Production and Role of IL-17 and Related Cytokines in Response to Respiratory Pathogens

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A thesis submitted to the College of Medicine, Veterinary and Life Sciences, University of Glasgow in fulfilment of the requirements for the degree of Doctor of Philosophy

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2012

Abstract

Background: Pneumonia is a disease of the lungs that is caused by a number of pathogens including Gram-negative *Pseudomonas aeruginosa* and Gram-positive *Streptococcus pneumoniae* [1]. These pathogens are prevalent causes of hospital-acquired pneumonia, and *P. aeruginosa* is particularly problematic with regards to chronic pulmonary infection in patients with Cystic Fibrosis (CF) [2]. Antibodies are known to provide a component of host-defence against this microbe, but recent evidence suggests that cells secreting the pro-inflammatory cytokine interleukin-17 (IL-17) [3, 4], namely T helper 17 (Th17) cells, are also significant in these responses [3, 5].

Aim: To investigate the sources of IL-17 and related cytokines during *P. aeruginosa* and *S. pneumoniae* infection, and to investigate if IL-1β has a role in Th17 formation during infection with these pathogens. Furthermore, to investigate the cytokine secretion of dendritic cells (DCs) derived from different sources following infection with these pathogens, and their roles at inducing Th-17 secretion from naive CD4+ T cells.

Methods & **Results:** Dendritic cells from mucosal sites were found to be better than GM-CSF derived bone marrow dendritic cells (BMDCs) at inducing Th17 cells from naive T cells. Th17 cells were derived in response to both *P. aeruginosa* and *S. pneumoniae*, with the role of IL-1β seeming to be negligible for *P. aeruginosa*. However, the role of IL-1β during Th17 cell induction during *S. pneumoniae* is unclear and needs further investigation. γδ T cells were found to be a source of IL-17 during *P. aeruginosa* infection in a IL-23 dependent manner. Furthermore, γδ T cells were also found to be a source of IL-22, yet the majority of cells were either IL-17 or IL-22 producing, not double producers as expected. *In vivo* infection with these pathogens identifed γδ T cells to be a main source of IL-17 during *P. aeruginosa* infection, with Th17 cells having more of a role during *S. pneumoniae* infection, yet the main sources of IL-17 still need to be identified. Preliminary infections with *S. pneumoniae* in IL-17RKO mice identified IL-17 as a key mediator in downstream inflammatory responses in the lung during infection.

Conclusions: This study demonstrates that IL-17 responses are induced in response to infection with the respiratory pathogens *P. aeruginosa* and *S. pneumoniae*. *Ex vivo,* mucosal DCs were found to induce more robust Th17 cell responses compared to GM-CSF derived BMDCS. *In vivo,* Th17 cells appear to have a role in *S. pneumoniae* infection, with γδ T cells appearing to be the dominant source of IL-17 during *P. aeruginosa* infection. Furthermore, *in vitro* investigations of γδ T cells found them to be differentially IL-17 and IL-22 producing in response to *P. aeruginosa* infection, in a DC contact independent manner.

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Acknowledgements

Firstly I must thank my supervisor Tom Evans for taking a chance on me and for his help and encouragement over my PhD. Especially his insistence that it would all be ok when I was panicking about having no results and it all going wrong-you were right, it has turned out ok. I must mention his catchphrase for whenever I was in doubt of my results: 'it is what it is'. This became my motto. I even have a keyring of this given to me by my special and daft friend Jen. Thanks Jen, for this and for being generally daft with me and helping me let off steam, for your understanding when I became a hermit and the worst friend ever while writing, and for listening to all my rants and placating me. You have been a real support.

Thanks to my wonderful Swedish meatball, Cecilia for teaching me everything I know, for her patience in teaching me and for a fantastic friendship! I miss you and hope to visit you in California soon. Thanks to many others in the GBRC for their help over the years, Jim and Shauna, Ashley G, Hannah, all of the Harnetts and Mowlings-far too many to mention individually, though I must mention my petite chou-fleur Aude and give a mega thank you to ex-mowling Ellie! Thanks to Ananda for being optimistic (even when she's so pessimistic about her own work-unnecessarily) and to Bob for being realistic! Thanks for being my 'bench buddies', and for making work a fun place to be. Thanks to Lucy, and Carole 'Roseyposey' for this also- Rosey, I can't wait to meet your little rose bud!

A special mention must go to Ryan for without his help I wouldn't have my last chapter and a possibility of a future publication. Thanks for putting up with me Ryan and for all your help, even when you had so much on your plate already.

I must also mention my little nephew Harry, seeing you every Friday made the weeks of writing bearable. You are such an amazing wee guy and you make me so happy. x

Finally a massive thank you to Sean for all his love and support, for his encouragement and persistence (which wasn't always graciously accepted, sorry). For putting up with my late nights, early mornings, bad moods, bad meals, and for being MIA for a few months. I know everyone says this about someone but I really couldn't have got through the last few years, and especially the last few months, without you! I love you and our new home! This is for you xxx

Author's Declaration

I declare that, except where explicit reference is made to the contribution of others, that this thesis is the result of my own work and has not been submitted for any other degree at the University of Glasgow or any other institution.

Signature……

Printed name……

Abbreviations

2-ME	2-mercaptoethanol
Ab	Antibody
Abx	Antibiotics
AhR	Aryl hydrocarbon receptor
AM	Alveolar macrophage
APC	Allophycocyanin
APCs	Antigen presenting cells
BAL	Bronchoalveolar lavage
Bcl	B cell lymphoma
BHI	Brain heart infusion
BIR	Baculovirus inhibitor of apoptosis protein repeat
BLP	Bacterial lipo-protein
BMDC	Bone marrow derived dendritic cells
BSA	Bovine serum albumin
CARD	Caspase activating and recruitment domain
CCL	CC chemokine ligand
CCR	CC chemokine receptor
CD	Cluster of differentiation
CF	Cystic Fibrosis
CFTR	Cystic fibrosis transmembrane conductance regulator
CFU	Colony forming units
CXCL	CXC chemokine ligand
CXCR	CXC chemokine receptor
DC	Dendritic cell
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EAE	Experimental autoimmune encephalomyelitis
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence activated cell sorting
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
FSC	Forward scatter

G-CSF	Granulocyte colony stimulating factor
GM-CSF	Granulocyte macrophage colony stimulating factor
H&E	Hematoxylin and eosin stain
HBSS	Hank's buffered salt solution
HRP	Horseradish peroxidise
IFN	Interferon
IL	Interleukin
iNKT cells	Invariant Natural Killer T cell
IVIS	*In vivo* imaging system
KO	Knock-out
LB	Luria Bertani
LPS	Lipopolysaccharide
LRR	Leucine rich repeats
LTi cell	Lymphoid tissue inducer cell
MACS	Magnetic-Activated Cell Sorting
MHC	Major histocompatability complex
MIC	Minimum inhibitory concentration
MOI	Multiplicity of infection
MS	Multiple sclerosis
NALT	Nasal associated lymphoid tissue
NK cell	Natural Killer cell
NKT cell	Natural Killer T cell
NLR	Nucleotide oligomerization domain and leucine rich repeat containing proteins
PAMP	Pathogen associated molecular pattern
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PE	Phycoerythrin
Percp	Peridinin Chlorophyll Protein Complex
PFA	Paraformaldehyde
P.I	Post infection
PLY	Pneumolysin
PMA	Phorbol 12-myristate 13-acetate
PRR	Pattern recognition receptor
PYD	Pyrin domain

RA	Rheumatoid arthritis
RBC	Red blood cell
RNA	Ribonucleic acid
RT	Room temperature
SSC	Side scatter
TC	Tissue culture
TCR	T cell receptor
TGFβ	Transforming growth factor beta
Th	Helper T cell
TIR	Toll-like Interleukin-1 receptor like
TLR	Toll like receptor
TMB	Tetramethylbenzidine
TNFα	Tumor necrosis factor alpha
WT	Wild type

1 Introduction

1.1 Introduction

Pneumonia is a disease of the lungs that is caused by a number of pathogens including Gram-negative *Pseudomonas aeruginosa* and Gram-positive *Streptococcus pneumoniae* [1]. These pathogens are prevalent causes of hospital-acquired pneumonia and *P. aeruginosa* is particularly problematic with regards to chronic pulmonary infection in patients with Cystic Fibrosis (CF) [2]. Antibodies (Abs) are known to provide a component of host-defence against this microbe, but recent evidence suggests that cells secreting pro-inflammatory cytokine interleukin-17 (IL-17) [3, 4], namely T helper 17 (Th17) cells, are also significant in these responses [3, 5], and we wished to investigate this.

The work presented in this thesis explores the production of IL-17 secreting cells in response to the pneumonia causing Gram-negative pathogen *P. aeruginosa* and to a lesser extent, the Gram-positive pneumonia causing pathogen *S. pneumoniae*. This chapter provides an overview of these respiratory pathogens, of immune defences in the lung, and of IL-17 and its functions with emphasis on the cells known to produce IL-17 and the importance of IL-17 in other infections.

The aim of this chapter is to highlight the importance of IL-17 in inflammatory responses to bacterial infection, and to place in context the importance of the results presented in this thesis in the fight against common bacterial infections that are causative agents of pneumonia.

1.2 Pneumonia

Pneumonia is an inflammatory condition of the lungs, which leads to respiratory problems such as difficulty breathing, severe coughing, chest pain and fever. It is usually caused by an infection, such as bacterial or viral infection, but fungi and parasites can also be responsible for the inflammation that leads to pneumonia [6].

Immunocompetent individuals who suffer from a mild pneumonia with no other complications normally recover well, but to those with co-morbidities such as Chronic obstructive pulmonary disease (COPD), pneumonia can be deadly [7]. Pneumonia can lead to certain complications such as respiratory failure due the

inflamed tissue not being able to absorb enough oxygen. Pneumonia appears to be responsible for nearly half of intensive care unit infections in Europe [8], illustrating its magnitude.

Two causative agents of bacterial pneumonia are *P. aeruginosa* and *S. pneumoniae* and it is these respiratory pathogens that are the focus of this thesis.

1.3 *Pseudomonas aeruginosa*

P. aeruginosa is a Gram-negative rod shaped flagellated opportunistic bacteria that is a common cause of respiratory infections in immunocompromised and hospitalised individuals [9].

An environmental pathogen commonly found in soil and water, *P. aeruginosa* is highly resistant to antibiotics. Perhaps this is, in part, due to the fact that it can be found in areas where antibiotic sources also grow, such as the soil, and so has developed factors to survive alongside these antibiotics. It can infect a number of life forms including amoebas [10], nematodes [11], insects [12, 13], plants [14] and mammals and is thus extremely adaptable to endure in these various environments where all of these organisms exist. It is a robust pathogen that has been shown to survive virtually oxygen free environments, even though it is classified as an aerobic bacterium [15], and so is known as a facultative anaerobe. This adaptability and potential to survive extremes of environment contribute to its resilient nature making it a very difficult pathogen not only to avoid, but to eradicate once it has been encountered.

P. aeruginosa is not detrimental to those who have a fully competent immune system, and may be found in the throat and nasal mucosa of these individuals [16], making them carriers. In these individuals, barrier functions of the lung (discussed in section 1.5 of this chapter) appear to be enough to ensure effective removal of the pathogen from the airways, and disruption of colonization. It is however a major source of infection in immunocompromised individuals such as patients undergoing chemotherapy and patients with compromised defences for instance burns patients, and CF sufferers [2]. In these

individuals it can cause a myriad of complications which will be discussed in the following sections.

1.3.1 P. aeruginosa pneumonia and hospital acquired infections

Hospital acquired infections (HAIs) or nosocomial infections (NIs) are infections acquired by individuals upon admission to hospital. *P. aeruginosa* is an important cause of HAIs as it is an opportunistic pathogen that seems to readily colonise those with other co-morbidities, worsening prognosis. It has been found that, of all HAIs, *P. aeruginosa* is responsible for 1 in 10 [17, 18], and is a leading cause of NIs worldwide.

P. aeruginosa is a major cause of urinary tract infections [19]; eye infections- it is the main cause of bacterial keratitis, an inflammation of the cornea [20]; wound infections [21], in addition to respiratory infections which are the main focus of this thesis. Generally *P. aeruginosa* can infect any mucosal surface or any instance where physical barriers have been breached such as wounds and burns.

As *P. aeruginosa* is an environmental pathogen, entry to the lungs is not particularly difficult. For example it colonizes the throat and nasal mucosa of carriers [16], and may be made airborne upon coughing and sneezing. Equally, if a source of *P. aeruginosa* infected water becomes disturbed and water particles become airborne, it allows the organism to be inhaled readily, leading to entry into the lung and possible colonisation.

In the lungs of immunocompromised individuals *P. aeruginosa* may colonize and form biofilms in the airway, reducing the conduction of oxygen in these airspaces. Biofilms are aggregates of the pathogen contained in a sticky matrix [22], which aid in the pathogens adherence to surfaces and survival as the sticky matrix is virtually impermeable to the hosts immune response [22]. The pathogen may also release pathogenic components leading to cellular destruction [23] and inflammation in the tissue. This may further impede oxygen diffusion in the lung, making the patient breathless and causing weakness in the individual due to lack of sufficient oxygen consumption.

P. aeruginosa can colonise plastics where it forms these biofilms, which is why it can be a problematic organism in the hospital environment where patients have indwelling urinary catheters or are intubated to aid with their breathing, thereby allowing *P. aeruginosa* to readily colonize the urinary tract or the airways. Intubation also inhibits normal clearance of respiratory secretions [24, 25], increasing the likelihood of respiratory infections.

1.3.2 P. aeruginosa infection in individuals with CF

CF is a recessive genetic disorder that results from a mutation in a protein called the cystic fibrosis transmembrane conductance regulator (CFTR) which is responsible for regulation of components of mucus, sweat and other body secretions [26]. It affects many body organs but is most well known for the difficulties it causes in respiratory function of sufferers.

CF patients have over production of mucus in the lung and reduced mucociliary function, meaning that this excess mucus cannot be removed from the lung effectively. As the mucus produced cannot be effectively removed, it becomes a breeding ground for pathogens, as it is a perfect moist warm environment and multiple infections can ensue, a primary source being *P. aeruginosa* [2], known to be a colonizer of structurally abnormal lung tissue. In CF individuals *P. aeruginosa* will eventually colonize 80% of adult patients [27, 28]. There is great inflammation in the lungs of these individuals, characterized by neutrophil infiltration [5], which can lead to fibrosis and result in further decline of lung function.

An additional complication of lack of functional CFTR, is that is has been shown to be a receptor for lipopolysaccharide (LPS) [29], a conserved component of bacterial cell walls and strong immunogenic factor. Functional CFTR may bind to LPS on pathogens and allow recognition and downstream immune responses to the pathogen [29]. In CF patients where this receptor is disrupted, this immune component is lacking and therefore the bacteria may thrive, as they are not recognised and eliminated.

Studies have shown elevated IL-17 in sputum samples of CF patients colonized with *P. aeruginosa* [3, 5] indicating that IL-17 is produced in response to *P.*

aeruginosa infection in CF patients. IL-17 may contribute to host defence but this immune response is also potentially pathological in CF individuals, as IL-17 is known to aid in recruitment of neutrophils [3, 30] which cause inflammation and fibrosis in the lung to the detriment of the host. Conceivably IL-17 may responsible for the persistent neutrophilia in the airways of CF patients [5] and consequently inflammation. One study showed that IL-17 in CF lungs may prime airway epithelial cells to up-regulate pattern recognition receptors [30], thus increasing bacterial recognition and enhancing the inflammatory response. Therefore attenuation of IL-17 responses in CF individuals may be beneficial.

1.4 *Streptococcus pneumoniae*

S. pneumoniae is a Gram-positive pathogen, commonly referred to as the pneumococcus. It is a commensal pathogen of humans, usually found in the nasopharynx. It may spread from the nasopharynx and cause a variety of infections including meningitis and pneumonia, and is thought to be responsible for 20-60% of bacterial cases of community acquired pneumonias [31, 32]. The pneumonia suffered by *S. pneumoniae* infection is clinically not that much different from that of the pneumonia induced by *P. aeruginosa* infection, discussed earlier, though one key difference between *S. pneumoniae* and *P. aeruginosa* is that *S. pneumoniae* can infect healthy individuals. A further pitfall of *S. pneumoniae* infection is, like *P. aeruginosa*, strains of *S. pneumoniae* exist that are resistant to common antibiotics [32], making it a difficult pathogen to control. Due to its antibiotic resistance *S. pneumoniae* is also a prominent source of HAIs, and is thought to responsible for early onset infections as opposed to *P. aeruginosa* which is a caustive agent of late onset pneumonia [33].

1.4.1 Pneumolysin (PLY)

Pneumolysin (PLY) is a toxin in *S. pneumoniae* that is very toxic to cells. It is a haemolysin, meaning it lyses red blood cells (RBCs), and is probably the most well studied of the pneumococcus virulence factors and is produced by a majority clinical isolates of *S. pneumoniae* [34]. PLY has been shown to induce CXCL8, a well known neutrophil attractant [35], from nasopharyngeal epithelia cells [36] and thus may play a role in neutrophil recruitment during *S. pneumoniae* infection in the lung. It has also been shown to be vital for

secretion of cytokines dependent on caspase-1 activation such as IL-18 and IL-1β [37, 38], which is discussed in section 1.6.5 of this introduction. Infection of macrophages with PLY deficient *S. pneumoniae* mutants had significantly reduced secretion of these pro-inflammatory cytokines indicating importance of PLY in production of caspase-1 dependent cytokines [37]. Recognition of PLY is considered to be via an innate pattern recognition receptor (PRR) named Toll-like receptor 4 (TLR4) [39], and this interaction is thought to be critical for immune responses to *S. pneumoniae* [39]. (TLRs and PRRs are described in greater depth later in the chapter). However recent studies have found that PLY is able to stimulate downstream production of pro-inflammatory cytokines in a TLR4 independent manner [40] and that the role of TLR4 in immune resistance to *S. pneumoniae* may be dose dependent [41]. Thus the roles of TLR4 and PLY interaction in *S. pneumoniae* infection remain to be fully elucidated.

1.5 Defences to lung infection

1.5.1 Barrier defences

The lungs are organised in such a way to protect them from an invading pathogens. Such defensive features of the lung include mucus, and mucus clearance by the cilia of the lungs [42]. If these are impaired or the pathogen manages to overcome them, then the very make up of the cells in the conducting zone, the area of the respiratory system that is in contact with the outside world, acts as barrier. The epithelia of the lung are stratified and connected by tight junctions that are somewhat impermeable to pathogens [43]. Colonizing *P. aeruginosa* may overcome this however by secretion of toxins that break down plasma membranes in cells [44], leading to cell death.

If in the instance that *P. aeruginosa* has colonised the lung by overcoming these defensive strategies then the innate, and eventually the adaptive, immune responses come into play.

1.5.2 Innate immunity

Innate immunity is the first arm of the immune system that a pathogen may encounter. It is broad and unspecific, acting against conserved pathogenic signals that can be found in many types of similar pathogens; for example

conserved components of cell walls in bacteria. These conserved components are called pathogen associated molecular patterns (PAMPs) and are recognised by PRRs, of which there are many types found on various cell types such as neutrophils, macrophages and epithelial cells. The most well-known of the PRRs are TLRs and NLRs (nucleotide oligomerization domain and leucine rich repeat containing proteins) discussed in more depth below.

1.5.2.1 TLRs

TLRs are the most 'famous', most studied and hence probably most well understood of the PRRs. TLRs are transmembrane spanning with an extracellular region of leucine rich repeats (LRR) which are used for recognition of PAMPs, and an intracellular TIR (Toll-like Interleukin-1 receptor like) domain, used for signalling and initiating of appropriate responses [45]. This intracellular toll-like region is also shared by IL-1R family, hence the name toll-like, interleukin-1-receptor like domain.

There are 10 TLRs in humans known to date, some extracellular, some intracellular, each with their own distinct ligand (see Table 1-1).

Table 1-1 TLRs and examples of ligands

TLR	Ligand
TLR1/2	Bacterial lipoprotein (BLP) from bacteria cell walls [46]
TLR2/6	MALP-2 (macrophage activating lipopeptide) [46]
TLR3	Double stranded RNA [46]
TLR4	Lipopolysaccharide (LPS) [46]
TLR5	Flagellin [46]
TLR7/8	Single stranded RNA [46]
TLR9	CpG DNA [46]
TLR10	Unknown [46]

With the exception of TLR3, all TLRs signal through the NFκB pathway [47] and thus may lead to induction of pro-inflammatory cytokines such as IL-6 [48]. Furthermore, TLR4 recognition of LPS by antigen presenting cells (APCs) such as

dendritic cells (DCs) leads to secretion of IL-23 [49], an important cytokine for maintenance of IL-17 secreting cells as discussed in section 1.6.4.

1.5.2.2 NLRs

NLRs are the main intracellular sensors of bacterial PAMPS and include Nucleotide-binding oligomerization domain-containing protein 1 (Nod1), Nod2 and NLRC4, whose ligands are illustrated in Table 1-2.

Table 1-2 NLRs and examples of ligands

NLR	Ligand
NOD1 (NLRC1)	γ-D-glutamyl-mesodiaminopimelic acid containing peptide from Gram-negative and some Gram-positive bacteria [50]
NOD2 (NLRC2)	Muramyl dipeptide (MDP) [50]
NLRC4	Flagellin [50]
NLRP3	PLY [51]

NLRs are composed of 3 structures, 2 of which are conserved among the NLRs- a C terminal LRR (like TLRs) for recognition of bacterial products, and a central NOD domain that mediates self-association. The NLRs vary in their N terminal structure which may be either a pyrin domain (PYD) and the subsequent NLR is named a NLRP; a caspase activating and recruitment domain (CARD) named NLRC; or a baculovirus inhibitor of apoptosis protein repeat (BIR), named NLRB [52], as well as others.

1.5.2.3 Neutrophils

Neutrophils are granular multi-lobed nucleated cells that are one of the first cells to be recruited to a site of infection. They are known to have a major role in bacterial pneumonia [53] and considered to be a hallmark indicator that the disease is present [54]. Neutrophils have been found to be indispensible in *P. aeruginosa* disease as it has been shown that in neutrophil depleted animals there is excessive mortality upon *P. aeruginosa* infection [55].

Neutrophils provide several bacteriocidal components to provide protection during bacterial infection such as proteinases [56] and defensins [57]. Neutrophils also provide lactoferrin, a bacteriocidal component that scavenges iron [58], thus depleting the pathogens source of this element, required for *P. aeruginosa* proliferation. Neutrophils are not the sole source of lactoferrin, as epithelia cells are known to secrete it also [59].

Neutrophils are recruited to the lung by chemokines, chemotactic cytokines which may be named CC or CXC depending on their spacing of cysteine residues. Chemokines act with adhesion molecule and intergrin interactions to allow the neutrophils to enter the airspace. Recognition of pathogens with PRRs such as TLRs lead to downstream production pro-inflammatory cytokines, which may include CXCL8 [59, 60], from cells in the bronchiole space and thus recruitment of neutrophils into the lung. It has recently been shown that IL-17 can also induce production of CXCL8 [61] and thus Th17 cells may be a candidate for upstream initiation of neutrophil recruitment in the lung.

1.5.2.4 Epithelial cells

As discussed epithelial cells are important at providing a tight barrier to lung invading pathogens, however epithelial cells are not just barrier cells but provide innate immune responses also. Epithelia cells express TLRs [59], and this leads to downstream activation of NFκβ pathway and production of various inflammatory cytokines such as TNFα, IL-1β, IL-6 [47] and chemokines such as CCL5 (RANTES) and CCL2 (MCP-1) [62]. TNFα, IL-1β, IL-6 are pro-inflammatory cytokines that play various role in infection and inflammatory responses, and CCL5 and CCL2 are known to recruit leukocytes to the site of inflammation. Essentially, engagement of TLRs on epithelia cells leads to robust localised inflammatory responses. Epithelial cells also secrete anti-microbial products such as β defensins and lactoferrin, which inhibit bacterial growth [59].

1.5.2.5 Macrophages

Macrophages are the resident leukocyte of the lung alveoli as can be observed by histology of uninfected animals and are so named alveolar macrophages (AMs). They act as scavengers in the lung, patrolling the tissue and phagocytosing foreign material [63]. Macrophages are not only innate effector cells but APCs

[64], cells which collect ‘debris’ in the tissue and carry it to immune sites to present to T cells. If the T cells do not recognise and respond to the presented ‘debris’ then the macrophage carries on its scavenging, however if the macrophage presents to a T cell something which it recognises, a cascade of responses are initiated inducing the adaptive immune response, making macrophages key to the branch of innate and adaptive immunity.

The role of alveolar macrophages during *P. aeruginosa* pneumonia appears to differ upon time point during infection. Studies have shown that early on in infection, approximately 8 hours, AMs contribute to neutrophil recruitment and associated tissue destruction. Thus deletion of AMs abrogates this deleterious effect [65]. This would make blockade of AMs an attractive prospect in the first few hours of infection. However, it appears that lack of AMs in the long term reduces long term survival of *P. aeruginosa* infected animals [65]. These findings indicate the AMs play a key role in animal survival during *P. aeruginosa* induced pneumonia.

1.5.3 Adaptive immunity

1.5.3.1 Antibodies

Antibodies (Abs) have been shown to be made in response to *P. aeruginosa* infection [66] but it appears that these Abs alone are not enough to allow bacterial clearance [66]. It has in fact been suggested that the humoral response to *P. aeruginosa* in CF individuals could have adverse effects and actually be detrimental to lung function and therefore worsen clinical outcome [67]. However, it is also thought that anti-pseudomonal Abs play a role in prevention of systemic spread of *P. aeruginosa* impeding bacteraemia and sepsis [54].

1.5.3.2 T cells

The role of T cells in *P. aeruginosa* infection is at present unclear though many are attempting to elucidate the roles of various T cells during this infection[68-70]. It is the purpose of our study to look at T cells during *P. aeruginosa* infection, specifically IL-17 producing T cells, both conventional αβ T cells and ‘unconventional’ γδ T cells, investigating if they do in fact have a role in *P. aeruginosa* infection and if so is that role indispensible.

1.6 Interleukin 17 (IL-17)

IL-17 is a pro-inflammatory cytokine of which there are several family members, named IL-17A-F, with varying sources listed in Table 1-3.

Table 1-3 IL-17 family members and their sources

IL-17 family member	Homology between murine and human	Secreted by
IL-17A (IL-17)	62%	Th17 cells [71] γδ T cells [72] Neutrophils [73, 74] CD8 cells [49, 75] iNKT cells [76, 77] mast cells [73] LTi-like cells [78]
IL-17B	88%	Chondrocytes [79] Neurons [80]
IL-17C	83%	Epithelial cells in psoriasis [81]
IL-17D	78%	Unknown
IL-17E (IL-25)	81%	Th2 cells [82] Mast cells [83] Eosinophils and Basophils [84]
IL-17F	77%	As IL-17A [85]

IL-17A is simply referred to as IL-17, and was the founding member of the family and probably the best understood. IL-17F is the closest family member to IL-17A, sharing 50% identity [86]. They are thought to have similar functions, but to bind with different affinity to their receptor, as discussed in the next section. IL-17 presence is observed in many extra-cellular bacterial infections [87, 88], autoimmune diseases [81, 89, 90], and is associated with inflammation. Some of the downstream targets of IL-17 are up-regulation of pro-inflammatory chemokines such as CXCL1 and CXCL8 [3, 61]; up-regulation of pro-inflammatory cytokines such as IL-1β and IL-6, and the colony stimulating cytokines G-CSF and GM-CSF [91], and up-regulation of anti-microbial peptides such as β defensins and mucins [92, 93]. These all contribute to the attraction and generation of immune cells and aid in inflammation. The role and source of each element up-regulated by IL-17 during lung infection is described more thoroughly in Table 1-4.

Table 1-4 Targets of IL-17, and their effects on immune responses

Downstream IL-17 target (and aliases)	**Effect on immune response during lung infection**
CXCL1 (melanoma growth stimulating activity, alpha (MSGA-α))	Chemokine with neutrophil attracting and activating properties [35]
CXCL8 (IL-8, Monocyte-derived neutrophil chemotactic factor (MDNCF)) (Mouse functional homolog MIP2)	Chemokine that is a potent neutrophil chemoattractant [94]
IL-1β	Pro-inflammatory cytokine with roles in cell migration, cell differentiation and fever [95]
IL-6	Involved in acute phase response [96] and Th17 differentiation [97]
Granulocyte colony stimulating factor (GCSF)	Regulates neutrophil release from the bone marrow [98], enhances cytotoxicity of neutrophils[99]
Granulocyte-macrophage colony stimulating factor (GM-CSF)	Neutrophil activating factor [100], enhances cytotoxicity of neutrophils [99]
α-defensins	Peptides from neutrophils with microbicidal properties [101]
Mucins	Component of mucus and mucus secretion and therefore physical trapping and eradication of pathogen [93]

The family member IL-17E, also known as IL-25, actually acts to inhibit IL-17 production from IL-17 producing cells and so is a negative regulator. It does via downstream blocking of the cytokine IL-23 [102] which is known to be important for IL-17 producing cell maintenance and is discussed in section 1.6.4 of this chapter. IL-17E treatment is linked to expression of IL-13, an anti-inflammatory Th2 cytokine, which in turn blocks DC secretion of IL-23, therefore hindering IL-

17 producing cell population survival [102, 103]. IL-17E has Th2 associations, perhaps further evidence of its IL-17/Th17 cell negative regulation properties as Th2 cells are known to compete with and negatively regulate Th1 and Th17 cells as they originate from the same precursors. The main sources of IL-17E considered to be memory Th2 cells [104], mast cells [83], eosinophils and basophils [84].

Recent studies have identified IL-17C as having a role in innate immune functions of epithelial cells [86] and regulation of Th17 cells in the auto-immune disease experimental autoimmune encephalomyelitis (EAE) [77] a mouse model of multiple sclerosis (MS).

Far less is known about the remaining IL-17 family members IL-17B and D. These molecules are much less studied and at present their functions are unknown.

1.6.1 IL-17 receptors

IL-17 signals through the IL-17 receptor (IL-17R). Although the term IL-17R is commonly used this is not just one structure, but a dimer composed of 2 units from a possible family of 5 subunits name IL-17RA-E, which are listed with the cells that they are expressed on in Table 1-5.

Table 1-5 IL-17 Receptor family members and their cells of expression

IL-17 receptor unit	Found on	Binds to
IL-17RA	Most cell types studied to date [105]	IL-17A
IL-17RB (IL-25R)	Th9 cells [106] Th2 cells [84] iNKT cells [107] basophils [108]	IL-17E (IL-25)
IL-17RC	Adipocytes [105] Chondrocytes [105] Epithelial cells [105]	IL-17A, IL-17F
IL-17RD	Epithelial and endothelial cells [109]	unknown
IL-17RE	Epithelial cells [86]	IL-17C

IL-17A signals through a heterodimeric complex of IL-17RA and IL-17RC. IL-17F also signals through this receptor complex, but IL-17A is known to respond more vigorously than IL-17F [110]. This IL-17RA/RC dimer is commonly referred to as IL-17R. It can be found on epithelial cells and fibroblasts [105] and so is an ideal

candidate for immune responses in the lung. In fact IL-17RA is claimed to be on all cells [105] so the potential for IL-17 signalling on all cells exists. Mice lacking in IL-17R have reduced G-CSF, reduced neutrophil recruitment, greater bacterial burden and thus a higher rate of fatalities following infection [61] indicating that IL-17 signalling is vital during infection.

IL-17E signals via a complex of IL-17RA and IL-17RB, also known as IL-25R. IL-17RB can be found on Th2 cells [84] and basophils [108] and so IL-17E appears to act on the cells it secretes, possibly indicative of a positive feedback loop.

1.6.2 IL-17 in infection and disease

IL-17 has major roles in infection and disease and seems to be a key player in many autoimmune diseases that were once considered to be Th1 dominated, such as rheumatoid arthritis (RA) [89]. Studies have shown IL-17 to be very important in anti-microbial responses such as *Staphylococcus aerus* [111] and *Klebsiella pneumoniae* [87, 112]. It was the role of IL-17 in these bacterial responses which has led us to investigate the role of IL-17 in *P. aeruginosa* infection, for if there is a similar IL-17 requirement for host survival and bacterial clearance, this quality could be utilized with administration of IL-17, or IL-23 to bolster IL-17 responses, during infection.

1.6.3 Cellular sources of IL-17

There are both innate and adaptive cellular sources of IL-17 which respond in different manners to different stimuli. Innate IL-17 secreting cells such as γδ T cells produce IL-17 in a fast non-specific manner with no specific antigen recognition. They respond to cytokines produced by activated DCs, or ligation of PRRs on their surface. Adaptive responses are pathogen specific, require presentation and co-stimulation by APCs and initially take longer to develop, but respond rapidly upon re-infection with the specific pathogen. Both the innate and adaptive IL-17 sources are discussed below.

1.6.3.1 Th17 cells

Th17 cells are CD4+ helper T cells that characteristically secrete IL-17 and IL-22 [71]. They are adaptive immune response cells identified as being IL-17 and IL-22

secreting, CD4+, CD3+ IL-23R+, positive for the transcription factor RORγt [113], and aryl hydrocarbon receptor (AhR) [114]. AhR is a cytosolic transcription factor that has been shown to be involved in Th17 cell development [115]. Th17 cells are thought to have many roles in disease such as RA [89] and are appearing evidently more important in bacterial infections [111]. As the name suggests, they can 'help' other immune cells and responses. Th17 cells have been shown to aid B cell responses aiding in B cell proliferation and antibody class switching [116]. However, their main role is in IL-17 secretion, which is the focus of this thesis.

1.6.3.1.1 Development of Th17 cells

Th17 cells are thought to be induced from the same Th0 CD4+ helper T cell precursor as that of the better known Th1 and Th2 cells. Induction of these cells to their helper class is under the influence of various cytokines. IL-12 and IFNγ induce Th1 cells [117], IL-4 induces Th2 cells [118]. The process of Th17 induction is only somewhat understood with differing roles for different cytokines found in humans and mice. Typically 3 cytokines are regarded to be inducers of Th17 cells; TGFβ, IL-6 and IL-1β. In mice TGFβ and IL-6 are thought to have the main roles in Th17 cell induction [97, 119, 120] with IL-1β believed to enhance the Th17 development from naive CD4+ precursors [121]. However there is now evidence that all 3 cytokines are not necessary, as Ghoreschi et al show that Th17 cells can be induced in the absence of TGFβ signalling [122]. In humans the process is less well understood with suggestions that Th17 development in humans is donor dependent [123]. IL-17 has been observed from naive CD4+ cells under the influence of IL-1β and IL-6 but not TGFβ [124]. However studies in humans show that TGFβ contribution to Th17 induction may be dose dependent with doses of approximately 10ng/ml allowing Th17 induction in combination with pro-inflammatory cytokines, but doses such as 50ng/ml inhibiting Th17 [125] and allowing for regulatory T cells (Tregs) instead. Other studies show that other pro-inflammatory cytokines such as IL-21 may play a role in Th17 cell generation in humans [125]. It is clear from previous studies that there are differences in Th17 development in humans and mice with IL-1β looking to play an important role in humans but less so in mice.

A key difference in Th17 cell induction compared to that of Th1 and Th2 is that it appears to be plastic. Once Th1 and Th2 cells are committed they remain that class of cell even upon exposure to cytokines that induce other helper T cell phenotypes such as TGFβ and IL-6 that induce Th17 cells [126]. However, Th17 cells can be switched, *in vitro,* to become Th1 cells upon exposure to Th1 inducing cytokines such as IL-12 [127], though this may be an artefact of *in vitro* culture where all biological IL-17 cell inducing and maintenance factors are not present. There is evidence that Th17 cells have the capacity to remain plastic *in vivo* under certain conditions [126, 128] and that the capacity for a Th17 cell to change its fate depends on the type of infection, whether it is acute or chronic, and the speed of resolution of infection [128]. In a study of EAE, it was shown that Th17 cells down-regulate their IL-17A production and up-regulate production of IFNγ, in what appears to be an IL-23 dependent manner [128]. It was shown that IL-23p19 deficient mice lacked IFNγ+ cells and displayed reduced T-bet [128], a transcription factor considered necessary for Th1 induction. This indicates that Th1 cell formation may be IL-23 dependent in this circumstance. This IL-23 dependent switch to a Th1 like cell is interesting as IL-23 is thought to enhance Th17 responses and has been shown to allow IL-17 secretion from γδ T cells in combination with IL-1β [129] (discussed in section 1.6.4). Thus it is remarkable that IL-23 may have such a role in IL-17 secreting cell maintenance but may also switch its phenotype to IFNγ producing under certain conditions.

Another interesting difference is that the signature cytokines that Th1 and Th2 cells secrete, IFNγ and IL-4 respectively, directly have positive feedback on themselves and limit the induction of the other phenotypes, whereas IL-17 production from Th17 cells is not known as yet to amplify Th17 cells directly, or to limit Th1, Th2 or Tregs. However, IL-21 secreted from Th17 cells acts in an autocrine manner to amplify Th17 cells [130]. This paradigm is illustrated below in Figure 1-1.

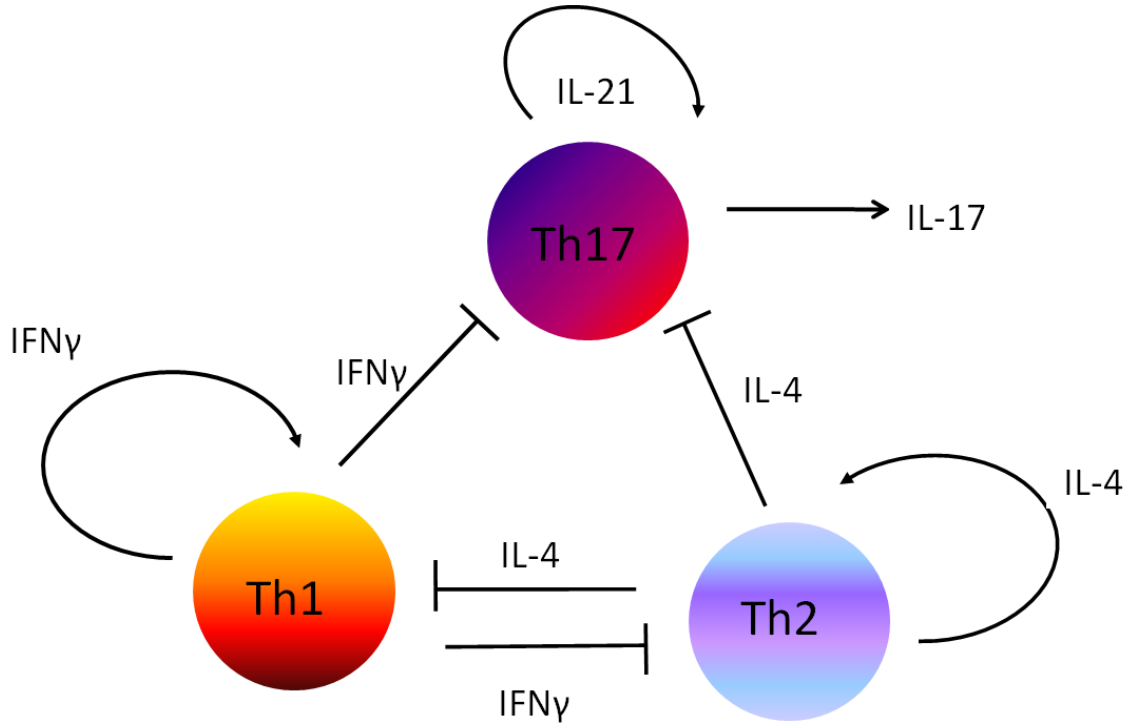

Figure 1-1 Helper T cell paradigm

Signature cytokines of Th1 and Th2 feedback directly on themselves to amplify their own cell type and block formation of other helper T cell. IL-17 has not as yet been shown to have similar direct abilities.

Another factor that distinguishes Th17 cells from other CD4+ T helper subsets is the expression of transcription factor RORγt. Transcription factors T-bet and GATA3 are expressed in Th1 [131] and Th2 [132] cells respectively, whereas RORγt is expressed in Th17 cells. Expression of RORγt is said to be essential for production of Th17 cytokines IL-17A, IL-17F, IL-21 and IL-22, and RORγt expression is adequate to induce expression of these cytokines from the cells [133].

1.6.3.2 γδ T cells

γδ T cells are 'innate like' T cells found mainly at mucosal sites. They have been mostly studied in the gut but they are important in the lung also having been found to reside in the subepithelium of the alveolar and non-alveolar regions of the lung [134]. Th17 cells have previously been considered to be the trademark IL-17 producing cell, yet it has been shown that there are instances where γδ T cells are the main source of IL-17, for example in *Mycobacterium tuberculosis* [135], *Staphylococcus aureus* [111] and *E.coli* [136]. Thus γδ T cells must be considered as an important source of IL-17. γδ T cells have been described as

either IL-17A alone expressing or dual expressers of IL-17A and IL-22 [72] akin to Th17 cells. Further to this, γδ T cells that produce IL-17 bear other similarities to Th17 cells; they are both positive for the transcription factor RORγt and have IL-23R on their surface along with AhR [72]. What sets γδ T cells apart from conventional αβ T cells is that they have PRRs on their surface and thus do not need antigen presentation from another cell, allowing responses to be faster. TLR2, a receptor for BLP, which is a component of Gram-positive bacteria cell walls, has been found on the surface of γδ T cells, as has dectin-1 [72], a receptor for β glucans, molecules found in plants, yeast and fungi. IL-17+ γδ T cells can also produce IL-17 in a fast antigen independent manner, with CD3 ligation of the T cell receptor (TCR) being enough stimuli for a γδ T cell to secrete IL-17 [137]. Furthermore IL-1β and IL-23 binding to their receptors on these cells is enough to allow IL-17 secretion in the absence of any engagement of CD3 [129].

αβ T cells have CD markers on their surface which identify them as CD4+ helper T cells or CD8+ cytotoxic T cells, and these CD markers ligate respectively to major histocompatability complex II (MHCII) and MHCI [138] on APCs when they recognise specific antigen presented via these MHC molecules. This process is vital for naive T cell induction of an immune response and occurs in an antigen specific manner. γδ T cells are mainly CD4- and CD8- [139] and thus do not recognise antigen in the typical way via MHC and APCs. As discussed they have PRRs on their surface and so may recognise antigen directly [72], eliminating the need for APCs. This saves time during the immune response as they can respond a lot faster than other T cells and are therefore thought to be more of an innate like cell. However, γδ T cells express CD62L (L-selectin) on their surface when they are inactivated which is down-modulated upon detection of certain stimuli [140, 141]. CD62L is an adhesion molecule found on naive lymphocytes which acts as a lymphoid homing receptor, responding to various ligands such as GlyCAM-1 [142], allowing naive lymphocytes to home to secondary lymphoid structures such as the lymph nodes. In these secondary lymphoid structures the naive lymphocyte may meet its corresponding antigen and become an effector cell, down-regulating its CD62L expression, as it no longer needs to home to these sites. Thus presence of CD62L is generally regarded as a marker of naive T cells, and consequently cells that lack CD62L are memory T cells. Due to the

presence and down-modulation of this molecule it could be suggested that γδ T cells are an adaptive cell type. CD44 is another cell marker commonly used to identify naive and memory cells. It is a cell surface glycoprotein that is known to be involved in cell to cell interactions and participating in lymphocyte activation and so is regarded as a marker for memory cells [143]. IL-17 producing γδ T cells have been shown to be CD44+ [144] further classifying them as a memory cells and therefore an adaptive cell as opposed to an innate cell. Thus it is difficult to categorize γδ T cells fully, though due to their ability to recognise antigen directly and respond in a fast non-specific manner, they behave more like an innate like T cell.

In αβ T cells the T cell receptor (TCR) is important during development of functional T cells in the thymus, but the γδ TCR may not play such a role in functional γδ T cell development. Developing αβ T cells in the thymus that recognise self antigen via their TCR and MHC molecules with high affinity are deleted as they would be self-reactive, a process known as negative selection [145, 146]. T cells that recognise antigen with moderate avidity are rescued from this deletion in a process known as positive selection, and proceed into the periphery [145-147]. The role of the γδ TCR and antigen recognition in development appears to differ however. γδ T cells are created regardless of whether they recognise antigen or not, but it appears those that do recognise antigen in the thymus are IFNγ producing γδ T cells, and those that are antigen naive are IL-17 producing γδ T cells [137]. Unlike αβ T cells that have TCRs with a vast repertoire of recognition, γδ TCR range of recognition is more reduced and scarcely understood [148] and this limited scope of recognition further likens them to innate cells.

1.6.3.3 Other sources of IL-17

There are many other cells that secrete IL-17 under different conditions including CD8 T cells [49, 75], neutrophils [73, 74], NKT cells [77, 149], mast cells [73, 150] and LTi-like cells [78]. LTi cells are lymphoid tissue inducer cells that are important in lymphoid organogenesis during foetal development. They express the transcription factor RORγt [151, 152], which made them subject of investigation in relation to IL-17, yet are negative for CD3 [153] and hence are not T cells. In adults cells exist that are phenotypically similar to LTi cells and

are termed LTi-like cells. LTi-like cells exist in the spleen that bear a similar profile Th17 cells. They produce IL-17 and IL-22, express RORγt, and have IL-23R and AhR on their surface [78]. LTi-like cells have been shown to secrete IL-17 when exposed to IL-23 [78] further confirming the role of IL-23 in stimulation of IL-17 secreting cells which is discussed in more depth below. Invariant NKT cells (iNKT) cells have been shown to be a source of IL-17 in neutrophillic inflammation [76, 77] and may have thus may have roles during *P. aeruginosa* and *S. pneumoniae* lung infections, though these cells are not investigated in this thesis.

1.7 IL-23

IL-23 is a pro-inflammatory cytokine that is known to amplify IL-17 responses in both αβ [154] and γδ [129] T cells. Amplification of CD4+ Th17 cells in response to IL-23 requires engagement of their TCR [129]. γδ T cells however, produce IL-17 in response to IL-23 in combination with IL-1β, in the absence of TCR engagement [129], further confirming γδ T cells ability to secrete IL-17 in a non-specific manner. Additionally, IL-23 also aids in γδ T cell survival [72, 155]. Although IL-23 is classically known to enhance Th17 responses, there is evidence that IL-23 may also have roles in driving Th17 cells to be IFNγ secreting under certain conditions [128], and therefore the role of IL-23 during Th17 responses requires further study.

IL-23 is composed of two subunits, IL-23p19 and IL-12p40 [156]. As the name suggests, IL-12p40 is a subunit of the Th1 inducing cytokine IL-12, along with IL-12 subunit IL-12p35 [157]. It is this sharing of the IL-12p40 subunit that caused confusion of IL-12 and thus Th1 cells role in disease where IL-23 and Th17 cells were responsible. For years studies suggested that IL-23 was necessary for Th17 cell induction, but this is not the case. IL-23 is essential for Th17 cell population maintenance, but not its induction [154]. Thus the importance of IL-23 in maintaining a functional Th17 cell population makes presence of IL-23R on their cell surface another characteristic of Th17 cells.

In *P. aeruginosa* lung infection, IL-23 has been shown to regulate airway inflammation but it appears it is not required for control of bacterial infection [158]. This was demonstrated using IL-23p19-/- mice where lack of IL-23 reduces

pro-inflammatory cytokines and chemokines in the airspace, but shows no difference in bacterial dissemination [158]. The source of IL-23 in infection is mainly thought to be macrophages and DCs, with IL-23 production from DCs shown to be TLR dependent, shown using mice with dysfunctional TLR4 signalling [49].

In the lung, AMs have been shown to be a source of IL-23 [159] and so these cells may be of great importance at inducing IL-17 responses to *P. aeruginosa* in the lung.

1.8 IL-1β

IL-1β is a pro-inflammatory cytokine produced by macrophages and DCs, and is considered the hallmark cytokine of inflammation. It is a member of the IL-1 superfamily which includes the members IL-1α, IL-18, IL-33 and IL-36. These other members will not be discussed further.

IL-1β has been suggested to contribute to the induction of IL-17 response in humans [124] and enhancement of IL-17 responses in mice [121]. IL-1β exerts its effects through binding to its receptors, IL-1R, of which there are 2, named type I and type II. IL-1RI is a functional receptor, whereas IL-1RII is non-functional and thus acts as a decoy receptor [160]. Upon ligation of IL-1β and IL-1RI, a further molecule is recruited to site, known as IL-1RAcP. Together these 3 molecules make a functional signalling complex that recruits MyD88 and downstream phosphorylation of NFκβ, leading to transcription of pro-inflammatory cytokines [47]. Thus IL-1β is just the first cytokine in a cascade of inflammation.

As IL-1β is so inflammatory there are natural measures in check to prevent its constant assault. One is the 2 step process of its release, as discussed in the next sub-section detailing inflammasome activation. This is a failsafe method to make sure IL-1β is only released when there are both extra and intracellular signals indicating a real threat, and therefore signifies an actual assault on the host cell. A further method is the expression of IL-1RII on cells, mentioned briefly previously. This receptor lacks the TIR signalling domain and therefore lacks any downstream signalling of the IL-1β molecule [160]. It acts as a decoy receptor

and essentially ‘mops up’ any left-over cytokine. A third measure is the presence of further IL-1 superfamily member that is a naturally occurring IL-1β receptor antagonist (IL-1Ra). IL-1Ra binds to IL-1R, preventing binding of IL-1β to its functional receptor and thus abrogating any signalling [161, 162]. These measures have all been put in place naturally by the immune system and thus demonstrate the robust and inflammatory nature of an IL-1β response if it must so be kept in check.

1.8.1 Inflammasome and IL-1β secretion

The processing and secretion of IL-1β is a complex process that involves 2 necessary steps: the production of the pro-form of IL-1β and then the cleavage and activation of pro-IL-1β into mature IL-1β.

The first step involves ligation of a PRR such as a TLR, which leads to production of pro-IL-1β in the cell [163]. As illustrated previously in Table 1-1 there are many different TLRs that recognise different pathogenic components, allowing formation of pro-IL-1β to a multitude of pathogens. This accumulates in the cell waiting to be cleaved by active caspase-1, when a true threat is encountered. The second step involves caspase-1 activation from pro-caspase-1, by a complex called the inflammasome. The inflammasome is activated by an intracellular PRR such as an NLR [164]. There are many types of inflammasome, reflected by their differing NLRs, thus allowing many bacteria types to activate the inflammasome, activating IL-1β and initiating inflammatory responses when needed [165]. Thus both extracellular and intracellular PRRs are important for IL-1β secretion. Furthermore, the inflammasome can be activated by endogenous factors such as ATP binding to the P2X7 receptor [166].

1.8.1.1 The inflammasome

The inflammasome is an intracellular complex composed of several proteins that leads to the eventual release of active IL-1β via activation of casapse-1. The inflammasome is complied of various proteins such as a protein called ASC (apoptosis-associated speck like protein containing a CARD (caspase recruitment domain)), the pro-form of caspase-1, and an intracellular PRR for example an NLR. This is illustrated below in Figure 1-2.

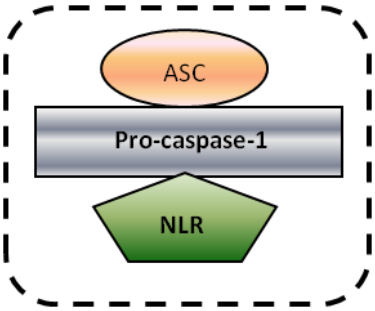

Figure 1-2 The inflammasome.
A complex of proteins composed of pro-caspase-1, an NLR and ASC

Together this complex acts as an intracellular sensor of bacterial invasion, and a response unit. Essentially a bacterial component is recognised intracellularly by an NLR in the inflammasome complex, and this leads to cleavage of pro-caspase-1 into its active form caspase-1. Caspase-1 is then able to cleave the pro-form of IL-1β that exists in the cell, produced upon TLR stimulation, into active IL-1β and allow its secretion from the cell. This full process is diagrammed below (Figure 1-3).

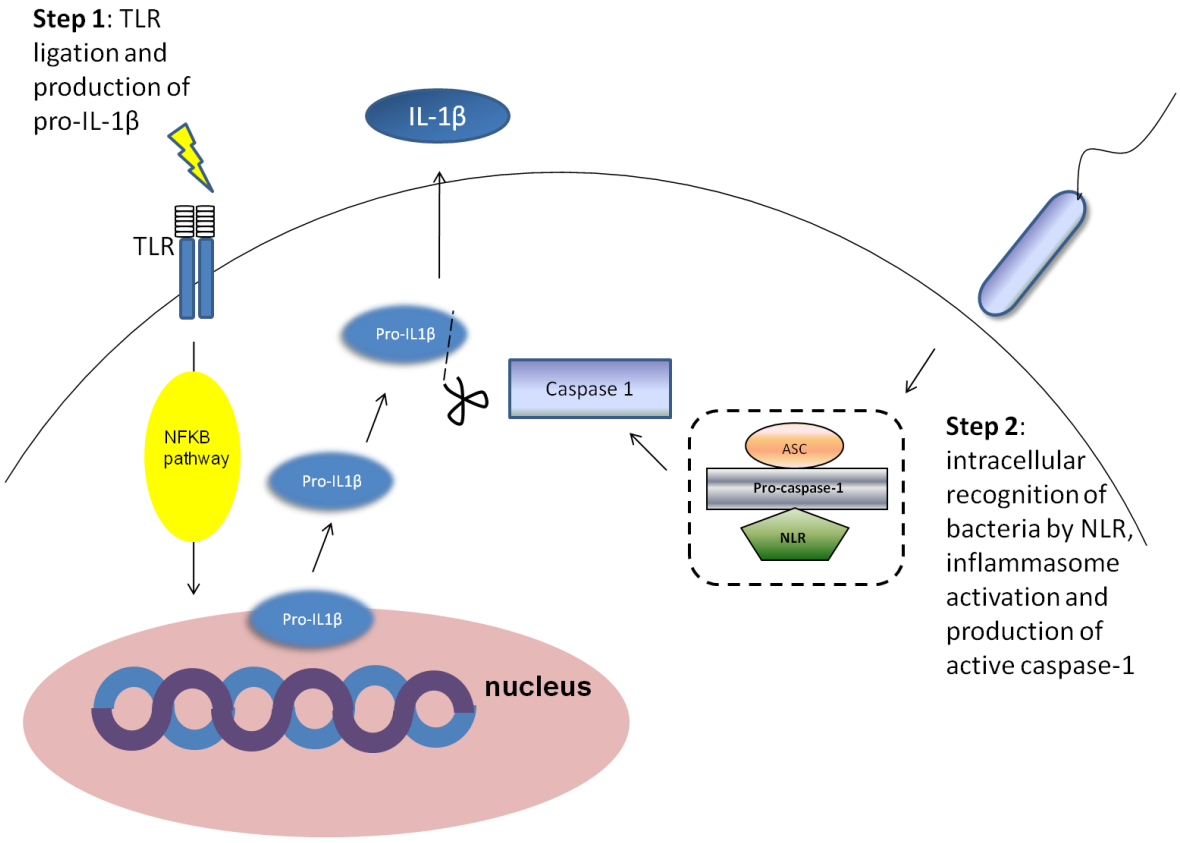

Figure 1-3 IL-1β processing by the inflammasome

1.8.2 Type III secretion system (T3SS)

Gram-negative bacterial strains have a number of systems in place to aid them with secretion of their toxins and virulence, named secretion systems of which there are 6 [167], numbered by roman numerals. *P. aeruginosa* has a type III secretion system (T3SS) that allows attachment of the bacteria to host cells and injection of toxins such as ExoU and ExoT [168]. The T3SS is a needle like complex, called the injectisome, that is composed of an extracellular needle and a base of circular rings that spans the bacterial membrane [169]. This is illustrated in Figure 1-4

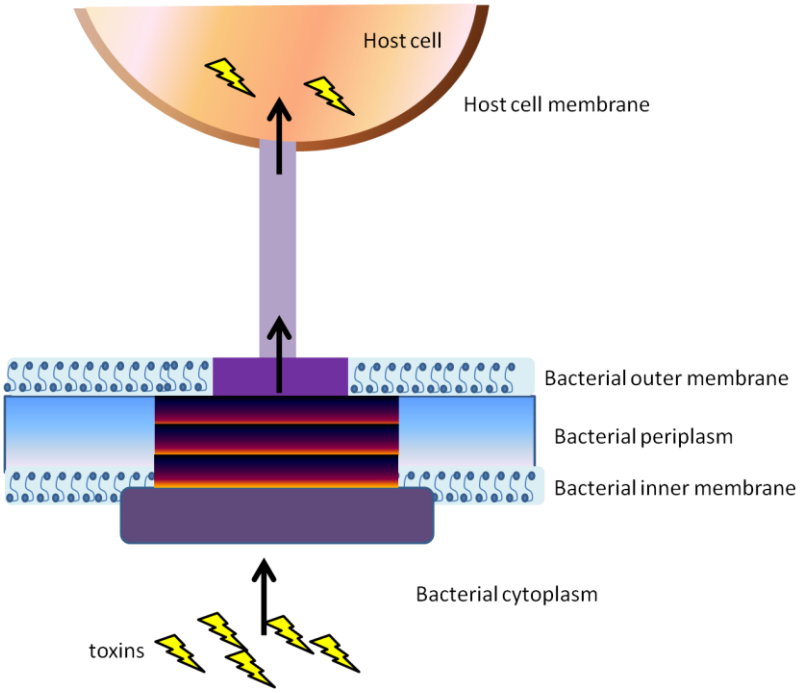

Figure 1-4 Schematic representation of the type III secretion system

The injectisome is said to have evolutionary similarities to the flagella of pathogens [170], a external tail like structure that allows motility [171]. The T3SS is one of the ways *P. aeruginosa* exerts its pathogenic effects by allowing attachment of the pathogen to host cells and passing effector toxins through three membranes, two of the bacteria and one of the host. Passage of these toxins into the host allows them to take effect within the host cells destroying host cell membranes [44], while subverting host responses such as phagocytosis [23]. Phagocytosis of bacteria by host cells, although an immune response protective mechanism, also allows entry of the effector toxins into host cells where they may exert pathogenic effects, yet this would not occur in non-

phagocytic cells. Thus the T3SS is an important virulence factor for bacteria as it allows pathogens to get into these non-phagocytic host cells and exert their harmful effects [170]. However, during this process the pathogen unknowingly allows passage of conserved proteins that are not harmful to the host such as flagellin, the protein component of flagella. These conserved proteins are recognised by intracellular PRRS in the host, for instance flagellin is recognised by NLRC4 [50], thus allowing the host to respond to the pathogen assault. This then may lead to inflammasome activation.

1.9 IL-22

IL-22 is a cytokine that is a member of the IL-10 family [172] that is also thought to be characteristically secreted by Th17 cells and thus is associated with IL-17 secretion. IL-22 is helical in structure and binds to a receptor composed of 2 subunits, IL-22R1 and IL-10R2, a IL-10 receptor subunit [173]. Human and mouse IL-22 share 79% homology [174]. A soluble receptor for IL-22 also exists, named IL-22 binding protein (IL-22BP) [175, 176]. As it is soluble it does not lead to any downstream IL-22 effects and thus is an antagonist to IL-22 signalling. IL-22R1 is found on non-lymphoid tissues such as the skin, small intestine, colon, liver and kidneys, but is absent on lymphoid tissues such as the thymus and spleen [177]. Furthermore IL-22R expression is not be found on of peripheral blood mononuclear cells (PBMCs), the pool of cells in the blood where immune cells reside [177], indicating that IL-22 does not exert effects on immune cells.

1.9.1 IL-22 Functions

IL-22 is thought to be a tissue protective cytokine, aiding in regeneration of epithelia [178] and has been shown to protective roles during a model of hypersensitivity pneumonitis by inhibiting excessive lung fibrosis [179]. IL-22 is thought to exert these tissue repair effects via increased expression of pro-survival molecules Bcl-2 and Bcl-xl, increased production of genes muc1 and muc3 that contribute to mucus production, and increased production of matrix metalloproteinases, enzymes that break down the extracellular matrix and have roles in tissue remodelling [180].

Evidence does exist however for a pro-inflammatory role for IL-22. For example it has been shown to increase transcription of inflammatory genes in hepatocytes (liver cells) during hepatitis B virus infection [181]. High IL-22 is seen in those with psoriasis and it is IL-22 that is thought to be responsible for the pathological inflammation in the skin of these individuals [182]. IL-22 also been shown to be the driving cytokine in bleomycin induced lung injury as blockade of this cytokine prevents further injury, indicating another pro-inflammatory role for IL-22. This study proposes that the pro-inflammatory activities of IL-22 are regulated by IL-17 and in the absence of IL-17, IL-22 promotes tissue repair [183]. Thus the role of IL-22 as a tissue repair or inflammatory cytokine appears to be dictated by the surrounding cytokine milieu. Its role in bacterial infection seems to be that of protective function, with IL-22 being responsible in host defense during, *Klebsiella pneumoniae* infection [112]. It may possibly have a similar function in *P. aeruginosa* infections, and it has been shown that there is increased IL-22 from T cells found in lung draining lymph nodes in CF patients with *P. aeruginosa* infection [112].

1.9.2 Cellular sources

As with IL-17 it appears there are several sources of IL-22, albeit perhaps not as many as IL-17. The main regarded source of IL-22 has traditionally been Th17 cells but there has been evidence in recent years to shed light on other cells that have IL-22 producing capabilities.

1.9.2.1 Th17

As stated Th17 cells are a widely known source of IL-22, with Th17 cells regarded as both IL-17 and IL-22 producing [71]. However there is evidence of a CD4+ helper T cell subset that produces IL-22 but is IL-17 negative. These cells have been aptly named Th22 cells and are discussed below.

1.9.2.2 Th22

10 years ago Th17 cells were discovered and proclaimed as a source of IL-22 also, but recently papers have described a CD4+ cell that secretes IL-22 but not IL-17 [184, 185]. These cells have been identified in the skin of individuals with inflammatory skin disorders such a psoriasis [186]. It was already known that IL-

22 played key role in such disorders but was assumed to be from Th17 cells. The emergence of this new subset identifies a cell type with the anti-inflammatory properties of a Th17 cell but lacking the pro-inflammatory properties of IL-17 (although IL-22 can be pro-inflammatory also under certain circumstances discussed earlier). In Th17 cell dominated infection and disease this may be a cell that could regulate Th17 cell inflammatory responses.

1.9.2.3 γδ T cells

γδ T cells are also a source of IL-22, with evidence seen of IL-22+ IL-17+ γδ T cells [72]. It has been observed during infection with *Bacillus subtilis*, that in the absence of γδ T cells, IL-17A levels remain the same due to compensation of Th17 cells but IL-22 levels are reduced, indicating that γδ T cells are an important source of IL-22 [179].

1.9.2.4 Other sources of IL-22

IL-22 can be made by other cells such as natural killer (NK) cells [187] and the afore mentioned LTi-like cells [78], but these are not investigated and thus not discussed further.

1.10 Hypothesis and aims

Hypotheses of the project are as follows:

1. DCs are activated by *P. aeruginosa* and *S. pneumoniae* to secrete cytokines that may contribute to Th17 cell responses, and mucosal DCs may respond more genuinely *in vitro* than artificially derived DCs in response to mucosal pathogens *P. aeruginosa* and *S. pneumoniae*.

2. *P. aeruginosa* and *S. pneumoniae* primed DCs that produce IL-1β and IL-23 contribute to generation of Th17 responses from naive CD4+ T cells and *ex vivo* mucosal DCs may induce more long-lasting Th17 responses than artificially derived DCs from bone marrow progenitors

3. γδ T cells produce IL-17 in response to DC derived cytokines in a DC independent manner.

4. IL-17+ cells may be pronounced in the lung and surrounding areas during *P. aeruginosa* and *S. pneumoniae* infection and that IL-17 may be responsible for neutrophil recruitment in these spaces.

The overall objectives of this research will be to:

1. Investigate the best method of DC induction from bone marrow progenitors, and use these DCs to investigate IL-1β and IL-23 production from the DCs during infection with *P. aeruginosa* and *S. pneumoniae*. Furthermore, to investigate the isolation of mucosal DCs from the lung and nasal associated lymphoid tissue (NALT) and investigate their responses to these pathogens. This will be addressed in Chapter 4.

2. Use these pathogen primed DCs investigated in Chapter 4, to investigate the consequences of co-culture of pathogen primed DCs with naive CD4+ T cells to see if Th17 responses are formed in response to *P. aeruginosa* and *S. pneumoniae*. This will be addressed in Chapter 5.

3. Investigate the contribution of γδ T cells in IL-17 responses to *P. aeruginosa* primed DCs. This is the focus of Chapter 6.

4. Investigate *in vivo* infections of *P. aeruginosa* and *S. pneumoniae* with focus on IL-17 producing cells in the lung and surrounding area. Preliminary investigations were also performed in IL-17RKO animals to see if IL-17 responses to the pathogens were beneficial or detrimental to the host. This is addressed in Chapter 7.

The following chapters of this thesis will deal with each separate objective, with a brief introduction of the topic and full discussion of results in context with the literature. All chapters will be summarized and linked together in the general discussion, which will also suggest how this research can be further directed.

2 Materials and methods

2.1 Mouse strains

2.1.1 C57BL/6

C57BL/6 mice purchased from Harlan were housed in University of Glasgow Biological Services facilities. Mice were used aged 6-12 weeks.

2.1.2 IL-17R KO

IL-17R KO mice on a C57BL/6 background were obtained from Jay Kolls, Louisiana State University Health Sciences Centre and bred in house.

2.2 Cell culture

2.2.1 Cell lines

2.2.1.1 X63 cells for GM-CSF

X63 cells are a murine cell line derived from a mouse myeloma that produces Granulocyte macrophage colony stimulating factor (GM-CSF) in culture [188]. Cells were obtained from Prof Maggie Harnett, University of Glasgow, and cultured in order to obtain GM-CSF for culturing of bone marrow derived dendritic cells (BMDCs). The cells were cultured in RPMI 1640 supplemented with 10% FCS, 100µg/ml Streptomycin, 100 U/ml Penicillin and 2mM L-glutamine (all Invitrogen). For the first week cells were also cultured in 0.5mg/ml Geneticin (G418)(Invitrogen). After this time cells were cultured in large volumes of G418 free media in large tissue culture (TC) flasks and cultured over a period of 2 weeks, closely monitored and being split when cells looked too dense or media became yellow. After a 2 week period the supernatant was removed by centrifugation to pellet cells and leave supernatant cell free. This was sterile filtered and stored at -20˚C until required. Batches of X63 media were tested by flow cytometry, staining for CD11c, a dendritic cell (DC) marker. Typically results were 50-70% CD11c+ cells cultured by this means from bone marrow derived progenitors (data not shown). These cells were also shown to be MHCII high and are not autofluorescent, a characteristic of macrophages [189, 190], (data not shown) thus we are confident we have culture DCs.

2.2.2 Bone-marrow derived DCs

C57BL/6 mice were scarified aged 6-12 weeks by cervical dislocation or by CO_2 gas, and femurs and tibias were dissected. Bone marrow progenitors were isolated similarly to previously described by others [191]. Briefly the ends of the bones were cut off using dissection scissors and the bone marrow was flushed into fresh media using a 21g needle and 5ml syringe. The bone marrow was then broken up by suction up and down the needle and then passed through sterile nitex mesh to remove any debris.

