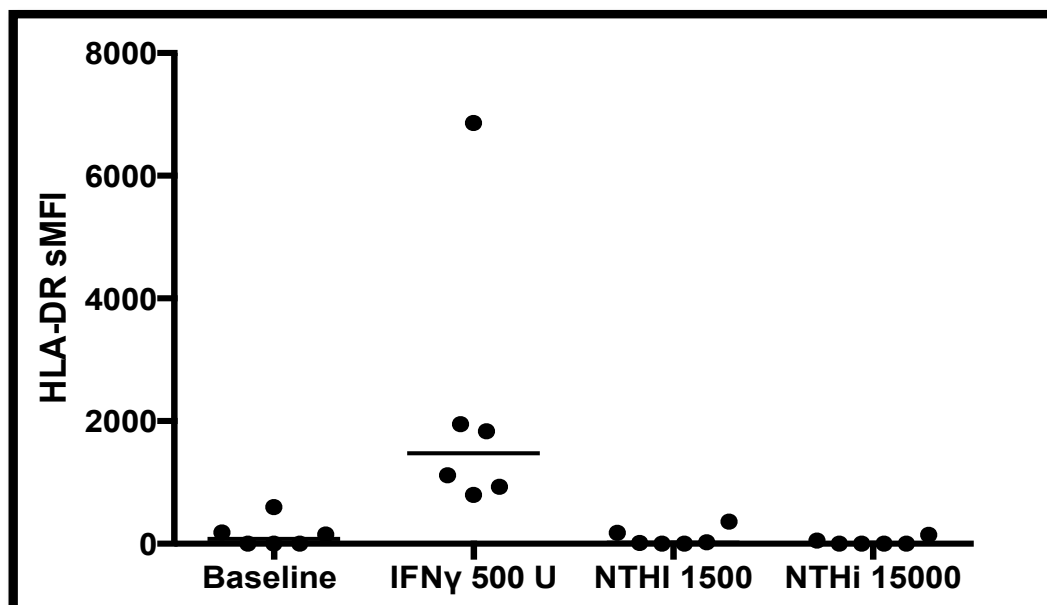
Figure 4-12- Expression of HLA-DR by human lung fibroblasts after exposure to heat killed NTHi: Fibroblasts obtained through growth from tissue explants were exposed to a heat-killed form of the common lung bacteria NTHi at either 1500 or 15000 heat killed bacteria per ml. Patient-matched fibroblast populations were concurrently exposed to IFNγ (500U/ml) to provide a reference as this has been shown to drive upregulation in this system (see previous figures). Shown above are the specific mean fluorescence intensity (sMFI) values for the expression of HLA-DR at baseline, after 48h stimulation with IFNγ or with increasing amounts of heat-killed NTHi. Bars represent median values. N=6

4.2.4.2 Expression of immune synapse stimulatory molecules CD80 and CD86 in response to interferon gamma

Results indicate fibroblasts possess the ability to express MHC class II. While this is critical for antigen-presentation to T helper cells, other stimulatory immune synapse molecules, such as CD80 and CD86, can also deliver activation signals. Therefore, lung fibroblasts CD80 and CD86 expression at baseline and after 48 hours exposure to IFNγ (500U/ml) was measured by flow cytometry. Results clearly show that both CD80 (figure 4-13A) and CD86 (Figure 4-13B) are not expressed at baseline or after IFNγ treatment
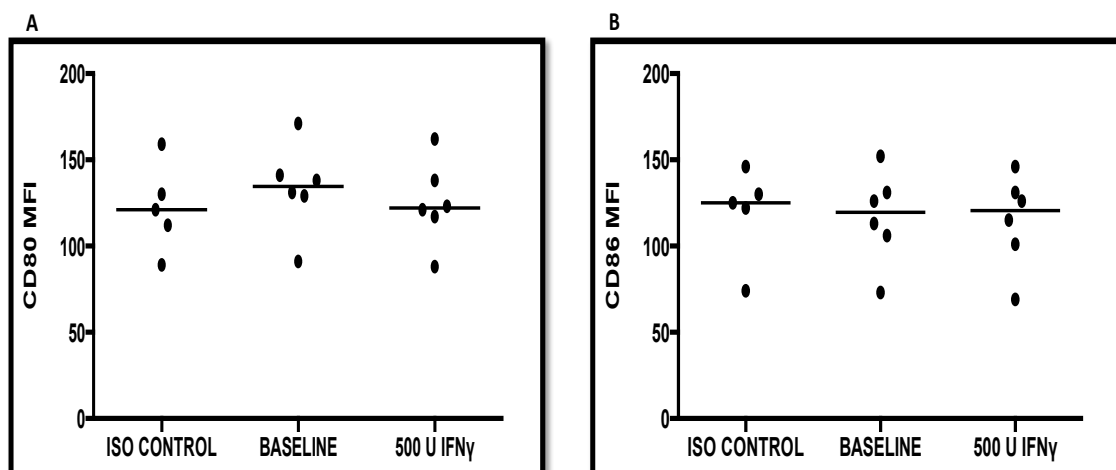
Figure 4-13- Expression of CD80 and CD86 on human lung fibroblasts: Fibroblasts obtained from growth from tissue explants were examined for the expression of costimulatory molecules CD80 and CD86 at baseline and after 48h stimulation with IFNγ (500U/ml). Shown above are the raw mean fluorescence intensity values (MFI) for the isotype control, the baseline level of expression and expression of CD80 (A) and CD86 (B) after 48h of stimulation. N=6

4.2.4.3   Upregulation of Intracellular adhesion molecule 1 by interferon gamma, interleukin 1 alpha and heat killed *Haemophilus Influenzae*

As well as examining MHC class II, CD80 and CD86 expression; ICAM-1 upregulation in response to proinflammatory factors was investigated. ICAM-1 provides cell-to-cell stability between the APC and interacting T cell, indirectly promoting T cell activation [172]. In contrast to MHC class II expression, ICAM-1 was detectable on cultured fibroblasts at baseline (Figure 4-14) and similar to MHC class II, there was a dose-dependent upregulation in response to increasing IFNγ exposure.
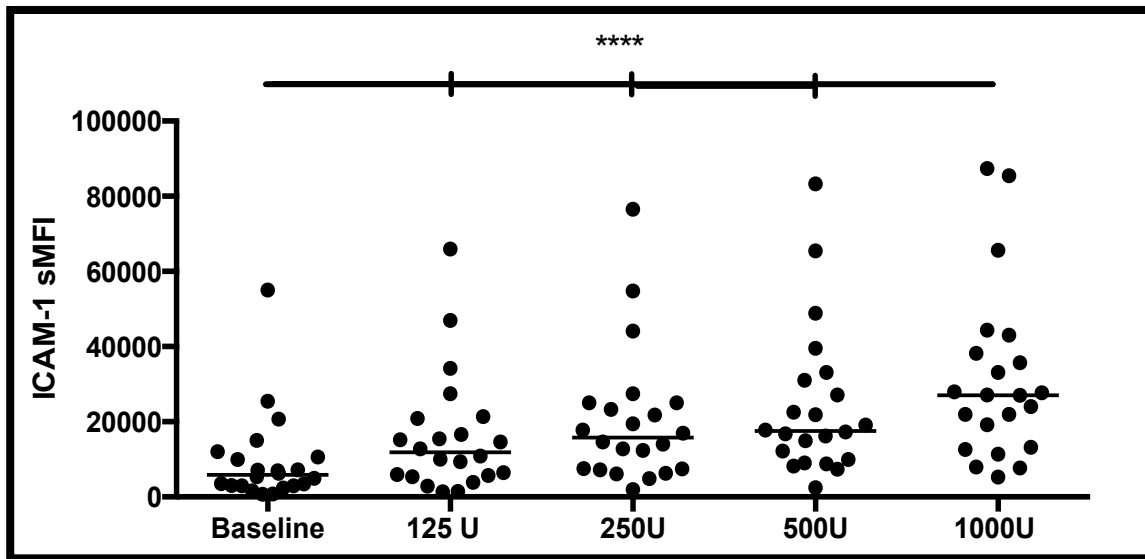
Figure 4-14- Dose-response increase in expression of ICAM-1 in response to IFNγ exposure: Fibroblasts obtained through growth from tissue explants were analysed for the expression of the adhesion molecule ICAM-1 in the resting state (baseline expression) or in response to increasing levels of the proinflammatory mediator IFNγ. Cells were treated with increasing levels of IFNγ from 125U/ml up to a maximum of 1000U/ml. Fibroblast cultures were incubated for 48 hours to allow upregulation. Expression was measured via flow cytometry. The specific mean fluorescent intensity (sMFI) of ICAM-1 expression for each patient is shown. Bars represent median values Statistical analysis was done through a Wilcoxon paired test. **** P<0.0001. N=22

Fibroblasts exposed to IL-1α were also examined for upregulation of ICAM-1. In contrast to the inability of IL-1α to drive MHC class II upregulation, IL-1α readily upregulated ICAM-1 expression (figure 4-15).
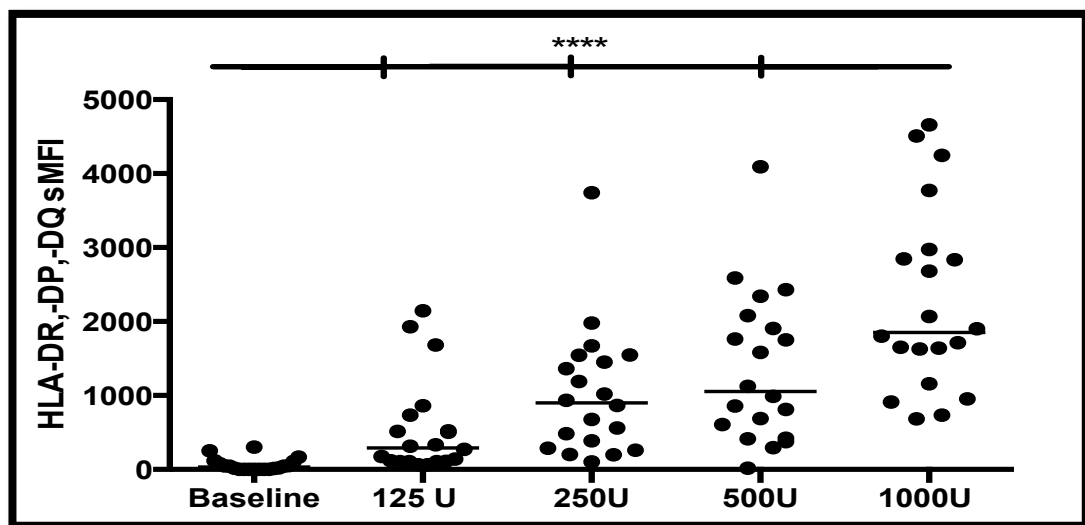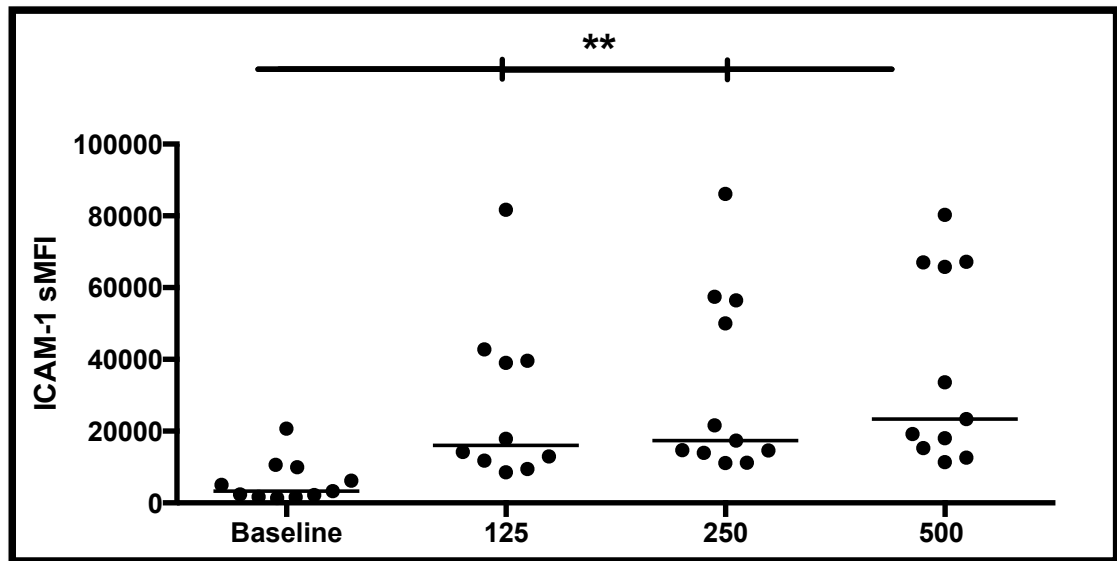
Figure 4-15- Dose-response increase in expression of ICAM-1 in response to IL-1a exposure: Fibroblasts obtained through growth from tissue explants were analysed for the expression of the adhesion molecule ICAM-1 in the resting state (baseline expression) or in response to increasing levels of the inflammatory cytokine IL-1α. Cells were treated with increasing levels of IL-1α from 125U/ml to a maximum of 500U/ml. Fibroblasts were incubated in the presence of cytokine for 48 hours to allow upregulation. Expression was measured via flow cytometry. The specific mean fluorescence intensity (sMFI) value for the expression of ICAM-1 is shown. Bars represent median values. Statistical analysis was done through a Wilcoxon paired test, ** P<0.005, N=12

Similar to the analysis carried out with MHC class II, it was possible to perform a unit-for-unit comparison of the ability of both IL-1α and IFNγ in driving ICAM-1 expression. This was performed on a cohort of matched fibroblasts that were simultaneously exposed to either cytokine and analysed together. As figure 4-16 shows, both cytokines are able to drive some degree of ICAM-1 upregulation, however IL-1α exposure provoked greater expression compared to IFNγ, based upon a unit-for-unit comparison.
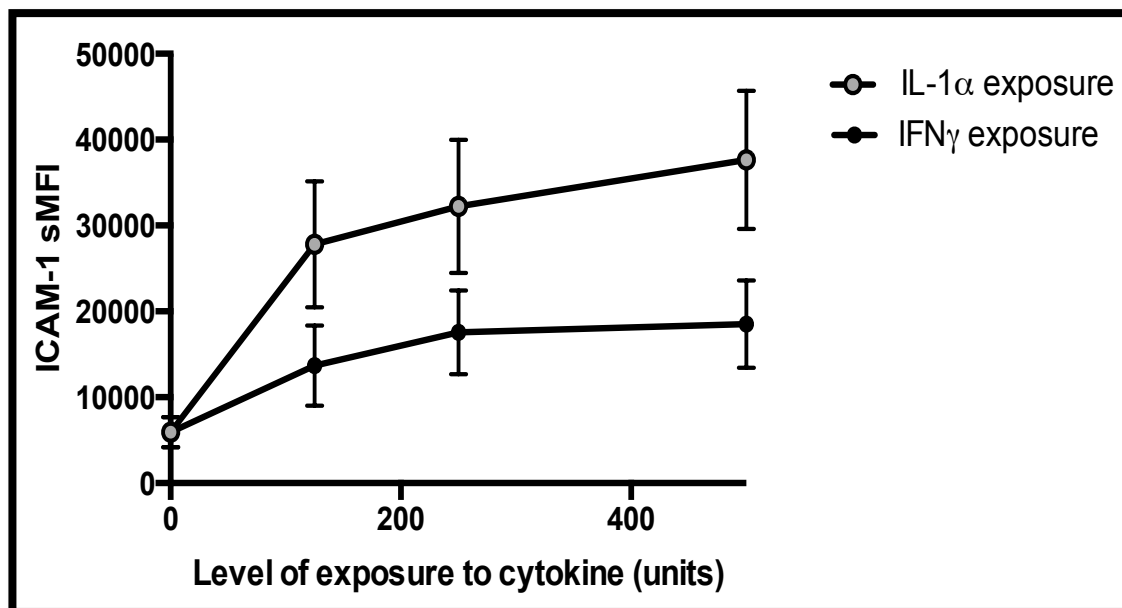
Figure 4-16- Comparison of the ability of IFNγ and IL-1α to drive upregulation of ICAM-1: The comparative ability of IFNγ (closed circles) and IL-1α (open circles) on the upregulation of ICAM-1 was examined. Fibroblasts were cultured with either cytokine for 48 hours before molecule expression was measured via flow cytometry. The ability of both cytokines to increase expression is shown as the specific mean fluorescent intensity (sMFI) value. Bars represent the SEM value. N=12

The ability of heat-killed NTHi to drive ICAM-1 upregulation was examined (figure 4-17). Exposure to either 1500 or 15000 heat-killed bacteria is sufficient to drive expression of ICAM-1, and was more potent than IFNγ (500U/ml) in driving upregulation. Overall, these results demonstrate that ICAM-1 expression can be driven by all three factors that IL-1α is more potent than IFNγ, and NTHi is able to drive greater upregulation than relatively large amounts of IFNγ.
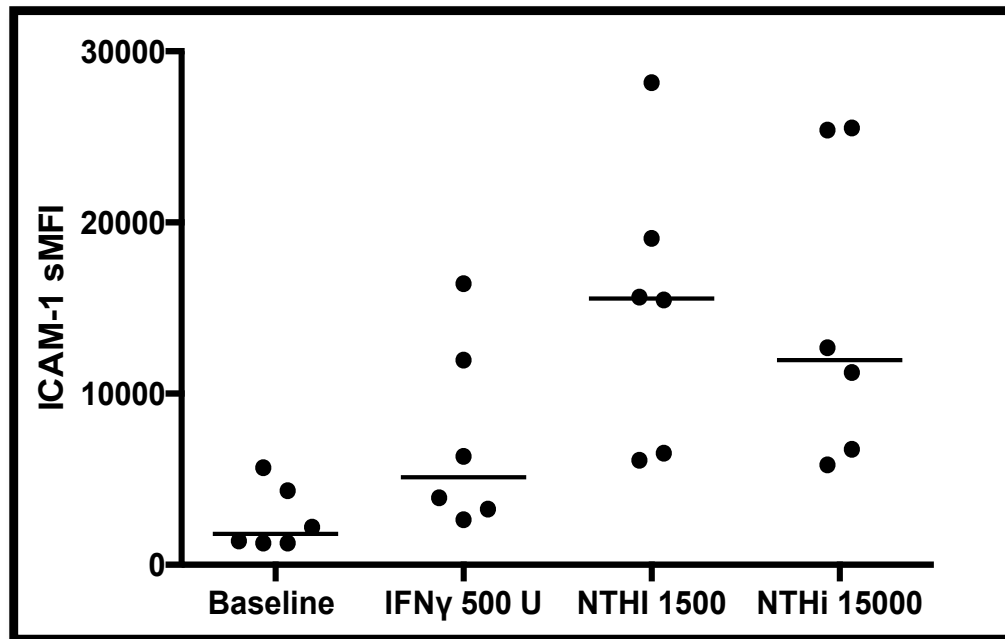
Figure 4-17- Expression of ICAM-1 by human lung fibroblasts after exposure to heat-killed NTHi: Fibroblasts obtained through growth from tissue explants were exposed to a heat-killed form of the common lung bacteria NTHi at either 1500 or 15000 heat killed bacteria per ml. Patient-matched fibroblast populations were concurrently exposed to IFNγ (500U/ml) to provide a reference as this has been shown to drive upregulation in this system (see previous figures). Shown above are the specific mean fluorescence intensity (sMFI) values for the expression of ICAM-1at baseline, after 48h stimulation with IFNγ or with increasing amounts of heat-killed NTHi. Bars represent median values. N=6

4.2.4.4 Upregulation of ICAM-1 and HLA-DR when both heat-killed nontypeable *Haemophilus Influenzae* and interferon gamma are administered together

IFNγ has been shown to upregulate both MHC class II and ICAM-1 while NTHi appears to only upregulate ICAM-1. Consequently, both factors would need to be administered together- NTHi as a source of antigen and IFNγ in order to drive HLA-DR expression and subsequent antigen presentation. Therefore it was necessary to verify exposure to both factors together did not impact expression of immune synapse molecules. Cultured fibroblasts were exposed to 500U/ml IFNγ, 1500 NTHi, or both together for 48 hours and the expression of HLA-DR and ICAM-1 measured by flow cytometry. As shown in figure 4-18, HLA-DR upregulation by IFNγ is maintained when exposed alongside NTHi. Similarly, ICAM-1 expression was upregulated in the

presence of both factors. Therefore, administering both factors together does not seem to prevent upregulation of HLA-DR or ICAM-1.
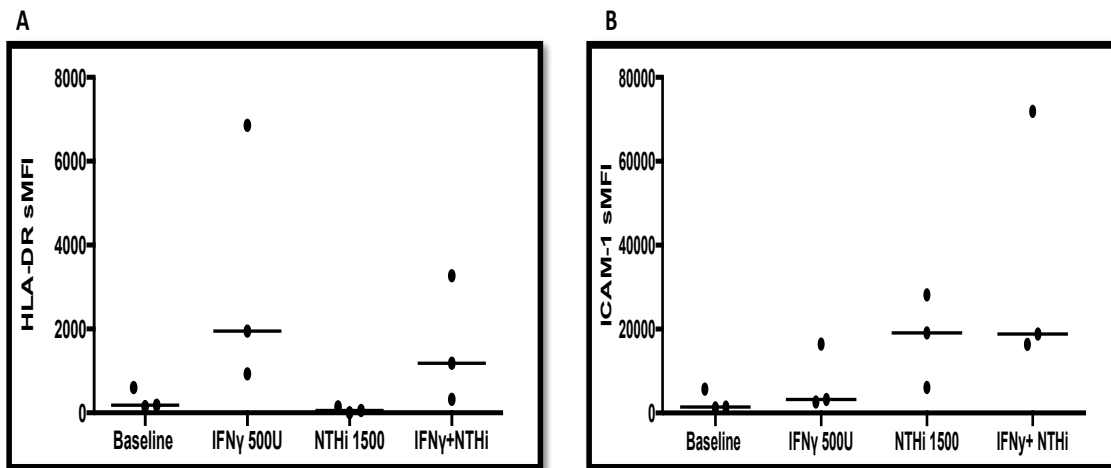
Figure 4-18- Expression of HLA-DR and ICAM-1 by human lung fibroblasts when exposed to both NTHi and IFNγ: Fibroblasts obtained via growth from tissue explants were exposed to IFNγ (500U/ML) alone, NTHi (1500 heat killed bacteria) alone, or both for 48 hours. Cells were then analysed for expression of HLA-DR and ICAM-1 via flow cytometry and results taken at baseline as well as after exposure to respective stimulatory factors. Results are displayed as specific mean fluorescent (sMFI) value. Bars represent median values. N=3

4.2.5   The ability of lung fibroblasts to internalize environmental particles

In order to process and present antigen upon MHC class II molecules, a cell must be able to internalize exogenous particles. Environmental antigen obtained in this manner is trafficked though a specific internal pathway, ultimately leading to its loading onto Class II MHC molecules. To measure the ability of lung fibroblasts to perform this function, cultured fibroblasts were incubated with FITC-albumin or FITC-dextran molecules as candidate antigen for up to 4 hours and internalization measured via flow cytometry (representative gating outline in figure 4-19). Experimental setups were run at 37°C and controls ran at 4°C, allowing a measure of the FITC-tagged molecules surface binding alone, as cells do not internalize at this temperature. Samples run at 37°C were also treated with trypan blue immediately before analysis to quench surface-bound and extracellular FITC, allowing an accurate reading of internalized signal. Fibroblasts readily internalized both FITC-dextran (figure 4-20) and FITC-albumin (figure 4-21) particles over a four-hour period. Results are expressed as percentage increase in fluorescence from control samples where cells were immediately exposed to FITC-tagged antigen and immediately washed off.
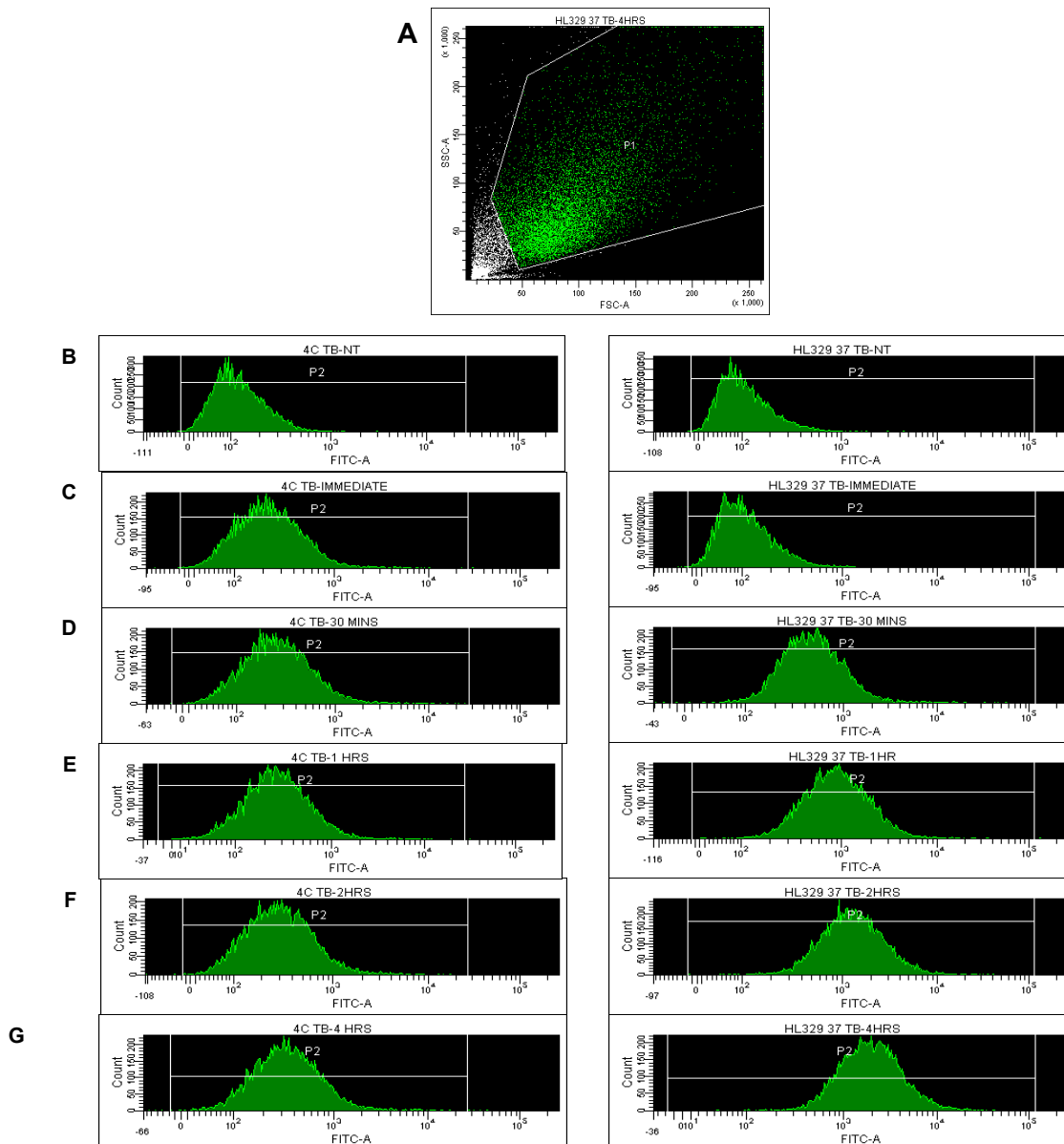
Figure 4-19- Gating strategy for the analysis of human lung fibroblasts ability to internalize exogenous fluorescently labelled particles *in vitro*: Fibroblasts obtained through *in vitro* growth from tissue fragments were tested for the ability to internalize environmental particles. Cells were exposed to 1mg/ml FITC tagged albumin or dextran for up to 4 h and subsequent internalized particles measured via flow cytometry. Samples were run at 4°C (B-G left column) or 37°C (B-G right column). Figure A shows initial gating of fibroblasts via size and granularity. Figure B-G shows the level of internalized particle as measured by fluorescent signal. Shown above are untreated cells (B) and immediately treated and washed controls (C). Measurements were taken at 30 minutes (B), 1h (C), 2h (D), and 4 h (E).
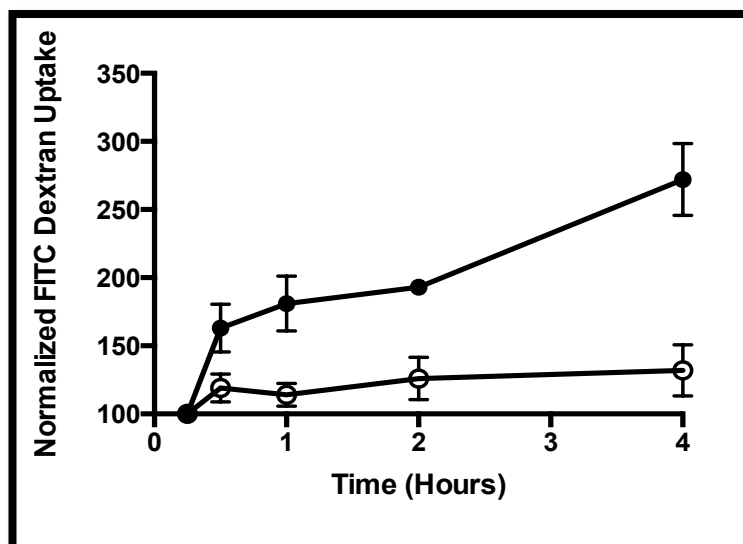
Figure 4-20- Internalization of FITC-Dextran particles by lung fibroblasts: Human lung fibroblasts were incubated with 1mg/ml FITC-dextran for up to four hours at either 37°C (filled circles) or 4°C (open circles). The resultant level of internalization over this time was measured through flow cytometry. The normalized level of internalization is shown above. Normalization is based upon deducting the level of internalization as measured against fibroblasts exposed to FITC-dextran, which is then immediately washed off. Results shown have surface-bound FITC signal deducted though treatment of cells with trypan blue prior to analysis, which quenches surface-bound FITC molecules. N=5.
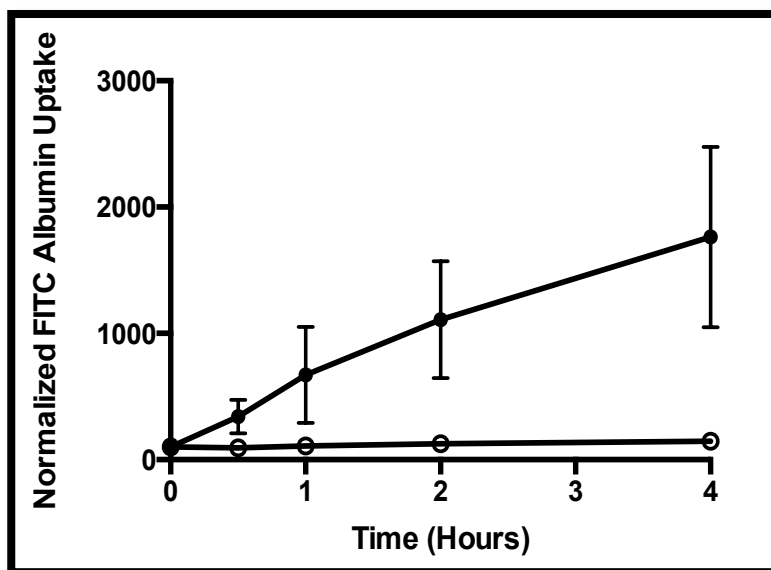
Figure 4-21- The uptake of FITC-albumin particles by lung fibroblasts: Human lung fibroblasts were incubated with 1mg/ml FITC-albumin for up to four hours at either 37°C (filled circles) or 4°C (open circles). The resultant level of internalization over this time was measured through flow cytometry. The normalized level of internalization is shown above. Normalization is based upon the level of internalization as measured against fibroblasts exposed to FITC-albumin, which is then immediately washed off. Results shown have surface-bound FITC signal deducted though treatment of cells with trypan blue prior to analysis, which quenches surface-bound FITC N=3.

