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Activation and function of mucosal $\gamma\delta$ T cells

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B.A. (Mod) Biochemistry with Immunology



A thesis submitted to

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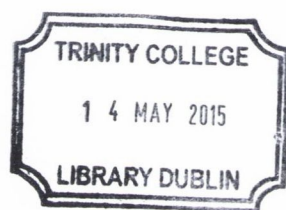
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2015



Thesis 10530

Declaration of Authorship

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ALICJA MISIAK

Abstract

$\gamma\delta$ T cells are innate-like lymphocytes characterised by rapid activation following infection or tissue injury. They are enriched in the skin and epitheliae of various organs, where breach of the barrier is likely to occur. $\gamma\delta$ T cells appear to segregate into functional subsets according to the TCR they express and cells with specific V γ V δ combinations preferentially home to particular sites in the body. While a lot is known about peripheral $\gamma\delta$ T cells, very little work has been done on tissue resident $\gamma\delta$ T cells, which are more likely to play a role in the initiation phase of the immune response.

The current study investigated the activation requirements of gut and lung resident $\gamma\delta$ T cells in comparison to their peripheral counterparts. The immune responses of lung $\gamma\delta$ T cells and their role in protection against *B. pertussis* infection were also investigated. *Bordetella pertussis* is a Gram negative bacterium which causes whooping cough, a severe respiratory disease in children, the incidence of which is increasing despite widespread use of vaccination.

Total intestinal intraepithelial lymphocytes (IEL) cells were found to produce IL-22 and IFN γ but no IL-17 in response to stimulation with IL-1 β +IL-23. However, in contrast to lymph node cells, $\gamma\delta$ IEL did not express IL-1, IL-18 and IL-23 receptors and consequently failed to respond to stimulation with IL-1 β +IL-23 or IL-18+IL-23. Furthermore, the majority of $\gamma\delta$ IEL were found to be CD27⁺ suggesting they are likely to secrete IFN γ upon activation.

Lung $\gamma\delta$ T cells, similar to lymph node $\gamma\delta$ T cells, were found to respond strongly to stimulation with IL-1 β +IL-23 to produce IL-17 and IL-22. The responding subsets of $\gamma\delta$ T cells were V γ 4 and V γ 6, with V γ 6 cells accounting for the majority of IL-17⁺IL-22⁺ double positive cells. In line with the strong IL-17 responses, the majority of lung $\gamma\delta$ T cells lacked the expression of CD27. Lung, but not lymph node or spleen, $\gamma\delta$ T cells produced IL-17 in response to live (LBP) and to a lesser extent to heat killed *B. pertussis* (HKBP). V γ 6 $\gamma\delta$ T cells were found to be the major producers of IL-17 in response to stimulation with LBP. Caspase 1 inhibitor strongly suppressed LBP-induced IL-17 production by $\gamma\delta$ T cells in the lung. Furthermore, $\gamma\delta$ T cells from IL-1R1^{-/-} mice failed to respond to LBP stimulation indicating that IL-17 production by

these cells is absolutely dependent on IL-1RI signalling. Lung $\gamma\delta$ T cells were found to produce IL-17 in response to supernatants from LBP-stimulated lung and spleen APC. However, no IL-23 was detected in APC supernatants suggesting that LBP-induced IL-17 production may be IL-23 independent.

$\gamma\delta$ T cells were found to secrete IL-17 at the early stages of *B. pertussis* infection, before the appearance of antigen-specific Th17 cells. Both V γ 4 and V γ 6 $\gamma\delta$ T cells contributed to IL-17 production in the lungs of infected mice. IL-22 production was found to peak in the lungs on day 7 post infection and $\gamma\delta$ T cells and other innate-like lymphocytes contributed to its production. IL-17 and IL-22 were found to contribute to the control of *B. pertussis* growth and its clearance from the lungs. Furthermore, IL-22^{-/-} mice had more severe inflammation and greater IL-17 responses suggesting that IL-22 may have an anti-inflammatory role in the lung during *B. pertussis* infection. Lung V γ 4 $\gamma\delta$ T cells from infected, but not naive mice were found to produce IL-17 in an antigen-specific manner. This correlated with enhanced IL-17 production by $\gamma\delta$ T cells in the lungs of convalescent mice upon reinfection.

Taken together, the current study has shown that $\gamma\delta$ T cells have differential capacity to respond to proinflammatory stimuli depending on the tissue in which they reside. Lung resident $\gamma\delta$ T cells were found to mount innate and memory IL-17 responses against *B. pertussis*. Further studies are required to clarify the contribution memory $\gamma\delta$ T cells to protective immunity against *B. pertussis*. The mechanism of IL-22 mediated protection should also be investigated.

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Publications

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Ross PJ, Sutton CE, Higgins S, Allen AC, Walsh K, **Misiak A**, Lavelle EC, McLoughlin RM, Mills KH. Relative contribution of Th1 and Th17 cells in adaptive immunity to *Bordetella pertussis*: towards the rational design of an improved acellular pertussis vaccine. *PLoS Pathog*. 2013 Apr;9(4):e1003264. doi: 10.1371/journal.ppat.1003264. Epub 2013 Apr 4.

Abbreviations

ACT	adenylate cyclase toxin
AhR	aryl hydrocarbon receptor
APC	antigen presenting cell
<i>B. pertussis</i>	<i>Bordetella pertussis</i>
BCR	B cell receptor
BrfA	Brefeldin A
CD	cluster of differentiation
CpG	Cytosine-guanine dinucleotide
DAMP	danger-associated molecular pattern
DC	dendritic cell
ELISA	enzyme-linked immunosorbent assay
FACS	Fluorescent-activated cell sorting
FCS	foetal calf serum
FICZ	6-formylindolo[3,2-b]carbazole
FMO	fluorescence minus one
FoxP3	Forkhead box P3
HKBP	heat killed <i>B. pertussis</i>
IBD	inflammatory bowel disease
IFN	interferon
IL	interleukin
iono	ionomycin
LBP	live <i>B. pertussis</i>
LG	lung
LN	lymph node
LPS	lipopolysaccharide
MACS	magnetic activated cell sorting
MHC	major histocompatibility complex
mono	monocyte
MØ	macrophage
NK	natural killer
Pa	acellular pertussis vaccine
PAMP	pathogen associated molecular pattern
p.i.	post infection
PBS	phosphate buffered saline
PMA	phorbol myristate acetate
PRR	pattern recognition receptor
PT	pertussis toxin
Pw	whole cell pertussis vaccine
RA	retinoic acid
RNA	ribonucleic acid
ROR	retinoic acid receptor-related orphan receptor