Culture media used was IMDM+glutamax supplemented with 10% heat inactivated FCS, 100µg/ml Streptomycin, 100 U/ml Penicillin, 2.5µg/ml Fungizone (amphotericin B) (all Invitrogen). Bone marrow mononuclear phagocytic precursors were plated into 9cm low adherence petri dishes (Sterlin) at a concentration of between 3-4x10^6 cells/plate. Media was additionally supplemented with 20% X63 media or 10ng/ml recombinant GM-CSF (Peprotech).

Cells were cultured for 7 days with feeds of 7ml complete media supplemented with X63 media or GM-CSF after days 3 and 6. Mature DCs were used between days 7-10. Mature DCs were scraped off plates using ice cold phosphate buffered saline (PBS) and harvested into 50ml tubes (Invitrogen). Cells were counted, washed in PBS and resuspended at desired concentrations for experiments.

2.2.3 Primary cell isolation

2.2.3.1 Splenocyte isolation

Spleens were dissected and mashed through 40um nitex into sterile media to obtain single cell suspensions. Cells were spun down at 300g for 10 minutes and pellet was resuspended in 5ml of red cell lysis buffer per spleen and left to incubate at room temperature (RT) for 10 minutes after which time cells were drenched with PBS and spun down again at 300g. Cells were resuspended in PBS, a sample was taken for counting and cells were spun down again giving them a final wash. Cells were then treated for specific isolation depending on experiment.

2.2.3.2 T Cell isolation using Magnetic-Activated Cell Sorting (MACS)

2.2.3.2.1 Naive T cell isolation

Naive Cells were sorted on the basis of CD4 and CD62L to isolate CD4+, CD62L+ naive populations and CD4+, CD62L- memory populations using antibody conjugated magnetic beads from Miltenyi and following manufacturer's instructions.

CD4 cells were negatively selected, whereby every other cell in the preparation was labelled and retained in the magnetic column allowing untouched CD4 cells to pass through. It is important to note that γδ T cells are not retained by this kit, and therefore come through in the flow through. Removal can be achieved by adding γδ beads, from a γδ positive selection kit, at time of bead incubation. CD62L cells were positively selected, with the naive CD4+ cells being in the CD62L+ prep retained on the column.

2.2.3.2.2 γδ T cell isolation

An enriched but not completely pure population of γδ T cells could be observed in the CD4+ preparation of splenocytes treated with the CD4 MACS negative selection kit. This was due to the kit not retaining γδ T cells as mentioned previously. This preparation was used for a majority of the γδ T cell work as γδ T cells make up such a small percentage of splenocytes that pure γδ T cell isolation allowed for very few conditions. However to test our hypothesis with γδ T cells some experiments were performed with a purified γδ T cells population, isolated using a γδ positive selection MACS kit and following manufacturer's instructions.

2.2.3.3 Nasal associated lymphoid tissue (NALT) isolation

NALT is a small tertiary lymphoid structure in mice above the hard palate. This tissue is so small it is difficult to identify by eye, so it is best just to remove the hard palate and isolate any cells associated with it. NALT was dissected by removing the head and jaw and using fine sharp scissors or a scalpel to cut around the hard palate, on the inside of the teeth. The hard palate was then removed very gently using a pair of tweezers and was agitated in a vial of sterile PBS to allow the cells of the tissue to become free. The hard palate can further

be mashed through nitex but it is recommended doing so after agitation as there are very few cells and some may get lost on the nitex.

2.2.3.4 Lung cell isolation

Lungs were digested using 50µl/ml of 26units/ml stock liberase (Roche) and 10µg/ml DNase (Roche) in RPMI. Lungs were chopped into small pieces in a bijoux and then 2ml of liberase/DNase digestion mix was added to each tube. Tissues were incubated at 37°C with gentle agitation for 1 hour before being passed through nitex to obtain a single cell suspension.

2.2.3.4.1 NALT and lung DC isolation

NALT or lung cells were isolated as stated, and DCs were removed using CD11c positive selection MACS beads (Miltenyi) following manufacturer's instructions, with the additional step of density centrifugation required for lung cells before CD11c MACS treatment.

2.2.3.4.2 Density centrifugation

After digestion of lung tissue the cells were spun down at 300g for 10 minutes to obtain a cell pellet. The 'volume' of cell pellet was estimated and an equal measure of Hanks buffered salt solution (HBSS) was added and mixed well. On top of this 2ml of ficoll-paque (GE healthcare) was gently laid and preparations were spun at 300g for 30 minutes with the brake off. This allows the distribution of cell populations as can be observed in the diagram below (Figure 2-1).

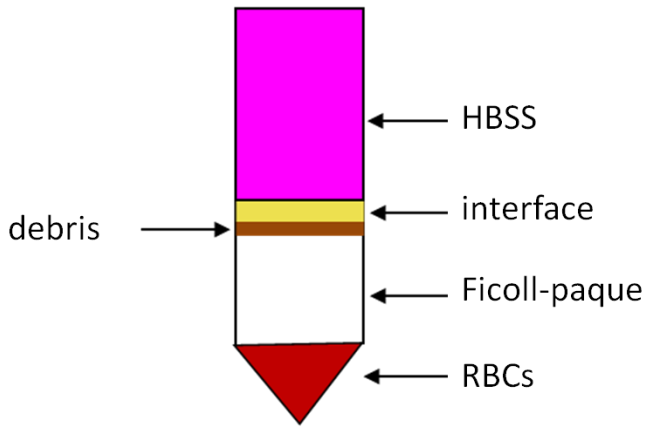

Figure 2-1 Illustration of density centrifugation of lung tissue

The interface, which contains immune cells, was isolated by gently pippeting without disturbing the other layers, before being washed twice and treated for DC isolation using MACS as previously described.

2.2.4 DC infection

$2.5x10^5$ DCs were set up in 48 well plates, in 500µl at a concentration of $5x10^5$ cells/ml and infected with the pathogens at various multiplicity of infections (MOIs) depending on pathogen, for various times depending on experiment. Antibiotics were added to cultures after desired time to kill pathogen: 10ng/ml gentamicin (Invitrogen) was added to *P. aeruginosa* infected cultures and 100µg/ml Streptomycin, 100 U/ml Penicillin was added to *S. pneumoniae* infected cultures. DCs were then incubated at 37°C overnight before being harvested for co-culture.

2.2.5 Co-culture set up

$1x10^5$ T cells were set up with $1x10^4$ DCs in their respective medias in 96 well round bottom plates. Wells were anti-CD3 coated (2.5µg/ml) (Ebioscience) for naive set up, but not memory. Where DCs alone in absence of conditioned media were cultured with T cells, the DCs were washed out of their conditioned media and resuspended in fresh media, before culturing with the T cells in fresh media. Where DC condition media was used alone in the absence of DCs, the DCs were spun out of the media, and the T cells spun down also, with the T cells resuspended in the DC condition media. Where both DCs and condition media were present, T cells were treated as above and DCs were just carried over from treated well in condition media in whatever volume contained $1x10^4$ DCs. Naive T cell co-cultures were left for 6 days, γδ T cell co-cultures for 3 days, after which times supernatants were harvested for analysis with ELISA, and cells were stained for flow cytometry.

2.3 Bacterial strains

2.3.1 P. aeruginosa

2.3.1.1 PA103

PA103 is a cytotoxic strain of *P. aeruginosa* commonly used in research [192]. It is non-flagellated, making it less motile, and has recently been found to activate the inflammasome, a platform that allows eventual IL-1β release (described in the introduction), via pilin [193]. Lack of flagellin may decrease bacterial recognition somewhat as flagellin is recognised by TLR5 [46], however there are other PRRs on DCs that detect *P. aeruginosa,* such as TLR4 recognition of LPS [46], and therefore lack of TLR5 is not a concern for our experimental set-up. Furthermore, in Chapter 4 we demonstrate that PA103 stimulates DCs effectively, measured by various outputs discussed in Chapter 4. PA103 possesses the toxins ExoU and ExoT and is very cytotoxic to cells upon infection. PA103 is the focus of our *in vitro* studies with *P. aeruginosa*, of which we use 2 strains PA103 ΔUΔT and PA103 ΔpcrV.

2.3.1.1.1 PA103 ΔUΔT

The symbol Δ denotes a lack of the following element. In this case this bacterial strain lacks the pseudomonal toxins ExoU and ExoT. Lack of these toxins, inhibits the cytotoxic properties of this pathogen, thus making it a useful model for investigation of immune recognition of the pathogen. Use of the wild type (WT) of this strain would simply kill the cells with which it was cultured making analysis challenging. Furthermore ExoU inhibits inflammasome activation [194] which is important for IL-1β secretion as discussed in the introduction. As IL-1β is of interest to our studies, ExoU deficient strains must be used in these experiments to allow inflammasome activation.

2.3.1.1.2 PA103 ΔpcrV

PcrV is an essential component of the Type III secretion system (T3SS) [169,195] and thus important for the pathogenic functions of *P. aeruginosa*. PA103 ΔpcrV lacks of this essential component resulting in lack of functional T3SS, and therefore obliterates any downstream effects such as inflammasome activation and IL-1β secretion.

2.3.1.2 PAO1

P. aeruginosa strain PAO1 is an invasive strain that is commonly used in experiments studying *P. aeruginosa* and is fully sequenced [196].

PAO1 can be purchased from calliper with a bioluminescent label, named Xen41, and is the focus of our *in* vivo studies with *P. aeruginosa*, where we wish to visualise colonisation of the pathogen in the lung using an *in vivo* imaging system (IVIS).

2.3.2 S. pneumoniae

2.3.2.1 D39

D39 is a capsulated virulent strain of *S. pneumoniae*, commonly used in laboratory experiments, whose genome has been mapped [197]. D39 is the focus of our *in vitro* studies with *S. pneumoniae*, of which we use 2 strains D39 WT and D39 ΔPLY.

2.3.2.1.1 D39 ΔPLY

D39 ΔPLY has an absent PLY toxin and is less virulent than its WT counterpart.

2.3.2.2 TIGR4

TIGR4 is a capsulated *S. pneumoniae* which can be purchased with a bioluminescent label from Calliper, named Xen35, and is the focus of our *in vivo* studies with *S. pneumoniae*, where we wish to visualise colonisation of the pathogen in the lung using an IVIS.

2.4 Bacterial cultures

2.4.1 P. aeruginosa

A single colony or 50µl of a 25% glycerol stock was added to 10ml of Luria-Bertani (LB) media (Invitrogen) and grown for 15 hours overnight at 225rpm at 37˚C with caps loose to allow aeriation. After 15 hours, the overnight culture was diluted 1:30 in LB and grown for another 90 minutes or until the OD600 was between 0.4 and 0.6 for this is when they are in exponential growth phase.

Bacteria were the centrifuged at 3000g for 10 minutes at 4˚C and washed twice in sterile PBS. Bacteria cell pellets were then resuspended in a volume of antibiotic free media to a concentration of approximately $1x10^6$ cfu/µl. The volume was obtained using this formula:

(OD600) / 0.4 x 1.8 = volume (ml) to resuspend pellet in, to obtain $1x10^6$ cfu/µl.

2.4.2 S. pneumoniae

For *S. pneumoniae*, a scraping of 25% glycerol stock was plated on blood agar plates and incubated at 37˚C overnight. A few colonies were selected and put into 5ml of brain heart infusion (BHI) broth under completely sterile conditions and grown in a 37˚C water bath for approx 8-12 hours or until 0D600 was between 0.3 and 0.5. Bacteria were the centrifuged at 3000g for 10 minutes at 4˚C and washed twice in sterile PBS and finally resuspended in the initial volume of antibiotic free media. Number of bacteria in suspension was ascertained by:

$$e\ (OD600x8.5) = cfu\ x10^7/ml$$

2.4.3 Pathogen minimum inhibitory concentration (MIC) assay

To test the lower limit of antibiotic needed to effectively kill pathogen a MIC assay was performed. A volume of pathogen at a known concentration was set up in wells in a 96 well plate. Antibiotics were added at doubling dilutions in duplicate and plates were incubating overnight. MIC was measured by visualising wells that were clear and so antibiotic was effective at killing pathogen, and wells that were cloudy, where antibiotic had not been effective and pathogen had multiplied. By this means a point of effectivity of antibiotic could be observed. An example of this is seen in Figure 2-2, a MIC assay of *P. aeruginosa* at an MOI of 10 testing the lower limit of gentamicin to use to effectively kill the pathogen. This figure shows an MIC of 12.5µg/ml gentamicin is effective at killing *P. aeruginosa* MOI 10.

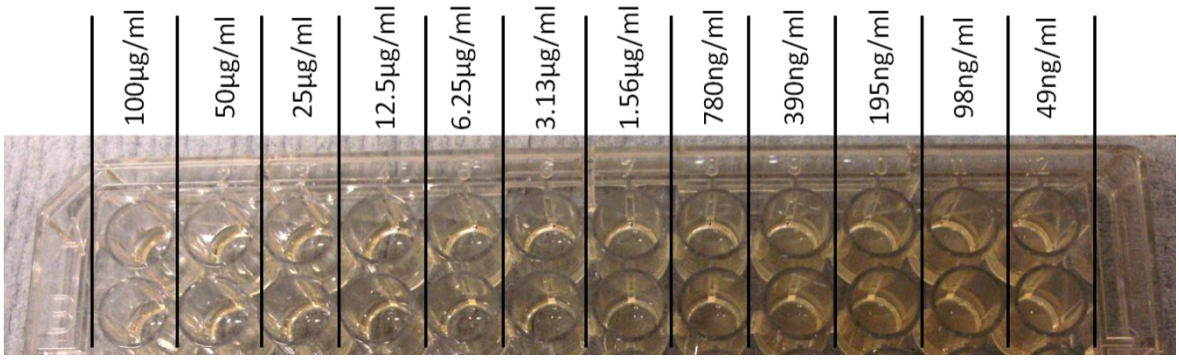

Figure 2-2 MIC assay of gentamicin killing of *P. aeruginosa*

2.5 Flow cytometry

Flow cytometry, or Fluorescence-activated cell sorting (FACS), was performed using antibodies purchased from Biolegend. Cell marker fluorochromes, their appropriate isotypes and the concentration at which they were used are documented in Table 2-1.

Table 2-1 Antibodies for flow cytometry

Marker	Clone	Isotype	Fluorescence label	Concentration used
CD4	RM4-5	Rat IgG2a, κ	FITC	5 µg/ml
CD8	53-6.7	Rat IgG2a, κ	FITC	5 µg/ml
γδ	GL3	Armenian Hamster IgG	FITC	2 µg/ml
γδ	GL3	Armenian Hamster IgG	APC	2 µg/ml
IL-22	poly5164	Polyclonal Goat IgG	PE	100µl/ml
Gr-1	RB6-8C5	Rat IgG2b, κ	PE/Cy7	2 µg/ml
IL-17A	TC11-18H10.1	Rat IgG1, κ	Alexafluor647	5 µg/ml
CD11c	N418	Armenian Hamster IgG	APC	2 µg/ml
CD40	HM40-3	Armenian Hamster IgM	FITC	5 µg/ml
CD86	GL-1	Rat IgG2a, κ	FITC	5 µg/ml

Extracellular staining was performed after cell harvest by washing cells twice in PBS at 300g to rinse off media. Cells were then incubated with appropriate dilutions of antibody, mentioned in Table 2-1, in 50µl FACS buffer for 30mins at 4°C. After this time cells were washed and acquired or fixed for intracellular staining.

Intracellular staining for IL-17 and IL-22 was performed on the cultured T cells, and harvested *in vivo* infected lung cells. Cells were stimulated with phorbol 12-myristate 13-acetate (PMA) (50ng/ml) and Ionomycin (500ng/ml) (both Sigma) in the presence of Brefeldin A (10µg/ml) (Sigma) for 5 hours, after which time cells were harvested and washed twice in PBS and extracellular staining was performed as described above.

After extracellular staining, cells were washed 3 times in PBS before being fixed using 200µl fixation solution (Biolegend) for 20 minutes at RT in the dark. After fixation cells were washed 3 times with 1ml permeablising solution (Biolegend) and stained for intracellular markers at appropriate dilution mentioned in Table 2-1 in the permeablising solution, and allowed to incubate at RT for 20 minutes in the dark. Cells were then washed 3 times in 1ml permeablising solution and resuspended in FACS buffer and kept on ice before being acquired on FACSCalibur (BD bioscience) where 20,000 gated events were recorded for each sample. Data was analysed using FlowJo software (Tree Star) comparing positive markers to negative isotypes. Cells were gated on forward scatter (FSC) and side scatter (SSC) to isolate the live population, regarded as cells not clustered on the axis. An example of this gating can be observed in Figure 2-3. The viability of these cells was verified using 7AAD (BD biosciences)(data not shown).

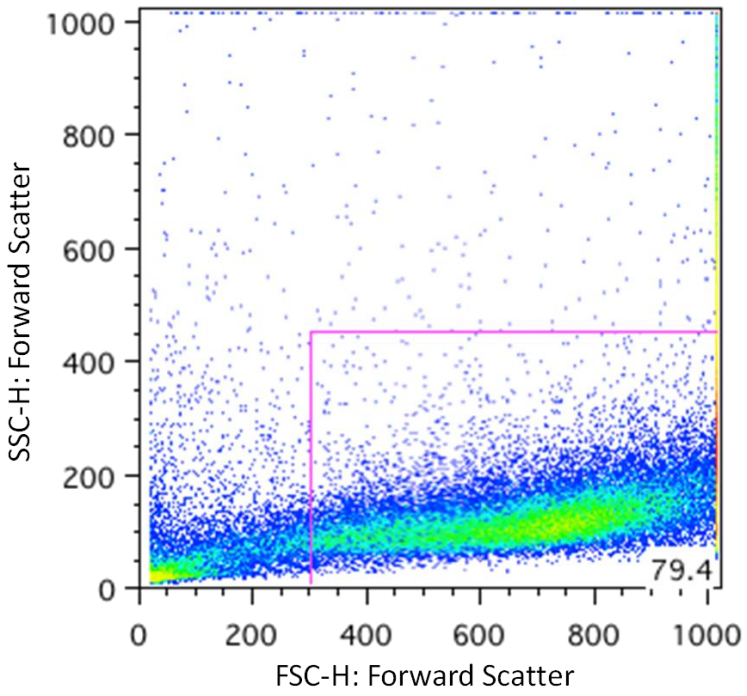

Figure 2-3 Example of standard gating for flow cytometry analysis.

This gating strategy was used when investigating *in vitro* DC cultures, *in vitro* T cell-DC co-cultures and *in vivo* CD4, CD19, γδ and IL-17 cell populations. The only exception to this gating was when Gr-1 positive cells were investigated during the *in vivo* infection experiments where the gate was extended up the side axis to encompass more granular cells as this is where Gr-1 cells would be found but no the other cells of interest would be situated here.

2.5.1 7AAD

7-Aminoactinomycin D (7AAD) is a fluorescent compound that can be detected by flow cytometry in fluorescence channel 3. It strongly binds to DNA and cannot penetrate cell membranes and so only binds to the DNA of dead cells, making it a useful live/dead cell marker. Dead cells are identified as 7AAD high cells and live cells are 7AAD low. 7AAD is available to buy commercially from BD, and 3µl of this was added to samples right before they were analysed on the FACSCalibur. An unstained sample of cells and a 7AAD single stained sample were recorded also for gating and compensation purposes.

2.5.2 CFSE

Carboxyfluorescein diacetate succinimidyl ester (CFSE) is a fluorescent dye that labels cells and is used as a measure of cell proliferation as it can be detected by flow cytometry. Cells take up CFSE and pass it on to daughter cells during cell division, thereby halving the concentration of CFSE present in each cell. This division can be observed by flow cytometry by decreasing peaks of CFSE shifting to the left. CFSE treatment of T cells was performed using a CellTrace™ CFSE Cell Proliferation Kit (Invitrogen), following manufacturer's instructions.

2.6 Enzyme-linked immunosorbant assay (ELISA)

ELISA duosets from R&D were used to determine the concentration of cytokines in cell culture supernatants, following manufactures instructions with slight variations. Generally high affinity 96 well flat bottom plates (VWR) were coated with 50µl capture antibody at recommended concentration in PBS with no carrier protein, overnight at RT. They were then washed four times with wash buffer (PBS, 0.05% Tween 20), blocked using 300µl appropriate block buffer (see Table 2-2) and allowed to incubate at RT for one hour. The plates were washed before

applying the samples and standards, which were incubated for 2 hours at RT or overnight at 4°C. Standards were provided by manufacturer and were performed in duplicate, with doubling dilutions. The plates were washed before the detection antibody conjugated to biotin was added and incubated for 2 hours at RT, or overnight at 4°C. Plates were washed again and then 50µl of streptavidin peroxidase dilution (1:200) in required reagent diluent (see Table 2-2) was applied and plates were incubated at RT for 20 minutes in the dark. The plates were washed, and 50µl TMB (KPL) substrate added to each well. Cytokine presence was indicated by substrate turning blue. During this reaction the plates were incubated in the dark, time dependent on the assay as some turned blue faster than others. Plates were removed from dark and 50µl of stop solution (KPL) was added when standards appeared to be of a satisfactory colour of blue, usually until blue had slightly appeared in standard dilution 7. Plates were then measured at 570nm using a plate reader (Tecan, sunrise) running Magellan software, with values calculated by software using standard curve generated by software. Table 2-2 illustrates the kits used, along with the concentrations of each antibody for each kit, the reagent diluents used and the standards measured.

Table 2-2 R&D Duoset antibody and standard concentrations

Cytokine	Capture Antibody (µg/ml)	Detection Antibody (µg/ml)	Reagent diluent	Blocking buffer	Top standard (pg/ml)	Lower Limit of Detection (pg/ml)
IL-1β	720	450	0.1% BSA, 0.05% Tween 20 in Tris-buffered Saline	PBS,1% BSA	1000	1.9
IL-17	144	72	PBS,1% BSA	PBS,1% BSA	1000	1.9
IL-22	360	108	PBS, 5% Tween 20	PBS, 5% Tween 20, 0.05% NaN3	2000	3.9
IL-23	720	18	PBS,1% BSA	PBS,1% BSA	1000	1.9

Unless otherwise stated a volume of 50µl per well was used, and antibodies, standards and samples were diluted in appropriate reagent diluents dependent on kit and cytokine (Table 2-2).

2.7 *In vivo* experiments

2.7.1 Intranasal lung infections of mice with bacteria to induce pneumonia

Pneumonia was induced in C57BL/6 mice using Gram-positive *S. pneumoniae* TIGR and Gram-negative *P. aeruginosa* PA01. Bioluminescent strains of these pathogens were purchased from calliper, named Xen35 and Xen41 respectively.

Bioluminescent pathogens were observed using the IVIS, a spectrum imager that can be used for *in vivo* disease monitoring to see if pathogens had been inhaled and if they had proceeded to the lung. When using bioluminescent pathogens

and the IVIS, white BALB/c mice are usually used and the mice can be simply photographed, but the fur of black C57BL/6 mice disrupts with the images and so C57BL/6 mice must be shaved first to expose the skin so the bioluminescence can be viewed.

Pathogens were given intranasally by anesthetising the mice using isoflurane and then gently pippeting 25µl of pathogen at desired concentration in PBS, $5x10^6$ for Xen 35 and $2x10^7$ for Xen 41, following methodology to induce acute pneumonia as developed by the group of TJ Mitchell, University of Glasgow and as tested by us. This was pipetted into the nostrils of the animal where the animal will inhale it by reflex, hopefully bypassing the swallowing reflex and allowing it to reach the lung. Mice are photographed in the IVIS before and after infection to show that bacteria have in fact reached the lung and we are confident pneumonia will be induced. IVIS use requires a qualified technician.

Animals were divided into 3 groups; control, *P. aeruginosa* infected and *S. pneumoniae* infected. Infected mice were treated as above and control mice were untreated completely. Mice were left until 48 hours and weight and health were monitored. 20µl of blood was taken by tail bleed after 24 hours to see if pathogen had become systemic as yet. Mice were culled as near to 48 hours as possible within regulations of home office license.

2.7.2 Measuring bacteria for intranasal infection

Pathogen concentration for infection was measured by plating out stocks of pathogen in various dilutions on agar plates, LB agar for *P. aeruginosa* and blood agar for *S. pneumoniae*. Colonies were counted and multiplied by dilution factor to work out how many pathogens/ml a stock contained in order to calculate working concentrations of pathogen for infections. Stocks were washed twice in PBS, spun at 4000 rpm and resuspended finally in PBS. Aliquots of the infecting sample must be taken before and after animal infection, re-plated and counted, to ensure that the desired amount of pathogen was given.

2.7.3 In vivo sample harvest

After cull, a bronchoalveolar lavage (BAL) was performed and was used for bacterial cultures, cytospin with Haemotoxylin&Eosin (H&E) staining and cytokine analysis by ELISA.

Pleural washes were collected and treated the same as the BALs.

Lungs were dissected and divided into 3 for 3 separate treatments. One section was placed in PBS for homogenising for plating for bacteriology; another was placed in PBS for digestion to obtain single cell suspensions for FACS staining to identify cell populations, and another placed in buffered formalin for fixation and histology.

Blood was taken from chest cavity, nasal washes were performed and brain and lung were taken for homogenising, all for plating for bacteriology.

2.7.3.1 BAL collection for bacteriology and cytospin

Animals were culled using CO_2 at 48 hours or when deemed too sick to continue according to home office regulations. Skin from around the throat was cut to expose the trachea, being careful not to disrupt the trachea or blood vessels. Holding the trachea slightly aloft, a small hole was cut at the front of the trachea and a pipette cut at an angle to create a bevelled tip, was inserted into this hole and 1ml PBS is flushed through the lungs twice.

100µl of this is used for plating for bacteriology, the remaining sample is treated first for a cytospin and then the remaining liquid is harvested for ELISA. We recognise the limitations of a single time point with reference to our cytokine measurements as it may not accurately portray cytokine levels in this space as it would fluctuate during infection. However, the resources were not available to us to consider other time points.

2.7.3.2 Pleural wash collection for bacteriology and cytospin

The chest cavity was exposed by removing the skin around it, careful not to pierce the pleura. The liver was removed carefully to allow exposure of the

diaphragm, which must be released to allow full distension of the pleural space. This was done by gently snipping the flaciform ligament of the liver which is attached to the diaphragm. Pleural washes were taken using 5ml of calcium free PBS, by inserting 18g needle between the ribs, which was removed using a 23g needle inserted under the animals right side of the diaphragm. These washes were treated the same as the BALs.

2.7.3.3 Lung tissue

The lungs of each animal were divided into 3 equal parts for various treatments described below.

2.7.3.3.1 Lung section for Flow cytometry

A section of lung was placed in PBS and then treated with a digestion mix (described previously) and passed through nitex to obtain a single cell suspension as described previously in lung cell isolation section. Cells were then stimulated with 50µg/ml PMA and 500µg/ml ionomycin in the presence of brefeldin A for 5 hours after which time they were harvested and stained for γδ, CD4, Gr-1, CD19 and IL-17 and analysed by flow cytometry.

2.7.3.3.2 Lung section for histology

A section of lung was embedded in paraffin, sectioned and stained with H&E to stain for neutrophils and macrophages.

2.7.3.3.3 Lung Homogenate for bacterial load measurements

Sections of lung were rinsed in ethanol and PBS before being homogenized in 1ml sterile PBS. The homogenizer was disinfected between samples using 70% ethanol to prevent contamination of subsequent samples. After ethanol clean, homogenizer was rinsed with PBS to remove any remaining ethanol that could prevent inhibition of bacterial growth of subsequent samples.

2.7.3.4 Blood collection for bacteriology

Blood was collected from tail bleeds mid experiment at 24 hours and from the chest cavity after culling. 20µl was collected and diluted 1:10, 8 times and then

plated in triplicate on agar plates to allow them to grow to count bacteria in the blood to investigate if the infection was systemic.

2.7.3.5 Nasal wash collection for bacteriology

Nasal washes performed by removing the head and flushing 1ml PBS through the nasal cavity, using a pastette, and collecting the liquid via the nose into an eppendorf.

2.7.4 Bacterial culture

Homogenates and washes collected as described above for bacteriology were serially diluted in sterile PBS and cultured, 20µl in triplicate, on appropriate media plates, LB agar plates for *P. aeruginosa* infection and blood agar plates for *S. pneumoniae* infection and incubated overnight at appropriate conditions, 37°C incubator for *P. aeruginosa*, and in an anaerobic cabinet at 37°C for *S. pneumoniae*. Bacterial colony counts from each animal were counted, averaged and compared between groups. Cfu/ml was calculated by average cfu, multiplied by the dilution, multiplied by 50 (as bacteria were plated in 20µl, 20µlx50=1000µl=1ml).

2.7.5 Cytospin

Cytopsins were performed of the BAL and pleural washes. For each sample cells were counted and resuspended at a concentration of $1x10^6$/ml in PBS so that a consistent $1x10^5$ cells would be applied in 100µl to all slides. Positively charged glass slides were labelled and placed into the metal holders of the cytospin apparatus, with cardboard filters and the cuvettes, to which an aliquot of 100µl of sample was added. Slides were then spun down at 400rpm for 6 minutes after which time cytospin apparatus was removed carefully as to not smudge the cell spot, and slides were allowed to air dry overnight. Once dry, cells were stained using rapid romanowsky and mounted using DPX and coverslips.

2.7.5.1 Rapid romanowsky staining of cytospins

Rapid romanowsky staining of cytospin slides was performed using a kit from Thermo scientific and following manufactures instructions.

2.7.6 Histology

Tissues were harvested and fixed in buffered formalin for 48 hours after which time they were transferred to 70% ethanol until they were embedded in paraffin. Tissues were processed using a Shandon citadel 1000 tissue processor (Thermo scientific). After this, tissues were transferred to blocks and submerged in hot wax and left to cool. Wax embedded tissues were cut onto slides using a microtome, before being left to dry and then stained for H&E and histologically analysed and photographed.

2.7.6.1 Hematoxylin&eosin staining of lung tissue

To H&E stain paraffin wax sections, wax was softened in a 60°C for 35 minutes and the following protocol was followed:

Dewax

1. Submerged in Xylene for 3 minutes X 2
2. 100% ethanol for 3 minutes X 2
3. 90% ethanol 3 minutes x 2
4. 70% ethanol 3 minutes x 2
5. Running water 3 minutes

Stain

1. Harris Haematoxylin for 2 minutes
2. Running water for 2 minutes

Differentiation

1. 1% Acid/Alcohol for a few seconds
2. Quick rinse in running water
3. Scotts Tap Water Substitute
4. Quick rinse in running tap water

Counter stain

1. 1% Eosin for 2 minutes
2. Running water

Dehydrate sections

1. 70% alcohol for 30 seconds
2. 90% alcohol for 30 seconds
3. 100% alcohol for 1 minute x 2

4. Xylene 3 minutes x 2

Mount coverslip over tissue section from Xylene with DPX mountant (Sigma).

2.8 Reagents

T cell media - IMDM+glutamax supplemented with 10% heat inactivated FCS, 100µg/ml Streptomycin, 100 U/ml Penicillin (all Invitrogen)

DC media – as T cell media with 2.5µg/ml Fungizone (amphotericin B), supplemented with 10ng/ml GM-CSF

PBS - 8g/L of NaCl, 0.2g/L of KCl, 1.44g/L of Na_2HPO_4, 0.24g/L of KH_2PO_4 (all Sigma) in distilled water.

Wash buffer - PBS, 0.05% Tween20

FACS buffer - 2% FCS in PBS, 0.09% NaN3 (Sigma)

MACS buffer - PBS, pH 7.2, 0.5% BSA, and 2 mM EDTA (Sigma)

Red cell lysis buffer - 0.15M NH_4Cl, 10mM $KHCO_3$, 0.1mM EDTA (all Sigma) in distilled water, pH 7.4

2.9 Statistical analysis

All ELISA results were analysed by paired T test, 1-way ANOVA or 2-way ANOVA using Graphpad Prism 4 software. Results were displayed as mean ± standard error of mean. A p value of < 0.05 was considered statistically significant.

3 Methods developed

3.1 Isolation of single cell suspension from lung

Several methods of obtaining single suspensions of lung cells have been described [198-200]. In general, cells are treated with digestive enzymes and then mashed through nitex to obtain single cell suspensions. Most protocols describe the use of DNase and collagenase D [200]. We initially used this combination for our digestion mix (both purchased from Roche) but we found that our single cell suspensions contained a lot of debris, and that further cell isolations using MACS isolation were less than 20% pure (data not shown). Others in the lab have used liberase (Roche) for lung tissue digestion instead of DNase and so this was tried also. We then compared to the quality of cell populations liberated by use of either collagenase D or liberase, both with DNase.

Use of liberase allows a much cleaner population with less debris than when collagenase D is used, as can be identified when looking at cells on the haemocytometer on the microscope (data not shown). Furthermore, use of liberase in preparation for CD11c+ dendritic cell (DC) isolation with MACS gives a enhanced CD11c+ population (Figure 3-1) indicating that a better single cell suspension has been produced by this means.

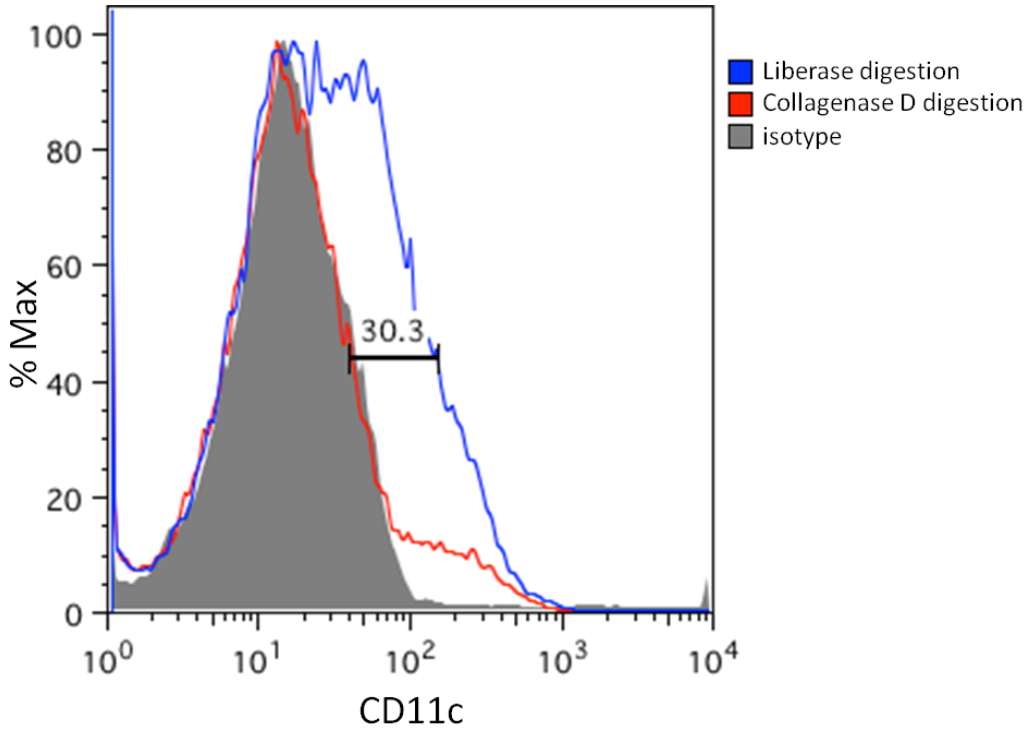

Figure 3-1 CD11c+ isolation using liberase to isolate lung cells is more effective than use of Collagenase D

Lung cells were isolated using a digestion mix of DNase with liberase (blue line) or Collagenase D (red line) and then treated with CD11c+ MACS selection. Cells stained for CD11c and analysed by flow cytometry, compared to isotype (filled histogram). Cells were gated on live cells based on their forward scatter (FSC) and side scatter (SSC) as described in materials and methods. Data representative of 3 experiments.

A 30% CD11c+ cell increase is evident with liberase preparation compared to 12% with collagenase D. Due to this, liberase was used for all experiments that required lung cell isolation.

3.2 Lung DC isolation

Isolation of lung DCs was performed by obtaining a single cell suspension of lung tissue and then using a CD11c+ positive selection MACS kit (Miltenyi).

Some studies recommend that to increase CD11c+ cell acquisition from the lung, cells should be incubated on tissue culture (TC) plates for 1 hour at 37°C after lung cells have been liberated, after which time non-adherent cells should be removed by gently pipetting off half the volume. Cells should be further incubated on TC plates overnight, with loosely adherent cells harvested the next day as it is this population that is believed to contain the CD11c+ cells [201].

This will be referred to therein as the adherent population, with removed cells referred to as the non-adherent population. The adherent cell preparation should then be harvested and treated with CD11c+ MACS kit to give a CD11c+ enriched population.

To test this phenomenon, we also kept aside the non-adherent populations for isolation and staining also to confirm that this population of cells did not contain CD11c+ DCs. Using both the adherent and non-adherent cell preparation with the CD11c MACS positive selection kit and subsequent staining with CD11c antibodies to test purity of CD11c isolated populations, we observed that there were just as many CD11c+ cells in the non-adherent preparation as the adherent (Figure 3-2).

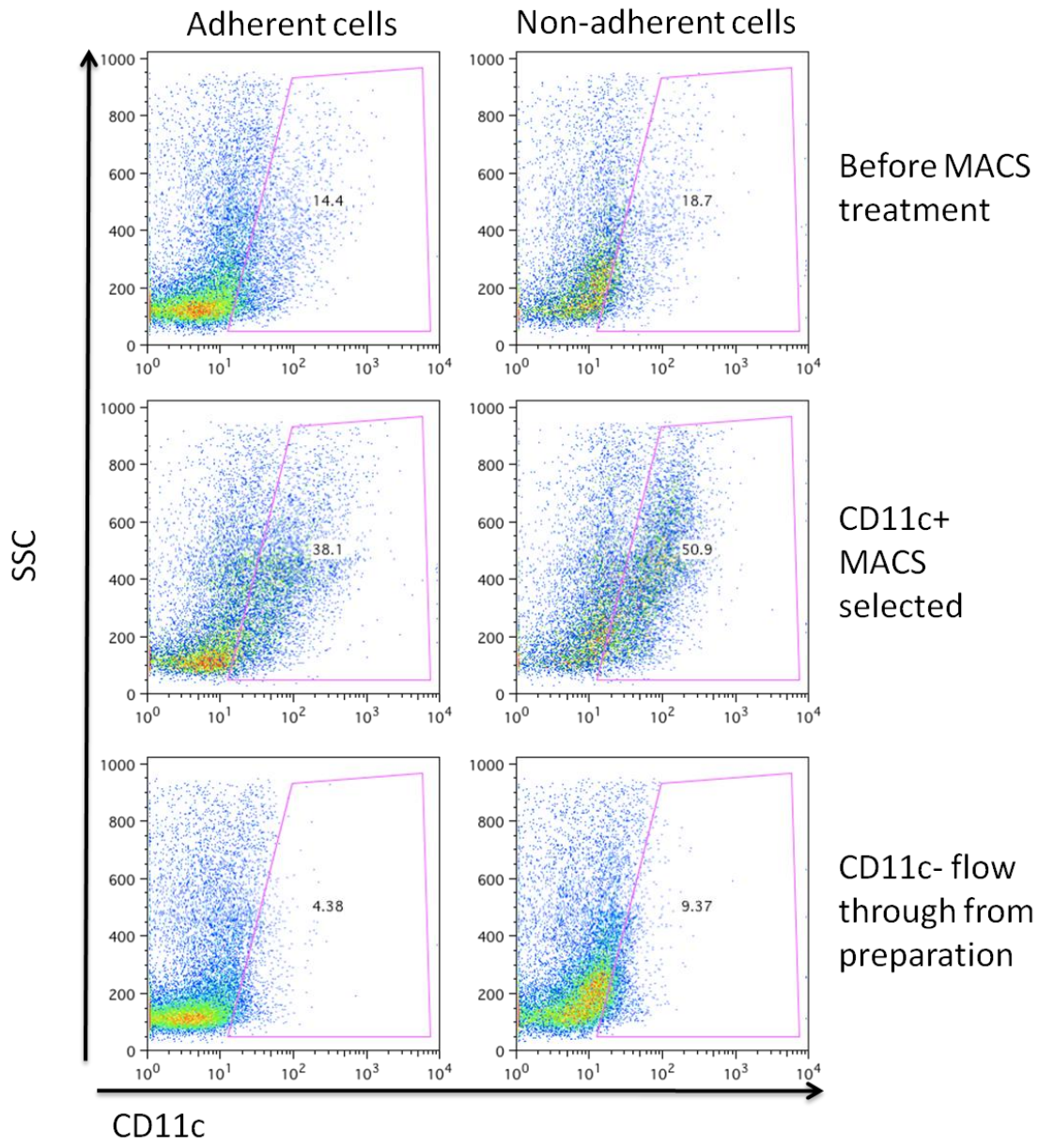

Figure 3-2 CD11c+ cells are not only contained in the adherent cell population as others have claimed

Lungs were digested and plated to obtain adherent and non-adherent cells as described. Both adherent and non-adherent cells were treated with CD11c MACS kit and both the positively retained cells and the flow through cells were stained for CD11c, compared to isotype, to test efficacy of kit and, and compared to cells before treatment. Cells were gated on live cells based on FSC and SSC as described in materials and methods. Data representative of 3 experiments.

The lung homogenates were tested before CD11c positive selection MACS preparation to give an idea of how many CD11c+ cells reside in the lung before purification. We see approximately 14% and 18% of CD11c+ cells in both the adherent and non-adherent preparations respectively, indicating that the non-adherent cell preparation not only contained CD11c+ cells but in fact marginally more than the adherent population (Figure 3-2). Both of these preparations were

then treated with CD11c+ MACS kit and both the positively selected cell preparation retained in the column and the CD11c negative preparation flow-through were stained with CD11c antibodies and analysed by flow cytometry to ensure no CD11c+ cells are escaping into this CD11c- preparation.

Upon CD11c positive selection we see 38% and 50% CD11c+ in the adherent and non-adherent cell preparations respectively, indicating that there are CD11c+ cells in the non-adherent cell preparation indeed and this should not be disregarded as others have suggested. Furthermore it is evidence that the CD11c positive selection kit is not highly pure as manufacturers claim. However, it appears that CD11c cells are not being lost in the flow-through, as the flow-through preparations of both the adherent and non-adherent cells appear negative for CD11c+ cells (Figure 3-2). This indicates that the problem is retention of other cell types in the column and not just the CD11c cells as desired. We believe this is due to the 'sticky' nature of epithelial cells in the lung and devised strategies to omit these cells such as density centrifugation pre-treatment discussed in the next section.

To further investigate if incubation of lung homogenates and selection of adherent cells increases CD11c+ cell isolation, we tested 3 methods of incubation on the same isolated lung homogenate preparations (Figure 3-3). Lung tissue was digested and passed through nitex to obtain a single cell suspension and then:

A) Treated with CD11c positive selection MACS kit with no incubations or removal of cells

B) Cells were incubated for 1 hour at 37°C on TC plates, with non-adherent cells removed and treatment of only the adherent cells with CD11c positive selection MACS kit

C) Cells were incubated for 1 hour at 37°C, with non-adherent cells removed and adherent cells further incubated overnight as other protocols suggest with further removal of non-adherent cells the next day and treatment of only the adherent cells with CD11c positive selection MACS kit

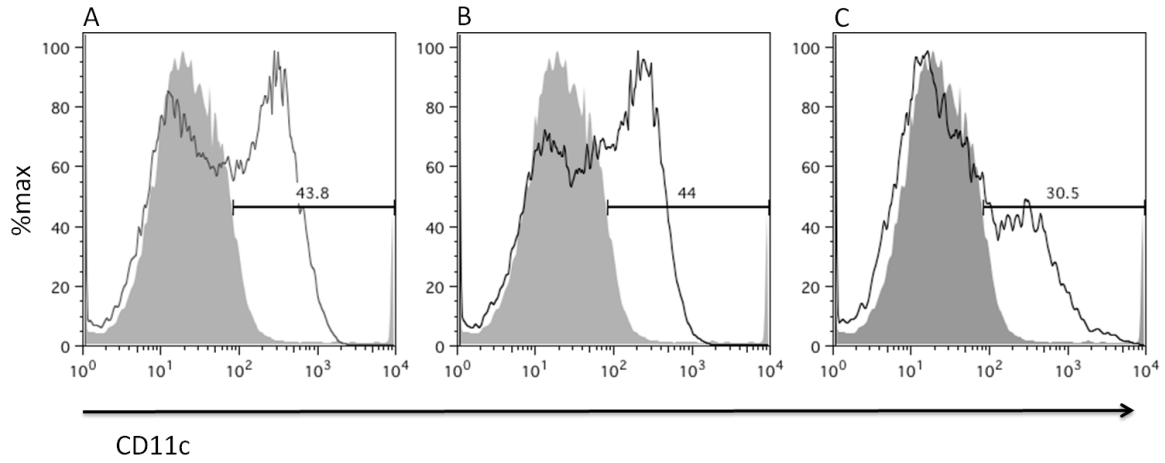

Figure 3-3 CD11c+ cells from different treatments of lung DC isolation
Lung DCs were isolated using CD11c+ MACS treatment after A) digestion and isolation by nitex; B) as A but with prior incubation for 1 hour at 37°C and removal of non-adherent cells; C) as B but with a further overnight incubation and second removal of non-adherent cells before MACS treatment. Cells were stained for CD11c (black line) and analysed by flow cytometry compared to isotype (filled histogram). Cells were gated on live cells based on FSC and SSC as described in materials and methods. Data representative of 3 experiments.

It appears that the recommended practice of 2 incubations and 2 steps of removal of non-adherent cell as described above, gives the worst CD11c+ cell population for the lung tissue of all 3 methods, with only 30% CD11c+ cells observed after positive selection (Figure 3-3C).

The best CD11c+ population is observed where there is no prior treatment or removal of non-adherent cells (Figure 3-3A), further indicating that the non-adherent preparation that others suggest we remove may contain CD11c cells and thus must be included. Due to this we performed all lung DC isolations on whole lung cell homogenates.

3.2.1 Density centrifugation

We have observed that upon isolation of CD11c+ positively selected lung DCs using MACS does not give rise to a highly pure population (Figure 3-2 and Figure 3-3), indicating contamination with other cells. We believe this contamination is probably epithelial cells as they are plentiful in the lung and sticky and so may stick to the column and be retained along with the positively selected cells. Due to this we introduced a further step of density centrifugation, as some studies suggest [200], before MACS treatment to separate the DC containing interface

from other cells and tissue. This should allow a purer population not contaminated with epithelial cells, before MACS preparation, thus allowing better selection of CD11c+ cells.

Ficoll-Paque (GE healthcare) is a density centrifugation medium commonly used for isolation of mononuclear cells from the blood, that allows all cells to be separated by density into layers [202, 203]. The technique of density centrifugation is normally used for blood and so is divided into plasma, peripheral blood mononuclear cells (PBMCs) and red blood cells (RBCs). We are not sure exactly how the cells in the lung would be separated, as there are more cells in lung tissue than in blood such as epithelial cells and connective tissue, and this has not been documented to present. When the lung homogenate was mixed with Hanks buffered saline solution (HBSS) and centrifuged over Ficoll-Paque the cells in the tissue become layered as can be seen in Figure 3-4.

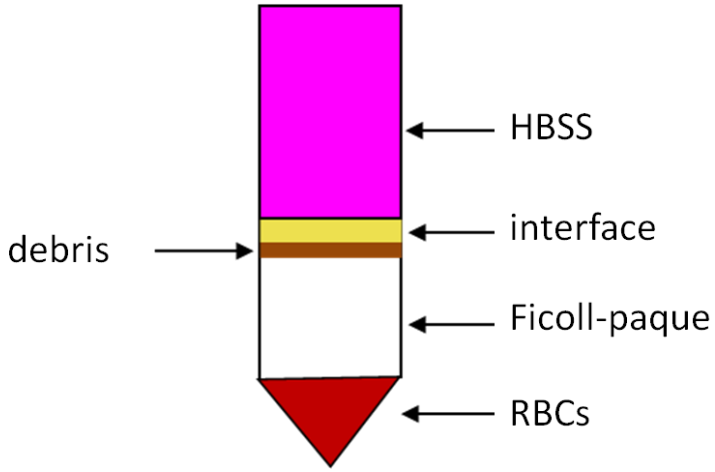

Figure 3-4 Diagram of density centrifugation of lung tissue homogenate

The very dense RBCs sink to the bottom and the ‘lighter’ interface, which contains immune cells such as lymphocytes, monocytes and macrophages sits nearer the top just under the buffer solution.

We believe the epithelial cells to be in the debris layer as we have named it, as this layer looked very ‘tissue like’ and was very sticky and congealed. Upon insertion of a pipette into the tube to liberate the interface, this ‘debris’ layer wasn’t disturbed or dispersed as the interface would be, and appeared to stick to the plastic and moved as a whole. This indicates that this debris layer could be the ‘stickiness’ that we believe is preventing pure CD11c isolation with MACS.

DNA is also regarded as ‘sticky’ and this layer is where we believe the disturbed DNA would reside, further confirming that removal of this layer may reduce the ‘stickiness’ of the preparation thus allowing a purer CD11c population.

The interface was removed as carefully as possible and once fully isolated this interface cell preparation was treated with CD11c positive selection MACS kit and the preparation was stained with CD11c antibodies and analysed by flow cytometry as before (Figure 3-5).

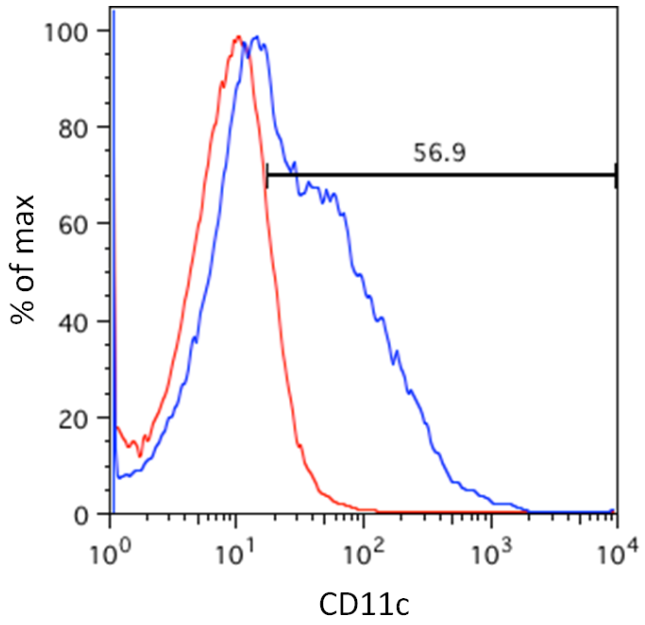

Figure 3-5 Density centrifugation treatment of lung tissue increases the CD11c+ populations isolated from the lung by CD11c+ MACS treatment

Lung tissue was digested and then separated using density centrifugation to isolate the CD11c+ cell containing interface which was then treated by CD11c+ MACS, and stained for CD11c for analysis by flow cytometry (blue line) compared to isotype (red line). Cells were gated on live cells based on FSC and SSC as described in materials and methods. Data representative of 2 experiments.

Pre-treatment with density centrifugation results in a CD11c+ cell population of 57% after CD11c+ MACS treatment. This was the purest CD11c+ population we observed although it is still not near as highly pure as the providers of these positive selection kits claim.

3.3 Conclusion

Liberation of a single cell suspension from lung tissue and further CD11c+ cell isolation proved difficult with no perfect technique found as yet. We found that use of liberase with DNase gives a much cleaner single cell suspension upon lung tissue digestion than that of Collagenase D with DNase, and so liberase is recommended when digesting lung tissue to obtain a single cell suspension for any purpose. CD11c+ isolation using CD11c positive selection MACS kit proved to be less than successful with many variations on how to best obtain maximum CD11c+ yield from the tissue tested, such as differing digestion enzymes, culturing of cells before treatments and density centrifugation. We found that incubation of the cells and CD11c+ treatment of the adherent cells only did not give a very pure CD11c+ population and furthermore was found to actually eliminate CD11c+ cells that could be found in the non-adherent cell preparation. Due to this, the whole lung homogenate was treated for CD11c+ cell isolation during our experiments to maximise CD11c+ DC number. CD11c+ populations were enhanced when the immune cells of the lung were separated by density gradient centrifugation prior to MACS selection, although this technique still did not give the highly pure CD11c+ population as we had hoped. We have exhausted all theories as how to increase this yield and thus decided that for our experiments where we wished to isolate lung CD11c+ DCs to use whole lung homogenates, prepared by density centrifugation before CD11c positive MACS selection and to take into account that the preparation may only be under 60% pure and that other cells may contribute to the responses.

4 Dendritic cell activation by *Pseudomonas aeruginosa* and *Streptococcus pneumoniae*

4.1 Introduction

Antigen presenting cells (APCs) present antigen to other cells for recognition and induction of immune responses. These cells uptake antigen in the periphery and carry them to secondary lymphoid tissues where they present antigen to T cells for recognition. If the T cell recognises an antigen, it proliferates and secretes cytokines to co-ordinate an immune response to that antigen, in the presence of co-stimulation which is also provided by the APC [204]. There are 3 types of APC; Dendritic cells (DC), macrophages and B cells [205], with DCs being the only APC whose sole function is dedicated to antigen presenting. Macrophages are also involved in innate immunity and phagocytosis, and B cells are involved in humoral immunity and antibody production [205].

DCs are commonly identified using the surface protein CD11c, with DCs being CD11c+. Though the use of the molecule solely as a DC marker is a subject of debate [206], it is a widely applied marker of murine DCs and is the DC marker of choice in our set up (these CD11c+ cells were verified to be DCs by other means as discussed in materials and methods which are not mentioned futher in this thesis). DCs can be activated by numerous stimuli which produces a range of phenotypic changes, such as up-regulation of co-stimulatory markers, major histocompatability complex (MHC) molecules and secretion of chemokines and cytokines [207]. In the work described in this thesis, we follow activation of DCs by measuring up-regulation of the co-stimulatory molecules CD40 and CD86, two molecules that would conjugate with receptors CD40L (CD154) and CD28 on T cells to allow T cell activation. We also follow production of the pro-inflammatory cytokines IL-23 and IL-1β as evidence that the DC has recognised the pathogen and has been activated by it via direct infection. As discussed in the introduction, IL-1β secretion involves intracellular recognition of a pathogen to activate the inflammasome and allow IL-1β release, and thus production of IL-1β may indicate that the pathogen has infected the cell. IL-23 secretion however just requires ligation of a TLR, most likely TLR4 [49], on the surface of the DC. DCs recognise pathogens by use of pathogen recognition receptors (PRRs) on their surface which recognise pathogen associated molecular patterns (PAMPs), on microorganisms. The most famous of the PRRs are the TLRs as discussed in the introduction. Recognition of bacterial components by the TLRs leads to DC activation [207], and thus we will use addition of recombinant

lipopolysaccharide (LPS) to cultures which is recognised by TLR4 [208] as a positive control to show DCs can be activated by TLR stimulation.

This chapter investigates different methods of DC induction from bone marrow progenitor cells, activation of these DCs by the pathogens *P. aeruginosa* and *S. pneumoniae* and investigates the use of the bacterial strains of *P. aeruginosa* which activate and do not activate the inflammasome, to represent a model of IL-1β presence and absence respectively. It also investigates isolation of DCs from mucosal sites and looks at their activation upon stimulation and infection to see if they have the potential to be better T cells stimulators than bone marrow derived DCs (BMDCs), which may induce Th17 cells in response to these respiratory pathogens.

4.2 BMDC induction by Granulocyte macrophage colony stimulating factor (GM-CSF)

4.2.1 Recombinant GM-CSF induces more CD11c+ cells from bone marrow progenitors than X63 media

GM-CSF is a cytokine known to induce bone marrow progenitor cells to become DCs under the correct culture conditions [209]. GM-CSF is available as a recombinant cytokine or can be found in the supernatant of cultures of the cell line X63, a mouse myeloma cell line that produces GM-CSF and secretes it into the supernatant [210, 211]. We sought to find out if recombinant GM-CSF or X63 media is more effective at inducing DCs from bone marrow and the activation status of these DCs upon stimulation with LPS.

Bone marrow was isolated as described in materials and methods and cultured with media supplemented with 10ng/ml recombinant GM-CSF or 20% X63 media.

A larger yield of CD11c+ cells is observed when recombinant GM-CSF is used as opposed to media supplemented with X63 (Figure 4-1) represented by a higher peak on the % max axis; 80% compared to 40% of 20,000 events recorded.

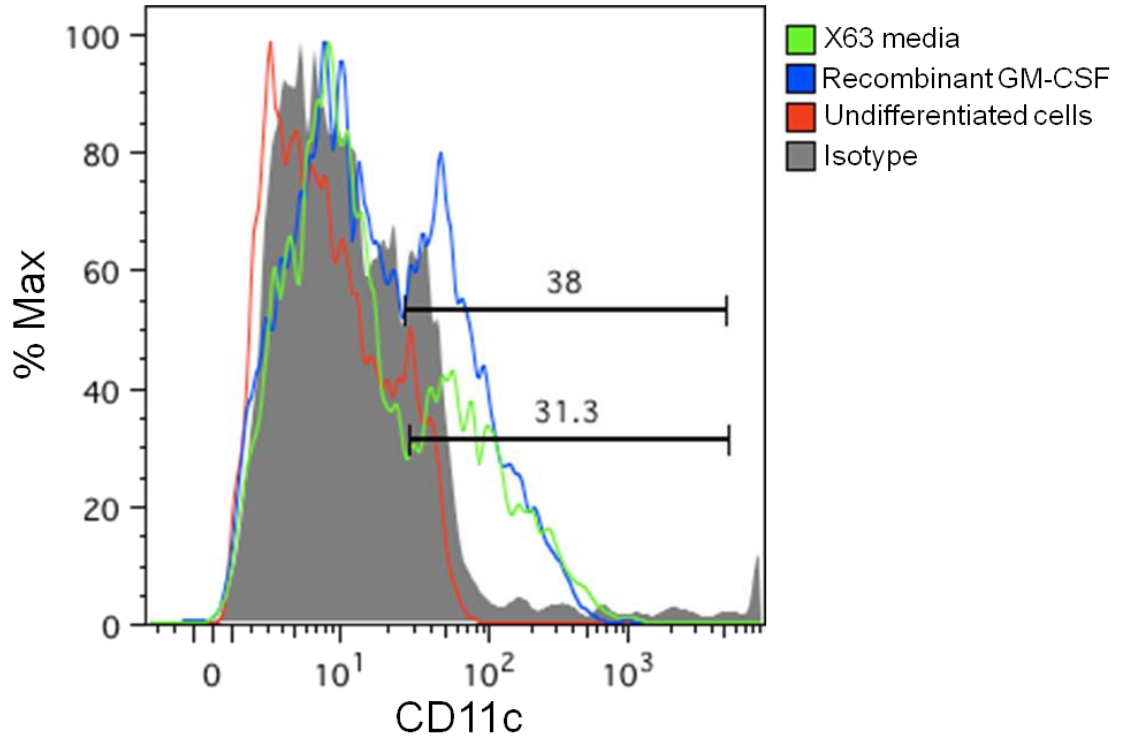

Figure 4-1 Recombinant GM-CSF induces higher numbers of CD11c+ populations from bone marrow than X63 media

Bone marrow was isolated from femurs of C57BL/6 mice and cells were cultured with recombinant GM-CSF (blue line) or X63 media (green line) for 7 days and compared to undifferentiated cells (red line) and CD11c isotype (filled histogram). Cells gated on live cells based on forward scatter (FSC) and side scatter (SSC) as described in material and methods. Histograms are representative of culture set up in triplicate. Data representative of 3 experiments.

Using recombinant GM-CSF gives a more consistent population of CD11c+ cells, as a calculated concentration of GM-CSF is added to culture.

4.2.2 Recombinant GM-CSF induces functional CD11c+ cells from bone marrow progenitors based on CD40 and CD86 up-regulation

Using up-regulation of co-stimulatory molecules CD40 and CD86, it is apparent that recombinant GM-CSF induced DCs are more readily activated on the basis of up regulation of their co-stimulatory molecules upon stimulation of LPS (Figure 4-2). LPS is a component of Gram-negative bacterial cell walls that is a potent activator of DCs due to its ability to be recognised by TLR4 on the surface of DCs [208].

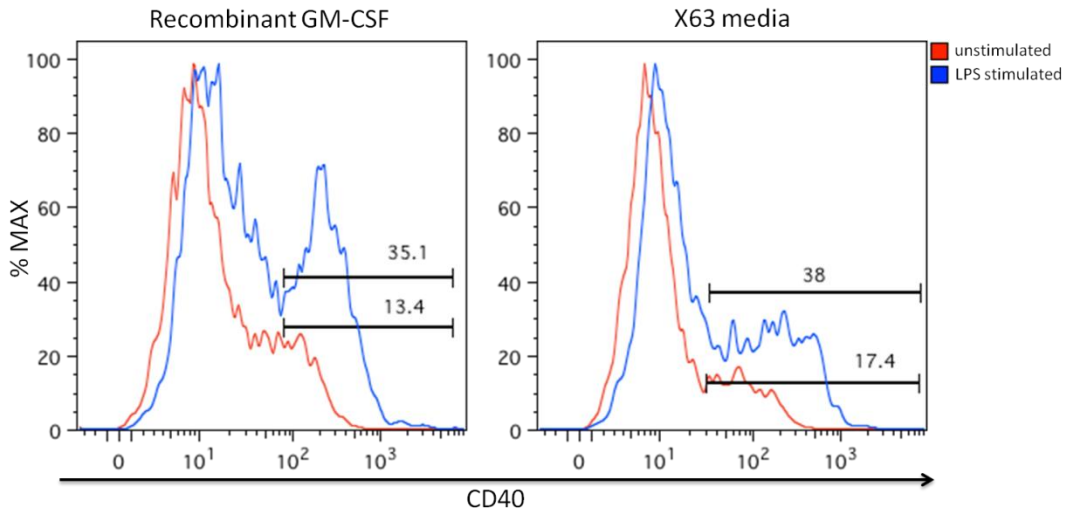

Figure 4-2 Recombinant GM-CSF induced DCs up-regulate more CD40 than X63 media induced DCs

DCs differentiated by recombinant GM-CSF or X63 media were left unstimulated (red line) or stimulated with LPS for 90 minutes (blue line) after which time cells were harvested, stained with CD40 and analysed by flow cytometry. Cells gated on live cells based on FSC and SSC as described in materials and methods, and positive gates for CD40 were set on isotypes on each cell type. Histograms are representative of culture set up in triplicate. Data representative of 3 experiments.

Use of recombinant GM-CSF to skew DCs from bone marrow progenitors, allows a higher yield of cells that up-regulate of CD40 than DCs initiated by X63 media, represented by a larger peak on the % max axis relative to 20,000 events recorded for each condition. This is also evident with the co-stimulatory marker CD86 (data not shown). These results indicate that recombinant GM-CSF is a better method of inducing DCs as it allows a more functional cell type that can be activated upon stimulation, and thus proceed to stimulate T cells for activation. Taken together these results indicate that recombinant GM-CSF not only generates a higher yield of CD11c+ cells(Figure 4-1) but also induces a better quality of DC gauged on activation capacity, compared to that of the X63 supernatant (Figure 4-2).

4.2.3 X63 and GM-CSF induced DCs do not differ in IL-1β secretion

A higher number of cells that up-regulate CD40 and CD86 upon LPS stimulation indicates that recombinant GM-CSF derived DCs have a greater capacity to generate inflammatory activated DCs. To confirm this we measured the ability of

the two different DC populations to produce IL-1β following infection with *P. aeruginosa*. Both types of DC produced very similar levels of IL-1β with no significant difference observed (Figure 4-3). This indicates that DCs induced with X63 media are infected and respond in the same manner as GM-CSF derived DCs, with regards to IL-1β production.

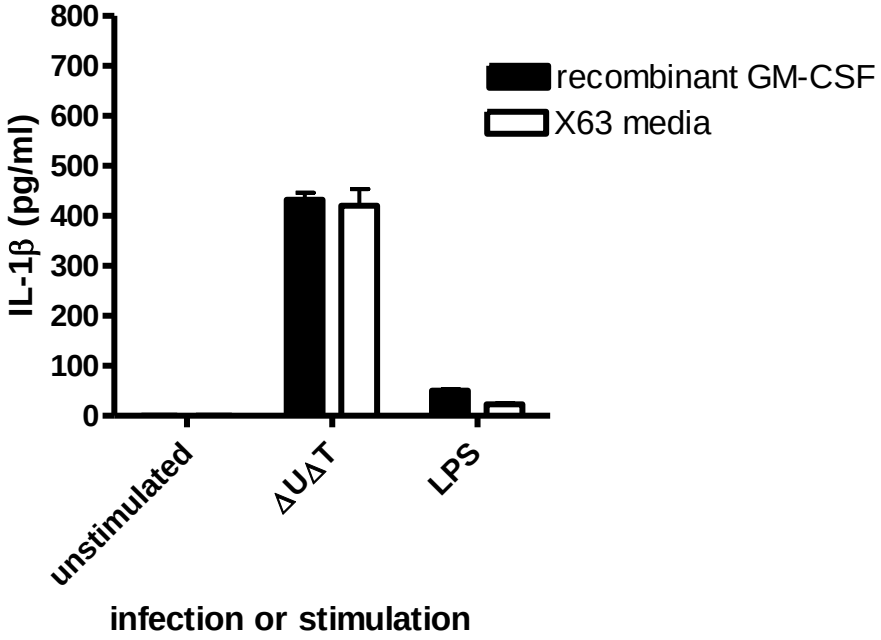

Figure 4-3 IL-1β output from recombinant GM-CSF and X63 induiced DCs does not differ
DCs differentiated by recombinant GM-CSF or X63 media were left unstimulated, infected for 90 minutes with *P. aeruginosa* ΔUΔT after which time antibiotics were added to kill the pathogen, or LPS stimulated. Cells were incubated overnight before supernatants were harvested and assayed for IL-1β by ELISA. All error bars represent mean ± standard error of the mean (SEM). Data are representative of 3 separate experiments of experimental triplicates.

4.2.4 Functional CD11c+ cell generation using Flt3 ligand

Fms-like tyrosine kinase 3 ligand (Flt3l) is a haematopoietic growth factor which is also known to induce DCs from bone marrow progenitors [212]. We tested the use of Flt3l during BMDC induction, and compared the process of BMDC induction by Flt3l and GM-CSF to investigate which method induced a better CD11c+ population. We found that at day 8 of generation, the day on which GM-CSF DCs are harvested and used in our experiments, Flt3l DCs expressed less CD11c than DCs generated by GM-CSF exposure (Figure 4-4).