4.2.6　　　Intracellular transport of internalized particles to the lysosome

In order for internalized particles to ultimately form a complex with MHC class II molecule, internalized material must be trafficked into a defined intracellular pathway, characterized by expression of specific vesicle markers. Therefore we aimed to investigate whether fibroblasts direct internalized antigen particles into vesicles expressing the lysosome-associated marker cathepsin-S via confocal microscopy. Due to the nature of this experiment it was necessary to control for the background non-specific binding of the secondary antibody to cell populations. Figure 4-22 displays the level of background staining generated by the secondary antibody alone.
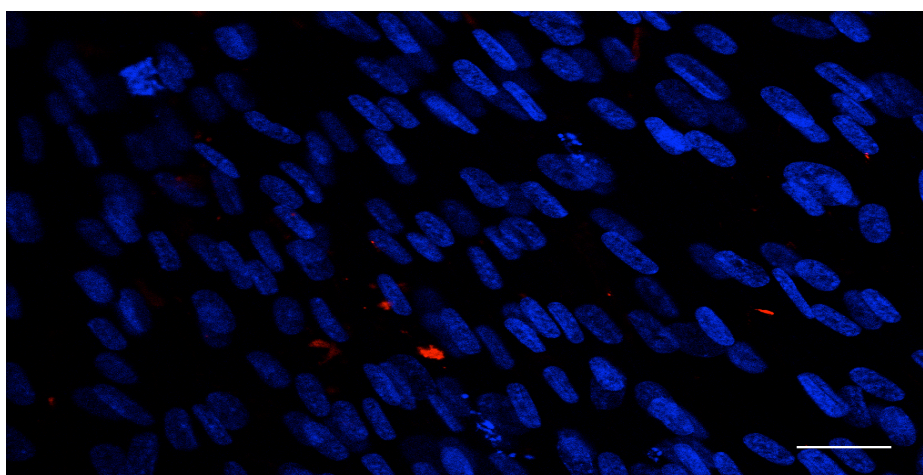
Figure 4-22- Level of non-specific staining by secondary antibody: Fibroblast populations were allowed to grow on glass cover slides before fixation and staining. Populations were stained with DAPI nuclear dye and the secondary Alexafluor 647-conjugated antibody without any prior primary antibody staining. Figure 28 shows the level of non-specific signal generated by the secondary antibody. Scale bar represents 25um

Fibroblast populations were tested for the co-localization of cathepsin-s and FITC-albumin in their resting state (no activation factors administered) or post-exposure to IFNγ. Figure 4-23 demonstrates that unstimulated fibroblasts internalize FITC albumin to within the cell. Unstimulated fibroblasts did not appear to have cathepsin-s signal co-localize with this internalized FITC-albumin.
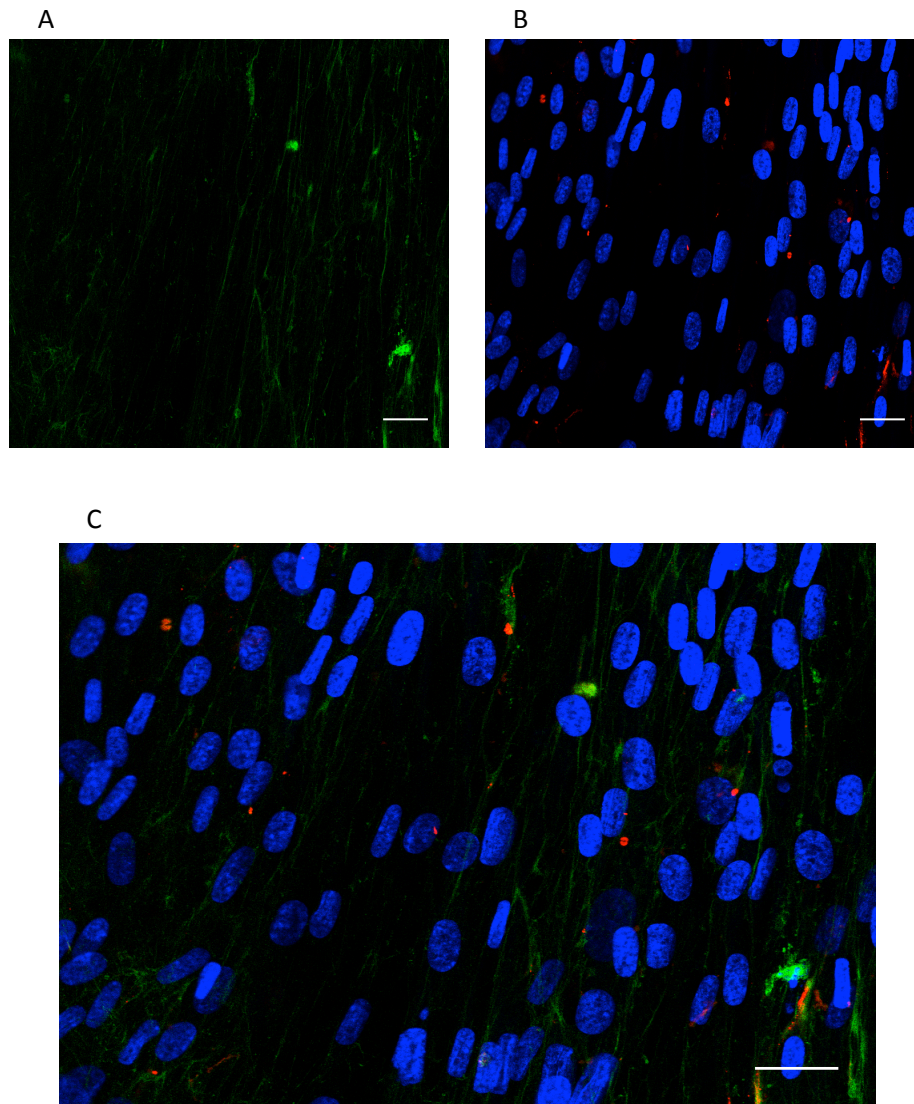
Figure 4-23- Resting state fibroblast level of internalization and cathepsin-S expression:
Fibroblast populations were allowed to adhere to glass cover slips at a density of 150,00-200,00 cells per slide. Fibroblast cultures were incubated in serum free media for 48 hours without the addition of any inflammatory cytokines (therefore these cells represent resting levels of cathepsin-s expression and FITC-albumin internalization). Cells were then cultured for 4 hours with 1mg/ml FITC-albumin. Cells were then washed and fixed with 4% PFA. Fixed cells were stained with DAPI nuclear dye (blue) and anti Cathepsin-S antibody (red) after previously being allowed to internalize FITC-albumin (green). Co-localized FITC-albumin and cathepsin-S appears yellow if present. Figure **A** shows the FITC signal alone while figure **B** shows cathepsin-s with DAPI. **C** depicts all three signals merged. Scale bar represents 25um. N=1

IFNγ conditioned fibroblasts again were able to internalize FITC albumin particles to within the cell body (figure 4-24). Furthermore, exposure to IFNγ appears to cause an increase in internal cathepsin-s expression, and a proportion of the internalized FITC-albumin particles co-localize with this cathepsin-s signal within vesicle-like formations

(figure 4-24C and figure 4-26). This suggests IFNγ conditioned fibroblasts upregulate internal cathepsin-s. Furthermore, the co-localization of internalized FITC-albumin with these cathepsin-s vesicles may indicate fibroblasts can traffic internalized particles into a MHC class II loading internal pathway.
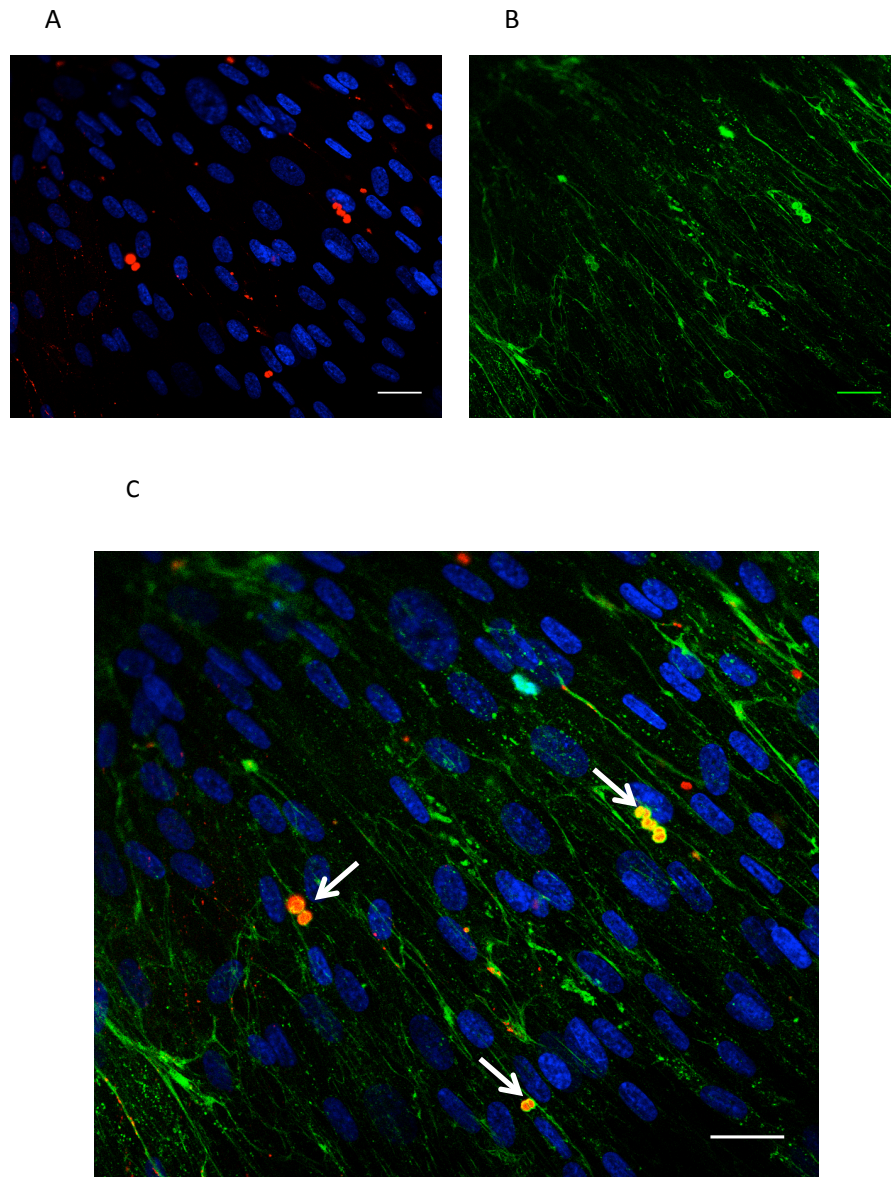
Figure 4-24- Activated fibroblast level of internalization and cathepsin-s expression: Fibroblast populations were allowed to grow on glass cover slips. Populations of 150,000-200,000 cells were cultured in serum free media for 48 hours in the presence of 500U/ml IFN- γ (therefore these cells represent activated fibroblast levels of cathepsin-s expression and FITC-albumin internalization). Cells were then cultured for 4 hours with 1mg/ml FITC-albumin before washing and fixation. Cells were then stained with DAPI nuclear dye (blue) and anti Cathepsin-S antibody (red). Co-localized FITC-albumin and cathepsin-S appears yellow if present. Figure A shows the FITC signal alone while B shows cathepsin-s with DAPI. Figure **C** depicts all three signals merged. Scale bar represents 25um. N=1
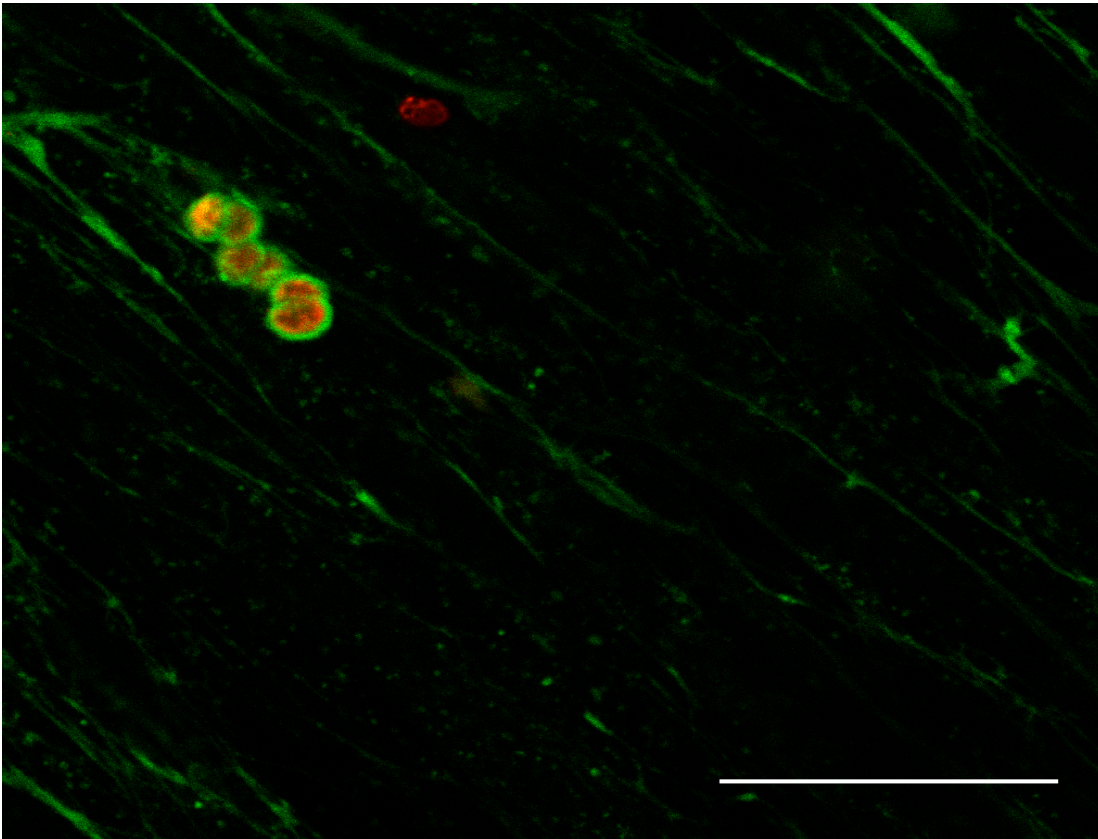
Figure 4-25-Fibroblast populations preactivated with IFNγ exhibit areas of FITC-albumin and cathepsin-S co-localized signal: Fibroblast populations were allowed to grow on glass cover slips. Populations of 150,000-200,000 cells were cultured in serum free media for 48 hours in the presence of 500U/ml IFNγ (therefore these cells represent activated fibroblast levels of cathepsin-s expression and FITC-albumin internalization). Cells were then cultured for 4 hours with 1mg/ml FITC-albumin. Cells were then washed and fixed in 4% PFA. Fixed cells were stained with DAPI nuclear dye (blue) and anti Cathepsin-S antibody (red) after being allowed to internalize FITC-albumin (green). Vesicle formations containing both FITC-albumin (green) and 647-tagged cathepsin-s (red) were visible in fibroblasts activated through IFNγ exposure for 48 hours. Scale bar represents 25um. N=1

4.3   DISCUSSION

The results of this chapter focus on the potential for fibroblasts to act as an APC for T helper cells. This involved examining the cell-surface expression of critical immune synapse molecules using both *in vitro* and *ex vivo* techniques. Initial *ex vivo* screening demonstrated HLA-DR expression upon both macrophages and epithelial cells (both confirmed APCs) as well as lower expression upon lung fibroblasts, strongly suggesting fibroblasts are engaged in antigen presentation to T helper cells in the lung environment. Similar works using animal models have demonstrated that many CD45-nonhaematopoietic cells are MHC class II+ cells via microscopy techniques [173], but did not examine the relative contribution of fibroblasts. While flow cytometry analysis provides evidence of HLA-DR expression by human lung fibroblasts, alternate techniques such as microscopy would also be beneficial. Investigating HLA-DR upon human lung sections via microscopy would allow examination of fibroblast HLA-DR expression in the context of the lung environment. This would allow visualization of HLA-DR expressing fibroblasts interacting with tissue resident T cell populations within the mesenchymal zones of the tissue. This could again be further extended to examining the nature of the T cell interaction with fibroblasts (e.g The cytokine production profile of T cells interacting with fibroblast). A drawback of this approach would be that fibroblast antigen-presentation may only occur in specific disease scenarios, primarily infiltration of the mesenchyme by infectious agents. Both COPD and bacterial pneumonia are examples of conditions where this may occur [117, 118]. Human distal lung samples are typically obtained from patients in a steady state of disease (as a prerequisite to undergoing surgery). Therefore the likelihood of identifying these interactions using such tissue samples may be unlikely. Mouse models of pneumonia, COPD and related conditions would allow a more detailed examination of fibroblast-T cell dynamics during these specific bacterial invasion events.

In order to further analyse lung fibroblast expression of immune synapse molecules and functionally assess fibroblast APC capabilities, an *in vitro* model was required. For this, adherent cells outgrown from tissue explants were used. These cells were characterised via flow cytometry to confirm fibroblast lineage, with all cells bearing a classic fibroblast-like phenotype with virtually no contamination from other APC populations, which would compromise the suitability of this isolation method.

Furthermore, this culture process did not promote the differentiation of myofibroblasts, which can occur due to culturing fibroblasts on different mediums [174]. These results indicate the outgrowth method is a reliable technique for isolating lung fibroblast populations. Cultured fibroblast expression of HLA-DR was completely lost (most likely due to loss of local conditioning factors). As a result, fibroblasts were exposed to known immunogenic mediators to examine what upregulates this critical molecule. Exposure to IFNγ was the only tested factor capable of driving HLA-DR upregulation, with IL-1α and NTHi having no effect on expression levels. Importantly, this ability to drive HLA-DR exposure is retained even when NTHi is co-administered. This is critical as we hypothesised both factors will be needed for fibroblast antigen presentation; NTHi as the antigen source and IFNγ to drive HLA-DR expression.

In contrast to HLA-DR upregulation by IFNγ alone, all three factors tested were able to promote ICAM-1 expression, with IL-1α and NTHi having particularly strong effects. ICAM-1 expression was examined only on *in vitro* cultured fibroblasts. While useful for investigating upregulation of ICAM-1 to specific inflammatory mediators, an *ex vivo* examination of expression (similar to the *ex vivo* detection of HLA-DR) would be useful. This would demonstrate the level of ICAM-1 expression by fibroblasts in the human lung environment. In addition to examining HLA-DR and ICAM-1 expression *in vitro*, expression of the co-stimulatory molecules CD80 and CD86 was investigated. Results indicate fibroblasts do not express either CD80 or CD86 at baseline or after IFNγ exposure. As well as examining cell surface immune synapse molecules upon lung fibroblasts, the ability to internalize environmental particles and their subsequent internal trafficking was investigated.

Flow cytometry analysis demonstrates time-dependent internalization of fluorescently tagged particles by *in vitro* fibroblasts, This analysis included experiments conducted at 4°C to arrest cellular processes involved in internalization (therefore giving a "baseline" measure of surface binding) as well as treating 37°C samples with trypan blue in order to quench surface-bound and extracellular fluorescent signal [175, 176]. These results demonstrate lung fibroblasts are able to internalize candidate antigen into discreet vesicle-like structures. This trait is essential for the uptake and presentation of environmental factors such as bacterial antigens. Fibroblasts were also investigated for the ability to selectively traffic antigen into late endosome or lysosome vesicles, in which antigen processing and loading onto MHC class II occurs. The

results show an apparent increase in cathepsin-S expression in IFNγ activated fibroblasts, which co-localizes with exogenous antigen containing vesicles.

IFNγ is considered the canonical APC activating cytokine, driving antimicrobial effector functions in macrophages and is associated with protection against intracellular infections [177]. Our results indicate that in the presence of this potent activating factor, fibroblasts adopt an APC phenotype that may result in the ability to presentation antigen to T helper cells. Considering the role of IFNγ in antimicrobial responses, it is a prime candidate for activating fibroblasts during local bacterial events in the lung. While IFNγ is produced by specialized immune cells, IL-1 family cytokines can be produced by a wider range of cells [178, 179]. Studies have shown that lung epithelial cells are a potent source of this cytokine, where it is stored as a preformed, biologically active molecule that is released upon cell stress and death. IL-1α is therefore a rapidly acting danger associated molecular pattern (or alarmin) for local fibroblast populations. Previous work has demonstrated that IL-1α elicits the release of IL-6 and IL-8 from fibroblasts, allowing fibroblasts to contribute to responses in cases where epithelial cell damage occurs (E.g cigarette smoke exposure and bacterial pneumonia). We show in the current study that lung fibroblasts also respond to IL-1α exposure through upregulation of ICAM-1.

Expression of ICAM-1 is associated with the retention of T cell populations in both the endothelia and parenchymal regions. Blockade of the ICAM-1/LFA-1 binding partnership has been shown to reduce inflammatory cell recruitment significantly and ameliorates inflammation in mouse models of disease [180]. This suggests a role whereby lung fibroblasts, responding to local epithelial trauma, promote the recruitment and retention of lymphocyte populations to the area. There is evidence suggesting ICAM-1 may also have a signalling role, as impairment of ICAM-1 LFA-1 signalling impairs the ability of naïve cells to form central memory cells during activation [181]. Whether this is due to direct signalling mechanism through ICAM-1-LFA-1, or an indirect effect due to unstable antigen-TcR interaction as a result of impaired immune synapse stability is still unclear. Finding such as this highlight the importance of ICAM-1 in APC- T cell interactions. Furthermore, we demonstrate fibroblasts respond directly to bacteria through strong upregulation of ICAM-1. Microbes such as NTHi contain a number of innate-receptor ligands, and the response generated by fibroblasts strongly suggests they express receptors for one, if not more, of these bacterial factors

[182]. Overall these results indicate fibroblasts have a general ability to upregulate ICAM-1 to a number of factors, but require IFNγ in order to express the critical MHC class II molecules.

While fibroblasts were capable of upregulating both ICAM-1 and HLA class II molecules, they were unable to upregulate co-stimulatory molecules CD80 or CD86 in response to IFNγ. Even at the highest levels of exposure there was negligible increase in expression from background levels. It is well documented that naïve T cells require both a TcR signal (obtained through interaction with pMHC) as well as costimulation signals in order to correctly activate and go on to mediate proinflammatory immunity. This costimulation signal is transmitted primarily though T cell CD28 interaction with CD80/86, both of which are expressed highly upon activated professional APCs [183]. Failure to provide this costimulation signal results in clonal anergy, deletion or the imprinting of an induced regulatory phenotype on the naïve T cell (reviewed in [184]. However, the requirement of co-stimulation for T memory and effector cells is less clear. Evidence suggests that CD80 and CD86 stimulation is not be required to activate memory populations [185], with the protein:MHC signal and other co-stimulatory signals providing adequate stimulation [46, 66].