RT-PCR	real time polymerase chain reaction
STAT	signal transduced and activator of transcription
sup	Supernatant
Tbet	T box expressed in T-cells
TCR	T cell receptor
TGF	transforming growth factor
Th	T helper
TLR	toll like receptor
TNF	tumour necrosis factor
Treg	regulatory T cell

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Chapter 1: General introduction

Chapter 1: General introduction

The immune system functions to protect our bodies against the pathogenic insult. It is commonly divided into innate and adaptive arm. The innate immune response is characterised by prompt activation but relatively low specificity towards the eliciting agent. The adaptive response, on the other hand, takes longer to develop but is highly specific and results in generation of a long lasting memory which will allow for a quicker pathogen clearance on subsequent encounter. However, the type of the adaptive immune response and its strength is dictated by signals coming from the innate immune cells.

The majority of studies investigating immune responses during infectious and non-infectious diseases focus primarily on peripheral blood, splenic and lymph node cells. While the differences in the surface markers expressed by those cells and the cytokines they produce can be a useful read-out for disease progression or the effectiveness of a treatment, they are often not reflective of the responses that take place in the tissues, such as lung, liver and intestines. Furthermore, the type of response that needs to be induced may not be the same in the periphery and in tissues. The cells that first come in contact with pathogens are the mucosal innate and adaptive immune cells as well as epithelial cells. The inflammatory mediators these cells produce play an important role in shaping the overall immune response and consequently the outcome of the disease. Therefore, it is important to understand how these cells respond to infection and other perturbations of the homeostatic state.

The major effector cells of the innate immune system are phagocytic cells, including dendritic cells (DC), macrophages and neutrophils. These cells survey the body and ingest and destroy the pathogen as well as produce proinflammatory cytokines. These in turn activate and attract other cells to the site of infection. The pathogens usually gain entry to the body via mucosa of various organs. Barrier breach results in rapid activation of the local innate immune system. Innate immune cells recognise molecules conserved among different classes of pathogens (PAMPS; pathogen associated molecular patterns) and host derived danger signals (DAMPS; danger

associated molecular patterns) by means of germline encoded pattern recognition receptors (PRRs). When PRR signalling is triggered in an antigen presenting cell (APC) it induces upregulation of genes required for efficient antigen presentation and T cell priming such as MHC class I and II and the costimulatory molecules CD86/86. In addition, PRR stimulation induces APC to secrete various cytokines and chemokines which leads to recruitment of other cell types to the site of infection. In addition to phagocytes, many other cell types, including NK, NKT cells and $\gamma\delta$ T cells, play an important role in the early immune responses. These cells become activated by cytokines produced by the APC or directly by innate receptors expressed on their surface. Cytokines produced by APC and other innate-like cells decide the type of adaptive immune response that will follow.

The adaptive response offers an advantage of a highly specific protection and immunological memory. This means that a given pathogen is very unlikely to cause a disease in the same host twice as the immune response is much faster and specific upon subsequent encounter of that same pathogen, a fact exploited in vaccination. The adaptive immune response relies on the function of T and B cells, which express rearranging, antigen-specific receptors, which in theory are capable of recognition of an infinite number of antigens. However, although the specificity and strength of the adaptive immune response depend on the affinity of T and B cell receptor for their cognate antigens, the nature of the response is largely dictated by the signals from innate immune cells. Furthermore, different local and systemic immune responses may be required to clear any given pathogen. It is crucial to understand what type of response is beneficial in a particular disease setting and how one can go about inducing it systemically or locally.

1.1 T helper cells

$\alpha\beta$ T cells become activated when they encounter their cognate antigens in the context of MHC molecules. The cytokine milieu generated by the APC polarises T helper cells into Th1, Th2 Th17 or Treg phenotypes. T helper cells are subdivided into these subsets based on effector molecules they secrete as well as the transcription factors they express (Zhu & Paul, 2008). Th1 cells secrete IFN γ and are important for defences against intracellular pathogens. Naive T cells differentiate into

Th1 cells in the presence of IL-12 and IFN- γ (Hsieh et al, 1993). IFN- γ secreted by the cells of the innate immune system induces a Th1 specific transcription factor T-bet, in naive T cells. Activation of T-bet induces IFN- γ secretion by T cells as well as expression of IL-12R β 2 chain, which makes the cells responsive to IL-12. Stimulation by IL-12 leads to activation of STAT-4, another transcription factor specific for this lineage, which further upregulates IFN γ secretion and thus strengthens the Th1 commitment (Murphy & Reiner, 2002). Cytokines produced by these cells are important for macrophage activation and cytotoxic T cell memory. Inappropriate Th1 responses contribute to pathology of autoimmune disorders such as rheumatoid arthritis or multiple sclerosis, as well as allergic disorders such as delayed-type hypersensitivity (DTH) and contact hypersensitivity (CHS) (Bartunkova et al, 2009; Ishizaki et al, 2007).

Th2 cells are important for humoral responses and mediate protection against helminth parasites. They are characterised by secretion of cytokines such as IL-4, IL-5 and IL-13 and are induced to differentiate when IL-4 is present (Le Gros et al, 1990). IL-4 signalling leads to activation of Th2 specific transcription factors STAT-6 and GATA-3 which induce IL-4 secretion in T cell and thus further strengthen the lineage commitment (Zhu & Paul, 2008). Immunopathologies associated with inappropriate Th2 responses include atopic dermatitis and airway hyperactivity (Bartunkova et al, 2009).