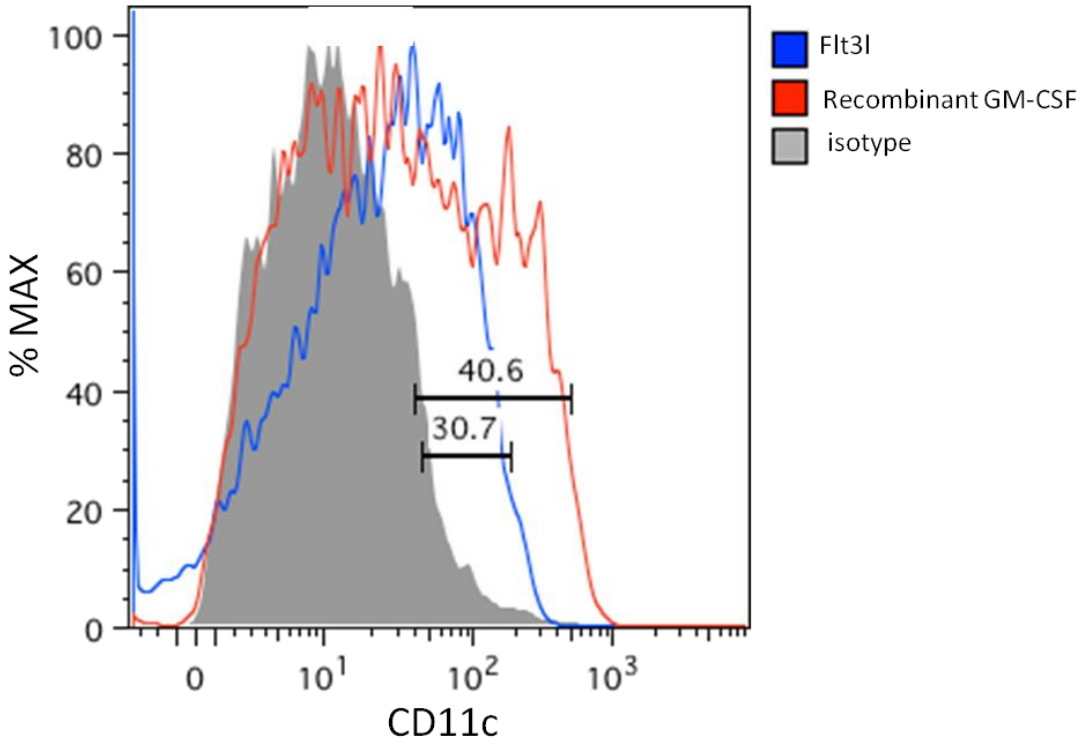

Figure 4-4 CD11c expression by DCs derived using Flt3l is lower than CD11c expression of DCs derived by recombinant GM-CSF

Bone marrow was isolated from femurs of C57BL/6 mice and cells were cultured with recombinant GM-CSF (red line) or Flt3l (blue line) for 8 days, after this time the cells were harvested and stained for CD11c and compared to CD11c isotype (filled histogram). Cells gated on live cells based on FSC and SSC as described in materials and methods. Histograms are representative of culture set up in triplicate. Data representative of 3 experiments.

However, we found that Flt3l DCs were more immature when unstimulated as represented by little to no CD40 or CD86 expression compared to recombinant GM-CSF derived DCs that have a small population of CD40/CD86 positive cells under unstimulated conditions (Figure 4-5). This small population of CD40/CD86+ cells that we see in unstimulated recombinant GM-CSF derived DCs is most likely due to some activation of cells upon agitation during preparation, by moving and washing in cold PBS, and upon adherence to tissue culture treated plastics, as these DCs are found to be quite sensitive to activation by these means. Upon stimulation with LPS, there is a distinct increase in CD40 and CD86 expressing cell populations, and percentages of cells in these populations, in GM-CSF derived DCs (Figure 4-5). In comparison Flt3l DCs, which had no CD40+ or CD86+ cells under unstimulated conditions, appear to all become activated illustrated by a full CD40/CD86 shift upon activation. Furthermore, it appears as if there is an both intermediate and a positive population of both markers, as 2 populations

are evident that are both increased from the CD40- and CD86- populations observed when Flt3l induced DCs are unstimulated (Figure 4-5).

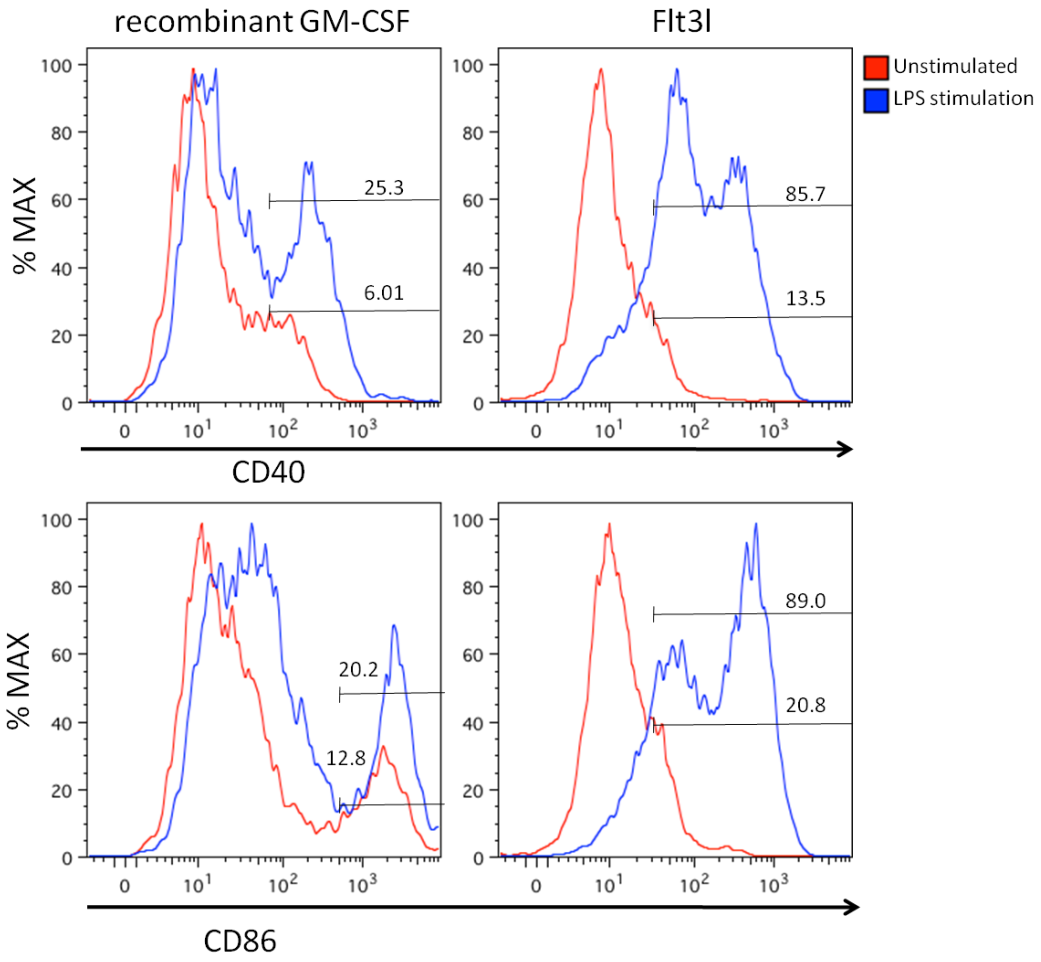

Figure 4-5 Activation of Flt3l derived DCs using LPS

DCs differentiated by recombinant GM-CSF or Flt3l media were stimulated with LPS (blue line) or left unstimulated (red line) for 90 minutes after which time cells were harvested, stained for CD40 and CD86 and analysed by flow cytometry. Cells gated on CD11c and positive gates for CD40 and CD86 were set on isotype for each marker on each cell type. Histograms are representative of co-culture set up in triplicate. Data representative of 3 experiments.

These results show that unstimulated recombinant GM-CSF DCs have a small CD40/CD86+ populations that are up-regulated upon LPS stimulation, showing them to become activated with this stimulus. Whereas, Flt3l derived DCs show little CD40 or CD86 when unstimulated, with large CD40 and CD86 populations generated upon stimulation with LPS.

Flt3l derived DCs show a better up-regulation of the co-stimulatory molecules with both an intermediate and a positive population indicating that they are

better at activating upon stimulation than recombinant GM-CSF cells, but as CD11c populations in Flt3l derived DCs are low, they cannot be readily identified as DCs according to the literature.

It is claimed that Flt3l derived DCs gain CD11c expression as they age, with CD11c expression appearing around day 9 and increasing to 100% CD11c+ cells by day 14 [213], and so we tested Flt3l derived DCs at a later stage, day 14, to see if CD11c had been up-regulated on these cells. We did not see this claimed increase in CD11c but a decrease, along with reduced CD40 and CD86 (data not shown). Due to this we examined the cells at this time point for their viability, based on the uptake of 7-AAD, a fluorescent dye that binds to DNA liberated from cells upon death. Day 14 GM-CSF derived DCs show an 80% live population in unstimulated conditions whereas live cells at day 14 in unstimulated Flt3l derived DCs are less than half of this, at 39% (Figure 4-6).

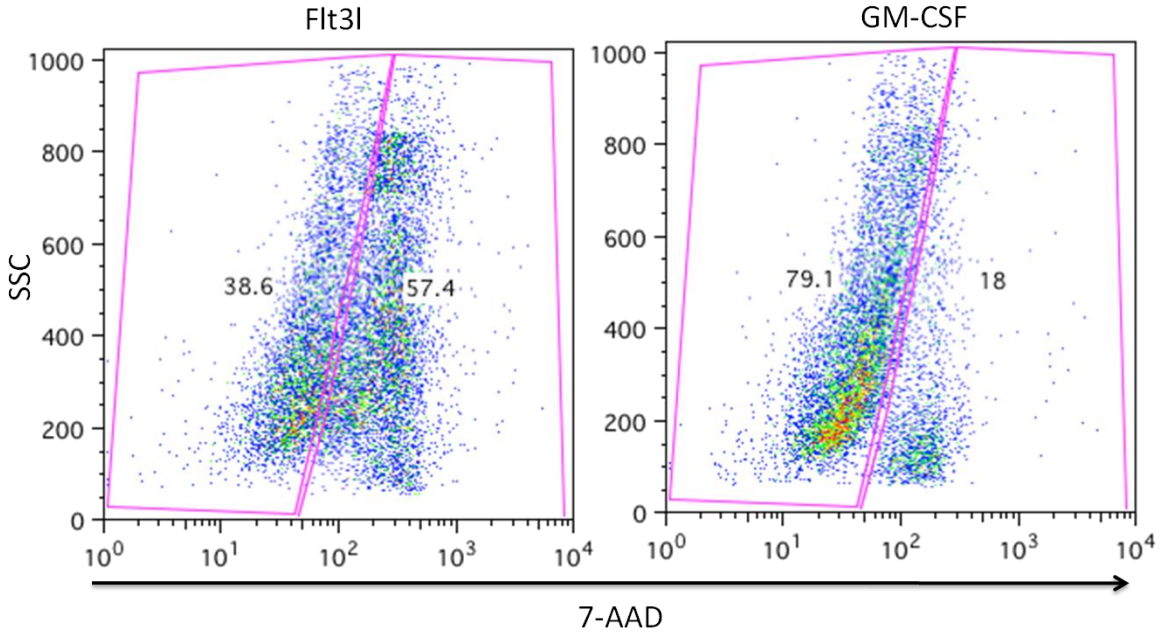

Figure 4-6 Cell death with day 14 Flt3l derived DCs

DCs differentiated by recombinant GM-CSF or Flt3l media were harvested at day 14 were stained for 7-AAD to investigate cell death. Data representative of 2 experiments.

These results illustrate that Flt3l DCs do not increase their CD11c+ expression overtime and in fact more than half of the cells are not viable by this time point. Due to these combined observations, recombinant GM-CSF derived DCs were used at day 8 for all experiments.

4.3 DCs from frozen bone marrow

Others in the group have observed previously that bone marrow progenitor cells can be frozen in 10% DMSO and 90% FCS at -80°C for a number of weeks to months, before being thawed and washed out of the DMSO mix, which are still functional in induction of macrophages. We wished to investigate if DCs could also be induced from bone marrow previously frozen and treated to induce DCs using GM-CSF as was performed for fresh DCs, as described in materials and methods.

A CD11c+ population can still be observed when frozen and thawed bone marrow is induced to be DCs using GM-CSF (Figure 4-7).

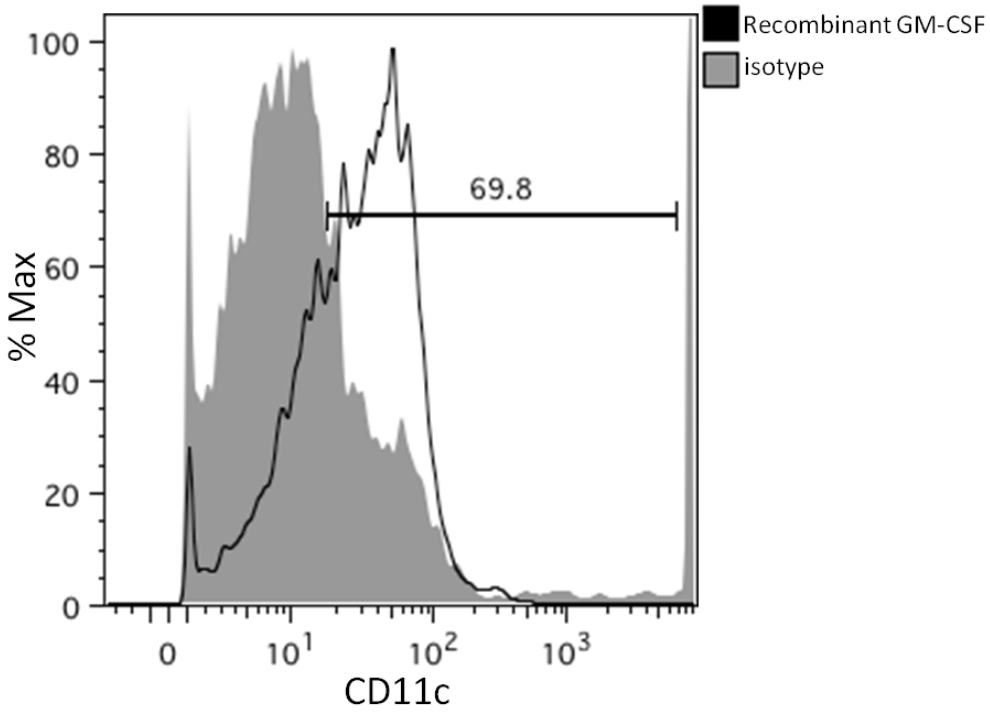

Figure 4-7 DCs from frozen bone marrow progenitors

Bone marrow isolated from femurs of C57BL/6 mice was frozen in 10% DMSO and 90% FCS at -80 °C for several weeks, after which time it was defrosted and cells were cultured with recombinant GM-CSF for 7 days to induce DCs. Cells were harvested and stained for CD11c (black line) and analysed by flow cytometry compared to isotype (filled histogram). Cells gated on live cells based on FSC and SSC as described in materials and methods. Data representative of 3 experiments.

The presence of a CD11c+ population indicates that bone marrow progenitors that have been frozen and thawed, retain their capacity to become our cells of

interest under the right culture conditions as has been observed for macrophages by others in the group previously.

However, upon LPS stimulation these cells do not have any up-regulation of co-stimulatory molecule CD86 indicating that they are not functional (Figure 4-8).

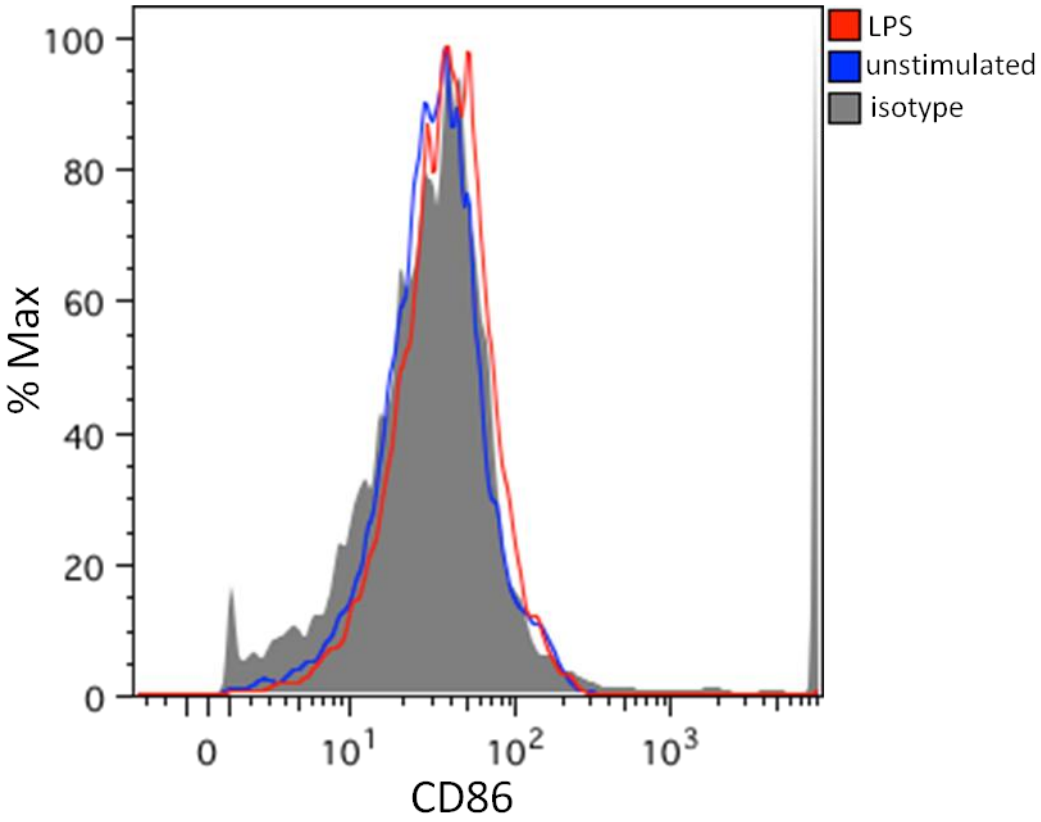

Figure 4-8 Lack of activation from DCs from frozen bone marrow progenitors
Bone marrow isolated from femurs of C57BL/6 mice was frozen in 10%DMSO and 90% FCS at -80 °C for several weeks, after which time it was defrosted and cells were cultured with recombinant GM-CSF for 7 days to induce DCs. Cells were left unstimulated (blue line) or stimulated with LPS for 90 minutes (red line), harvested, stained for CD40 and analysed by flow cytometry compared to isotype (filled histogram). Cells gated on live cells based on FSC and SSC as described in materials and methods. Data representative of 3 experiments.

Lack of CD86 up-regulation in response to potent immunogenic LPS indicates that DCs induced from frozen bone marrow progenitors are not functional. Due to this frozen bone marrow was not used and fresh bone marrow was used for all DC cultures.

4.4 *P. aeruginosa* activates BMDCs

The main aim of our experiments is to co-culture *P. aeruginosa* activated DCs with naive T cells to see if there is an induction of Th17 cells and use two *P. aeruginosa* strains, ΔUΔT and ΔpcrV, one with and one without IL-1β secretion inducing capacities respectively, to investigate if IL-1β plays a role in Th17 induction to *P. aeruginosa*. The difference in IL-1β production in each strain is due to the presence or absence of a functional type 3 secretion system (T3SS) which, as discussed in the introduction, is important for allowing intracellular recognition of pathogens, inflammasome activation and thus IL-1β production.

First, however, we wished to ascertain that *P. aeruginosa* does in fact activate DCs and induce up-regulation of important co-stimulatory molecules CD40 and CD86 that would aid in T cell activation. BMDCs were harvested and stimulated with pathogen for 90 minutes before addition of antibiotics to kill the pathogen. DCs were then left overnight to allow maturation and up-regulation co-stimulatory molecules. Cells were then harvested and stained for CD11c, CD86 and CD40 and measured by flow cytometry.

Upon infection with both *P. aeruginosa* strains ΔUΔT and ΔpcrV, DC activation is evident as illustrated by an increase in CD11c+ CD40+ cells and CD11c+ CD86+ cells (Figure 4-9) from unstimulated conditions, confirming that both strains of the pathogen activate DCs.

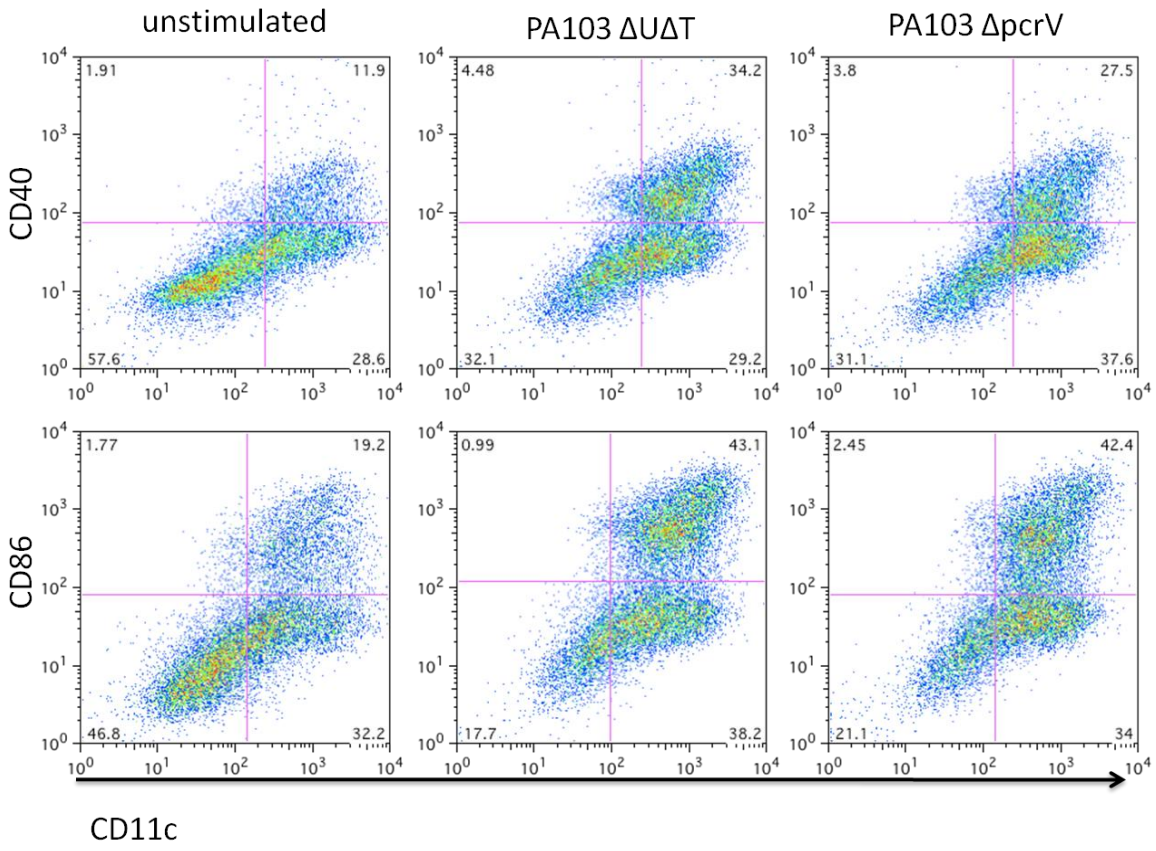

Figure 4-9 *P. aeruginosa* infection activates DCs to up-regulate co-stimulatory markers CD40 and CD86

DCs differentiated by recombinant GM-CSF were left unstimulated or infected with *P. aeruginosa* strains ΔUΔT or ΔpcrV for 90 minutes before the addition of antibiotics to kill the pathogen, and cells were incubated overnight. After this time cells were harvested and stained for CD11c, CD40 and CD86 and analysed by flow cytometry. Cells gated on live cells based on FSC and SSC as described in material and methods. Positive gates were set on isotype for each marker on PA103 ΔUΔT stimulated cells. Dot plots are representative of culture set up in triplicate. Data representative of 3 experiments.

As before a population of CD40+ CD11c+ cells and CD86+ CD11c+ cells, 12% and 19% respectively, can be observed when the DCs are unstimulated. This percentage of CD40+, CD86+ cells is probably due to the fact that disturbing the DCs, with spins, and washes with cold PBS, may allow some activation as discussed earlier. However, an increase in both co-stimulatory markers can be observed upon *P. aeruginosa* infection with both strains of PA103 ΔUΔT and ΔpcrV. This indicates that presence or absence of an operational T3SS is not necessary for DC activation as based on up-regulation of CD40 and CD86. From these results we can thus conclude that infection of DCs with both stains of live *P. aeruginosa* allows activation of the cells observed by up-regulation of the co-stimulatory molecules CD40 and CD86.

4.5 Initial IL-1β production from DCs upon *P. aeruginosa* infection is T3SS dependent

As discussed more fully in the introduction, IL-1β processing and secretion is dependent on a complex called the inflammasome. Briefly, pro-IL-1β is made in cells upon TLR stimulation, and IL-1β is only released when pro-IL-1β is cleaved and converted into active IL-1β by products of inflammasome activation. *P. aeruginosa* activates the inflammasome unintentionally when injecting toxins into the host cells using its T3SS. During this process *P. aeruginosa* also inadvertently passes bacterial proteins into the host cell that the inflammasome complex recognises, activating the inflammasome and allowing downstream cleavage of pro-IL-1β and eventual IL-1β release.

We used 2 bacterial strains of *P. aeruginosa* PA103, one with a functional T3SS but no T3SS exotoxins, PA103 ΔUΔT, and a strain with a non-functional T3SS, PA103 ΔpcrV, to demonstrate the contribution of an intact T3SS in inflammasome activation and therefore IL-1β output.

After infection of DCs for 90 minutes and resting for 2 hours after antibiotic additon, PA103 ΔUΔT induces IL-1β release from the DCs, but as expected PA103 ΔpcrV does not (Figure 4-10). This confirms that the presence of a T3SS system is important for inflammasome activation and downstream IL-1β release from DCs in response to the pathogen *P. aeruginosa*.

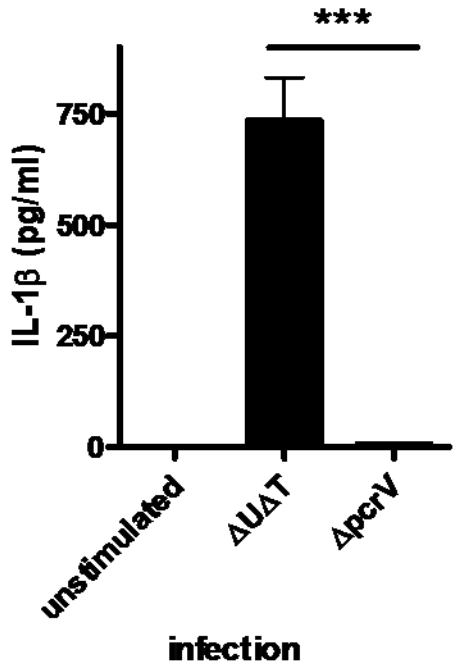

Figure 4-10 IL-1β is secreted from DCs infected with T3SS competent PA103 ΔUΔT but not T3SS deficient PA103 ΔpcrV

DCs differentiated by recombinant GM-CSF were left unstimulated or infected for 90 minutes with *P. aeruginosa* strains ΔUΔT and ΔpcrV before addition of antibiotics to kill the pathogen. After 2 hours incubation, supernatants were harvested and analysed for IL-1β by ELISA. All error bars represent mean ± SEM. Data are representative of 3 separate experiments of experimental triplicates. ***p<0.001.

This was considered to be standard as had also been shown previously by our group in macrophages [193], and experiments were continued under the assumption that the strain ΔUΔT allowed IL-1β release and ΔpcrV did not. However, if the cells were given antibiotics to kill the pathogen after the 90 minutes and then left overnight to mature, as is our set up for the co-culture of infected DCs with naive T cells, it appears other factors activate the inflammasome and allow IL-1β increase from both bacterial strains (Figure 4-11) making this bacterial model, representing IL-1β presence or absence, ineffective.

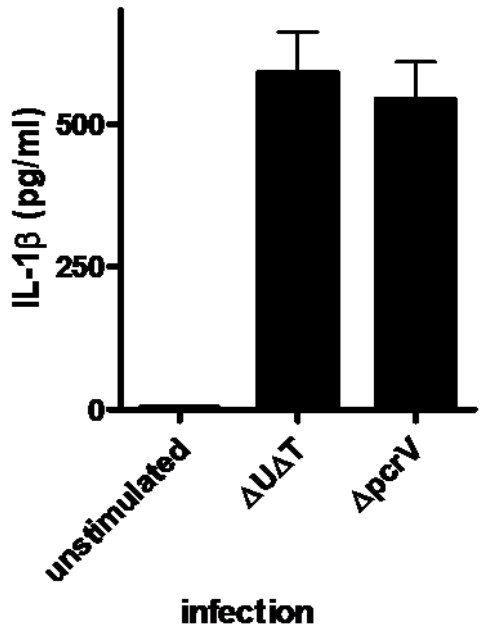

Figure 4-11 IL-1β is secreted from DCs infected with both PA103 strains after overnight incubation, regardless of T3SS contribution

DCs differentiated by recombinant GM-CSF were left unstimulated or infected for 90 minutes with *P. aeruginosa* strains ΔUΔT and ΔpcrV before addition of antibiotics to kill the pathogen. Cells were incubated overnight before supernatants were harvested and analysed for IL-1β by ELISA from experimental triplicates. All error bars represent mean ± SEM. Data are representative of 3 separate experiments of experimental triplicates.

These other factors that lead to overnight IL-1β secretion from DCs infected with T3SS deficient ΔpcrV are unknown and require investigation.

DC activation at an earlier time point was then investigated to see if DCs infected for 90 minutes and then given antibiotics and allowed to activate for 2 hours, which do show a difference in IL-1β between the 2 strains (Figure 4-10), could be used for co-culture. However, T cells co-cultured with DCs only allowed 2 hours to mature after infection all died giving no measurable results. We thus conclude that overnight incubation of 90 minute infected DCs is necessary after antibiotic addition to allow full activation of the DCs and thus full co-operation with T cells, allowing T cell activation and survival.

It appears there is a balance to be met in the timing of the experiment. Freshly infected DCs show a difference of IL-1β from the pathogens and would be useful tool to show the contribution of IL-1β to Th17 generation. However these DCs

are lacking in co-stimulatory molecules and thus will not co-operate with and activate T cells, thereby passively allowing the T cells to die. Upon careful consideration it was decided that as we are searching for a specific response from T cells to that of *P. aeruginosa* and therefore DC presentation and co-stimulation is important, that the overnight process would remain intact. We could then use this set up to investigate if naive IL-17 responses can be induced using pathogen primed DCs but we cannot make any assumptions about IL-1β contribution using this experimental setup. Therefore bacterial strains are not compared on their contribution to IL-1β in our experiments.

4.6 DC activation by *P. aeruginosa* occurs early with few bacteria, and increases over time and MOI

We wished to test how quickly *P. aeruginosa* infected and activated the DCs, and the influence of different multiplicity of infections (MOI) on DC activation and IL-1β secretion. DCs were infected with T3SS competent ΔUΔT at different MOIs ranging from 0-30, at 30 minute intervals from 0-150 minutes, following which antibiotics were added and cultures were left for an additional 2 hours to allow time for some activation. Following infection and resting for 2 hours, significant concentrations of IL-1β were detected at as little as 30 minutes infection, which appears to remain somewhat stable till 2.5 hours of infection (Figure 4-12). Also it appears that an MOI of as little as 2 is enough to induce IL-1β secretion, indicating that it only takes a few bacteria in this setting to infect host cells and induce IL-1β secretion.

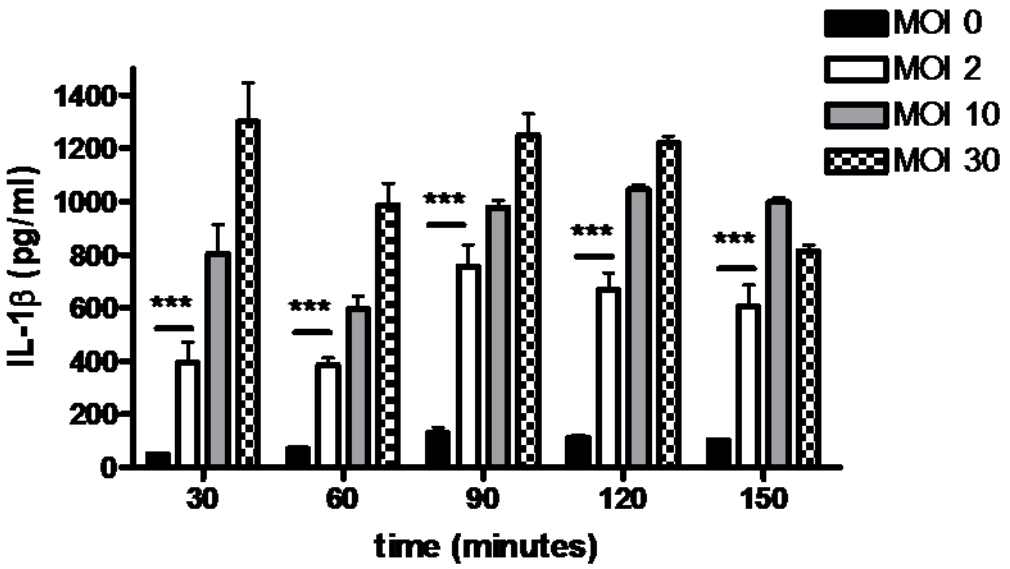

Figure 4-12 *P. aeruginosa* infection of DCs over time at various MOIs
DCs differentiated by recombinant GM-CSF were infected with *P. aeruginosa* strain ΔUΔT at 30 minute intervals at various MOIs before addition of antibiotics to kill the pathogen. Cells were incubated for 2 hours before supernatants were harvested and analysed for IL-1β by ELISA. All error bars represent mean ± SEM. Data are representative of 3 separate experiments of experimental triplicates.***$p<0.001$.

At earlier time points a high MOI appears to induce more IL-1β but as time goes on it appears that the MOIs 2-30 may not differ significantly. At the latest time point measured, 150 minutes, it appears that IL-1β concentrations from the highest MOI of 30 decrease from that of the same MOI 30 minutes previously. For our experiments an MOI of 10 and infection time of 90 minutes was sufficient. This MOI and time point was carried through the remaining experiments.

4.7 IL-23 output from *P. aeruginosa* infected DCs

As we are investigating IL-17 from cells with a primary interest in Th17 cells, we wished to look at IL-23 secretion from *P. aeruginosa* infected DCs as IL-23 is an essential maintenance factor for Th17 cells [154]. The supernatants of *P. aeruginosa* DCs infected for 90 minutes and left overnight to incubate after additon of antibiotics were harvested and IL-23 was measured by ELISA.

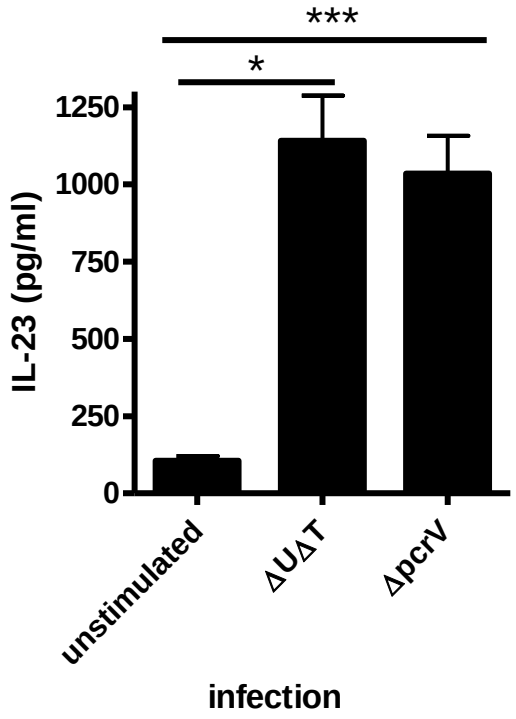

Figure 4-13 IL-23 secretion from PA103 ΔUΔT and ΔpcrV infected DCs does not differ
DCs differentiated by recombinant GM-CSF were left unstimulated or infected for 90 minutes with *P. aeruginosa* strains ΔUΔT and ΔpcrV before addition of antibiotics to kill the pathogen. Cells were incubated overnight before supernatants were harvested and analysed for IL-23 by ELISA. All error bars represent mean ± SEM. Data are representative of 3 separate experiments of experimental triplicates. * $p<0.05$, *** $p<0.005$.

Upon *P. aeruginosa* infection both strains, PA103 ΔUΔT and PA103 ΔpcrV, induce secretion of substantial amounts of IL-23 from DCs (Figure 4-13), with no significant difference between strains indicating that IL-23 production from DCs is independent of T3SS.

4.8 *Streptococcus pneumoniae* activates BMDCs

To investigate if IL-17 induced responses differed between Gram-positive and Gram-negative bacterial strains, Gram-positive *S. pneumoniae* was used to infect the DCs in the same manner as the *P. aeruginosa* had been used. Two strains of *S. pneumoniae* were used, D39 wild type (WT) and D39 ΔPLY, a variant of the WT *S. pneumoniae* that lacks the PLY toxin. As discussed in the introduction PLY is very cytotoxic, and so it is expected that the WT may just kill all the cells whereas the ΔPLY mutant will not kill the cells and may allow the DCs to process the pathogen allowing activation.

It is important to confirm that as with *P. aeruginosa*, *S. pneumoniae* does activate DCs and allows up-regulation of the co-stimulatory markers CD40 and CD86, and therefore has the potential to activate T cells and induce a Th17 response. Preliminary studies by others in the lab suggest that *S. pneumoniae* infection of DCs with both strains D39 WT and D39 ΔPLY, requires a much lower MOI than required for *P. aeruginosa* to induce activation of DC. They found that the MOI of 10 that we use for *P. aeruginosa*, kills all DCs when this MOI of *S. pneumoniae* is applied in these *in vitro* conditions.

Initial work suggests use of MOIs below 10 for *S. pneumoniae* infection. We tested this by infecting DCs with differing MOIs of *S. pneumoniae*, 0.5, 1, 2 and 5 and looking at the activation of DCs via up-regulation of co-stimulatory markers CD40 and CD86. We also see, as others in the group have, that lower MOIs of *S. pneumoniae* infection are better at inducing up-regulation of CD40 (Figure 4-14) and CD86 (data not shown) on DCs, with higher MOIs showing no up-regulation of these co-stimulatory markers at all.

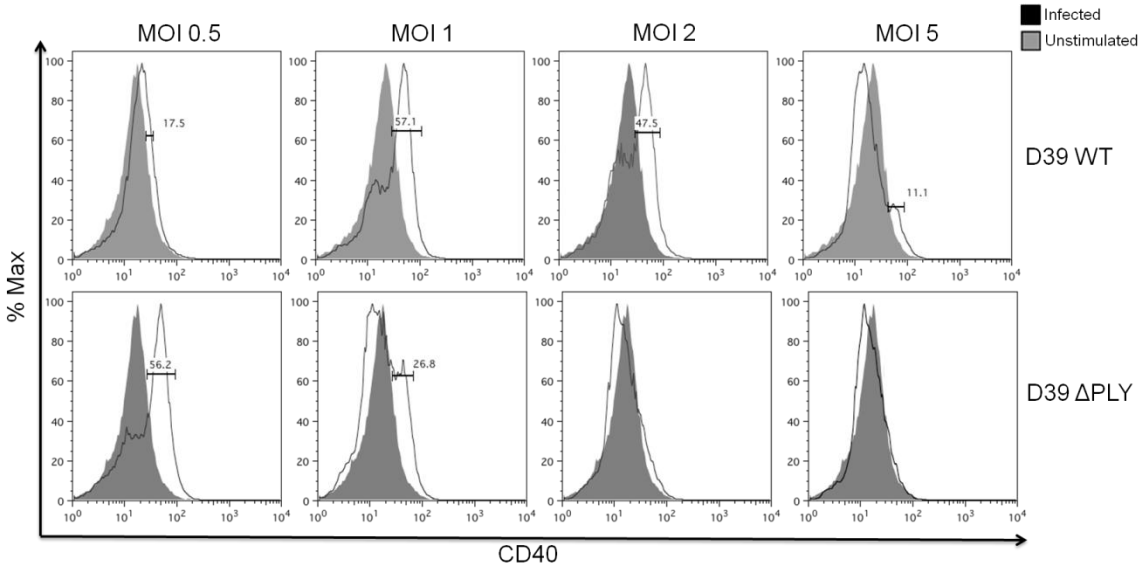

Figure 4-14 DC activation with *S. pneumoniae* occurs at low MOIs

DCs differentiated by recombinant GM-CSF were left unstimulated (filled histogram) or infected with *S. pneumoniae* (black line) for 90 minutes before the addition of antibiotics to kill the pathogen and cells were incubated overnight. After this time cells were harvested and stained for CD40 and analysed by flow cytometry. Cells gated on live cells based on FSC and SSC as described in materials and methods. Positive gates were set on isotype on D39 WT stimulated cells. Histograms are representative of culture set up in triplicate. Data representative of 3 experiments.

With D39 WT an MOI of 1 and 2 appears to induce the best up-regulation of CD40, with a higher MOI of 5 appearing to induce very little up-regulation of this marker at all (Figure 4-14). For the PLY deficient strain, D39 ΔPLY, MOIs of 0.5 and 1 appear to be the most effective at DC activation with regards to up-regulation of CD40. This confirms what others in the group have seen, that lower MOIs are more effective at activating DCs during *S. pneumoniae* infection. Due to this lower MOIs were used for *S. pneumoniae* infection experiments.

4.8.1 S. pneumoniae activation of DCs requires overnight incubation

As with *P. aeruginosa* infection, proper DC activation requires overnight incubation of the DCs after the 90 minute infection to allow sufficient time for co-stimulatory markers to be up-regulated. DCs infected for 90 minutes before antibiotic addition, only allowed to incubate for 2 hours before being harvested and stained, showed no up-regulation of CD40 (Figure 4-15) and CD86 (data not shown) in any of the infection conditions.

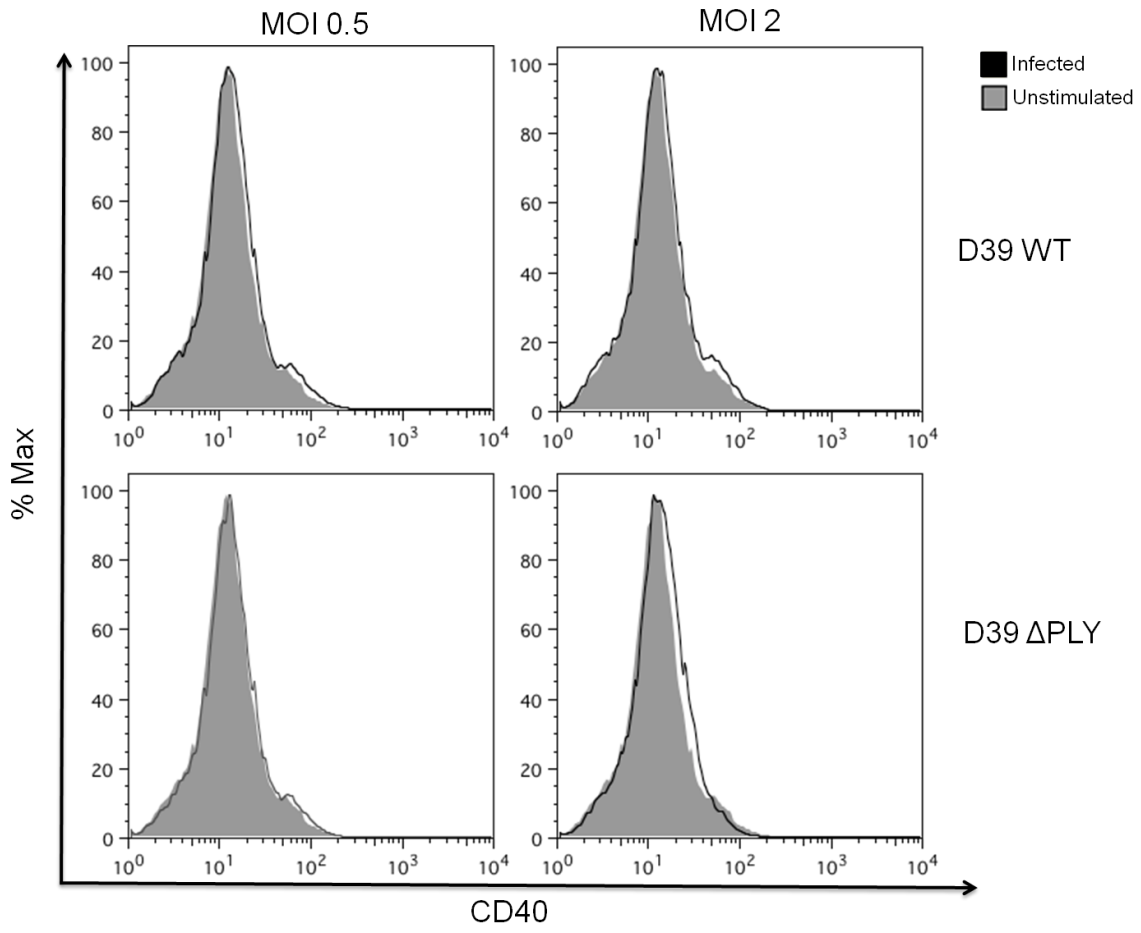

Figure 4-15 DC activation with *S. pneumoniae* harvested after 2 hours after 90 minute infection and addition of antibiotics

DCs differentiated by recombinant GM-CSF were left unstimulated (filled histogram) or infected with *S. pneumoniae* for 90 minutes before addition of antibiotics to kill the pathogen, and incubated for 2 hours. After this time cells were harvested and stained for CD40 (black line) and analysed by flow cytometry compared to isotype. Cells gated on live cells based on FSC and SSC as described in materials and methods. Histograms are representative of culture set up in triplicate. Data representative of 3 experiments.

Thus, 2 hours is not sufficient time after destruction of pathogen for activation of DCs demonstrated by up-regulation of CD40 and CD86.

With these results we confirm that lower MOIs should be used for *S. pneumoniae* infection of DCs such as MOI 1 and 2, and that these infected DCs must be allowed to incubate overnight to allow sufficient up-regulation of co-stimulatory molecules to thus allow proper T cell activation.

4.9 IL-1β output from *S. pneumoniae* infected DCs

DCs were infected with *S. pneumoniae* in the same manner as *P. aeruginosa*, and the supernatants analysed for IL-1β by ELISA to see the effects of *S. pneumoniae* pathogen on IL-1β secretion from DCs. We wished to investigate if there was any difference in IL-1β secretion between the WT and PLY deficient strains of D39, as it has been shown that PLY deficient *S. pneumoniae* strains show significantly less IL-1β production from phagocytes [37, 38].

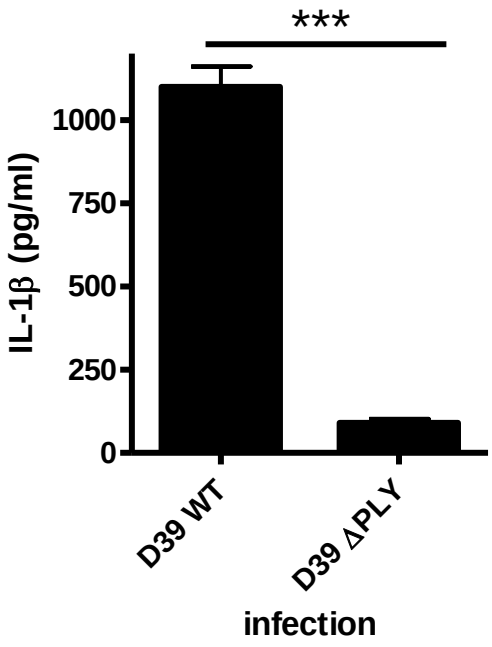

Figure 4-16 *S. pneumoniae* D39 WT allows IL-1β secretion from DCs whereas the PLY deficient mutant D39 ΔPLY does not

DCs differentiated by recombinant GM-CSF were infected for 90 minutes with *S. pneumoniae* strains D39 WT and D39 ΔPLY before addition of antibiotics to kill the pathogen. Cells were incubated overnight before supernatants were harvested and analysed for IL-1β by ELISA. All error bars represent mean ± SEM. Data are representative of 3 separate experiments of experimental triplicates. ***p<0.005.

We observe IL-1β secretion from DCs infected with *S. pneumoniae* WT strain D39 but not when DCs are infected with PLY deficient mutant D39 ΔPLY (Figure 4-16), indicating the key role of PLY in IL-1β secretion from DCS.

4.10 IL-23 from *S. pneumoniae* infected DCs

IL-23 release is thought to be TLR4 dependent [49]. The primary ligand for TLR4 is LPS, a bacterial cell wall component present in Gram-negative bacteria but not Gram-positive pathogens such as *S. pneumoniae*, therefore indicating that *S. pneumoniae* would not lead to release of IL-23. However PLY is suggested to be a TLR4 ligand [39] and so IL-23 may be produced by infection with PLY competent D39 WT, but not with PLY deficient D39 ΔPLY. We investigated this by infecting DCs with both *S. pneumoniae* strains and analysing the supernatants for IL-23 by ELISA (Figure 4-17).

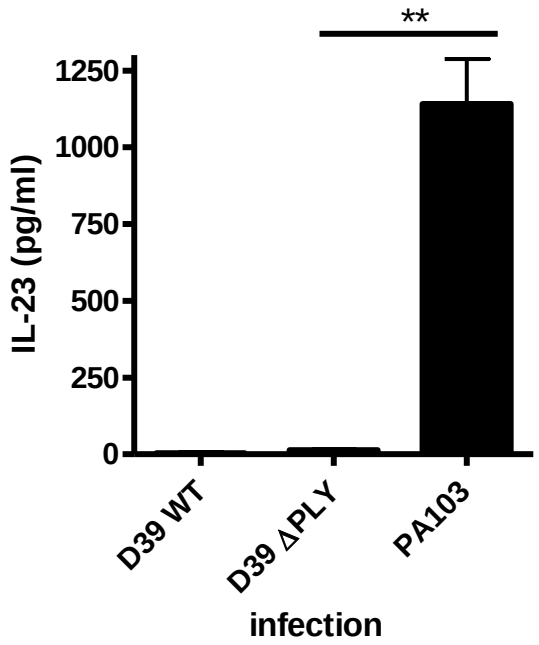

Figure 4-17 Infection with *S. pneumoniae* strains D39 WT and D39 ΔPLY does not lead to IL-23 release from DCs

DCs differentiated by recombinant GM-CSF were infected for 90 minutes with *S. pneumoniae* strains D39 WT and D39 ΔPLY before addition of antibiotics to kill the pathogen. Cells were incubated overnight before supernatants were harvested and analysed for IL-23 by ELISA. Supernatants of PA103 ΔUΔT infected DCs, previously shown to secrete IL-23 (Figure 4-13), were also analysed as a positive control. All error bars represent mean ± SEM. Data are representative of 3 separate experiments of experimental triplicates. **p<0.005.

Negligible IL-23 is produced by DCs infected with either strain of *S. pneumoniae* (Figure 4-17), as compared to IL-23 production by P. *aeruginosa* infected DCs. The lack of IL-23 observed from DCs during *S. pneumoniae* infection indicates that if IL-23 production is TLR4 dependent, perhaps PLY is not in fact a TLR4

ligand as no IL-23 is observed during infection with PLY competent strain D39 WT. Thus, the role of PLY as a TLR4 ligand requires further investigation.

4.11 DCs do not produce IL-17 in response to *P. aeruginosa*

Our primary goal is to see if naive T cells co-cultured with infected DCs secrete IL-17, the characteristic cytokine of Th17 cells, and it is therefore important to ascertain if DCs themselves secrete IL-17 and so could be a source of IL-17 in our co-culture. The supernatant of infected DCs was measured for IL-17A by ELISA, using naive T cells induced to become Th17 cells by culture with TGFβ, IL-6 and IL-1β as a positive control.

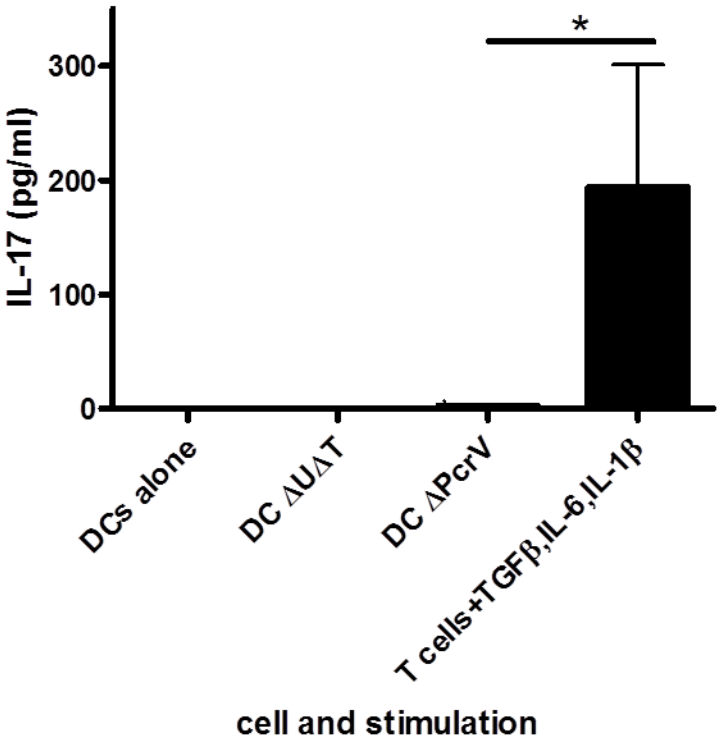

Figure 4-18 DCs do not secrete IL-17 during *P. aeruginosa* infection
DCs were left unstimulated, or infected with *P. aeruginosa* strains ΔUΔT and ΔpcrV for 90 minutes before addition of antibiotics and left overnight to mature. After this time supernatants were harvested and measured of IL-17 by ELISA. Supernatants of T cells cultured with Th17 inducing cytokines TGFβ, IL-16 and IL-1β were also analysed for IL-17, as a positive control. All error bars represent mean ± SEM. Data are representative of 3 separate experiments of experimental triplicates.* $p<0.05$.

Figure 4-18 shows that *P. aeruginosa* infected DCs do not secrete IL-17 and therefore we can be confident that any IL-17 we do see from our cultures is from T cells stimulated by DCs infected with pathogen, and not the infected DCs themselves.

4.12 Isolation of mucosal tissue DCs

BMDCs were used for cultures as they are easy to make and they are plentiful. We however wanted to investigate the use of tissue DCs from mucosal areas in which the respiratory pathogens *P. aeruginosa* and *S. pneumoniae* would encounter. It was hypothesised that these mucosal DCs may have properties or pre-dispositions to secrete certain cytokines upon defence to the respiratory pathogens, and induce cell types equipped to respond to mucosal pathogens effectively. We hypothesised that mucosal DCs may possess key differences that may allow them to respond more effectively to these pathogens than BMDCs. For example, mucosal DCs have been shown to display an immature phenotype [214], unlike BMDCs which we have shown to be somewhat mature due to some expression of CD40 and CD86 when unstimulated (Figure 4-9).

We wanted to investigate if DCs isolated from mucosal tissues could induce Th17 cells from splenic naive T cells upon pathogen infection, and this is explored in the next chapter. First we had to ascertain how to isolate DCs from mucosal tissues, and their properties of activation.

To investigate this phenomenon we isolated DCs from the lung, the direct site where an inhaled pathogen would colonise and set up infection therefore encountering DCs, and from the nasal (or nasopharynx) associated lymphoid tissue (NALT), a small tertiary lymphoid structure in mice above the hard palate. Murine NALT is apparently equivalent to tonsils in humans [215, 216]. Mice do not have tonsils and humans were always thought not to have NALT, though recent studies suggest NALT tissues may exist in humans after all [217]. It is a tertiary lymphoid tissue, defined as a tissue not normally involved in day to day immune responses, that takes on an immune role when challenged [218, 219].

4.12.1 Immune cells in NALT

As *P. aeruginosa* and *S. pneumoniae* are common lung pathogens that enter via inhalation, NALT tissue is optimally placed to respond to such inhaled antigens. The NALT was dissected from naive mice and cells were liberated from the tissue as described in materials and methods. These cells were then stained with antibodies to identify which immune populations may be present and in what percentages.

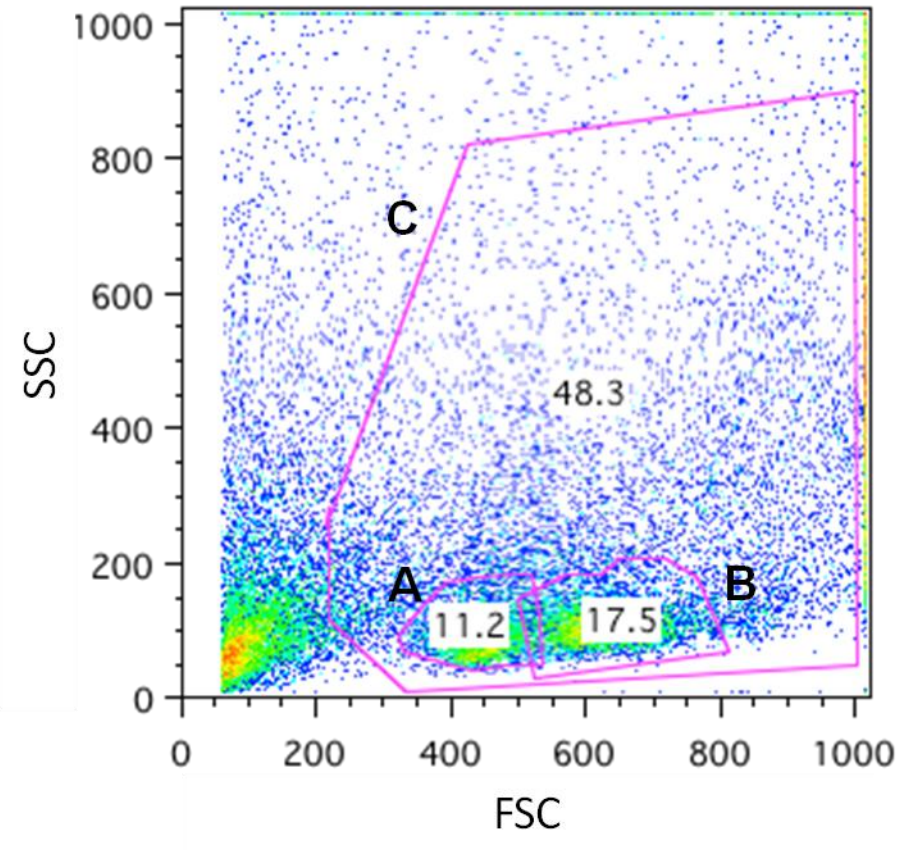

Figure 4-19 Cell populations from NALT

NALT cells were isolated from dissected NALT and populations were investigated by flow cytometry. Plot representative of 3 experiments.

Three populations of cells were identified in the NALT based on their FSC and SSC (Figure 4-19). Two small and very dense populations can be observed, labelled A and B. Both these population have similar low SSC, indicating that they are not granular, but population B has a higher forward scatter and therefore is a population of larger cells, as FSC represents size. A third, larger and more granular population that encompasses these 2 smaller populations was

also gated, and labelled as C. These 3 populations were investigated as a whole and individually to see where the CD11c+ DCs and CD4+ T cells, if any, resided.

It appears that population B, the population of non-granular larger cells as based on forward scatter, contains a small, but distinct compared to isotype, population of CD11c+ cells and CD4+ cells (Figure 4-20 and Figure 4-21). Looking at population B alone, and staining for CD4 we see 6% CD4 cells are contained within it (Figure 4-20). This population is small but extremely distinct from the CD4- cells and isotype, indicating that it is a true CD4+ T cell population.

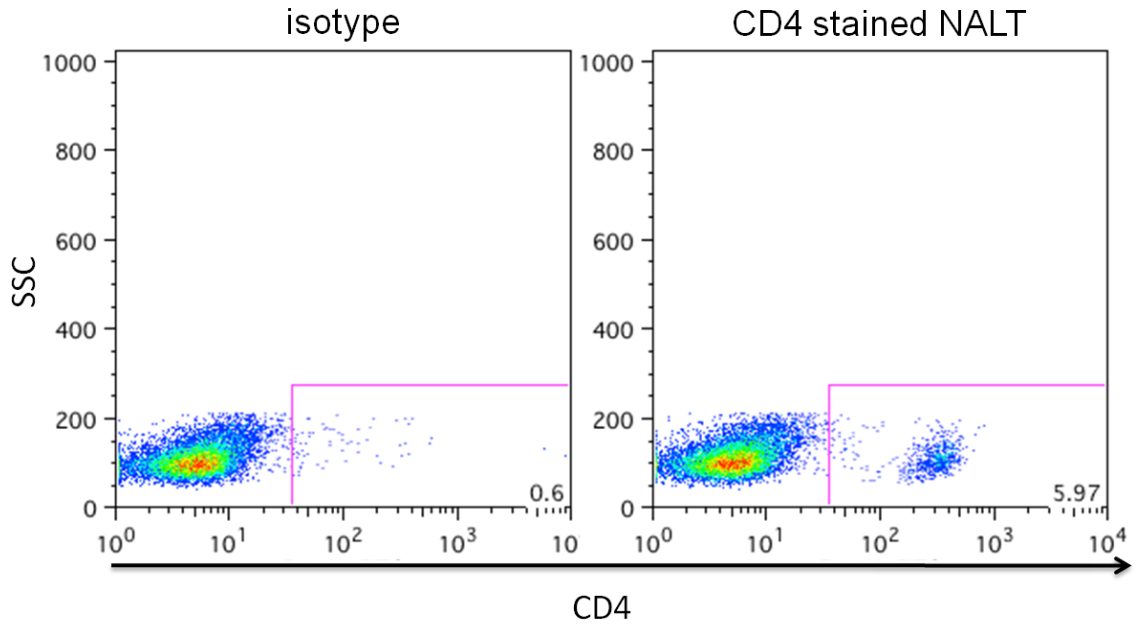

Figure 4-20 CD4 population of naive NALT

NALT cells were isolated from dissected NALT and cells were stained for CD4 and analysed by flow cytometry compared to isotypes. Cells gated on population B identified in Figure 4-19. Data representative of 3 experiments.

The actual number of CD4 cells in the unstimulated NALT however is much smaller than this figure represents. This is CD4+ population is 6% of a 17.5% cell population (this is the percentage of all NALT cells that population B represents), making this CD4+ population less than 1% of the total NALT cells. However, it must be considered that the animals from which the NALT was dissected were naive, and as the NALT is a tertiary immune structure that responds to immune challenge, perhaps cell populations would be larger during infection. This is of consideration for future experiments.

CD11c populations in this tissue were also small, with 3.4% of cells in population B being CD11c+ (Figure 4-21). This population is not as distinct as that of CD4+ cells, but a CD11c+ population is still evident in the NALT.

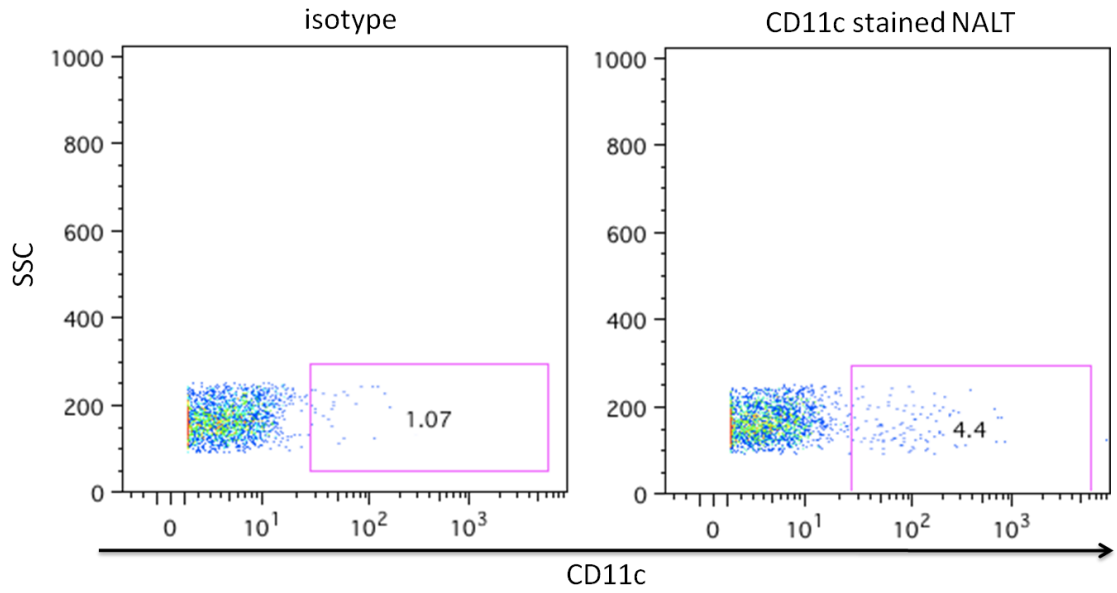

Figure 4-21 CD11c+ NALT cells

NALT cells were isolated from dissected NALT and cells were stained for CD11c and analysed by flow cytometry compared to isotypes. Cells gated on population B identified in Figure 4-19. Data representative of 3 experiments.

These results confirm that the tissue we isolated was a lymphoid tissue that contains DCs, and thus DCs may be isolated from this tissue for further experimentation investigating the roles of mucosal DCs.

4.12.2 Isolation of mucosal DCs

Isolation of CD11c+ cells from these tissues was performed by digestion of the tissue and then using CD11c+ MACS kit to isolate the CD11c+ cells. Positive selection involves the retention of positively labelled cells on the magnetic column which are then removed by flushing of the column once removed from the magnetic field. Unfortunately this isolation is not very pure, with a positive yield of less than 50% on average (see Chapter 3 Methods Developed Figure 3-2 and Figure 3-3). As discussed in Chapter 3, we believe it to be due to the rest of the cells in the tissue, epithelia cells, goblet cells and such that make the preparation very sticky and therefore stick to the magnetic column with the positively selected cells, only to then be mechanically flushed through as part of

the positive prep. Various methods of lung digestion and preparation before CD11c+ isolation were tested to maximise this selection process that are not discussed here (see Chapter 3 Methods Developed). Once a method of maximum yield was elucidated we found that we could isolate a >50% CD11c+ population from the lung with this positive selection kit (Figure 4-22).

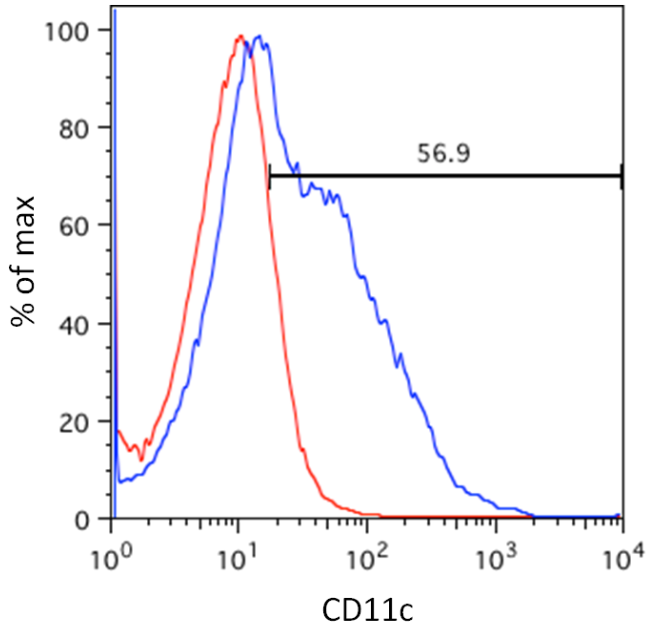

Figure 4-22 DC isolation from lung using positive selection MACS
Lung tissue was digested and treated with CD11c+ MACS isolation kit to isolate CD11c+ cells, which were stained with CD11c (blue line) and analysed by flow cytometry compared to isotype (red line) ,to test efficacy of kit. Data representative of 3 experiments.