A number of other costimulatory factors may act alongside HLA-DR antigen presentation to mediate T cell activation. Surface based molecules such as Inducible T-cell stimulator ligand (ICOSL) are known to be upregulated upon fibroblasts after TNFα treatment, and LPS exposed lungs upregulate ICOSL expression in experimental models [186]. ICOSL can deliver a costimulatory signal to previously activated T cells, which express ICOS after initial activation. ICOS is not expressed on naïve cells; therefore this mechanism represents a memory-cell specific form of stimulation [187]. It is possible that after activation with an inflammatory factor (eg IFNγ or NTHi bacterial lysate), lung fibroblasts upregulate this stimulatory molecule and this provides a costimulatory signal along with the primary p:MHC signals to cause memory T cell activation

CD86 expression by fibroblasts should not result in an inability to activate lung T helper cells- particularly as the vast majority of T cells in the lung appear to have a memory cell phenotype (see chapter 3). Functional assays involving fibroblast-T memory cell co-culture, as performed in chapter 5, address the necessity or redundancy

136

of CD80 and CD86 in this situation. In addition to CD28-based ligands, other surface-based molecules may affect T cell activation, including CD40. Fibroblasts have already shown to express CD40 both in the resting state and upregulated upon exposure to inflammatory mediators [92, 188]. Therefore expression of other co-stimulatory molecules may be expressed upon fibroblasts and allow activation of memory-type T cells.

The current study has clearly highlighted the ability of lung fibroblasts to express human class II MHC molecules and ICAM-1, two of the fundamental molecules of the immune synapse. This suggests the possibility that under inflammatory conditions (i.e exposure to IFNγ) fibroblasts potentially act as APCs for memory T helper cells. The examination of immune synapse molecules using *in vitro* cultured fibroblasts does have a number of limitations. Using monocultured fibroblasts and administering singular cytokines is a greatly simplified model of what is a complex environment *in vivo*. Fibroblasts are likely to be subject to conditioning from multiple cytokines produced by the diverse range of cells present within the lung environment. Studies have shown that co-exposure to multiple cytokines can have different effects from adding single cytokines alone [189]. Of particular relevance is the observation that IFNγ and tumour necrosis factor-alpha (TNFα) together can drive greater MHC class II expression upon structural cells than either factor administered alone [189]. Furthermore, we examine a small number of potential cytokines that may influence fibroblasts expression of immune synapse molecules. While the ability of IFNγ is well established in driving MHC class II [36], we cannot rule out that other factors may also drive upregulation on fibroblasts. A more comprehensive examination of fibroblast responses to inflammatory mediators and lung-based PAMPs would be required to determine if IFNγ is the only factor able to drive MHC class II on fibroblasts.

In order for internalized particles to be presented upon MHC class II they need to be trafficked towards the lysosome. Particles are first internalized into early endosomal vesicles and then moved through a series of progressively more acidic compartments until finally they reach the lysosome, where exogenous material is processed and loaded onto MHC molecules. Lysosomes are characterised by the presence of cathepsin family proteins, which cleave the invariant chain molecule that sits in the MHC binding pocket, allowing subsequent loading of internalized antigen onto the MHC molecule [190]. Co-localization of FITC-tagged molecules with the 647-tagged cathepsin-S would indicate

that internalized particles have been trafficked into the MHC class II pathway. Cathepsin-S was selected as it has been shown to be upregulated by professional APCs such as B cells and macrophages in response to IFNγ activation [23, 101] and in epithelial cell lines [100]. Our results indicate that there was an increase in the expression of cathepsin-S in IFNγ exposed fibroblasts when compared to unexposed populations. Furthermore, activated populations appear to have distinct formations of cathepsin-S and FITC-albumin in large vesicle-like structures that were not apparent in inactive fibroblast populations (figure 4-27). While this result may indicate a level of processing via a cathepsin-s lysosomal pathway, there are a number of issues to be addressed. Firstly, results are based upon a single experiment, and further repetition of these assays is required to confirm this result. While the secondary antibody control experiments shows only small areas of non-specific antibody binding, it is possible that these same areas also bind FITC-albumin and may account for co-localized signal.
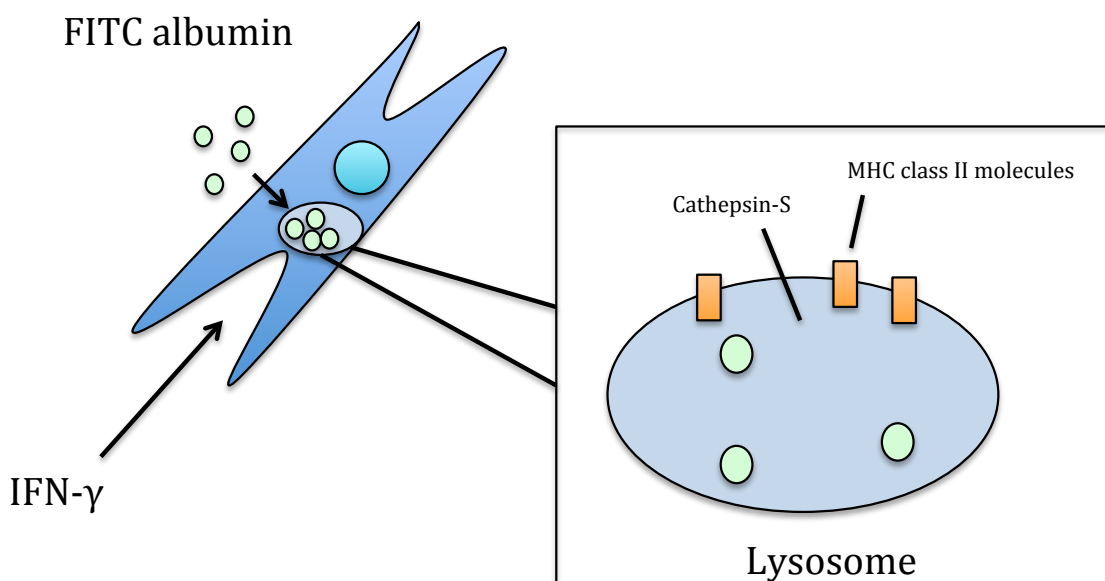
Figure 4-26- The internal trafficking of exogenous particles for loading upon MHC molecules in the lysosome: Environmental particles can be internalized by fibroblasts and trafficked into internal vesicles. After stimulation with proinflammatory mediators such as IFN-γ, cells can activate in such as way that molecules that facilitate the formation of MHC:protein complexes (such as cathepsin-s) are upregulated in the lysosome vesicle. This ultimately can result in an increase in the cells ability to present exogenous antigen molecules upon it surface via MHC class II molecules

There is also evidence to suggest that cathepsin family molecules have non-redundant roles in MHC and antigen processing, therefore cathepsin-S may not be the main mechanism involved in fibroblast antigen processing [23]. Further examination of the expression of other cathepsin family molecules and perhaps other lysosomal markers such as the lysosomal associated membrane protein 1 (LAMP-1) via alternate techniques such as quantitative PCR may give a clearer indication of the processing pathway used by fibroblasts. A caveat to using PCR would be the inability to identify if exogenous particles co-localize into these pathways. While the results regarding cathepsin-S expression within human lung fibroblasts are currently inconclusive from this study, the ability of the cells to express HLA-DR upon its surface would strongly suggest that a MHC class II based pathway is operational within the cell. Studies examining human lung fibroblasts have demonstrated their ability to express the class II transactivator, a master transcription cofactor that mediates the upregulation of MHC class II expression (Fang et al 2013). Evidence such as this lends itself to the argument that fibroblasts have the correct internal machinery to load antigen and surface-present peptide-MHC complexes. Therefore, the current results indicate that fibroblasts may be able to adopt an APC phenotype under inflammatory conditions when considering their ability to internalize and process antigen. However more work would be required to identify the exact mechanisms and pathway these cells utilize to produce peptide-antigen loaded MHC class II.

Using human lung samples, we show that fibroblasts express HLA-DR within the lung environment, strongly suggesting the capacity to present antigen to T helper cells. Further *in vitro* experiments demonstrate IFNγ as being a key cytokine required for HLA-DR expression. While there is no apparent expression of the co-stimulatory molecules CD80 and CD86, the role these molecules play in activation of antigen-experienced memory cell populations is debateable and more emphasis may be placed upon HLA-DR-TcR interactions. Studies examining cellular interaction within the human lung are limited by the fact samples come from individuals in a stable state of disease. As such, fibroblast antigen-presentation to lung T cells during bacterial invasion of the mesenchyme would most likely not be observed in these lung subjects. Therefore, to examine lung fibroblast presentation of bacterial antigen, an *in vitro* co-culture method was designed. This focused upon the T cell activation state after

interaction with NTHi pulsed, IFNγ activated autologous fibroblasts. The findings from this investigation are outlined in chapter 5.

5. ANTIGEN PRESENTATION OF NONTYPEABLE *HAEMOPHILUS INFLUENZAE* BY HUMAN LUNG FIBROBLASTS TO AUTOLOGOUS T HELPER CELLS

5.1   Introduction

Work in previous chapters suggests lung fibroblasts may act as an antigen-presenting cell for T helper cells under inflammatory conditions. The expression of MHC class II by fibroblasts strongly suggests an antigen presenting capability. A lack of CD80 and CD86 expression should not impede T cell activation by fibroblasts due to the fact the majority of lung cells exhibit a memory phenotype and do not require this level of stimulation [46]. To test this hypothesis, a co-culture system pairing autologous lung fibroblasts and T helper cells was designed. This utilized lung T cells which had been exposed to a heat killed lung microbe (NTHi) to drive the activation and proliferation of NTHi-responding T cells, providing a large pool of antigen-specific cells for experimental use. NTHi is a common lung microbe, and high frequencies of NTHi-specific T cells can be found within distal lung samples [130], making it an ideal candidate antigen for generating T cell lines from lung cell preperations. Furthermore, NTHi is relevant in a number of disease processes including COPD [122, 191], although the exact means by which NTHi contributes to inflammation are still being elucidated.

Previous work examining fibroblast antigen presentation have focused on general markers of activation, such as T cell proliferation [149]. It has not been demonstrated which T helper subsets are activated by antigen-presenting lung fibroblasts, and if fibroblasts can process and present lung bacterial antigens. T cell activation was measured by the production of cytokines associated with 4 major T helper subsets. Antimicrobial IFNγ-producing Th1 and IL-17A-producing Th17 cells were screened for, along with the allergic inflammation associated IL-13 producing Th2 cell and the immunoregulatory IL-10 producing Treg. We hypothesis that cultured lung fibroblasts will require IFNγ activation in order to present bacterial antigen to T helper cells and provoke cytokine production.

5.2   Results

5.2.1   Initial T cell response to NTHi

Leukocyte populations were isolated from human distal lung via migration from tissue fragments (see section 4.2). This yields lung leukocytes containing tissue-resident T

cell and APC populations. These were used to gauge the initial T cell response to heat-killed NTHi. For this, cultures of $2x10^5$ leukocytes were exposed to heat-killed NTHi, allowing tissue-resident APCs to process and present bacterial antigens to antigen-specific T cells, and activation measured by intracellular cytokine production associated with 4 major T cell subsets (Th1-IFNγ, Th2 IL-13, Th17 IL-17A and Treg IL-10). PMA and ionomycin treatment was also used in certain cultures to cause nonspecific activation to gauge the overall T cell cytokine profile. Figure 5-1A shows the initial PMA and Ionomycin activation of T helper cells, while 5-1B shows the initial T helper cell response to NTHi.
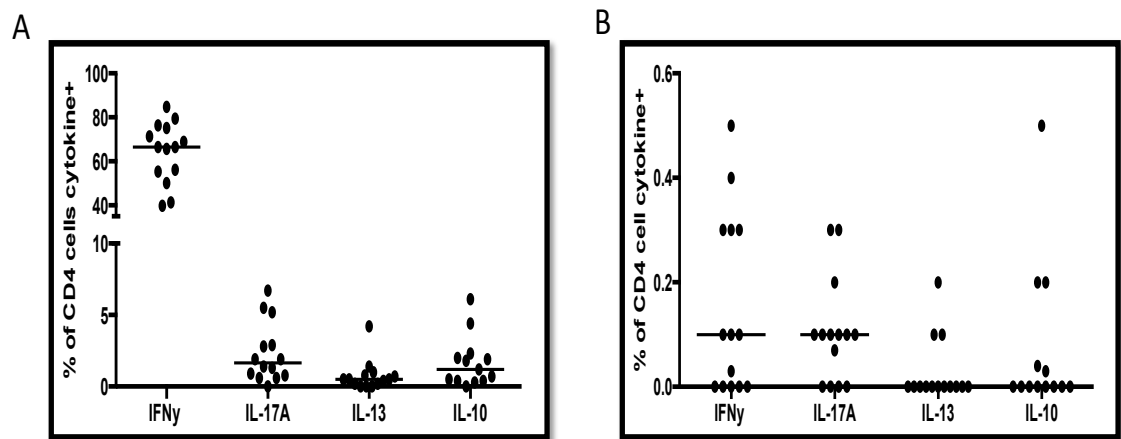
Figure 5-1- Initial T helper cell subsets and T helper cell response to heat-killed NTHi: Lung leukocyte populations were exposed to either PMA and ionomycin to cause polyclonal activation of T helper cells (A) or to 1500 heat-killed NTHi (B). The T helper cell response was measured via the production of intracellular cytokines IFNγ, IL-13, IL-10 and IL-17A. The results show the percentage of the CD4+ T helper cells positive for one cytokine corrected for the percentage of unstimulated cells producing that particular cytokine.

Figure 5-1 shows that the overall T helper population is predominantly made up of Th1, IFNγ producing cells. Smaller populations of IL-17A and IL-10 producing populations are also apparent, while there are very few, if any, IL-13 producing cells in most subjects. Challenge with heat-killed NTHi (figure 5-1B) shows the response is dominated by IFNγ and IL-17A producing cells. The majority of subjects do not respond to NTHi via production of IL-10 or IL-13.

It was also possible to examine CD8 cytotoxic T cell and CD4-CD8- ("double negative") response to heat-killed NTHi. PMA and ionomycin stimulation show that the vast majority of cytotoxic T cells produce IFNγ, with a smaller population of IL-13 producing cells also apparent. There were very few IL-17A and IL-10 producing cytotoxic T cells (figure 5-2A). When exposed to heat-killed NTHi, there was a predominant IFNγ and IL-13 response, with smaller IL-17A and IL-10 responses (figure 5-2B).
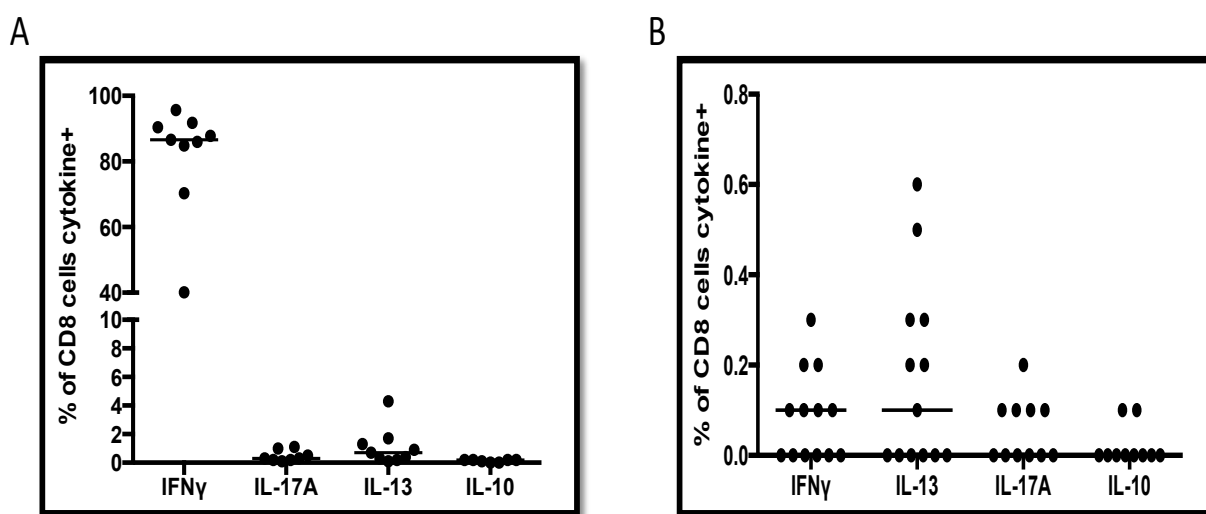
Figure 5-2-Initial cytotoxic T cell subsets and response to heat-killed NTHi: : Lung leukocyte populations were exposed to either PMA and ionomycin (A) or to 1500 heat-killed NTHi (B). Subsequent activation of cytotoxic T cells was examined via the production of intracellular cytokines IFNγ, IL-13, IL-10 and IL-17A. The results show the percentage of the CD8+ cytotoxic T cells positive for one of these cytokines corrected for the percentage of unstimulated cells producing that particular cytokine.

Likewise, the majority of DN T cells produce IFNγ when stimulated with PMA+ionomycin, with sizeable populations of IL-10 and IL-17A producing cells also apparent and only a small IL-13 producing population (Figure 5-3A). When exposed to heat-killed NTHi, the DN T cell response featured production of all four cytokines, with IFNγ production being dominant (Figure 5-3B).
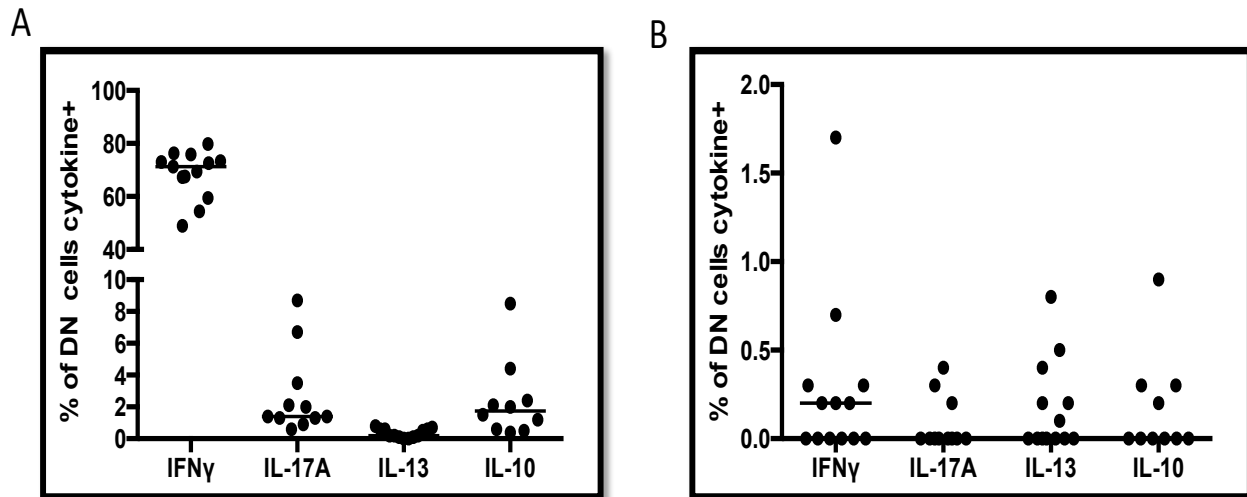
Figure 5-3- Initial double negative T cell subsets and response to heat-killed NTHi: Lung leukocyte populations were exposed to either PMA and ionomycin (A) or to 1500 heat-killed NTHi (B). Subsequent activation of double negative T cells was examined via the production of intracellular cytokines IFNγ, IL-13, IL-10 and IL-17A. The results show the percentage of the CD4-CD8- T cells positive for one of these cytokines corrected for the percentage of unstimulated cells producing that particular cytokine.

5.2.2   Development of an NTHi-specific lung-derived T helper cell line

To examine antigen-presentation and activation of T cells by fibroblasts, a population of T helper cells with a known antigen-specificity is required. As fibroblasts take up to 4 weeks to reach sufficient numbers in culture, this period of time was used to develop a large population of autologous, lung microbe specific T helper cells to use in these assays. Lung leukocyte populations were used to develop these NTHi-specific T cell lines. Briefly, lung leukocytes were exposed to heat-killed NTHi for 5 days before receiving the growth cytokine IL-2 (to boost proliferation of activated T cells). Cultures were then left up to 4 weeks to allow the proliferation of NTHi-specific T cells (see section 2.4 for detailed methodology). Due to this antigen being exogenous, this method should preferentially promote the expansion of T helper cells. In order to verify this and understand the effects of culture upon T cell populations as a whole, NTHi exposed cultures were screened for CD4 (T helper cells), CD8 (cytotoxic cells) and proliferation marker Ki67 after 10 days of culture with NTHi (with IL-2 treatment at day 5). Figure 5-4 shows representative gating for the analysis of T cell proportions and Ki67 staining. Results clearly indicate that NTHi and IL-2 treated cultures contain

predominantly CD4 T cells, and that these cells express highest Ki67 when exposed to both NTHI and IL-2 (figure 5-5A and B respectively). Taken together, these results indicate that exposure of lung leukocytes to heat-killed NTHi results in a predominantly CD4 T helper population.
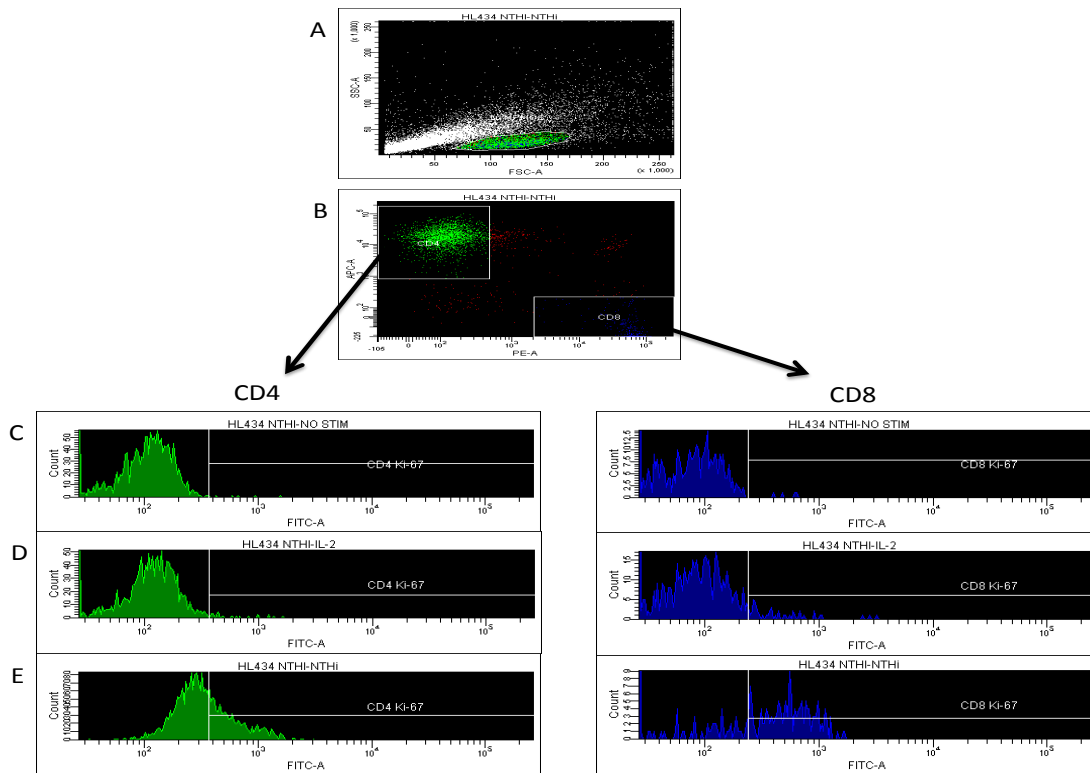
Figure 5-4- **Screening NTHi-exposed T cells for CD4 and CD8 proportions and expression of proliferation marker Ki-67:** Lung leukocyte populations were exposed to heat-killed NTHi for 5 days. IL-2 was administered after 5 days and cultures left for a further 5 days. Cells were then analysed for proportions of CD4 and CD8 cells as well as for the proliferation marker Ki-67. Shown above is the initial gating strategy for lymphocyte-sized cells (A) and CD4 and CD8 expression (B). Cells were separated into CD4 (left column) and CD8 (right column) cells before being screened for KI-67.   C depicts unstimulated controls, D shows IL-2 treated alone and E shows NTHi and IL-2 treated cells. Representative of 5 experiments.
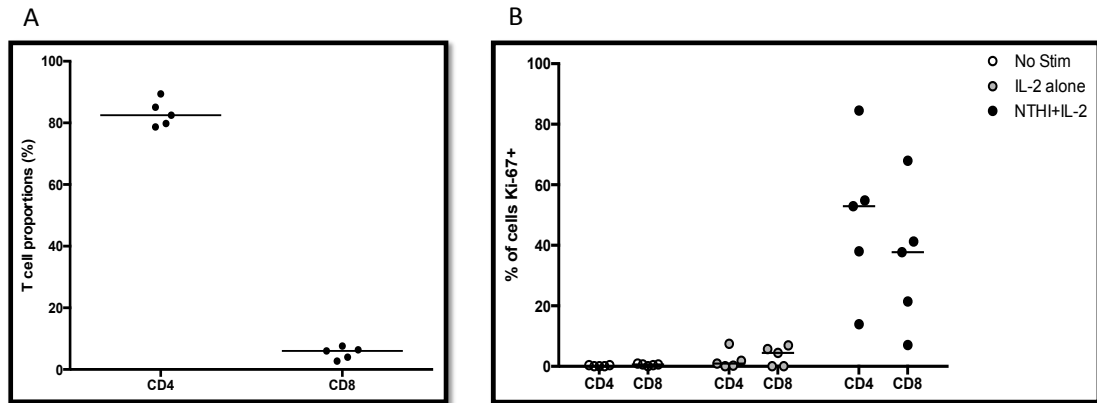
Figure 5-5- The proportion of CD4 and CD8 cells and Ki67 expression within each population after exposure to heat-killed NTHi and/or IL-2: Lung Leukocyte cultures exposed to both NTHi and IL-2 or to IL-2 alone were examined for the proportions of CD4 and CD8 cells and the expression of Ki67 within each population. A shows the percentage of cells within the lymphocyte-sized gate expressing CD4 or CD8. B shows the level of Ki67 expression within each population. N=5

Exposing T cells to NTHi in a long-term culture can potentially affect T helper cell subset proportions. T helper cells were therefore exposed to PMA+ionomycin after 4 weeks in culture and examined for cytokine production (figure 5-6). This analysis reveals long-term cultured T helper cells are predominantly IFNγ producers, with an expanded population of IL-17A producing cells compared to initial cytokine profile reads (Figure 5-1A). In contrast, the IL-10 and IL-13 producing cells (which were initially small populations) appear to be lost.
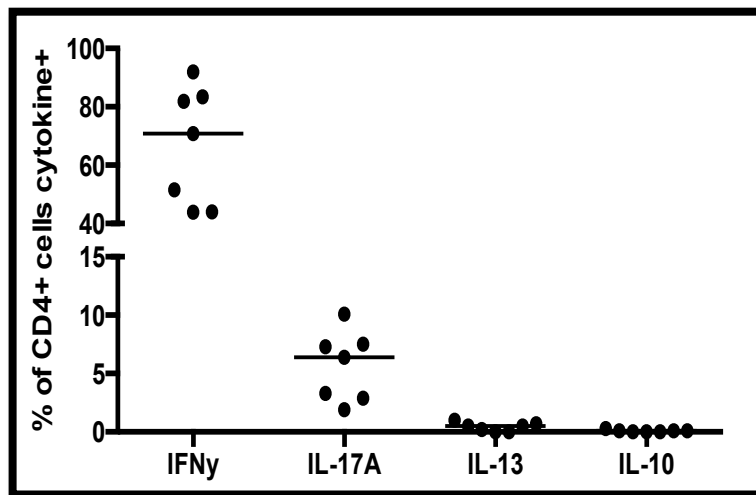
Figure 5-6- Examining the effector subsets present in NTHi-specific T helper cell lines:
Cells that were exposed to 1500 heat-killed NTHi and growth cytokines for up to 4 weeks were exposed to PMA and ionomycin to activate cytokine production in all T helper cell subsets. Cytokines relating to the four major T helper cell subsets were screened for. The results show the percentage of the T helper cell population positive for one of these cytokines corrected for the percentage of unstimulated cells producing that particular cytokine. N=7