Th17 cells secrete IL-17A, IL-17F, IL-21, IL-22, TNF- α and GM-CSF. Naive T lymphocytes differentiate into Th17 cells in the presence of TGF- β and IL-6 or IL-1 β and IL-23. ROR γ t is the transcription factor specific for this lineage (Bettelli et al, 2006; Ivanov et al, 2006). Th17 cells are thought to mediate protective immunity against extracellular bacteria and fungi but have also been implicated in causing the pathology of inflammatory and autoimmune diseases, alongside Th1 cells, and in some disease models were found to be the prevalent pathogenic cell type (Korn et al, 2007; Zhu & Paul, 2008).

Finally, Treg cells play an important role in maintenance of homeostasis, limiting the collateral damage associated with inflammation and preventing autoimmunity. Two sorts of inducible regulatory T (Treg) cells can be distinguished based on their production of TGF β or IL-10. Their differentiation is induced in the periphery in

response to the same cytokines and FoxP3 is the transcription factor characteristic for this lineage. However, FoxP3 negative cells producing Treg cytokines have also been observed. Moreover, IL-10 production can be induced in CD4⁺ cells in response to TGFβ, IL-27 and IL-10 itself (Zhou et al, 2009; Zhu & Paul, 2008).

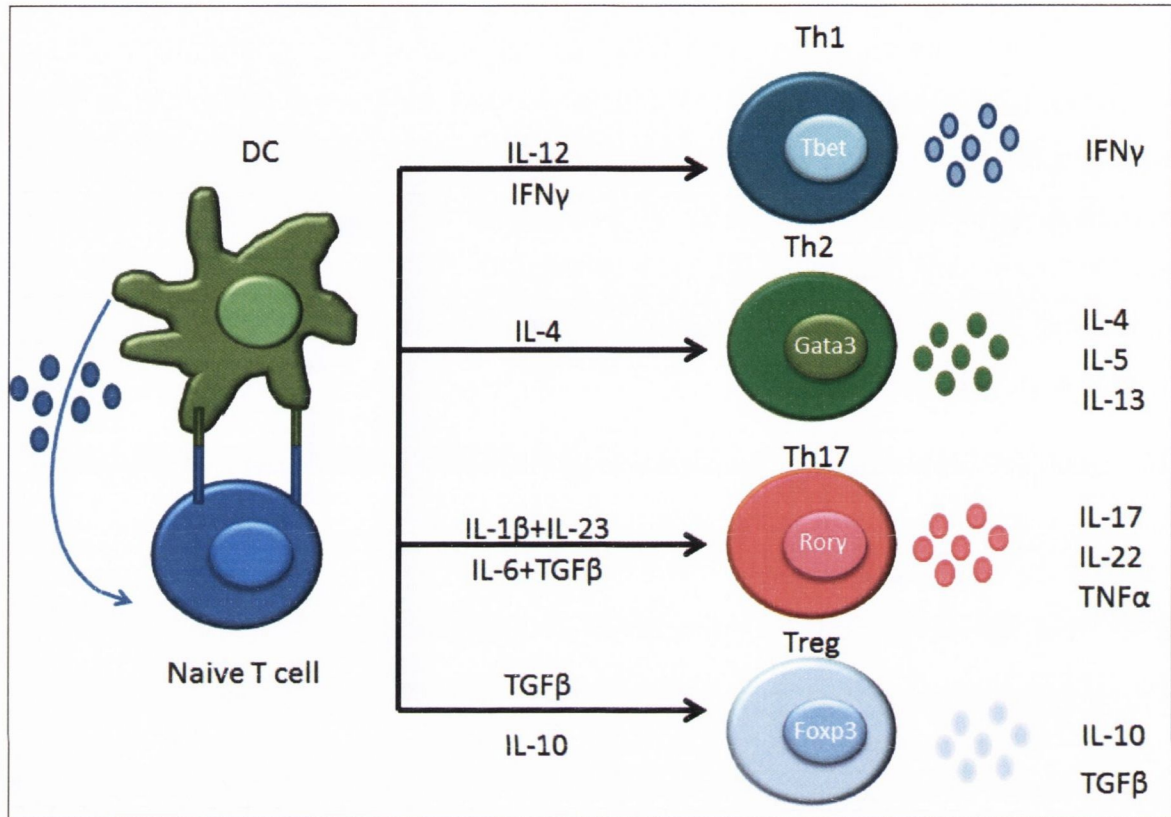


Fig. 1.1 T helper cell polarisation

1.2 Cytotoxic (CD8⁺) T cells

Cytotoxic T cells express the CD8 co-receptor and recognise antigens in the context of the ubiquitously expressed MHC class I molecule. They derive their name from their main function – killing of infected or malignant cells. Upon recognition of a cognate antigen, CD8⁺ T cells kill the presenting cell through production of cytotoxic molecules (perforin and granzyme) or by FAS signalling which induces a signalling cascade culminating in the target cell apoptosis. Similar to Th1 cell, CD8⁺ T cells are an important source of IFNγ and TNFα (Mosmann et al, 1997). However, they can also produce Th2 and Th17 cytokines (Huber et al, 2013; Mosmann et al, 1997; Woodland & Dutton, 2003). In order to become efficiently activated, naive CD8⁺ T cells require help from Th cells in the form of cytokines, in particular IL-2 and IFNγ, and CD40-CD40L dependent APC activation (Behrens et al, 2004). Furthermore, T

helper cells play an important role in generation of cytotoxic T cell memory (Bevan, 2004). CD8 T cells are required for clearance of viruses and intracellular bacteria as well as for anti-tumour immunity (Gattinoni et al, 2012; Keppler et al, 2009; Klenerman & Hill, 2005). Inappropriate, CD8 T cell activation can lead to immunopathology or autoimmunity, a prime example of a disease where CD8 T cell play a pathogenic role being type I diabetes (Lamont et al, 2014)