Unfortunately this is the purest population of CD11c+ cells we could isolate from the lung via several methods and alterations. Methods to isolate purer populations need further investigation.

4.12.3 Activation of lung DCs with P. aeruginosa infection

CD11c+ lung DCs were infected with *P. aeruginosa* to test their ability to respond to and to become activated by *P. aeruginosa* as measured by up-regulation of CD40 on CD11c+ cells.

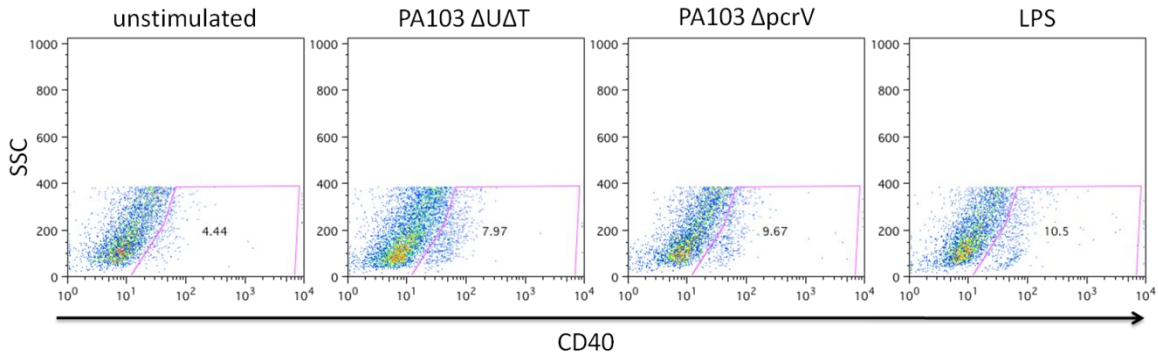

Figure 4-23 Lung DCs are activated by *P. aeruginosa* infection
CD11c+ cells selected by positive MACS (known to be approximately 50% pure) were infected with *P. aeruginosa* strains ΔUΔT and ΔpcrV for 90 minutes before antibiotic addition to kill pathogen, and incubation overnight. After this time cells were harvested and stained for CD11c and CD40 and analysed by flow cytometry. Cells gated on CD11c. Positive gates were set on isotype for LPS stimulated cells. Dot plots are representative of culture set up in triplicate. Data representative of 3 experiments.

Upon infection with both *P. aeruginosa* strains ΔUΔT and ΔpcrV, an up-regulation of CD40 can been observed from unstimulated conditions (Figure 4-23), indicating that *P. aeruginosa* activates lung DCs to up-regulate co-stimulatory markers. This is also observed with LPS stimulation. This confirms that DCs isolated from lung can be activated in a similar manner to BMDCs as we have seen before and therefore they can also be used in co-culture to activate naive T cells.

CD11c+ NALT cells were also purified using the CD11c+ MACS kit. Due to their reduced number, and therefore reduced availability, these cells were not stained for CD11c or tested for activation as lung CD11c+ isolated cells were, and were used directly for co-cultured experiments detailed in Chapter 5.

4.13 Discussion

There have been a number of studies looking into the contribution of pathogen infected APCs in their contribution to Th17 responses [220, 221] and we wanted to investigate this also in response to respiratory pathogen *P. aeruginosa* and to a lesser extent *S. pneumoniae*. This study first investigated various methods of DC induction from bone marrow progenitors to elucidate the best methods of DC induction for the outputs we required; up-regulation of CD40 and CD86, and IL-1β output. Sufficient up-regulation of CD40 and CD86 will allow T cell activation,

and secretion of IL-1β may contribute to Th17 induction. We compared GM-CSF from two sources, recombinant GM-CSF as manufactured and purchased from a company, and 'naturally' occurring GM-CSF as harvested from a cell line, X63, that secretes GM-CSF into its supernatant and that others use routinely [210, 211]. We found that recombinant GM-CSF gives a better CD11c+ population, as measured by a higher CD11c+ population and a higher percentage of cells present in this CD11c+ population. One reason for the higher CD11c+ population of cells observed in recombinant GM-CSF may be that the concentration of GM-CSF in the X63 media isn't known and may vary from batch to batch of harvested supernatant and consequently DC quality may vary in cultures with X63 media. We also found recombinant GM-CSF derived DCs to be better at up-regulation of CD40 and CD86 upon LPS stimulation. This indicates that recombinant GM-CSF may induce 'better' DCs than X63 media. However this is only with regards to CD11c+ populations and CD40 and CD86 up-regulation as we found both GM-CSF and X63 derived DCs to be similar in their IL-1β secretion in response to *P. aeruginosa* infection.

We also investigated the use of Flt3l at DC induction, as it was suggested to induce better DC populations by another group in the lab. We found that Flt3l induced cells are more immature when unstimulated with no CD40 or CD86 expression, and that these DCs are almost completely activated upon LPS stimulation with whole shifts to CD40 and CD86+ cells. In fact, using CD40 and CD86 stained unstimulated populations to represent negative populations, it appears that upon LPS stimulation of Fltl3 DCs there are positive and intermediate populations of CD40 and CD86. Why this is so is unclear, and was not explored any further as our main focus was finding a fully functional CD11c+ DC population and it appeared that Flt3l DCs were mostly CD11c-. We found that CD11c+ population with Flt3l were reduced, with GM-CSF giving better CD11c+ populations and thus, more DCs as defined by the literature. Due to these observations recombinant GM-CSF induced DCs were used throughout BMDC experiments. It appears in our set up that Flt3l DCs do not survive till 14 days to become 100% CD11c+ cells as some studies have claimed [213]. Our culture technique for Flt3l derived DCs, along with the Flt3l itself, was provided by another group in the lab who use Flt3l to derive DCs from bone marrow progenitors on a regular basis. However, this group use the cells at day 7-9 of

generation, also reporting cell death after day 9. This probably reflects why they have not suffered with this protocol beyond that time point and confirms our observation of cell death at day 14.

We decided that due to their continued use in the literature to continue with GM-CSF derived DCs for our experiments. However, the rapid up-regulation of co-stimulatory molecules on early Flt3l derived DCs make them an attractive target of future studies where co-stimulation is of concern.

We also confirmed that better and more functional CD11c+ populations are induced using freshly isolated bone marrow progenitors compared to frozen and thawed progenitors as the frozen and thawed bone marrow induced DCs are CD11c+ but show no up-regulation of co-stimulatory molecules CD40 or CD86. Thus it is better to use fresh bone marrow where co-stimulation is important.

Once a DC induction method was decided upon we investigated activation of these DCs by the pathogen *P. aeruginosa* as it was important to ascertain the effect *P. aeruginosa* had on DCs before we cultured them with naive T cells to see if Th17 responses could be induced. We confirm that DCs infected with *P. aeruginosa* are activated, as demonstrated by up-regulation of CD40 and CD86 and secretion of IL-23. *P. aeruginosa* strain ΔUΔT also allows secretion of IL-1β. Thus *P. aeruginosa* primed DCs may have factors in place that may allow induction of Th17 cells from naive T cells, as IL-1β is considered to have a role in enhancing Th17 development [121] and IL-23 has been shown to be important for Th17 maintenance [154]. Here recognition of bacterial components by TLRs is probably enough to up-regulate CD40 and CD86, and that may be why we do not see a difference in infection as both strains of *P. aeruginosa* will be recognised in the same manner as they only differ in their T3SS presence. For effective up-regulation of CD40 and CD86, DCs infected with *P. aeruginosa* for 90 minutes before addition of antibiotics had to be left overnight to fully mature as cells harvested after 2 hours of maturation that were subsequently co-cultured with naive T cells did not allow T cell survival. We therefore confirm that for maximum co-stimulation capabilities infected DCs must be left overnight to mature after 90 minutes infection and antibiotics addition.

We wanted to investigate the contribution of IL-1β in Th17 induction and used 2 strains of *P. aeruginosa* to show this. PA103 ΔUΔT has a competent T3SS and so leads to inflammasome activation and downstream IL-1β release when infecting DCs. PA103 ΔpcrV lacks a functional T3SS and so lacks ability to activate the inflammasome and thus does not lead to IL-1β secretion from DCs that it infects. These strains can be used as a model of IL-1β contribution to Th17 induction when DCs infected with these bacterial strains are co-cultured with naive T cells. We confirmed that infection of DCs with ΔUΔT allows IL-1β release and infection of DCs with ΔpcrV does not. We discovered however that this observation is time dependent. IL-1β presence with ΔUΔT infection, and absence with ΔpcrV infection was observed when DCs were infected for 90 minutes and supernatants were harvested and analysed for IL-1β after 2 hours incubation. For our co-culture DCs needed to be left overnight to mature and allow full up-regulation of CD40 and CD86, as has been discussed above. We found that DCs infected with *P. aeruginosa* strains ΔUΔT and ΔpcrV for 90 minutes before antibiotic addition and left overnight did not differ in their IL-1β secretion indicating that over this time other factors lead to inflammasome activation and IL-1β production. For example, dead and dying cells release factors called danger associated molecular patterns (DAMPS) [222] that may be recognised by PRRs that activate the inflammasome when a microbial pathogen is present [223], and ATP may activate the inflammasome via ligation to the P2X7 receptor [166]. Due to time constraints, the factors that lead to inflammasome activation and IL-1β secretion during PA103 ΔpcrV infection over time were not investigated further. Due to the observation that these *P. aeruginosa* strains do not show a difference in IL-1β secretion when left overnight after 90 minute infection and addition of antibiotics to kill the pathogen as was required for our co-culture set up, no assumptions could be made about the contribution of IL-1β in our following co-culture experiments, and therefore we could not use this model to investigate the contribution of IL-1β to Th17 induction in the next chapter. As not the primary focus of our study the contribution of IL-1β was not further investigated. However, if role of IL-1β was a focus of study with these pathogens one could use recombinant IL-1Ra to block IL-1β signalling.

We found that *P. aeruginosa* infection of DCs occurs in a rapid manner, as demonstrated by IL-1β secretion observed from DCs in 30 minutes after

infection. In this time, to allow IL-1β release, the DC must recognise *P. aeruginosa* extracellularly with a PRR to allow downstream production of pro-IL-1β, and intracellularly with a NLR to activate the inflammasome. This inflammasome activation cleaves and activates caspase-1 which in turn cleaves and activates IL-1β, allowing its secretion. We have shown that this complex process may occur in as little as 30 minutes indicating the pathogens ability to infect quickly but also the host's ability to respond rapidly.

We found that *P. aeruginosa* infected DCs also secreted IL-23 with both *P. aeruginosa* strains, indicating that IL-23 secretion from DCs is T3SS independent. The ability of *P. aeruginosa* to allow IL-23 secretion from DCs may contribute to Th17 population maintenance and IL-17 responses to the pathogen which is investigated in further chapters.

We also wanted to investigate DC infection with the Gram-positive pathogen *S. pneumoniae* to see if there was a difference in DC activation and IL-1β and IL-23 secretion in Gram-negative and Gram-positive respiratory pathogens. We found that, as with *P. aeruginosa*, *S. pneumoniae* activation of DCs required overnight incubation after infection and antibiotic addition to allow proper induction of CD40 and CD86, as 2 hours maturation after antibiotic addition showed no up-regulation of these co-stimulatory molecules. Infection with *S. pneumoniae* requires lower MOIs than *P. aeruginosa* for adequate infection and stimulation of the DCs, as an MOI higher than 5 led to no activation as measured by CD40 and CD86 up-regulation. Furthermore, at MOI 5 cells appeared to be dead as could been observed by clustering of cells at the axis (data not shown). Thus under these conditions *S. pneumoniae* is a much more potent pathogen than *P. aeruginosa* as fewer bacteria per host cell are needed to cause a reaction and cell death is observed at a much lower MOI of *S. pneumoniae* than *P. aeruginosa*.

IL-1β was produced by DCs infected with *S. pneumoniae* but significant differences were observed between the PLY competent strain D39 WT and the PLY deficient strain D39 ΔPLY indicating, as others have seen [37], that PLY has a key role in secretion of IL-1β from DCs during *S. pneumoniae* infection. This leads us to hypothesise that if IL-1β plays a key role in Th17 development that infection with *S. pneumoniae* D39 WT will induce a strong Th17 response,

whereas PLY deficient stain ΔPLY will not. This is investigated in the next chapter.

IL-23 from *S. pneumoniae* is negligible, perhaps confirming the theory that IL-23 secretion from DCs may be TLR4 dependent [49]. The 'famous' ligand of TLR4 is LPS, a bacterial wall component in Gram-negative pathogens. Gram-positive *S. pneumoniae* lacks LPS and therefore will not activate TLR4 by this means and this may explain the lack of IL-23 secretion from DCs infected with *S. pneumoniae*. However, it has been claimed that PLY is a TLR4 ligand [39] and as TLR4 leads to IL-23, we hypothesised that we may see IL-23 from the PLY competent strain D39 WT and not from its PLY deficient counterpart D39 ΔPLY. However we see no IL-23 from either strain. This may indicate that PLY is not a TLR4 ligand as others have suggested [39]. However, it may also indicate that IL-23 is not TLR4 dependent and thus both these theories require further investigation.

Mucosal DCs are DCs found at mucosal sites where these respiratory pathogens *P. aeruginosa* and *S. pneumoniae* would inhabit, and so we wanted to investigate if these DCs had abilities to induce Th17 responses from naive T cells when these DCs were infected with *P. aeruginosa*. Our tissues of interest were the lung, as *P. aeruginosa* is a respiratory pathogen that can infect and colonize the lung in immunocompromised individuals [66], and the NALT, a tertiary immune structure that lies between the mouth and nose and so will respond to inhaled *P. aeruginosa* into the lung. NALT is very small and difficult to identify for isolation, especially in naive uninfected mice, as by definition a tertiary immune structure is not involved in day to day immune responses but responds when challenged [219]. Hence we believe, as with all immune tissues when under challenge, that the NALT may increase in size when challenged and therefore be easier to identify and dissect. Isolation of NALT (as described in Chapter 2 Materials and Methods) was difficult as NALT tissue is not readily visible by eye, but we have shown by flow cytometry that we do liberate immune cells, including CD4+ and CD11c+ cells, and thus we are confident that we are liberating NALT as others have reported use of this technique for NALT isolation also [224]. The mice from which the NALT was dissected were naive in the sense that they had not been treated with infection, and so the NALT is small and has low lymphoid cell numbers. It would be interesting to see if the size of this

organ increased upon *in vivo* infection, and if the lymphoid cell numbers differed.

Isolation of DCs from the lung using positive selection MACS appeared a tricky process that never gave a better than 50% CD11c+ population. However, we did see that CD11c+ populations from the lung are activated by *P. aeruginosa* infection and LPS stimulation indicating that there is the potential that they could co-stimulate naive T cells in response to this pathogen, making them of interest for co-culture studies.

CD11c+ NALT cells were never stained for CD11c and investigated by flow cytometry to check kit purity as NALT cells were so few to begin with and thus so reduced after CD11c+ selection that there were only enough numbers left to proceed with culture. We however imagine percentages would be similar if not better than that of lung as there are fewer cell types in the NALT tissue that may contaminate the CD11c+ preparation. As stated, with reference to lung tissue, selection of DCs using CD11c positive MACS selection appears to only be 50% effective, however we proceeded with infection and co-culture to give us a sense of what may happen using cells from this mucosal site. These results can be found in chapter 5 of this thesis. Perhaps other DC isolation techniques such as sorting by flow cytometry may give a cleaner and purer CD11c+ population from these sites. This must be considered by groups hoping to isolate CD11c+ DCs from these sites in the future.

In conclusion the results presented in this chapter demonstrate that recombinant GM-CSF may be the best method of DC differentiation with regards to CD11c+ populations. However, if using CD11c to identify DCs was not the most important quality in an experiment, the results in this chapter suggest that Flt3l derived DCs may be an attractive target for studies where co-stimulation is of importance. We found Flt3l DCs to be in a completely immature state when unstimulated, that show 2 positive populations of CD40 and CD86 when stimulated with LPS, showing better up-regulation of these markers than GM-CSF derived DCs do. We also showed that *P. aeruginosa* activates DCs and allows up-regulation of co-stimulatory molecules CD40 and CD86 to allow activation of T cells. *P. aeruginosa* infection of DCs also leads to release of IL-1β and IL-23 which may contribute to Th17 induction as is investigated in the next chapter. It

was shown that strains of *P. aeruginosa* with a functional T3SS allow secretion of IL-1β from DCs, whereas strains without functional T3SS do not, in a time dependent manner as this is observed after 90 minutes but not when infected cells have been incubated overnight after antibiotic addition to kill pathogen, indicating other factors may play a role in inflammasome activation over this time. Gram-positive *S. pneumoniae* was also shown to activate DCs and allow IL-1β production in a manner that is amplified by PLY presence.

Thus this chapter confirms that the pathogens *P. aeruginosa* and *S. pneumoniae* activate DCs, allowing up-regulation of co-stimulatory markers and secretion of pro-inflammatory cytokines. Therefore these DCs may be used in co-culture with T cells to investigate the contribution of these pathogen primed APCs during Th17 induction.

5 *In vitro* IL-17 responses induced by the mucosal pathogens *Pseudomonas aeruginosa* and *Streptococcus pneumoniae*

5.1 Introduction

Recently a novel subset of CD4+ helper T cells entitled Th17 cells, named due to their characteristic secretion of IL-17 [225], have been described that have varying roles in disease as described in the introduction of this thesis. Th17 cells are identified by CD markers CD3 and CD4, cytokine receptors IL-23R and IL-1βR, chemokine receptor CCR6 and by production of cytokines IL-17A, IL-17F, IL-21 and IL-22 [113]. It must be noted that not all CD4+ cells that secrete IL-22 are Th17 cells, as a new subtype of helper T cells that secrete IL-22 but not IL-17 have recently been identified. These cells are named Th22 cells [184, 185] and have been discussed more thoroughly in the introduction.

The process of Th17 cell induction is a somewhat understood, with TGFβ and IL-6 thought to have the main roles at inducing Th17 cells from naive CD4+ progenitors in mice [97, 119, 120] with pro-inflammatory cytokine IL-1β thought to amplify this process [121]. In humans Th17 cell induction is less understood and is still under investigation. As with mice, the 3 cytokines TGFβ, IL-6 and IL-1β are thought to be the classic combination at Th17 cell induction used in culture but studies are showing that these 3 cytokines are not always necessary in humans. For example, studies have shown that Th17 cell populations have been induced from naive CD4+ T cells in an IL-1β and IL-6 dependent manner, independent of TGFβ [124]. There is also evidence that other pro-inflammatory cytokines such as IL-21 may play roles in Th17 induction also [125]. The role of IL-1β in Th17 cell induction in human and mice seems to differ and is worthy of investigation.

As discussed in the previous chapter, *P. aeruginosa* strain PA103 ΔUΔT is able to activate the inflammasome due to the presence of a functional Type III Secretion System (T3SS) and therefore allows IL-1β release from dendritic cells (DCs), whereas PA103 ΔpcrV does not lead to IL-1β release due to lack of a functional T3SS. We wished to investigate if naive CD4+ T cells became Th17 cells in response to co-culture with *P. aeruginosa* infected DCs, and to see if IL-1β from the infected DCs contributed to this Th17 induction. However, as can be seen in the previous chapter, our culture technique of DCs leads to IL-1β secretion from both *P. aeruginosa* strains and thus IL-1β contribution can no longer be investigated by this means.

The aim of this chapter is to investigate if Th17 cells can be induced in response to *P. aeruginosa*, and if DC presence is important or only that of the cytokines produced by the infected DCs, as studies have shown *in vitro* that exposure to cytokines TGFβ, IL-6 and IL-1β can induce Th17 cells in mice [97]. Do the *P. aeruginosa* infected DCs secrete these factors, and are they sufficient to induce a strong Th17 cell response? We showed in the previous chapter that *P. aeruginosa* infected DCs secrete IL-1β and IL-23, and thus *P. aeruginosa* infected DCs are a source of these pro-inflammatory cytokines in culture. A source of TGFβ in this culture is unknown but we would predict that T cells themselves may secrete this, as T cells are a source of TGFβ [226]. Thus, many of the components are there for a Th17 cell response. Others have shown that bacteria primed APCs can promote induction of Th17 cells in humans [220, 221] and we wished to explore if this occurs in mice also.

5.2 Co-culture of CD4+ T cells with *P. aeruginosa* infected DCs allows T cell proliferation

We wanted to investigate if upon co-culture with *P. aeruginosa* infected DCs, total CD4+ splenic T cells were induced to proliferate indicating that the cell types have co-operated and led to T cell activation. We investigated this using CFSE incorporation. As expected, co-culture of total CD4+ T cells with DCs infected with both stains of *P. aeruginosa* leads to T cell proliferation (Figure 5-1).

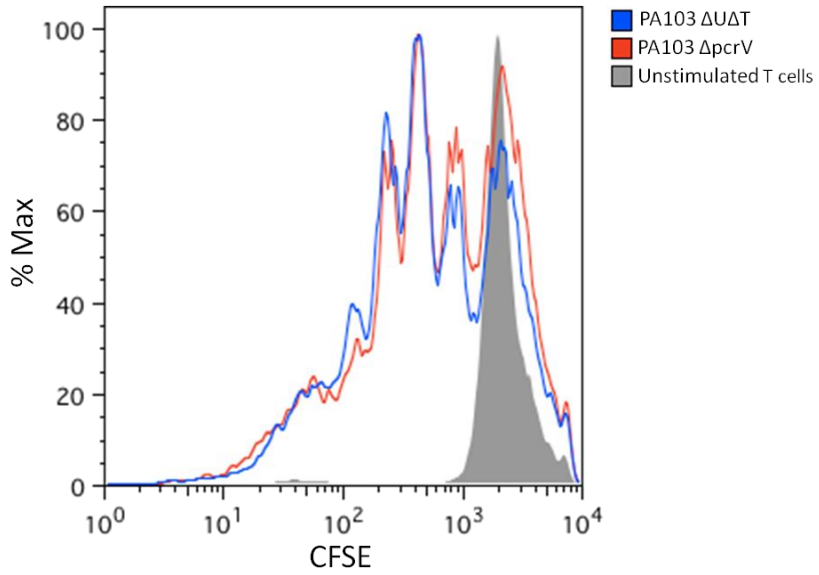

Figure 5-1 T cells co-cultured with *P. aeruginosa* infected DCs are induced to proliferate
MACS sorted total CD4+ T cells were pre-treated with CFSE and left unstimulated (filled histogram) or co-cultured with *P. aeruginosa* ΔUΔT (blue line) or ΔpcrV (red line) infected DCs and cultured for 6 days before harvest and analysis by flow cytometry. Data representative of 3 experiments.

We confirmed that T cells cultured with *P. aeruginosa* infected DCs proliferate and thus the DCs are interacting with the T cells and activating them and therefore we may investigate these cells for IL-17 production knowing that they are activated by the *P. aeruginosa* infected DCs. No difference in T cell proliferation can be seen between PA103 strains, indicating that T cell proliferation when stimulated by an active DC is T3SS independent.

5.3 Co-culture of CD4+ T cells with *P. aeruginosa* infected DCs allows IL-17 production

We confirmed that in mice, as others have observed in with other pathogens in humans [220, 221], that co-culture of total CD4+ T cells with *P. aeruginosa* primed DCs leads to IL-17 secretion. We show that CD4+ T cells sorted by negative selection MACS co-cultured with *P. aeruginosa* infected DCs but not unstimulated DCs induces production of an IL-17+ population (Figure 5-2).

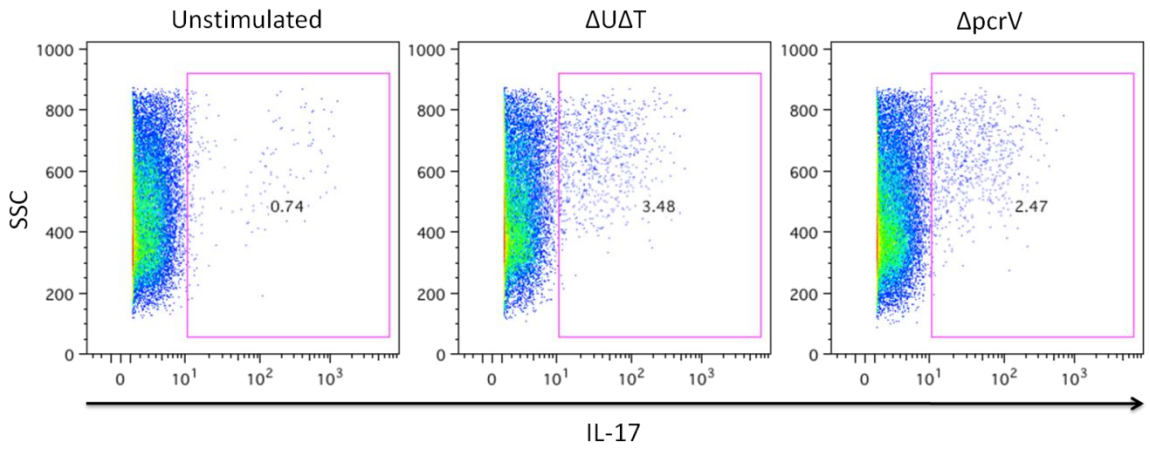

Figure 5-2 Culture with *P. aeruginosa* infected DCs induces IL-17+ populations from MACS isolated CD4+ T cells

MACS sorted CD4+ T cells were co-cultured with *P. aeruginosa* infected DCs for 6 days, after which time cells were stimulated with PMA and ionomycin in the presence of brefeldin A for 5 hours. Cells were then harvested and stained for IL-17 and analysed by flow cytometry. Cells gated on live cells based on forward scatter (FSC) and side scatter (SSC) as described in materials and methods. Positive gates were set on isotype on ΔUΔT stimulated cells. Dot plots are representative of co-culture set up in triplicate. Data representative of 3 experiments.

Thus it appears that Th17 cells can be induced from CD4+ T cells co-cultured with *P. aeruginosa* infected DCs.

5.4 IL-2 diminishes the IL-17 producing population observed upon *P. aeruginosa* infection

IL-2, formerly known as lymphocyte activating factor, is a T cell cytokine known to be important in the survival and proliferation of T cells in culture [227], but in the case of Th17 cells it is considered to be inhibitory [228]. We wanted to investigate if IL-2 addition reduced IL-17 secretion from CD4+ T cells co-cultured with *P. aeruginosa* infected DCs, indicating a reduction in Th17 cells, and to investigate if our cultures survive until day 6 to allow sufficient time for induction of Th17 cells. Stimulating anti-CD3 antibody was added in these experiments to activate the T cell receptor, as we wished to maximise any response from naive T cells which would require T cell activation; the DCs would provide accessory molecule stimulation.

Total CD4+ T cells were co-cultured with unstimulated or *P. aeruginosa* infected DCs, in the presence or absence of IL-2 for 6 days after which time the supernatants of the culture were analysed for IL-17 by ELISA. With the addition

of IL-2, the concentration of IL-17 from T cells cultured with *P. aeruginosa* infected DCs is significantly reduced (Figure 5-3).

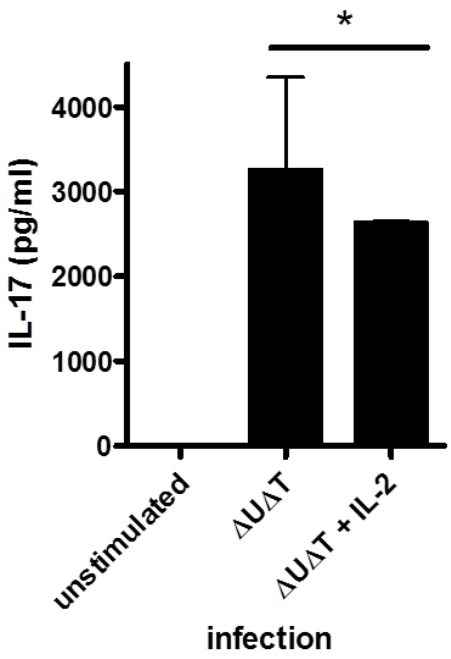

Figure 5-3 IL-2 reduces IL-17 production from MACS isolated CD4+ T cells
MACS sorted CD4+ T cells were co-cultured with unstimulated or *P. aeruginosa* infected DCs with or without addition of IL-2 for 6 days, after which time supernatants were harvested and analysed for IL-17 by ELISA. All error bars represent mean ± SEM. Data are representative of 3 separate experiments of experimental triplicates. * $p<0.05$.

We further wanted to investigate if this was just a reduction in IL-17 production by the IL-17 secreting cells or if there were reduced IL-17 secreting populations upon addition of IL-2 to culture. To investigate this we analysed the IL-17 populations in this culture by flow cytometry. Co-culture of T cells with DCs infected with *P. aeruginosa* in the presence of 50 units/ml of IL-2, which is the recommended concentration for optimal T cell survival, shows a population of 1.5% IL-17+ cells. In the absence of IL-2, the IL-17+ population more than doubles to 3.6% IL-17+ cells (Figure 5-4).

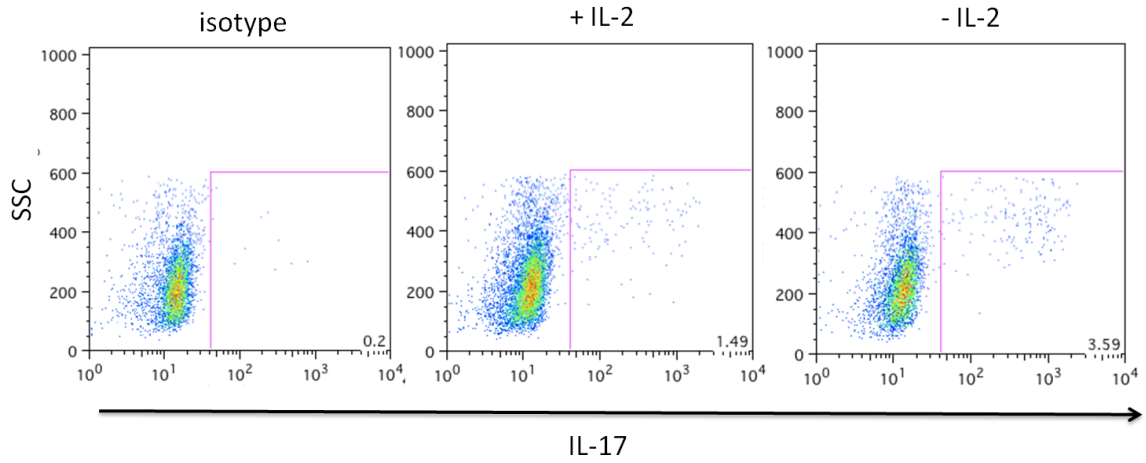

Figure 5-4 IL-2 reduces IL-17+ populations

MACS sorted CD4+ T cells were co-cultured with PA103 ΔUΔT infected DCs in the presence or absence of exogenous IL-2 for 6 days, after which time cells were stimulated with PMA and ionomycin in the presence of brefeldin A for 5 hours. Cells were then harvested and stained for IL-17 and analysed by flow cytometry. Cells gated on live cells based on FSC and SSC as described in materials and methods. Positive gates were set on isotype on ΔUΔT stimulated cells. Dot plots are representative of co-culture set up in triplicate. Data representative of 3 experiments.

This shows that IL-2 addition does not only block IL-17 secretion from IL-17+ cells but reduces IL-17 secreting populations. These results confirm that addition of IL-2 reduces production of IL-17 cells. We also witness that IL-17+ cells in our set-up survive up to 6 days in culture without exogenous IL-2 addition. However the T cells themselves make IL-2 which will aid in T cell survival and thus we cannot discount the effect of endogenous IL-2. We were concerned that lack of additional T cell survival signals would lead to cell death after 6 days, but this is not observed suggesting addition of anti-CD3, and factors secreted in the culture are enough to ensure T cell survive for the time period. Due to these observations IL-2 was not added to subsequent experiments.

5.5 IL-17 producing cells observed during co-culture of CD4 MACS sorted cells with *P. aeruginosa* infected DCs are CD4-

We have established that co-culture of *P. aeruginosa* infected DCs with total CD4+ T cells isolated by negative selection MACS leads to an IL-17+ population (Figure 5-2). However, co-staining with CD4 antibodies to confirm that these cells are CD4+, and thus Th17 cells, revealed that this IL-17+ population is not a CD4+ population as others have seen and hence is not a Th17 cell (Figure 5-5).

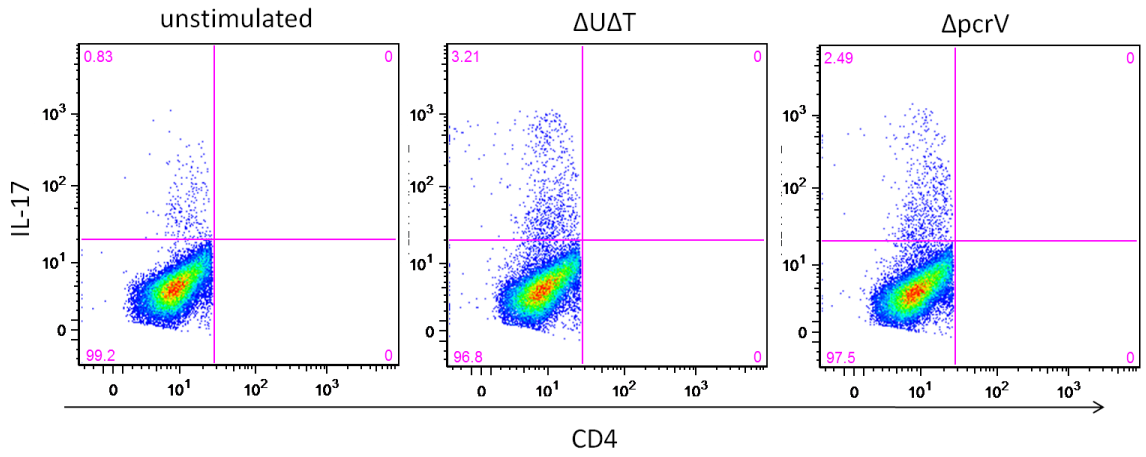

Figure 5-5 IL-17+ populations during co-culture with *P. aeruginosa* infected DCs are CD4- MACS sorted CD4+ T cells were co-cultured with *P. aeruginosa* infected DCs in the presence or absence of IL-2 for 6 days, after which time cells were stimulated with PMA and ionomycin in the presence of brefeldin A for 5 hours. Cells were then harvested and stained of IL-17 and analysed by flow cytometry. Cells gated on live cells based on FSC and SSC as described in materials and methods, and further gated on the IL-17+ cell containing population. Dot plots are representative of co-culture set up in triplicate. Positive gates were set on isotypes on ΔUΔT stimulated cells. Data representative of 3 experiments.

IL-17+ cell populations are apparent in both ΔUΔT and ΔpcrV infections indicating that both these bacterial strains can lead to IL-17 secretion from selected cells. Oddly, we see that this IL-17+ population is a CD4- population and not a CD4+ source. As we have selected CD4+ cells by MACS isolation, the observation that these cells are CD4- is confounding. As the CD4+ cells have been isolated using negative selection, a process whereby all other cells are labelled and retained and that the CD4+ cells pass through untouched, we wondered if we were perhaps not adding enough of the antibody cocktail stage that would deselect everything else. We tried several times adding more beads to retain more CD4- cells and still saw the same IL-17+, CD4- population upon co-culture with *P. aeruginosa* infected DCs. The antibody cocktail component that labels all other cells for retention contained antibodies to CD8 cells (CD8a), macrophages (CD11b), B cells (B220), NK cells (DX5) and red blood cells (ter-119). We noted that γδ T cells are not retained by this kit, whereas they are in the human kit, and so this prompted us to stain our cultures for γδ T cells to investigate they are responsible for our IL-17 positive populations observed upon co-culture with *P. aeruginosa* infected DCs.

5.6 γδ T cells are prolific producers of IL-17 and do so in an antigen independent manner

Upon investigation of this IL-17+ CD4- population that we see upon co-culture with *P. aeruginosa* infected DCs in a theoretically CD4+ isolated cell preparation, we find that the source of IL-17 in our co-culture is γδ T cells (Figure 5-6).

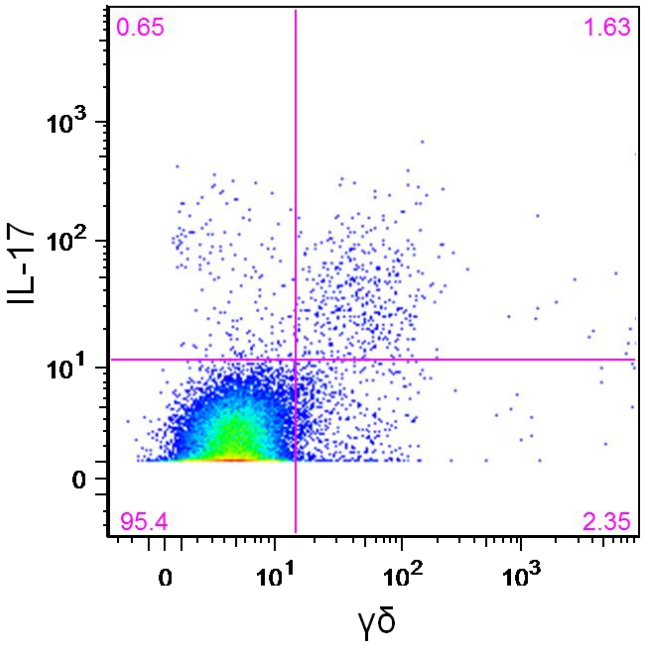

Figure 5-6 IL-17 observed upon co-culture with *P. aeruginosa* infected DCs is from γδ T cells
MACS sorted CD4+ T cells were co-cultured with *P. aeruginosa* infected DCs for 6 days, after which time cells were stimulated with PMA and ionomycin in the presence of brefeldin A for 5 hours. Cells were then harvested and stained of IL-17 and γδ and analysed by flow cytometry. Cells gated on live cells based on FSC and SSC as described in materials and methods. Positive gates were set on isotypes on ΔUΔT stimulated cells. Dot plots are representative of co-culture set up in triplicate.. Data representative of 3 experiments.

71% of the IL-17 producing cells observed upon co-culture with *P. aeruginosa* infected DCs are γδ T cells, and 40% of the γδ T cells in this preparation are IL-17+. This indicates that γδ T cells may have a big role to play in IL-17 production during *P. aeruginosa* infection. This relatively small 1.6% of γδ+ IL-17+ cells, along with a small population, 0.65%, of IL-17+ CD4- γδ- cells that have not been identified, make prolific amounts of IL-17 in this experimental set up with no significant difference seen between *P. aeruginosa* stains (Figure 5-7), indicating that γδ T cells are abundant sources of IL-17 during infection.

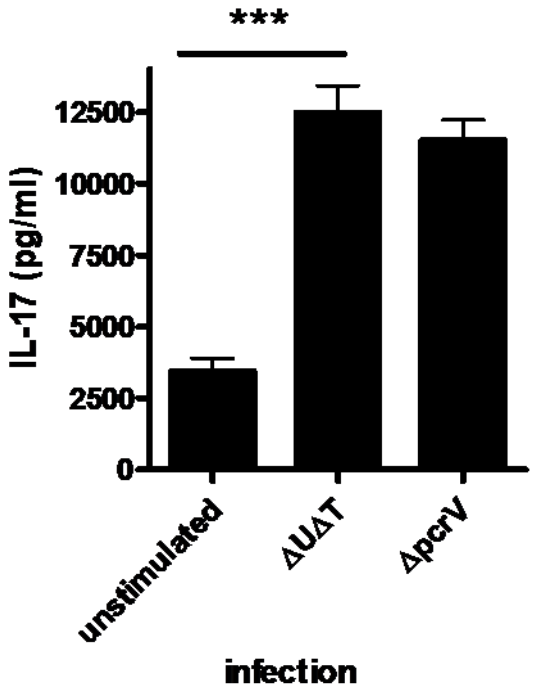

Figure 5-7 IL-17 from CD4+ γδ + cells co-cultured with *P. aeruginosa* infected DCs stimulated with anti-CD3

MACS sorted CD4+ T cells were co-cultured with *P. aeruginosa* infected DCs in the presence of anti-CD3 for 6 days, after which time supernatants were harvested and analysed for IL-17 by ELISA. All error bars represent mean ± SEM. Data are representative of 3 separate experiments of experimental triplicates. *** $p<0.001$.

In these cultures IL-17 can be observed even in unstimulated conditions, albeit significantly lower than in infection conditions. γδ T cells are considered to be a more innate like T cell and have been shown to secrete IL-17 upon stimulation of the CD3 receptor in the absence of other stimuli [137]. This explains the surge of IL-17 from T cells co-cultured with unstimulated DCs observed in culture. This led us to investigate the role of γδ T cells in IL-17 production during *P. aeruginosa* infection which is described in the next chapter.

5.7 CD44 and CD62L are interchangeable markers of T cell memory status and naive CD44- T cells become CD44+ memory T cells upon activation.

As our CD4 negatively selected cell preparation contained naive and memory CD4+ T cells as well as contamination from γδ T cells we decided to isolate naive T cells from this preparation and culture them with our *P. aeruginosa* infected DCs to determine if Th17 cells could be induced from naive T cells under these

conditions. Naive and memory cells can be identified using cell surface markers CD62L and CD44 respectively as discussed in the introduction. We used a CD62L MACS kit to positively select CD62L+ naive cells which we used for culture, and we stained the remaining CD62L- preparation (memory T cells) with antibodies to CD44 and analysed them using flow cytometry (Figure 5-8).

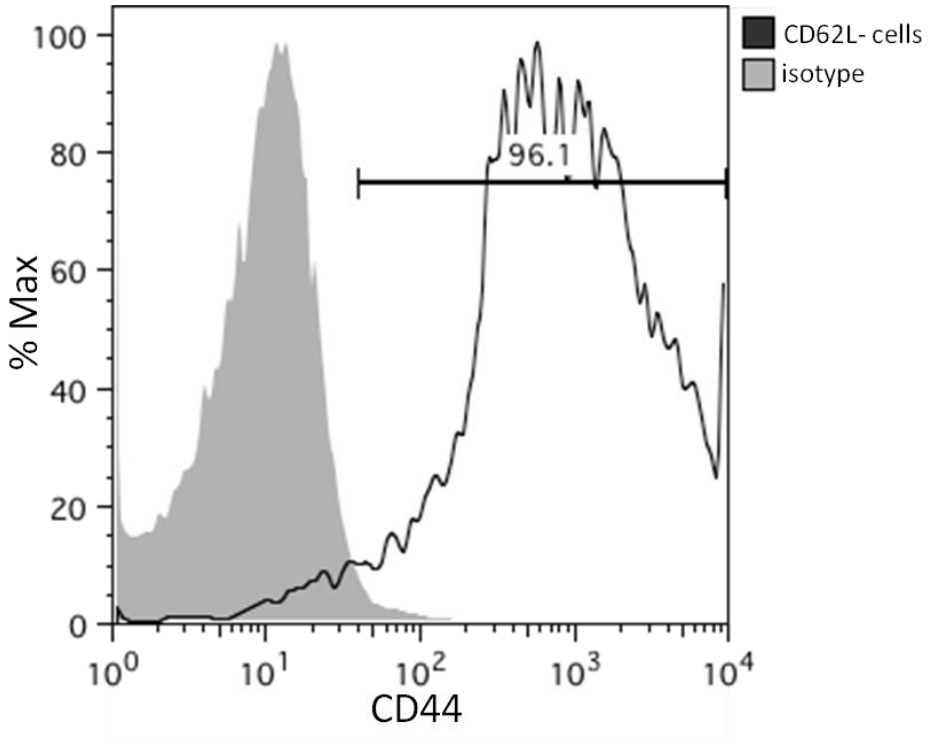

Figure 5-8 CD62L- cells are CD44+

CD62L- cells selected by MACS were stained for CD44 (black line) and analysed by flow cytometry against corresponding isotype (filled histogram) to investigate comparability of these 2 memory/naive T cell markers. Cells gated on live cells based on FSC and SSC as described in materials and methods. Histograms are representative of co-culture set up in triplicate. Data representative of 2 experiments.

96% of the CD62L- MACS isolated cells are CD44 positive, confirming that the purification protocol has resulted in a population of essentially pure naive and memory T cells.

We also confirmed that during co-culture CD62L+ CD44- naive cells are activated and become CD44+ cells further confirming use of this marker as a memory/naive indicator (Figure 5-9).

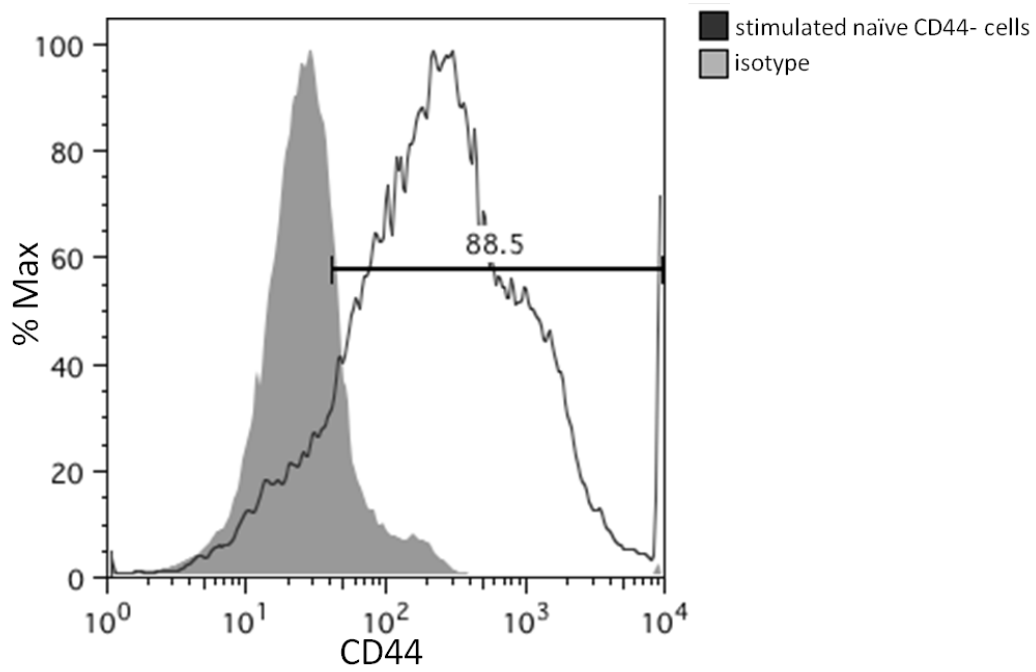

Figure 5-9 Naive CD44- cells become CD44+ upon activation

Naive CD62L+ CD44- CD4+ T cells were co-cultured with *P. aeruginosa* infected DCs for 6 days, after which time cells were harvested and stained for CD44 (black line) and analysed by flow cytometry against corresponding isotype (filled histogram). Cells gated on live cells based on FSC and SSC as described in materials and methods. Histograms are representative of co-culture set up in triplicate. Data representative of 3 experiments.

CD44 expression levels on CD62L+ naive cells was not measured before stimulation, as all CD62L+ cells were used for experimental set-up. But as CD44 and CD62L appear to be interchangeable markers as we have seen from CD44+ staining of CD62L memory cells, one may speculate that naive CD62L+ cells would be CD44- before stimulation, and that there is almost a complete shift to CD44+ cells during stimulation upon co-culture (Figure 5-9). This observation would be logical as these cells are now activated and CD44 is present on activated cells.

5.8 γδ T cells are memory like with regards to CD62L expression

As it is known that CD4+ T cell selection using negative selection MACS kit is not pure, we purified the CD4+ population further by dividing it into naive and memory populations. By selecting CD62L+ naive cells only we hope to eliminate the γδ T cells from the preparation, leaving only the naive CD4+ T cells and thus

allowing us to conclude that any IL-17 we see in our co-culture is from Th17 cells.

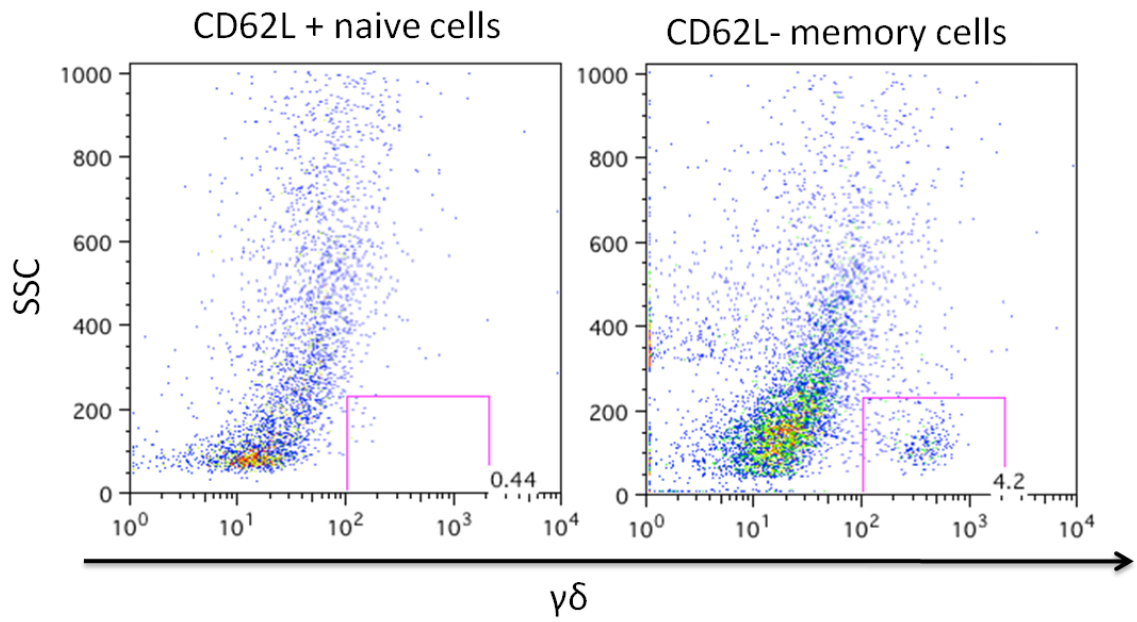

Figure 5-10 γδ T cells are CD62L- 'memory like' cells

MACS isolated CD4+ T cells were divided into naive and memory preparations on the basis of CD62L using MACS and stained for γδ to identify if γδ T cells are CD62L+ or CD62L -. Cells gated on live cells based on FSC and SSC as described in materials and methods. Dot plots are representative of co-culture set up in triplicate. Data representative of 3 experiments.

We confirm that γδ T cells are more 'memory like' with regards to surface marker expression of CD62L and do not express this marker as γδ T cells cannot be found in the CD62L+ preparation (Figure 5-10). Thus purifying the CD4 MACS selected cell preparation with CD62L MACS to isolate naive CD4+ T cells eliminates γδ T cell contamination in the preparation, leaving just naive CD62L+ CD4+ T cells. This allows us to see if upon culture with *P. aeruginosa* infected DCs Th17 cell responses can be induced from naive CD4+ T cells.

5.9 Naive CD4+ T cells become Th17 cells upon co-culture with *P. aeruginosa* infected DCs

Naive CD4+ T cells were isolated as described above and co-cultured with *P. aeruginosa* infected DCs in the presence of anti-CD3 to see if IL-17, and therefore Th17 cell responses, may be induced by *P. aeruginosa* primed DCs. We see that IL-17 is produced upon co-culture of naive T cells with *P. aeruginosa*

infected DCs, but not unstimulated DCs indicating that Th17 cell responses can be generated from naive CD4+ T cells in response to *P. aeruginosa* (Figure 5-11).

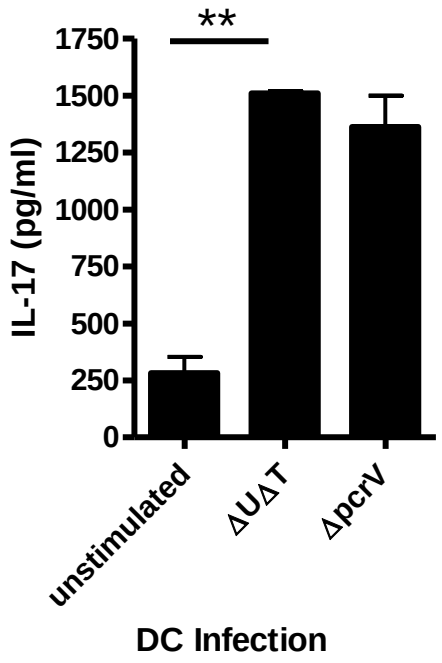

Figure 5-11 Co-culture with *P. aeruginosa* infected DCs induces naive CD4+ T cells to become Th17 cells

Naive CD62L+ CD4+ T cells were cultured with unstimulated or *P. aeruginosa* infected DCs for 6 days, after which time supernatants were harvested and analysed for IL-17 by ELISA. All error bars represent mean ± SEM. Data are representative of 3 separate experiments of experimental triplicates. ** $p < 0.005$.

An IL-17 producing population can be observed in both *P. aeruginosa* strains ΔUΔT and ΔpcrV with no significant difference in IL-17 production seen between strains indicating that IL-17 production is from these cells is T3SS independent. The presence of IL-17 from naive CD4+ T cells co-cultured with *P. aeruginosa* infected DCs indicates that Th17 cells can be induced upon infection with *P. aeruginosa*.

Analysis of the cells by flow cytometry show small, 0.5%, but clear IL-17+ IFNγ- populations from naive CD4+ T cells co-cultured with *P. aeruginosa* infected DCs, but not unstimulated DCs (Figure 5-12).

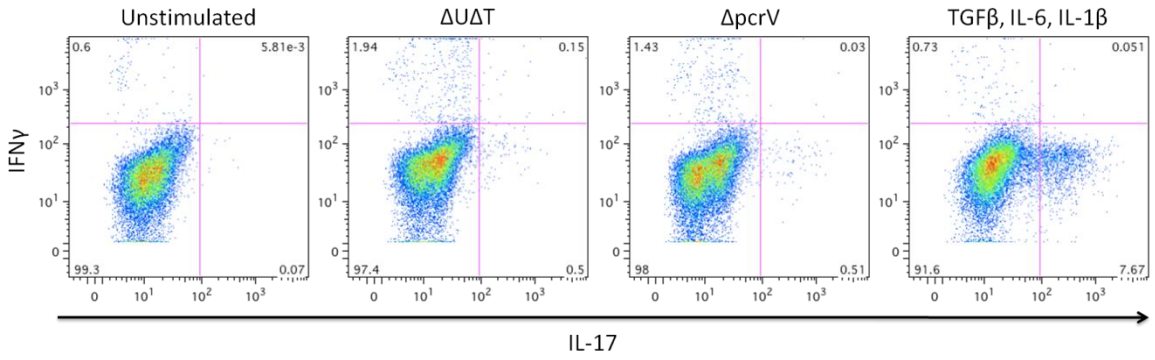

Figure 5-12 IL-17 producing populations upon co-culture with *P. aeruginosa* infected DCs
Naive CD62L+ CD4+ T cells were cultured with unstimulated or *P. aeruginosa* infected DCs for 6 days, after which time cells were stimulated with PMA and ionomycin in the presence of brefeldin A for 5 hours. Cells were then harvested and stained of IL-17 and IFNγ and analysed by flow cytometry. T cells cultured with Th17 inducing cytokines TGFβ, IL-16 and IL-1β were also analysed for IL-17, as a positive control to show techniques were sound. Cells gated on live cells based on FSC and SSC as described in materials and methods. Positive gates were set on isotypes on ΔUΔT stimulated cells. Dot plots are representative of co-culture set up in triplicate. Data representative of 3 experiments.

This population may not be large but is unquestionably increased from unstimulated, and taken together with the ELISA results (Figure 5-11) imply that Th17 cells are induced to *P. aeruginosa* infection during this co-culture.

5.10 IL-17 secretion from naive CD4+ T cells is DC dependent

The literature describes that Th17 cells can be induced from naive CD4+ Th0 precursors upon ligation of CD3 and CD28 in the presence of TGFβ, IL-6 [97, 120] and perhaps also IL-1β. In our culture we hypothesise that the DCs will provide some of these factors as we have previously seen IL-1β secretion and up-regulation of co-stimulation markers from the *P. aeruginosa* infected DCs (see previous chapter). Thus, we set out to determine if DCs secrete the cytokines needed to induce Th17 cell and is this sufficient for their induction. We have demonstrated that Th17 cells develop from naive T cells co-cultured with *P. aeruginosa* infected DCs, but wish to explore if the DCs role in Th17 cell induction is to provide only basic co-stimulation and cytokines or do they supply other factors.

We investigated this by culturing naive T cells the presence of DCs or in their absence with co-stimulation provided artificially by anti-CD28 addition. For conditions in the absence of DCs, T cells were cultured in the supernatants of the infected DC where all soluble factors and cytokines secreted from the DCs would be, referred to therein as conditioned media. For conditions in the presence of DCs, T cells were cultured with infected DCs carried over in their conditioned media. Both conditions received anti-CD3 to stimulate the TCRs, as without TCR stimulation the naive cells will not respond and will die without stimulation in culture over 6 days. Using this set-up we found that naive T cells only secrete IL-17 in the presence of both DCs and their conditioned media and not in DC absence even when co-stimulation was provided artificially (Figure 5-13).

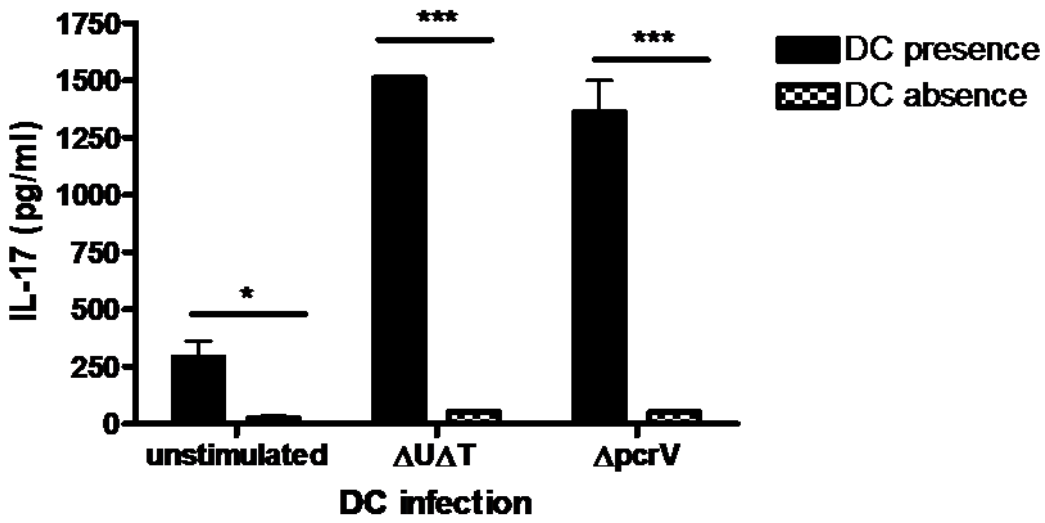

Figure 5-13 IL-17 secretion from naive T cells requires DC presence

Naive CD62L+ CD4+ T cells were cultured with DC conditioned media in the presence or absence of DCs, in presence of anti-CD28, for 6 days, after which time supernatants were harvested and assayed for IL-17 by ELISA. All error bars represent mean ± SEM. Data are representative of 3 separate experiments of experimental triplicates.*p <0.05 *** p<0.001.

The difference in IL-17 secreted from T cells in DC absence or presence in both infection conditions is significant. A small amount of IL-17 can be observed from unstimulated DCs but we have shown previously that even unstimulated DCs up-regulate some CD40 and CD86 and secrete some IL-23, upon aggravation upon plating (see previous chapter).

This result indicates that the DCs play a bigger role than just standard co-stimulation, which we provided with anti-CD28, and cytokines, which would have been present in the conditioned media.

5.11 Role of IL-1β contribution in Th17 cell development

We previously discovered that infection of DCs with both *P. aeruginosa* strains ΔUΔT and ΔpcrV, for 90 minutes before overnight incubation allowed better up-regulation of co-stimulatory markers but IL-1β from both strains when we hoped to use these strains as a model to represent the presence of or absence of IL-1β, (see previous chapter). Due to this we can no longer make any assumptions of role of IL-1β in Th17 cell induction with use of these strains in our co-culture, as no IL-1β difference can be observed between strains under our co-culture conditions. We wished to define the role of IL-1β in Th17 cell induction by skewing naive T cells to become Th17 cells in the presence of CD3, CD28, TGFβ and IL-6, with or without addition of IL-1β. We found no significant difference in IL-17 concentration in naive cells induced to be Th17 cells in the presence or absence of IL-1β, and similar percentages of IL-17+ populations, as based on isotype, from both cultures (Figure 5-14). Positive gates were set using the isotype control, set on TGFβ, IL-6 and IL-1β stimulated cells, to represent a negative population. We recognise that less stringent gating on this isotype would encompass a larger population in the positive samples and it thus may be insinuated that the majority of cells are IL-17+.

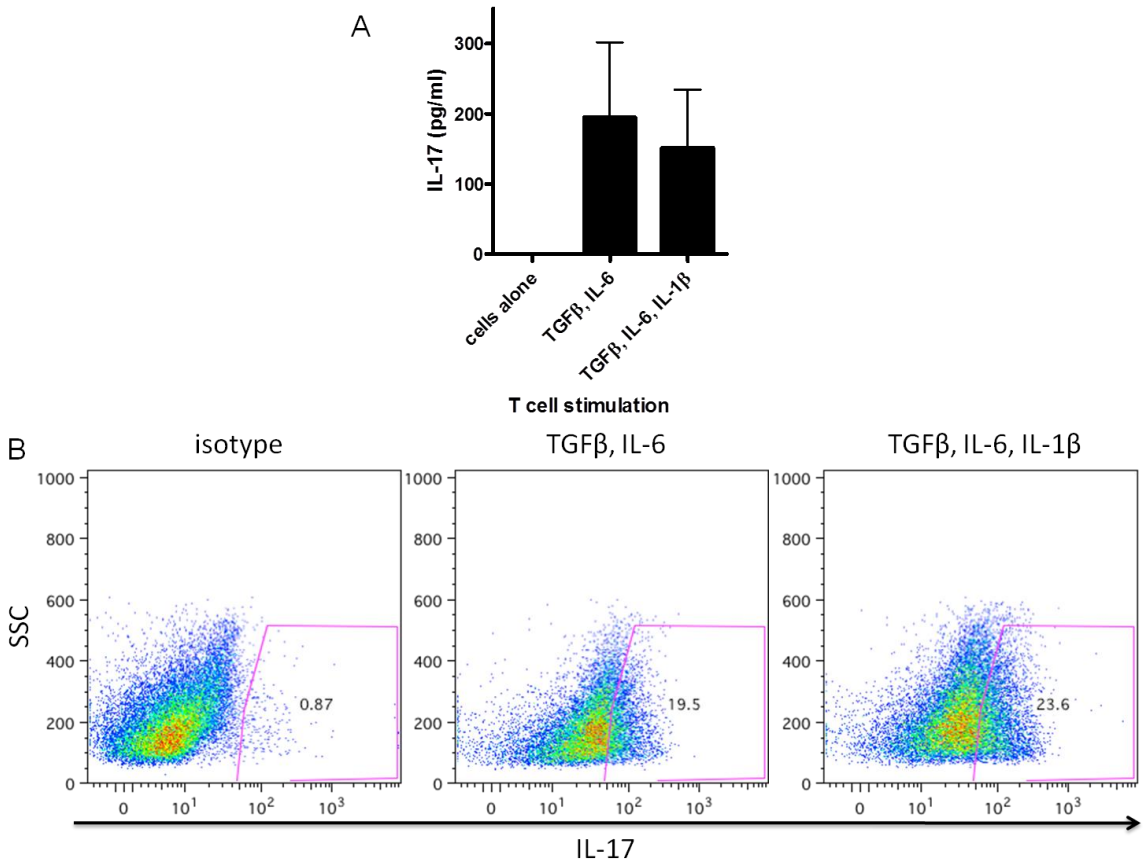

Figure 5-14 IL-17 production from naive T cells is IL-1β independent
Naive CD62L+ CD4+ T cells were cultured with anti-CD3, anti-CD28, TGFβ, IL-6 and with or without IL-1β for 6 days, after which time supernatants were harvested and analysed for IL-17 by ELISA (A). All error bars represent mean ± SEM. Data are representative of 3 separate experiments of experimental triplicates. Cells were stimulated with PMA and ionomycin in the presence of brefeldin A for 5 hours, after which time they were harvested and stained for IL-17 and analysed by flow cytometry compared to isotype control stained on T cells cultured with TGFβ, IL-6 and IL-1β (B). Cells gated on CD4+ cells. Dot plots are representative of co-culture set up in triplicate. Data representative of 3 experiments.

Thus we see that induction of IL-17 from naive CD4+ T cells isolated from the spleen, does not require IL-1β in our *in vitro* set up, although IL-1β addition may enhance IL-17+ populations.

5.12 IL-17 secreting cells induced from co-culture with *P. aeruginosa* do not co-express IL-22

Th17 cells are described as both IL-17 and IL-22 secreting [71] and we wished to investigate if the CD4+ IL-17+ cells that we see during co-culture with *P. aeruginosa* infected DCs were also IL-22+, representing 'true' Th17 cells. However, the IL-17 secreting cells we see here derived from naive CD4+ T cells

appear to secrete low levels of IL-22, in comparison to the levels we observed of IL-17 (Figure 5-11), as evident by ELISA analysis of cell culture supernatants (Figure 5-15).

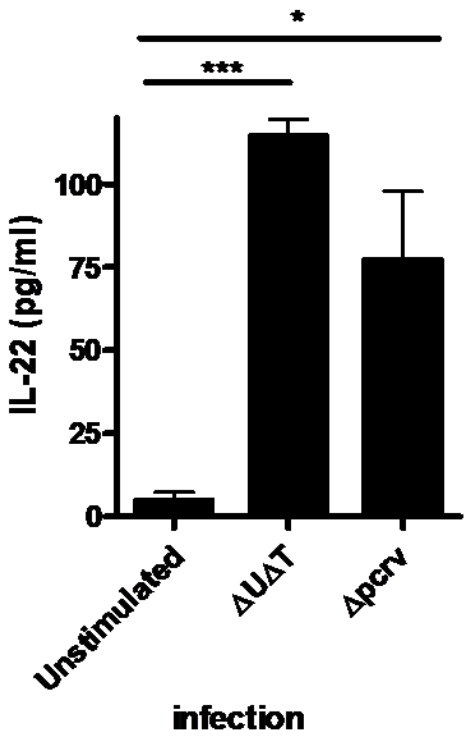

Figure 5-15 IL-22 secretion from naive T cells culture with *P. aeruginosa* infected DCs is low
Naive CD62L+ CD4+ T cells were co-cultured with unstimulated or *P. aeruginosa* infected DCs for 6 days, after which time supernatants were harvested and analysed for IL-22 by ELISA. All error bars represent mean ± SEM. Data are representative of 3 separate experiments of experimental triplicates. * $p<0.05$, *** $p<0.001$.