A small cohort of subjects PMA+ionomycin response immediately after isolation were compared to the response after 4-5 weeks of NTHi exposure and culture. Tracking individual subject samples in this way further demonstrates that driving NTHi-specific T cell lines favours IFNγ and IL-17A T helper cell development (figure 5-7). In all subjects, the proportion of IL-13 and IL10 producing cells decreased over the culture period.
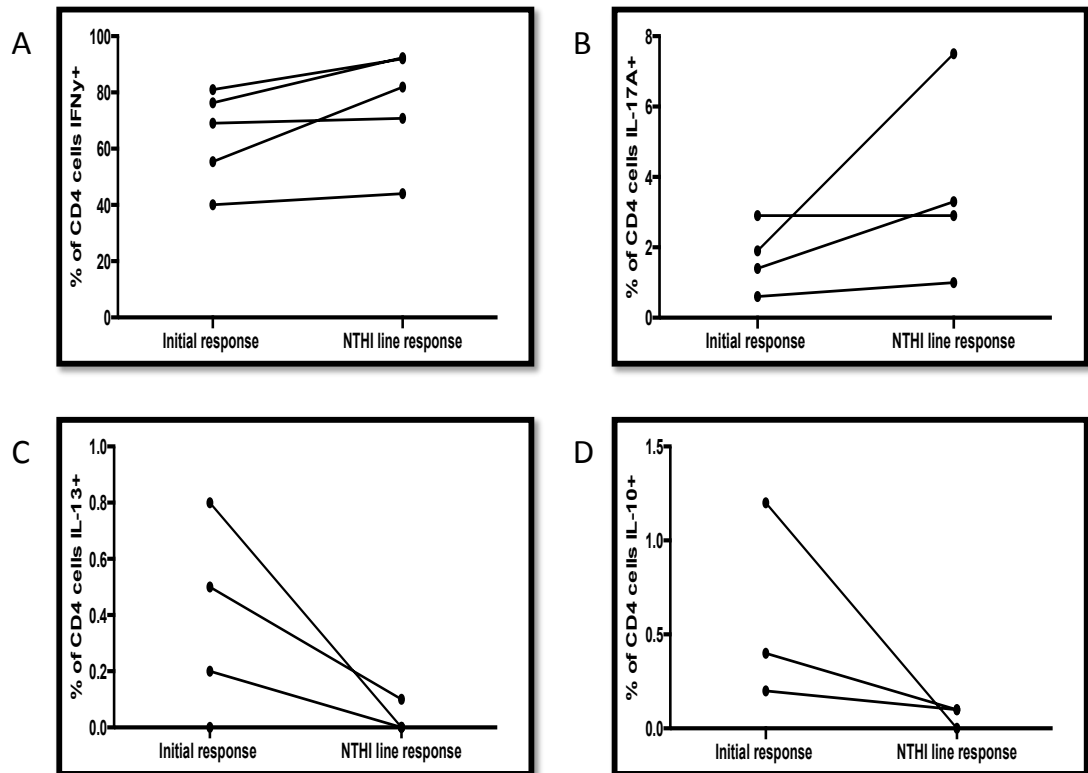
Figure 5-7- Comparison of initial T helper cell subsets against NTHi-exposed long term cultured T helper cells: T helper cells isolated from distal lung were exposed to PMA+ionomysin within 24 hours to activate cytokine production in all T helper cell subsets. This was compared to the PMA+ionomycin response by T helper cells after 4 weeks of culture after NTHi exposure to drive antigen-specific T cell proliferation. Shown is the production of IFNγ (A), IL17A (B), IL-13 (C) and IL-10 (D). Connected lines denote subject initial response to response after 4 weeks of culture. The results show the percentage of the T helper cell population positive for one of these cytokines corrected for the percentage of unstimulated cells producing that particular cytokine.

Taken as a whole, these results allow a greater understanding of the experimental model being used in this project. Through this long-term culture process, we are able to produce T cells lines that consist of Th1 and Th17 subsets with little to no Th2 and Treg subsets. The next stage in understanding these T helper cell lines is to demonstrate these cell s respond to NTH in an antigen-specific manner.

151

5.2.3 Activation of NTHi-specific T helper cells by antigen-pulsed monocyte derived dendritic cells

Generating T cell lines to NTHi drives the expansion of Th1 and Th17 cells, however it must be verified that these cells have expanded antigen-specifically and retain antigen-dependent activation to NTHi. Therefore a co-culture model using T helper cell lines and autologous professional APCs in the form of monocyte derived dendritic cells (moDCs) was designed. After 4 weeks of culture, subject NTHi-exposed lung cell cultures contained sufficient T helper cells for use in co-culture assays. It was possible to culture T cells with a professional APC in the form of IL-4 and GM-CSF conditioned moDCs. Briefly, PBMCs were obtained from autologous blood samples and stored at -80°C until T cell lines were ready for use. PBMCs were then defrosted and monocyte progenitors differentiated into moDCs (see section 2.7 for full protocol). In order to confirm freeze-thawing did not affect moDC generation, samples were screened for CD11c, CD123 and HLA-DR to confirm moDC lineage (with moDCs exhibiting a CD11c+ CD123- HLA-DR^{hi} phenotype, figure 5-8).
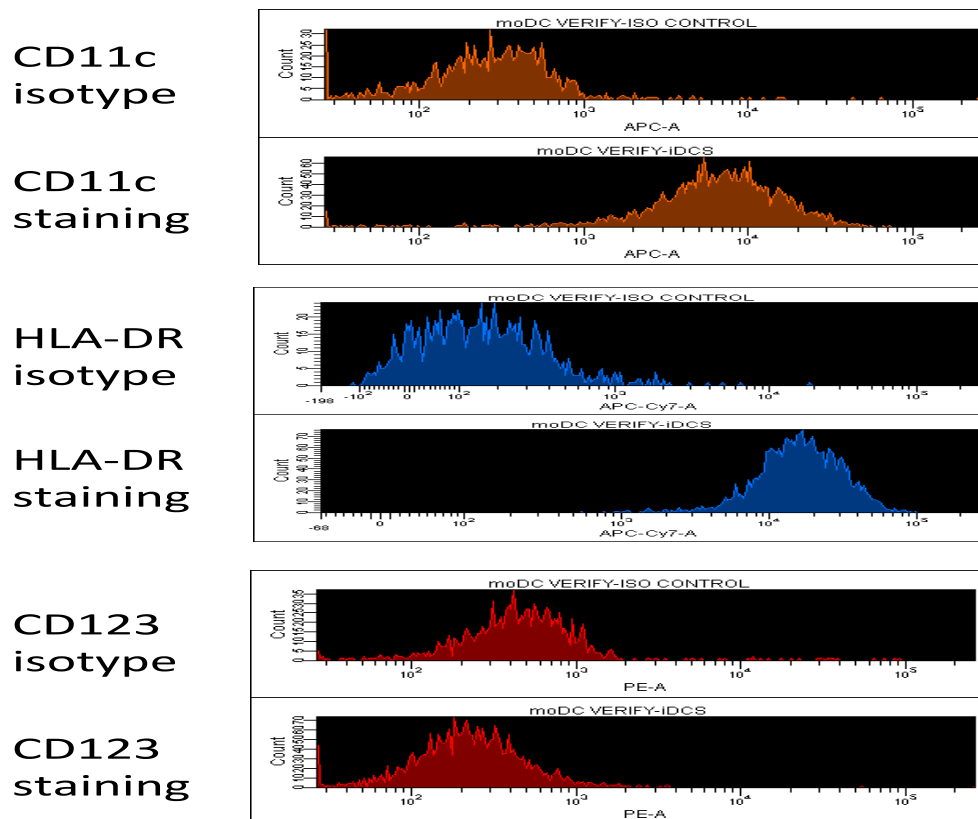
Figure 5-8- blood-derived monocytes exposed to IL-4 and GM-CSF differentiate into monocyte-derived dendritic cells: PBMCs were isolated from patients and immediately frozen until later use. After thawing, CD14+ cells were isolated via MACS to obtain monocyte cells. These were then cultured for 7 days in GM-CSF and IL-4 before use in co-culture assays. These cells were screened for the expression of CD11c, CD123 and HLA-DR, as shown above. Both isotype control and specific staining histograms are displayed. Representative of 3 experiments.

Flow cytometry clearly demonstrates these cells differentiate into CD11c+, CD123-, HLA-DR high-expressing moDC populations (Figure 5-8). The moDC represents a "gold standard" professional APC, providing a measure of optimal antigen-dependent activation of the T cells. Thus, lung T helper cells were cultured with autologous moDCs to verify that the NTHi-exposed T cell lines were antigen-specific and to provide a gauge of the response to optimal antigen-presentation. MoDCs were exposed to NTHi in the presence or absence of IFNγ for 48 hours prior to co-culture. T helper cells were purified from NTHi-exposed leukocyte cultures via MACS (see section 2.13 for full protocol) and rested overnight in cytokine-free media. Co-cultures were then established at a ratio of 100,000 T helper cells to 25,000 moDCs and incubated overnight in the presence of brefeldin A. T helper cells were then screened for the

production of intracellular cytokines. Brefeldin A is necessary to cause intracellular
build-up of cytokines, but can be toxic to cells over prolonged periods. Therefore a cell
viability dye was used to assess cell death due to brefeldin-A exposure in this model.
This demonstrated T helper cells were 95%+ viable after this process. To assess if any
T helper cell activation was antigen-specific, NTHi exposed moDC cultures were also
treated with HLA-DR blocking antibodies or corresponding isotype control.
Representative flow cytometry plots for IFNγ and IL-13 (cytokine staining panel 1), and
IL-10 and IL-17A (cytokine panel 2) are shown in figures 5-9 and 5-10 respectively.
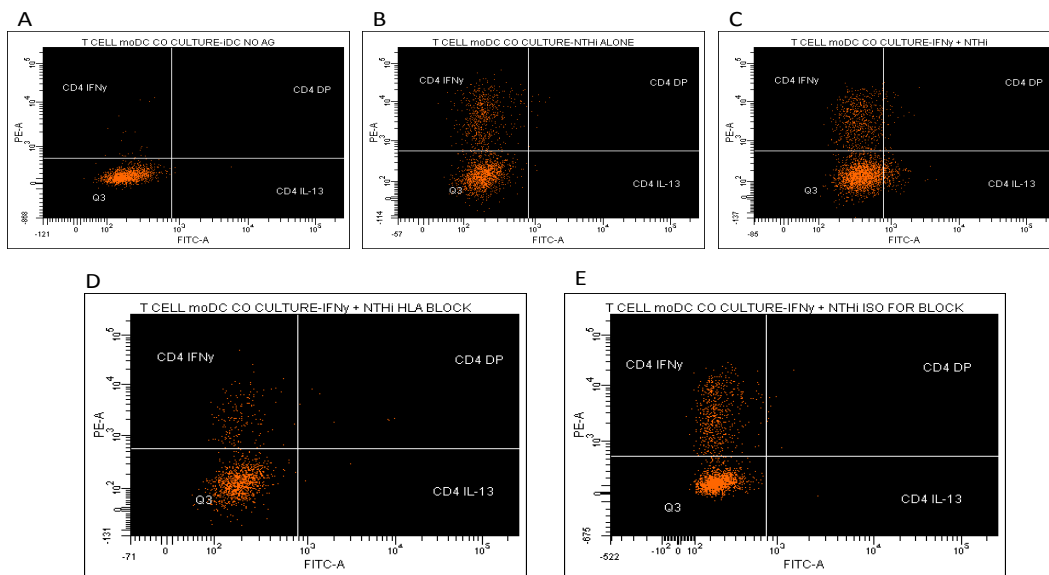
**Figure 5-9-Representative flow cytometry plots of T helper cell IFN-γ and IL-13
production after culture with monocyte-derived dendritic cells:** T helper cells were cultured
overnight with moDCs which had been left unstimulated (A), exposed to heat-killed NTHi alone
(B) or both heat-killed NTHi and IFNγ together (C). Cultures were also exposed to both heat-
killed NTHi and IFNγ and then administered HLA-DR blocking antibodies (D) or
corresponding isotype control for blocking antibodies (E). T helper cell cytokine production
was then measured via flow cytometry. Shown above is the production of IFN-γ and IL-13 by T
helper cells. Representative of 3 experiments.
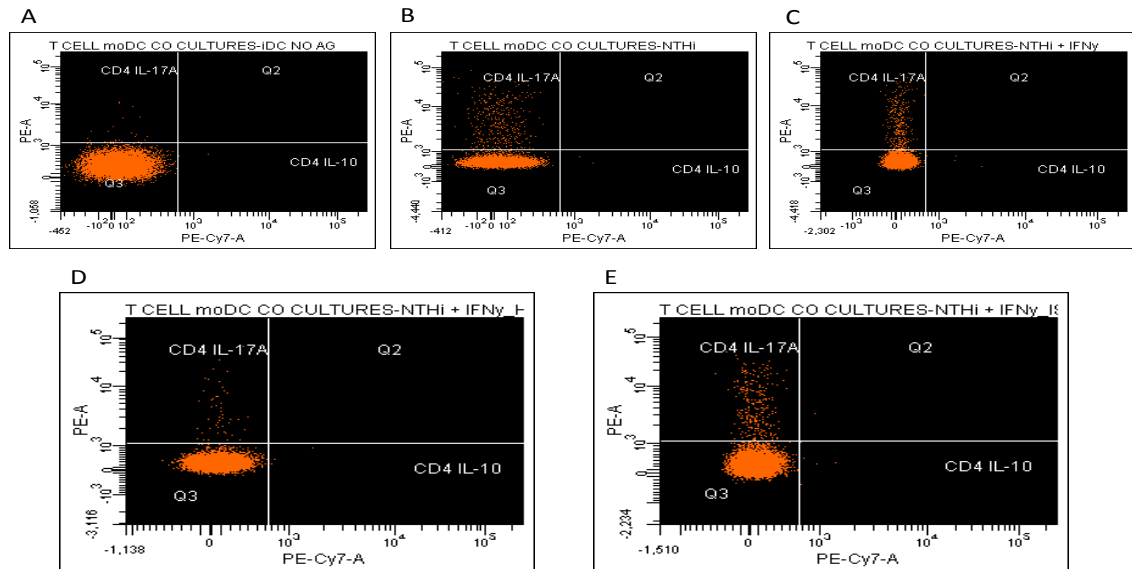
Figure 5-10- Representative flow cytometry plots of T helper cell IL-10 and IL-17A production after culture with monocyte-derived dendritic cells: T helper cells were cultured overnight with moDCs which had been unstimulated (A), exposed to heat-killed NTHi alone (B) or both heat-killed NTHi and IFNγ together (C). Cultures were also exposed to both heat-killed NTHi and IFNγ and then administered HLA-DR blocking antibodies (D) or corresponding isotype control for the blocking antibody (E). T helper cell cytokine production was then measured via flow cytometry. Shown above is the production of IL-10 and IL-17A by T helper cells. Representative of 3 experiments

After overnight culture with moDCs pre-exposed to NTHi, T helper cells produced IFNγ (figure 5-11A) and IL-17A (Figure 5-11B). Furthermore, production of these cytokines was suppressed via the specific action of HLA-DR blockade, indicating activation is due to antigen-presentation.. Isotype control antibody cultures indicate this was a direct effect of blocking HLA-DR and not through the introduction of an antibody to the experimental system. Although this trend appears in all setups, the low number of repetitions (N=3) would not expect to reach significance

A

| IFNγ (500U) | ✖ | ✖ | ✔ | ✔ | ✔ |
| NTHi (1500) | ✖ | ✔ | ✔ | ✔ | ✔ |

B

| IFNγ (500U) | ✖ | ✖ | ✔ | ✔ | ✔ |
| NTHi (1500) | ✖ | ✔ | ✔ | ✔ | ✔ |
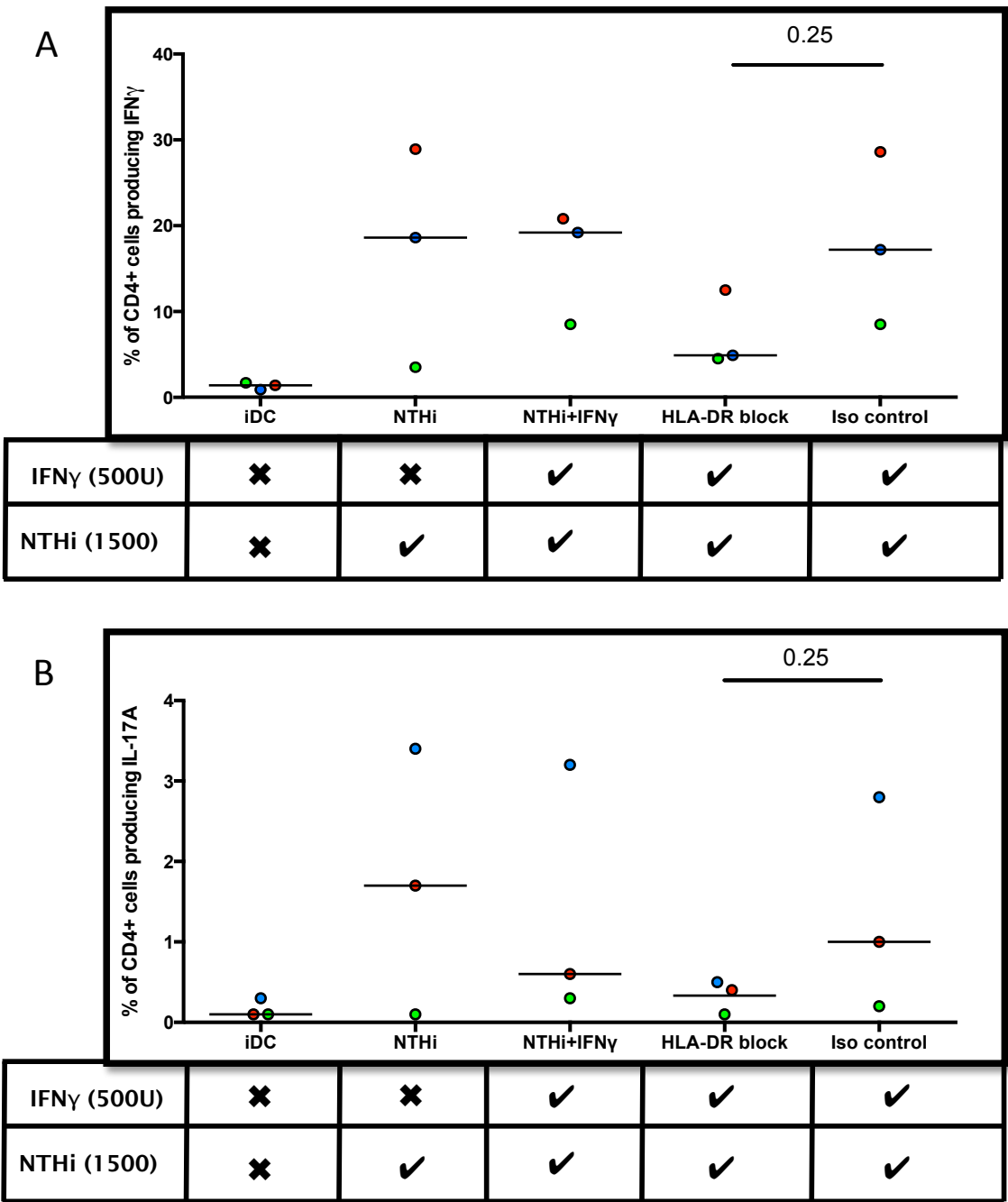
Figure 5-11-Production of cytokines IFNγ and IL-17A by lung T helper cells after co-culture with NTHi-pulsed moDCs: MoDCs were exposed to heat-killed NTHi or heat-killed NTHi+IFNγ for 48 hours. After exposure, autologous CD4 T helper cells were introduced to cultures at a ratio of 25,000 moDCs to 100,000 T cells. In some cases, HLA-DR blocking antibody or corresponding isotype control (iso control) were introduced to NTHi+IFNγ exposed moDC cultures. Figure A shows the production of IFNγ while B shows IL-17A production by T helper cells after co-culture. Statistical comparison performed with a Wilcoxon paired test. N=3

In contrast, there was very little production of IL-13 (fig 5-12A) or IL-10 (fig 5-12B). This is likely due to the culture process causing loss of IL-10 and IL-13 producing T helper cells (see section 5.7).
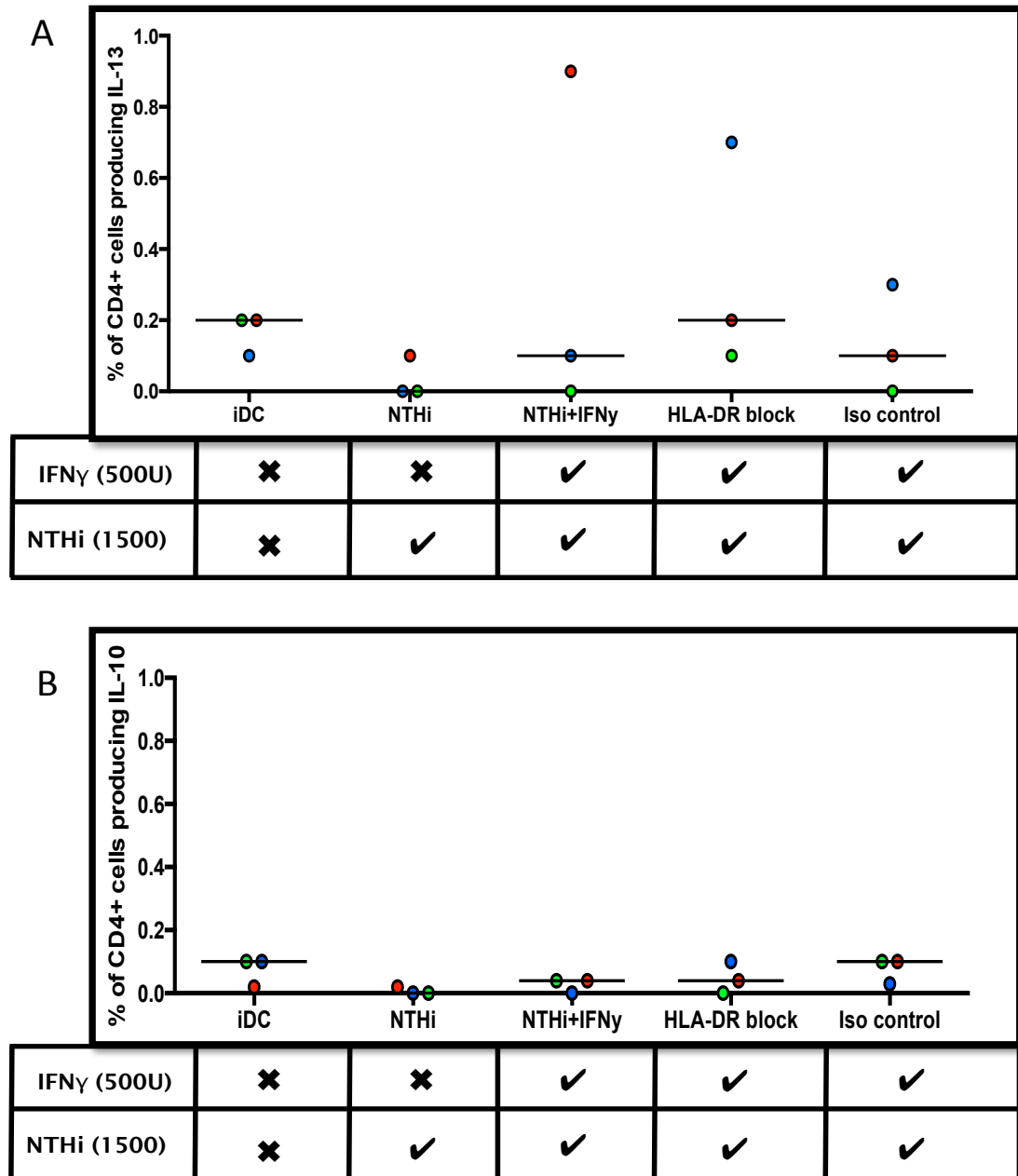
Figure 5-12- The production of cytokines IL-13 and IL-10 by lung T helper cells after co-culture with NTHi-pulsed moDCs: MoDCs were exposed to heat-killed NTHi or heat-killed NTHi+IFNγ for 48 hours. After exposure, autologous CD4 T helper cells were introduced to cultures at a ratio of 25,000 moDCs to 100,000 T cells. In some cases, HLA-DR blocking antibody or corresponding isotype control (iso control) were introduced to NTHi+IFNγ exposed moDC cultures. Figure A shows the production of IL-13 while B shows IL-10 production by T helper cells after co-culture. N=3.

Taken together, these results demonstrate that driving T cell lines via heat-killed NTHi exposure results in antigen-specific T helper cells of a Th17 and Th1 phenotype. As moDCs are a professional APC, the response generated by T helper cells can be considered optimal antigen-dependent activation. The culture process involved in generating NTHi-specific T cells results in a loss of IL-10 and IL-13 producing T cells. Therefore this model is not suitable for examining activation of Th2 or Treg cells.

5.2.4 Co-culture of lung lymphocyte populations with autologous human lung fibroblasts

MoDC-T cell co-culture experiments confirm that exposing lung leukocytes to NTHi drives development of antigen-specific T cell lines. With this model validated, NTHi T cell lines were then used to explore the antigen-presentation function of lung fibroblasts. Similar to moDC cultures, fibroblasts were exposed to NTHi, IFNγ or both factors for 48 hours prior to co-culture. NTHi+IFNγ cultures were also administered HLA-DR blocking antibodies or corresponding isotype controls to confirm if any activation was antigen-specific. Initial fibroblast co-culture experiments made use of unsorted T cell lines (i.e CD4 purification was not carried out). As a result, small numbers of CD8 cytotoxic cells were also cultured alongside CD4 T helper cells. Similar to previous moDC co-cultures, cells were co-cultured overnight and T cell activation measured via intracellular production of cytokines IFNγ and IL-17A only (see 2.13 for full protocol).