1.3 IFN γ

IFN γ is the effector cytokine mainly associated with Th1, CD8 T cells and NK cells, but can be produced by many other cell types, including macrophages, type 1 innate lymphoid cells (ILC1), and $\gamma\delta$ T cells (Fuchs et al, 2013; Olson et al, 2009; Puddu et al, 2005; Puddu et al, 1997). NK cells respond to IL-12 or IL-12 and IL-18 to produce IFN γ (Aste-Amezaga et al, 1994; Chakir et al, 2001; Kosaka et al, 2012; Nomura et al, 2002; Trinchieri, 1997). IFN γ acts both on immune cells and on non-hematopoietic cells (Schreiber & Farrar, 1993; Valente et al, 1992). It has been shown to be important in responses against viruses, bacteria and tumours (Brown et al, 2012; LaCasse et al, 2011; Thirion & Coutelier, 2009). It induces activation and maturation of macrophages and DC, which results in upregulation of MHC class II and costimulatory molecules by these cells and enhanced antigen presentation (Goldszmid et al, 2012). Furthermore, IFN γ induces production of nitrogen oxide by macrophages (Xing et al, 2000). The production of IFN γ by Th1 cells is required for clearance of intracellular pathogens. The importance of IFN γ is highlighted by studies in mice and clinical data from humans with defects in IFN γ signalling. Human IFN γ R1 deficiency results in a life-threatening autosomal recessive immune disorder and affected children die due to disseminated mycobacterial infections unless bone marrow transplant is performed (Edeer Karaca et al, 2012; Marazzi et al, 2010; Vinh et al, 2009). Similar to humans, mice deficient in IFN γ can develop disseminating bacterial infections (Bao et al, 2000; Cooper et al, 1993; Mahon et al, 1997). However, increased IFN γ has also been implicated in the pathogenesis of various autoimmune diseases (Browne et al, 2013; Butrimiene et al, 2004; Damsker et al, 2010; Fuss et al, 1996; Rafa et al, 2010).

1.4 IL-17

IL-17 (or IL-17A) is the signature cytokine of Th17 cells. However, many other cell types have also been shown to be capable producing IL-17 and in certain situations they secrete concentration exceeding those produced by CD4 T cells. Other IL-17-producing cell types include $\gamma\delta$ T cells, NK cells, NKT cells as well as ILC3. IL-17 receptor is expressed ubiquitously and therefore IL-17 can act upon many cell types in the body. IL-17 plays an important role in immune responses to extracellular pathogens. The main outcome of IL-17 signalling is to promote neutrophil recruitment to the site of infection. IL-17 promotes secretion of proinflammatory cytokines, such as IL-6 and G-CSF and chemokines such as CXCL2, CCL20, and CCL7. IL-17 induces production of antimicrobial peptides, such as β -defensins, and upregulation of adhesion molecules (Aujla et al, 2007; Liang et al, 2006; Steinstraesser et al, 2011). It can contribute to protective mucosal responses by promoting antibody class switching (Jaffar et al, 2009). Furthermore, IL-17 can promote tissue remodelling by induction of matrix metalloproteinases. IL-17 has been shown to have an essential role in defences against oropharyngeal candidiasis, caused by the commensal fungus *Candida albicans*, by promoting neutrophil recruitment and antimicrobial defences (Conti et al, 2009). IL-17 has been implicated in the granuloma formation during *Mycobacterium tuberculosis* infection (Yoshimura et al, 2009). Inappropriate IL-17 responses, on the other hand, have been associated with the induction of autoimmune diseases, such as arthritis or inflammatory bowel disease (Do et al, 2011; Roark et al, 2007b).

1.5 IL-22

IL-22 is a member of IL-10 cytokine family originally identified as IL-10-related T cell-derived inducible factor (IL-TIF)(Dumoutier et al, 2000a). Cells that produce IL-22 include activated Th1 cells, Th17 cells, NK cells, NKT cells, ILC, and $\gamma\delta$ T cells (Bachmann et al, 2010; Chung et al, 2006; Ivanov et al, 2013; Paget et al, 2012; Satoh-Takayama et al, 2011). The gene encoding IL-22 is located in close proximity to IFN γ gene, both in mice and humans (Dumoutier et al, 2000b). IL-22 receptor complex consists of IL-22R1 and IL-10R2 chains (Kotenko et al, 2001a). Although IL-22 is produced mostly by immune cells, no expression of functional IL-22 receptor has been detected on lymphocytes. Instead, IL-22R has been found to be expressed on various cells of epithelial origin, including intestine, lung, and skin epithelial cells

as well as keratinocytes and hepatocytes (Dumoutier et al, 2000b; Wolk et al, 2004). Furthermore, a natural antagonist of IL-22 has been identified – IL-22 binding protein (IL-22BP) – which is a soluble version of the IL-22R1 receptor chain. However, it is not a truncated version of the receptor but a soluble protein encoded on a separate gene (Kotenko et al, 2001b). Recently, subsets of DC in the human and mouse intestine and lymphoid tissue were found to produce IL-22BP in the in steady-state conditions (Huber et al, 2012; Martin et al, 2013). Functions of IL-22 include antimicrobial defence by induction of production of antimicrobial peptides, such as β -defensins, RegIII γ , RegIII β and S100 proteins as well as control of keratinocyte growth and differentiation (Gläser et al, 2005; Zheng et al, 2008). IL-22 has been shown to play a protective role in bacterial and viral infection, as well as in models of tissue injury by contributing to tissue repair (Ivanov et al, 2013; Pociask et al, 2013; Simonian et al, 2010). Aberrant IL-22 production, on the other hand, has been associated with inflammatory diseases such as psoriasis or systemic lupus erythematosus (Qin et al, 2011; Zheng et al, 2007).

1.6 Common γ chain cytokines: IL-2, IL-7 and IL-15.

IL-2, IL-7 and IL-15 are members of the common γ chain cytokine family and play an important role in differentiation, proliferation and survival of T cells. They signal through heterodimeric (IL-7) or heterotrimeric (IL-2 and IL-15) receptors all of which comprise the common γ receptor chain. In addition, IL-2 and IL-15 also share the β chain and thus differ from each other only by the α chain of the receptor. Other members of the common γ chain cytokine family include IL-4, IL-9 and IL-21. The gene encoding the common γ chain is mutated in humans with X-linked severe combined immunodeficiency (Fugmann et al, 1998).