Our initial observation is that IL-22 production from naive T cells co-cultured with *P. aeruginosa* infected DCs is low compared to the concentrations of IL-17 we have observed. IL-22 concentrations of only 100pg/ml are found, whereas IL-17 levels reached 1000pg/ml (Figure 5-11). However, the IL-22 secreted from naive cells upon culture with infected DCs is significantly different statistically to that of culture with unstimulated DCs (Figure 5-15) indicating that this is a real difference. However, no IL-22+ stained populations are observed by flow cytometry in these cultures (Figure 5-16).

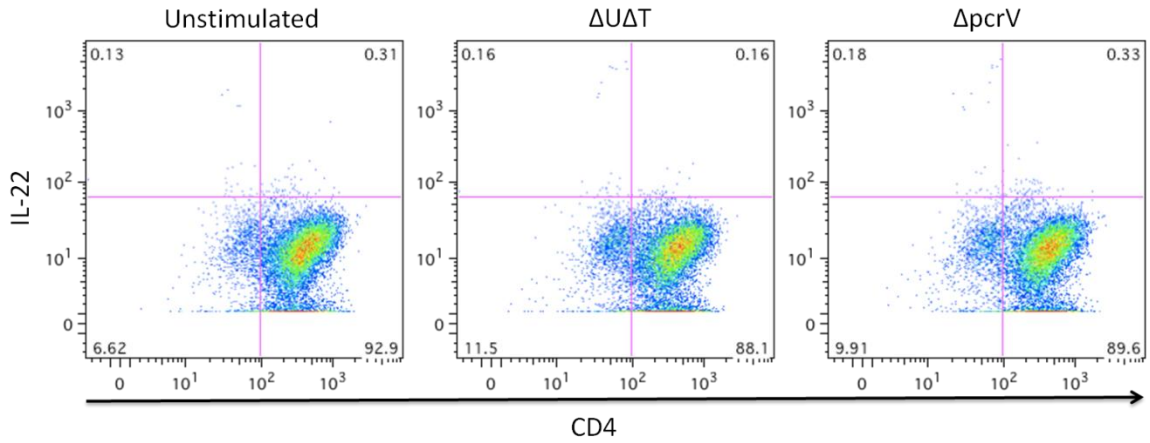

Figure 5-16 IL-22+ cells are not induced from naive CD4+ T cells co-cultured with *P. aeruginosa* infected DCs

Naive CD62L+ CD4+ T cells were cultured with unstimulated or *P. aeruginosa* infected DCs for 6 days, after which time cells were stimulated with PMA and ionomycin in the presence of brefeldin A for 5 hours. Cells were then harvested and stained for CD4 and IL-22 and analysed by flow cytometry. Cells gated on live cells based on FSC and SSC as described in materials and methods. Positive gates were set on isotype on ΔUΔT stimulated cells. Dot plots are representative of co-culture set up in triplicate. Data representative of 3 experiments.

Furthermore, naive T cells induced to be Th17 cells by stimulation with CD3 and CD28 in the presence of IL-1β, TGFβ and IL-6, secrete IL-17 but not IL-22 as can be observe by both flow cytometry and ELISA (Figure 5-17). We are confident that our IL-22 antibody is functional as we see IL-22 staining with the same antibody when investigating γδ T cells in chapter 6.

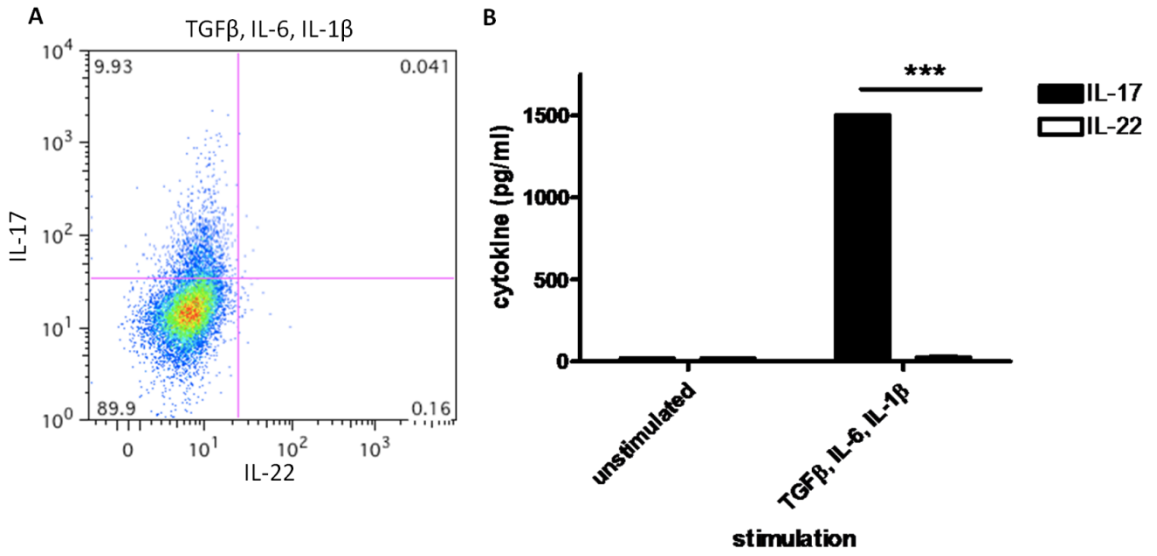

Figure 5-17 Cytokine induced Th17 cells are IL-17+ and IL-22-

Naive CD62L+ CD4+ T cells were induced to become Th17 cells in the presence of TGFβ, IL-6 and IL-1β for 6 days, after which time supernatants were harvested and cells were stimulated with PMA and ionomycin in the presence of brefeldin A for 5 hours. Cells were then harvested and stained of IL-17 and IL-22 and analysed by flow cytometry (A). Cells gated on live cells based on FSC and SSC as described in materials and methods. Positive gates were set on isotypes on TGFβ, IL-6, IL-1β stimulated cells. Dot plots are representative of co-culture set up in triplicate. Data representative of 5 experiments. Supernatants were analysed for IL-17 and IL-22 by ELISA (B). All error bars represent mean ± SEM. Data are representative of 5 separate experiments of experimental triplicates. *** $p<0.001$.

Thus, it appears that our CD4+ IL-17+ cells derived from naive cells co-cultured with *P. aeruginosa* DCs, or in the presence of Th17 inducing cytokines, are IL-22- and not archetypal Th17 cells.

5.13 CD4+ naive T cells can be induced to secrete IL-17 when cultured with *S. pneumoniae* infected DCS

We wished to investigate if Th17 cells could be induced following infection with a Gram-positive respiratory pathogen such as *S. pneumoniae*. Gram-positive and Gram-negative pathogens fundamentally differ in their cell walls and as this is a surface that the DCs would directly come in contact with and potentially recognise, this cell wall difference may well lead to a different response. LPS is a main component of Gram-negative bacterial walls and has been used previously as a non-specific control of activation for the *P. aeruginosa* experiments. Gram-positive bacterial strains do not have LPS, making use of LPS as a comparison void. Bacterial lipoprotein (BLP) is a component of Gram-

positive and Gram-negative bacteria cell walls, recognised by TLR2 that can be used as a positive DC activation control for Gram-positive *S. pneumoniae*.

In preliminary experiments DCs were infected with two *S. pneumoniae* strains; D39 WT and D39 ΔPLY, a variant of D39 that lacks cytotoxic PLY toxin and thus will be less cytotoxic to the DCs. We have shown in the previous chapter that infection of DCs with *S. pneumoniae* leads to DC activation, some IL-1β secretion but no IL-23 secretion as was witnessed with infection with Gram-negative *P. aeruginosa*. IL-1β secretion differs between the *S. pneumoniae* strains D39 WT and D39 ΔPLY, with low secretions of IL-1β during infection with PLY deficient D39 ΔPLY, and significantly higher IL-1β concentrations from infection with PLY competent D39 WT. Thus if IL-1β does contribute considerably to Th17 induction, a difference will be observed between infection with these *S. pneumoniae* strains during co-culture. *S. pneumoniae* infected DCs and their conditioned media were co-cultured with naive T cells as has been described for *P. aeruginosa* infected DCs and IL-17 presence was investigated by ELISA (Figure 5-18) and flow cytometry (Figure 5-19).

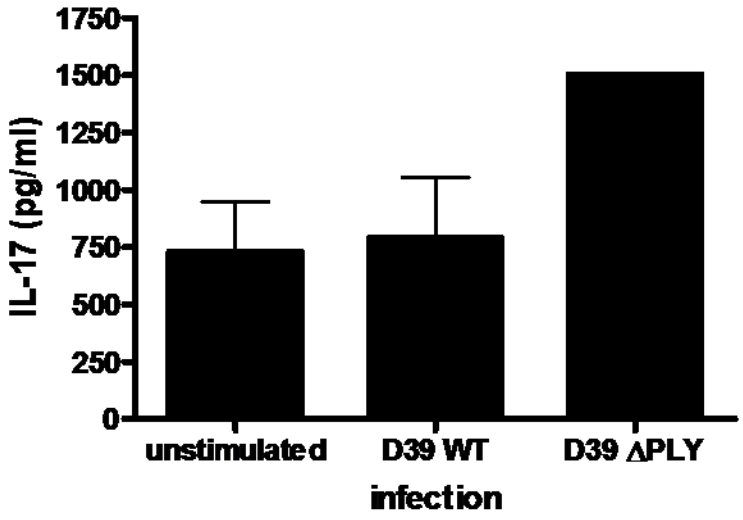

Figure 5-18 Co-culture with PLY deficient *S. pneumoniae* infected DCs induces naive CD4+ T cells to become Th17 cells

Naive CD62L+ CD4+ T cells were cultured with unstimulated or *S. pneumoniae* infected DCs for 6 days, after which time supernatants were harvested and analysed for IL-17 by ELISA. All error bars represent mean ± SEM. Data are representative of 3 separate experiments of experimental triplicates.

In these sets of experiments, the unstimulated DCs unusually gave a high background level of IL-17 production (Figure 5-18). However, a non significant trend (p=0.0601) can be observed upon D39 ΔPLY infection, whereby IL-17 production is increased from that of unstimulated. IL-17 production by naive T cells co-cultured with unstimulated DCs is further observed by flow cytometry as a 2.2% IL-17+ population can be observed (Figure 5-19). An increase in IL-17+ population to 3.79% is seen upon infection with D39 WT. However infection with ΔPLY which appears to allow better IL-17 secretion (Figure 5-18) shows a reduced IL-17+ population from unstimulated, 1.13% (Figure 5-19). Co-culture of naive T cells with BLP stimulated DCs seems to give the largest population of IL-17+ cells as measured by flow cytometry (Figure 5-19) suggesting induction of IL-17+ cells in this set up is not *S. pneumoniae* specific.

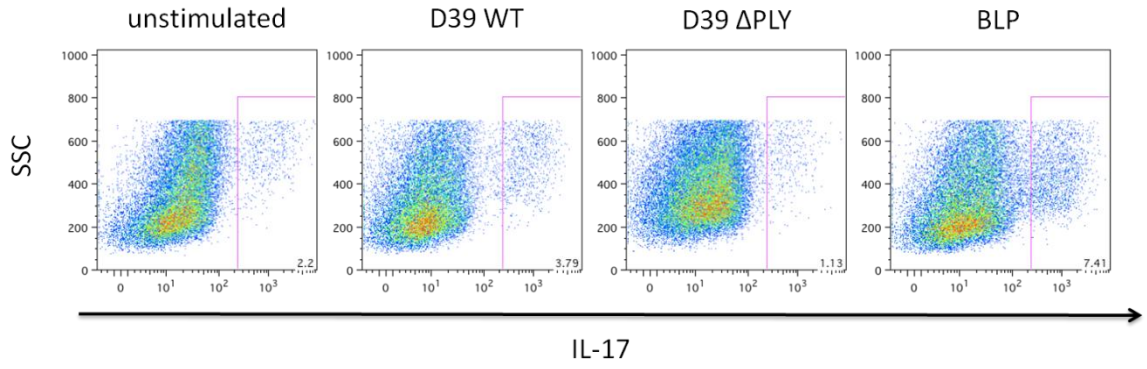

Figure 5-19 Th17 cell populations are induced from naive T cells co-cultured with *S. pneumoniae* infected DCs

Naive CD62L+ CD4+ cells were co-cultured with *S. pneumoniae* infected DCs for 6 days, after which time cells were stimulated with PMA and ionomycin in the presence of brefeldin A for 5 hours. Cells were then harvested and stained for IL-17 and analysed by flow cytometry. Cells gated on live cells based on FSC and SSC as described in materials and methods. Positive gates were set on isotype on D39 WT stimulated cells. Dot plots are representative of co-culture set up in triplicate. Data representative of 3 experiments.

Therefore the FACS data does not correlate with the ELISA data. The ELISA implies that D39 ΔPLY induces better IL-17 production with concentrations observed appreciably different from that of 'unstimulated', however the IL-17 populations seen when investigating these co-cultures by flow cytometry would indicate that there are fewer IL-17 producing cells in D39 ΔPLY than unstimulated or D39 WT infection, and an increase of IL-17+ cells from unstimulated under D39 WT infection. This disagreement between FACS and

ELISA, and IL-17+ population and secretion observed in unstimulated conditions was observed 3 times and is discussed further in the discussion.

5.14 Naive CD4+ T cells become Th17 cells upon co-culture with *S. pneumoniae* infected DCs, and require DC-T cell contact for optimal IL-17 production

As with *P. aeruginosa*, we wished to investigate if IL-17 production from naive T cells co-cultured with *S. pneumoniae* infected DCs was DC contact dependent. We explored this by culturing naive T cells either with *S. pneumoniae* infected DC conditioned media in the presence of artificially provided anti-CD28, or by co-culturing naive T cells with *S. pneumoniae* infected DCs washed out of their conditioned media and resuspended in fresh media. As with *P. aeruginosa*, *S. pneumoniae* induction of IL-17 from naive CD4+ T cells is dependent on presence of DCs in culture as naive CD4+ T cells cultured with *S. pneumoniae* infected DC conditioned media express significantly lower levels of IL-17 than when DCs are present in culture (Figure 5-20).

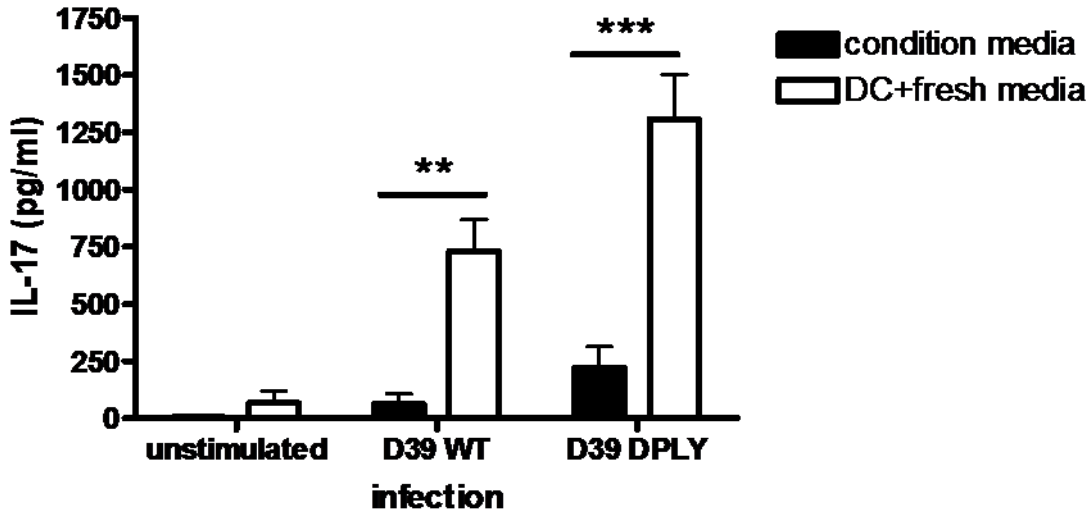

Figure 5-20 IL-17 secretion from naive T cells co-cultured with *S. pneumoniae* infected DCs is DC contact dependent.

Naive CD62L+ CD4+ T cells were co-cultured with unstimulated or *S. pneumoniae* infected DCs washed out of their conditioned media and resuspended in fresh media, or cultured in the conditioned media of these DCs for 6 days, after which time supernatants were harvested and analysed for IL-17 by ELISA. All error bars represent mean ± SEM. Data are representative of 3 separate experiments of experimental triplicates. ** $p<0.005$, *** $p<0.001$.

It would appear that *S. pneumoniae* infected DC conditioned media may have some contribution as it can be observed upon D39 ΔPLY infection IL-17 production is increased from that of unstimulated (Figure 5-20). However, these concentrations are reduced in comparison to those observed where DCs are present, indicating DC presence is important.

These observations are confirmed on analysis of the cells by flow cytometry (Figure 5-21).

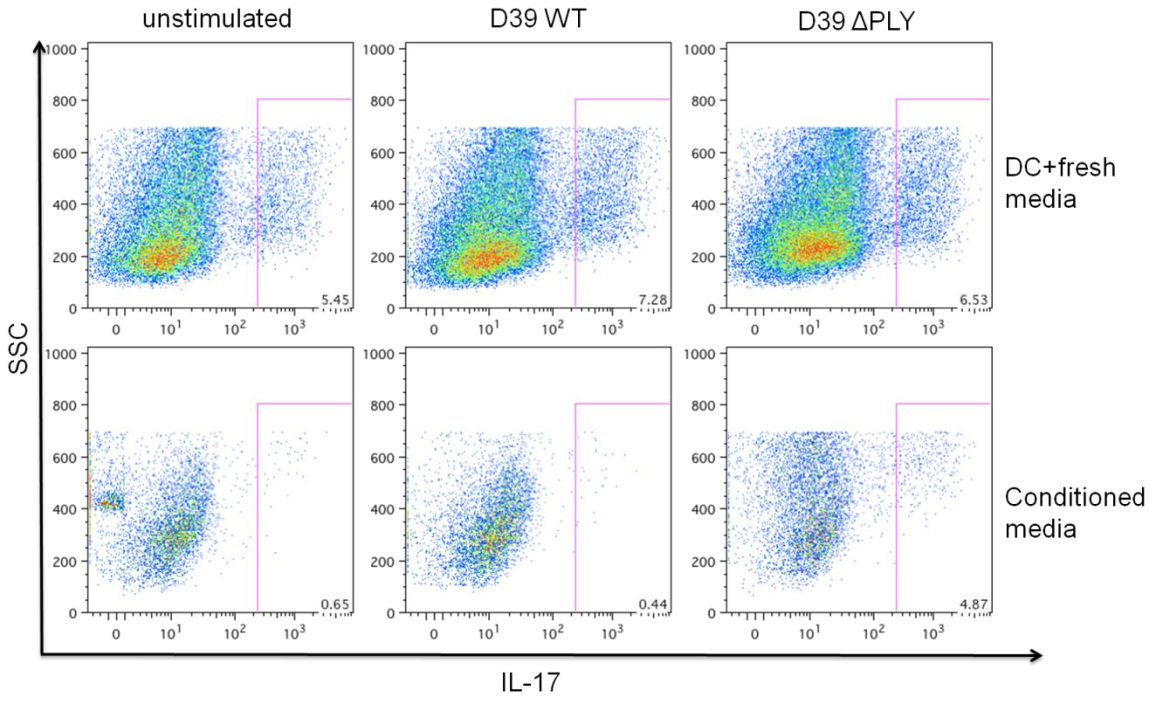

Figure 5-21 Production of IL-17+ populations from naive T cells co-cultured with *S. pneumoniae* infected DCs is DC contact dependent

Naive CD62L+ CD4+ cells were co-cultured with *S. pneumoniae* infected DCs in fresh media, or in *S. pneumoniae* infected DC conditioned media alone, for 6 days, after which time cells were stimulated with PMA and ionomycin in the presence of brefeldin A for 5 hours. Cells were then harvested and stained for IL-17 and analysed by flow cytometry. Cells gated on live cells based on FSC and SSC as described in materials and methods. Positive gates were set on isotype on D39 WT stimulated cells. Dot plots are representative of co-culture set up in triplicate. Data representative of 3 experiments.

IL-17+ cell populations are observed in DC presence, and these populations are reduced when DCs are absent and T cells are cultured solely in conditioned media. In agreement with the ELISA data (Figure 5-20) it appears that even in the absence of DCs co-culture of naive T cells with the conditioned media of D39 ΔPLY allows production of IL-17+ populations, although reduced from populations

observed when DCs are present (Figure 5-21). It is unclear why D39 ΔPLY may lead to IL-17 producing populations in a DC contact independent manner and this was not investigated further as was not a focus of this thesis.

These results indicate that DC presence is important for IL-17 secretion from naive cells in response to *S. pneumoniae* and it appears that D39 ΔPLY infected DCs are better than its WT counterpart at inducing such responses.

5.15 IL-17 may be induced from naive CD4+ T cells when cultured with *P. aeruginosa* infected mucosal DCs.

As *P. aeruginosa* is a mucosal pathogen, colonising lungs and mucosal surfaces, we wished to investigate if mucosal DCs were superior to the 'artificial' GM-CSF derived BMDCs that we induced at stimulating IL-17 responses from naive T cells when co-cultured.

5.15.1 P. aeruginosa infected NALT DCs induce IL-17 responses from naive CD4+ T cells

Co-culture with *P. aeruginosa* infected NALT DCs but not uninfected NALT DCS allows production of an IL-17 secreting population from naive splenic CD4+ T cells (Figure 5-22).

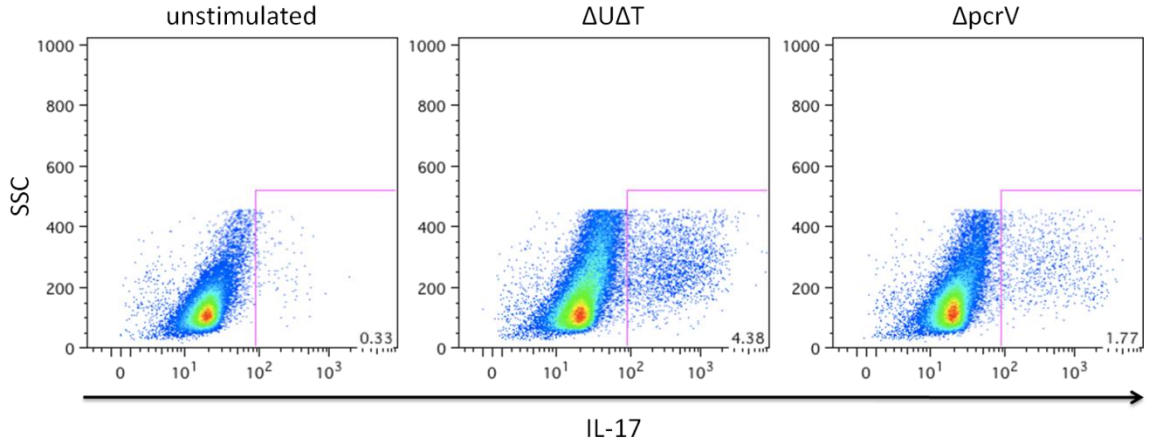

Figure 5-22 Naive T cells co-cultured with *P. aeruginosa* infected NALT DCs become IL-17+
Naive CD62L+ T cells were co-cultured with unstimulated or *P. aeruginosa* infected NALT DCs for 6 days, after which time cells were stimulated with PMA and ionomycin for 5 hours. Cells were then harvested and stained for IL-17 and analysed by flow cytometry. Cells gated on CD4+ cells. Positive gates were set on isotype on ΔUΔT stimulated cells. Dot plots are representative of co-culture set up in triplicate. Data representative of 2 experiments.

Naive T cells cultured with *P. aeruginosa* infected DCs show 4.4% and 1.8% IL-17+ populations with ΔUΔT and ΔpcrV respectively, whereas none is observed when T cells are cultured with unstimulated DCs. This indicates that *P. aeruginosa* infected NALT DCs have the capacity to induce IL-17 responses from naive T cells. We observe that PA103 ΔUΔT induces a much higher IL-17+ population than PA103 ΔpcrV (Figure 5-22) although upon investigation of IL-17 in the culture supernatant by ELISA, both strains appear to secrete similar levels of IL-17 (Figure 5-23).

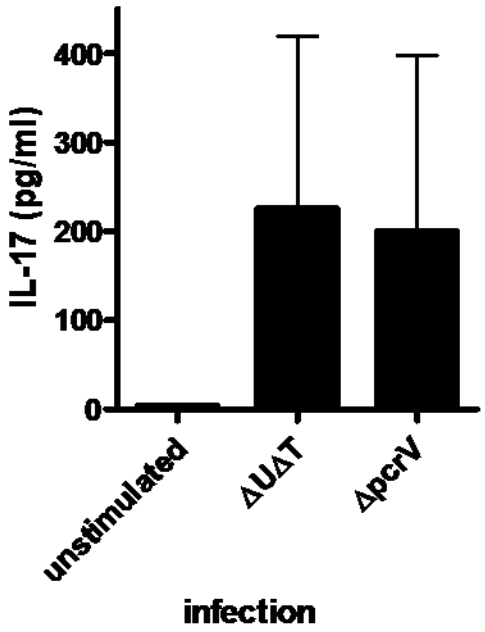

Figure 5-23 IL-17 production from co-culture of naive T cells with *P. aeruginosa* infected NALT DCs.

CD62L+ Naive CD4+ T cells were co-cultured with unstimulated or *P. aeruginosa* infected NALT DCs for 6 days after which time supernatants were harvested and analysed for IL-17 by ELISA. All error bars represent mean ± SEM. Data are representative of 2 separate experiments of experimental triplicates.

The same results were observed on repeating the experiment. Ideally this would have been repeated several more times to confirm this result but due to scarcity of material further analysis was not performed.

We digested whole NALT tissue and stimulated it with anti-CD3 and anti-CD28 to mimic the presence of DCs and co-stimulation to see if an innate IL-17+

population exists in the naive NALT tissue that we liberate and could it be responsible for IL-17 production upon anti-CD3 stimulation (Figure 5-24).

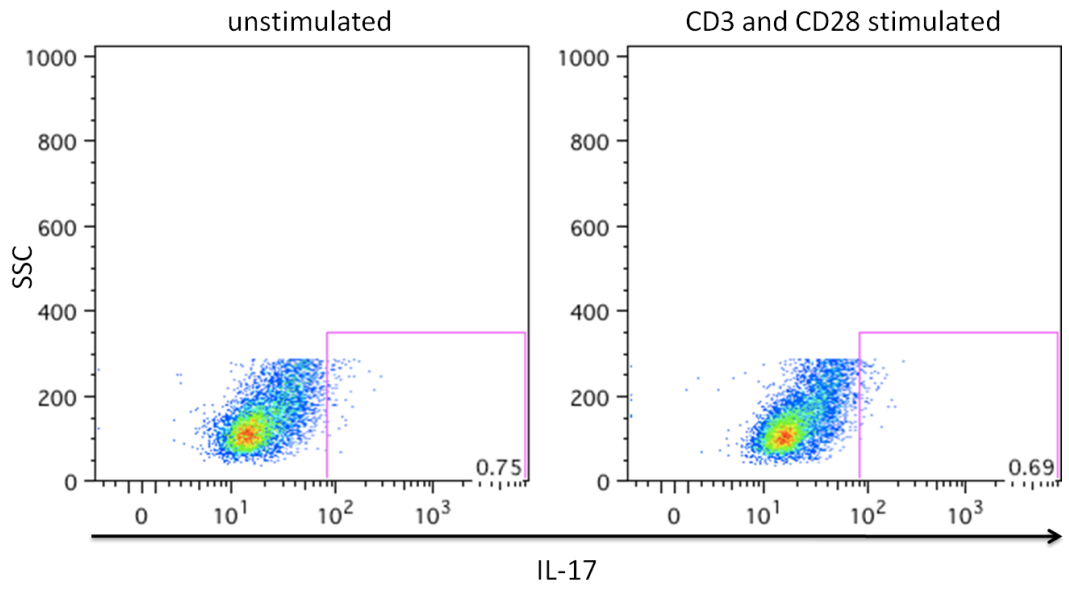

Figure 5-24 Stimulated whole NALT does not contain an innate IL-17 producing population
Whole NALT was stimulated with anti-CD3 and anti-CD28 for 3 days before being stimulated with PMA and ionomycin in the presence of brefeldin A for 5 hours. After this time cells were harvested and stained for IL-17 and analysed by flow cytometry. Cells gated on live cells based on FSC and SSC. Positive gates were set on isotype on anti-CD3 and anti-CD28 stimulated cells. Dot plots are representative of co-culture set up in triplicate. Data representative of 2 experiments.

Whole NALT tissue stimulated with anti-CD3 and anti-CD28 does not lead to emergence of an IL-17+ population (Figure 5-24) indicating that there is not an IL-17 producing innate population in this tissue that is activated by anti-CD3 and we therefore consider the IL-17+ population we see from naive CD4+ T cells upon co-culture with *P. aeruginosa* infected NALT DCs (Figure 5-22) to be from the development of Th17 cells. These observations indicate that NALT derived DCs may be better at inducing IL-17 responses from naive T cells upon *P. aeruginosa* infection as opposed to GM-CSF derived BMDCS. This may be as NALT DCs are biological DCs placed at sites of *P. aeruginosa* invasion and thus they may have factors that aid in responses to respiratory pathogen. This requires further investigation but could be a potential area of interest for further *P. aeruginosa* infection and DC studies.

5.15.2 *P. aeruginosa* infected lung DCs induce IL-17 responses from naive CD4+ T cells

When analysing our BMDC/splenic T cell co-cultures by flow cytometry we normally see one population of cells that has a low side scatter and a middling to high forward scatter. It is this population that we select to represent our live cells, as everything else is on the axis, and this is where we have previously observed our IL-17+ cells. However when looking at cultures with lung derived DCs we see a new population in our co-culture based on forward scatter that we do not normally see when culturing naive splenic T cells with BMDCs and did not witness when culturing with NALT DCs either. We gate this new population separate to our usual 'live' population and brand it 'activated' as it is not see in unstimulated conditions but only in infection conditions (Figure 5-25).

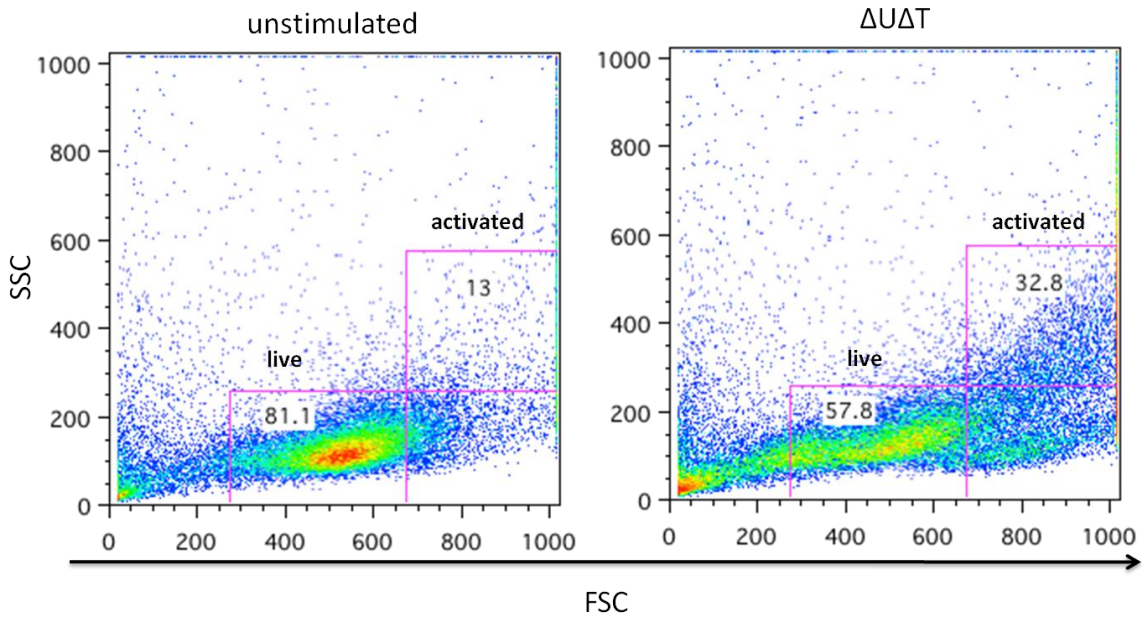

Figure 5-25 A new 'activated' population of T cells is observed when naive T cells are co-cultured with *P. aeruginosa* infected lung DCs

Naive CD62L+ T cells were co-cultured with unstimulated or *P. aeruginosa* infected lung DCs for 6 days, after which time cells were then harvested and stained for and analysed by flow cytometry. Dot plots are representative of co-culture set up in triplicate. Data representative of 3 experiments.

Usually for analysis we gate on the whole 'live' population as marked in Figure 5-25 to encompass all live cells and ensure we include the IL-17+ population which is normally found here. If we gate on 'live' in the lung DC- splenic T cell co-cultures we see a 1.74% IL-17+ population upon ΔUΔT infection up from 0.12% of unstimulated (Figure 5-26) indicating that there is a population of IL-17+ cells

present in this co-culture and confirming that IL-17+ T cells can be induced upon co-culture of naive CD4+ T cells with *P. aeruginosa* exposed lung DCs. However if we focus on this new 'activated' population which isn't normally observed in our co-culture set up and is clearly a visible addition from unstimulated to infected based on FSC, we see that there are 1.05% IL-17 cells in unstimulated but an increase to 7.15% upon ΔUΔT infection (Figure 5-26). It would thus appear that all our IL-17 producing cells are in this 'activated' population.

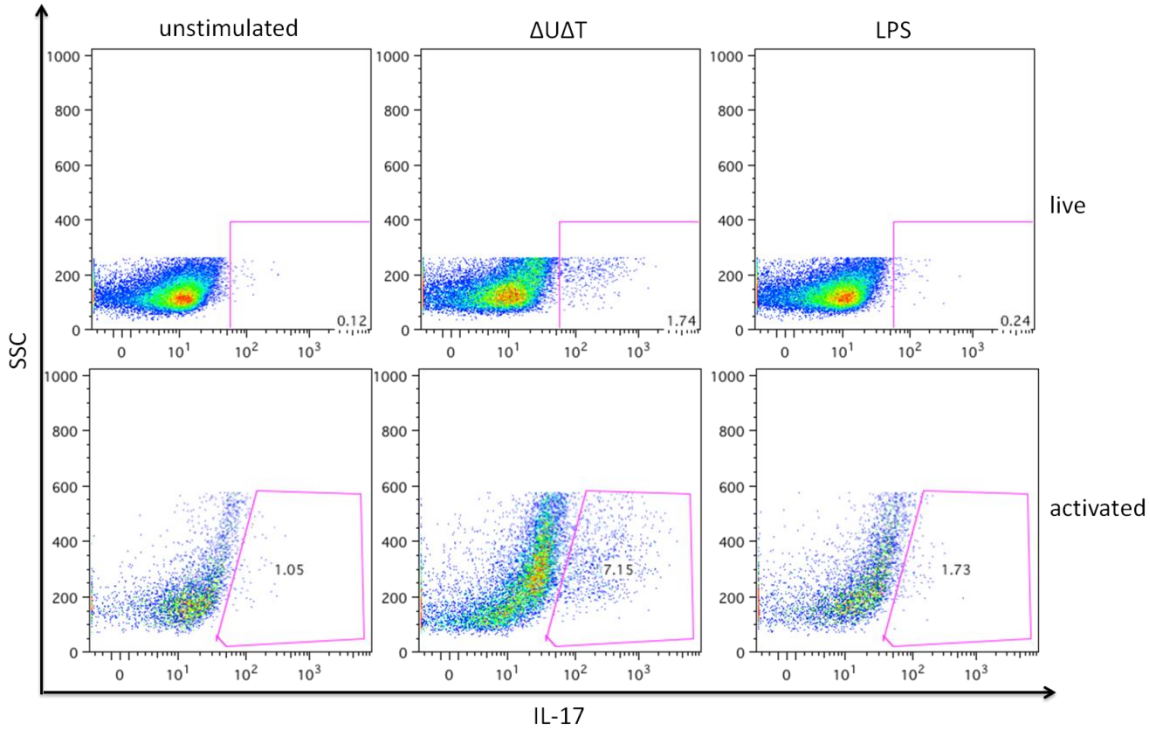

Figure 5-26 'Live' and 'activated' T cell populations from co-culture with lung DCs contain IL-17+ populations

Naive CD62L+ T cells were co-cultured with unstimulated or *P. aeruginosa* infected lung DCs for 6 days, after which time cells were stimulated with PMA and ionomycin for 5 hours. Cells were then harvested and stained for IL-17, and analysed by flow cytometry. Plots gated on 'live' and 'activated' populations identified by FSC and SSC. Positive gates were set on isotype on ΔUΔT stimulated cells. Dot plots are representative of co-culture set up in triplicate. Data representative of 3 experiments.

Although small, as 7.5% of 30% of a total population only amounts to approx 2% of all cells, there is still clearly a Th17 cell induction upon culture of naive T cells with *P. aeruginosa* infected lung DCs. These IL-17+ cell populations are not observed when lung DCs were treated with LPS (Figure 5-26), indicating that it is not just an activated DC, but a *P. aeruginosa* infected DC that is leading to this production of IL-17 from the naive T cells and so a specific response to *P.*

aeruginosa may be induced by lung DCs co-cultured with naive CD4+ T cells. Production of IL-17 from T cells co-cultured with *P. aeruginosa* infected lung DCs is further confirmed by ELISA and analysis of the culture supernatants for IL-17 where a significant concentration of IL-17 is observed with infected DCs compared to unstimulated DCs (Figure 5-27).

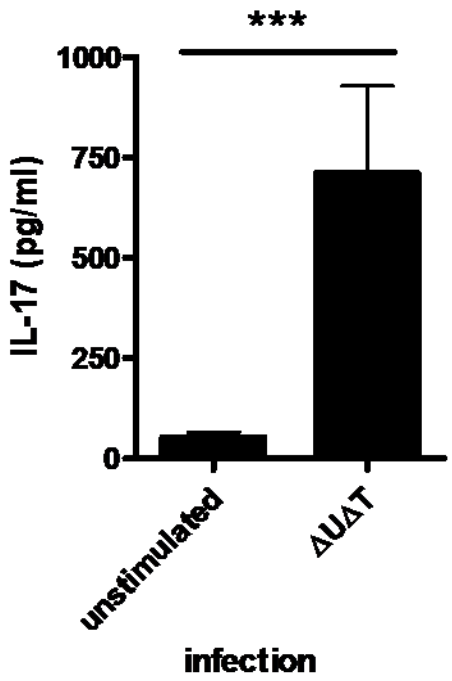

Figure 5-27 Naive T cells co-cultures with *P. aeruginosa* infected lung DCs are IL-17 secreting.

Naive CD62L+ CD4+ T cells were co-cultured with unstimulated or *P. aeruginosa* infected lung DCs for 6 days, after which time supernatants were harvested and analysed for IL-17 by ELISA. All error bars represent mean ± SEM. Data are representative of 3 separate experiments of experimental triplicates. *** $p<0.001$.

These results indicate that mucosal DCs induce IL-17 responses from naive T cells when infected with *P. aeruginosa*, and comparison to populations observed when using BMDCs suggest mucosal DCs may induce these responses in a more robust manner.

5.15.3 IL-22 responses from naive CD4+ T cells co-cultured with P. aeruginosa infected mucosal DCs.

IL-22 levels were also analysed by ELISA and flow cytometry in the above experiments with mucosal DCs to investigate if IL-17+ cells observed in these co-

cultures co-expressed IL-22, as IL-22 was not evident from IL-17+ cells induced by culture with BMDCs (Figure 5-15 and Figure 5-16).

Very small, but significant, levels from IL-22 can be observed from naive T cells cultured with *P. aeruginosa* infected lung DCs compared to culture with unstimulated DCs (Figure 5-28).

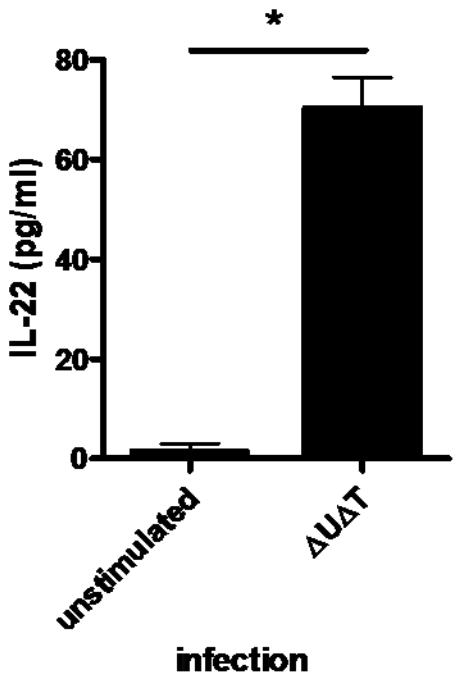

Figure 5-28 IL-22 secretion from naive T cells co-cultured with *P. aeruginosa* infected lung DCs is low

Naive CD62L- CD4+ T cells were co-cultured with unstimulated or *P. aeruginosa* infected lung DCs for 6 days, after which time supernatants were harvested and analysed for IL-22 by ELISA. All error bars represent mean ± SEM. Data are representative of 3 separate experiments of experimental triplicates. * $p<0.05$.

Co-culture with NALT infected DCs gave even smaller concentrations of IL-22 that did not differ significantly from unstimulated and therefore are considered to be negligible.

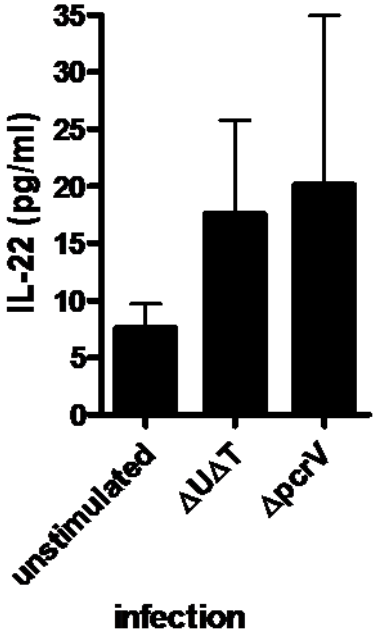

Figure 5-29 Naive T cells co-cultured with *P. aeruginosa* infected NALT DCs do not secrete IL-22

Naive CD62L+ CD4+ T cells were co-cultured with unstimulated or *P. aeruginosa* infected NALT DCs for 6 days, after which time supernatants were harvested and analysed for IL-22 by ELISA. All error bars represent mean ± SEM. Data are representative of 3 separate experiments of experimental triplicates.

No IL-22+ populations could be observed by flow cytometry for culture with either mucosal DC (data not shown).

These results indicate that IL-22 is not co-expressed by IL-17+ cells induced by co-culture with mucosal DCs suggesting that, as was observed with co-culture with *P. aeruginosa* infected BMDCs, these cells are not archetypal Th17 cells.

5.16 Discussion

It has been shown that Th17 cells can be induced in human studies from APCs that are primed with a variety of innate immune stimuli [220, 221]. We wished to investigate this phenomenon in mice in relation to the respiratory pathogen *P. aeruginosa*, while also looking at the contribution of IL-1β in Th17 cell induction as its importance in mice is still subject of debate.

Our original model for the investigation of IL-1β contribution was to use 2 bacterial strains of *P. aeruginosa,* one with a competent T3SS that leads to IL-1β release from DCs upon infection, PA103 ΔUΔT, and another strain that does not

have a functional T3SS and thus does not lead to IL-1β release from the DCs during infection, named PA103 ΔpcrV. We showed however in the previous chapter that overnight incubation of *P. aeruginosa* infected DCs after killing of the live pathogen, to allow full DC maturation for T cell activation- a necessity in our co-culture set up, led to IL-1β secretion from both strains and therefore this model could no longer be used to investigate IL-1β contribution to Th17 cell induction. We then sought to investigate the contribution of IL-1β in Th17 cell generation by simple induction of Th17 cells from naive CD4+ T cells with the cytokines TGFβ and IL-6 in the absence or presence of IL-1β. We saw no significant difference between IL-17 secretion upon IL-1β absence or presence, and similar IL-17+ populations as ascertained by flow cytometry, indicating that IL-1β is not needed for Th17 cell induction in mice. We thus imagine that if the overnight culture of DCs with both bacteria strains did allow a model of IL-1β presence or absence that we may have in fact not observed any difference in IL-17 induction between strains in this set-up. This agrees with previous studies that IL-1β contribution may not be as important in mice as it is in humans [97, 120, 121, 124]. Another possibility to investigate the role of IL-1ß during culture would be the use of recombinant IL-1Ra to block IL-1β signalling, but as this was not the main focus of our study this was not investigated.

We confirmed that addition of exogenous IL-2 to culture reduces IL-17 production, not only by reducing IL-17 secretions by these cells but by reducing the IL-17 secreting populations themselves. Studies show that IL-2 signalling via stat 5 is what inhibits Th17 cell generation as deletion of Stat 5 restored a previously absent Th17 cell population [228]. We did not investigate this however as we wanted only to confirm that addition of IL-2 would inhibit our IL-17 cultures and thus we should not add IL-2 to our culture to maximise IL-17 populations. We were curious as to how lack of IL-2 would affect survival of T cells in our culture as it is known to be vital for T cell survival [227]. It appears artificial IL-2 addition is not necessary for Th17 cell survival in our culture as our cells appear survive till day 6 without addition of IL-2 as gauged on scatter of cells by flow cytometry. However the T cells themselves may produce endogenous IL-2 which may contribute to T cell survival, thus we cannot claim complete absence of IL-2 in our culture.

We discovered that CD4 negative isolation using MACS was not pure and was contaminated with γδ T cells which upon stimulation of their TCRs with anti-CD3 could be induced to secrete vast amounts of IL-17 that was enhanced upon co-culture with *P. aeruginosa* infected DCs and their supernatants. γδ T cells are considered to be a more innate like T cell that respond in a different manner to Th17 cells and must be considered and treated differently. For Th17 induction from a naive CD4+ T cell, stimulation through the CD3 receptor is necessary. Stimulation of the CD3 receptor in the presence of antigen activated DCs and appropriate cytokines leads to formation of an activated CD4+ helper T cell. However in the absence of TCR stimulation, naive cells are not activated and no response is induced. In contrast, stimulation of the CD3 receptor alone in γδ T cells is enough to allow IL-17 secretion in the absence of other stimuli [137]. IL-23 addition with IL-1β, is also enough to induce IL-17 secretion from γδ T cells [129] in the absence of TCR stimulation and addition of both these cytokines in combination of TCR stimulation has a synergistic effect which we believe to be seeing here in infection conditions as we have already shown in the previous chapter that *P. aeruginosa* infected DCs secrete IL-23 and IL-1β, and we have stimulated our culture with anti-CD3. This explains the surge of IL-17 from T cells co-cultured with unstimulated DCs observed in culture, and the significant increase seen during infection conditions. This led us to further investigate γδ T cells and their IL-17 secretion as can be seen in the next chapter.

We overcame this obstacle of γδ contamination of our CD4+ population by using CD62L to isolate naive CD62L+ CD4+ T cells, as we found γδ T cells to be contained in the CD62L- memory preparation. We found that upon culture of these naive cells with *P. aeruginosa* infected DCs IL-17 responses could be induced with no significant difference observed between *P. aeruginosa* strains indicating that the presence or absence of a T3SS system may not effect such induction, although assumptions of IL-1β contribution cannot be made. Furthermore T cell proliferation does not appear to differ between strains indicating that T cell proliferation in response to *P. aeruginosa* infected DCs is T3SS independent.

Flow cytometry of IL-17+ populations in these cultures indicate that IL-17 producing populations are small, approx 0.5%, yet we see lots of IL-17 secreted from these populations with concentrations higher than 1000pg/ml when naive T

cells are co-cultured with *P. aeruginosa* infected DCs. We believe this lack of detection of a distinct IL-17+ population by flow cytometry in these cultures, where IL-17 is clearly observed by ELISA, is a kinetics problem as the cells are left in culture for 6 days with no additional stimulation. This may lead to a down-regulation of their IL-17 producing capability by some means or perhaps a shift in IL-17 producing capacity of these cells due to increased presence of IFNγ. As discussed in the introduction Th17 cells are plastic and may shift to becoming IFNγ producing Th1 cells under influence of Th1 cytokines [127], losing their IL-17 producing capabilites. We do observe some IFNγ cells from our co-cultures (Figure 5-12) and thus presence of these cells may down-regulate IL-17. One way to investigate if there had been a substantial IL-17+ cell population at some point during the culture, would be to use recently developed reporter mice that express enhanced yellow fluorescense protein (eYFP) with IL-17a. These mice permanently label IL-17a producing cells and these can be identified as such by flow cytometry, even if they no longer make IL-17 upon restimulation [128]. Use of these mice would allow identification of cells that had capacity to produce IL-17 at any stage, therby allowing us to investigate if the IL-17 producing population in this set up had been considerably larger before our point of detection.

Co-culture of naive CD4+ T cells with *P. aeruginosa* infected DCs leads to induction of Th17 cells in a DC dependent manner. Culture of naive T cells in the absence of DCs but still in the presence of their conditioned media with artificial co-stimulation provided by addition of anti-CD28 did not allow secretion of IL-17 from culture indicating that the DCs provide something more during Th17 cell induction rather than just delivery of cytokines such as IL-23 and IL-1β, and co-stimulation via CD28. What these other factors that DCs supply may be are unclear. It could be a number of things from extra co-stimulation of the T cell provided by the DC, for example CD40 ligation has been found to be important for Th17 development [220], to presentation and recognition of antigen. If antigen recognition is the extra factor that DC presence provides for production of Th17 cells during *P. aeruginosa* infection, this would indicate a specific Th17 response to *P. aeruginosa*, although this was not investigated and so no conclusions can be made.

IL-22 concentration levels from these co-cultures were low, at around 100pg/ml, in comparison to that of IL-17 which displayed concentrations of over 1000pg/ml. We wonder, as standard, are secreted IL-22 concentrations during *P. aeruginosa* infection lower than that of IL-17? Considering IL-22 secretion alone, we see that upon culture with *P. aeruginosa* infected DCs, naive cells secrete statistically significant amounts of IL-22 compared to co-culture with unstimulated DCs. This would indicate that the IL-22 secreted from the T cells upon co-culture with infected DCs, albeit low, is real. However we do not see any IL-22+ populations as identified by flow cytometry. Do these IL-22+ populations not restimulate their production of IL-22 upon polyclonal stimulation with PMA and ionomycin as IL-17 production does? Or are there no IL-22 populations present and the IL-22 concentrations we see from our ELISA negligble? Investigation of Th17 cells induced by cytokines TGFβ, IL-6 and IL-1β in the presence of anti-CD3 and anti-CD28, a well documented technique for Th17 cell induction which we have shown to be IL-17+ via flow cytometry and ELISA, showed no IL-22 from these cells by either output. However, upon further investigation into the background of IL-22 production by Th17 cells it is claimed that IL-22 from Th17 induced cells IL-23 dependent [229]. IL-23 is not added to these Th17 inducing positive control cultures, and may explain the lack of IL-22 here where we see well-defined IL-17+ populations. It is also claimed that secretion of IL-22 could be considered to be a measure of maturation stage of Th17 cells [229]. This indicates that Th17 cells are not always IL-22+, but may gain expression of the cytokine during development. During our infection co-culture the IL-17+ CD4+ cells we see are single IL-17+ producers that do not appear to secrete IL-22 but this cannot be attributed to lack of IL-23 as we know IL-23 is present in the conditioned media of *P. aeruginosa* infected DCs as we have shown in the previous chapter. Therefore lack of IL-22 from our co-cultures cannot be explained by lack of IL-23, and the reasons are unclear. IL-22 secreted from a CD4+ cell has generally been regarded to be from Th17 cells as these cells are considered double producers of IL-17 and IL-22 [71]. However recent studies have unveiled the existence of a CD4+ IL-22+ IL-17- cell, named Th22 [184]. This poses the question that conversely could there be a CD4+ IL-17+ cell population that does not secrete IL-22. Studies have shown a single CD4+ IL-17+ cell in humans in responses to *Mycobacterium* [230], thus existence of such a cell during *P. aeruginosa* infection may not be unreasonable.

Co-culture of naive CD4+ T cells with Gram-positive *S. pneumoniae* also appears to lead to induction of IL-17+ cells. Curiously the flow cytometry and ELISA data disagree about the contributions of the 2 strains D39 WT and D39 ΔPLY to IL-17 induction, with ELISA giving evidence that D39 ΔPLY is better at IL-17 secretion while flow cytometry observations suggest that D39 WT induced better IL-17+ populations. We are more inclined to believe the ELISA data as it is an accumulative observation of IL-17 secreted over the 6 days whereas the FACS data is a snap shot on day 6 where the cells are non-specifically re-stimulated and induced to secrete cytokines, and so may not be an actual representation of what went on but what could have occurred upon this non-specific stimulation. Though it is hard to make definite assumptions with this data due to the high background of IL-17 seen when DCs are uninfected, which may suggest some contamination, IL-17 secretion from co-culture with D39 ΔPLY infected DCs is signifigantly higher than the 'basal' contaminated level and so we regard this to be real. Due to this we are inclined to say that, as with *P. aeruginosa* infection, IL-17 is induced from naive CD4+ T cells upon co-culture with *S. pneumoniae* infected DCs. It appears that infection with D39 ΔPLY leads to higher IL-17 secretion that D39 WT which could be due to the reduced cytotoxicity of D39 ΔPLY to cells. Reduced cytotoxicity results in reduced cell death in D39 ΔPLY infected DCs which results in more live cells, more cytokine secretion and more activation to naive T cells in culture, than its toxic WT counterpart. Furthermore as D39 ΔPLY seems to induce better IL-17 secretion than D39 WT, it suggests that the role of IL-1β during Th17 induction in response to *S. pneumoniae* is negligble as we have shown in the previous chapter that IL-1β production from D39 ΔPLY is low, whereas D39 WT produces significant concentrations of the cytokine. Thus high IL-17 concentrations where low IL-1β concentrations are present indicates that IL-1β is not neccessary for Th17 induction. The IL-17 secretion from these cells does not appear to be specific to *S. pneumoniae* as co-culture of naive T cells with DCs stimulated with BLP, a component of *S. pneumoniae* cell walls, also induced IL-17+ T cells. This may indicate that co-stimulation and cytokines provided by an activated DC that has recognised bacterial components, may be enough to induce a Th17 cell response. However upon culture of the naive T cells with *S. pneumoniae* infected DC conditioned media in the presence of co-stimulation provided by antibodies, reduced IL-17 is observed from these cells. This is also true for *P. aeruginosa*. This indicates that DCs do not only just

provide basic CD28 co-stimulation and cytokines but some other physical factor that requires contact, which has been discussed above. However unlike *P. aeruginosa,* where culture of naive T cells with both strains did not lead to IL-17 production when cultured with conditon media alone, *S. pneumoniae* strain D39 ΔPLY may activate DCs in such a manner that they allow naive T cells to become Th17 cells in DC absence. In DC absence cultures with this strain small levels of IL-17 secretion and a fairly substantial IL-17+ populations were observed, albeit significantly lower than when DCs were present. This suggests that Th17 cells may be induced from secreted factors produced by D39 ΔPLY infected DCs such as cytokines, and that this population is enhanced when DCs are present. As this was observed with Gram-positive *S. pneumoniae* and not Gram-negative *P. aeruginosa* this could be a Gram-postive/Gram negative difference in Th17 cell induction, or it may be specific to *S. pneumoniae*. This requires further investigation to establish if *S. pneumoniae* and other Gram-positive but not Gram-negative pathogens may induce Th17 cells from naive T cells when cultured with their infected DC conditioned media in absence of physical DC-T cell contact.

Interestingly we observe 'better' populations of Th17 cells induced from mucosal DCs *ex vivo* than artificially derived DCs induced with commercial GM-CSF. This was measured by the ability to see a large and distinct IL-17+ population by flow cytometry from NALT and lung isolated DC co-cultures on day 6, which was not observed in BMDC co-cultures. We believe mucosal DCs are more effective than BMDCs at inducing robust Th17 responses that survive to point of detection and retain their IL-17 secreting capabilities. We struggled to see IL-17+ populations as demonstrated by flow cytometry during out naive T cell/infected BMDC co-cultures even where we observed IL-17 concentrations in the supernatant and thus know an IL-17+ population has been present. The observation that we can see a clearer IL-17+ cell populations by flow cytometry with mucosal DC co-culture than with BMDC co-culture, even with exact same harvest, stimulation and staining protocols, suggests that *P. aeruginosa* infected mucosal DCs induce a much more robust population of Th17 cells that survive 6 days in culture to be re-stimulated and stained. Thus it would appear that mucosal NALT and lung DCs, which would encounter *P. aeruginosa* upon infection of an animal, may have the potential to induce IL-17 responses in a much more robust manner than

in vitro induced BMDCs can. In 2009 a few studies elucidated the importance of lung and NALT DCs in inducing Th17 cell responses. A study in mice investigating immunisation with cholera toxin indicates that lung DCs may be induced to, under this stimulation, secrete Th17 cell inducing cytokines in the lung [231]. Another study in humans investigated the contribution of lung DCs to Th1 and Th17 cell responses in emphysema [232]. NALT DCs have been shown to secrete more IL-6 than DCs isolated from cervical lymph nodes and spleens of intranasal immunised mice with OVA [233] indicating that by this production they may be better equipped at inducing Th17 cells than other DCs. Thus evidence exists that mucosal DCs may be equipped to allow efficient, robust Th17 cell induction during infection, and we see this also.

As with co-culture of *P. aeruginosa* infected BMDCs with naive T cells, co-culture with *P. aeruginosa* infected mucosal NALT and lung DCs lacked IL-22 secretion or presence of IL-22+ populations. This again suggests that the IL-17+ cells we observe during *P. aeruginosa* infection are not archetypal Th17 cells and could possibly belong to a CD4+, IL-17+, IL-22- subtype. Together these observations suggest that this CD4+, IL-17+, IL-22- cell population is generated from naive CD4+ splenocytes regardless of type and origin of DC used to induced them.

It would be interesting to see if IL-17 production increases in our co-cultures if the lung DC population wasn't contaminated with other cells due to erroneous positive selection, which was discussed in the previous chapter. Would better Th17 cell responses be observed if the DC population was pure, and exclusively committed to being activated and potentially inducing Th17 cells? Or are there cells carried over from the lung and NALT in these contaminated preparations that aid in contribution to Th17 responses, and so reduced Th17 cell populations would be observed if the co-culture was solely naive T cells and infected DCs? It poses the question, could one of these contaminated cells be responsible for IL-17 production and so elimination of it may eradicate IL-17+ populations. This requires further investigation of all cells present in the 'DC' isolated cultures by MACS from mucosal sites, and perfecting of DC isolation techniques to obtain purer populations. It is also of interest to co-culture lung derived DCs with lung derived T cells, thereby isolating both cell types from the site where pathogen would encounter and a response would be induced. To isolate lung DCs perfectly and to also isolate naive CD4+ T cells from the lung would be a time consuming

process that unfortunately we could not commit to and therefore could not investigate, but we believe this to be an interesting area for those investigating respiratory pathogens in the future.

In conclusion we demonstrated that Th17 cell responses can be induced to Gram-negative *P. aeruginosa* and Gram-positive *S. pneumoniae* in a DC contact dependent manner. The contribution of IL-1β during these *P. aeruginosa* infections is unclear, but we have shown with *S. pneumoniae* strains that the contribution of IL-1β to Th17 induction is negligible. Furthermore, *in vitro* addition of IL-1β to naive T cells cultured with TFGB and IL-6, does not make a significant difference to IL-17 secretion from naive T cells. IL-22 secretion is not evident in these cultures suggesting that the cells are a CD4+, IL-17+ and IL-22- subtype, still to be legitimately identified. Mucosal DCs isolated from lung and NALT were found to induce more robust IL-17+ cell populations than BMDCs and are of great interest for future studies investigating the role of DCs in inducing immune responses to pathogens.

6 IL-17 secretion from γδ T cells following *P. aeruginosa* infection

6.1 Introduction

γδ T cells are a subset of T cells that differ from conventional αβ T cells at their T cell receptor (TCR). They are much less common in peripheral blood than αβ T cells and as a consequence have been much less studied, though this appears to be changing with discoveries of the emerging importance of γδ T cells in immune responses and a shift of focus of T cell studies in immunity. The main quality that makes γδ T cells stand out from their αβ counterparts is that unlike αβ T cells, γδ T cells do not require antigen presented by major histocompatability complexes (MHCs) to become activated, as they lack CD4 and CD8 [139]. γδ T cells do not need antigen presentation from antigen presenting cells (APCs), have a limited TCR repertoire, and can recognise poorly defined polymorphic structures directly. Additionally, they have pattern recognition receptors (PRRs) on their surface such as Toll-like receptor 2 (TLR2) [72] which recognises bacterial lipo-protein (BLP) a component of cell walls in Gram-positive bacteria, and dectin-1 [72] a receptor that recognises β-glucans, components of plants, yeast and fungi. Therefore γδ activation occurs in a faster manner, as antigen does not need to be processed. Furthermore, it has been shown that addition of pro-inflammatory cytokine IL-23 is sufficient to induce pro-inflammatory IL-17 responses from γδ T cells [72, 129] without the involvement of the TCR. However, ligation of their TCR by anti-CD3 also known to be adequate stimulus for IL-17 secretion in the absence of IL-23 [137]. Due to this ability to directly to recognise unprocessed antigen and to induce immune responses with lack of a co-stimulatory cell, γδ T cells are thought to be a more ‘innate’ like type of cell. However, they do express CD44 and are CD62L- as we have confirmed in the previous chapter, classifying them as having a ‘memory’ phenotype on that basis. Classification of cells as naive and memory is normally reserved for adaptive immune cells, making γδ T cells ‘memory like’ ‘innate like cells’. It is due to these spectrum of qualities that γδ T cells are frequently referred to as a bridge between innate and adaptive immune responses [234].

In the previous chapter, during our investigation with CD4+ T cells cultured with *P. aeruginosa* infected dendritic cells (DCs) and their conditioned media we found that the source of IL-17 in these cultures was γδ T cells. This was due to contamination of the CD4+ cells with γδ T cells as the negative selection MACS kit that was used to isolated CD4+ T cells did not retain γδ T cells and thus they

could be found in the CD4+ cell preparation also. The discovery that γδ T cells were responsible for our IL-17 production upon culture with *P. aeruginosa* infected DCs led us to investigate this cell population in more depth.

As γδ T cells have been found in the sub-epithelium of alveolar and non-alveolar regions of the lung [134] and are thought to be involved in the regulation of immune responses to microbial pathogens [235] this implies that we may find γδ T cells in the response to *P. aeruginosa,* a respiratory pathogen of the lung.

This chapter aims to look at γδ T cells in culture with *P. aeruginosa* infected DCs to see if DC presence is important for IL-17 production from γδ T cells as it was for naive CD4+ T cells, and to look at IL-22 secretion from these cells as it could not be found in IL-17 secreting CD4+ T cells in the previous chapter. We investigated this by either culture of memory CD62L- preparation from the CD4+ negatively selected splenocytes fraction that we know to contain γδ T cells as these cells and their IL-17 production could be identified using flow cytometry, or isolation of γδ T cells alone from the spleen using γδ positive isolation MACS which we show to be over 75% effective at isolating a pure γδ population. The CD62L- CD4+ γδ containing preparation was used in some instances as γδ T cells represent such a small percentage of cells in the spleen, that to isolate them alone would give very small numbers thereby reducing the number of conditions we could set up. By using the CD4+ CD62L- 'memory' preparation, which we know contains our cells of interest that can be readily identified by flow cytometry; we increase our cell number and therefore our conditions. These cells were cultured with *P. aeruginosa* infected DC and/or their conditioned media as the naive cells had been, but in the absence of anti-CD3 as CD3 ligation is enough to induce γδ T cells to secrete IL-17 [137] in an uncontrolled and unspecific manner.

6.2 γδ T cells are contained in the CD62L- memory preparation of CD4+ negatively selected cells by MACS, and proliferate upon stimulation

In the previous chapter we confirmed that γδ T cells are memory-like in their expression of CD44 and thus can be found in the CD62L- memory preparation of CD4 MACS negatively isolated T cells. Upon culture with unstimulated DCs a

small 1.8% population of γδ T cells can be observed in this CD62L- 'CD4+' preparation, but upon co-culture with *P. aeruginosa* infected DCs this γδ population increases to 4.2% demonstrating that γδ T cells proliferate upon stimulation (Figure 6-1).

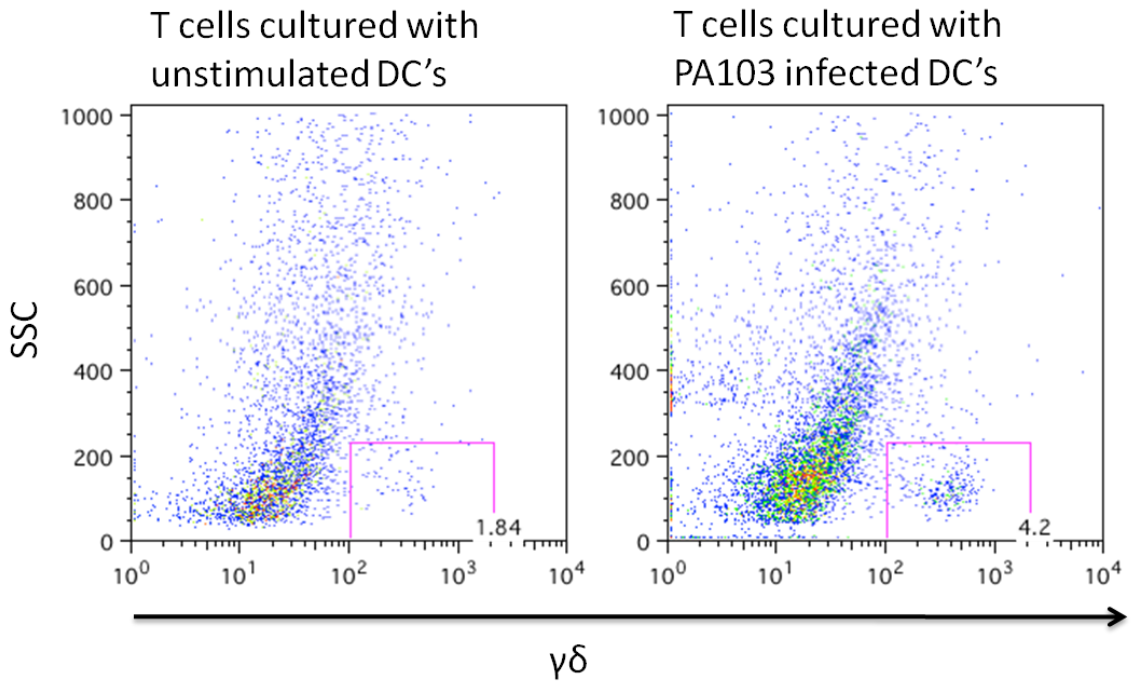

Figure 6-1 γδ T cells proliferate upon stimulation

γδ T cells, as part of the memory CD62L- CD4+ preparation, were co-cultured with *P. aeruginosa* infected DCs carried over in their conditioned media for 6 days. After this time cells were harvested and stained for γδ and analysed by flow cytometry. Cells gated on live cells based on forward scatter (FSC) and side scatter (SSC) as described in materials and methods. Dot plots are representative of co-culture set up in triplicate. Data representative of 3 experiments.