A

	No stim	NTHi alone	IFNy alone	NTHi+IFNy	HLA-DR BLOCK	Isotype
IFNγ (500U)	✘	✘	✔	✔	✔	✔
NTHi (1500)	✘	✔	✘	✔	✔	✔

B

	No stim	NTHi alone	IFNy alone	NTHi IFNy	HLA-DR BLOCK	Isotype
IFNγ (500U)	✘	✘	✔	✔	✔	✔
NTHi (1500)	✘	✔	✘	✔	✔	✔
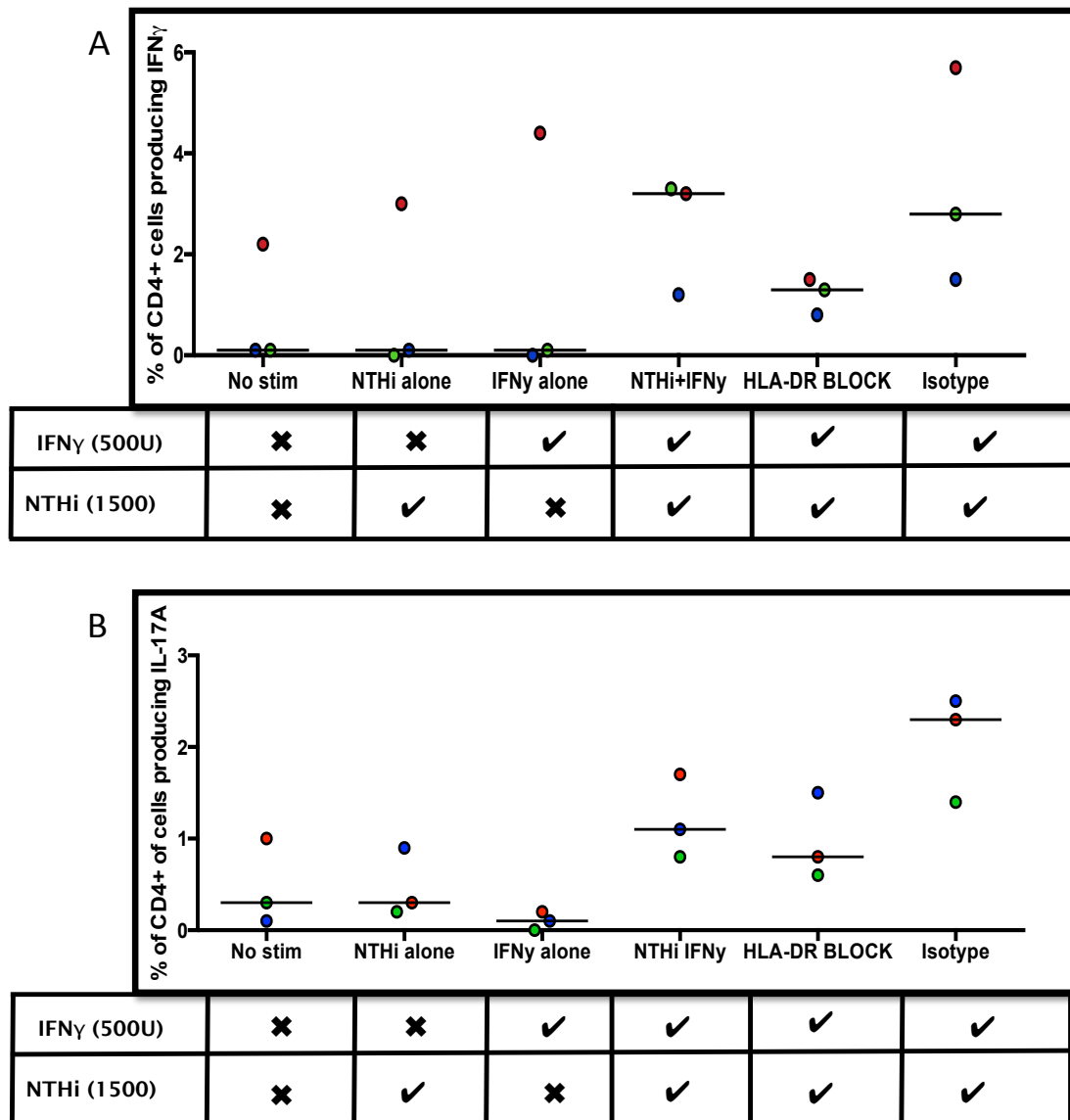
Figure 5-13-The production of IFNγ and IL-17A by T helper cells from unsorted co-culture with autologous lung fibroblasts: Human lung fibroblasts were exposed to heat-killed NTHi and/or IFNγ for 48 hours. After exposure, autologous NTHi-exposed lymphocyte lines containing both CD4 and CD8 T cells were cultured with fibroblasts at a ratio of 25,000 fibroblast to 100,000 T cells. In some cases, HLA-DR blocking antibody or corresponding isotype control was introduced to NTHi+IFNγ exposed fibroblast cultures. Shown above is the resultant production of IFNγ (A) and IL-17A (B) by T helper cells after co-culture. N=3

CD4 T helper cells show noticeable production of both IFNγ (figure 5-13A) and IL-17A (figure 5-13B) when paired with fibroblasts previously exposed to NTHi and IFNγ. Cytokine production was again suppressed by the presence of HLA-DR blocking antibodies, indicating activation is due to antigen-presentation. Unlike T helper cells, cytotoxic T cell cytokine production was not optimal when fibroblasts were pre-exposed

to both IFNγ and NTHi and the magnitude of response was smaller (Figure 5-14A-B). Cytotoxic T cells demonstrated no HLA-DR dependent activation, indicating they are not responding to antigen presentation via MHC class II.

	No stim	NTHi alone	IFNy alone	NTHi IFNy	HLA-DR BLOCK	Isotype
IFNγ (500U)	✘	✘	✔	✔	✔	✔
NTHi (1500)	✘	✔	✘	✔	✔	✔

	No stim	NTHi alone	IFNy alone	NTHi IFNy	HLA-DR BLOCK	Isotype
IFNγ (500U)	✘	✘	✔	✔	✔	✔
NTHi (1500)	✘	✔	✘	✔	✔	✔
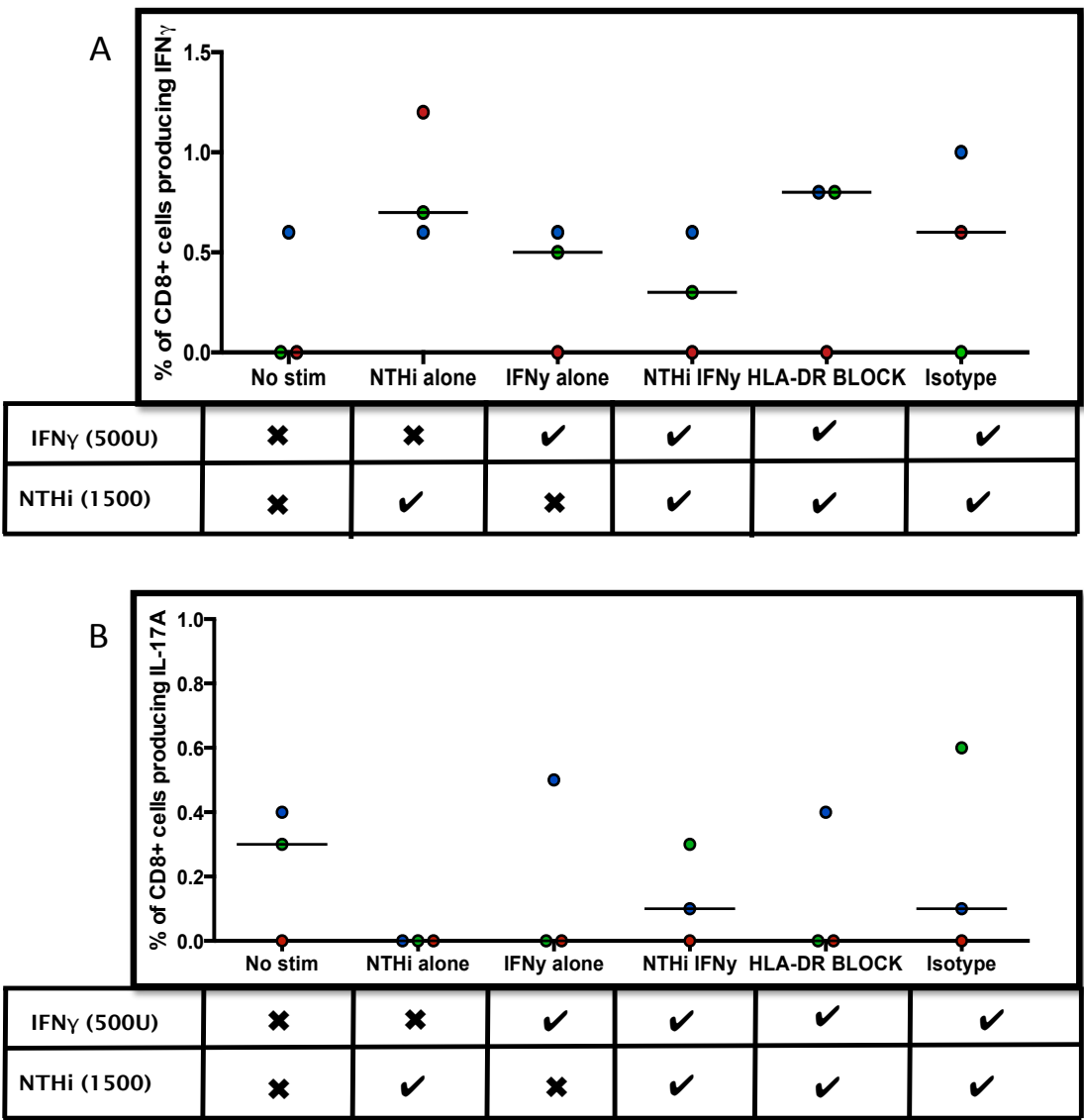
Figure 5-14-The production of IFNγ and IL-17A by cytotoxic T cells from unsorted co-culture with autologous lung fibroblasts: Human lung fibroblasts were exposed to heat-killed NTHi and/or IFNγ for 48 hours. After exposure, autologous NTHi-exposed lymphocyte lines containing both CD4 and CD8 T cells were cultured with these fibroblasts at a ratio of 100,00 T cells to 25,000 fibroblasts**.** In some cases, HLA-DR blocking antibody or corresponding isotype control were introduced to NTHi+IFNγ exposed fibroblast cultures. Shown above is the resultant production of IFNγ (A) and IL-17A (B) by cytotoxic T cells after co-culture. N=3

5.2.5 Co-culture of lung CD4 T helper cells with autologous human lung fibroblasts

Co-cultures using unsorted lung lymphocytes provide evidence that fibroblasts can activate T helper cells via antigen-presentation. However, as well as containing T helper cells, the NTHi exposed lung cultures may contain small numbers of tissue-resident APCs that persist in culture (for example, B cells). T helper cells were therefore purified via MACS and used in co-culture with autologous lung fibroblasts; ensuring results are reflective of fibroblast-T helper cell interaction alone. Fibroblasts were exposed to NTHi and/or IFNγ 48 hours before culture. T helper cells were then incubated alongside fibroblasts overnight and subsequent production of intracellular cytokines IFNγ, IL-13 (representative gating shown in figure 5-15) IL-17A and IL-10 (representative gating shown in figure 5-16) examined.
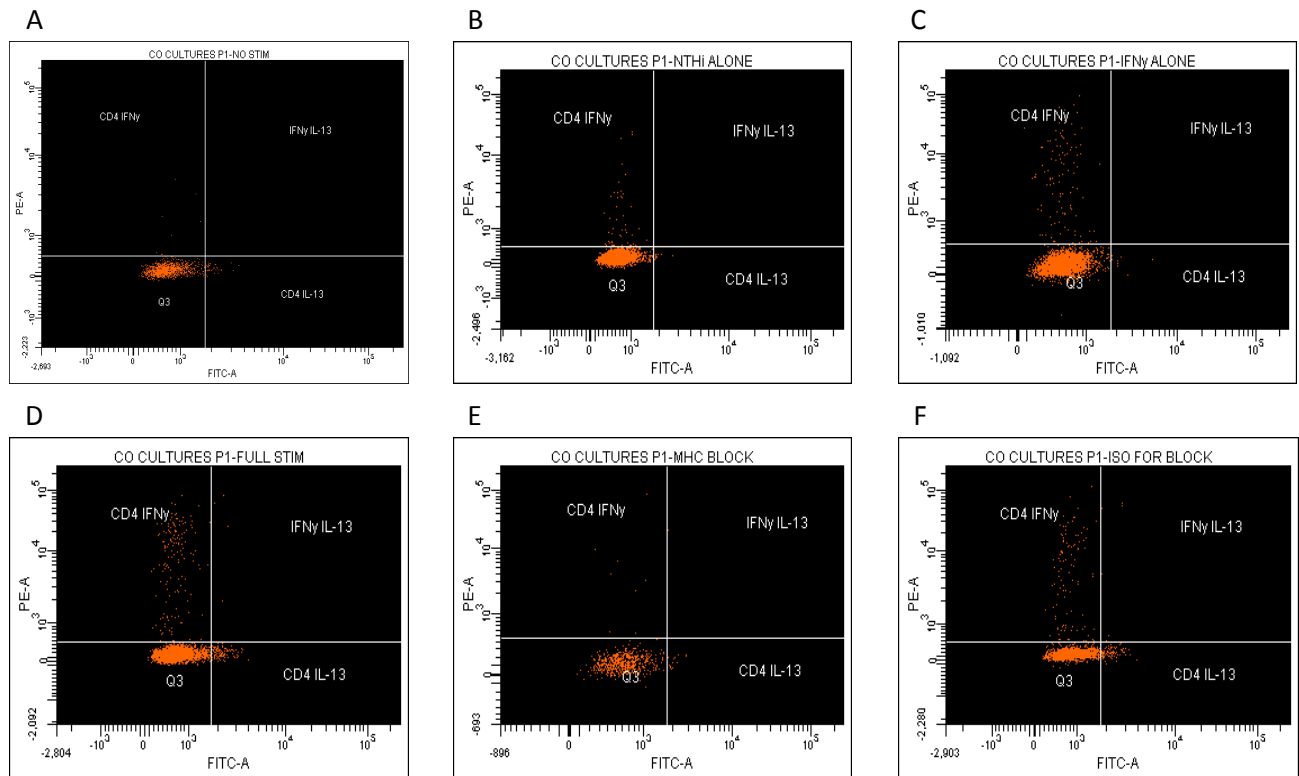
Figure 5-15- Representative flow cytometry plots of T helper cell IFN-γ and IL-13 production after culture with autologous lung fibroblasts: T helper cells were cultured overnight with lung fibroblasts which were unstimulated (A), exposed to heat-killed NTHi alone (B), IFNγ alone (C) or both heat-killed NTHi and IFNγ together (D). Cultures were also exposed to both NTHi and IFNγ and then administered HLA-DR blocking antibodies (E) or corresponding isotype control for blocking antibodies (F). T helper cell production of cytokines IFNγ and IL-13 was then measured via flow cytometry. Representative of 6 experiments
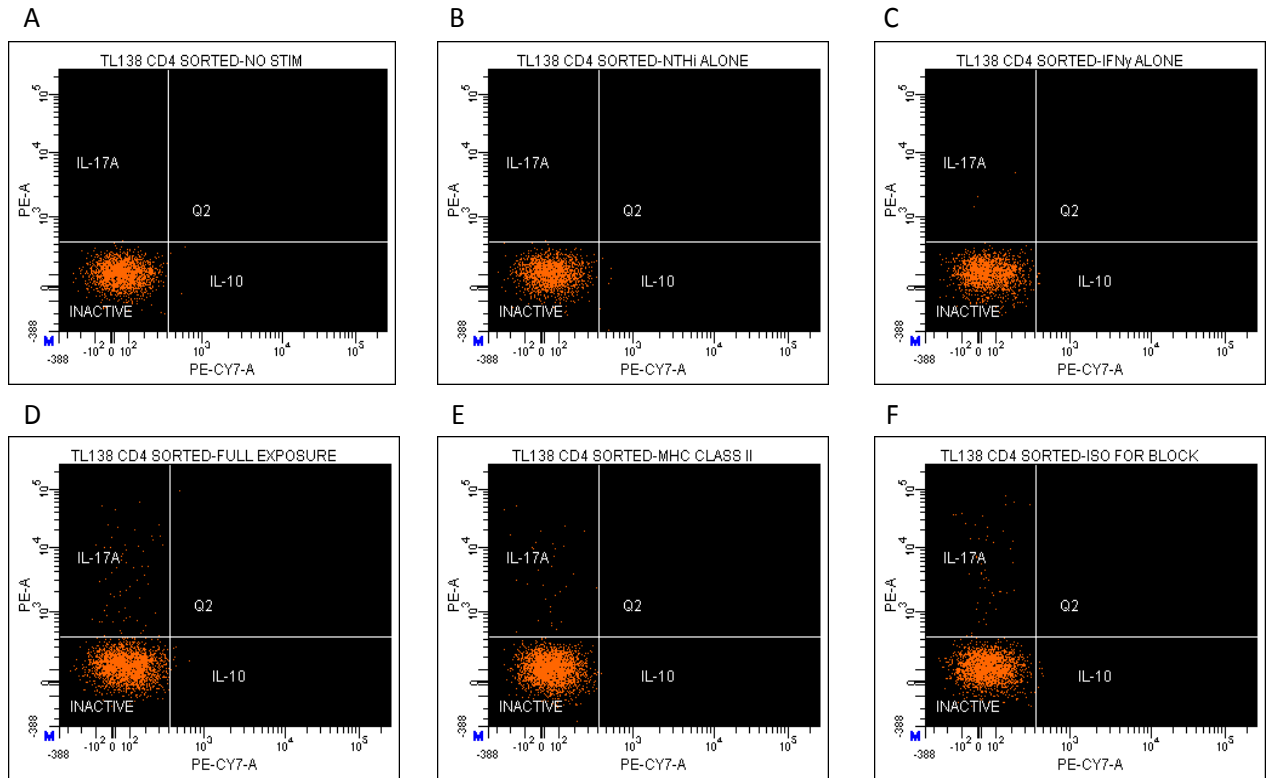
Figure 5-16- Representative flow cytometry plots of T helper cell IL-17A and IL-10 production after culture with autologous lung fibroblasts: T helper cells were cultured overnight with lung fibroblasts which were unstimulated (A), exposed to heat-killed NTHi alone (B), IFN γ alone (C) or both heat-killed NTHi and IFNγ together (D). Cultures were also exposed to both NTHi and IFNγ and then administered HLA-DR blocking antibodies (E) or corresponding isotype control for blocking antibodies (F). T helper cell production of cytokines IFNγ and IL-13 was then measured via flow cytometry. Representative of 6 experiments

As in previous co-cultures, HLA-DR blocking antibodies were used to examine if T helper cell activation is due to fibroblast antigen-presentation. Similar to unsorted T cell populations in co-culture with fibroblasts, optimal production IL-17A and IFNγ occurs when fibroblasts were pre-exposed to both NTHi and IFNγ (figure 5-17A and B). Blocking HLA-DR causes a significant decrease in the production of both IL-17A and IFNγ when compared to isotype control setups, demonstrating activation is due to fibroblast presentation of NTHi antigens. In contrast, NTHi+IFNγ exposed fibroblasts do not cause antigen-specific activation of IL-10 or IL13 producing cells (figure 5-18A and 5-18B). It should once again be noted that the IL-10 and IL-13 producing Treg and Th2 populations appear to be lost due to the culture process involved in developing NTHi-specific T cells. Therefore it is not possible to conclude if fibroblasts can or cannot activate Th2 or Treg cells via antigen presentation is this experimental model. Overall, these results confirm that IFNγ conditioned lung fibroblasts can present NTHi antigens Th1 and Th17 subsets.
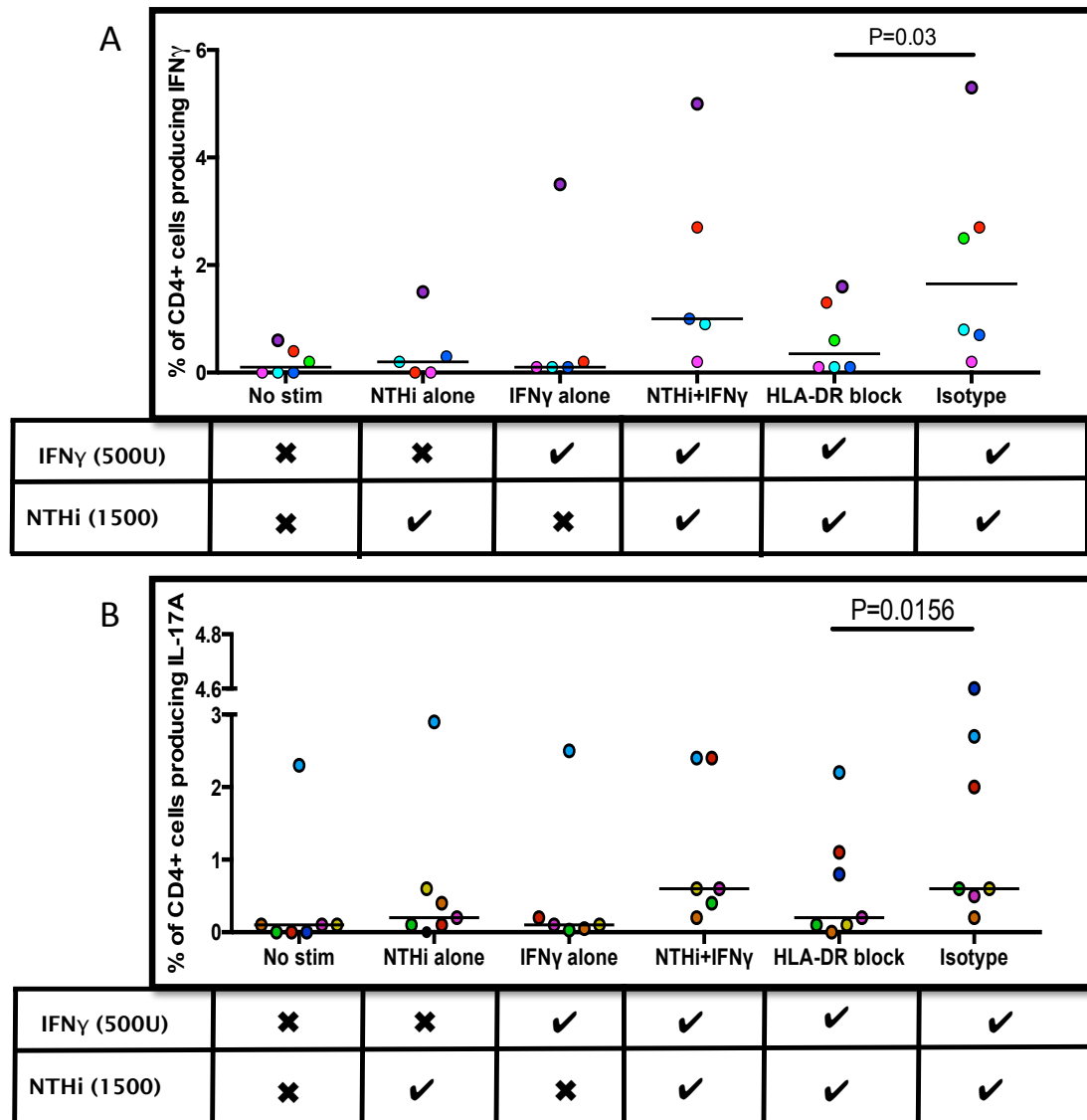
Figure 5-17-The production of IFNγ and IL-17A by CD4 sorted lung T helper cells with autologous lung fibroblasts: Human lung fibroblasts were exposed to heat-killed NTHi and/or IFNγ for 48 hours. After exposure, autologous lung-derived, NTHi-exposed T helper cells (previously purified via MACS sorted for CD4 expression) were cultured with these fibroblasts at a ratio of 25,000 fibroblast to 100,000 T cells. In some cases, HLA-DR blocking antibody or corresponding isotype control were introduced to NTHi+IFNγ exposed fibroblast cultures. Shown above is the resultant production of IFNγ (A) and IL-17A (B) by T helper cells after co-culture. Statistical analysis performed with Wilcoxon signed-rank test
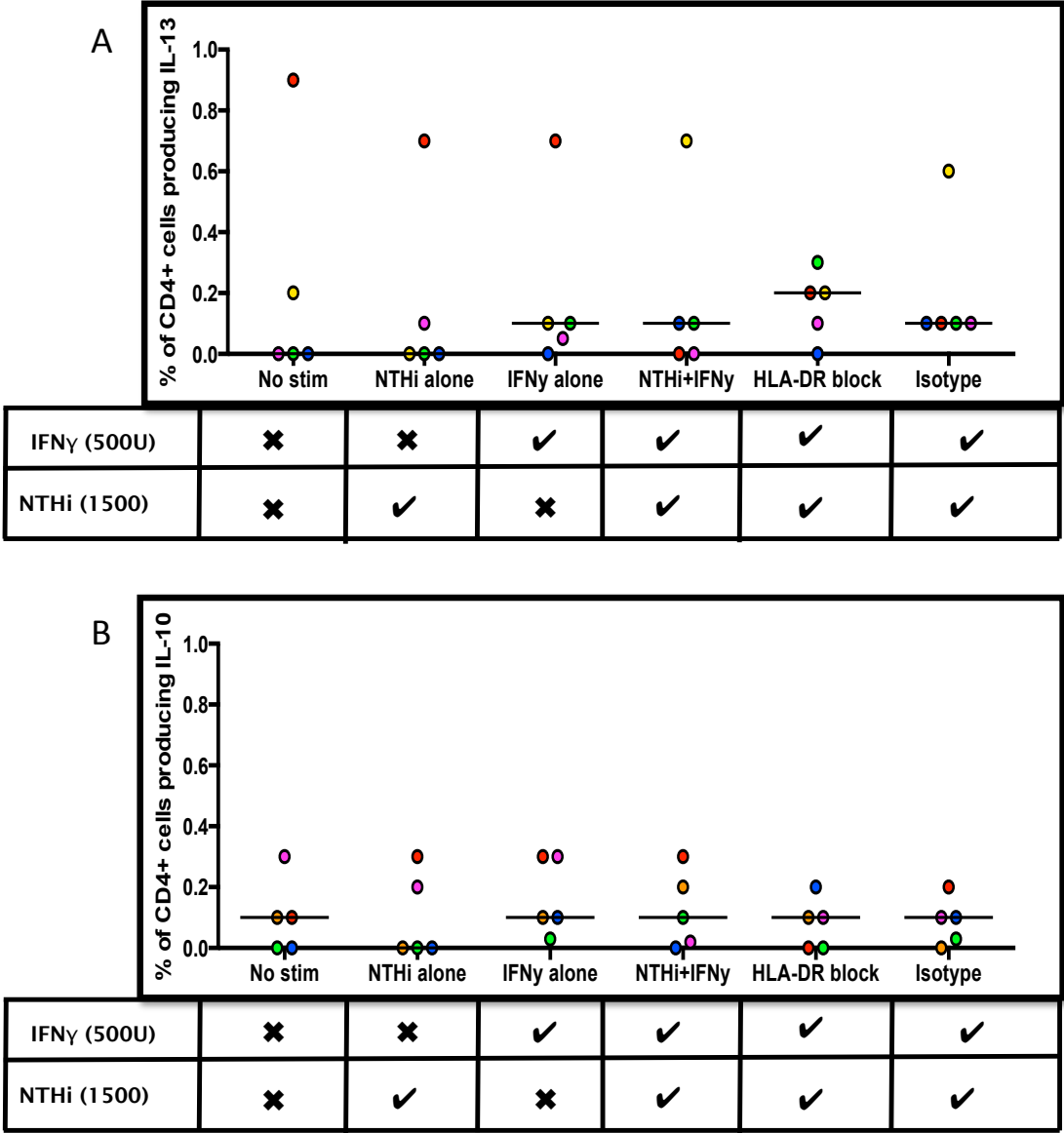
Figure 5-18-The production of IL-13 and IL-10 by CD4 sorted lung T helper cells with autologous lung fibroblasts: Human lung fibroblasts were exposed to heat-killed NTHi and/or IFNγ for 48 hours. After exposure, autologous lung-derived, NTHi-exposed T helper cells (previously purified via MACS sorted for CD4 expression) were cultured with these fibroblasts at a ratio of 25,000 fibroblast to 100,000 T cells. In some cases, HLA-DR blocking antibody or corresponding isotype control were introduced to NTHi+IFNγ exposed fibroblast cultures. Shown above is the resultant production of IL-13 (A) and IL-10 (B) by T helper cells after co-culture.