IL-2 is a multifunctional cytokine which promotes proliferation, differentiation and survival of T cells as well as NK cell cytotoxicity. IL-2 is produced primarily by activated CD4 T cells. The expression of the three chains of IL-2R varies between cell types and their differentiation stages (Kim et al, 2006). Furthermore, different IL-2 receptor chain combinations can bind IL-2 with different affinity. IL-2R α is expressed on activated T cells and binds IL-2 weakly. The IL-2R β/γ heterodimer present on NK cells and memory T cells binds IL-2 with intermediate affinity while the heterotrimeric receptor, expressed on activated T cells and Treg cells, has the

highest affinity for IL-2. Engagement of the intermediate and high affinity IL-2 receptors results in activation of phosphoinositol 3-kinase/AKT, Ras-MAP kinase, and JAK-STAT pathways (Nakamura et al, 1994; Nelson et al, 1994). The main JAK-STAT molecules involved in IL-2 signalling are JAK1, JAK3, and STAT5.

IL-15 shares many characteristics of IL-2 and plays an important role in development and homeostasis of NK cells, NKT cells, and CD8 T cell memory (Kennedy et al, 2000). It is also crucial for the development and maintenance of CD8 $\alpha\alpha$ intestinal intraepithelial lymphocytes (IEL) (Ma et al, 2009; Malamut et al, 2010). IL-15 can be produced by many cell types. In contrast to IL-2, it is not secreted but expressed on the cell surface in complex with IL-15R α (Dubois et al, 2002). The main mode of IL-15 signalling involves transpresentation of IL-15/IL-15R α complexes to neighbouring cells (Castillo & Schluns, 2012). Upon interaction of IL-15/IL-15R α with a IL-2/IL-15R β 1/ γ_c expressing cell JAK1 and JAK3 become activated, which in turn leads to phosphorylation of STAT5 (Johnston et al, 1995). IL-15 can also be cleaved from the cell surface and exist in soluble form, but it is unclear whether soluble IL-15 complexes can signal *in vivo* (Castillo & Schluns, 2012).

The main source of IL-7 are the stromal cells of the lymphoid tissues (Jiang et al, 2005). However, it can also be produced by epithelial cells in the thymus, liver and intestine, as well as by other non-hematopoietic cells. IL-7 stimulates proliferation of lymphoid progenitor cells as well as survival and proliferation of naïve and memory T cells (Namen et al, 1988; Schluns et al, 2000; Tan et al, 2001). It has also been shown to control the homeostatic proliferation of $\gamma\delta$ T cells and IL-7R deficiency results in complete absence of these cells. $\alpha\beta$ T cells, on the other hand, are still present in IL-7R $^{-/-}$ mice, albeit at reduced numbers (Baccala et al, 2005; Maki et al, 1996). Binding of IL-7R induces activation of signalling molecules belonging to the JAK/STAT family. In particular JAK1, JAK3 and STAT5 are activated downstream of IL-7 receptor (Lin et al, 1995).

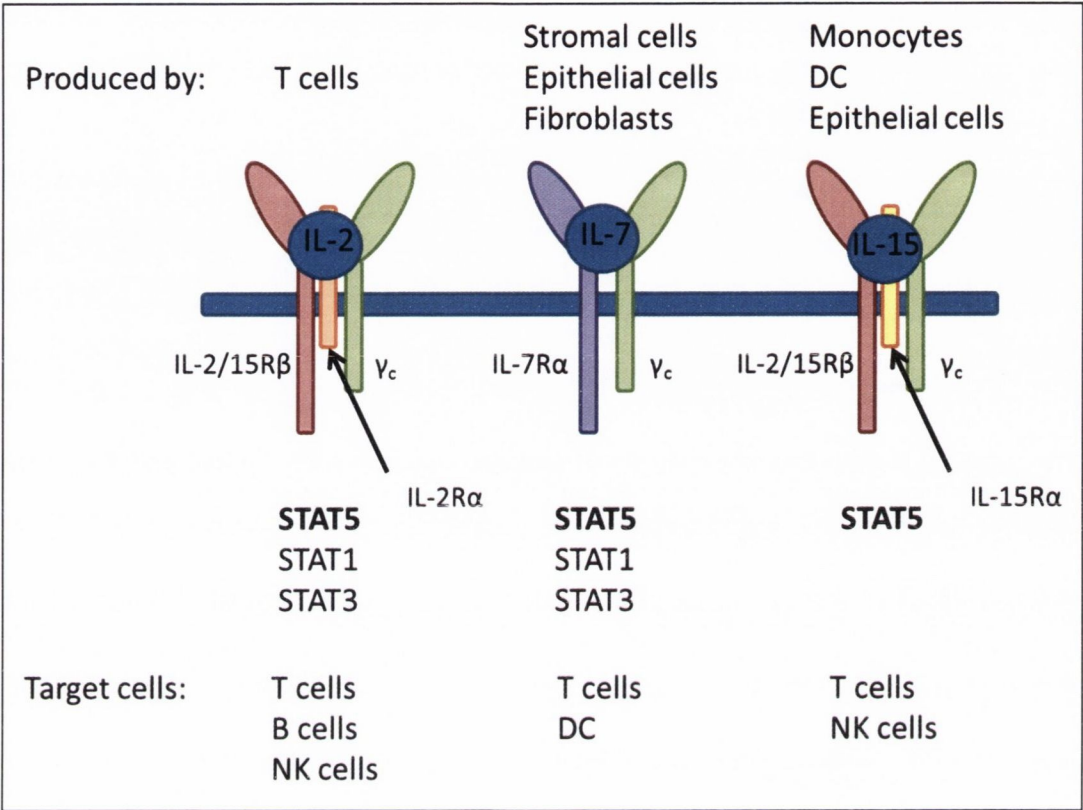


Figure 1.2. IL-2, IL-7 and IL-15 receptor.

Receptor chains that come together to form IL-2, IL-7 and IL-15 receptor as well as the STAT molecules activated upon receptor engagement are shown. Predominantly activated STATs are in bold. DC-dendritic cell, NK cell- natural killer cell. Figure adapted from Liao et al. 2011

1.7 IL-1 family cytokines: IL-1α, IL-1β and IL-18

The IL-1 family cytokines are potent proinflammatory mediators which play an important role in orchestrating antimicrobial responses. Disregulation of their signalling can lead to inflammatory and autoimmune diseases. Multiple layers of

control are in place to prevent this from happening. IL-1b and IL-18 are produced as inactive proteins termed pro-cytokines and need to be enzymatically cleaved in order to become fully functional. Proteolytic processing of IL-1b and IL-18 is dependent on the assembly and activation of a multiprotein complex consisting of pattern recognition receptor, caspase-1 and adaptor proteins called the inflammasome (Schroder & Tschopp, 2010). The main caspase involved in IL-1b and IL-18 processing is caspase-1 and it itself needs to be cleaved in order to become active. The requirement for the inflammasome activation adds an additional control step in the secretion of these cytokines. In contrast to IL-1 β and IL-18, IL-1 α is produced as an active protein, does not contain a caspase cleavage site and consequently does not require caspase-1 dependent processing. However, recently it has been shown that although caspase-1 cannot directly cleave IL-1 α , active IL-1 α secretion can be both caspase-1 dependent and independent (Gross et al, 2012).