These cultures were set up in the absence of IL-2, to increase IL-17+ populations as IL-2 has been described to reduce IL-17+ cells [228] which we confirmed in the previous chapter. Thus, proliferation of γδ T cells is independent of exogenous IL-2, though as stated previously we cannot account for endogenous IL-2 made by the T cells themselves.

6.3 γδ isolation using MACS

We have shown in the previous chapter that γδ T cells reside in the CD4+ preparation using negative selection MACS, and that these cells can be identified by flow cytometry for their IL-17 secretion upon culture with *P. aeruginosa* infected DCs. We wanted to investigate this splenic γδ population alone to see if

it responded to DCs and their conditioned media infected with *P. aeruginosa* in the same manner as γδ T cells as part of the 'CD4+' preparation do. We were interested to see if the induction of IL-17+ secretion from γδ T cells was solely due to culture of γδ T cells and the infected DCs, or if memory CD4+ T cells in the preparation may contribute somehow to IL-17 secretion from γδ T cells, and thus needed to find an effective way to isolate γδ T cells. To isolate γδ T cells for these experiments we used a positive selection MACS kit which labels the γδ T cells and retains them in the magnetic column, allowing all other unlabelled cells to pass through. After MACS treatment samples of these γδ + cells were stained for γδ and investigated by flow cytometry to check the purity of the kit before further experiments were carried out (Figure 6-2).

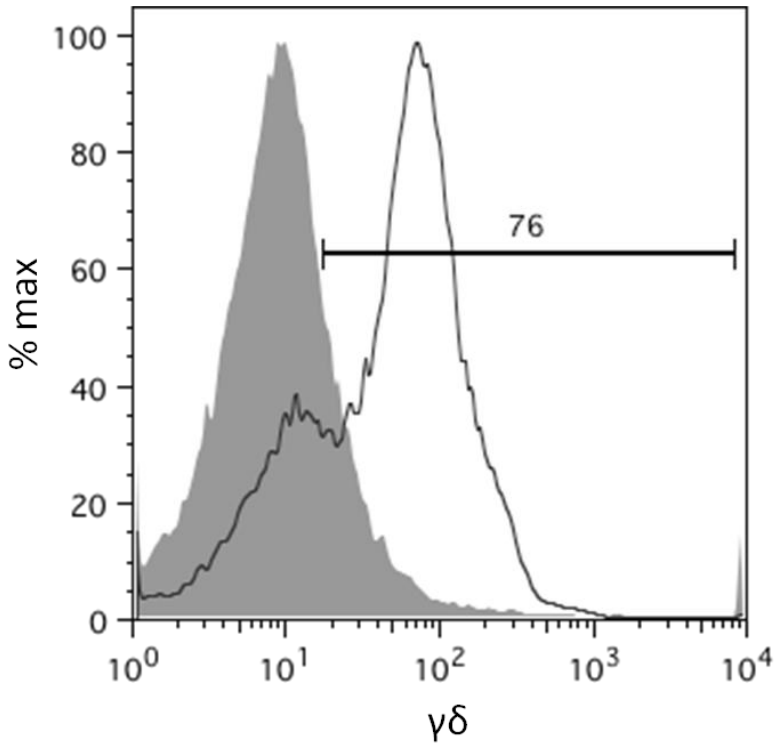

Figure 6-2 γδ T cells isolated using positive selection MACS

Splenocytes were treated using γδ positive selection MACS kit and stained for γδ T cells (black line) to test kit efficacy, and analysed by flow cytometry against isotype control (filled histogram). Data representative of 5 experiments.

The γδ positive selection MACS kit appears over 75% effective and we are comfortable to proceed knowing we have isolated mainly γδ + T cells but will still confirm this population by flow cytometry upon experimentation before making any claims about its IL-17 secreting activity.

6.4 γδ T cells are IL-17 producing upon co-culture with *P. aeruginosa* infected DC conditioned media in a DC contact independent manner.

In the previous chapter we discovered that Th17 cells differentiated from naive CD4+ T cells need stimulation of their TCR and co-stimulation provided by DCs in order to secrete IL-17. We wanted to investigate if γδ T cells respond in the same DC dependent manner. γδ T cells do not have CD4 or CD8 on their surface and thus lack MHC recognition, meaning they do not require DC presence in the same manner as CD4 and CD8 T cells; however, they may still require co-stimulation provided by DCs to allow adequate activation. We were curious as to how γδ T cells would respond to DC absence, and therefore absence of co-stimulation. We have already observed in the previous chapter that CD3 ligation is enough to allow IL-17 secretion from γδ T cells, and that γδ T cells survive till day 6 without anti-CD3 addition where naive CD4+ T cells do not, and thus CD3 stimulation is not supplied in these co-cultures. We have discovered previously, while looking for Th17 responses, that γδ T cells co-cultured with *P. aeruginosa* infected DCs and their conditioned media induce IL-17 secretion, and so we wanted to investigate if isolated γδ T cells cultured with DC conditioned media in the absence of DC's, CD3 and CD28 stimulation would still secrete IL-17. As shown in Figure 6-3, when purified γδ T cells were cultured with conditioned media from infected DCs they were activated to secrete IL-17 in a CD3 independent, DC contact independent manner.

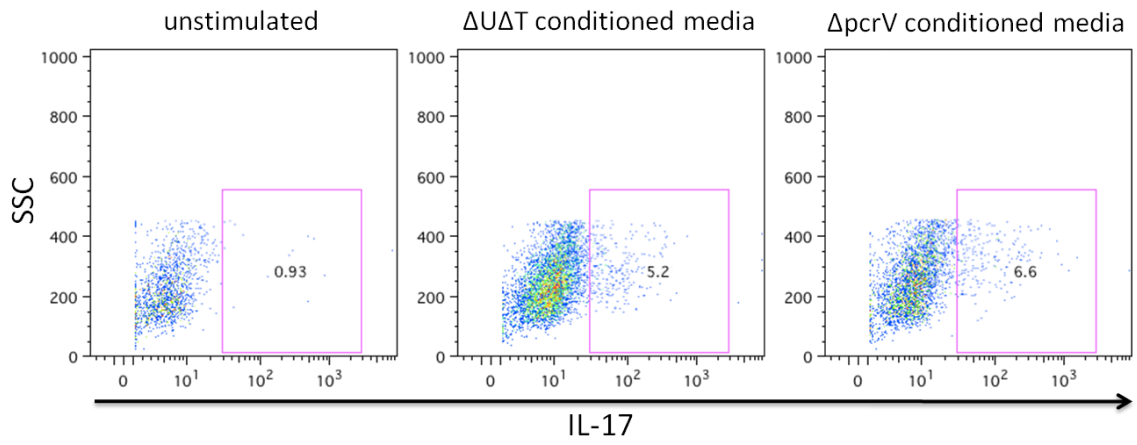

Figure 6-3 γδ T cells cultured in *P. aeruginosa* infected DC conditioned media in the absence of DCs secrete IL-17

γδ T cells were isolated using MACS and cultured in the conditioned media of *P. aeruginosa* infected DCs for 6 days in the absence of DCs and any other co-stimulation, before being stimulated with PMA and ionomycin in the presence of brefeldin A for 5 hours. After this time cells were harvested and stained for IL-17 by flow cytometry. Cells gated on live cells based FSC and SSC as described in materials and methods. Positive gates were set on isotype on ΔUΔT conditioned media stimulated cells. Dot plots are representative of co-culture set up in triplicate. Data representative of 3 experiments.

These results show that culture of γδ T cells alone with *P. aeruginosa* infected DC conditioned media allows IL-17 secretion cells and that unlike Th17 cells, γδ T cells do not need the presence of DCs or even artificial co-stimulation provided by commercial anti-CD28 to induce IL-17 production. To confirm the IL-17 secreted during this co-culture set-up was in fact from γδ T cells the cells were double stained with γδ and IL-17 (Figure 6-4).

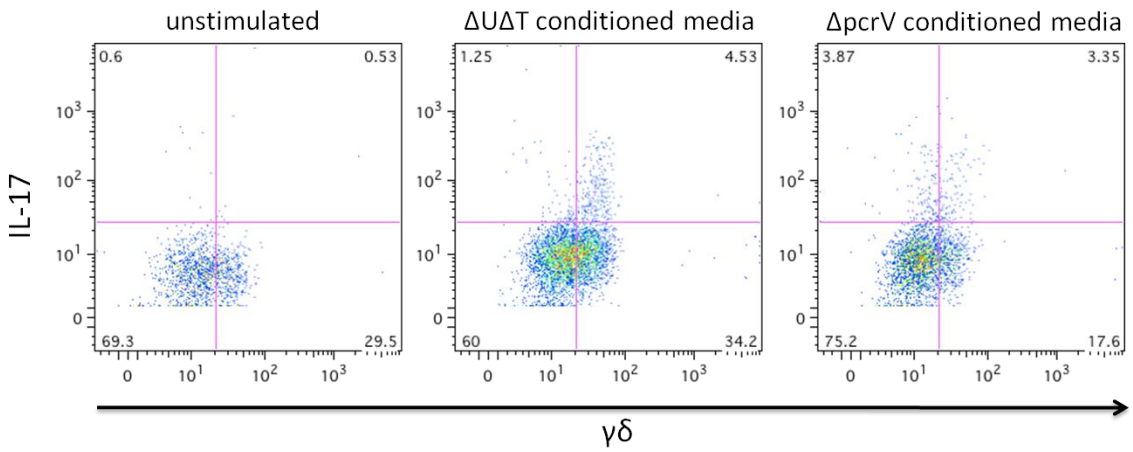

Figure 6-4 IL-17 cells observed during γδ T cell cultured in *P. aeruginosa* infected DC conditioned media are γδ+

γδ T cells were isolated using MACS and cultured in the conditioned media of *P. aeruginosa* infected DCs for 6 days in the absence of DCs and any other co-stimulation, before being stimulated with PMA and ionomycin in the presence of brefeldin A for 5 hours. After this time cells were harvested and stained for IL-17 and γδ and analysed by flow cytometry. Cells gated on live cells based FSC and SSC as described in materials and methods. Positive gates were set on isotypes on ΔUΔT conditioned media stimulated cells. Dot plots are representative of co-culture set up in triplicate. Data representative of 3 experiments.

We see from Figure 6-4 that the majority of IL-17+ cells are γδ+. A further notable observation from this figure is the reduction in γδ+ populations. We have previously shown that γδ isolation from spleens results in over 75% γδ + T cells (Figure 6-2), so why are large γδ + T cell populations not observed in the co-culture analysis? The γδ staining to test the efficacy of the kit was performed immediately after isolation, whereas the results above are after 3 days in culture, and so we suppose that not all cells have survived the 3 days, and this is why we see a reduction in γδ+ T cells. We are happy however that the above flow cytometry plots show that the IL-17+ population is γδ+, confirming that the IL-17+ cells we see in our cultures with isolated γδ T cells are in fact IL-17+ γδ T cells.

These results indicate that γδ T cells are more innate like, not needing DC co-stimulation and lacking specific antigen recognition as they can be induced to secrete IL-17 in response to LPS stimulated DC conditioned media.

The contribution of DCs in IL-17 induction from γδ T cells was further investigated by culturing γδ T cells with *P. aeruginosa* infected DCs carried over in their conditioned media to represent the contribution of DCs plus cytokines and secreted factors, or with *P. aeruginosa* infected DCs washed out of their conditioned media and resuspended in fresh media to represent the sole contribution of the DCs alone, albeit they may continue to activate and secrete factors during culture time under the correct stimulus (Figure 6-5).

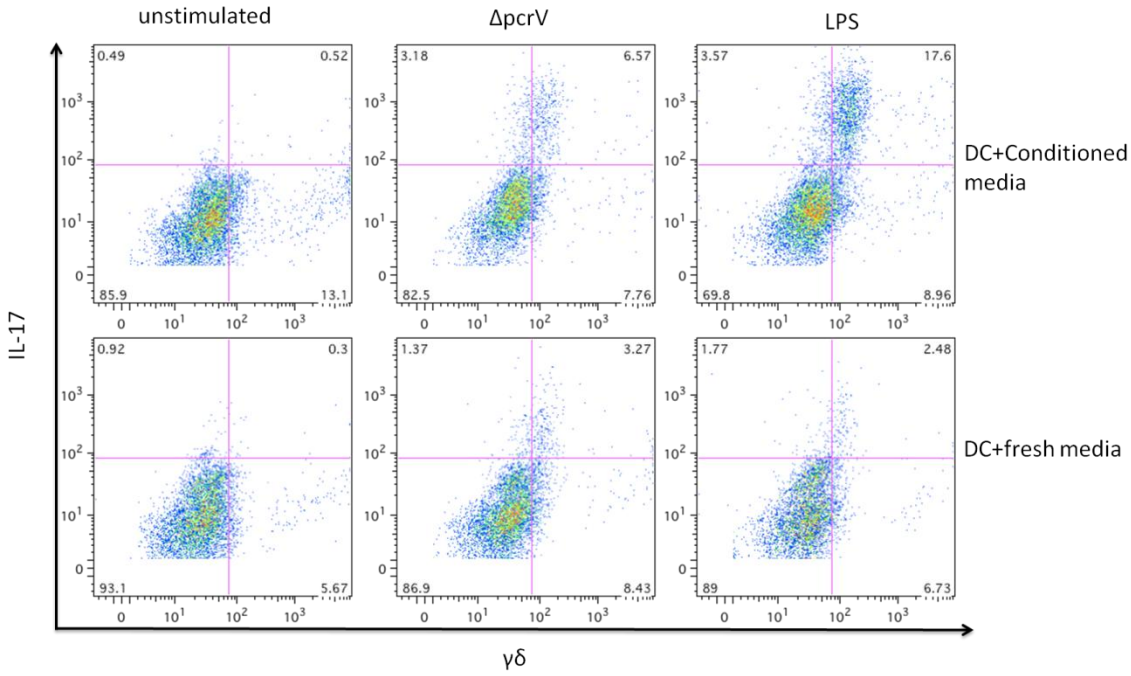

Figure 6-5 Secreted factors from activated DCs are more important than DC contact at inducing production of IL-17 from γδ T cells

γδ T cells were co-cultured with DCs carried over in their conditioned media or DCs washed out of their conditioned media and resuspended in fresh media for 6 days. After this time cells were stimulated with PMA and ionomycin in the presence of brefeldin A for 5 hours before being harvested, stained for IL-17 and γδ and analysed by flow cytometry. Cells gated on live cells based FSC and SSC as described in materials and methods. Positive gates were set on isotypes on LPS conditioned media stimulated cells. Dot plots are representative of co-culture set up in triplicate. Data representative of 2 experiments.

In the presence of the DCs washed of their conditioned media and resuspended in fresh media a small percentage, 3.27%, of IL-17 can be observed from γδ T cells cultured with *P. aeruginosa* infected DCs (Figure 6-5). However this IL-17+ population is much larger when DC conditioned media is present also, 6.57%, indicating that DC conditioned media from infected DCs plays a much bigger role in IL-17 secretion from γδ T cells than the DCs do themselves (Figure 6-5). This implies that it is cytokine contribution from DCs rather than a cellular

interaction with γδ T cells that to induce IL-17 from these cells. This response is seen to be non-specific to *P. aeruginosa* as stimulation of DCs with LPS induces much larger IL-17+ populations, 17.6%, (Figure 6-5) indicating that an activated DC by a non-specfic means is sufficient to secrete factors that induce IL-17 from γδ T cells. The contribution of conditioned media versus DCs is still evident where LPS has been used to stimulate DCs.

This observation was confirmed by analysing the supernatants of the co-culture for IL-17 secreted by the cells, analysed by ELISA (Figure 6-6). Significantly higher concentrations of IL-17 are observed in the cell culture when DC conditioned media is present, as opposed to just stimulated DCs.

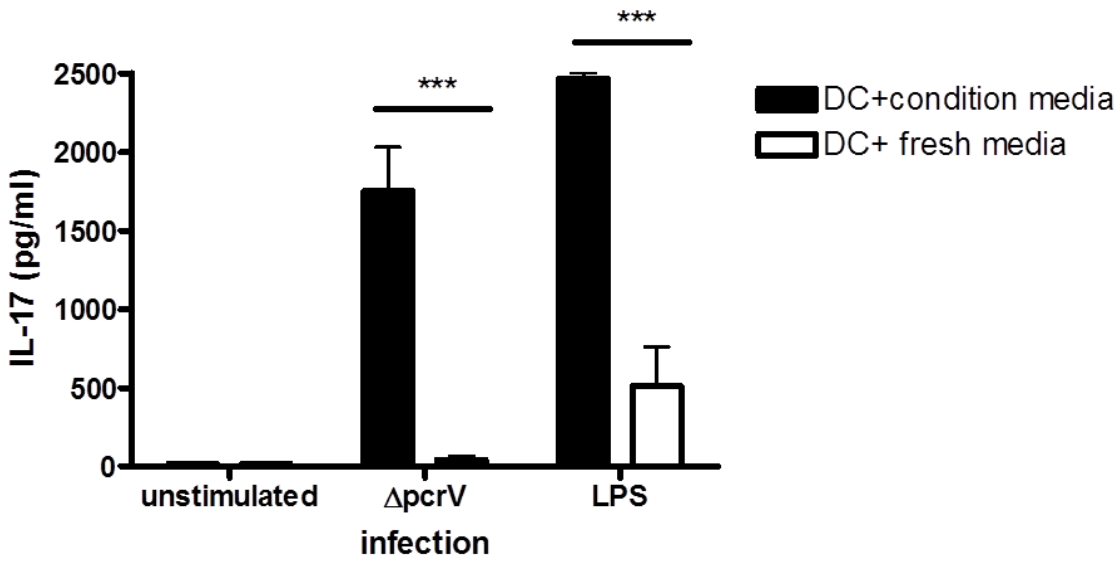

Figure 6-6 Production of IL-17 from γδ T cells requires conditioned media from stimulated DCs

γδ T cells were co-cultured with DCs carried over in their conditioned media or DCs washed out of their conditioned media and resuspended in fresh media for 6 days. After this time supernatants were harvested and analysed for IL-17 by ELISA. All error bars represent mean ± SEM. Data are representative of 3 separate experiments of experimental triplicates. *** $p < 0.001$.

6.5 γδ T cells do not directly recognise *P. aeruginosa*

γδ T cells express PRRs on their surface such as TLR2 and dectin-1 [72]. *P. aeruginosa* would not be recognised by dectin-1, and although TLR2 can recognise *P. aeruginosa* components it has been shown to have a more limited role in inducing immune responses to *P. aeruginosa* than TLR4 does [236]. We

wished to investigate if γδ T cells could recognise *P. aeruginosa* directly via TLR2, or perhaps by way of a currently unidentified receptor. To test this γδ T cells were directly infected with *P. aeruginosa* for 90 minutes before addition of antibiotics (Figure 6-7). γδ T cells were also set up with LPS stimulation, as it has been shown to be a strong inducer of IL-17 from these cells when γδ T cells are cultured with conditioned media of LPS stimulated DCs (Figure 6-5). This was to investigate if LPS had a direct effect on γδ T cells although it is known that γδ T cells do not have TLR4 [72], the best characterised recognition receptor for LPS. As a positive control to check the that the γδ T cells cultured were functional and had IL-17 producing capabilities, γδ T cells were cultured in *P. aeruginosa* infected conditioned media, as we have previously shown in Figure 6-3 such culture leads to IL-17+ production from γδ T cells.

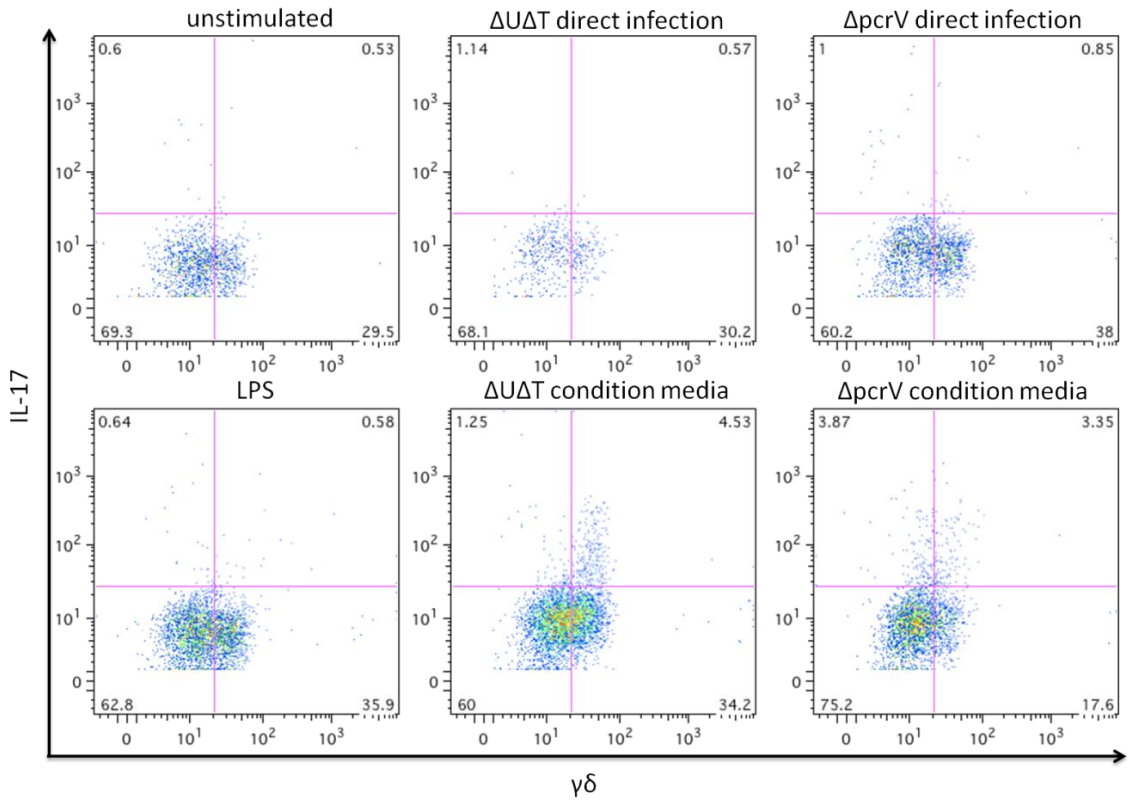

Figure 6-7 Direct infection of γδ T cells with *P. aeruginosa* does not induce IL-17+ γδ+ T cell populations

γδ T cells were left unstimulated, stimulated with LPS, directly infected with live *P. aeruginosa*, or cultured with *P. aeruginosa* infected DC conditioned media for 6 days. After this time cells were stimulated with PMA and ionomycin in the presence of brefeldin A for 5 hours before being harvested, stained for IL-17 and γδ and analysed by flow cytometry. Cells gated on live cells based FSC and SSC as described in materials and methods. Positive gates were set on isotypes on ΔUΔT conditioned media stimulated cells. Dot plots are representative of co-culture set up in triplicate. Data representative of 3 experiments.

As can be observed from Figure 6-7, direct infection of γδ T cells with *P. aeruginosa* does not induce IL-17 secretion from these cells indicating that γδ T cells do not directly recognise *P. aeruginosa,* and respond to direct infection with the pathogen by way of IL-17 secretion. TLR2 on γδ T cells may recognise *P. aeruginosa* in this set up but not induce IL-17 responses, a relationship that would require more investigation. The inclusion of γδ T cells cultured with *P. aeruginosa* infected DC conditioned media that does allow IL-17 secretion shows that the cells used in these experiments are functional and have the capacity to secrete IL-17, confirming that the fact that direct infection of γδ T cells with *P. aeruginosa* does not induce IL-17, as opposed to the lack of IL-17 due to quality of cells or experimental set up. Furthermore, there is no IL-17 response from γδ T cells directly stimulated with LPS confirming the lack of TLR4 on γδ T cell surface and any other means of LPS recognition. These results are confirmed when looking at IL-17 secretions from these cells in the supernatant (Figure 6-8).

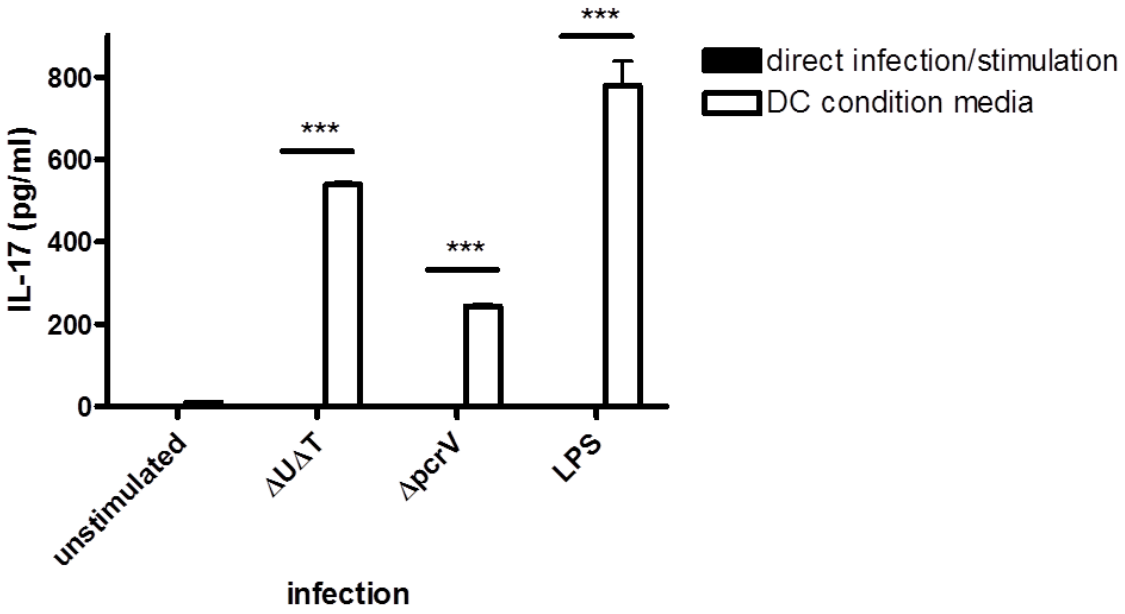

Figure 6-8 Direct infection of γδ T cells with *P. aeruginosa* does not lead to IL-17 secretion
γδ T cells were left unstimulated, stimulated with LPS, directly infected with live *P. aeruginosa*, or cultured with *P. aeruginosa* infected DC conditioned media for 6 days. After this time supernatants were harvested and analysed for IL-17 by ELISA. All error bars represent mean ± SEM. Data are representative of 3 separate experiments of experimental triplicates.*** $p<0.001$.

Together these results confirm that γδ T cells do not directly recognise *P. aeruginosa* or LPS, and respond via IL-17 production. They also suggest that IL-17 secretion from γδ T cells occurs in a DC dependent manner.

We have shown previously that IL-17 may be secreted from γδ T cells in a DC contact independent manner (Figure 6-4 and Figure 6-5). Yet the data here suggests DCs are still neccessary at some point during culture to secrete the factors that drive IL-17 secretion from γδ T cells, as absolute DC absence from co-culture leads to lack of IL-17 secretion from γδ T cells. Thus, IL-17 secretion from γδ T cells may be described as DC dependent but DC contact independent.

6.6 γδ T cells produce IL-17 in an IL-23 dependent manner

We have previously shown in chapter 4 that *P. aeruginosa* infected DCs secrete IL-23, a well known Th17 maintenance factor, and we have shown in this chapter that factors secreted by the DCs into their media are sufficient and important for IL-17 induction. We wished to investigate if IL-23 from the DCs could be responsible for IL-17 production from the γδ T cells as it has been shown to have an important role by others [129]. To investigate if IL-23 was responsible for IL-17 production from γδ T cells, we set up cultures of γδ T cells as part of a CD4+ memory preparation isolated by CD4 negative MACS selection, as it is also known to contain γδ T cells. γδ T cells are clearly identified by flow cytometry to be the source of IL-17 during these cultures and CD4+ cells are shown to be IL-17 negative under these conditions (see previous chapter) so we are confident that any IL-17 we see in this set up is coming from IL-17+ γδ T cells. Cells were set up with unstimulated and *P. aeruginosa* infected conditioned media as this has been shown to be enough to induce an IL-17 response (Figure 6-3), in the absence or presence of a commercially purchased functional IL-23 blocking antibody. These conditions are named untreated or blocked, referring respectively to the absence of presence of this blocking antibody. As a control an isotype matched antibody was used to show that the contribution of the IL-23 blocking antibody was specific. In the IL-23 block untreated conditions large concentrations of IL-17 are observed from γδ T cells cultured with conditioned media from *P. aeruginosa* infected DCs but not in the condition where media from uninfected DCs was used. Addition of IL-23 blocking antibody to these cultures significantly lowers the concentration of IL-17 observed in the positive cultures (Figure 6-9). Whereas use of an antibody of the same isotype as the blocking antibody does not reduce IL-17, indicating that the reduction in IL-17 from the γδ T cells is due to IL-23 blocking and not non-specific antibody addition. In fact, isotype addition

to culture appears to increase IL-17 secretion in this culture. This phenomenon was not further explored and therefore we do not know why this is.

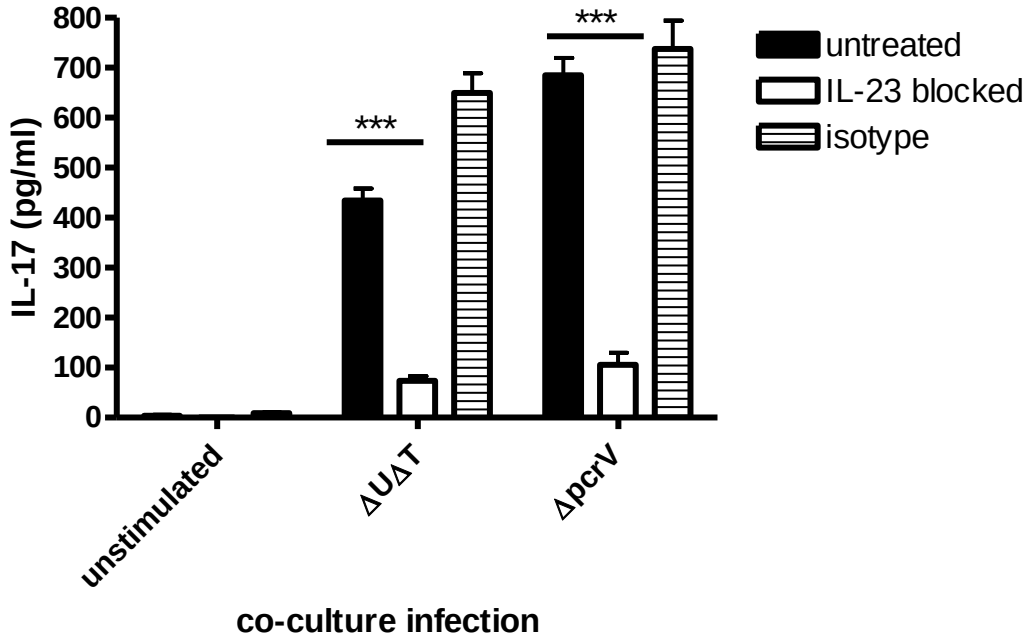

Figure 6-9 IL-17 secretion from γδ T cells is IL-23 dependent
MACS sorted CD4+ T cells that are known to contain γδ T cells, which were identified to be the IL-17 secreting population by flow cytometry in previous experiments, were cultured with unstimulated or *P. aeruginosa* infected DC conditioned media in the absence or presence of IL-23 blocking antibody for 6 days before supernatants were harvested and analysed for IL-17 by ELISA. An isotype control was also set up for these conditions. All error bars represent mean ± SEM. Data are representative of 3 separate experiments of experimental triplicates. *** $p<0.001$.

This confirms that IL-23 is important in IL-17+ γδ T cell maintenance, and that IL-23 contribution from DCs plays a key role in IL-17 secretion from γδ T cells.

6.7 γδ T cells are IL-22 producing upon co-culture with *P. aeruginosa* infected DC conditioned media in a DC contact independent and IL-23 dependent manner.

IL-17 secreting CD4 cells, Th17 cells, are also considered to be IL-22 secreting, though we did not observe this in the previous chapter exploring IL-17+ CD4+ cells during *P. aeruginosa* infection. However we wanted to investigate if IL-22 was secreted from IL-17+ γδ cells, and if so were the same γδ T cells responsible for both IL-17 and IL-22 secretion. This was investigated by culturing γδ T cells with *P. aeruginosa* infected DC condition media as previously described and

analysing for IL-22 by intracellular flow cytometry staining and ELISA analysis of culture supernatants.

We observe IL-22+ γδ T cells when isolated γδ T cells are cultured with *P. aeruginosa* infected DC conditioned media. This indicates, as was observed with IL-17 secretion from γδ T cells, that IL-22 production from γδ T cells is DC contact independent (Figure 6-10).

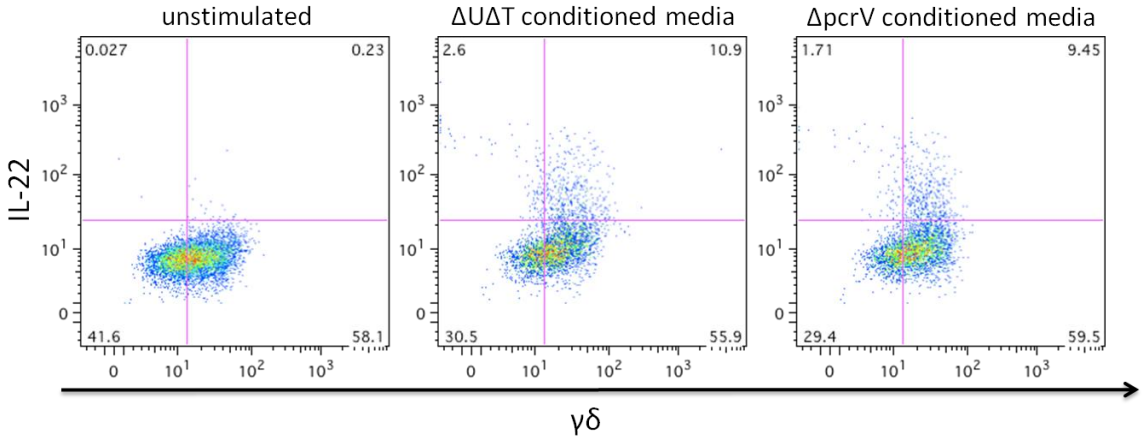

Figure 6-10 γδ T cells cultured in *P. aeruginosa* infected DC conditioned media in the absence of DCs secrete IL-22

γδ T cells were cultured with conditioned media of *P. aeruginosa* infected DCs for 6 days in the absence of DCs and any other co-stimulation. After this time cells were stimulated with PMA and ionomycin in the presence of brefeldin A for 5 hours, harvested and stained for IL-22 and γδ and analysed by flow cytometry. Cells gated on live cells based FSC and SSC as described in materials and methods. Positive gates were set on isotypes on ΔUΔT conditioned media stimulated cells. Dot plots are representative of co-culture set up in triplicate. Data representative of 3 experiments.

Figure 6-10 demonstrates that production of IL-22 from γδ T cells does not require DC presence and thus occurs upon exposure to cytokines and secreted factors produced by *P. aeruginosa* infected DCs. As with IL-17+ γδ T cells we wished to investigate if IL-23 secreted into the *P. aeruginosa* infected DC supernatant was responsible for IL-22 secretion from γδ T cells, and done so by analysing the supernatants of the γδ T cells cultured in the conditioned media of infected DCs in the presence of absence or IL-23 blocking antibody, which showed reduction in IL-17, by IL-22 ELISA (Figure 6-11). (Isotype control was not analysed for IL-22 production).

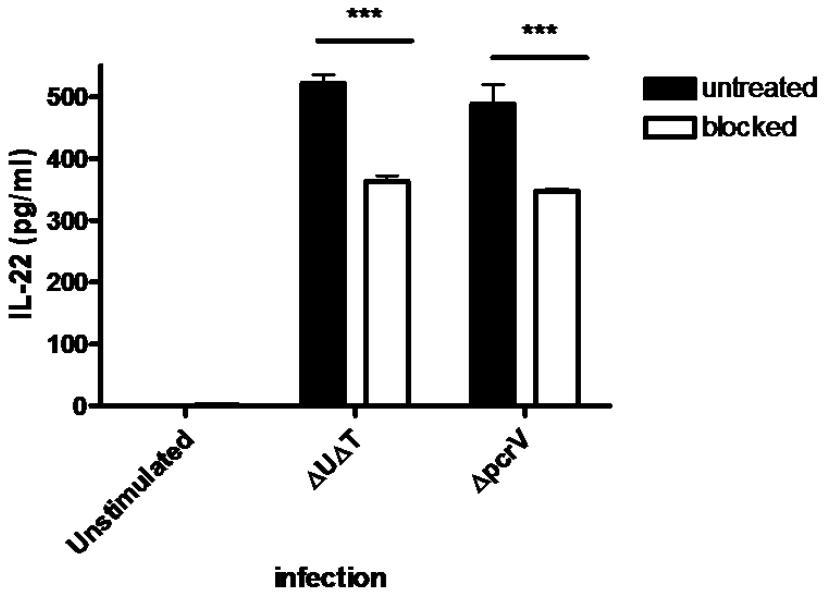

Figure 6-11 IL-22 secretion from γδ T cells is partly IL-23 dependent
MACS sorted CD4+ T cells that are known to contain γδ T cells were cultured with uninfected or *P. aeruginosa* infected DC conditioned media in the absence or presence of IL-23 blocking antibody for 6 days before supernatants were harvested and analysed for IL-22 by ELISA. All error bars represent mean ± SEM. Data are representative of 3 separate experiments of experimental triplicates. *** $p<0.001$.

The observed reduction in IL-22 from γδ T cells is not as dramatic as it appears to be for IL-17 upon IL-23 blocking, indicating that cytokines other than IL-23 may stimulate IL-22 production. However the reduction of IL-22 upon IL-23 blocking is still statistically significant (Figure 6-11) indicating IL-22 production from γδ T cells is in part IL-23 dependent, a theory considered further in the discussion of this chapter.

6.8 The majority of IL-17 secreting cells induced from co-culture with *P. aeruginosa* do not co-express IL-22

Upon further investigation of these IL-17 and IL-22 populations obtained from isolated γδ T cells cultured with *P. aeruginosa* infected DC conditioned media, we see that although some of these cells may be IL-17+ IL-22+ double producers, the majority of each appear to be single producers (Figure 6-12). Traditionally Th17 cells are both IL-17 and IL-22 secreting, but the IL-17 secreting cells we observed in the previous chapter derived from naive CD4+ T cells do not appear to secrete IL-22 at all, as shown by FACS and ELISA. γδ T cells are considered to be an innate form of a Th17 cell as they share so many characteristics, and as

Th17 cells are generally regarded as IL-17 and IL-22+, we hypothesized that the IL-22+ population that we see upon culture of γδ T cells with *P. aeruginosa* infected DC conditioned media would be the same cell population as the IL-17+ cells seen under these circumstances. However, the IL-22+ γδ T cell population we see upon culture with *P. aeruginosa* infected DC condition media appears to be a mainly separate population from that of the IL-17 producing γδ population based on flow cytometric analysis of intracellular cytokine production (Figure 6-12).

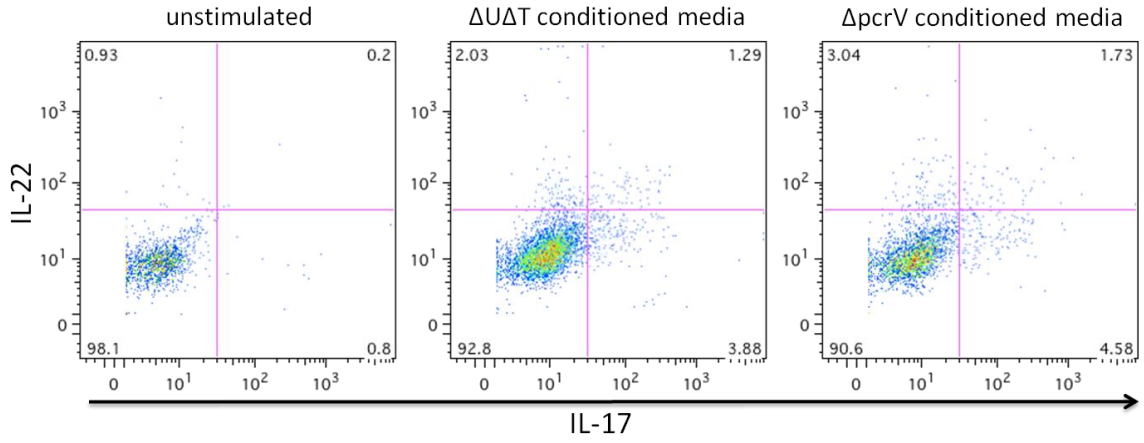

Figure 6-12 IL-17+ and IL-22+ γδ populations are distinct

γδ T cells were cultured with conditioned media of *P. aeruginosa* infected DCs for 6 days in the absence of DCs and any other co-stimulation. After this time cells were stimulated with PMA and ionomycin in the presence of brefeldin A for 5 hours, harvested and stained for IL-17 and IL-22 and analysed by flow cytometry. Cells gated on live cells based FSC and SSC as described in materials and methods. Positive gates were set on isotypes on ΔUΔT conditioned media stimulated cells. Dot plots are representative of co-culture set up in triplicate. Data representative of 3 experiments.

Upon culture of γδ T cells with PA103 ΔUΔT infected condition media we see 38% of the IL-22+ cells are also IL-17+, and 24% of the IL-17+ cells are also IL-22 producing. With PA103 ΔpcrV infection conditioned media a similar pattern is observed with 36% of IL-22+ cells also being IL-17+, and 27% of IL-17+ cells also IL-22+. However, in both conditions the majority of cells are single IL-17 or IL-22 producers. This indicates in both infections that the majority of IL-17+ and IL-22+ γδ T cells are single producers and so IL-17 and IL-22 production from γδ T cells is distinct.

6.9 Discussion

γδ T cells exist in small populations in tissues but respond in a prolific manner to pro-inflammatory stimulus such as IL-23 and IL-1β [129].

The results in this chapter show that γδ T cells are differentially IL-17 and IL-22 producing in response to *P. aeruginosa* in a DC dependent yet DC contact independent manner, that is dependent on IL-23. The necessary physical presence of DCs for inducing IL-17 from γδ T cells was shown to be negligible, with γδ T cells cultured in the conditioned media of *P. aeruginosa* infected DCs shown to be sufficient at allowing abundant secretion of IL-17 from γδ T cells. Culture of γδ T cells with infected DCs washed out of their conditioned media and resuspended in fresh media to represent the contribution of DCs alone, still induced a small percentage of IL-17+ cells compared to culture with uninfected DCs, indicating that the cells retain some capacity to secrete the important cytokines. But as culture of γδ T cells with these infected DCs and their conditioned media increased IL-17+ populations by double, this indicates that it is early on and while still exposed to the pathogen/stimulus that DCs secrete their IL-17 inducing cytokines such as IL-23. The importance of IL-23 at IL-17+ producing cell maintenance was verified by almost complete blockade of IL-17 when anti-IL-23 was used. This confirms that γδ T cells secrete IL-17 in response to IL-23, in the absence of TCR stimulation, and that an important IL-17 producing factor produced by DCs and found in the conditioned media is IL-23.

IL-22 secretion from γδ T cells was also seen to be independent of DC contact but dependence of IL-23 is uncertain. IL-22 was significantly reduced in these cultures upon IL-23 blockade as is evident by statistical analysis, yet this reduction was not as dramatic as that observed with IL-17 where IL-23 blockade almost abolished secretion of the cytokine. This indicates that IL-22 production by γδ T cells may not be IL-23 dependent, but merely IL-23 may enhance IL-22 secretion from γδ T cells.

Controversially we found that IL-17 and IL-22 γδ+ T cells may be principally different cells. IL-17 γδ T cells have been shown to be double producers of IL-17 and IL-22 with addition of artificial aryl hydrocarbon receptor (AhR) ligands [72] but we see single producers of IL-17 and IL-22 during culture with *P. aeruginosa*

infected DCs, with a small fraction of double producers. Studies of bacterial infection by others have also shown differential subsets of IL-17 and IL-22 production during infection with Gram-positive *Bacillus subtilis* [179]. Interestingly in this study IL-17 and IL-22 producers appear to differ in their γδ expression, with IL-22 producers appearing to have lower γδ expression than that of IL-17 producers [179]. This suggests functional differentiation of γδ T cells, and is of interest for future studies. Furthermore other groups in the lab have seen single IL-17 and IL-22 producers in other models such as collagen induced arthritis (CIA) (unpublished data) indicating that IL-22 and IL-17 single producing γδ T cells exist and need further investigation. The observation by others in the lab of single producers in CIA signifies that these cells are not limited to pathogen infection as CIA is an animal model of autoimmune disease, and so may play a bigger role in immunity than previously realised. Both IL-17 and IL-22 were observed to be significantly reduced when IL-23 contribution to γδ T cell stimulation was blocked. Although both cytokines showed a significant reduction statistically, the reduction in IL-17 is more dramatic with an almost complete block, whereas IL-22 showed only a reduction. The emergence of IL-22 single producing γδ T cells that dominate the culture over IL-17+IL-22+ double producers, poses the question that perhaps the IL-22 reduction with IL-23 block is not as dramatic as observed for IL-17, as IL-22 is mainly secreted by a single IL-22 secreting producer that may have different dynamics and respond differently to IL-23, if at all. Perhaps the reduction we see in IL-22 with IL-23 blockade is from the IL-17+ IL-22 double producers and these IL-22 single producers continue to produce IL-22 in an IL-23 independent manner. This is all speculation at present and requires further investigation, but it would be of great interest to isolate these 3 cytokine producing populations, single IL-17+, single IL-22+ and double producing IL-17+ IL-22+cells and investigate the effect of IL-23 blockade on secretion of IL-17 and IL-22 in these individual γδ populations. These distinct populations, and the effect of IL-23 on them, are of great interest and must be investigated further.

The IL-17+ responses from γδ T cells were found not to be specific to *P. aeruginosa* as culture of γδ T cells with LPS stimulated DC condition media induced greater IL-17+ populations than that of infection. This indicated that it may in fact be DC recognition of LPS on *P. aeruginosa* cell walls that leads to DC

activation. In chapter 4 we demonstrated that IL-23 secretion from DCs stimulated with LPS is higher than that of *P. aeruginosa* infection, giving an explanation for the higher IL-17+ γδ T cell population we see here during culture with LPS stimulation.

These results confirm that γδ T cells are more innate like as they do not need DC co-stimulation, and lack specific antigen recognition as they can be induced to secrete IL-17 in response to LPS stimulated DC conditon media.

Although these IL-17 responses were found to be DC contact independent, they are still found to be dependent on DCs to supply IL-17 secreting factors such as IL-23. Direct infection of γδ T cells or culture with LPS, in the absence of DCs, showed no IL-17 in the supernatants and no IL-17 producing populations as identified by flow cytometry. This shows that γδ T cells do not directly recognise and respond to *P. aeruginosa* or LPS by way of IL-17 production, and in fact IL-17 secretion from γδ T cells does depend on DCs. DCs are needed to be stimulated by the pathogen or stimulus to become activated and secrete IL-23 and other factors to induce γδ T cells to become IL-17 secreting. Thus although γδ T cells may produce IL-17 independently of DC presence and a physical DC interaction, DCs are still needed to provide γδ T cell activating cytokines. *In vivo* DCs may not play as large a role, as IL-23 can also be supplied by macrophages exposed to *P. aeruginosa* [237] and thus IL-17 secretion from γδ T cells may be able to occur in a completely DC independent manner. However in our set up DCs are the source of cytokines that drive IL-17 responses from γδ T cells in response to *P. aeruginosa* infection.

The lack of need for DC contact and co-stimulation provided by these cells indicates that we would be able to use our IL-1β presence and absence model using *P. aeruginosa* strains ΔUΔT and ΔpcrV, previously described in Chapter 4 and 5, to investigate the contribution of IL-1β to IL-17 production by γδ T cells. Briefly ΔUΔT allows IL-1β secretion, where ΔpcrV does not, as it has a competent T3SS, a feature necessary for inflammasome activation, which that leads to IL-1β production and secretion. ΔpcrV has a disrupted non-functional T3SS and thus does not activate inflammasome activation or allow downstream production and release of IL-1β. The obstacle which we came up against with previous use of this model while trying to investigate the contribution of IL-1β during initiation

of Th17 responses from naive CD4+ T cells was that the IL-1β difference seen between strains was only evident in the supernatants after a few hours post infection and addition of antibiotics. This was not adequate time to allow sufficient up-regulation of co-stimulatory molecules CD40 and CD86 to co-stimulate the naive T cells. Overnight maturation of the infected DCs after antibiotic addition lead to adequate up-regulation of CD40 and CD86 which would stimulate naive T cells, but other unknown factors during this time lead to inflammasome activation and IL-1β secretion by both strains making the model of IL-1β presence and absence ineffective in this case. However as we have shown that γδ T cells secrete IL-17 in a DC contact independent manner, adequate up-regulation of CD40 and CD86 is unnecessary for γδ T cell culture. Thus we could mature DCs for a shorter time after antibiotic addition allowing a difference in IL-1β to be observed, and may use this model to investigate IL-1β contribution at IL-17 induction from γδ T cells. Unfortunately this observation was made later in the course of experiments and due to time constraints this was never investigated but is of interest for future work investigating the contribution of IL-1β in IL-17 induction from γδ T cells. IL-17+ γδ T cells in the spinal cords of mice have themselves have been shown to secrete IL-1β, thus indicating that IL-1β may play a role [238].

Overall this chapter shows that γδ T cells are prolific producers of IL-17 in a DC dependent but DC contact independent manner and that IL-17 secretion from these cells is IL-23 dependent. IL-22 is also secreted from γδ T cells, but in what looks to be a different γδ T cell from IL-17+ γδ T cells where contribution of IL-23 needs further explored. These findings make these cells an interest of further study especially when looking at *in vivo* infections to see if these cells are sources of IL-17 and IL-22 during *P. aeruginosa* infection.

7 *In vivo* responses to *Pseudomonas aeruginosa* and *Streptococcus pneumoniae*

7.1 Introduction

We have observed IL-17 responses to both *P. aeruginosa* and *S. pneumoniae* in our *in vitro* set up but we wished to explore these responses *in vivo*, investigating if IL-17 is produced by cells in the lung *in vivo* upon infection with these pathogens and if so, does it occur in the manner that we have observed *in vitro*, and are the same cells responsible for *in vivo* and *in vitro* IL-17 production.

Our aim was to investigate IL-17 producing cell populations in the lung following induction of pneumonia with the Gram-negative bacterium *P. aeruginosa* and the Gram-positive organism *S. pneumoniae* to see if the response varied with infection with these two pathogens. This was carried out by infecting wild-type (WT) mice with the pathogens intranasally to induce acute pneumonia. Mice were left for 48 hours or until they had became sick and had to be sacrificed (according to home office regulations). The strains of *P. aeruginosa* and *S. pneumoniae* used for the *in vitro* trials differed from that of the *in vivo* work. *P. aeruginosa* PA103 was used for the *in vitro* work, and PAO1 was used in the *in vivo* work, and *S. pneumoniae* D39 was used in *in vitro* work, with TIGR4 being used for *in vivo* work. The *in vitro* stains of each pathogen used are commonly used experimental strains and this is the reason for their use. The stains for the *in vivo* work differed as we wanted to use bioluminescent strains that we would be able to visualise with the IVIS (*in vivo* imaging system) to see if the bacteria had colonised the lung, and it was only these strains of the pathogen that were available for this purpose. This is not ideal as these pathogen strains differ in some ways (not discussed); however, we are confident that both strains of each pathogen effectively show genuine responses of the immune system to *P. aeruginosa and S. pneumoniae* and thus the results are a good basis for future work.

A preliminary trial was also completed with IL-17 receptor (IL-17R) knock out (KO) mice to analyse the effects of IL-17 signalling in pneumonia. IL-17 signalling via the IL-17R is thought to aid in neutrophil recruitment [3, 61, 239]. Thus in the absence of IL-17R, one may predict that there may be less recruitment of neutrophils at the site of infection. This is what we wished to investigate in relation to *P. aeruginosa* and *S. pneumoniae* infection in the lung. As only one

experiment was performed using the KO mice (N=3), with *S. pneumoniae* infection, no firm assumptions can be made but it gives indications of what may be happening *in vivo* and how to perhaps proceed with repeats and future experiments.

IL-17 producing cell populations were investigated in the lung tissue using flow cytometry, IL-17 concentrations in the bronchoalveolar lavage (BAL) and pleural space were measured by ELISA, overall lung tissue homology was inspected by histology and H&E staining, and cellular infiltrates in the BAL and pleural space were studied by cytospins and staining using rapid Romanowsky kit.

This work was completed in collaboration with TJ Mitchell and his group, University of Glasgow.

7.2 Establishment of pneumonia infection using *P. aeruginosa*

The number of pathogens to induce acute pneumonia with *S. pneumoniae* has previously been established by the Mitchell group as $5x10^6$ colony forming units (cfu), and so this figure was used in experiments with this pathogen. The dose of *P. aeruginosa* to use to deliver a similar level of infection was however was less clear. There is evidence that too high a dose will kill the mice very rapidly, but too little and the animals will clear the pathogen with little effect [55]. Thus, a pilot experiment was performed to test the number of pathogens to infect with intranasally to induce acute pneumonia.

3 groups of 2 animals were infected intranasally with $5x10^6$, $2x10^7$ and $1x10^8$ cfu and left for 48 hours. As predicted the animals given the highest dose of pathogens, $1x10^8$ cfu, had to be sacrificed within a matter of hours. At the 48 hour time point the animals that had been given the lowest dose, $5x10^6$ cfu, appeared to have been outwardly unaffected by the bacteria and were still active and bright eyed with no weight loss, indicating that they were healthy. The animals that had been infected at $2x10^7$ were moribund, and so it was concluded that pneumonia had been induced and had taken effect on the health of the animal. Due to these observations an infectious dose of $2x10^7$ was used for all subsequent *P. aeruginosa* infections.

However, in subsequent experiments the $2x10^7$ cfu dose of *P. aeruginosa* induced a rather more severe infection, necessitating sacrifice of infected mice after 24-36 hours. We nonetheless have analysed the results and have made preliminary conclusions that may be considered when looking at future experiments.

7.3 *P. aeruginosa* and *S. pneumoniae* intranasal infection induces lung disease

The clinical health of the animals was monitored regularly over the course of infection and recorded at certain time points; 0 hours post infection (p.i), 24 hours p.i and 30 or 48 hours p.i for *P. aeruginosa* and *S. pneumoniae* respectively. Health was gauged on weight loss and external appearance. Animals that were lively and active with shiny coats indicated good health, whereas sickness was indicated by animals being lethargic, starey, matted coats, hunched backs and finally moribund-meaning near death.

Unfortunately use of the IVIS to detect that the pathogen had entered the lung and was colonizing this space was hindered due to the dark fur and thick skin of the C57BL/6 WT mice used. The laser of the IVIS does not readily penetrate the fur and skin of black C57BL/6 mice as it would white BALB/c animals. We tried removing the hair of these animals to aid in imaging but still found the pathogen difficult to detect, and due to this we cannot comment on pathogen colonization with imaging. However, bacteriology of the blood, BAL and homogenised lung confirmed presence of the infectious pathogens in the animals (data not shown). Furthermore, due to clinical manifestations we were sure we had induced pneumonia in these animals.

7.3.1 P. aeruginosa and S. pneumoniae infection induce hematomas in the lung.

Following infection, it can be clearly observed that the dissected lungs of the infected animals, compared to that of the controls, are consolidated and hemorrhagic and thus indicate that pneumonia has ensued (Figure 7-1).

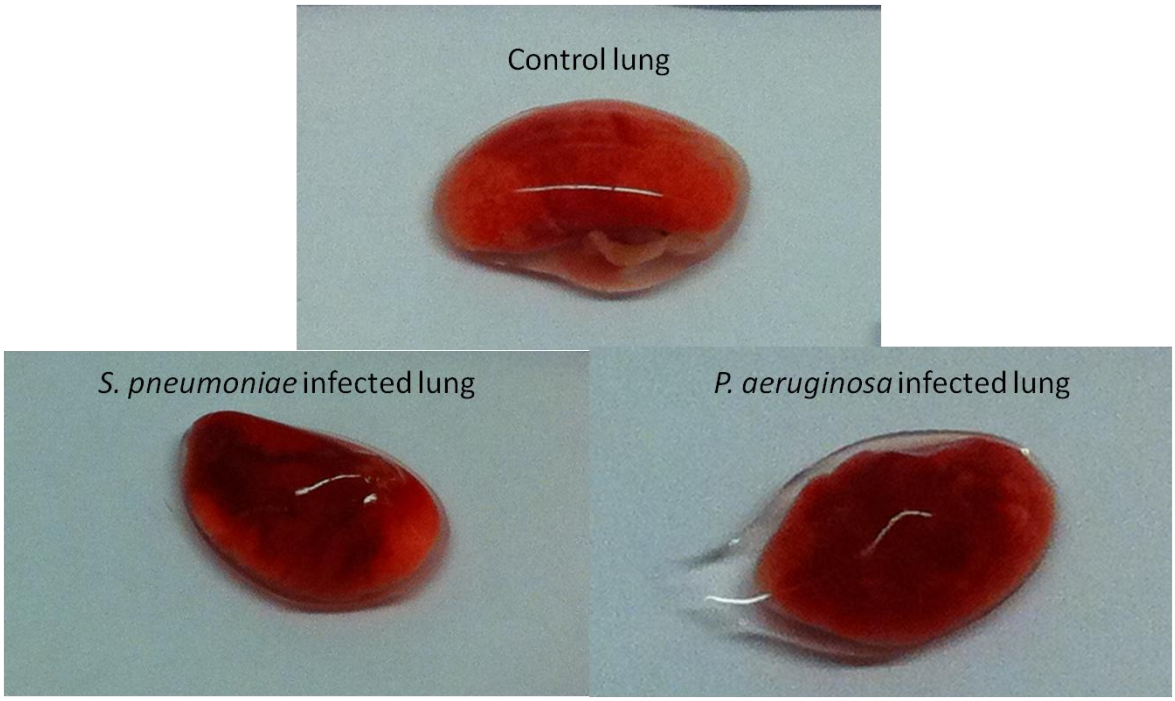

Figure 7-1 Dissected lungs from *in vivo* model

Mice were uninfected (control), or intranasally infected with *P. aeruginosa* or *S. pneumoniae* to induce pneumonia. After 30-48 hours depending on infection, mice were culled and lungs were dissected and photographed to illustrate the haemorrhagic appearance of infected lungs. Pictures are representative of 3 individual animals per group. Data are representative of 3 experiments.

The lungs of the infected animals are very consolidated and haemorrhagic, compared to the pinkish healthy lungs of the controls. These hematomas are indicative of lung tissue destruction, thus showing that the infections have developed. Pneumonia is associated as a hemorrhagic disease [240, 241] and thus these observations confirm that disease was induced.

7.3.2 P. aeruginosa and S. pneumoniae both induce severe lung inflammation indicative of acute pneumonia.

Looking at the histology of the infected lungs, it is evident that both pathogens induced inflammation in the lung and there are major morphological changes in the lung tissue, compared to the control, that are characteristic of pneumonia [242].

Healthy animal histology displays many alveolar spaces with thin walls, thin walled major airways, blood vessels with small surrounding spaces and no cellular infiltrate (Figure 7-2).

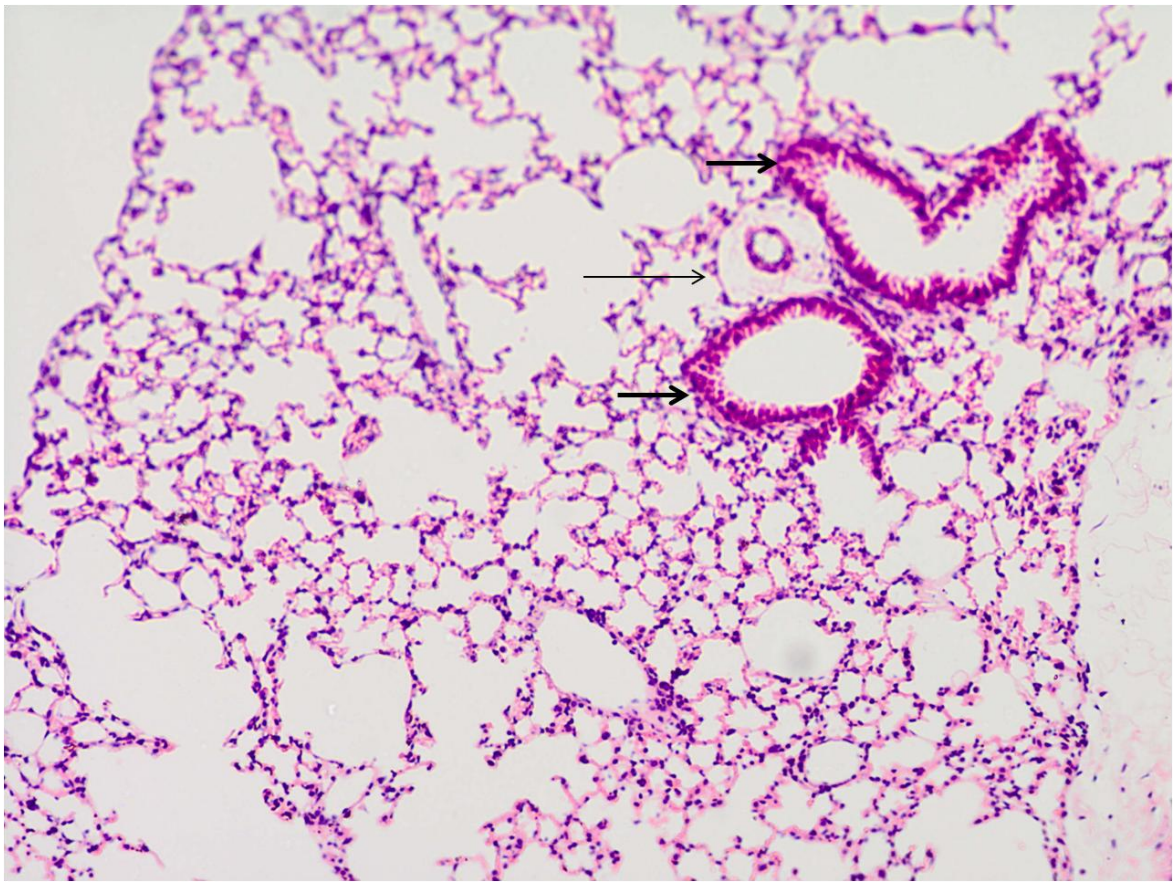

Figure 7-2 Section of healthy control lung stained with H&E

Lungs of uninfected control animals sacrificed at 48 hours were fixed in paraffin, before being sectioned and stained with H&E. The thick arrow indicates airways and the thin arrow indicates blood vessels. Magnification, x40. Pictures are representative of 3 individual animals per group. Data are representative of 3 experiments.

In the *P. aeruginosa* infected mice this morphology is greatly distorted. Large areas of inflammation are evident, with immune cell infiltration throughout the tissue, loss of alveolar spaces, airway wall thickening, blood throughout the tissue and cellular infiltrate in the spaces surrounding blood vessels and within airways (Figure 7-3).

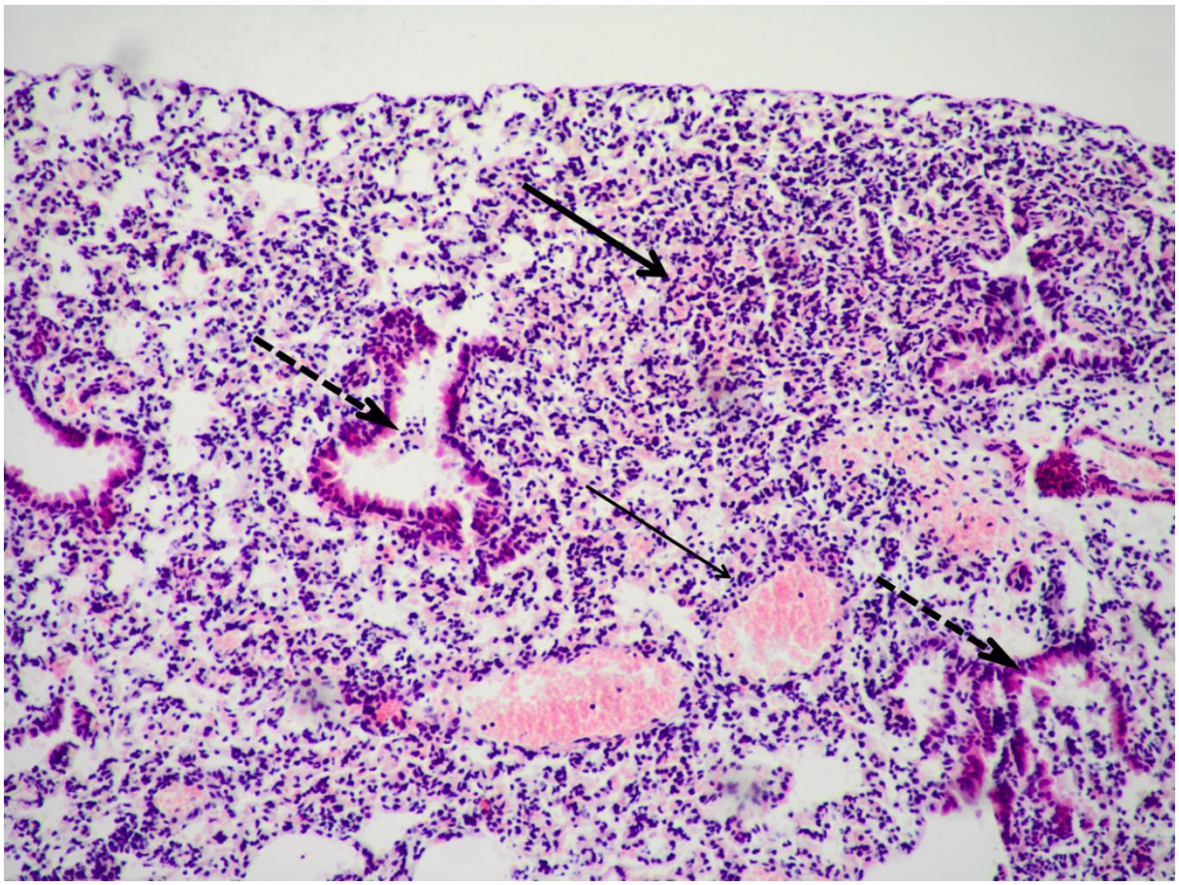

Figure 7-3 Section of *P. aeruginosa* infected lung stained with H&E
Animals were infected with $2x10^7$ cfu *P. aeruginosa*, sacrificed at 30 hours and lung tissue was dissected and fixed in paraffin, before being sectioned and stained with H&E. The thick arrow indicates areas of cellular infiltrate, inflammation and haemorrhage. The thin arrow indicates areas of parenchymal haemorrhage. The dashed arrow indicates areas where cells are shed in the airway. Magnification x40. Pictures are representative of 3 individual animals per group. Data are representative of 3 experiments.