5.3   DISCUSSION

The results in this chapter demonstrate fibroblasts are able to function as inducible APCs under inflammatory conditions. The experimental model used to examine this interaction involves generating NTHi-specific T helper cells from human lung samples. In designing this model, T cell responses to NTHi were tracked from initial isolation from the lung, up to restimulating resultant T cell lines after allowing expansion of NTHi-specific T cells. The initial response to NTHi after isolation is characterised primarily by IFNγ production by helper, cytotoxic and DN T cells. T helper cells also contained a population of IL-17A producing cells, indicating that both Th1 and Th17 subsets are responsive to NTHi. Both of these subsets go on to proliferate and become the dominant cell types present in T helper cell lines. These T helper cell lines were re-stimulated by co-culture with either NTHi-pulsed autologous moDCs or lung fibroblasts. Paring T helper cells with autologous moDCs confirms the antigen-specificity of the T cell lines generated though NTHi exposure, and provides a reference for optimal antigen-dependent activation. This level of activation can then be compared to the level of activation provoked by fibroblasts as APC, comparing the structural and professional APC types.

One of the primary strengths of this study is the use of lung-derived lung fibroblast and T cell populations together. This more accurately represents the cellular interaction occurring in the human lung *in vivo*. A major caveat to this approach the availability of human tissue. All lung fibroblasts were obtained from subjects undergoing resection surgery, and while small amounts of tissue suffice for fibroblast outgrowth (see section 2-6), larger samples were required for both fibroblast and T cell isolation. Particularly for the generation of lung T cell lines to NTHi, larger tissue samples were required in order to isolate sufficient numbers of T cells. The use of human lung samples for these assays also presents issues regarding underlying pathological conditions that may impact results. Patients in this cohort were classed as COPD or non-COPD based upon lung function data, and while analysis of differences between these groups shows no difference (chapter 3), studies with larger cohorts do report significant T cell responses to NTHi in COPD subjects compared to non-COPD equivalents [130]. Therefore we cannot rule underlying lung pathology, such as COPD or similar, impacting the co-culture results observed in this study. Further co-culture

analyses using clearly defined, well-characterised subject cohorts may be able to examine differences between disease states in this model. However, due to time limitations and the scarcity of lung samples, all samples of adequate size and negative for major infectious (for example, HIV or tuberculosis) were used as a source of fibroblasts and lung T cells in this study.

MoDC-T helper cell co-culture demonstrated a strong Th1 response as seen by the production of IFNγ, with a smaller population of IL-17A producing Th17 cells also activating. In both cases, activation was dependent upon the presence of NTHi and was suppressed by HLA-DR blocking antibodies, confirming antigen presentation of bacterial antigens via MHC class II. Co-cultures using fibroblasts as APCs showed a similar trend, with both Th1 and Th17 cells activating as a result of antigen presentation. In this case, fibroblasts required both IFNγ and NTHi exposure to cause T cell activation, whereas moDCs required only NTHi. This is most likely due to fibroblasts requiring IFNγ to express HLA-DR, whereas moDCs have constitutive baseline expression of this critical molecule [192]. In cases where CD8 T cells were cultured with fibroblasts, activation did not follow any discernable trend in terms of HLA-DR sensitivity. Activation of CD8 T cells may have occurred due to presentation of antigens via MHC class I, which fibroblasts are known to express [193], however this was not examined in our co-culture model.

Comparing the magnitude of T helper cell responses between either APC shows moDCs have a greater ability to stimulate Th1 cells, while both APCs appear to stimulate Th17 cells to a similar degree. While this may suggest fibroblasts preferentially activate Th17 cells over Th1 cells, and to the same degree as a professional APC, it is important to note that a direct comparison between fibroblasts and moDCs from the same subject, using the same T cell line, was not carried out. Performing this head-to-head analysis is the only way to conclusively compare the ability of each APC to differentially activate T helper subsets. In all co-cultures there was little (if any) activation of IL-13 and IL-10 producing cells. This is most likely due to the fact that the long-term cultured T cell lines contains no IL-10 or IL-13 producing cells. Therefore, the experimental model used in this study is not suitable for examining the relationship between APC and NTHi-specific Treg and Th2 subset.

The initial CD4 and CD8 responses to NTHi demonstrated in this study are similar to previous results using a live-infection NTHi model [130]. Using similar lung-tissue isolation methods to our study, the response to live NTHi was characterised via intracellular cytokine production. Here, the T helper response was characterised by IL-17 (0.1% of CD4 cells), IFNγ (less than 0.01% CD4 cells) and IL-13 (0.01% of CD4 cells) cytokine production- values that are similar to the initial T helper response in our study using heat-killed NTHi. The CD8 response was also comparable, with predominantly IFNγ producing cells activating. Similar to the results in the current study, there was notable variability in the response to NTHi across all cytokine readouts. Of particular note is the observation that the response to NTHi what characterised by an increased T cell IL-13 signal in COPD subjects compared to healthy equivalents. Our findings show considerable spread in the CD8 IL-13 response, which in part may be due to underlying lung disease within some of these subjects. However, due to the relatively low numbers in the cohort examined, it is not possible to draw conclusions over the differences in responses due to lung disease (the study performed by King analysed a cohort of 30 non COPD and 39 COPD subjects. Our study contained a total of 14 subjects from all disease categories).

In addition to CD4 and CD8 T cells, there was a sizeable population of CD3+CD4-CD8- "double negative" T cells. This cell fraction has been shown to be involved in responses to intracellular pathogens. A murine model of pulmonary *Francisella tularensis* infection demonstrated that DN T cells rapidly respond via robust (and ultimately protective) IL-17 and IFNy production. *F.tularensis* is a facultative intracellular bacteria known to infect macrophages [194], similar to NTHi. Further work has demonstrated that DN cells responding to *F.tularensis* contain the recently described MAIT cells, which respond to bacterial-based vitamin B metabolites [49]. Furthermore, these MAIT cells are also involved in combating tuberculosis, another lung-based intracellular pathogen [195]. In chapter 3 we demonstrate that a proportion of DN T cells express the MAIT cell markers CD161 and T cell receptor Vα7.2 chain, suggesting MAIT cells are present within these human distal lung samples. Taken together, it is possible that NTHi-responding DN populations in our study contain a number of these MAIT cells.

After initial characterisation of the T cell response to NTHi, lung leukocytes were exposed to heat-killed bacteria to drive the proliferation of NTHi-specific T cells over a 4-week period. CD4 T helper and CD8 cytotoxic T cells are differentially activated depending on the means by which APCs receive antigen. Cytosolic-based antigens (such has intercellular pathogens actively infecting an APC) favour a CD8 response [196]. Exogenous antigens that must be internalized typically promote CD4 responses [197, 198]. Therefore administering NTHi- exogenously should preferentially drive T helper cell activation and proliferation. As our results show, exposure to NTHi and IL-2 results in expansion of T helper cells over cytotoxic T cells. It should be noted that these early experiments are based upon using only IL-2 to boost T cell proliferation- later cultures used both IL-2 and IL-7 to boost NTHi-specific proliferation to maximise cell numbers and survival [199]. However, when these NTHi exposed IL-2+IL-7 boosted cultures are examined immediately prior to co-culture, they still contain predominantly T helper cells, indicating this culturing technique still preferentially drives T helper cell proliferation.

This culture process provided a large number of autologous T helper cells for use in co-culture assays. However, it may promote the expansion of certain T helper subsets over others, skewing the overall T helper population subset profile. Therefore it was necessary to examine the subset proportions after long-term culture after NTHi exposure. Though using PMA+ionomycin stimulation to nonspecifically activate all T cells from long-term cultures exposed to NTHi, Th1 cells appear to be the dominant subset present. There is also an increase in the proportion of IL-17A producing cells. In contrast, the IL-10 and IL-13 producing populations are no longer present. Further analysis by tracking individual subject T cell lines from initial activation to the final T cell line used in co-culture reveal almost all IL-10 and IL-13 producers are no long present, even if initially detectable in a particular subject. This may be explained by the dominance of initial IFNγ and IL-17A responses to NTHi, which then go on proliferate and overgrow Treg and Th2 cells. Ideally, all subject responses to NTHi and PMA+ionomycin would be tracked from initial isolation up to 4 weeks of culture after line development. This would allow longitudinal tracking of the subjects T helper subsets as the T cell line develops. However, due to limitations on isolating cells from human tissue, this was not possible for all cases. Using whole heat-killed bacteria may also skew the T helper cell subsets. Bacterial-based membrane lipoproteins have been

shown to promote IL-17 production from T helper cells in response to a specific antigen [200]. Interestingly, the mechanism driving this appears to be due to activation of the APC interacting with the T helper cell rather than directly upon the T cell itself. A similar mechanism with NTHi may promote the expansion and/or development of lL-17A T cells in our culture model.

Therefore, a major caveat to this study was NTHi T cell line generation resulted in the loss of Th2 and Treg cells. As a result, this experimental model was unsuitable to demonstrate fibroblast antigen presentation and activation of Th2 or Treg cells. To explore fibroblast interaction with Treg or Th2 cells, altering the culture conditions during exposure to antigen and development of the T cell line may be required. For example, exposing T cells to a candidate antigen in the presence of exogenous IL-4 or thymic stromal lymphopoiten (TSLP) may drive the proliferation and development of Th2 cells [201, 202]. Whether this approach would work on T cells isolated upon the lung is unclear. Memory T cells that have been committed to a Th1 phenotype long-term may be refractory to alterations in their phenotype and essentially terminally differentiated to their initially primed subset [40, 203]. An alternative method would be to prime blood-derived, naïve T cells to a specific antigen under Th2 or Treg polarizing conditions. Naive T cells, which have not committed to any T helper effector lineage, are much more receptive to conditioning factors [40]. Therefore priming of naïve T cells by blood-derived APCs such as moDCs to an antigen in the presence IL-4 of should promote formation of Th2 cells [204] while exposure to TGFβ should drive Treg polarization [37, 205]. Using polarized blood-derived T cells in a fibroblast co-culture model like the one used in this study may allow an examination of fibroblast antigen presentation to Th2 and Treg cells. A drawback of this approach would be that any conditioning of the T cells that occurs in the lung tissue would be lost. Local conditioning in the lung affects the phenotype of the T cell populations. For example, resident memory T cell populations, which appear to form after entry into the lung environment and persist their long-term, would not be present in blood-derived T cell cultures [60]. The loss of such phenotypically distinct memory T cell subsets may impact the ability to accurately investigate lung T cell-fibroblast interactions *in vitro*.

The ability of fibroblasts to activate Th1 and Th17 cells in response to NTHi suggests fibroblasts are able to promote anti-microbial responses. A robust response to NTHi is known to require the activity of neutrophil and macrophage populations [206],

both of which are influenced by Th1 and Th17 cells respectively. We also demonstrate possible feedback mechanisms between these T helper subsets and lung fibroblasts. In our study, IFNγ clearly drives MHC Class II expression on human lung fibroblasts (see section 4-9). Critically, lung fibroblasts do not seem to be able to present antigen without conditioning with this cytokine. In this regard, interaction between T helper cells and fibroblasts resulting in IFNγ production may represent a positive feedback loop, promoting further MHC class II expression on both the interacting fibroblast and other cells in the immediate vicinity. A reciprocal mechanism may also exist between fibroblasts and Th17 cells. IL-17 mediates neutrophilic responses primarily through acting upon non-haematopoietic cells such as fibroblasts. Exposure to IL-17 provokes the release of IL-6, IL-8, G-CSF and if exposed in conjunction with TNF- α, the release of GM-CSF [207]. All of these cytokines are heavily involved in the recruitment and activation of neutrophils to the local area. Cytokines such as IL-6 and TGFβ produced by fibroblasts are also known to promote Th17 development and activation, and antigen presentation by these cells may skew interacting T cells into a Th17 phenotype. This is particularly relevant for T reg cells, which share similar cytokine requirements to Th17 cells and significant plasticity is though to exist between these cells, driven by cytokines in the local environment [208]. The ability of lung fibroblasts to activate and interact with IL-17A producing Th17 cells would allow the same fibroblasts to drive neutrophillic inflammation in the local area. This interaction may represent a key mechanism in promoting neutrophilic responses to microbial insults in the human lung.

6. DISCUSSION AND FUTURE WORK

6.1   Introduction

This study aimed to address whether lung fibroblasts possess the capability to present bacterial antigen to lung-derived T helper cells. Through using autologous, lung-derived populations we were able to accurately investigate this interaction, overcoming issues surrounding the use of allogenic models or PBMC derived T cells. Therefore the experimental model used in this study represent more closely the interaction occurring in the human lung environment. The results presented in this thesis demonstrate that human lung fibroblasts are able to present antigen to T helper cells, causing activation of proinflammatory Th1 and Th17 cells as measured though cytokine production. Here I will discuss these finding and the possible implications in the wider context of lung immunology as well as highlight key areas where this work could be further developed.

6.2   Memory T cells are abundant in the lung

Our analysis shows the vast majority of lung T cells are CD45RO expressing memory cells, primarily CD45RO+CCR7- effector memory type cells. Results indicate virtually no naïve cells present in the distal lung environment. This corroborates findings from similar human lung studies [69] and other nonlymphoid organs like the skin [209] that show these peripheral tissues are rich sources of memory-type T cells. Memory cells are generated after naïve T cells are presented with their cognate antigen and activated by professional APCs. Therefore there are a huge number of memory T cells stationed in the lung (1 x 10^{10} T cells during the steady state [67] which have antigen-specificity for common lung pathogens like *influenza* responders [60, 69], NTHi [130] and RSV [210]. Comparing the relative responder frequencies of both lung and blood T cells to *influenza* challenge demonstrates that lung-pathogen specific T cells are highly enriched in the lung compared to splenic and skin populations [69]. These vast numbers of antigen-specific T cells require interaction with local APC populations to effectively mediate immunity in the organ

6.2.1   Lung tissue resident memory populations and fibroblast interaction

Recent developments have revealed the existence of tissue resident-memory T cell populations. These long-lived T cells appear to lodge within the lungs and do not

recirculate as other effector-memory T cells are thought to [211, 212]. Tissue resident T cells do not rely upon replenishment from circulatory sources and exist in distinct microenvironmental niches within the lung, where they are responsible for rapid and protective responses to lung pathogens [60, 67]. Generation of these highly specialized T cell subsets appears to require infection via the respiratory pathway rather than via the systemic route [213]. Perhaps most interestingly of all, resident memory T cells do not seem to exist in ectopic, organized lymphoid structures (such as bronchus-associated lymphoid tissue), which are known to occur during certain acute infections [5, 214]. Rather, resident memory cells appear to be more diffusely stationed within the tissue, particularly in the submucosal and immediate subepithelial zones [60].

This growing body of work shows that tissue resident memory T cells must be reliant upon cells of the local microenvironmental niche in which they are stationed in order to function correctly. Complex interplay between multiple cell types here may be required for effective responses in these distal locations. For example, the production of IFNγ by T helper cells appears to be critical in generating an effective CD8 resident memory T cell response to *influenza* and recruitment of these cells to the correct areas of the lungs [65, 215]. Antigen-specific activation by a tissue APC is most likely required in order to cause IFNγ production by T helper cells in this scenario. As fibroblasts reside within the submucosal areas where these resident memory cells are stationed, fibroblast antigen-presentation may contribute to activation, regulation and maintenance of this critically important class of T cell. Functional assays in our study demonstrate that fibroblasts present antigen the IFNγ producing Th1 subset. Activation of Th1 cells via fibroblast antigen-presentation may represent a critical step in conditioning the local environment in early phases of an immune response in the lung.

6.3 Fibroblasts contribute to activation of T helper cells by antigen presentation

In chapter 4 we demonstrate through *ex vivo* examination that lung fibroblasts express HLA-DR within the human lung environment.. While lower than expression levels upon macrophages and epithelial cells, it does suggest fibroblasts are engaged in antigen presentation within the human lung environment. Expression by both fibroblast and epithelial cells here corroborates previous work on mice, demonstrating that many CD45- nonhaematopoietic cells are MHC class II+ cells [173]. We go on to

demonstrate that fibroblast possess the ability to activate T helper cells antigen-specifically. What we do not show is how the nature of the T cell response generated by fibroblasts is affected by different antigen sources.

Professional APCs, like dendritic cells, are able to modulate the nature of T cell activation though releasing different cytokines at the same time as presenting antigen. These cytokines are variably produced depending upon the pathogen encountered, and influence the activity of the T helper cell interacting with the APC. For example, microbial lipopeptides from *B.burgdoferi* (the cause of Lymes disease) promote antigen-specific imprinting of a Th17 phenotype [200], while listeria exposed APCs preferentially drive Th1 phenotype though IL-12 release [34]. It seems possible from our results that a constituent of NTHi promotes the activation and expansion of Th1 and/or Th17 cells (as seem by PMA+Ionomycin activation of NTHi exposed cultures after 4 weeks- figure 5-6). As initial expansion of T cell lines was done on whole lung cell preparations, this effect may have been initially orchestrated through NTHi effecting a number of different tissue-resident APCs during T cell line development stage. However our co-culture results indicate that NTHi-activated fibroblasts also are able to readily promote and activate Th1 and Th17 cells, suggesting fibroblasts are also sensitive to NTHi-derived factors and promote activation in a similar manner. The ability of an APC to respond to varying pathogens encountered is mediated through innate receptors such as TLRs. Ligation of different TLRs instructs the APC to selectively release cytokines that go on to influence T cell activation. Bacterial pathogens are known to have varying abilities to trigger TLRs and cause inflammatory activation of cells [216], and such mechanism may affect fibroblast APC behaviour. NTHi constituents such as the outer membrane protein P6 and P2, both highly conserved lipoprotein factors, are known to act on macrophages resulting in the production of TNFα and IL-8 [217]. Conversely, neither of these membrane proteins appears to stimulate IL-12 production, suggesting they are not responsible for promoting Th1 responses [40]. As expansion of Th17 cells appears to occur after NTHi exposure and long-term culture (figure 5-6), this may suggest a NTHi-derived factor promotes activation of Th17 cells.

6.3.1 Fibroblasts may promote a switch from regulatory to T helper 17 cells

The switch in T helper phenotype from one effector subset to another is known as T cell plasticity. The ability of T helper cells to switch from one subset profile to another may be important in the transition from an anti-inflammatory to proinflammatory state during a pathogenic insult. Treg cells are thought to be particularly sensitive to phenotypic switch in peripheral tissue zones [218]. Treg cells usually promote tolerance and suppress inflammation via mechanisms like the release of IL-10 [219]. In the context of the mucosal environment, this may allow the immune system to restrain inflammation to commensal-type bacteria during the steady state. However, it is thought that Treg cells are able to convert to the potent inflammatory Th17 subset in certain situations. A rapid switch to a proinflammatory state would allow Tregs to quickly respond to pathogenic insults in the peripheral areas. [218]. Evidence suggests IL-6 produced by fibroblast-like cells of the joints mediates the switch from Treg to Th17 phenotypes [220]. Lung fibroblasts are also known to produce significant amounts of IL-6 in response to stimulation with the bacterial constituent LPS and the cytokine TNFα [221], suggesting NTHi exposed fibroblasts would be capable of driving Treg to Th17 transition through this mechanism. Furthermore, stromal fibroblast populations in the gastric mucosa have been shown to promote the generation of Th17 cells through cytokine production after infection with the bacteria *Helicobacter pylori [222]*. We show that fibroblasts readily activate Th17 cells in response to a specific bacteria. As NTHi is considered a colonizing bacterium in most individuals with no underlying lung pathology [223], it is possible that Treg cells restrain inflammatory responses under normal circumstances to prevent chronic inflammation [224]. The switch from regulatory to a proinflammatory phenotype may be mediated in part by fibroblasts in the mesenchyme. Uptake of NTHi and the effect NTHi exposure has upon the fibroblasts cytokine release profile would allow antigen-specific conversion of Treg cells to Th17 cells in circumstances where NTHi gains access to the fibroblast compartment.

6.4 Fibroblasts may have a preferential capacity to activate Th17 cells

Fibroblasts present bacterial antigen and cause activation of Th1 and Th17 cell types. However, activation by antigen-bearing moDCs causes markedly greater activation of Th1 cells (figure 5-11). This may be due to increased HLA-DR;antigen complexes

upon the moDC surface, or due to production of soluble or cell-bound factors by the moDC that facilitate Th1 activation. Interestingly, Th17 cells are activated to a similar degree by both APCs. This may suggest fibroblasts have a specialized ability to activate Th17 rather than Th1 cells. The overall Th17 population used for these assays is much smaller than the Th1 population after NTHi exposure and line development (figure 5-6), and the small yet similar levels of stimulation by both APCs may be reflective of the fact there simply aren't as many of the cells present. Due to limited T cell numbers, it was not possible to do both a fibroblast and moDC co-culture using the same T cell line. This head-to-head comparison would allow a direct comparison of Th17 activation by both APCs. Work done in other anatomical locations demonstrate fibroblasts readily produce cytokines such as IL-6, IL-21, IL-1β and TGFβ in response to bacterial infection, resulting in development of Th17 cells [222]. Asthmatic bronchial fibroblasts also appear to promote Th17 development through release of similar factors [225]. The production of these cytokines suggests fibroblasts facilitate activation of Th17 cells.

6.5 The potential for fibroblast interaction with novel cytotoxic CD4 cytotoxic T cells

The ability to express MHC class II allows antigen-specific interaction with cells expressing CD4, while CD8 expressing cells are restricted to MHC class I bound antigens. CD4 T cells are conventionally thought to exert control of infections through directing the wider immune response via cytokine production. However, studies are continuing to demonstrate novel effector subsets of CD4 T cells, including cytotoxic CD4 T cells [99, 226, 227]. Initial observations demonstrated that antigen-activated, mature CD4 T cells lose expression of the transcription factor "Th inducing POZ-Kruppel factor" (ThPOK) in response to microbial factors in the intestinal mucosa of animals [226]. Loss of ThPOK, which normally commits the cells to a T helper phenotype, resulted in upregulation of cytotoxic-related factors including CD244 [228], indicating ThPOX- CD4+ T cells are cytotoxic. This cytotoxic CD4 phenotype has also been shown in human cases of latent chronic tuberculosis infection [227]. Tuberculosis-specific CD4 cells in latently infected individuals expressed a ThPOX- phenotype, and were notably increased in latently infected individuals compared to healthy controls.

This suggests a situation whereby chronic inflammation promotes deregulation of conventional CD4 T cells and allows formation of a cytotoxic CD4 subset. Further evidence of CD4 cytotoxic T cells in human lung disease come from *influenza* studies [99]. Previously unexposed, seronegative individuals generated CD4 T cells responding to *influenza* challenge, which correlated with less severe symptoms and viral shedding. This suggests CD4 T cell mediated immunity is an important factor in viral defence in unexposed individuals with no pre-existing antibody-based immunity. A number of CD4 T cells in this model express CD107a after antigen challenge *ex vivo*, indicating recent degranulation by these cells [106] and CD4 T cells were demonstrated to kill *influenza*-pulsed APCs [99]. These studies collectively suggest an important role of CD4 cytotoxic T cells in providing protective responses to pathogens through direct killing of infected cells.

The expression of CD4 limits the T cell response to MHC class II bound antgeins, suggesting only MHC class II expressing cells are the targets of these cytotoxic CD4 T cells. *Influenza* infects lung epithelial cells, and drives increased expression of MHC class II on these cells [99], which taken alongside the previous evidence of cytotoxic capabilities of these CD4 T cells, suggests cytotoxicity is directed against infected epithelial cells in this model. Through exposure to IFNγ, lung fibroblasts express MHC class II and are functionally capable at presenting antigen to CD4 T cells. This strongly suggests that fibroblasts may also be a target of CD4 cytotoxic action during infection of a fibroblast. Investigations into this area may indicate a novel interaction involved in lung immune responses, similar to the emerging role of cytotoxic CD4 cells in targeting epithelia cells.

6.6   The lung fibroblast as a phagocyte in the mesenchyme

Through both flow cytometry and confocal microscopy, we show that fibroblasts readily internalize both sugar and protein candidate antigen particles. Internalization of environmental antigen in this way typically results in loading onto MHC class II molecules and thus favours T helper responses [197, 229]. Previous work has shown that fibroblasts are capable of phagocytosing senescent neutrophils to a similar degree as macrophage populations [230]. Phagocytosis of scenescent neutrophils most likely

plays an important role in the resolution phase of an immune response. However uptake of apoptotic cells has also shown to provide a source of antigen for the internalizing cell; effectively "delivering" antigen to APCs that is subsequently presented [231]. A similar mechanism may contribute to lung fibroblasts obtaining antigen for presentation. This form of uptake may be particularly relevant in situations where macrophages (which also uptake dying neutrophils) are overwhelmed or impaired and neutrophils are recruited into the mesenchyme.

Fibroblasts have also been demonstrated to be an infectious target for the bacteria *Staphylococcus aureus* via a fibronectin dependent mechanism [232, 233]. This receptor-mediated form of internalization has been postulated as a means by which bacteria gain protection from antibiotic clearance and long-term reservoirs of infection. However, this mechanism may also provide a means by which fibroblasts obtain bacterial antigen for subsequent presentation. We demonstrate fibroblasts are able to present NTHi antigens and activate T helper cells. While internalization of NTHi is not specifically demonstrated, NTHi is known to express fibronectin adhesins, similar to *S.aureus* [234]. Therefore NTHi may be internalized by fibroblasts in a fibronectin-dependent manner. Further studies examining this may indicate the means by which NTHi invades the mesenchyme and is taken up by fibroblasts for antigen presentation.

6.7 Lung fibroblasts may not process antigen and MHC class II may bind extracellular proteins directly

We have demonstrated that fibroblasts upregulate surface MHC class II after IFNγ exposure, and that this is critical for antigen presentation to T helper cells. However our efforts to examine intracellular processing of antigen were inconclusive. It has been shown that immature dendritic cells contain large amount of cell-surface MHC class II with no bound antigen, and can go on to directly bind extracellular proteins and stimulate antigen-specific T cell responses [235] These assays make use of defined, short peptide sequences that may not require processing in order to bind the MHC class II molecule. In contrast, whole heat-killed bacteria such as the NTHi used in our study may require internal processing to generate the correct sized antigens for MHC class II loading. Therefore we cannot rule out direct binding of NTHi antigens to surface MHC class II, although this seems highly unlikely. Considering the fact professional APCs have been demonstrated to use surface loading of MHC class II and go on to stimulate T

cells, a similar mechanism may also exist in fibroblasts and contribute to immune responses in the lung.

6.8 Fibroblast antigen presentation may contribute towards inflammation in bacterial pneumonia and in COPD

Subjects with COPD and smoking history are known to have increased Th17 lung activity [236] as well as a stronger Th17 cell response to NTHi challenge [130]. While NTHi is known to associate with disease exacerbations, it is not understood fully how it contributes to the pathogenic process. Additionally, studies into secondary bacterial pneumonia have demonstrated bacterial invasion of the mesenchyme coincides with massive neutrophil recruitment [17]. Viral infection in this case appears to prime the lung environment for bacterial pneumonia though impairing conventional immune defences such as alveolar macrophages [237, 238] We demonstrate that fibroblasts are able to present NTHi and activate Th17 cells. Even though the percentage of Th17 cells reactivated by fibroblasts is small, the Th17 populations in general are considered proportionally minor (See section X in chapter 3- IL-17A proportions)[239-241]. Despite their small numbers, Th17 cells are known to be potent mediators of neutophilic inflammation [42]. Taken together, this may suggest that fibroblast antigen presentation to Th17 cells during bacterial invasion of the mesenchyme is a major mechanism in recruiting the large numbers of neutrophils observed in pneumonia. This may represent a final layer of immune defence after alveolar macrophages and the lung epithelium. Beyond the fibroblast layers, infection is able to reach the circulation and may lead to systemic infection if left unimpeded. As such, a robust and massive neutrophilic response driven in part by fibroblasts may act as a last-resort response by the immune system to prevent dissemination of infection systemically.