IL-1 α and IL-1 β bind the same receptors, namely IL-1 receptor I (IL-1RI) and IL-1RII. IL-1RI is the true IL-1 receptor while IL-1RII is a decoy receptor that allows for the control of the amount of IL-1 that is available to bind to IL-1RI. Further level of regulation of IL-1 signalling comes in form of the IL-1 receptor antagonist (IL-1RA), which is the natural inhibitor of IL-1RI. IL-1RA binds to the IL-1RI and thus prevents IL-1 α and IL-1 β from binding. Although IL-1 β has been regarded as the main driver of inflammation, studies involving IL-1 α /IL-1 β single or double knock out and IL-RI deficient mice suggest that both of these cytokines are important for the overall proinflammatory response and in some diseases they may have opposing effects (Bersudsky et al, 2014; Joosten et al, 2010; Kamari et al, 2011; Vonk et al, 2006)

IL-18 signals through its own unique receptor, IL-18R. Similar to IL-1R, IL-18R signalling is controlled via production of an inhibitor protein. IL-18R signalling is inhibited by IL-18 binding protein, which in contrast to IL-1RA does not bind the receptor, but IL-18 itself and thus neutralises it (Novick et al, 1999).

IL-1R/IL-18R binding leads to activation of the signalling cascade involving MyD88, IRAK, TRAF6 and NF κ B molecules and ultimately results in the induction of proinflammatory cytokine and chemokine secretion from innate cells (Cohen, 2014). Furthermore, both IL-1 and IL-18 play important roles in T cell polarisation (Dinarello, 1999; Sutton et al, 2006). Although there are many similarities between IL-1R and IL-

18R signalling, there are also differences between their end point effects exist. IL-1 but not IL-18 signalling results in fever induction and only IL-18 promotes IFN γ production by T cells and NK cells (Dinarello, 1999; Lee et al, 2004).

1.8 IL-12 cytokine family: IL-12 and IL-23

IL-12 cytokine family comprises of structurally related heterodimeric proteins with overlapping subunit usage. To date four members have been identified, namely IL-12, IL-23, IL-27 and IL-35. IL-12 is composed of the p40 subunit and the IL-12p35 subunit. The p40 subunit is shared by IL-23 which also consists of a unique p19 subunit. IL-27 is composed of Ebi3, which is structurally related to IL-12p40, and the p35 and p19 related subunit - p28. Ebi3 subunit can also pair with the p35 subunit of IL-12 to form IL-35. IL-12, IL-23 and IL-27 are mainly produced by myeloid cells upon TLR stimulation while the main source of IL-35 are the natural Treg cells (Collison et al, 2007). IL-12 and IL-23 are proinflammatory cytokines, IL-27 has been reported to have both pro- and anti-inflammatory effects while IL-35 is chiefly anti-inflammatory. The receptors recognising the members of the IL-12 cytokine family are also heterodimeric proteins and similarly share their receptor subunits. IL-12 receptor is composed of IL-12R β 1 and IL-12R β 2 chain. IL-23 receptor utilises the IL-12R β 1 chain paired with the IL-23R α chain. IL-27 signals through a receptor composed of the IL-6 gp130 chain and the IL-27 receptor-specific WSX-1 chain. The IL-35 receptor is composed of the IL-12R β 2 and the gp130 chain. However, partial IL-35 function has been detected in mice lacking either gp130 or IL-12 β 2 but not both of these chains suggesting that IL-35 can also signal through IL-12R β 2 and gp130 homodimers, albeit with a lesser potency (Collison et al, 2012).

IL-12 is an important player in the cellular immunity as it activates NK cells and initiates the differentiation of naïve T cells into Th1 cells (Gately et al, 1998; O'Garra et al, 1995; Parihar et al, 2002). Furthermore, it stimulates IFN γ production by NK cells, T cells and ILC1 (Bernink et al, 2013; Gately et al, 1998). IL-12 is crucial for anti-tumour responses as it promotes tumour killing via induction of cellular immunity and suppresses its growth by inhibiting angiogenesis (Del Vecchio et al, 2007;

Portielje et al, 2003). Inappropriate IL-12 responses can lead to development of autoimmune diseases. Although the pathological role of IL-12 in some of the immune disorders has been deduced from the early studies involving IL-12p40 neutralisation or IL-12p40 knock out mice. After the discovery of IL-23 some of the effects originally ascribed to IL-12, were found to be in fact mediated by IL-23 (Becher et al, 2002; Cua et al, 2003). IL-23 promotes the induction of Th17 cells which are important in for mucosal immunity, but can become highly pathogenic if not properly controlled (Kolls, 2010; Langrish et al, 2005). IL-23 can also synergise with IL-1 β or IL-18 to promote innate IL-17 production by $\gamma\delta$ T cells (Lalor et al, 2011; Sutton et al, 2009b). Increased levels of IL-12 and IL-23 can be detected in the skin of psoriasis and intestinal mucosa of IBD patients and monoclonal antibodies targeting these cytokines have shown positive therapeutic effects in clinical trials (Fuss et al, 2006).