In *S. pneumoniae* infected mice an inflammatory cell infiltrate in the lung tissue was also evident mainly centred around the bronchioles and associated blood vessel area (Figure 7-4).

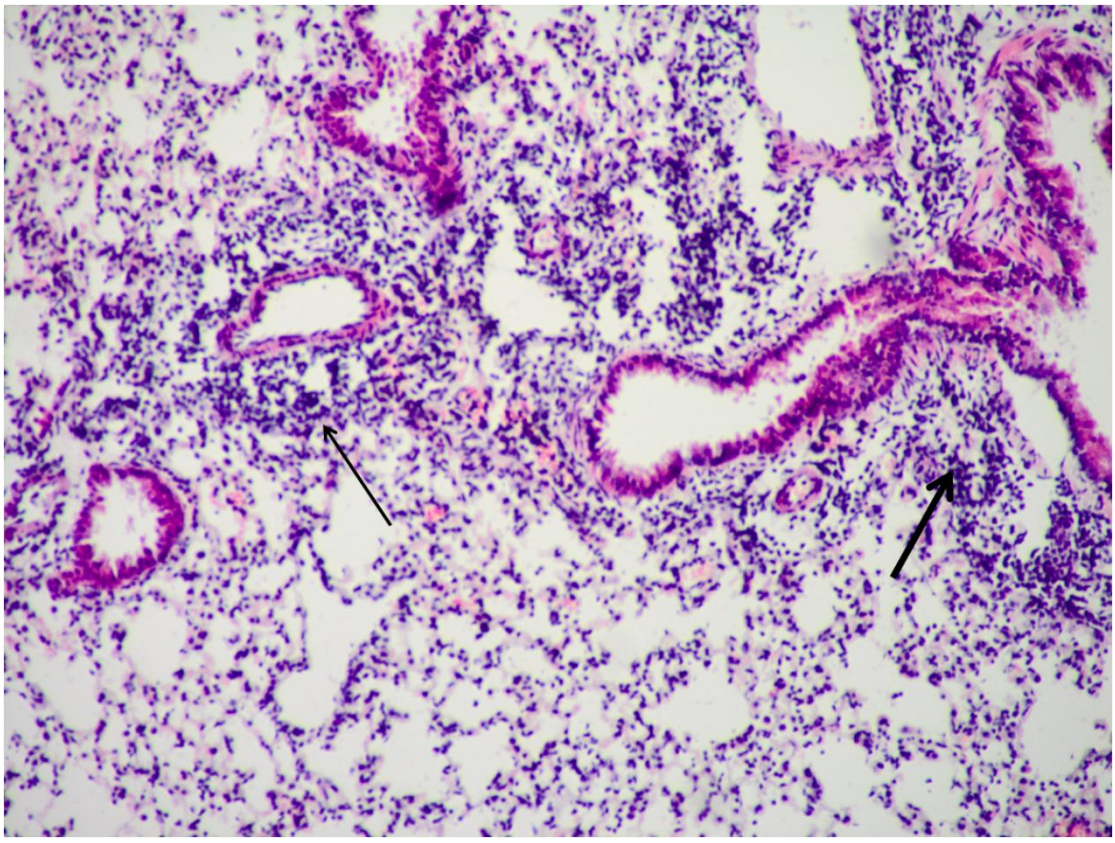

Figure 7-4 Section of *S. pneumoniae* infected lung stained with H&E

Animals were infected with $5x10^6$ cfu *S. pneumoniae*, sacrificed at 48 hours and lung tissue was dissected and fixed in paraffin, before being sectioned and stained with H&E. The thick arrow indicates areas of cellular infiltrate and inflammation in the tissue. The thin arrow indicates areas of cellular infiltrate in the peribronchial and perivascular spaces. Magnification x40. Pictures are representative of 3 individual animals per group. Data are representative of 3 experiments.

These observations confirm that inflammation, and thus pneumonia, has been induced in the infections. It is difficult to assess fully the phenotype of these cells as the sections are orientated so that the cells lie upon one another making identification difficult, but using a higher magnification it can be seen that the densely stained cells are multi-lobed nuclei neutrophils, some of which are highlighted in Figure 7-5.

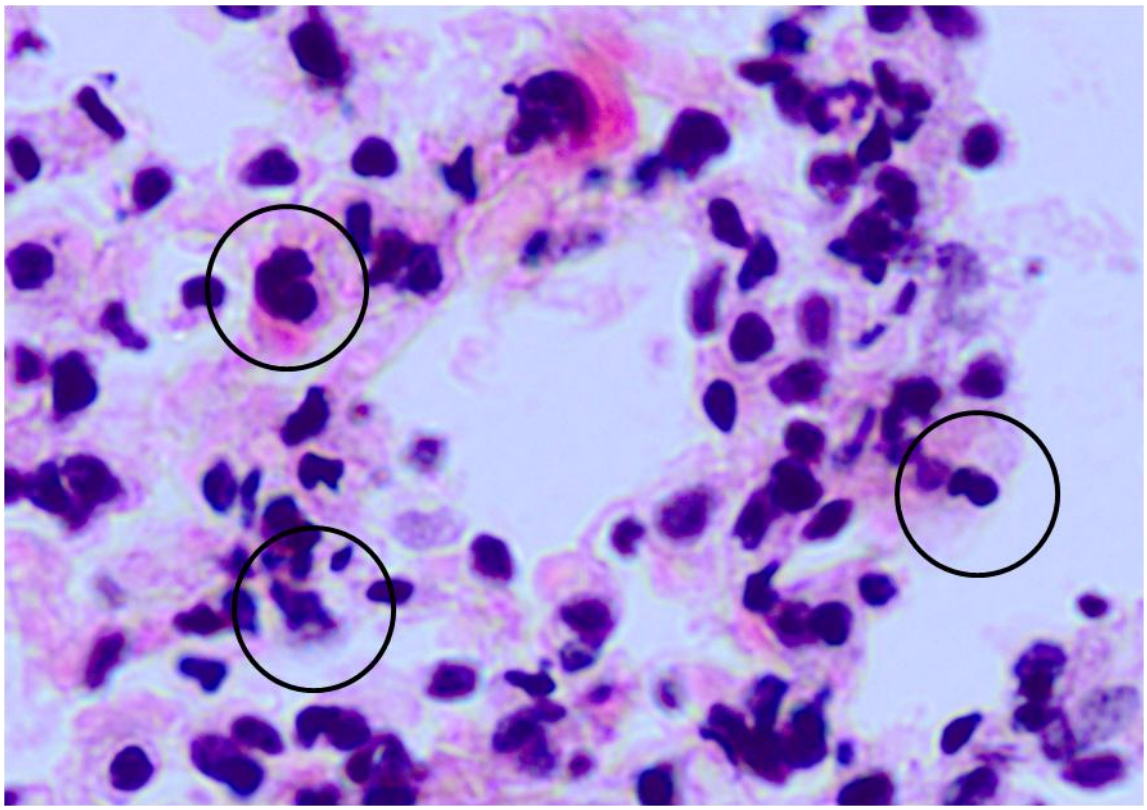

Figure 7-5 Inflammation in the lung during bacterial pneumonia is due to neutrophils
Animals were infected with $5x10^6$ cfu *S. pneumoniae*, sacrificed at 48 hours and lung tissue was dissected and fixed in paraffin, before being sectioned and stained with H&E. Neutrophils responsible for this inflammation are highlighted above. Magnification x100. Pictures are representative of 3 individual animals per group. Data are representative of 3 experiments.

This infiltrate of neutrophils is further confirmation that pneumonia has been induced.

Examining the histology of lung tissue to compare the two infections, (Figure 7-3 and Figure 7-4), we see no real evidence as such that the infections differ in their inflammatory response. Both appear to have vast areas of inflammation characterised by neutrophils. Direct comparison is hindered as the *P. aeruginosa* infection was much more severe, as could be seen clinically, and so these sections cannot be compared as similar levels of infection and sickness. However, both infections have induced inflammation and pneumonia and thus we could investigate the cellular composition of *P. aeruginosa* and *S. pneumoniae* induced pneumonia further by examining cytospins of the BAL and pleural wash, and by flow cytometry of the lung homogenates.

7.4 Neutrophils dominate the cellular population in the BAL of infected animals

Further evidence of infection and pneumonia is a strong neutrophil infiltrate in the BAL of the infected animals.

In healthy control animals macrophages are the predominant cell in the BAL. Following infection, we expect to see a large infiltration of neutrophils, the hallmark of inflammation during pneumonia [53, 54]. Using rapid Romanowsky staining, macrophages are identified as large cells with one large dark stained nucleus and abundant cytoplasm. Neutrophils are smaller, appear to have less cytoplasm and identified by dark stained multi-lobed nuclei (Figure 7-6).

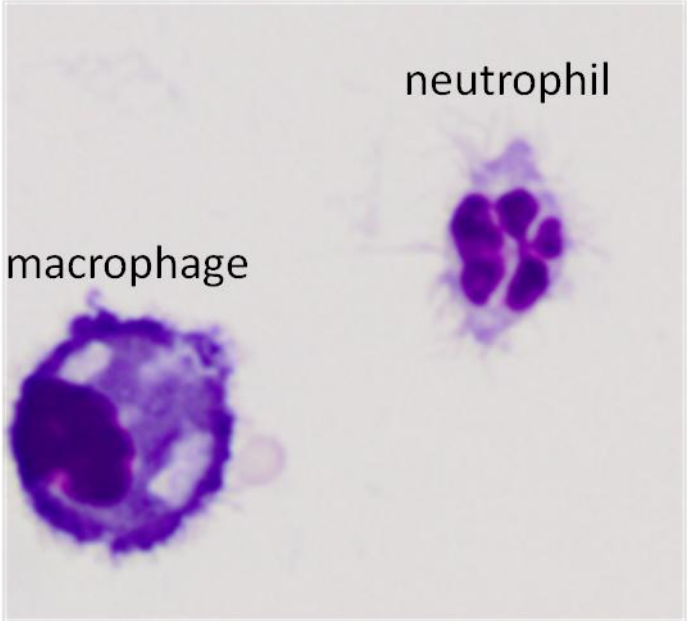

Figure 7-6 Examples of a macrophage and neutrophil identified by rapid Romanowsky staining

Cells from a BAL were stained with rapid Romanowsky to identify macrophages and neutrophils. The macrophage (left) is larger with one nucleus and abundant cytoplasm, whereas the neutrophil (right) is smaller, has multi-lobed nuclei and less cytoplasm.

The BAL of the control animals consists of mainly macrophages, whereas upon infection with both in *P. aeruginosa* and *S. pneumoniae* the cells in the BAL are mainly multi-nucleated neutrophils (Figure 7-7).

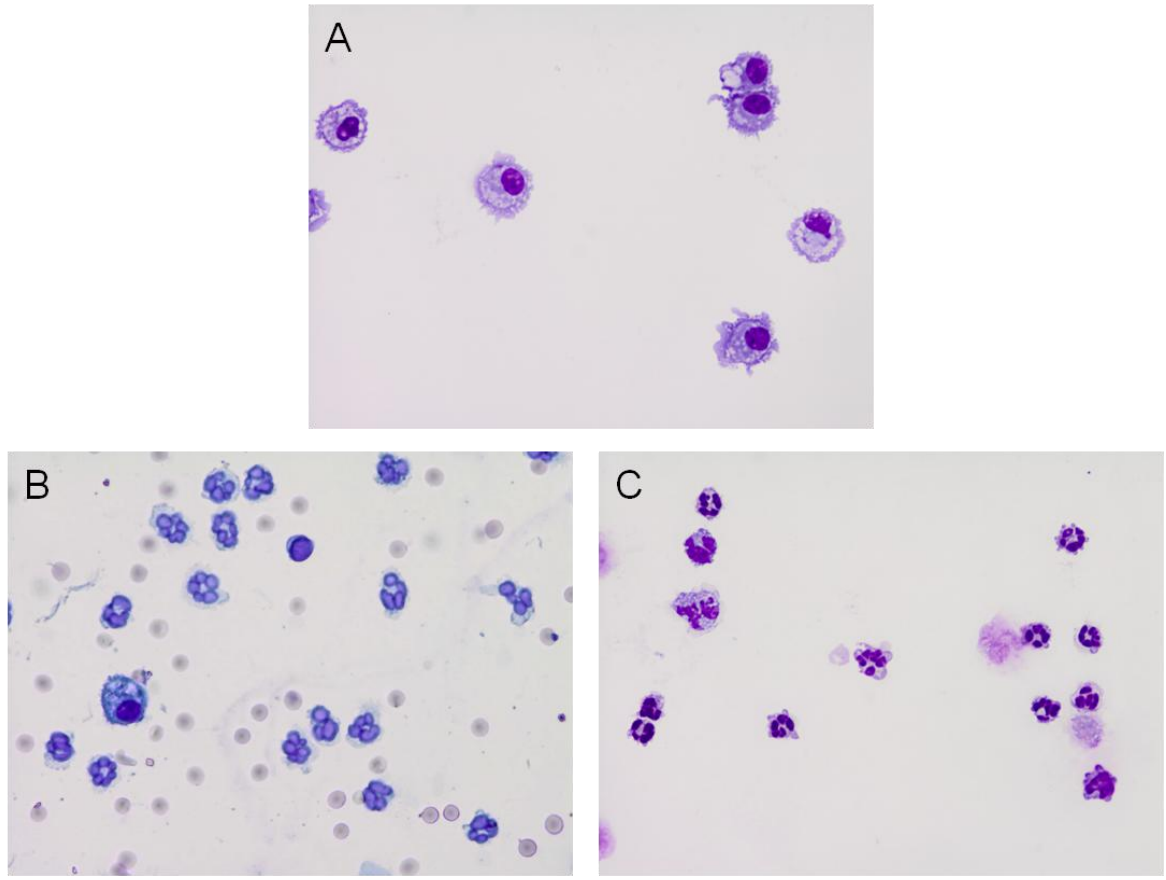

Figure 7-7 Cytospin of cells from BAL of control lung, *P. aeruginosa* infected lung and *S. pneumoniae* infected lung stained with rapid Romanowsky

Animals were left uninfected (A), or infected with $2x10^7$ *P. aeruginosa* (B) or $5x10^6$ cfu *S. pneumoniae* (C), sacrificed at 30 or 48 hours according to animal health, and BALs were performed. Aliquots of the BAL were spun down onto slides and stained with rapid Romanowsky to show cellular differentiation of cell population. BAL of control animals (A) show mainly macrophages whereas upon infection with *P. aeruginosa* (B) and *S. pneumoniae* (C) this space is mainly occupied by neutrophils. Pictures are representative of 3 individual animals per group. Data are representative of 3 experiments.

To investigate if this presence of neutrophils was due to recruitment of more cells or a change in cellular percentage of the cells present in the BAL the cells collected were counted, ratios of differentiated cells were counted and percentages of neutrophils, macrophages, lymphocytes and other cells were calculated. Total cells counted versus neutrophil number is graphed below (Figure 7-8).

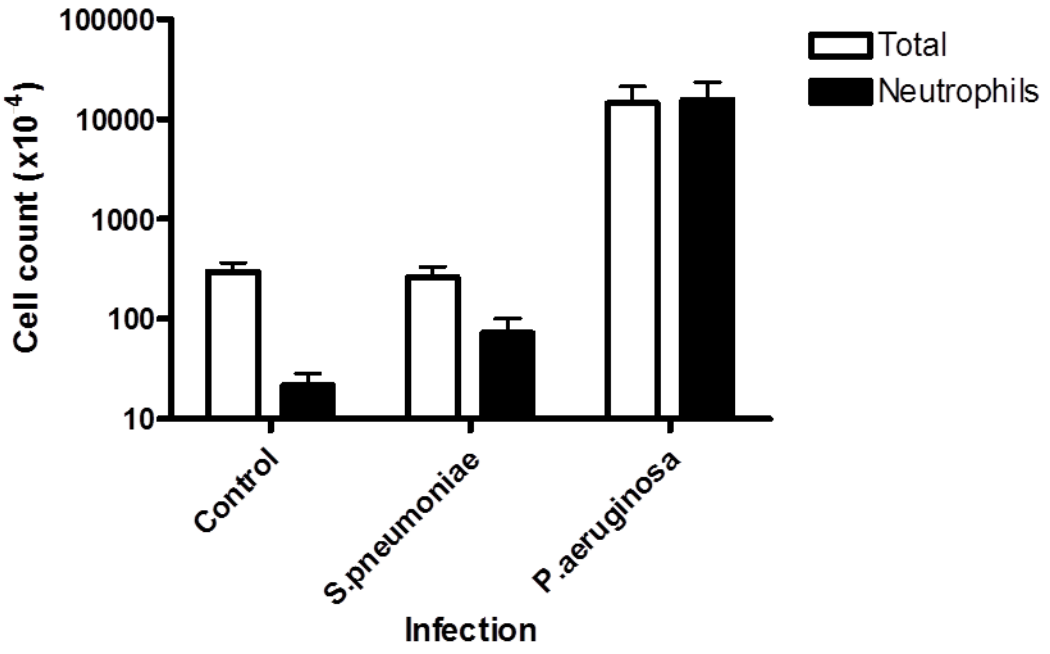

Figure 7-8 Counts of neutrophil populations in BAL

Animals were left uninfected, or infected with 2x10^7 *P. aeruginosa* or 5x10^6 cfu *S. pneumoniae*, sacrificed at 30 or 48 hours according to animal health, and BALs were performed. Total cell counts in the BAL were obtained for all conditions. Differential counts of a sample of BAL spun onto slides were performed and percentages of cell populations present in the BAL were calculated. This percentage was then applied to the total to estimate how many of the total cells neutrophils were. All error bars represent mean ± SEM from 3 individual animals per group. Data are representative of 3 separate experiments.

In the control animals it appears that approximately one tenth of the whole cell population obtained from the BAL are neutrophils, with the remaining cells being macrophages (Data not shown). The neutrophil number increases upon *S. pneumoniae* infection, although the total cell number does not appear to have changed indicating a shift to neutrophils of the cells that occupy this space. In *P. aeruginosa* infection however, there appears to be an increase in cell number which appears to be totally dominated by neutrophils. These observations are evidence that pneumonia has been induced.

7.5 IL-17 concentrations in the BAL

We hypothesised that IL-17 and IL-17R signalling may be responsible for neutrophil recruitment in the lung during bacterial infection and so we measured the concentrations of IL-17 in the BAL to see if there was a correlation between increased IL-17 and increased neutrophils in this space (Figure 7-9).

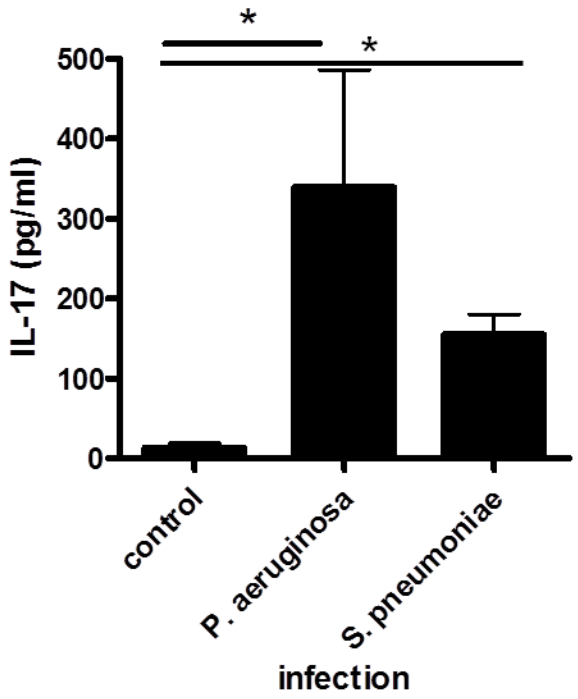

Figure 7-9 IL-17 concentrations in the BAL during *P. aeruginosa* and *S. pneumoniae* infection

Animals were left uninfected, or infected with $2x10^7$ *P. aeruginosa* or $5x10^6$ cfu *S. pneumoniae*, sacrificed at 30 or 48 hours according to animal health. BALs were performed and cells were spun out, leaving the BAL wash which was analysed for IL-17 by ELISA. All error bars represent mean ± SEM from 3 individual animals per group. Data are representative of 3 separate experiments. *$p<0.05$.

IL-17 is significantly increased from control during both infections, in correlation with dominance of neutrophils in this space as witnessed in the cytospin (Figure 7-7). IL-17 concentrations are increased greatly during *P. aeruginosa* infection, which correlates with increased cell numbers that were dominated by neutrophils (Figure 7-8). This correlation suggests that IL-17 may be responsible for increased neutrophil numbers in the BAL.

7.6 *S. pneumoniae* infection induces inflammation at the pleural edge that is not observed in *P. aeruginosa* infection

The edge of the lung where it comes into contact with the chest wall is covered in a membrane, the visceral pleural membrane. This is a thin layer of connective tissue covered in a flattened squamous epithelium, the mesothelium, that can be observed in Figure 7-10A.

We observed that in *S. pneumoniae* infection (Figure 7-10C) but not *P. aeruginosa* infection (Figure 7-10B) the visceral pleura is grossly inflamed, mainly with neutrophils (Figure 7-10 D).

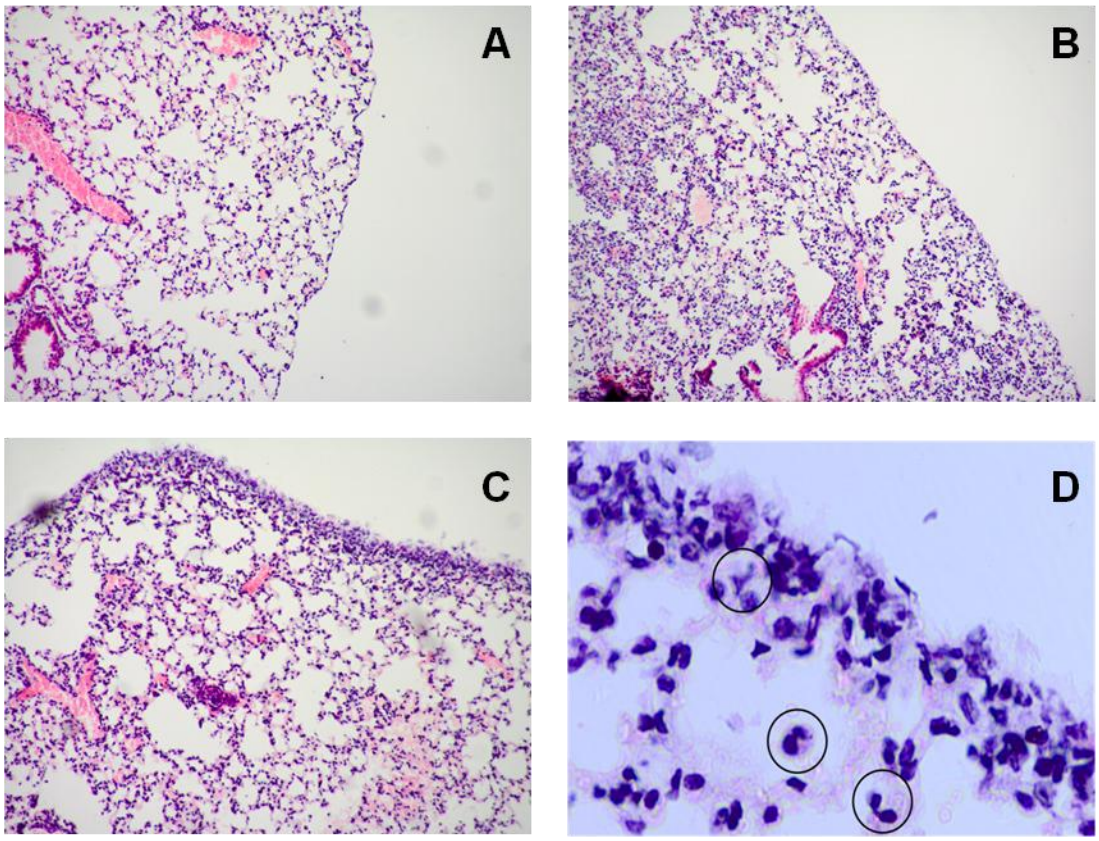

Figure 7-10 Pleural edge of *S. pneumoniae* mice is inflamed compared to control and *P. aeruginosa* infected animal

Animals were left uninfected (A), or infected with $2x10^7$ *P. aeruginosa* (B) or $5x10^6$ cfu *S. pneumoniae* (C), sacrificed at 30 or 48 hours according to animal health, and lung tissue was dissected and fixed in paraffin, before being sectioned and stained with H&E. Magnification x40. A severely inflamed pleural edge, indicated by gross cellular infiltrate is evident in *S. pneumoniae* infected lung (C) compared to that of an uninfected animal (A) and a *P. aeruginosa* infected lung (B), that is composed of mainly neutrophils (D) which are highlighted. Magnification x100. Pictures are representative of 3 individual animals per group. Data are representative of 3 experiments.

The most interesting observation here is that it is the visceral pleura of the moderately clinically sick *S. pneumoniae* infected mice is grossly inflamed whereas that of the extremely clinically sick *P. aeruginosa* infected mice is not, despite these animals having a worse prognosis and showing greater inflammation and cellular infiltrate in the lung parenchyma (Figure 7-3) as opposed to that of *S. pneumoniae* infection (Figure 7-4). Why this is, is unclear and led us to investigate the cells present in the pleural space during these

infections, to see if they differ between *P. aeruginosa* and *S. pneumoniae* infection.

7.7 Immune cells migrate into the pleural space during *S. pneumoniae* infection

To investigate this pleural inflammation, washes of the pleural space to liberate cells from this compartment were performed, cells were counted and ELISAs, cytospins and flow cytometry of the infiltrate were analysed. This was only performed on one experiment, with 4 animals per group and so results must be interpreted with caution, but as all animals in each group responded in the same manner these results may give preliminary indications as to what is going on in this pleural space upon infections and indicates a site of interest for future studies.

7.7.1 More cells are found in the pleural space of infected mice than control animals

We observe that upon infection more cells can be found in the pleural space of infected animals than are found in uninfected control animals (Figure 7-11). No significant difference in total cell number can be observed between *P. aeruginosa* and *S. pneumoniae* infection which is intriguing as gross inflammation at the pleural edge was not observed in the case of *P. aeruginosa* infection, only that of *S. pneumoniae* infected mice. Yet it would appear that many cells infiltrate this space during both infections (Figure 7-11).

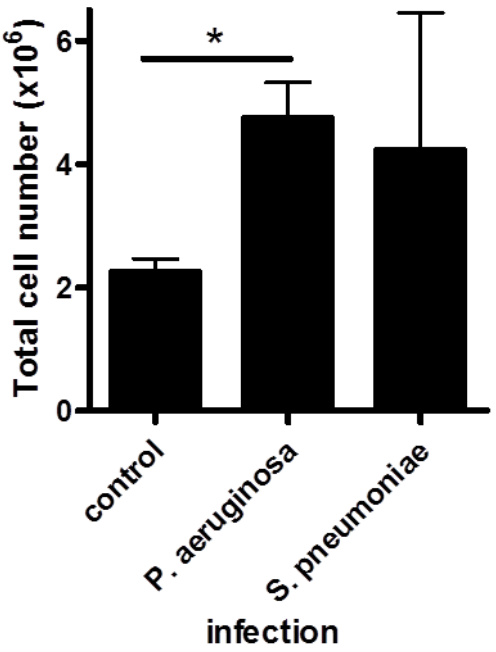

Figure 7-11 Total cell numbers in pleural space of uninfected and infected animals
Animals were left uninfected, or infected with $2x10^7$ *P. aeruginosa* or $5x10^6$ cfu *S. pneumoniae* and sacrificed at 30 or 48 hours according to animal health. Pleural washes of animals were performed and cells were counted to give totals of cells liberated from this area. All error bars represent mean ± SEM from 4 individual animals per group. Data are representative of 1 experiment. * $P< 0.05$.

There is an approximate 2-fold increase in cells in the pleural space during infection, indicating that upon both *P. aeruginosa* and *S. pneumoniae* infection cells are recruited to this site.

7.7.2 Upon infection there is a cellular infiltrate of neutrophils into the pleural space

We see from the cytospins of the cellular populations washed out of the pleural space that in control mice the pleural space is occupied by macrophages with a small presence of neutrophils (Figure 7-12A), unlike in the BAL where in the control mainly macrophages can be observed (Figure 7-7A). Upon infection the cellular infiltrate in this pleural space increases dramatically. In *P. aeruginosa* infection, the cells are both macrophages and neutrophils, as identified by their densely stained nuclei (Figure 7-12B). Following *S. pneumoniae* infection, the cellular infiltrate is almost exclusively neutrophils (Figure 7-12C).

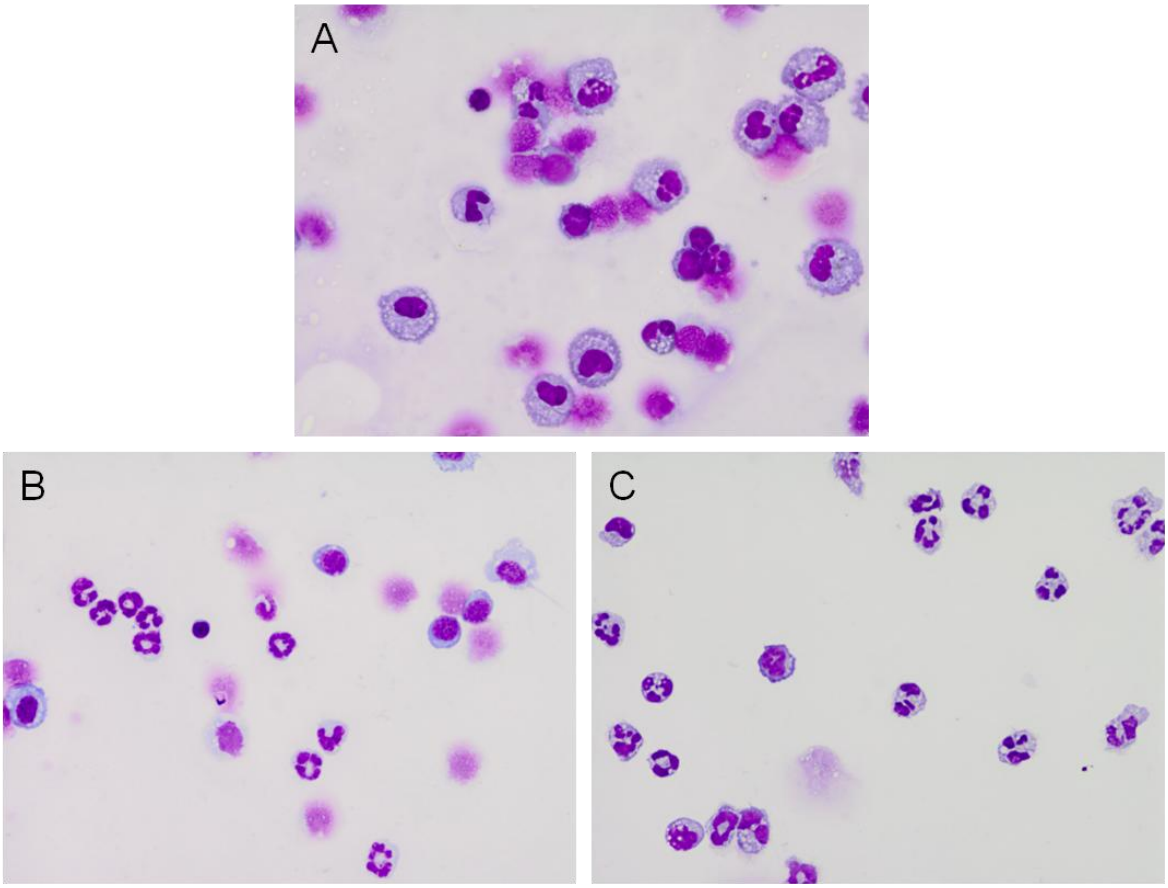

Figure 7-12 Cytospins of lung pleural washes stained with H&E

Animals were left uninfected, infected with $2x10^7$ *P. aeruginosa* or $5x10^6$ cfu *S. pneumoniae*, and sacrificed at 30 or 48 hours according to animal health. Pleural washes of the animals were performed, and aliquots of the pleural wash were spun down onto slides and stained with H&E to show cellular differentiation of cell population. Pleural washes of control animals (A) show mainly macrophages with small neutrophil presence, whereas upon infection with *P. aeruginosa* (B) and *S. pneumoniae* (C) there is recruitment of neutrophils to this space more so in *S. pneumoniae* infection. Pictures representative of 4 individual animals per group. Data representative of 1 experiment.

Differential cell counts of the cells liberated from the pleural space were performed and are graphed below in Figure 7-13.

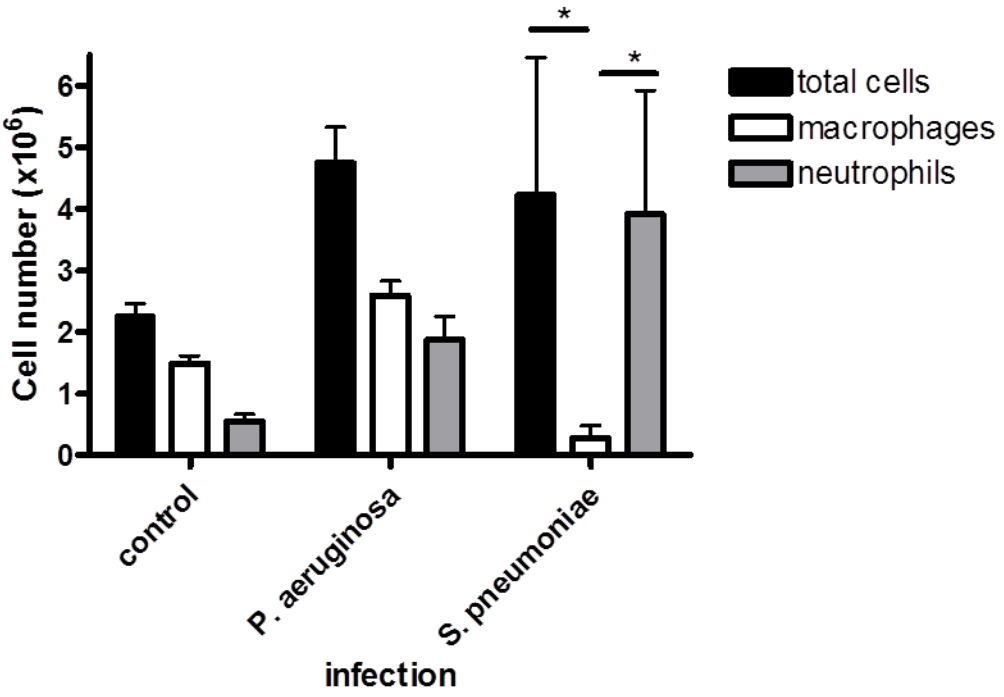

Figure 7-13 Neutrophils dominate the pleural space during *S. pneumoniae* infection
Animals were left uninfected, infected with $2x10^7$ *P. aeruginosa* or $5x10^6$ cfu *S. pneumoniae*, and sacrificed at 30 or 48 hours according to animal health. Pleural washes of the animals were performed, and cells were counted to obtain total cell counts. Aliquots of the pleural wash were spun down onto slides and stained with H&E to show cellular differentiation of cell population, and percentages of neutrophils and macrophages were counted and calculated. Pleural washes of control animals (A) show mainly macrophages whereas upon infection with *P. aeruginosa* (B) cell numbers are increased which are neutrophils and macrophages, wheras during infection with *S. pneumoniae* (C) this space is mainly occupied by neutrophils. All error bars represent mean ± SEM from 4 individual animals per group. Data are representative of 1 experiment. * $p< 0.05$.

During both infections neutrophils are increased in the pleural cavity; however, the contribution of neutrophils during each infection is different. During *P. aeruginosa* infection, cells in the pleural space appear to be represented equally by macrophages and neutrophils. During *S. pneumoniae* infection this space appears to be completely dominated by neutrophils, and this may explain the gross inflammation observed at the visceral pleura during *S. pneumoniae* infection.

7.7.3 IL-17 in the pleural space

As we wished to investigate the role of IL-17 in the lung during these infections and suspect that IL-17 may act upstream to attract neutrophils, we measured the concentrations of IL-17 in the washes of the pleural space (Figure 7-14).

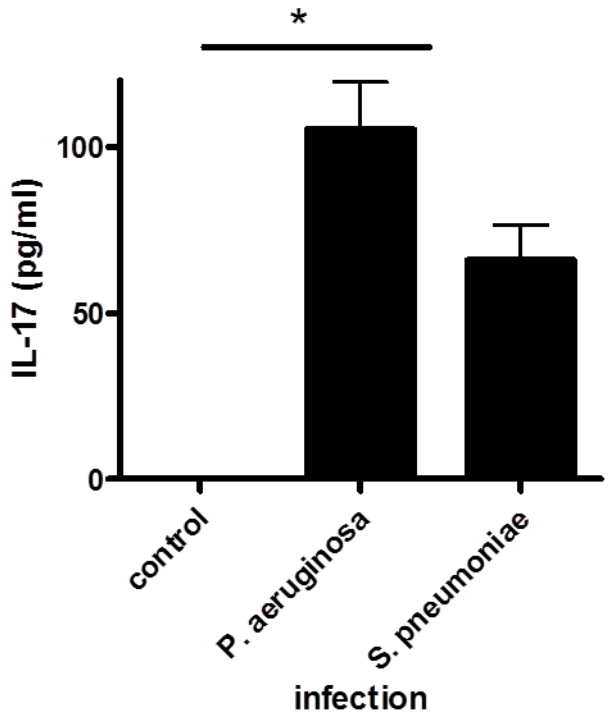

Figure 7-14 Concentrations of IL-17 are found in the pleural space during bacterial infection Animals were left uninfected, infected with $2x10^7$ *P. aeruginosa* or $5x10^6$ cfu *S. pneumoniae* and sacrificed at 30 or 48 hours according to animal health. Pleural washes of the animals were performed and these were analysed for IL-17 by ELISA. All error bars represent mean ± SEM from 4 individual animals per group. Data are representative of 1 experiment. * $p< 0.05$.

Although somewhat low, concentrations of IL-17 differ significantly in the pleural space during infection compared to the control. Concentrations of IL-17 do not differ significantly in this space between infections, indicating that IL-17 responses in this space may not be Gram-negative or Gram-positive pathogen specific. This was observed in all 4 animals, but as this is only one experiment, must be repeated before conclusive results can be made.

7.7.4 Sources of IL-17 in pleural space

To investigate the source of IL-17 in the pleural space, cells in the pleural space were stimulated with PMA and ionomycin in the presence of brefeldin A to retain

any IL-17 produced by the cells for intracellular cytokine staining. Unfortunately no IL-17+ cells are observed in this experiment by this means. However we do notice that in the pleural space there are shifts in immune cell populations and these were investigated in the next section.

7.7.5 Immune cells are increased in the pleural space upon infection

Cytological examination cannot differentiate well all the different leukocytes present in the pleural cavity. Thus, the remaining cells harvested from the pleural space were stained for extracellular markers CD4, γδ, Gr-1 and CD19 to investigate the populations of CD4+ cells, γδ T cells, neutrophils and B cells in the pleural space respectively. We wished to examine if populations of these cells were present in the pleural space normally and if they altered during *P. aeruginosa* and *S. pneumoniae* infection. Note cells are gated on a live population of low side scatter where we have previously shown IL-17 cells to be located as these are our primary cells of interest, with exception of gating for Gr-1 cells, as described in materials and methods.

In the pleural space of the uninfected animals there appears to be a high percentage of CD4+ T cells in the live gated cells, 64%, that increases slightly upon infection with *P. aeruginosa*, 68%, and appears to decrease slightly during *S. pneumoniae* infection to 58% (Figure 7-15A). However it must be considered that in the pleural space of the infected animals the cell numbers in this space are vastly increased (Figure 7-11) so in *S. pneumoniae* infection where CD4+ T cell percentage has dropped from that of control there are still many more CD4+ T cells present upon infection.

γδ T cell presence in the pleural space is low with less than 3% γδ T cells found in this space, on gated cells, in the uninfected control animals. Comparatively this may not be a low percentage as γδ T cell populations in non-lymphoid tissues are generally regarded to be below 5% of T cell population [243]. This γδ population in the pleural space appears to decrease to approximately half, 1.5%, upon infection with both *P. aeruginosa* and *S. pneumoniae* (Figure 7-15B). Due to total cell numbers in this space being double upon infection, a percentage population of half would give the same number of γδ T cells in both uninfected

and infected animals. So it would appear that γδ T cell numbers are unchanged in the pleural space during both infections indicating that γδ T cells are not important at this site during infection with *P. aeruginosa* and *S. pneumoniae*.

Analysis of the total cells found in the pleural space confirm that in *S. pneumoniae* infection, where we see gross inflammation at the visceral pleura, this inflammation may be caused by neutrophils. In the control animals approximately 3% of neutrophils can be observed when looking at all cells in this space. This increases slightly to 4.85% upon *P. aeruginosa* infection but increases dramatically to 28% upon *S. pneumoniae* infection (Figure 7-15C). This confirms the observation that many neutrophils are observed in the pleural space during *S. pneumoniae* infection as was observed on cytospins (Figure 7-12) and histology (Figure 7-10C) of these samples. It is worth noting that according to the cytospins and analysis of cells in this space by rapid Romanowsky staining, that during *P. aeruginosa* infection there appeared to be many neutrophils in this space, though not as many as *S. pneumoniae* infection. Yet this is not evident with the flow cytometry data and so the outputs conflict somewhat. More repeats must be performed before any firm assumptions can be made about neutrophil recruitment at this site during each infection.

It would appear that in uninfected animals approximately a third of cells in the pleural space are B cells, as identified by marker CD19, in our gated population of interest. During infection with both *P. aeruginosa* and *S. pneumoniae,* B cell percentages in the pleural space seem to decrease (Figure 7-15D). Again it must be noted that although B cell percentages are lower upon infection, the total number of cells in this space has doubled and so there are still more B cells in the pleural space of infected animals than uninfected control animals. However as a percentage of all cells found in this space, B cells seem to reduce upon infection indicating that they do not have a major role in the pleural space during infection with *P. aeruginosa* and *S. pneumoniae*.

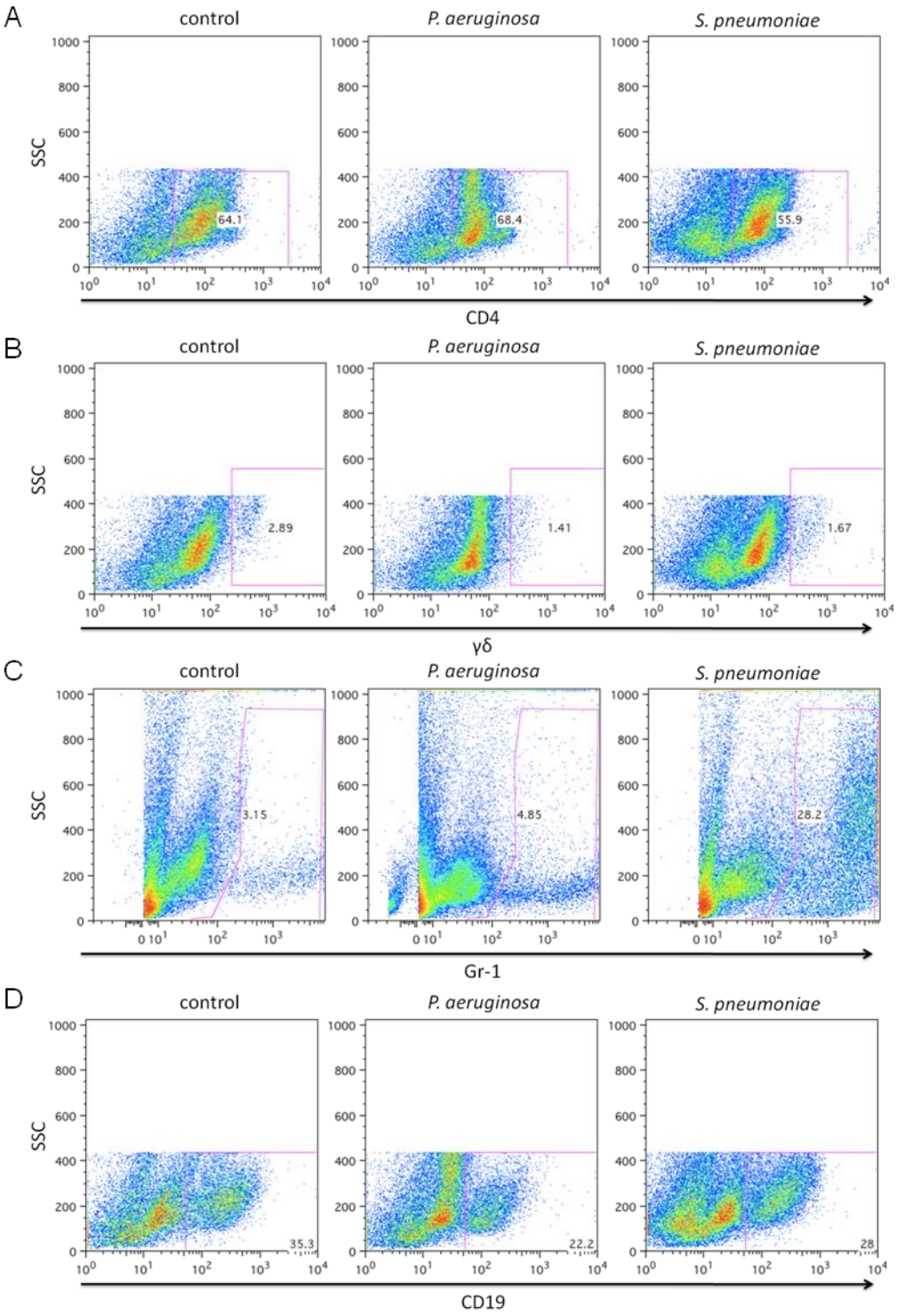

Figure 7-15 Immune cells in the pleural space during infection

Animals were left uninfected, infected with $2x10^7$ *P. aeruginosa* or $5x10^6$ cfu *S. pneumoniae* and sacrificed at 30 or 48 hours according to animal health. Pleural washes of the animals were performed and cells were isolated and stained with CD4 (A), γδ (B), Gr-1 (C) and CD19 (D), and analysed by flow cytometry compared to their respective isotypes. Cells gated on live cells based on FSC and SSC with a larger gate set for Gr-1 analysis as described in materials and methods. Positive gates were set on isotypes for markers on cells isolated from *P. aeruginosa* infected animals. Dot plots represent data pooled from 4 animals. Data representative of 1 experiment.

These observations of immune cells in the pleural space are of one experiment only and so must be taken with caution. However, they do suggest that the pleural space is an area of great interest for further studies, as others have investigated in Mycobacteria infection [244, 245], and should be considered as a site of cytokine secretion and cellular infiltrate upon infection, especially that of *S. pneumoniae* infection.

7.8 Immune cell populations in the lung increase during bacterial pneumonia

To investigate the immune response in the lung tissue, the site of infection during acute pneumonia infection to the pathogens *P. aeruginosa* and *S. pneumoniae*, the lungs of these animals were digested to obtain single cell suspensions and cells were stained to assay for CD4 cells, γδ T cells, neutrophils and IL-17 production by these cells, which were analysed by flow cytometry. Again it must be noted that cells are gated on a live population of low side scatter, as described in materials and methods.

During *P. aeruginosa* infection, an increase of almost double CD4+ cells from that of control was observed in our gated population. In *S. pneumoniae* infection the CD4+ cell population appears slightly reduced than that of control (Figure 7-16A).

γδ T cells increase approximately 2.5 times in this gated population from control upon both *P. aeruginosa* and *S. pneumoniae* infection (Figure 7-16B), illustrating γδ T cells play a role in the lung in pneumonia during both infections.

Gr-1 is a marker commonly used to identify neutrophils when analysing blood, which also appeared to effectively identify neutrophils in the pleural space (Figure 7-15). However in the lung Gr-1 expression is difficult to interpret, with Gr-1 populations in the control uninfected animals looking to be high (Figure 7-16C). We know that there are few neutrophils in the uninfected control animals as we have observed in both their BAL (Figure 7-7A) and washes of the pleural space (Figure 7-12A), and from the histology (Figure 7-2). This leads us to question the use of Gr-1 as a marker for neutrophils in the lung tissue. Monocytes may also be Gr-1+ [246], and as the lung is known to be full of

alveolar macrophages we are inclined to say that in the lung tissue we are staining for these and any other monocytic like cells that may be present, as Gr-1 is classed as a monocyte marker [246]. Thus, it would appear that Gr-1 cannot be used as a marker for neutrophils in the lung tissue and therefore we cannot make any assumptions about neutrophil contribution in the lung during infection by this means.

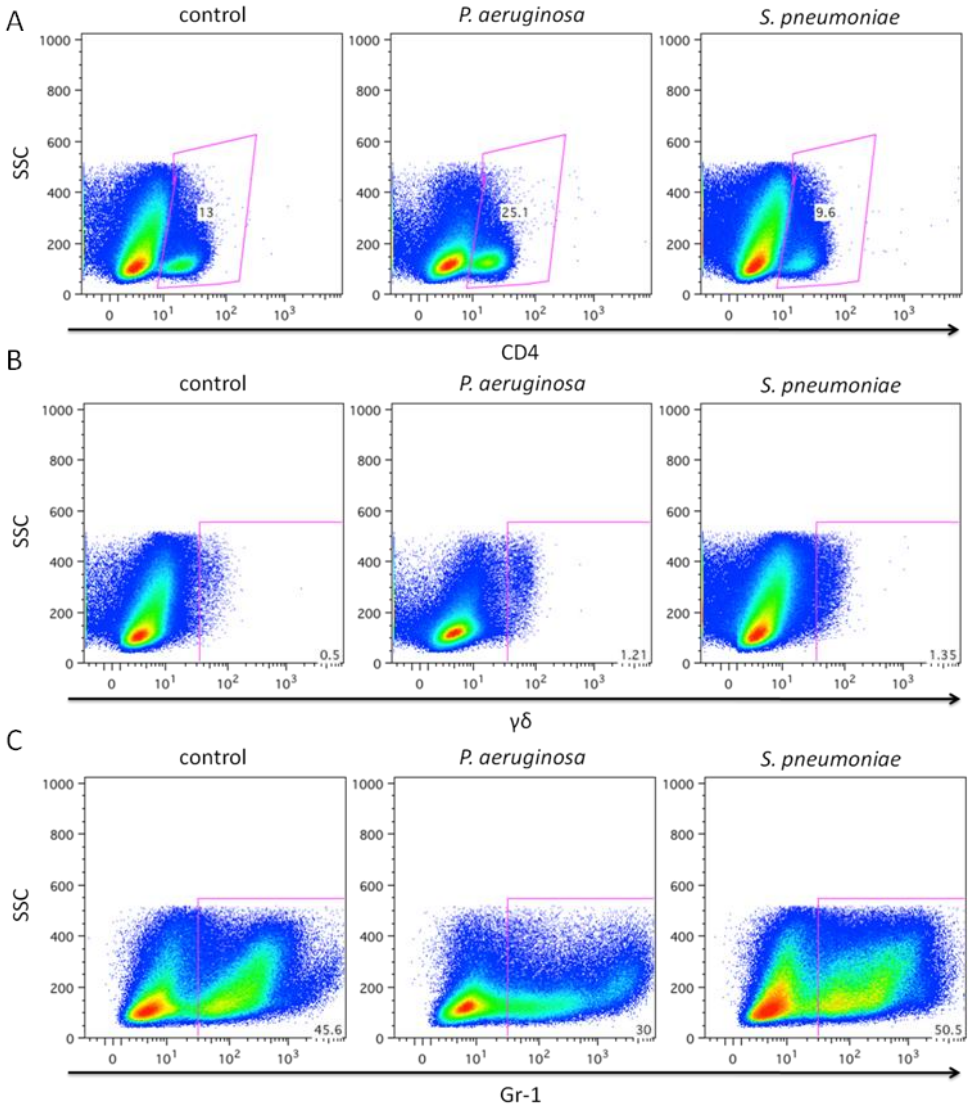

Figure 7-16 Immune cells in the lung during pneumonia

Animals were left uninfected, infected with $2x10^7$ *P. aeruginosa* or $5x10^6$ cfu *S. pneumoniae* and sacrificed at 30 or 48 hours according to animal health. Lungs of the animals were dissected and digested to obtain single cell suspensions. Cells were stained with CD4 (A), γδ (B) and Gr-1 (C) and analysed by flow cytometry compared to their respective isotypes. Cells gated on live cells based on FSC and SSC with a larger gate set for Gr-1 analysis as described in materials and methods. Positive gates were set on isotypes for markers on cells isolated from *P. aeruginosa* infected animals. Dot plots represent data pooled from 3 animals. Data representative of 3 experiments.

7.9 Sources of IL-17 in the lung during bacterial pneumonia

IL-17 production throughout the 3 *in vivo* experiments varied, and therefore it is difficult to make any firm conclusions based on these experiments alone. In one case we see no IL-17 in the uninfected control animals and an increase in IL-17+ cells during infection (data not shown). However an identical repeat of this experiment bore different results with IL-17 observed from uninfected animals also. It is worthy to note that PMA and ionomycin stimulation used to induce intracellular production of cytokines makes any cell that has been or is capable of secreting IL-17, secrete it again, and so the information we have obtained from the unstimulated lungs may not be very informative. We need a measure of cells that have produced IL-17 at some point during infection and this is discussed further in the discussion. However, where IL-17+ cells have been observed in the uninfected control animals we observe a shift in the cells that produce IL-17 during the infections and can formulate theories of IL-17 production and the cells responsible for IL-17 secretion during *P. aeruginosa* and *S. pneumoniae* infection, as we see the same source of IL-17 from controls and each infection every time. Due to the fluctuating nature of these experiments, numerous repeats of these experiments must be performed in order to come to any firm conclusions, but we have documented our preliminary findings below.

Upon *S. pneumoniae* infection, although less severe clinically than *P. aeruginosa* infection, more IL-17 is observed from cells in the lung tissue (Figure 7-17).

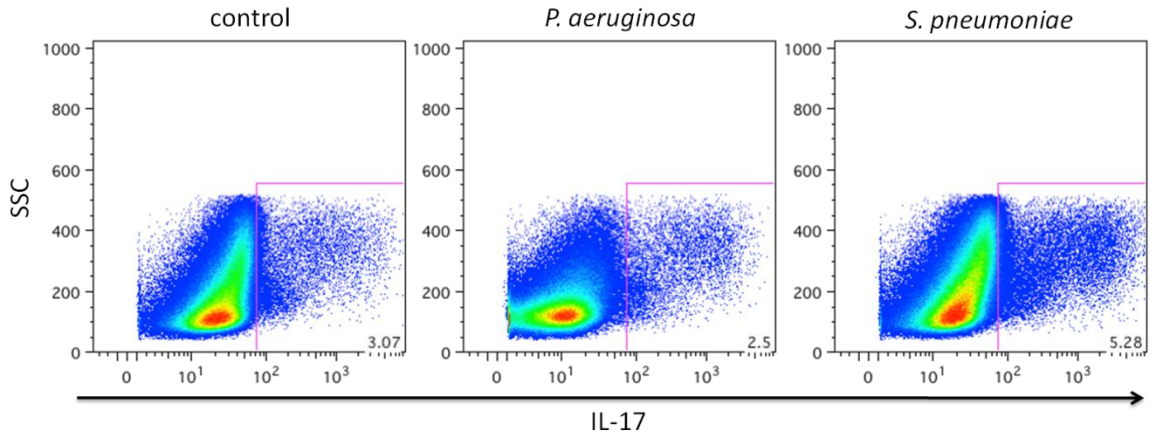

Figure 7-17 IL-17 cells are increased during *S. pneumoniae* but not *P. aeruginosa* infection
Animals were left uninfected, infected with $2x10^7$ *P. aeruginosa* or $5x10^6$ cfu *S. pneumoniae* and sacrificed at 30 or 48 hours according to animal health. Lungs of the animals were dissected and digested to obtain single cell suspensions. Cells were stimulated with PMA and ionomycin in the presence of brefeldin A for 5 hours, after which time cells were harvested and stained for IL-17 and analysed by flow cytometry compared to isotype. Cells gated on live cells based on FSC and SSC as described in materials and methods. Positive gates were set on isotypes for markers on cells isolated from *P. aeruginosa* infected animals. Dot plots represent data pooled from 3 animals. Data representative of 3 experiments.

This is a curious observation as *P. aeruginosa* infection appeared to be more clinically severe. This led us to look at the sources of IL-17 during these infections to see if they differed during the two infections.

In control mice 18% of the IL-17 producing cells are γδ T cells, and this increases to 42% upon *P. aeruginosa* infection showing a strong shift to IL-17 producing γδ T cells in *P. aeruginosa* infection. In *S. pneumoniae* infection γδ T cells are still only responsible for 18% of IL-17+ cells (Figure 7-18).

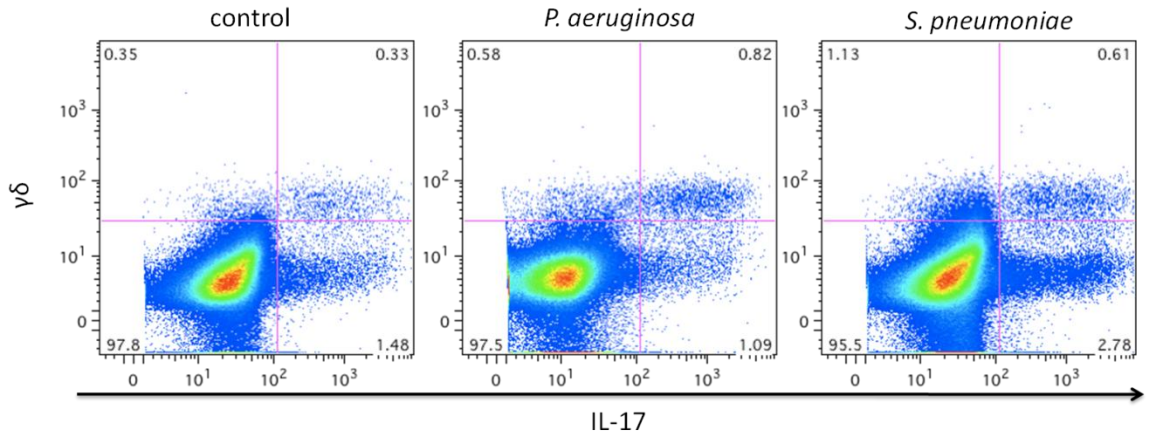

Figure 7-18 IL-17+ γδ T cells in the lung during *P. aeruginosa* and *S. pneumoniae* infection
Lungs of uninfected control mice, *P. aeruginosa* infected mice and *S. pneumoniae* infected mice were dissected and digested to obtain single cell suspensions. Cells were stimulated with PMA and ionomycin in the presence of Brefeldin A for 5 hours before being harvested and stained for γδ and IL-17, and analysed by flow cytometry compared to their respective isotypes. Cells gated on live cells based on FSC and SSC as described in materials and methods. Positive gates were set on isotypes for markers on cells isolated from *P. aeruginosa* infected animals. Dot plots represent data pooled from 3 animals. Data representative of 3 experiments.

This indicates that γδ T cells may be an important source of IL-17 during *P. aeruginosa* infection but not so during *S. pneumoniae* infection. It would appear that in correlation with this shift to IL-17 production from γδ T cells during *P. aeruginosa* infection the amount of IL-17 producing CD4 cells, Th17 cells, reduces during this infection (Figure 7-19).

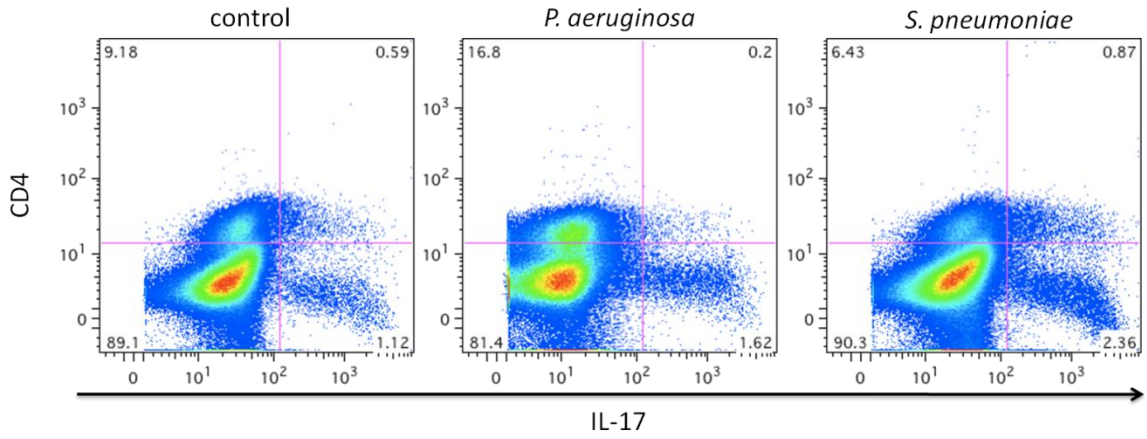

Figure 7-19 Th17 cells in the lung increase during *S. pneumoniae* bacterial pneumonia, yet decrease during infection with *P. aeruginosa*

Lungs of uninfected control mice, *P. aeruginosa* infected mice and *S. pneumoniae* infected mice were dissected and digested to obtain single cell suspensions. Cells were stimulated with PMA and ionomycin in the presence of Brefeldin A for 5 hours before being harvested and stained for CD4 and IL-17, and analysed by flow cytometry compared to their respective isotypes. Cells gated on live cells based on FSC and SSC as described in materials and methods. Positive gates were set on isotypes for markers on cells isolated from *P. aeruginosa* infected animals. Dot plots represent data pooled from 3 animals. Data representative of 3 experiments.

It appears that although CD4 T cells increase during *P. aeruginosa* infection (Figure 7-16A) they are not IL-17 producing, (Figure 7-19), and therefore not Th17. This indicates that another CD4+ helper T cell is recruited during *P. aeruginosa* infection, although what is unclear as we did not test for any other cytokines that could characterize other helper T cell subsets.

Th17 cells appear to increase slightly upon infection with *S. pneumoniae* but the majority of IL-17+ cells are CD4- (Figure 7-19).

Using Gr-1 as a tissue granulocyte marker and not as a marker solely for neutrophils, we have identified during bacterial pneumonia caused by *P. aeruginosa*, granulocytes, including neutrophils, are not responsible for production of IL-17 (Figure 7-20). From control to *S. pneumoniae* infection the percentage of Gr-1+ IL-17+ cells doubles and visibly looks to be a more prominent population, but this Gr-1+ IL-17+ is only responsible for 22% of IL-17 production according to percentages using gates that originate from clean isotypes, and this percentage does not differ from control or *P. aeruginosa*

infection. So it appears Gr-1+ cells including neutrophils and macrophages are not responsible for IL-17 production during these infections.

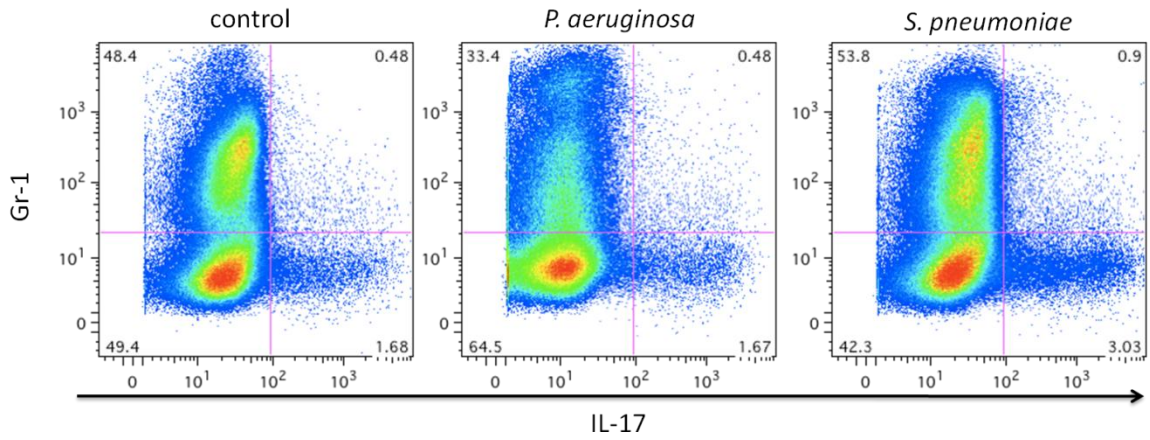

Figure 7-20 IL-17+ Gr-1+ cells in the lung during bacterial pneumonia

Lungs of uninfected control mice, *P. aeruginosa* infected mice and *S. pneumoniae* infected mice were dissected and digested to obtain single cell suspensions. Cells were stimulated with PMA and ionomycin in the presence of Brefeldin A for 5 hours before being harvested and stained for Gr-1 and IL-17, and analysed by flow cytometry compared to their respective isotypes. Cells gated on live cells based on FSC and SSC as described in materials and methods. Positive gates were set on isotypes for markers on cells isolated from *P. aeruginosa* infected animals. Dot plots represent data pooled from 3 animals. Data representative of 3 experiments.

From these results it appears that the sources of IL-17 during *P. aeruginosa* and *S. pneumoniae* infection differ. These results give evidence of γδ T cells being a key cellular source of IL-17 during *P. aeruginosa* infection but the source of IL-17 during *S. pneumoniae* infection is less clear. Small populations of IL-17+ γδ T cells, Th17 cells and IL-17+ Gr-1+ cells are evident during *S. pneumoniae* infection. However, the majority of IL-17+ cells appear to be CD4-, γδ- and Gr-1- cells. This difference in IL-17 producing cells between *P. aeruginosa* and *S. pneumoniae* infection may represent a difference in host response and IL-17 production between Gram-negative and Gram-positive bacteria species, or these responses may be pathogen specific. Many experiments with other Gram-negative and Gram-positive pathogens must be performed before any assumptions about sources of IL-17 during Gram-positive and Gram-negative bacteria can be made, but these results give a preliminary indicating that roles of IL-17 and sources of IL-17 during these infections may differ.

7.10 Effects of IL-17 signalling via its receptor IL-17R on the immune responses in the lung during *S. pneumoniae* pneumonia

Preliminary experiments with IL-17RKO mice were performed to investigate the role of IL-17 and signalling via its receptor IL-17R, during *S. pneumoniae* pneumonia, and the effects that this cytokine and receptor pairing may have on the immune response in the lung during this infection. It is well documented that neutrophils are one of the first immune cells on site during infection, and that IL-17 plays a role in their recruitment [3, 61, 239]. In bacterial pneumonia in the lung, IL-17 released by cells at this site during the infection may bind to IL-17R on epithelial cells in the lung, which in turn secrete the neutrophil attractant chemokine IL-8 [247] (otherwise known as CXCL8 with homologs KC or MIP-2 in mice [248]). IL-8 attracts neutrophils to the site of IL-17 release, and as a result to the site of infection. It is therefore supposed that in IL-17RKO mice, neutrophil recruitment will be severely reduced due to lack of IL-17 signalling and as a consequence the mice will be sicker as neutrophils, which are clearly considered to be necessary due to the rapid speed of their influx during infection, are not present. Sickness in the animals is measured clinically by weight loss and observations in behaviour such as matted coat, staggered gait, lethargy and finally becoming moribund-a state of near death. Due to time constraints and slow breeding of the IL-17RKO mice, only one experiment in triplicate was performed with *S. pneumoniae* infection in IL-17 KO and WT mice, and although not conclusive, data was analysed and hypotheses were formed to be used as a template for future experiments.