6.9 FUTURE WORK

A number of key experimental assays in this study contain low sample numbers, particularly APC-T cell co-cultures making use of lung tissue samples. Expanding the size of the experimental cohort for these co-cultures would increase the confidence of the results observed to date. Furthermore, through increasing the cohort size and carefully characterizing enlisted subjects for underlying lung pathologies or conditions, including (but not limited to) COPD endotypes, asthma, smoking history, steroid use

and exacerbation history, disease and condition specific differences in fibroblast-T cell interaction could be accounted for. Currently, it is not possible to discern the effects any of these factors may have on fibroblast APC function due to the low numbers of subjects analysed.

Co-culture assays using subject-matched lung T cells and APC populations should continue. A priority area should be a direct head-to-head comparison of fibroblast and moDCs capability to activate the same NTHi-specific T cell line. Data in chapter 5 suggests that fibroblasts may have a specialized role in activating Th17 cells to a similar degree as professional APCs. Through concurrently examining both APCS using the same T cell line, it would be possible to accurately compare of the differential ability to reactivate memory cell subsets by either APC.

MHC class II antigen presentation by fibroblasts is key to activating T helper cells as shown by blocking experiments in section 5-17. It is likely that a number of other co-stimulatory factors are also produced by fibroblasts that contribute to T cell activation. Using RNA seq transcriptome analysis, transcription of immune relevant factors by fibroblasts could be examined both at resting state and after IFNγ exposure. As fibroblasts only activate T helper cells after IFNγ preconditioning in our model, a comprehensive analysis of the proteins involved in antigen processing and presentation, cell surface T cell costimulation and soluble cytokines could be performed. As IFNγ stimulation drives the MHC class II processing pathway, upregulation of factors such as the cathepsin family molecules, LAMP-1 and other internal processing machinery critical to peptide loading could be examined via this technique. Simultaneous examination for the production of a broad number of cytokines by fibroblasts after IFNγ, NTHi and both factors could also be performed. This should focus on fibroblast production of cytokines involved in the activation and maintenance of Th1 and Th17 cells.

In addition to molecular techniques to gain a comprehensive understanding of fibroblast mechanisms relevant to T cell activation, flow cytometry analysis could also be used. Characterizing fibroblast expression of innate receptors such as the TLRs, which allow cells to detect and activate in response to PAMPs, would indicate how the cell is able to detect pathogens and modulate their activation state accordingly. As these receptors are expressed on cells in an inactive, steady state, they may not be actively

transcribed when unstimulated and therefore not detectable at baseline via RNA measurement. Therefore measuring protein expressing directly via flow cytometry would allow detection of these molecules on resting cells.

While presentation of NTHi by fibroblasts has been shown in this study, examining the ability of lung fibroblasts to present antigen from other lung pathogens would build a greater understanding of the contribution of fibroblasts to lung immune responses. Using similar T cell line development to specific pathogens (such as *influenza)* would demonstrate any for fibroblasts to promote distinct T cell responses depending on the nature of the pathogen encountered (similar to professional APCs).

Functional assays assessing the ability of lung CD4 T cells to exert cytotoxic killing of antigen-presenting fibroblasts would demonstrate a novel mechanism in lung immunology. As shown with multiple studies in the lung, cytotoxic CD4 T cells are important in responding to both bacterial [227] and viral [99] lung pathogens. Fibroblast MHC class II expression and functional antigen presentation have been shown in this current study. This presents the possibility of fibroblasts becoming a target for cytotoxic CD4 T cells as a result of this antigen-presenting capacity. Therefore, analysis of NTHi specific CD4 T cells for markers of cytotoxic functionality (eg loss of the transcription factor ThPOK) or recent degranulation after antigen challenge (CD107 expression) could be carried out via flow cytometry. A functional killing assays using chromium-51 could also be performed. Fibroblasts labelled with chromium-51 and allowed to present antigen to antigen-specific CD4 sorted T cells would allow accurate detection of CD4-mediated lysis via chromium-51 release [242]. This would require both characterisation of initial T cell responders *ex vivo,* as well as re-challenge models potentially using co-culture methodology similar to that used for measuring cytokine release. Thorough analysis of NTHi-specific lines would be necessary to examine if CD4 cytotoxic T cells are lost or expanded by driving T cell lines to specific antigen *in vitro*.

Human based systems do not allow for direct examination of T helper cell-fibroblasts interaction during events such as bacterial pneumonia. Gaining regular human samples from subjects with bacterial pneumonia to further examine fibroblast-T cell dynamics is highly unlikely due to the fact such individuals are not considered for surgery while suffering pneumonia. Microscopic studies examining bacterial invasion

in the human lung are limited to cadaveric samples [117]. The study by Sheng only investigated the targets of viral infection in the lung and bacterial populations during pneumonia, and did not scrutinize factors such as HLA-DR expression on structural cells or the localization of T cell populations relative to stromal cells. Therefore, animal models could be used to scrutinize fibroblast antigen-presentation to T helper cells during bacterial invasion in much more detail. Animal models of bacterial superinfection such as those used by Damjanovic et al would allow examination of the role of fibroblast antigen presentation in this scenario [17]. Microscopic investigation during bacterial invasion would allow a greater understanding of the contribution of fibroblast-T helper cell interaction in propagating inflammation.

Summary

The aim of this study was to examine if human lung fibroblasts could serve as an antigen-presenting cell for lung T helper cells. Through both *in vitro* and *ex vivo* techniques, fibroblasts are shown to exhibit traits associated with APC function. Co-culture assays finally demonstrate that fibroblasts are able to activate T helper cells in an antigen-dependent manner, presenting antigen from a common lung bacteria to lung T cells and eliciting cytokine production. This interaction resulted in activation of proinflamamtory Th1 and Th17 cells, however the scope of *in vitro* assays is limited, and the role of fibroblast antigen presentation may be much more complex *in vivo*. Therefore this thesis suggests further work exploring the contribution of fibroblast-T helper cell interaction towards immune responses in the lung may provide novel insight into how lung immunity is co-ordinated in the submucosal areas. This may be of particular importance in diseases characterised by pathogenic infiltration of the epithelial barriers and into the mesenchyme.

References

1.   Erb-Downward, J.R., et al., *Analysis of the lung microbiome in the "healthy" smoker and in COPD.* PLoS One, 2011. **6**(2): p. e16384.
2.   Bals, R. and P.S. Hiemstra, *Innate immunity in the lung: how epithelial cells fight against respiratory pathogens.* Eur Respir J, 2004. **23**(2): p. 327-33.
3.   Amin, K., *The role of mast cells in allergic inflammation.* Respir Med, 2012. **106**(1): p. 9-14.
4.   Gosman, M.M., et al., *Association of mast cells with lung function in chronic obstructive pulmonary disease.* Respir Res, 2008. **9**: p. 64.
5.   Hogg, J.C., et al., *The nature of small-airway obstruction in chronic obstructive pulmonary disease.* N Engl J Med, 2004. **350**(26): p. 2645-53.
6.   Green, G.M. and E.H. Kass, *THE ROLE OF THE ALVEOLAR MACROPHAGE IN THE CLEARANCE OF BACTERIA FROM THE LUNG.* J Exp Med, 1964. **119**: p. 167-76.
7.   Steinmuller, C., et al., *Local activation of nonspecific defense against a respiratory model infection by application of interferon-gamma: comparison between rat alveolar and interstitial lung macrophages.* Am J Respir Cell Mol Biol, 2000. **22**(4): p. 481-90.
8.   Losa Garcia, J.E., et al., *Evaluation of inflammatory cytokine secretion by human alveolar macrophages.* Mediators Inflamm, 1999. **8**(1): p. 43-51.
9.   Altman, J.D., et al., *Phenotypic analysis of antigen-specific T lymphocytes.* Science, 1996. **274**(5284): p. 94-6.
10.   Davis, M.M., et al., *Ligand recognition by alpha beta T cell receptors.* Annu Rev Immunol, 1998. **16**: p. 523-44.
11.   Guenot, M., et al., *Phosphoantigen Burst upon Plasmodium falciparum Schizont Rupture Can Distantly Activate Vgamma9Vdelta2 T Cells.* Infect Immun, 2015. **83**(10): p. 3816-24.
12.   Goldrath, A.W. and M.J. Bevan, *Selecting and maintaining a diverse T-cell repertoire.* Nature, 1999. **402**(6759): p. 255-62.
13.   Babbitt, B.P., et al., *Binding of immunogenic peptides to Ia histocompatibility molecules. 1985.* J Immunol, 2005. **175**(7): p. 4163-5.
14.   Starr, T.K., S.C. Jameson, and K.A. Hogquist, *Positive and negative selection of T cells.* Annu Rev Immunol, 2003. **21**: p. 139-76.
15.   Ramsey, C., et al., *Aire deficient mice develop multiple features of APECED phenotype and show altered immune response.* Hum Mol Genet, 2002. **11**(4): p. 397-409.
16.   Sim, B.C., et al., *Thymic skewing of the CD4/CD8 ratio maps with the T-cell receptor alpha-chain locus.* Curr Biol, 1998. **8**(12): p. 701-4.
17.   Damjanovic, D., et al., *Marked improvement of severe lung immunopathology by influenza-associated pneumococcal superinfection requires the control of both bacterial replication and host immune responses.* Am J Pathol, 2013. **183**(3): p. 868-80.
18.   Basu, S., et al., *Necrotic but not apoptotic cell death releases heat shock proteins, which deliver a partial maturation signal to dendritic cells and activate the NF-kappa B pathway.* Int Immunol, 2000. **12**(11): p. 1539-46.
19.   Iwasaki, A. and R. Medzhitov, *Toll-like receptor control of the adaptive immune responses.* Nat Immunol, 2004. **5**(10): p. 987-95.
20.   Lim, J.P. and P.A. Gleeson, *Macropinocytosis: an endocytic pathway for internalising large gulps.* Immunol Cell Biol, 2011. **89**(8): p. 836-43.

References

21.   Stuart, L.M., et al., *Response to Staphylococcus aureus requires CD36-mediated phagocytosis triggered by the COOH-terminal cytoplasmic domain.* J Cell Biol, 2005. **170**(3): p. 477-85.

22.   Platt, C.D., et al., *Mature dendritic cells use endocytic receptors to capture and present antigens.* Proc Natl Acad Sci U S A, 2010. **107**(9): p. 4287-92.

23.   Beers, C., et al., *Differential regulation of cathepsin S and cathepsin L in interferon gamma-treated macrophages.* J Exp Med, 2003. **197**(2): p. 169-79.

24.   Denzin, L.K., et al., *Right place, right time, right peptide: DO keeps DM focused.* Immunol Rev, 2005. **207**: p. 279-92.

25.   Blum, J.S., P.A. Wearsch, and P. Cresswell, *Pathways of antigen processing.* Annu Rev Immunol, 2013. **31**: p. 443-73.

26.   Schmid, D., M. Pypaert, and C. Munz, *Antigen-loading compartments for major histocompatibility complex class II molecules continuously receive input from autophagosomes.* Immunity, 2007. **26**(1): p. 79-92.

27.   Aichinger, M., et al., *Macroautophagy substrates are loaded onto MHC class II of medullary thymic epithelial cells for central tolerance.* J Exp Med, 2013. **210**(2): p. 287-300.

28.   Hrstka, R., et al., *Francisella tularensis strain LVS resides in MHC II-positive autophagic vacuoles in macrophages.* Folia Microbiol (Praha), 2007. **52**(6): p. 631-6.

29.   Schwartz, R.H., *A cell culture model for T lymphocyte clonal anergy.* Science, 1990. **248**(4961): p. 1349-56.

30.   Grakoui, A., et al., *The immunological synapse: a molecular machine controlling T cell activation.* Science, 1999. **285**(5425): p. 221-7.

31.   Bromley, S.K., et al., *The immunological synapse and CD28-CD80 interactions.* Nat Immunol, 2001. **2**(12): p. 1159-66.

32.   Meuer, S.C., et al., *Triggering of the T3-Ti antigen-receptor complex results in clonal T-cell proliferation through an interleukin 2-dependent autocrine pathway.* Proc Natl Acad Sci U S A, 1984. **81**(5): p. 1509-13.

33.   Mosmann, T.R., et al., *Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins.* J Immunol, 1986. **136**(7): p. 2348-57.

34.   Hsieh, C.S., et al., *Development of TH1 CD4+ T cells through IL-12 produced by Listeria-induced macrophages.* Science, 1993. **260**(5107): p. 547-9.

35.   Gao, J., et al., *An interferon-gamma-activated site (GAS) is necessary for full expression of the mouse iNOS gene in response to interferon-gamma and lipopolysaccharide.* J Biol Chem, 1997. **272**(2): p. 1226-30.

36.   Steimle, V., et al., *Regulation of MHC class II expression by interferon-gamma mediated by the transactivator gene CIITA.* Science, 1994. **265**(5168): p. 106-9.

37.   Ma, Y.L., et al., *IL-4-Producing Dendritic Cells Induced during Schistosoma japonica Infection Promote Th2 Cells via IL-4-Dependent Pathway.* J Immunol, 2015. **195**(8): p. 3769-80.

38.   Robinson, D.S., et al., *Predominant TH2-like bronchoalveolar T-lymphocyte population in atopic asthma.* N Engl J Med, 1992. **326**(5): p. 298-304.

39.   Desmouliere, A., et al., *Transforming growth factor-beta 1 induces alpha-smooth muscle actin expression in granulation tissue myofibroblasts and in quiescent and growing cultured fibroblasts.* J Cell Biol, 1993. **122**(1): p. 103-11.

40. Szabo, S.J., et al., *Regulation of the interleukin (IL)-12R beta 2 subunit expression in developing T helper 1 (Th1) and Th2 cells.* J Exp Med, 1997. **185**(5): p. 817-24.

41. Miyamoto, M., et al., *Endogenous IL-17 as a mediator of neutrophil recruitment caused by endotoxin exposure in mouse airways.* J Immunol, 2003. **170**(9): p. 4665-72.

42. Lin, L., et al., *Th1-Th17 cells mediate protective adaptive immunity against Staphylococcus aureus and Candida albicans infection in mice.* PLoS Pathog, 2009. **5**(12): p. e1000703.

43. Park, H., et al., *A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17.* Nat Immunol, 2005. **6**(11): p. 1133-41.

44. Korn, T., et al., *IL-17 and Th17 Cells.* Annu Rev Immunol, 2009. **27**: p. 485-517.

45. Arpaia, N., et al., *Metabolites produced by commensal bacteria promote peripheral regulatory T-cell generation.* Nature, 2013. **504**(7480): p. 451-5.

46. Fontenot, A.P., et al., *CD28 costimulation independence of target organ versus circulating memory antigen-specific CD4+ T cells.* J Clin Invest, 2003. **112**(5): p. 776-84.

47. Djuretic, I.M., et al., *Transcription factors T-bet and Runx3 cooperate to activate Ifng and silence Il4 in T helper type 1 cells.* Nat Immunol, 2007. **8**(2): p. 145-53.

48. Reantragoon, R., et al., *Antigen-loaded MR1 tetramers define T cell receptor heterogeneity in mucosal-associated invariant T cells.* J Exp Med, 2013. **210**(11): p. 2305-20.

49. Kjer-Nielsen, L., et al., *MR1 presents microbial vitamin B metabolites to MAIT cells.* Nature, 2012. **491**(7426): p. 717-23.

50. Kawachi, I., et al., *MR1-restricted V alpha 19i mucosal-associated invariant T cells are innate T cells in the gut lamina propria that provide a rapid and diverse cytokine response.* J Immunol, 2006. **176**(3): p. 1618-27.

51. Dusseaux, M., et al., *Human MAIT cells are xenobiotic-resistant, tissue-targeted, CD161hi IL-17-secreting T cells.* Blood, 2011. **117**(4): p. 1250-9.

52. Meierovics, A., W.J. Yankelevich, and S.C. Cowley, *MAIT cells are critical for optimal mucosal immune responses during in vivo pulmonary bacterial infection.* Proc Natl Acad Sci U S A, 2013. **110**(33): p. E3119-28.

53. Mikhak, Z., J.P. Strassner, and A.D. Luster, *Lung dendritic cells imprint T cell lung homing and promote lung immunity through the chemokine receptor CCR4.* J Exp Med, 2013. **210**(9): p. 1855-69.

54. Badovinac, V.P., B.B. Porter, and J.T. Harty, *Programmed contraction of CD8(+) T cells after infection.* Nat Immunol, 2002. **3**(7): p. 619-26.

55. Reinhardt, R.L., et al., *Visualizing the generation of memory CD4 T cells in the whole body.* Nature, 2001. **410**(6824): p. 101-5.

56. Michie, C.A., et al., *Lifespan of human lymphocyte subsets defined by CD45 isoforms.* Nature, 1992. **360**(6401): p. 264-5.

57. Sallusto, F., et al., *Two subsets of memory T lymphocytes with distinct homing potentials and effector functions.* Nature, 1999. **401**(6754): p. 708-12.

58. Chetoui, N., et al., *Interleukin-7 promotes the survival of human CD4+ effector/memory T cells by up-regulating Bcl-2 proteins and activating*

the JAK/STAT signalling pathway. Immunology, 2010. **130**(3): p. 418-26.

59. Stemberger, C., et al., *Lowest numbers of primary CD8(+) T cells can reconstitute protective immunity upon adoptive immunotherapy.* Blood, 2014. **124**(4): p. 628-37.

60. Turner, D.L., et al., *Lung niches for the generation and maintenance of tissue-resident memory T cells.* Mucosal Immunol, 2014. **7**(3): p. 501-10.

61. Mackay, L.K., et al., *The developmental pathway for CD103(+)CD8+ tissue-resident memory T cells of skin.* Nat Immunol, 2013. **14**(12): p. 1294-301.

62. Wherry, E.J., et al., *Lineage relationship and protective immunity of memory CD8 T cell subsets.* Nat Immunol, 2003. **4**(3): p. 225-34.

63. Chang, J.T., et al., *Asymmetric T lymphocyte division in the initiation of adaptive immune responses.* Science, 2007. **315**(5819): p. 1687-91.

64. Flossdorf, M., et al., *CD8(+) T cell diversification by asymmetric cell division.* Nat Immunol, 2015. **16**(9): p. 891-3.

65. Laidlaw, B.J., et al., *CD4+ T cell help guides formation of CD103+ lung-resident memory CD8+ T cells during influenza viral infection.* Immunity, 2014. **41**(4): p. 633-45.

66. Floyd, T.L., et al., *Limiting the amount and duration of antigen exposure during priming increases memory T cell requirement for costimulation during recall.* J Immunol, 2011. **186**(4): p. 2033-41.

67. Chapman, T.J. and D.J. Topham, *Identification of a unique population of tissue-memory CD4+ T cells in the airways after influenza infection that is dependent on the integrin VLA-1.* J Immunol, 2010. **184**(7): p. 3841-9.

68. Brown, D.W., et al., *Mechanisms of binding of cutaneous lymphocyte-associated antigen-positive and alphaebeta7-positive lymphocytes to oral and skin keratinocytes.* Immunology, 1999. **98**(1): p. 9-15.

69. Purwar, R., et al., *Resident Memory T Cells (TRM) Are Abundant in Human Lung: Diversity, Function, and Antigen Specificity.* PLoS One, 2011. **6**(1).

70. GeurtsvanKessel, C.H., et al., *Clearance of influenza virus from the lung depends on migratory langerin+CD11b- but not plasmacytoid dendritic cells.* J Exp Med, 2008. **205**(7): p. 1621-34.

71. del Rio, M.L., et al., *CD103- and CD103+ bronchial lymph node dendritic cells are specialized in presenting and cross-presenting innocuous antigen to CD4+ and CD8+ T cells.* J Immunol, 2007. **178**(11): p. 6861-6.

72. Kim, T.S., et al., *Distinct dendritic cell subsets dictate the fate decision between effector and memory CD8(+) T cell differentiation by a CD24-dependent mechanism.* Immunity, 2014. **40**(3): p. 400-13.

73. Nakano, H., et al., *Pulmonary CD103(+) dendritic cells prime Th2 responses to inhaled allergens.* Mucosal Immunol, 2012. **5**(1): p. 53-65.

74. Furuhashi, K., et al., *Mouse lung CD103+ and CD11bhigh dendritic cells preferentially induce distinct CD4+ T-cell responses.* Am J Respir Cell Mol Biol, 2012. **46**(2): p. 165-72.

75. Cai, Y., et al., *In vivo characterization of alveolar and interstitial lung macrophages in rhesus macaques: implications for understanding lung disease in humans.* J Immunol, 2014. **192**(6): p. 2821-9.

76. Chelen, C.J., et al., *Human alveolar macrophages present antigen ineffectively due to defective expression of B7 costimulatory cell surface molecules.* J Clin Invest, 1995. **95**(3): p. 1415-21.

77. Soroosh, P., et al., *Lung-resident tissue macrophages generate Foxp3+ regulatory T cells and promote airway tolerance.* J Exp Med, 2013. **210**(4): p. 775-88.

78. Ballinger, M.N., et al., *Role of granulocyte macrophage colony-stimulating factor during gram-negative lung infection with Pseudomonas aeruginosa.* Am J Respir Cell Mol Biol, 2006. **34**(6): p. 766-74.

79. Gardai, S.J., et al., *By binding SIRPalpha or calreticulin/CD91, lung collectins act as dual function surveillance molecules to suppress or enhance inflammation.* Cell, 2003. **115**(1): p. 13-23.

80. Fernandez, S., et al., *Inhibition of IL-10 receptor function in alveolar macrophages by Toll-like receptor agonists.* J Immunol, 2004. **172**(4): p. 2613-20.

81. Bonfield, T.L., et al., *Normal bronchial epithelial cells constitutively produce the anti-inflammatory cytokine interleukin-10, which is downregulated in cystic fibrosis.* Am J Respir Cell Mol Biol, 1995. **13**(3): p. 257-61.

82. Akbari, O., R.H. DeKruyff, and D.T. Umetsu, *Pulmonary dendritic cells producing IL-10 mediate tolerance induced by respiratory exposure to antigen.* Nat Immunol, 2001. **2**(8): p. 725-31.

83. Guillot, L., et al., *Involvement of toll-like receptor 3 in the immune response of lung epithelial cells to double-stranded RNA and influenza A virus.* J Biol Chem, 2005. **280**(7): p. 5571-80.

84. Groskreutz, D.J., et al., *Respiratory syncytial virus induces TLR3 protein and protein kinase R, leading to increased double-stranded RNA responsiveness in airway epithelial cells.* J Immunol, 2006. **176**(3): p. 1733-40.

85. Homma, T., et al., *Corticosteroid and cytokines synergistically enhance toll-like receptor 2 expression in respiratory epithelial cells.* Am J Respir Cell Mol Biol, 2004. **31**(4): p. 463-9.

86. Suwara, M.I., et al., *IL-1alpha released from damaged epithelial cells is sufficient and essential to trigger inflammatory responses in human lung fibroblasts.* Mucosal Immunol, 2013.

87. Darby, I., O. Skalli, and G. Gabbiani, *Alpha-smooth muscle actin is transiently expressed by myofibroblasts during experimental wound healing.* Lab Invest, 1990. **63**(1): p. 21-9.

88. Ronnov-Jessen, L. and O.W. Petersen, *Induction of alpha-smooth muscle actin by transforming growth factor-beta 1 in quiescent human breast gland fibroblasts. Implications for myofibroblast generation in breast neoplasia.* Lab Invest, 1993. **68**(6): p. 696-707.

89. Henrikson, R.C. and T.J. Smith, *Ultrastructure of cultured human orbital fibroblasts.* Cell Tissue Res, 1994. **278**(3): p. 629-31.

90. Hallgren, O., et al., *Altered fibroblast proteoglycan production in COPD.* Respir Res, 2010. **11**: p. 55.

91. Kotaru, C., et al., *Regional fibroblast heterogeneity in the lung: implications for remodeling.* Am J Respir Crit Care Med, 2006. **173**(11): p. 1208-15.

92. Brouty-Boye, D., et al., *Chemokines and CD40 expression in human fibroblasts.* Eur J Immunol, 2000. **30**(3): p. 914-9.

93. Hwang, C.J., et al., *Orbital fibroblasts from patients with thyroid-associated ophthalmopathy overexpress CD40: CD154 hyperinduces IL-6, IL-8, and MCP-1.* Invest Ophthalmol Vis Sci, 2009. **50**(5): p. 2262-8.

References

94.   Bartok, B. and G.S. Firestein, *Fibroblast-like synoviocytes: key effector cells in rheumatoid arthritis.* Immunol Rev, 2010. **233**(1): p. 233-55.
95.   Vancheri, C., et al., *Interaction between human lung fibroblasts and T-lymphocytes prevents activation of CD4+ cells.* Respir Res, 2005. **6**: p. 103.
96.   Figeac, F., et al., *Lung fibroblasts share mesenchymal stem cell features which are altered in chronic obstructive pulmonary disease via the overactivation of the Hedgehog signaling pathway.* PLoS One, 2015. **10**(3): p. e0121579.
97.   Teran, L.M., et al., *Th1- and Th2-type cytokines regulate the expression and production of eotaxin and RANTES by human lung fibroblasts.* Am J Respir Cell Mol Biol, 1999. **20**(4): p. 777-86.
98.   Cunningham, A.C., et al., *A comparison of the antigen-presenting capabilities of class II MHC-expressing human lung epithelial and endothelial cells.* Immunology, 1997. **91**(3): p. 458-63.
99.   Wilkinson, T.M., et al., *Preexisting influenza-specific CD4+ T cells correlate with disease protection against influenza challenge in humans.* Nat Med, 2012. **18**(2): p. 274-80.
100.   Beers, C., et al., *Cathepsin S controls MHC class II-mediated antigen presentation by epithelial cells in vivo.* J Immunol, 2005. **174**(3): p. 1205-12.
101.   Barrera, C., et al., *Expression of cathepsins B, L, S, and D by gastric epithelial cells implicates them as antigen presenting cells in local immune responses.* Hum Immunol, 2001. **62**(10): p. 1081-91.
102.   Gerber, A., et al., *Expression of cathepsins B and L in human lung epithelial cells is regulated by cytokines.* Adv Exp Med Biol, 2000. **477**: p. 287-92.
103.   Wiendl, H., et al., *Antigen processing and presentation in human muscle: cathepsin S is critical for MHC class II expression and upregulated in inflammatory myopathies.* J Neuroimmunol, 2003. **138**(1-2): p. 132-43.
104.   Lo, B., et al., *Alveolar epithelial type II cells induce T cell tolerance to specific antigen.* J Immunol, 2008. **180**(2): p. 881-8.
105.   Qian, X., et al., *The ICOS-ligand B7-H2, expressed on human type II alveolar epithelial cells, plays a role in the pulmonary host defense system.* Eur J Immunol, 2006. **36**(4): p. 906-18.
106.   Aktas, E., et al., *Relationship between CD107a expression and cytotoxic activity.* Cell Immunol, 2009. **254**(2): p. 149-54.
107.   Umetsu, D.T., et al., *Antigen presentation by human dermal fibroblasts: activation of resting T lymphocytes.* J Immunol, 1986. **136**(2): p. 440-5.
108.   Tran, C.N., et al., *Presentation of arthritogenic peptide to antigen-specific T cells by fibroblast-like synoviocytes.* Arthritis Rheum, 2007. **56**(5): p. 1497-506.
109.   Boots, A.M., A.J. Wimmers-Bertens, and A.W. Rijnders, *Antigen-presenting capacity of rheumatoid synovial fibroblasts.* Immunology, 1994. **82**(2): p. 268-74.
110.   Geppert, T.D. and P.E. Lipsky, *Dissection of defective antigen presentation by interferon-gamma-treated fibroblasts.* J Immunol, 1987. **138**(2): p. 385-92.
111.   Backhed, F., et al., *Host-bacterial mutualism in the human intestine.* Science, 2005. **307**(5717): p. 1915-20.
112.   Thompson, W.W., et al., *Mortality associated with influenza and respiratory syncytial virus in the United States.* JAMA, 2003. **289**(2): p. 179-86.