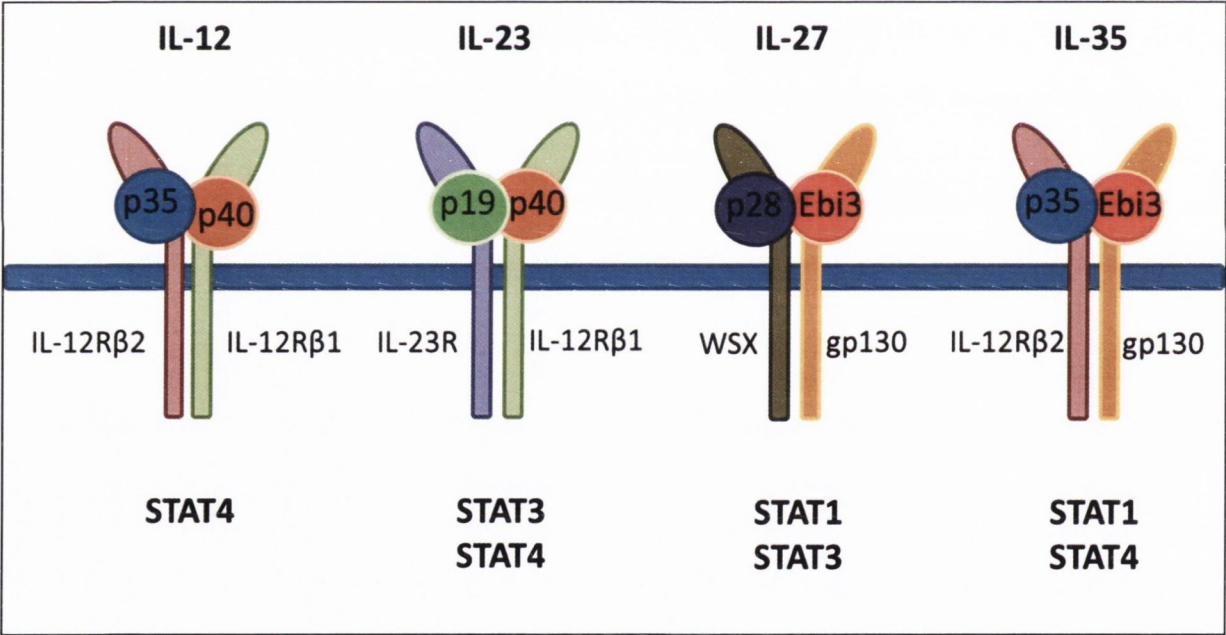


Figure 1.3. IL-12 cytokine family. Shown are cytokine subunits, receptor chains and predominant STAT molecules utilised by various members of the IL-12 cytokine family. Figure modified from (Olson et al, 2013)

1.9 $\gamma\delta$ T cells

The $\gamma\delta$ T cells are an unusual subset of lymphocytes which, despite belonging to the T cell lineage, shares many characteristics with the cells of the innate immune

system. They behave as memory-like cells in that they become rapidly activated upon recognition of cognate antigens or in response to cytokines. Similar to the conventional T cells, $\gamma\delta$ T cells perform their function through secretion of soluble mediators or cytotoxicity against target cells. However, $\gamma\delta$ T cell response is much quicker than that of conventional T cells and they recognise a different range of antigens, many of which are self-molecules. $\gamma\delta$ T cells are enriched in mucosal epithelia, where pathogens are first encountered, accounting for as much as 50% of the resident CD3⁺ cells in the intestine. This localisation of $\gamma\delta$ T cells allows them to participate in the initiation of the immune response, thus making it possible for them to influence the adaptive response that will follow. However, little is known about how tissue resident $\gamma\delta$ T cell become activated and what their function is during disease.

1.10 $\gamma\delta$ T cell development

$\gamma\delta$ and $\alpha\beta$ T cells develop in the thymus from a common CD4⁻CD8⁻ or double negative (DN) T cell progenitor (Shibata, 2012). The DN stages of T lymphocyte development are subdivided into four substages, DN1 through DN4, based on the expression of surface molecules CD117, CD44, and CD25 (Shibata, 2012). The decision on whether a cell is going to become an $\alpha\beta$ or a $\gamma\delta$ T cells is made somewhere between DN2 and DN3 stage, as these cells have the potential to develop into either type of a T cell, but DN4 cells can only progress to become an $\alpha\beta$ T cells or an NKT cell. Not much is known about the exact events that decide the fate of a DN thymocyte and a lot of effort is put into elucidating factors involved in this process. A number of models have been proposed that attempt to explain how the $\alpha\beta$ versus $\gamma\delta$ decision is made, some pointing towards the role of the TCR in the selection process, while other ones stress the importance of signals preceding the TCR expression.

One of these models, the stochastic model, argues that the fate of the cell is determined before the TCR is expressed and the cell will only progress through the subsequent stages of development if it succeeds to produce a TCR that matches its fate. Evidence supporting this model include the fact that the progenitor cells expressing certain surface molecules and transcription factors preferentially progress into one and not the other T cell lineage and manipulation of these molecules can alter the $\alpha\beta/\gamma\delta$ fate decision (Kang et al, 2001; Melichar et al, 2007). In particular, IL-

7R α and a high mobility group transcription factor, Sox13, have been implicated in promoting the development of $\gamma\delta$ T cells. In contrast to $\alpha\beta$ T cells, $\gamma\delta$ T cells require IL-7 signalling for the expression of a functional TCR as IL-7 receptor deficient mice lack these cells and IL-7R α expression on DN thymocytes correlates with the likelihood of the cell becoming a $\gamma\delta$ T cell (Kang et al, 2001; Maki et al, 1996). Similarly, Sox13 expression is higher in the DN cells biased to become $\gamma\delta$ T cells and maintained in mature $\gamma\delta$ T cells, but lost in double positive cells. Furthermore, Sox13 transgenic animals showed impairment in the development of $\alpha\beta$ T cells while $\gamma\delta$ T cells developed normally (Melichar et al, 2007). Accordingly, $\gamma\delta$ T cells were found to be largely reduced and defective in Sox13 knock out animals while no abnormality in the proportion of $\alpha\beta$ thymocytes was observed (Melichar et al, 2007).