7.11 IL-17RKO mice are larger, heavier and have more subcutaneous fat that WT mice

Firstly in this preliminary experiment the IL-17RKO mice infected with *S. pneumoniae* did not appear to be clinically sicker than the WT mice with the same infection. However, we are careful not to assume that this is due to IL-17R absence but could in fact be due to size differences between the strains as the IL-17KO mice were older and much larger, and therefore the number of pathogens given intranasally during infection may have had a lesser effect. Ideally this would have been accounted for before the experiment was carried

out and each animal would have been given a cfu to deliver a similar level of clinical sickness over the time. However, calculating the correct cfu to infect the larger mice with to obtain a similar level of sickness of these mice would have taken weeks and many animals to ascertain. Neither of these resources were available to us at this point and so it was decided that as a preliminary experiment the same number of pathogens would be used for both the WT and KO mice regardless of their age and size difference. The KO mice used were older by approximately 8 weeks, and were 16 weeks of age as opposed to 8 weeks of age of the WT mice. However, age difference in these animals was not our greatest concern though as opposed to size difference between animals. The KO mice were much larger, and not just larger than the WT mice but much larger than any mice we have ever observed regardless of age. The KO animals were significantly heavier and almost double the weight of the WT animals as can be observed from Figure 7-21.

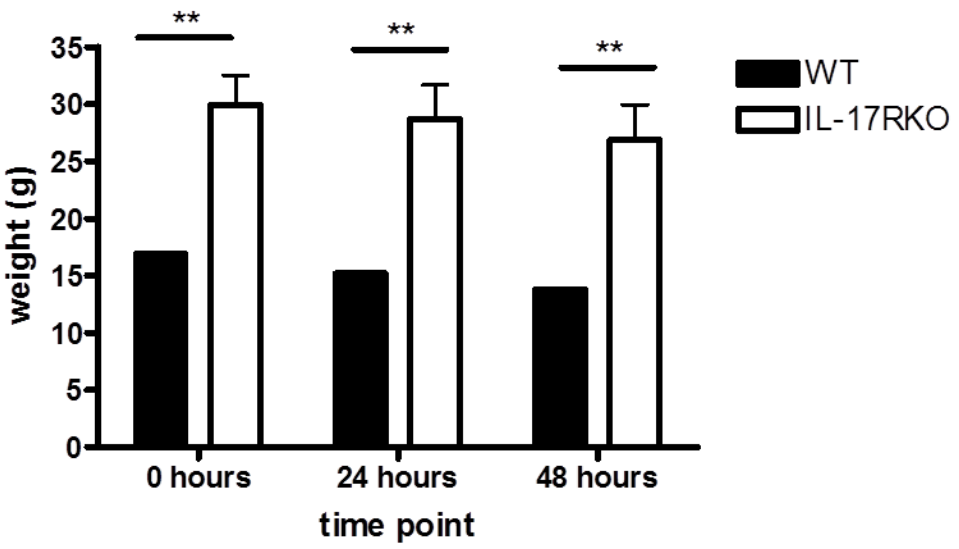

Figure 7-21 IL-17RKO animals are significantly heavier than their WT counterparts
The weight of IL-17RKO and WT animals infected with *S. pneumoniae* was monitored over a 24 hour period to illustrate the difference in weight between these mouse strains. All error bars represent mean ± SEM from 3 individual animals per group. Data are representative of 1 experiment. ** $p < 0.005$.

The IL-17RKO animals were not just significantly heavier and bigger in size but much fatter, with lots of subcutaneous fat that was observed when dissecting the animals to liberate our tissues of interest. Ordinarily upon dissection when abdominal skin is removed the organs in the peritoneum can all be seen though

the thin peritoneum membrane. In the KO animals there were several layers of fat directly underneath the skin to penetrate before organs could be seen. It was initially supposed that as these mice were older this may be the reason for their size and weight increase. However, others have noticed fat and weight gain in IL-17R KO mice also [249] and this is discussed further in the discussion.

During *S. pneumoniae* induced acute pneumonia, the IL-17RKO mice became sick, albeit not sick as the WT and unfortunately we cannot make any assumptions of whether this was down to IL-17R absence or not, due to such discrepancies in size as the pathogen cfu may have a lesser effect in the larger mice which have larger lungs. The apparent clinical sickness of the IL-17RKO mice, however, allows us to interpret our immunological findings in relation to the infection in the mice, albeit perhaps not directly in relation to infection in the WT.

7.12 IL-17 signalling via IL-17R may induce inflammation in the lung during *S. pneumoniae* infection

The histology of these preliminary experiments shows that the WT mice infected with *S. pneumoniae* have large areas of inflammation in the lung parenchyma with gross inflammation at the visceral pleura as previously discussed. The histology of the IL-17RKO *S. pneumoniae* infected lung almost looks like a healthy lung, with large spaces and little inflammation in the tissue and at the visceral pleura (Figure 7-22).

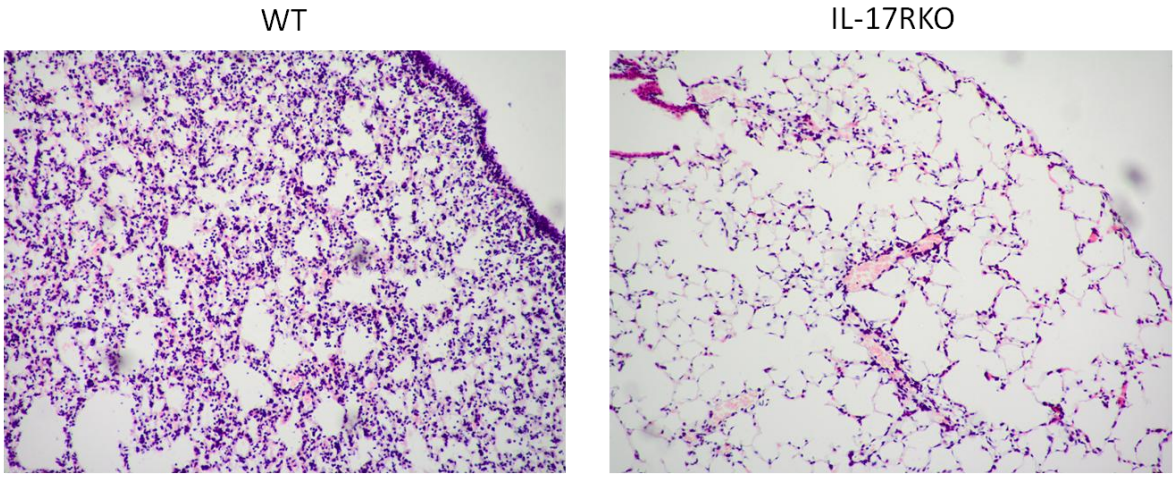

Figure 7-22 Histology of WT lung during *S. pneumoniae* infection is grossly inflamed whereas histology of IL-17RKO lung during the same infection is not
WT and IL-17RKO animals were infected with $5x10^6$ cfu *S. pneumoniae*, sacrificed at 48 hours and lung tissue was dissected and fixed in paraffin, before being sectioned and stained with H&E. magnification x40. . Pictures are representative of 3 individual animals per group. Data are representative of 1experiment.

This suggests a role for IL-17 signalling through IL-17R at inducing inflammation in both the parenchyma and at the visceral pleura in *S. pneumoniae* infection, as this inflammation is absent in IL-17RKO animals.

7.13 IL-17 and neutrophil recruitment in BAL in IL-17RKO animals

Previously we have observed considerable amounts of IL-17 in the BAL during *S. pneumoniae* infection (Figure 7-9), that are significantly increased from the control, appearing to correlate with an influx of neutrophils in this space (Figure 7-7C) indicating that IL-17 signalling may enhance neutrophil recruitment. Similar concentrations of IL-17 in the BAL were observed here during *S. pneumoniae* infection in the WT animals as we have observed before (Figure 7-9), with no significant difference observed in absence of IL-17R (Figure 7-23).

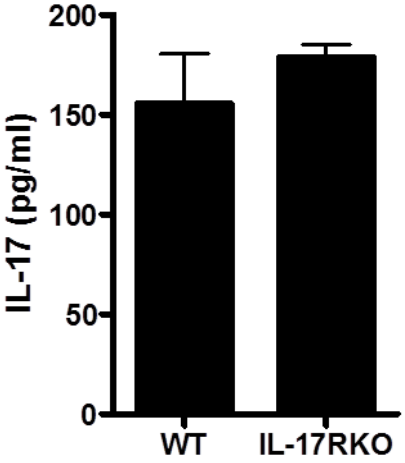

Figure 7-23 IL-17R does not regulate IL-17 concentrations in the BAL
WT and IL-17RKO animals were infected with $5x10^6$ cfu *S. pneumoniae*, and sacrificed at 48 hours. BALs were performed and analysed for IL-17 by ELISA.. All error bars represent mean ± SEM from 3 individual animals per group. Data representative of 1 experiment.

This indicates that IL-17 signalling via IL-17R may not regulate IL-17 secretion from cells in the lung during *S. pneumoniae* infection.

Although the two animal strains, WT and IL-17RKO, appear to secrete similar concentrations of IL-17 in the BAL (Figure 7-23), the cellular compositions in this space differ (Figure 7-24).

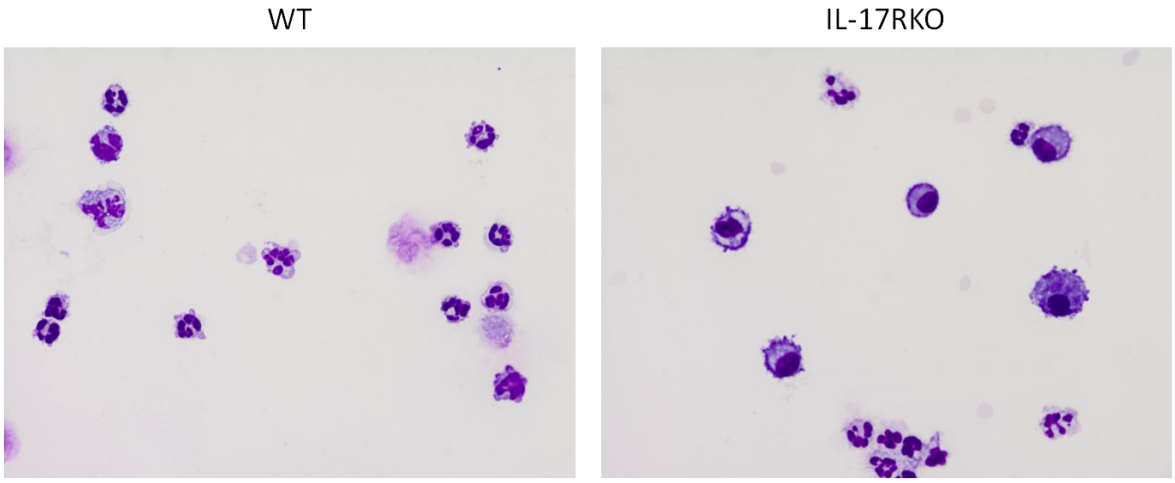

Figure 7-24 Cytospin of cells from BAL of WT and IL-17RKO lung during *S. pneumoniae* infection stained with rapid Romanowsky

WT and IL-17RKO animals were infected with $5x10^6$ cfu *S. pneumoniae*, sacrificed at 48 hours, and BALS were performed. Aliquots of the BAL were spun down onto slides and stained with rapid Romanowsky to show cellular differentiation of cell population. . Pictures are representative of 3 individual animals per group. Data are representative of 1 experiment.

In the WT animals neutrophils almost completely dominate the BAL during *S. pneumoniae* infection (Figure 7-24). However, during *S. pneumoniae* infection in the IL-17RKO animals although there is increased neutrophil numbers, there is also a definite macrophage presence (Figure 7-24). This neutrophil/macrophage mix may represent a less severe inflammatory cell infiltrate, which would correlate with reduced inflammation observed in the histology of the lungs in these animals (Figure 7-22). Fewer neutrophils in the BAL of IL-17RKO animals indicates that IL-17R signalling enhances neutrophil recruitment, further supporting our hypothesis that IL-17 signalling may aid in neutrophil recruitment in the lung during *S. pneumoniae* infection. However as these mice were larger and we are not clear of the extent of the infection due to the cfu that we have administered, further experiments will be required to confirm this finding.

7.14 IL-17 signalling via IL-17R may regulate immune cells in the pleural space during *S. pneumoniae* infection

7.14.1 Increased cells in the pleural space of IL-17RKO mice during S. pneumoniae infection

In *S. pneumoniae* infection in the WT animals an influx of cells has previously been observed in the pleural space as can be observed in Figure 7-11. We wished to investigate the cellular composition in this space during *S. pneumoniae* infection in the IL-17R KO mice, where there is a notable lack of inflammation at the pleural edge, thus investigating what role IL-17 and IL-17R may play at recruiting cells to this space during *S. pneumoniae* infection.

First we observe that there appears to be many more cells in the pleural space of the KO mouse, than the WT mouse (Figure 7-25).

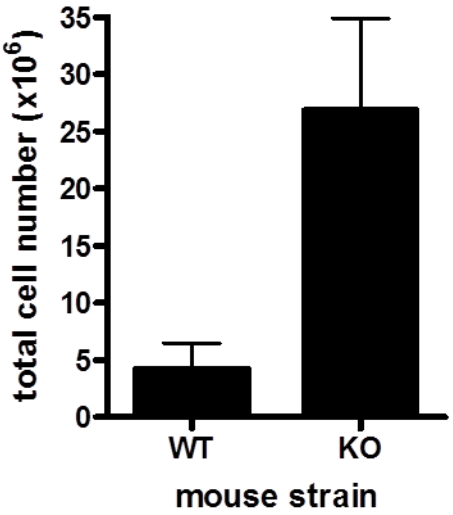

Figure 7-25 Cell numbers are increased in pleural space during *S. pneumoniae* infection in IL-17RKO animals

WT and IL-17RKO animals were infected with $5x10^6$ cfu *S. pneumoniae*, and sacrificed at 48 hours according to animal health. Pleural washes of the animals were performed, and cells were counted to obtain total cell counts. All error bars represent mean ± SEM from 3 individual animals per group. Data are representative of 1 experiment.

This is of interest as there is no inflammation at the pleural edge in IL-17RKO animals as there is in the WT (Figure 7-22). What are these cells and why would

there be more cells present in this space in the IL-17RKO animals if there is no inflammation at this site?

Upon analysis of cells in the pleural space by staining them use rapid Romanowsky, the cells in this space look to be mainly neutrophils as can be identified by their multi-nucleated appearance (Figure 7-26).

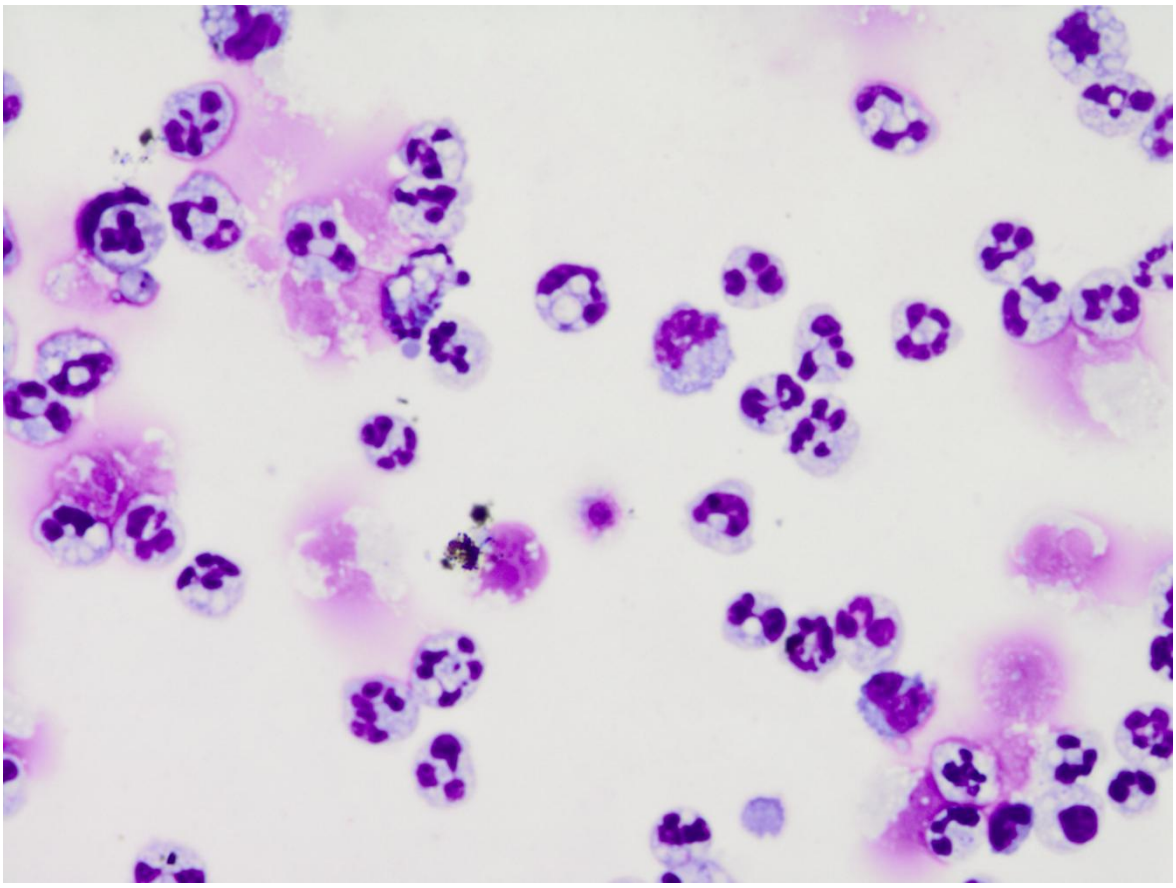

Figure 7-26 The pleural space of IL-17RKO animals is dominated by neutrophils during *S. pneumoniae* infection

WT and IL-17RKO animals were infected with 5x10^6 cfu *S. pneumoniae*, sacrificed at 48 hours Aliquots of the pleural wash from IL-17RKO animals were spun down onto slides and stained with rapid Romanowsky kit to show cellular differentiation of cell populations . Pictures are representative of 3 individual animals per group. Data are representative of 1 experiment.

Differential counts of cells were performed and percentages of macrophages and neutrophils were calculated. It appears as if the cellular infiltrate in the pleural space during *S. pneumoniae* infection in IL-17RKO animals is mainly neutrophils (Figure 7-27).

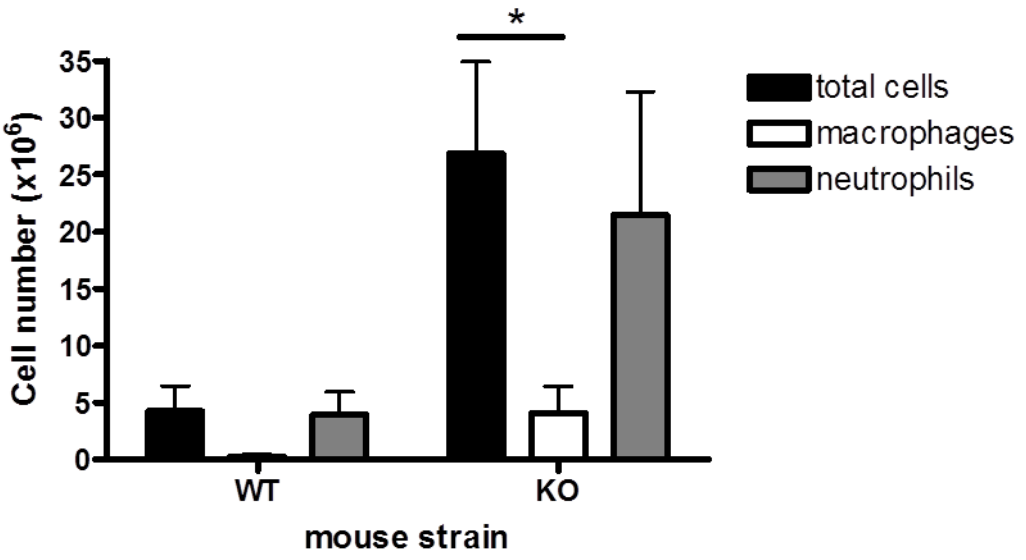

Figure 7-27 Neutrophils are increased in the pleural space during *S. pneumoniae* infection in IL-17RKO animals

WT and IL-17RKO animals were infected with $5x10^6$ cfu *S. pneumoniae*, and sacrificed at 48 hours according to animal health. Pleural washes of the animals were performed, and cells were counted to obtain total cell counts. Aliquots of the pleural wash were spun down onto slides and stained with rapid romanowsky to show cellular differentiation of cell population, and percentages of neutrophils and macrophages were counted and calculated. Pleural washes of both WT and IL-17RKO animals show mainly neutrophils, however the total number is significantly increased in IL-17RKO animals compared to WT animals. All error bars represent mean ± SEM from 3 individual animals per group. Data representative of 1 experiment. * $P < 0.05$

This observation is of interest as there is no inflammation at the visceral pleura during *S. pneumoniae* infection in IL-17RKO animals as observed by histology (Figure 7-22), and we believed that neutrophils would be responsible for this inflammation, as observed in WT animals during *S. pneumoniae* infection. Furthermore we believed that lack of IL-17R signalling would down-regulate recruitment of neutrophils, not increase it as has been observed here. This conflict of results is discussed further in the discussion.

7.14.2 IL-17R may regulate IL-17 concentrations in pleural space

We have previously observed small but significant concentrations of IL-17 in the pleural space during *S. pneumoniae* infection (Figure 7-14) and so we were interested to see if lack of IL-17R affected IL-17 concentrations in the pleural

space during this infection (Figure 7-28) as IL-17 secretion in the BAL appears to be IL-17R independent during *S. pneumoniae* infection (Figure 7-23).

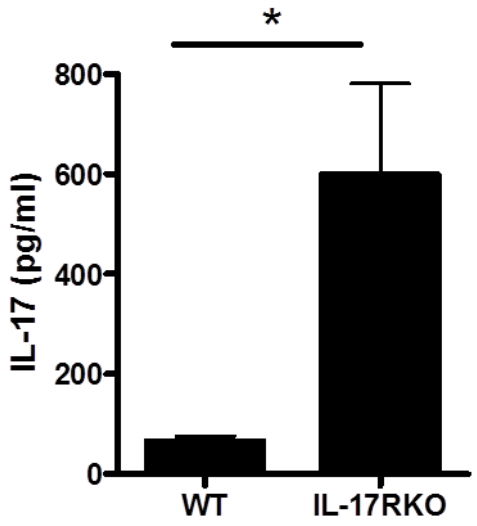

Figure 7-28 IL-17 is increased during *S. pneumoniae* infection in IL-17RKO animals
WT and IL-17RKO animals were infected with $5x10^6$ cfu *S. pneumoniae*, and sacrificed at 48 hours. Pleural washes were taken and analysed for IL-17 by ELISA. All error bars represent mean ± SEM from 3 animals. Data representative of 1 experiment. * $p< 0.05$.

From this preliminary experiment it appears that, unlike the BAL, presence of IL-17R regulates IL-17 concentrations in the pleural space as IL-17 concentration in the IL-17RKO mice are significantly larger from that during the same infection in the WT animals. This may indicate that IL-17 negatively regulates itself via signalling through IL-17R, or it could be that the same concentrations of IL-17 are produced regardless of presence or absence of IL-17R, but it is not consumed as it has no receptor to bind to. We did not investigate either of these theories and so cannot comment further.

7.14.3 IL-17R regulation of immune cells in pleural space

The pleural washes of these animals were analysed by flow cytometry to further investigate the cellular composition in this space and how it differed between WT and IL-17RKO animals during *S. pneumoniae* infection, so we may gain a better idea of the role of IL-17R, if any, in recruitment of cells to the pleural edge during this infection.

In the pleural space γδ T cells do not appear to differ between WT and IL-17R KO animals during *S. pneumoniae* infection (Figure 7-29A), indicating that γδ T cells are not regulated at this site by IL-17 signalling through IL-17R on cells. CD4+ cells increase from 68% in WT mice to 85% in IL-17RKO mice (Figure 7-29B). Increased CD4+ cells observed in IL-17R absence indicates that CD4+ recruitment in the pleural space in *S. pneumoniae* infection is regulated by IL-17 and IL-17R, with IL-17 signalling appearing to limit recruitment of CD4+ cells to this site. B cells increase from 22% in WT animals to 30% in the IL-17RKO animals, indicating that IL-17 presence may have a role in down-regulating B cell recruitment in the pleural space during *S. pneumoniae* infection. There is a small increase in the percentage of Gr-1 cells in this space in the absence of IL-17R with 28.2% Gr-1 cells observed in the WT animals and 34.1% in the IL-17RKO (Figure 7-29C). However, the absolute numbers of neutrophils as assayed by direct cell counting did show an increase (Figure 7-27).

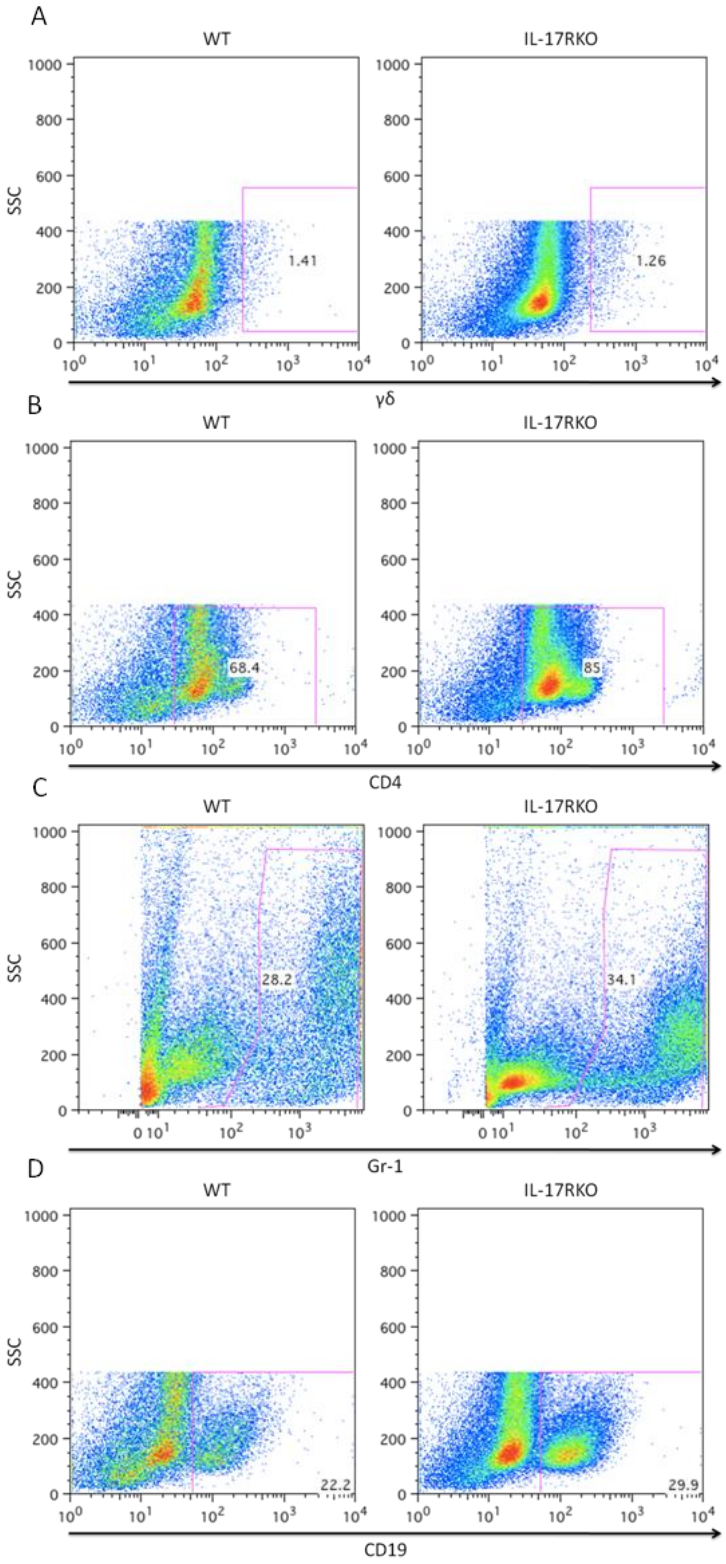

Figure 7-29 Immune populations in the pleural space of WT and IL-17RKO mice during *S. pneumoniae* infection

Lungs of WT and IL-17RKO *S. pneumoniae* infected mice were dissected and digested to obtain single cell suspensions. Cells were stained with CD4 (A), γδ (B) Gr-1 (C) and CD19 (D) and analysed by flow cytometry compared to their respective isotypes. Cells gated on live cells based on FSC and SSC with a larger gate set for Gr-1 analysis as described in materials and methods. Positive gates were set on isotypes for markers on cells isolated from *P. aeruginosa* infected animals. Dot plots represent data pooled from 3 animals. Data representative of 1 experiment.

For reasons unknown IL-17 could not be observed by flow cytometry and so the sources of IL-17, and if IL-17 producing cells differ at this site between the WT and IL-17RKO animals during *S. pneumoniae* infection, is presently unknown.

7.15 IL-17 signalling via IL-17R may regulate immune cells in the lung during *S. pneumoniae* infection

Infiltrate of immune cells in the lungs of IL-17RKO mice is not evident from the histology but analysis of the cells in the digested lung by flow cytometry show that immune cells are increased during *S. pneumoniae* infection in the IL-17RKO animals (Figure 7-30). Lungs of the WT and IL-17RKO animals infected with *S. pneumoniae* were digested to obtain single cell suspensions and stained for IL-17 investigate if IL-17 production differs between the WT and the IL-17RKO, and CD4 and γδ to indentify if these immune cells are sources of IL-17 in these animals. Looking at the surface markers, γδ T cells almost double (Figure 7-30A) and CD4+ cells appear to increase 30% in the IL-17RKO mice from WT (Figure 7-30B) indicating that IL-17 may down-regulate these populations to an extent, in the lung during *S. pneumoniae* infection.

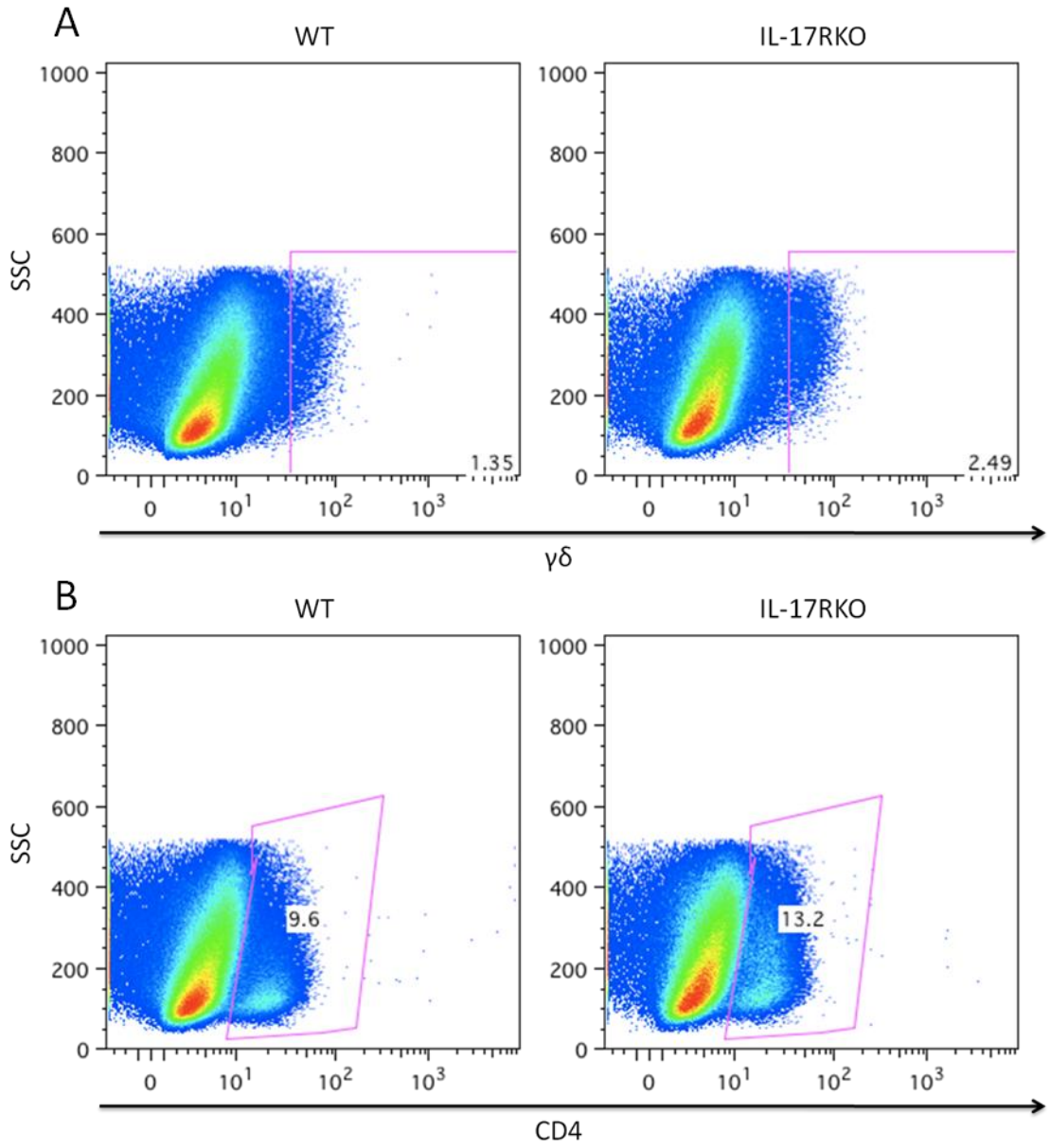

Figure 7-30 CD4 and γδ populations in WT and IL-17RKO mice during *S. pneumoniae* infection

Lungs of WT and IL-17RKO *S. pneumoniae* infected mice were dissected and digested to obtain single cell suspensions. Cells were stained for CD4 and IL-17, and analysed by flow cytometry compared to their respective isotypes. Cells gated on live cells based on FSC and SSC as described in materials and methods. Positive gates were set on isotypes for markers on cells isolated from *P. aeruginosa* infected animals. Dot plots represent data pooled from 3 animals. Data representative of 1 experiment.

The lung cells were also stained for Gr-1 to identify neutrophils, but the populations of Gr-1 in each animal did not greatly differ (data not shown). We have previously stated that Gr-1 may stain all granulocytes in the lung, and so use of this marker cannot give us any conclusions about neutrophil involvement in these infections, and so is not included in this thesis.

7.15.1 IL-17R negatively regulates IL-17+ cells in the lung during S. pneumoniae infection

Upon investigating the IL-17+ producing cells we see that IL-17 cell populations increase 1.7 times during *S. pneumoniae* infection in the IL-17RKO animals from WT (Figure 7-31).

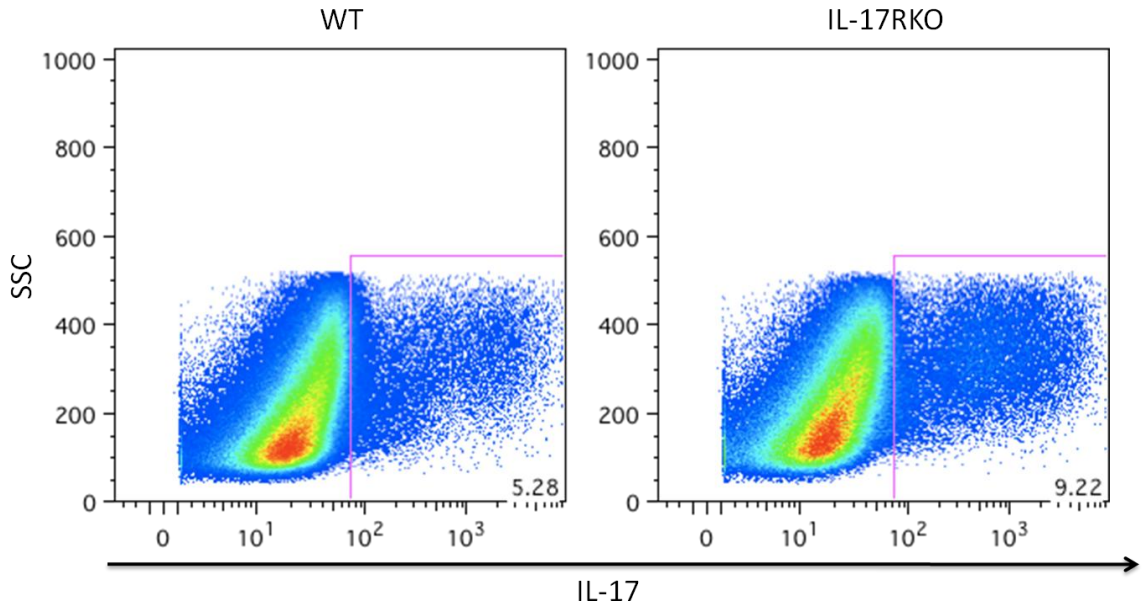

Figure 7-31 IL-17 producing populations are increased in *S. pneumoniae* infection in the absence of IL-17R

Lungs of WT and IL-17RKO *S. pneumoniae* infected mice were dissected and digested to obtain single cell suspensions. Cells were stimulated with PMA and ionomycin in the presence of brefeldin A for 5 hours then stained for IL-17, and analysed by flow cytometry compared to their respective isotypes. Cells gated on live cells based on FSC and SSC as described in materials and methods. Positive gates were set on isotypes for markers on cells isolated from *P. aeruginosa* infected animals. Dot plots represent data pooled from 3 animals. Data representative of 1 experiment.

As we observe increased IL-17+ cells in the IL-17RKO animals, compared to WT, we deduce that IL-17 signalling through IL-17R negatively regulates IL-17 producing cells, indicating IL-17 acts in a negative feedback loop on the cells that produce it. The sources of IL-17 in *S. pneumoniae* infection between the WT and IL-17R KO mice appear to be similar (Figure 7-32) just increased in the IL-17RKO mice, explaining the increased IL-17+ population observed in figure Figure 7-31.

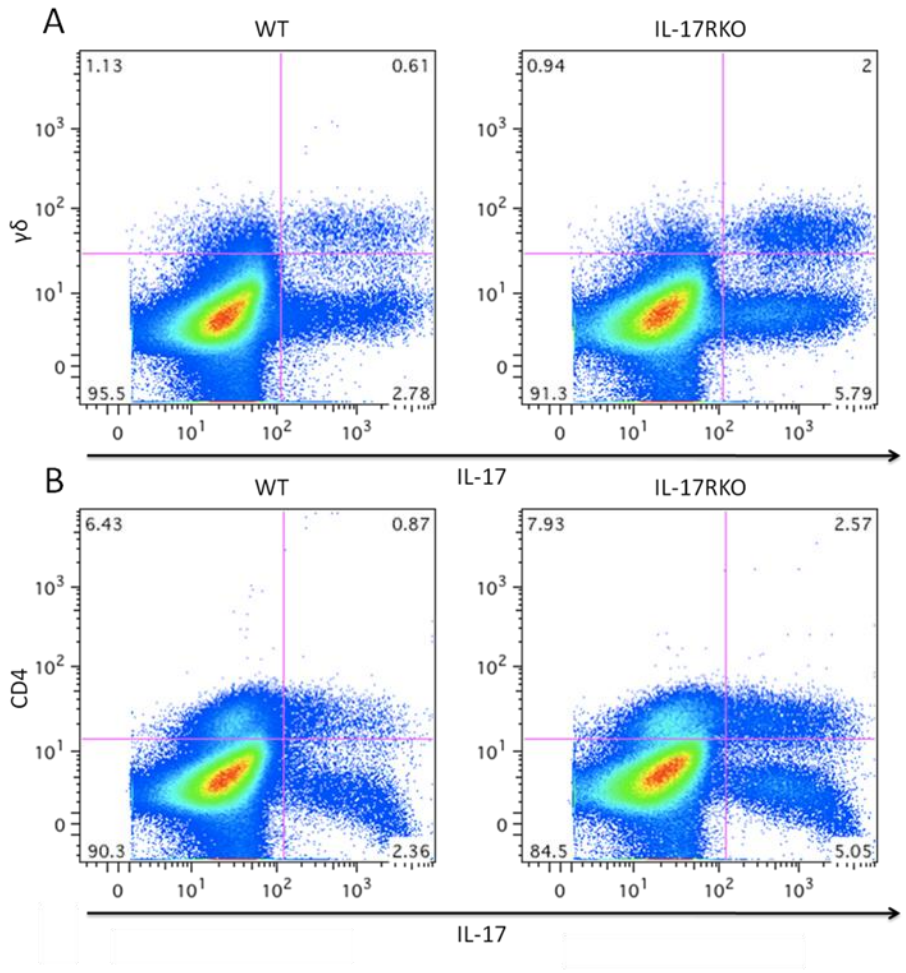

Figure 7-32 Sources of IL-17 during *S. pneumoniae* infection in WT and IL-17RKO mice
Lungs of WT and IL-17RKO *S. pneumoniae* infected mice were dissected and digested to obtain single cell suspensions. Cells were stimulated with PMA and ionomycin in the presence of brefeldin A for 5 hours then stained for CD4, γδ, Gr-1 and IL-17,and analysed by flow cytometry compared to their respective isotypes. Cells gated on live cells based on FSC and SSC as described in materials and methods. Positive gates were set on isotypes for markers on cells isolated from *P. aeruginosa* infected animals. Dot plots represent data pooled from 3 animals. Data representative of 1 experiment.

IL-17+ γδ T cells increase from 17% of IL-17+ cells in the WT animals to 25% of IL-17+ cells in IL-17RKO animals(Figure 7-32A). Th17 cells are responsible for 27% of IL-17 productions in the WT and this is increased to 34% in the IL-17RKO animals (Figure 7-32B). Gr-1 expressing cells were not investigated as a source of IL-17 as, has been discussed previously, it is an unsuitable marker for lung tissue staining.

7.16 Discussion

Pneumonia is induced in mice upon intranasal infection of both *P. aeruginosa* and *S. pneumoniae*. We have observed that these infections are similar in their response in some ways such as inflammation and haematomas, yet they differ in some distinct ways such as areas where inflammation is focussed and cell types responsible for IL-17 secretion in these infections.

Pneumonia has been observed as a haemorrhagic disease [240, 241] and this is evident in the lungs of the infected animals in our experiments. It can be observed by eye upon dissection and in the histology there is evidence in some areas, especially in the *P. aeruginosa* infection, of bloody infiltrate indicating that infection has ensued in the lungs of these animals. We therefore conclude that disease has developed in these animals and any following hypotheses we make may be genuine in reference to bacterial pneumonia infection in the lungs induced by these pathogens.

In both infections there is clear inflammation in the lung as evident from the histology of the tissues. In comparison to the control we observe great inflammatory cell infiltrate in both *P. aeruginosa* and *S. pneumoniae* infections which appears to be mainly neutrophil based upon further magnification, as identified by cells with multi-lobed, densely stained nuclei. Both infections show large areas of neutrophil based inflammation but sites where inflammation is concentrated varies between infections. In the *P. aeruginosa* infection, the inflammation appears to be all encompassing, most of the tissue appears to be heavily infiltrated with neutrophils and the open spaces of the bronchioles can no longer be observed. In the *S. pneumoniae* infection, a somewhat less severe infection in our experiment as measured by clinical observations, the inflammation appears to be focussed around the blood vessels and at the visceral pleura. This was observed in every individual *S. pneumoniae* infected animal in every experiment (N=12) indicating that it is a genuine response in these animals to *S. pneumoniae* infection in the lung. Extensive inflammation at the visceral pleura in *S. pneumoniae* infection and not *P. aeruginosa* infection, which we observed to be a clinically more severe infection, indicates a somewhat specific reaction to *S. pneumoniae* at the visceral pleura. Although during *P. aeruginosa* infection gross inflammation is not observed at the visceral

pleura, many immune cells are still found in the pleural space in both infections, as can be observed by the approximate 2-fold increase of cells at this site during infections. Analysis of cytospins of cells found in this space, stained with rapid Romanowsky to identify macrophages, neutrophils and lymphocytes, indicates that there is an influx of neutrophils in this space from control in both infections. So it appears that there is a similar number of cells in *P. aeruginosa* infection as there is in *S. pneumoniae* infection in the pleural space, and they both look to be dominated by neutrophils, yet only infection with *S. pneumoniae* shows inflammation at the visceral pleura. Why is this?

Upon further analysis of the cell population in the pleural space by flow cytometry, the percentage population of neutrophils, as identified by Gr-1 staining, appears to be only marginally increased from that of the control during *P. aeruginosa* infection, whereas the population of neutrophils in *S. pneumoniae* infection from control increases considerably. This result indicates that neutrophils are increased in the pleural space during *S. pneumoniae* but less so in *P. aeruginosa* infection and this would explain the gross inflammation observed in *S. pneumoniae* infection but not *P. aeruginosa* infection. Use of Gr-1 as a neutrophil marker is debatable, however cytospin analysis of cells confirms that there are many neutrophils in the pleural space during *S. pneumoniae* infection. Although total cell numbers were similar in the two different infections, the percentage of neutrophils was significantly higher in the *S. pneumioniae* infection (Figure 7-13).

During both infections the percentage of γδ T cells in the pleural space halves, but as there are double the amount of cells in the space during the infections, the total cell number of γδ T cells in the pleural space does not differ between uninfected control and both *P. aeruginosa* and *S. pneumoniae* infection. This implies that γδ T cells do not have a role in fighting *P. aeruginosa* and *S. pneumoniae* at this site, though they were observed to be a source of IL-17 in the lung tissue during *P. aeruginosa* infection as will be discussed later. CD4+ cell and B cell percentages are slightly reduced in the pleural space during the infections, but again as the total cell count in this space is increased 2-fold, even a small decrease in cell percentages still results in increased cell numbers of these populations in the pleural space during infection. It would thus appear that B cells and CD4+ cells are recruited to the pleural space during *P.*

aeruginosa and *S. pneumoniae* infection also, but do not play major roles as they do not represent major populations of cells at this site. Why is there such recruitment of immune cells to this site during these infections? Others in the research institute have observed with *S. pneumoniae* that there are many bacteria in the pleural space (unpublished data) that would correlate with the inflammation that we see. It is unclear how the pathogen reaches this space or the importance of this location in establishing infection. However, these data correlate closely with human lung infection with *S. pneumoniae*, where pleural involvement is common [250, 251].

We observed increased concentrations of IL-17 in the pleural space during both infections, more so in *P. aeruginosa* but this may just reflect the severity of this infection. Taking this observation of increased IL-17 concentrations during infection, along with the cytospin data where increased neutrophils were observed during both infections, one could suggest that increased IL-17 correlates with increased neutrophils in the pleural space, indicating that IL-17 aids in neutrophils recruitment.

No IL-17+ producing cells were observed in the pleural space by flow cytometry. It is unclear if this is a technical error or if the cells no longer produce IL-17. Staining for IL-17 on pleural cells was only performed on one experiment and so must be repeated before any assumptions about IL-17+ populations in the pleural space can be made. However neutrophils are known to be a source of IL-17 during airway inflammation [252, 253], and since we observe increased neutrophils and increased IL-17 in the pleural space during infection it is conceivable that a source of IL-17 may be neutrophils.

The BALs of these animals were also analysed. Cells in the controls appear to be composed of a macrophage and neutrophil mix as identified by rapid Romanowsky staining. During *S. pneumoniae* infection cell number in the BAL does not change but appears to be mainly composed of neutrophils when the lungs are infected. However, during *P. aeruginosa* infection, not only are cells in the BAL mainly neutrophils but there is an increase in cell number also. This indicates that the cellular milieu in the BAL changes upon bacterial infection, and during the more severe infection with *P. aeruginosa*, many more neutrophils are recruited. IL-17 is increased in the BAL during both infections, again more so

during *P. aeruginosa* infection. Increased IL-17 concentrations in the BAL and increased neutrophils as observed on the cytospins implies that IL-17 may be responsible for neutrophil recruitment at this site.

Looking at cells in the lung tissue, and so the site of infection, we see an increase in immune cell populations during both infections. γδ T cells appear to be increased during both infection and CD4+ cells seem to increase almost 2-fold during *P. aeruginosa* infection, but not so much during *S. pneumoniae* infection. We could not fully investigate neutrophil populations in the lung by flow cytometry as the selected marker for neutrophils Gr-1, which has been shown to be effective for identifying neutrophils in the blood by other groups, and in the pleural space as observed by us. However, the use of this marker as a marker for tissue neutrophils is flawed as all Gr-1+ cells which may contain alveolar monocytes/macrophages will be stained also [246]. Hence use of Gr-1 as a marker for neutrophils is the tissue was abandoned. Since these experiments were performed there has been development of a new antibody for identifying neutrophils by flow cytometry named 1A8. Gr-1 identifies both Ly-6G and Ly-6C, and thus may identify Ly-6C monocytic cells also. New clone 1A8 idenfies Ly-6G only which distinguishes neutrophils and thus use of this antibody is a consideration for furtue experiments. A further method to identify neutrophils are use of a myeloperoxidase (MPO) assay, which in future could be used to assay neutrophil accumulation in the tissue during *P. aeruginosa* and *S. pneumoniae* infection.

It is evident that infection with *S. pneumoniae* leads to a higher IL-17+ population than infection with *P. aeruginosa,* as total percent of IL-17+ cells during *S. pneumoniae* doubles from that of uninfected, whereas IL-17+ total population percentage with *P. aeruginosa* doesn't seem to vary from control. What does vary during *P. aeruginosa* infection is the shift in the cells that produce IL-17. During *P. aeruginosa* infection IL-17+ cells seem to shift from being from CD4+ cells as they are in the control, to γδ T cells, with 40% of the IL-17+ cells being γδ+ also. This indicates the presence of Th17 cells in the resting animal that can make IL-17 if stimulated. However upon *P. aeruginosa* infection, γδ T cells appear to be a predominant source of IL-17, with CD4+ IL-17+ cells decreasing. During *S. pneumoniae* infection, where we see increased IL-17+ cells, the main source of IL-17 is still unclear. We see an increase in Th17

cells, yet we did not see an increase in CD4+ cells indicating another shift of IL-17 production from control. However, it appears that the majority of IL-17+ cells in the lung are CD4-, γδ-, CD19- and Gr-1- (although we found use of Gr-1 to be dubious for tissue staining to represent neutrophils, we still show that our IL-17+ cells are Gr-1 and thus not neutrophils, alveolar monocytes/macrophages or any other Gr-1+ cell that may be in the lung). The main source of IL-17 during these infections thus remains unclear. We hypothesize that it must be some kind of memory or innate cell with the speed of response. We speculate, that IL-17 may be produced from lung epithelial cells, along with other pro-inflammatory cytokines such as IL-1β, IL-6 and TNFα [254].

A pitfall in our IL-17 analysis is the well documented use of polyclonal stimulation to induce the cells to secrete their cytokines so that they may be retained and analysed. This polyclonal stimulation is essentially allowing any cell that can and has ever been able to produce IL-17, once again produce it and be monitored. Thus, we will activate pre-existing IL-17 producing populations in the control animals. There have been suggestions of injecting the animals at the final stage of infection with brefeldin A, to capture cytokines intracellularly in the cells that are producing these cytokines during the assault, instead of non-specifically re-stimulating them. This would give a more accurate portrayal of what cells are producing IL-17 during infection, but will also be harmful to the animals and so must be tightly regulated. This is a consideration for future work.

The contribution of IL-17 signalling via its receptor IL-17R in controlling the immune response to *S. pneumoniae* infection in the lung was investigated during one experiment by use of IL-17R KO animals. These animals were deficient in IL-17RA and thus do not allow signalling of IL-17A or IL-17F. The IL-17RKO animals were a lot older than the WT animals, aged 16 weeks as opposed to 8 weeks. We acknowledge that using animals so different in age did not make an ideal experimental set up, but the previous *in vivo* infections had been continually set up with mice aged at 8 weeks and so these were the animals that were continually ordered and made available during experiments, and so were the only WT animals we had available when the IL-17RKO animals were available to us. As stated this was not a model set up as ideally animals should be age matched, however we considered that as both ages of animals are regarded as adults, there may not be too much immunological difference in response to the

pathogen and so decided to carry out a preliminary investigation with whatever resources were available to us. We observed that the IL-17RKO animals were much larger in size than we expected, and were twice the weight of the WT animals. As the IL-17RKO animals were older than the WT mice we expected a size difference. However, the IL-17RKO animals were much larger than we expected WT animal of 16 weeks to be. The size difference between these animals did not appear to be the normal difference that would have been observed between older and younger WT animals. A consequence of this size is the number of pathogens given to the mice may have a lesser effect on these animals before immunological differences are even taken into account. The lungs of the larger IL-17RKO mice are proportionally bigger, and so one would suppose that a cfu of $2x10^7$ would have a lesser effect in these animals as relatively there is a lower multiplicity of infection (MOI), compared to lungs of smaller animals. Using this infection of $2x10^7$ cfu the IL-17RKO mice still succumbed to infection to a point and displayed clinical manifestations of sickness such as lethargic behaviour, staggered gait and matted coats. This infection was not as severe as that of the WT animals with the same *S. pneumoniae* infection where animals displayed the same symptoms that were more pronounced. However, we conclude that infection has ensued in the IL-17RKO animals and therefore any observations we made were in response to *S. pneumoniae* infection.

Upon dissection it was observed that the large IL-17RKO mice had abundant subcutaneous fat. Older WT animals are generally found to be larger in size but not fatter. Other studies have found that IL-17RKO mice are heavier and fatter than WT mice [249]. It appears that IL-17 down-regulates leptin [249], a hormone that negatively regulates body weight [255]. High levels of plasma leptin correlates weight gain and conversely low levels of plasma leptin correlate with weight loss [255]. Thus if IL-17 down-regulates leptin as the study claims [249], IL-17RKO animals would have higher levels of leptin, as IL-17 is not signalling via IL-17R and down-regulating leptin production. Higher leptin levels correlates with weight gain which we observe in these IL-17RKO animals. Another study with IL-17KO mice further confirms the regulatory qualities of IL-17 with reference to adipose tissue formation claiming IL-17 inhibits adipogenesis [256]. This is further proof that IL-17 signalling, through IL-17R,

regulates fat deposition in mice and thus animals lacking in this signalling will be overweight with fatty deposits which we observe. This massive size and weight difference could affect the response to pathogen and so it is suggested that in future studies mice not be age matched but perhaps size and weight matched, to give a better idea of how these animals respond to *S. pneumoniae* and the contribution of IL-17 and IL-17R during this infection. An alternative would be to work out a model for cfu of pathogen per gram of weight to obtain similar levels of sickness.

It was hypothesised that IL-17, and its interaction with IL-17R, is a key source of downstream neutrophil recruitment during bacterial pneumonia and thus lack of IL-17R would lead to lack of neutrophils at the site of infection. This can be observed in the histology of the IL-17RKO lung where there is greatly reduced inflammation in the lung and we no longer see the gross inflammation that can be observed at the visceral pleura in *S. pneumoniae* infection in WT mice, thereby indicating that the hypothesis may be true, as we have shown that neutrophils contirubte greatly to this gross inflammation. However, this lack of neutrophils and inflammation in the infected IL-17RKO mice is interesting as the animals were clinically unwell, as measured by clinical parameters, but no more unwell than the WT. One would perhaps predict that in a setting where there is a lack of neutrophils, there would be inadequate fighting of infection and therefore the animal would be sicker as infection develops, but we do not see this. We observe that IL-17RKO mice are not as sick as the WT infected mice, and this would suggest that there may be other immune cells present at the site of infection to fight pathogen and to stop the mouse succumbing to infection, but we do not see this in the histology. We in fact see less cellular infiltrate in the IL-17RKO mice. The theory that cells have compensated for the lack of neutrophils in fighting this infection may be backed up by the observation that although there is no inflammation in the lung tissue with regards to morphology, more immune cells can be observed in the lung tissue when it is digested as observed by flow cytometry. Perhaps CD4+ memory cells and γδ T cells have compensated here, as we see an increase in both these populations in the lungs of IL-17RKO animal from the WT animal during *S. pneumoniae* infection. What is notable is that we see fewer neutrophils and less inflammation in the tissue as a consequence in the IL-17RKO animals, as viewed by histology of the lung tissue,

but there appears to be more neutrophils in the pleural space of these mice during *S. pneumoniae* infection. This is observed by flow cytometric analysis of cells in the pleural space obtained by pleural wash, and rapid Romanowsky staining of these cells also. Both techniques display neutrophil influx in this space in the IL-17RKO animals that is not observed in the lung tissue. This implies that the neutrophils are still present in the pleural space, but they are not migrating and causing inflammation in the lungs of the IL-17RKO animals. This suggests that IL-17 signalling is important in migration of neutrophils into lung but not pleural space. The reasoning for the flux of neutrophils into this space is not clear. It is possible that pleural cells subsequently migrate to the lung, however it is not common for cells to migrate into a tissue in such a manner, and so the direction of neutrophil flux will need to be examined in further experiments. Thus the role of IL-17 signalling in neutrophil recruitment in the lung and surrounding areas during *S. pneumoniae* infection is still unclear.

The histology and lack of neutrophils in the lung correlates with our hypothesis that IL-17 signalling via IL-17R may be responsible for neutrophil recruitment. But we also imagined these mice to be sicker which we do not witness, suggesting that perhaps neutrophils contribute to decline in host health with regards to lung function during acute infection. These conflicting results require further experimentation.

In conclusion, we see that sources of IL-17 differ during *P. aeruginosa* and *S. pneumoniae* infection, with γδ T cells appearing to be a key source of IL-17 during *P. aeruginosa* infection and CD4+ cells appearing to more important during *S. pneumoniae* infection then *P. aeruginosa,* although the main source of IL-17 during these infections seems to be γδ-, CD4- and Gr-1-. Further investigation is required to see what the main source of IL-17 is during *P. aeruginosa* and *S. pneumoniae* infection. The sites of inflammation during these infections differ with inflammation of whole lung observed during *P. aeruginosa* infection, but localized inflammation at the visceral pleura during *S. pneumoniae* infection. Why these pathogens differ in their location of inflammation and why *S. pneumoniae* has such a specific effect at the pleural surface is unclear and will be an interesting subject of future investigations.

Our preliminary experiment suggests that our hypothesis that IL-17 signalling via IL-17R may be responsible for neutrophil recruitment in the lung during this infection may be true, as reduced neutrophils are observed in the lung by histology and analysis of the BAL. Furthermore IL-17 and IL-17R signalling looks as if they may regulate other immune cells such as CD4+ cells and IL-17 production in the lung during *S. pneumoniae* infection. However, this must be fully investigated with age matched studies where the pathogen delivers a similar level of infection that can be directly compared, before any firm conclusions can be made about the role of IL-17 and IL-17R in the lung during this infection.

Overall our preliminary experiments investigating *in vivo* infection with *P. aeruginosa* and *S. pneumoniae* in mice give us good cause to proceed with the experiments and investigate our observations further in greater depth.

8 General Discussion

The main hypothesis of our research was that Th17 cells may be a major source of IL-17 in response to infections with the bacterial pathogens *P. aeruginosa* and *S. pneumoniae*, that IL-1β may influence induction of these Th17 cells during infection, and that IL-17 secreted by these cells may be a key attractant of neutrophils in the lung during bacterial pneumonia.

The results presented in this thesis indicate that *P. aeruginosa* and *S. pneumoniae* infect DCs to induce cytokines that may influence production and maintenance of Th17 and other IL-17 producing T cells during these infections, such as IL-1β, with *P. aeruginosa* infection of DCs also leading to production of Th17 maintenance cytokine IL-23.

Our primary aim with the *in vitro* experiments was to investigate the contribution of IL-1β in Th17 induction from naive CD4+ T cells. Our original strategy to investigate IL-1β contribution was to use 2 strains of each pathogen, one that allowed IL-1β secretion from DCs, and one that does not. In the case of *P. aeruginosa*, PA103 ΔUΔT is a strain that allows IL-1β secretion from DCs due to possesion of a fully functional T3SS that leads to activation of the inflammasome and IL-1β secretion. *P. aeruginosa* strain PA103 ΔpcrV does not allow IL-1β production from DCs as it lacks a functional T3SS, and so does not activate the inflammasome. We confirmed that these strains do and do not produce IL-1β from DCs respectively after 90 minutes infection before addition of antibiotics and incubation for 2 hours. However we found this difference in IL-1β secretion between these strains to be time dependent, as cells allowed to incubate overnight after antibiotic addition to allow full up-regulation of co-stimulatory molecules, produce IL-1β upon infection with both *P. aeruginosa* strains. So it appears that other factors, that we have not investigated, activate the inflammasome and lead to IL-1β release from the DCs infected with PA103 ΔpcrV when incubated overnight after antibiotic addition. Thus these *P. aeruginosa* strains cannot be used in our experimental set up to demonstrate the contribution of IL-1β absence and presence.

In the case of *S. pneumoniae*, we use strains D39 WT and a PLY deficient mutant of this D39 ΔPLY and found that, as has been suggested [37, 38], PLY is important at induction of significant IL-1β secretion from DCs and so the D39 ΔPLY represents our IL-1β absence situation. The PLY deficient mutant did not

completely abrogate IL-1β secretion from DCs but we do see that IL-1β secretion during infection of DCs with the PLY competent D39 WT is significantly higher. Use of *S. pneumoniae* and the differences observed in IL-1β production from the DCs indicate that, upon co-culture of naive CD4+ T cells with DCs infected with the *S. pneumoniae* strains, IL-1β does not appear to make a significant contribution to Th17 induction from naive T cells. We confirmed this by culture of naive CD4+ cells with Th17 inducing cytokines TGFβ and IL-6, set up in the presence of absence of IL-1β. We observed Th17 populations from naive CD4+ T cells both in IL-1β absence and presence, further indicating that IL-1β is not necessary for Th17 induction from naive CD4+ T cells. However, addition of IL-1β does appear to increase Th17 populations, and so although not important for Th17 induction, appears to have roles at enhancing these populations.

We found that an important source of IL-17 during *P. aeruginosa* infection was γδ T cells, which contaminated the MACS isolated CD4+ T cell preparation from mice. These cells produced IL-17 in a rapid IL-23 dependent, IL-1β independent manner. We found IL-23 was produced by DCs infected with *P. aeruginosa* but not *S. pneumoniae* in what is believed to be a TLR4 dependent manner [49], which may be backed up by the fact that *P. aeruginosa* possesses TLR4 ligand LPS and *S. pneumoniae* does not. However, *S. pneumoniae* toxin PLY has been claimed by some groups to be a TLR4 ligand [39], yet we do not see IL-23 production from DCs infected with PLY competent D39 WT, and so this questions the role of PLY as a TLR4 ligand, or the TLR4 dependence of IL-23 secretion, and must be investigated further.

We hypothesised that Th17 cells may be a major source of IL-17 during bacterial pneumonia, yet we find that the majority of IL-17+ cells during *P. aeruginosa* and *S. pneumoniae* infection are CD4-. Not only this, but they are mainly γδ- and Gr-1- indicating that the main source of IL-17 during these infections are not γδ T cells nor neutrophils. However, γδ T cells appear do to be a key source of IL-17 during infection with *P. aeruginosa*, as we see a shift in IL-17 production during *P. aeruginosa* infection to γδ+IL-17+ cells from CD4+ Th17 cells that are observed in healthy control animals. We hypothesise that the main source of IL-17 in the lung during this infection could be non-immune cells such as epithelial cells, as these cells may recognise antigen directly as they have PRRs on their surface [59] and are known to secrete other cytokines such as IL-1β, IL-6 and

TNFα [254]. Therefore in future experiments we believe epithelial cells should be investigated during bacterial pneumonia alongside immune cells and innate lymphoid cells.

We observe that both *P. aeruginosa* and *S. pneumoniae* infection induce pneumonia that manifest itself in haematomas, inflammation and neutrophil influx. A key difference in the lung inflammation in these infections is that *S. pneumoniae* infection, which manifested as a less severe infection clinically, has gross inflammation at the edge of the visceral pleura of the lung that is not observed in clinically severe *P. aeruginosa* infection. Preliminary experiments suggest that this inflammation may be IL-17 dependent as during *S. pneumoniae* infection in IL-17RKO animals this gross inflammation at the pleural edge was absent. Upon further inspection it appears as if neutrophils may be responsible for this inflammation at the pleural edge as is witnessed by histology, and as this is absent in IL-17RKO, this suggest that neutrophil recruitment in the lung during *S. pneumoniae* infection is enhanced by IL-17 and IL-17R signalling. However, our preliminary experiments imply neutrophils are enhanced in IL-17RKO animals in the pleural space during *S. pneumoniae* infection. This indicates that the role of IL-17 signalling in neutrophil recruitment may differ at different sites during bacterial pneumonia, and this is of great interest to future studies. It has also brought attention to the pleural space as an area to investigate in studies of bacterial pneumonia. Usually the BAL is used as an illustration of what is going on inside the lung during disease. The pleural wash is a wash of the pleura that surrounds the lung and this space is not usually investigated during *in vivo* bacterial infection models. However, our data suggests that this area could be of great interest and we suggest that others in the future consider this site also.

Taken together the results presented in this thesis indicate that IL-1β is not required for induction of Th17 cells during *P. aeruginosa* and *S. pneumoniae* infection, yet it may enhance Th17 populations during these infections *in vitro*. γδ T cells are confirmed to be a prolific source of IL-17 and produce the cytokine in an IL-23 dependent manner, independent of IL-1β. We show that bacterial pneumonia in the lungs differs with Gram-negative and Gram-positive pathogens in their sites of inflammation, with interest in further studying the pleural space in *S. pneumoniae* infection as this is a site of gross inflammation that has not as yet been thoroughly investigated. Preliminary data hints to the role of IL-17 in

neutrophil recruitment in the lung, but again must be investigate much more thoroughly before any conclusions can be made.

While these observations of the role of IL-17 and sources of IL-17 during *P. aeruginosa* and *S. pneumoniae* infection are informative, they are merely a basis for the in depth study of IL-17 cells and their roles in respiratory pneumonia. Future studies must further investigate all sources of IL-17 during pneumonia with these pathogens and focus on the pleural space as an area of interest alongside the BAL and lung tissue itself, to give a better idea of what is occurring at this site during infection. Further studies with IL-17RKO mice must be performed to affirm the role of IL-17R signalling during pneumonia, with care and attention taken to age match and size match the animals for comparison of infection to infection in WT animals. Additionally it would be of interest to investigate the histology of lung inflammation during infection with another Gram-positive pathogen, such as *Staphylococcus aureus* another causative agent of bacterial pneumonia, to investigate if inflammation at the pleural edge is a Gram-positive pathogen quality or a sole feature of *S. pneumoniae* infection. It is also of interest to investigate the role of IL-1β during Th17 induction *in vivo* as this may be different to its role, or lack thereof, during Th17 induction *in vitro*. This could be investigated with use of IL-1β KO animals, and investigation of Th17 and IL-17 secretion cells responses during infection.

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