113. Halperin, S.A., et al., *Pathogenesis of lower respiratory tract symptoms in experimental rhinovirus infection.* Am Rev Respir Dis, 1983. **128**(5): p. 806-10.

114. Kreijtz, J.H., et al., *Primary influenza A virus infection induces cross-protective immunity against a lethal infection with a heterosubtypic virus strain in mice.* Vaccine, 2007. **25**(4): p. 612-20.

115. Webster, R.G., et al., *Molecular mechanisms of variation in influenza viruses.* Nature, 1982. **296**(5853): p. 115-21.

116. Gething, M.J., et al., *Cloning and DNA sequence of double-stranded copies of haemagglutinin genes from H2 and H3 strains elucidates antigenic shift and drift in human influenza virus.* Nature, 1980. **287**(5780): p. 301-6.

117. Sheng, Z.M., et al., *Autopsy series of 68 cases dying before and during the 1918 influenza pandemic peak.* Proc Natl Acad Sci U S A, 2011. **108**(39): p. 16416-21.

118. Morens, D.M., J.K. Taubenberger, and A.S. Fauci, *Predominant role of bacterial pneumonia as a cause of death in pandemic influenza: implications for pandemic influenza preparedness.* J Infect Dis, 2008. **198**(7): p. 962-70.

119. Hilty, M., et al., *Disordered microbial communities in asthmatic airways.* PLoS One, 2010. **5**(1): p. e8578.

120. Ghoneim, H.E., P.G. Thomas, and J.A. McCullers, *Depletion of alveolar macrophages during influenza infection facilitates bacterial superinfections.* J Immunol, 2013. **191**(3): p. 1250-9.

121. Saetta, M., et al., *CD8+ T-lymphocytes in peripheral airways of smokers with chronic obstructive pulmonary disease.* Am J Respir Crit Care Med, 1998. **157**(3 Pt 1): p. 822-6.

122. Murphy, T.F., et al., *Persistent colonization by Haemophilus influenzae in chronic obstructive pulmonary disease.* Am J Respir Crit Care Med, 2004. **170**(3): p. 266-72.

123. Erwin, A.L. and A.L. Smith, *Nontypeable Haemophilus influenzae: understanding virulence and commensal behavior.* Trends Microbiol, 2007. **15**(8): p. 355-62.

124. Starner, T.D., et al., *Haemophilus influenzae forms biofilms on airway epithelia: implications in cystic fibrosis.* Am J Respir Crit Care Med, 2006. **174**(2): p. 213-20.

125. Ketterer, M.R., et al., *Infection of primary human bronchial epithelial cells by Haemophilus influenzae: macropinocytosis as a mechanism of airway epithelial cell entry.* Infect Immun, 1999. **67**(8): p. 4161-70.

126. Berenson, C.S., et al., *Impaired phagocytosis of nontypeable Haemophilus influenzae by human alveolar macrophages in chronic obstructive pulmonary disease.* J Infect Dis, 2006. **194**(10): p. 1375-84.

127. Aoshiba, K., J. Tamaoki, and A. Nagai, *Acute cigarette smoke exposure induces apoptosis of alveolar macrophages.* Am J Physiol Lung Cell Mol Physiol, 2001. **281**(6): p. L1392-401.

128. Ishida, T., et al., *Inhibition of immunological function mediated DNA damage of alveolar macrophages caused by cigarette smoke in mice.* Inhal Toxicol, 2009. **21**(14): p. 1229-35.

129. Lugade, A.A., et al., *Cigarette smoke exposure exacerbates lung inflammation and compromises immunity to bacterial infection.* J Immunol, 2014. **192**(11): p. 5226-35.

References

130. King, P.T., et al., *Lung T-cell responses to nontypeable Haemophilus influenzae in patients with chronic obstructive pulmonary disease.* J Allergy Clin Immunol, 2013. **131**(5): p. 1314-21 e14.

131. Spellberg, B. and J.E. Edwards, Jr., *Type 1/Type 2 immunity in infectious diseases.* Clin Infect Dis, 2001. **32**(1): p. 76-102.

132. Happel, K.I., et al., *Divergent roles of IL-23 and IL-12 in host defense against Klebsiella pneumoniae.* J Exp Med, 2005. **202**(6): p. 761-9.

133. Ye, P., et al., *Requirement of interleukin 17 receptor signaling for lung CXC chemokine and granulocyte colony-stimulating factor expression, neutrophil recruitment, and host defense.* J Exp Med, 2001. **194**(4): p. 519-27.

134. McLellan, A.D., et al., *Dermal dendritic cells associated with T lymphocytes in normal human skin display an activated phenotype.* J Invest Dermatol, 1998. **111**(5): p. 841-9.

135. Crabbe, E., et al., *The use of enzymatic procedures to recover testicular germ cells.* Hum Reprod, 1997. **12**(8): p. 1682-7.

136. Autengruber, A., et al., *Impact of enzymatic tissue disintegration on the level of surface molecule expression and immune cell function.* Eur J Microbiol Immunol (Bp), 2012. **2**(2): p. 112-20.

137. Mulder, W.M., et al., *Reduced expression of distinct T-cell CD molecules by collagenase/DNase treatment.* Cancer Immunol Immunother, 1994. **38**(4): p. 253-8.

138. Baume, D.M., et al., *Differential expression of CD8 alpha and CD8 beta associated with MHC-restricted and non-MHC-restricted cytolytic effector cells.* Cell Immunol, 1990. **131**(2): p. 352-65.

139. Campbell, J.P., et al., *Total lymphocyte CD8 expression is not a reliable marker of cytotoxic T-cell populations in human peripheral blood following an acute bout of high-intensity exercise.* Brain Behav Immun, 2008. **22**(3): p. 375-80.

140. Marshall, J.D., et al., *Induction of interferon-gamma from natural killer cells by immunostimulatory CpG DNA is mediated through plasmacytoid-dendritic-cell-produced interferon-alpha and tumour necrosis factor-alpha.* Immunology, 2006. **117**(1): p. 38-46.

141. Arase, H., N. Arase, and T. Saito, *Interferon gamma production by natural killer (NK) cells and NK1.1+ T cells upon NKR-P1 cross-linking.* J Exp Med, 1996. **183**(5): p. 2391-6.

142. Freeman, C.M., et al., *Basal gene expression by lung CD4+ T cells in chronic obstructive pulmonary disease identifies independent molecular correlates of airflow obstruction and emphysema extent.* PLoS One, 2014. **9**(5): p. e96421.

143. Appay, V., et al., *Memory CD8+ T cells vary in differentiation phenotype in different persistent virus infections.* Nat Med, 2002. **8**(4): p. 379-85.

144. Schiott, A., et al., *CD27- CD4+ memory T cells define a differentiated memory population at both the functional and transcriptional levels.* Immunology, 2004. **113**(3): p. 363-70.

145. Clark, R.A., et al., *A novel method for the isolation of skin resident T cells from normal and diseased human skin.* J Invest Dermatol, 2006. **126**(5): p. 1059-70.

146. Tang, Y., et al., *Increased numbers of NK cells, NKT-like cells, and NK inhibitory receptors in peripheral blood of patients with chronic obstructive pulmonary disease.* Clin Dev Immunol, 2013. **2013**: p. 721782.

147. Majo, J., H. Ghezzo, and M.G. Cosio, *Lymphocyte population and apoptosis in the lungs of smokers and their relation to emphysema.* Eur Respir J, 2001. **17**(5): p. 946-53.

148. Crowe, C.R., et al., *Critical role of IL-17RA in immunopathology of influenza infection.* J Immunol, 2009. **183**(8): p. 5301-10.

149. Apostolou, I., et al., *Origin of regulatory T cells with known specificity for antigen.* Nat Immunol, 2002. **3**(8): p. 756-63.

150. Sharma, S., et al., *T cell-derived IL-10 promotes lung cancer growth by suppressing both T cell and APC function.* J Immunol, 1999. **163**(9): p. 5020-8.

151. Fiorentino, D.F., M.W. Bond, and T.R. Mosmann, *Two types of mouse T helper cell. IV. Th2 clones secrete a factor that inhibits cytokine production by Th1 clones.* J Exp Med, 1989. **170**(6): p. 2081-95.

152. Maloy, K.J. and F. Powrie, *Regulatory T cells in the control of immune pathology.* Nat Immunol, 2001. **2**(9): p. 816-22.

153. Zielinski, C.E., et al., *Pathogen-induced human TH17 cells produce IFN-gamma or IL-10 and are regulated by IL-1beta.* Nature, 2012. **484**(7395): p. 514-8.

154. Gerosa, F., et al., *CD4(+) T cell clones producing both interferon-gamma and interleukin-10 predominate in bronchoalveolar lavages of active pulmonary tuberculosis patients.* Clin Immunol, 1999. **92**(3): p. 224-34.

155. Jung, T., et al., *Interleukin-13 is produced by activated human CD45RA+ and CD45RO+ T cells: modulation by interleukin-4 and interleukin-12.* Eur J Immunol, 1996. **26**(3): p. 571-7.

156. Zhu, Z., et al., *Pulmonary expression of interleukin-13 causes inflammation, mucus hypersecretion, subepithelial fibrosis, physiologic abnormalities, and eotaxin production.* J Clin Invest, 1999. **103**(6): p. 779-88.

157. Wills-Karp, M., et al., *Interleukin-13: central mediator of allergic asthma.* Science, 1998. **282**(5397): p. 2258-61.

158. Zheng, T., et al., *Inducible targeting of IL-13 to the adult lung causes matrix metalloproteinase- and cathepsin-dependent emphysema.* J Clin Invest, 2000. **106**(9): p. 1081-93.

159. Szabo, S.J., et al., *A novel transcription factor, T-bet, directs Th1 lineage commitment.* Cell, 2000. **100**(6): p. 655-69.

160. Raffin, C., et al., *Human memory Helios- FOXP3+ regulatory T cells (Tregs) encompass induced Tregs that express Aiolos and respond to IL-1beta by downregulating their suppressor functions.* J Immunol, 2013. **191**(9): p. 4619-27.

161. Zhang, D.H., et al., *Transcription factor GATA-3 is differentially expressed in murine Th1 and Th2 cells and controls Th2-specific expression of the interleukin-5 gene.* J Biol Chem, 1997. **272**(34): p. 21597-603.

162. Nascimbeni, M., S. Pol, and B. Saunier, *Distinct CD4+ CD8+ double-positive T cells in the blood and liver of patients during chronic hepatitis B and C.* PLoS One, 2011. **6**(5): p. e20145.

163. Crispin, J.C., et al., *Expanded double negative T cells in patients with systemic lupus erythematosus produce IL-17 and infiltrate the kidneys.* J Immunol, 2008. **181**(12): p. 8761-6.

164. Das, G., S. Sheridan, and C.A. Janeway, Jr., *The source of early IFN-gamma that plays a role in Th1 priming.* J Immunol, 2001. **167**(4): p. 2004-10.

References

165. Biron, C.A., *Activation and function of natural killer cell responses during viral infections.* Curr Opin Immunol, 1997. **9**(1): p. 24-34.

166. Dillon, S.M., et al., *The natural killer cell interferon-gamma response to bacteria is diminished in untreated HIV-1 infection and defects persist despite viral suppression.* J Acquir Immune Defic Syndr, 2014. **65**(3): p. 259-67.

167. Beauchamp, N.M., R.D. Yammani, and M.A. Alexander-Miller, *CD8 marks a subpopulation of lung-derived dendritic cells with differential responsiveness to viral infection and toll-like receptor stimulation.* J Virol, 2012. **86**(19): p. 10640-50.

168. Cooper, M.A., T.A. Fehniger, and M.A. Caligiuri, *The biology of human natural killer-cell subsets.* Trends Immunol, 2001. **22**(11): p. 633-40.

169. Rossi, G.A., et al., *Human ciliated bronchial epithelial cells: expression of the HLA-DR antigens and of the HLA-DR alpha gene, modulation of the HLA-DR antigens by gamma-interferon and antigen-presenting function in the mixed leukocyte reaction.* Am J Respir Cell Mol Biol, 1990. **3**(5): p. 431-9.

170. Baglole, C.J., et al., *Isolation and phenotypic characterization of lung fibroblasts.* Methods Mol Med, 2005. **117**: p. 115-27.

171. Momburg, F., et al., *Immunohistochemical study of the expression of a Mr 34,000 human epithelium-specific surface glycoprotein in normal and malignant tissues.* Cancer Res, 1987. **47**(11): p. 2883-91.

172. Dang, L.H., et al., *Role of ICAM-1 in antigen presentation demonstrated by ICAM-1 defective mutants.* J Immunol, 1990. **144**(11): p. 4082-91.

173. Kreisel, D., et al., *Cutting edge: MHC class II expression by pulmonary nonhematopoietic cells plays a critical role in controlling local inflammatory responses.* J Immunol, 2010. **185**(7): p. 3809-13.

174. Chia, H.N., M. Vigen, and A.M. Kasko, *Effect of substrate stiffness on pulmonary fibroblast activation by TGF-beta.* Acta Biomater, 2012. **8**(7): p. 2602-11.

175. Hed, J., et al., *The use of fluorescence quenching in flow cytofluorometry to measure the attachment and ingestion phases in phagocytosis in peripheral blood without prior cell separation.* J Immunol Methods, 1987. **101**(1): p. 119-25.

176. Van Amersfoort, E.S. and J.A. Van Strijp, *Evaluation of a flow cytometric fluorescence quenching assay of phagocytosis of sensitized sheep erythrocytes by polymorphonuclear leukocytes.* Cytometry, 1994. **17**(4): p. 294-301.

177. Yoshida, K., et al., *Protection against pulmonary infection with Klebsiella pneumoniae in mice by interferon-gamma through activation of phagocytic cells and stimulation of production of other cytokines.* J Med Microbiol, 2001. **50**(11): p. 959-64.

178. Shreeniwas, R., et al., *Hypoxia-mediated induction of endothelial cell interleukin-1 alpha. An autocrine mechanism promoting expression of leukocyte adhesion molecules on the vessel surface.* J Clin Invest, 1992. **90**(6): p. 2333-9.

179. Perrier, S., et al., *IL-1ra and IL-1 production in human oral mucosal epithelial cells in culture: differential modulation by TGF-beta1 and IL-4.* Clin Exp Immunol, 2002. **127**(1): p. 53-9.

180. Lundberg, A.H., et al., *Blocking pulmonary ICAM-1 expression ameliorates lung injury in established diet-induced pancreatitis.* Ann Surg, 2001. **233**(2): p. 213-20.

181. Parameswaran, N., et al., *Lack of ICAM-1 on APCs during T cell priming leads to poor generation of central memory cells.* J Immunol, 2005. **175**(4): p. 2201-11.

182. VanCompernolle, S.E., et al., *Expression and function of formyl peptide receptors on human fibroblast cells.* J Immunol, 2003. **171**(4): p. 2050-6.

183. Damoiseaux, J.G., et al., *Costimulatory molecules CD80 and CD86 in the rat; tissue distribution and expression by antigen-presenting cells.* J Leukoc Biol, 1998. **64**(6): p. 803-9.

184. Mueller, D.L., M.K. Jenkins, and R.H. Schwartz, *Clonal expansion versus functional clonal inactivation: a costimulatory signalling pathway determines the outcome of T cell antigen receptor occupancy.* Annu Rev Immunol, 1989. **7**: p. 445-80.

185. Croft, M., L.M. Bradley, and S.L. Swain, *Naive versus memory CD4 T cell response to antigen. Memory cells are less dependent on accessory cell costimulation and can respond to many antigen-presenting cell types including resting B cells.* J Immunol, 1994. **152**(6): p. 2675-85.

186. Swallow, M.M., J.J. Wallin, and W.C. Sha, *B7h, a novel costimulatory homolog of B7.1 and B7.2, is induced by TNFalpha.* Immunity, 1999. **11**(4): p. 423-32.

187. Hutloff, A., et al., *ICOS is an inducible T-cell co-stimulator structurally and functionally related to CD28.* Nature, 1999. **397**(6716): p. 263-6.

188. Fries, K.M., et al., *CD40 expression by human fibroblasts.* Clin Immunol Immunopathol, 1995. **77**(1): p. 42-51.

189. Gold, R., K.V. Toyka, and H.P. Hartung, *Synergistic effect of IFN-gamma and TNF-alpha on expression of immune molecules and antigen presentation by Schwann cells.* Cell Immunol, 1995. **165**(1): p. 65-70.

190. Riese, R.J., et al., *Essential role for cathepsin S in MHC class II-associated invariant chain processing and peptide loading.* Immunity, 1996. **4**(4): p. 357-66.

191. Bandi, V., et al., *Infectious exacerbations of chronic obstructive pulmonary disease associated with respiratory viruses and non-typeable Haemophilus influenzae.* FEMS Immunol Med Microbiol, 2003. **37**(1): p. 69-75.

192. Chapuis, F., et al., *Differentiation of human dendritic cells from monocytes in vitro.* Eur J Immunol, 1997. **27**(2): p. 431-41.

193. Abendroth, A., et al., *Varicella-zoster virus retains major histocompatibility complex class I proteins in the Golgi compartment of infected cells.* J Virol, 2001. **75**(10): p. 4878-88.

194. Clemens, D.L., B.Y. Lee, and M.A. Horwitz, *Francisella tularensis enters macrophages via a novel process involving pseudopod loops.* Infect Immun, 2005. **73**(9): p. 5892-902.

195. Gold, M.C., R.J. Napier, and D.M. Lewinsohn, *MR1-restricted mucosal associated invariant T (MAIT) cells in the immune response to Mycobacterium tuberculosis.* Immunol Rev, 2015. **264**(1): p. 154-66.

196. Jardetzky, T.S., et al., *Identification of self peptides bound to purified HLA-B27.* Nature, 1991. **353**(6342): p. 326-9.

197. Ziegler, H.K. and E.R. Unanue, *Decrease in macrophage antigen catabolism caused by ammonia and chloroquine is associated with inhibition of antigen presentation to T cells.* Proc Natl Acad Sci U S A, 1982. **79**(1): p. 175-8.

198. Unanue, E.R., *Antigen-presenting function of the macrophage.* Annu Rev Immunol, 1984. **2**: p. 395-428.

References

199. Kondrack, R.M., et al., *Interleukin 7 regulates the survival and generation of memory CD4 cells.* J Exp Med, 2003. **198**(12): p. 1797-806.

200. Infante-Duarte, C., et al., *Microbial lipopeptides induce the production of IL-17 in Th cells.* J Immunol, 2000. **165**(11): p. 6107-15.

201. Schulze-Koops, H., P.E. Lipsky, and L.S. Davis, *Human memory T cell differentiation into Th2-like effector cells is dependent on IL-4 and CD28 stimulation and inhibited by TCR ligation.* Eur J Immunol, 1998. **28**(8): p. 2517-29.

202. Wang, Y.H., et al., *Maintenance and polarization of human TH2 central memory T cells by thymic stromal lymphopoietin-activated dendritic cells.* Immunity, 2006. **24**(6): p. 827-38.

203. Murphy, E., et al., *Reversibility of T helper 1 and 2 populations is lost after long-term stimulation.* J Exp Med, 1996. **183**(3): p. 901-13.

204. Chen, W., et al., *Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3.* J Exp Med, 2003. **198**(12): p. 1875-86.

205. Coffman, R.L., *Immunology. The origin of TH2 responses.* Science, 2010. **328**(5982): p. 1116-7.

206. Foxwell, A.R., J.M. Kyd, and A.W. Cripps, *Nontypeable Haemophilus influenzae: pathogenesis and prevention.* Microbiol Mol Biol Rev, 1998. **62**(2): p. 294-308.

207. Fossiez, F., et al., *Interleukin-17.* Int Rev Immunol, 1998. **16**(5-6): p. 541-51.

208. Kimura, A. and T. Kishimoto, *IL-6: regulator of Treg/Th17 balance.* Eur J Immunol, 2010. **40**(7): p. 1830-5.

209. Clark, R.A., et al., *The vast majority of CLA+ T cells are resident in normal skin.* J Immunol, 2006. **176**(7): p. 4431-9.

210. Olson, M.R., S.M. Hartwig, and S.M. Varga, *The number of respiratory syncytial virus (RSV)-specific memory CD8 T cells in the lung is critical for their ability to inhibit RSV vaccine-enhanced pulmonary eosinophilia.* J Immunol, 2008. **181**(11): p. 7958-68.

211. Bromley, S.K., et al., *Recirculating memory T cells are a unique subset of CD4+ T cells with a distinct phenotype and migratory pattern.* J Immunol, 2013. **190**(3): p. 970-6.

212. Mackay, C.R., W.L. Marston, and L. Dudler, *Naive and memory T cells show distinct pathways of lymphocyte recirculation.* J Exp Med, 1990. **171**(3): p. 801-17.

213. Anderson, K.G., et al., *Cutting edge: intravascular staining redefines lung CD8 T cell responses.* J Immunol, 2012. **189**(6): p. 2702-6.

214. Moyron-Quiroz, J.E., et al., *Role of inducible bronchus associated lymphoid tissue (iBALT) in respiratory immunity.* Nat Med, 2004. **10**(9): p. 927-34.

215. Nakanishi, Y., et al., *CD8(+) T lymphocyte mobilization to virus-infected tissue requires CD4(+) T-cell help.* Nature, 2009. **462**(7272): p. 510-3.

216. Mogensen, T.H., et al., *Live Streptococcus pneumoniae, Haemophilus influenzae, and Neisseria meningitidis activate the inflammatory response through Toll-like receptors 2, 4, and 9 in species-specific patterns.* J Leukoc Biol, 2006. **80**(2): p. 267-77.

217. Berenson, C.S., et al., *Outer membrane protein P6 of nontypeable Haemophilus influenzae is a potent and selective inducer of human macrophage proinflammatory cytokines.* Infect Immun, 2005. **73**(5): p. 2728-35.

218. Lochner, M., et al., *In vivo equilibrium of proinflammatory IL-17+ and regulatory IL-10+ Foxp3+ RORgamma t+ T cells.* J Exp Med, 2008. **205**(6): p. 1381-93.

219. Vieira, P.L., et al., *IL-10-secreting regulatory T cells do not express Foxp3 but have comparable regulatory function to naturally occurring CD4+CD25+ regulatory T cells.* J Immunol, 2004. **172**(10): p. 5986-93.

220. Komatsu, N., et al., *Pathogenic conversion of Foxp3+ T cells into TH17 cells in autoimmune arthritis.* Nat Med, 2014. **20**(1): p. 62-8.

221. Zhang, J., L. Wu, and J.M. Qu, *Inhibited proliferation of human lung fibroblasts by LPS is through IL-6 and IL-8 release.* Cytokine, 2011. **54**(3): p. 289-95.

222. Pinchuk, I.V., et al., *Stromal cells induce Th17 during Helicobacter pylori infection and in the gastric tumor microenvironment.* PLoS One, 2013. **8**(1): p. e53798.

223. Turk, D.C., *The pathogenicity of Haemophilus influenzae.* J Med Microbiol, 1984. **18**(1): p. 1-16.

224. Round, J.L. and S.K. Mazmanian, *Inducible Foxp3+ regulatory T-cell development by a commensal bacterium of the intestinal microbiota.* Proc Natl Acad Sci U S A, 2010. **107**(27): p. 12204-9.

225. Loubaki, L., et al., *Co-culture of human bronchial fibroblasts and CD4+ T cells increases Th17 cytokine signature.* PLoS One, 2013. **8**(12): p. e81983.

226. Mucida, D., et al., *Transcriptional reprogramming of mature CD4(+) helper T cells generates distinct MHC class II-restricted cytotoxic T lymphocytes.* Nat Immunol, 2013. **14**(3): p. 281-9.

227. Arlehamn, C.L., et al., *Transcriptional profile of tuberculosis antigen-specific T cells reveals novel multifunctional features.* J Immunol, 2014. **193**(6): p. 2931-40.

228. Garni-Wagner, B.A., et al., *A novel function-associated molecule related to non-MHC-restricted cytotoxicity mediated by activated natural killer cells and T cells.* J Immunol, 1993. **151**(1): p. 60-70.

229. Castellino, F. and R.N. Germain, *Extensive trafficking of MHC class II-invariant chain complexes in the endocytic pathway and appearance of peptide-loaded class II in multiple compartments.* Immunity, 1995. **2**(1): p. 73-88.

230. Hall, S.E., et al., *Apoptotic neutrophils are phagocytosed by fibroblasts with participation of the fibroblast vitronectin receptor and involvement of a mannose/fucose-specific lectin.* J Immunol, 1994. **153**(7): p. 3218-27.

231. Blachere, N.E., R.B. Darnell, and M.L. Albert, *Apoptotic cells deliver processed antigen to dendritic cells for cross-presentation.* PLoS Biol, 2005. **3**(6): p. e185.

232. Usui, A., et al., *Conspicuous ingestion of Staphylococcus aureus organisms by murine fibroblasts in vitro.* Microbiol Immunol, 1992. **36**(5): p. 545-50.

233. Sinha, B., et al., *Fibronectin-binding protein acts as Staphylococcus aureus invasin via fibronectin bridging to integrin alpha5beta1.* Cell Microbiol, 1999. **1**(2): p. 101-17.

234. Fink, D.L., B.A. Green, and J.W. St Geme, 3rd, *The Haemophilus influenzae Hap autotransporter binds to fibronectin, laminin, and collagen IV.* Infect Immun, 2002. **70**(9): p. 4902-7.

References

235.   Santambrogio, L., et al., *Abundant empty class II MHC molecules on the surface of immature dendritic cells.* Proc Natl Acad Sci U S A, 1999. **96**(26): p. 15050-5.

236.   Di Stefano, A., et al., *T helper type 17-related cytokine expression is increased in the bronchial mucosa of stable chronic obstructive pulmonary disease patients.* Clin Exp Immunol, 2009. **157**(2): p. 316-24.

237.   Small, C.L., et al., *Influenza infection leads to increased susceptibility to subsequent bacterial superinfection by impairing NK cell responses in the lung.* J Immunol, 2010. **184**(4): p. 2048-56.

238.   Franke-Ullmann, G., et al., *Alteration of pulmonary macrophage function by respiratory syncytial virus infection in vitro.* J Immunol, 1995. **154**(1): p. 268-80.

239.   Li, S., et al., *Detection and significance of TregFoxP3(+) and Th17 cells in peripheral blood of non-small cell lung cancer patients.* Arch Med Sci, 2014. **10**(2): p. 232-9.

240.   Lowes, M.A., et al., *Psoriasis vulgaris lesions contain discrete populations of Th1 and Th17 T cells.* J Invest Dermatol, 2008. **128**(5): p. 1207-11.

241.   Conti, H.R., et al., *Th17 cells and IL-17 receptor signaling are essential for mucosal host defense against oral candidiasis.* J Exp Med, 2009. **206**(2): p. 299-311.

242.   Gotch, F., et al., *Identification of viral molecules recognized by influenza-specific human cytotoxic T lymphocytes.* J Exp Med, 1987. **165**(2): p. 408-16.