The instructive model, on the other hand, stresses the role of the TCR in the T cell development and argues that the type of the signal transmitted by $\gamma\delta$ and the pre-TCR, the complex of rearranged TCR β with pre-TCR α , during T cell differentiation is qualitatively different and thus the type of receptor expressed on the DN cell decides its fate. This model has evolved to what is now referred to as the signal strength model, which proposes that not the receptor itself, but the strength of the signal transmitted by that receptor decides the fate of the cell, with a strong signal promoting $\gamma\delta$ development and a weak signal promoting the $\alpha\beta$ fate (Haks et al, 2005; Hayes et al, 2005; Kreslavsky et al, 2008). Under normal conditions, $\gamma\delta$ TCR usually transmits a stronger signal than the pre- $\alpha\beta$ TCR (Hayes & Love, 2002). However, in the studies involving TCR $\gamma\delta$ transgenic mice, in which both $\gamma\delta$ and $\alpha\beta$ T cells are present, experimental manipulation of the signal strength allowed to divert DN thymocytes into the opposite lineage, even if the TCR has been already successfully expressed (Haks et al, 2005). This data supports the currently favoured idea that the signal strength, rather than the identity of the TCR expressed by the immature DN lymphocyte, decides its lineage commitment.

1.11 $\gamma\delta$ TCR

Similar to $\alpha\beta$ T cells, $\gamma\delta$ T cells generate T cell receptors through RAG gene dependent V(D)J recombinations in the thymus. However, the V γ V δ gene segment pool is much smaller than that of $\alpha\beta$ T cells. Furthermore, in contrast to the conventional T cells, $\gamma\delta$ T cells appear to restrict rather than expand their V γ V δ chain

repertoire during the recombination process, as not all of the V chains can pair to produce functional TCRs (Boucontet et al, 2009; Hayday, 2000; Pereira & Boucontet, 2004). A further level of restriction comes from the tissue distribution of $\gamma\delta$ T cells – particular V γ V δ subsets tend to preferably home to specific sites in the body, a fact that holds true both in mice and humans. For example, the V γ 7 chain expressing cells are the dominant subset of $\gamma\delta$ T cells in the murine gut, while in humans the intestinal intraepithelial compartment is dominated by V δ 1 and V δ 3 $\gamma\delta$ T cells (nomenclature after Heilig and Tonegawa) (Chowers et al, 1994; Kagnoff, 1998). In mice, $\gamma\delta$ T cells emerge from the thymus in a number of embryonic waves and sequentially populate distinct tissues. V γ 5V δ 1 $\gamma\delta$ T cells, which reside in the skin epithelia, are the first to leave the thymus, around embryonic day 14, followed by the V γ 6V δ 1 subset, which is mainly found in the epithelia of uterus, kidney and lung and other V γ subsets, which are more ubiquitously distributed. In addition, the development of certain V γ V δ subsets is restricted to the early embryonic and neonatal periods while others are detected in the thymus throughout the life of the animal. Despite the apparent limitations of their TCR, $\gamma\delta$ T cells have a potential to recognise a broad spectrum of antigens. This is due to diversity in the complementarity determining region 3 (CDR3), which can be generated through the unusual usage of V δ D gene segments (Davis & Bjorkman, 1988; Hayday, 2000; Rock et al, 1994). However, even with this potential for diversity, invariant $\gamma\delta$ T cells using the same CDR3 are present in certain tissues. For example, in mouse uterus and skin, 90% of resident $\gamma\delta$ T cells express invariant V γ 6V δ 1 and V γ 5V δ 1 TCRs, respectively (Asarnow et al, 1988; Hayday, 2000).

Table 1.1 Mouse $\gamma\delta$ T cell subsets nomenclature

Heilig and Tonegawa	Garman et al.	location
V γ 1	V γ 1.1	various
V γ 2	V γ 1.2	various
V γ 3	V γ 1.3	various
V γ 4	V γ 2	various
V γ 5	V γ 3	epidermis
V γ 6	V γ 4	tongue, female reproductive tract, lung, liver
V γ 7	V γ 5	small intestine

The tissue restriction of $\gamma\delta$ TCRs suggests that expression of certain $V\gamma V\delta$ combinations should be advantageous in particular site in the body, possibly for recognition of locally expressed stress-inducible antigens. It is therefore intuitive to assume that specific $\gamma\delta$ T cell subsets should be positively selected in the thymus. However, to date only one subset of $\gamma\delta$ T cells has been shown to be dependent on thymic expression of specific antigens, namely the skin resident murine $V\gamma 5V\delta 1$ $\gamma\delta$ T cells (Boyden et al, 2008). A number of studies show that thymic signalling decides the effector fate of $\gamma\delta$ T cells. $\gamma\delta$ T cells cells that have not encountered antigen in the thymus are thought to be poised towards IL-17 production, and on the other hand, the antigen experienced cells become IFN γ producers.

Species	Peripheral location	Predominant V gene segment usage
mouse	thymus	Diverse
	Spleen	$V\gamma 1$ and $V\gamma 4$
	Lymph nodes	Diverse
	Epidermis	$V\gamma 5V\delta 1$
	Liver	$V\gamma 1V\delta 6.3$, $V\gamma 4$ and $V\gamma 6$
	Gut epithelia	$V\gamma 7V\delta 4$, $V\gamma 7V\delta 5$, $V\gamma 7V\delta 6$
	Uterovaginal epithelia	$V\gamma 6V\delta 1$
	Lung epithelia	$V\gamma 4$ and $V\gamma 6$
human	Thymus	$V\delta 1$
	Peripheral blood	$V\gamma 9V\delta 2$
	Spleen	$V\delta 1$
	Liver	$V\delta 1$ and $V\delta 3$
	Gut epithelia	$V\delta 1$ and $V\delta 3$
	Dermis	$V\delta 1$
	Uterine epithelia	$V\delta 1$

Table 1.2. Tissue distribution of TCR V subsets of $\gamma\delta$ T cells in mice and humans.

In contrast to conventional $\gamma\delta$ T cells which can be divided into subsets based on the effector molecules they secrete (IFN γ , IL-4 or IL-17) or the end result of their activation (death of the target cell), $\gamma\delta$ T cells are often divided according to the $V\gamma$ and $V\delta$ chains they express (nomenclature after Heilig and Tonegawa). Cells with specific $V\gamma V\delta$ combinations seem to secrete limited number of cytokines. For

example, mouse V γ 4⁺ cells have been shown to be the major producers of IL-17 in a number of disease models (Roark et al, 2007a) while V γ 1⁺ cells seem to be skewed towards IFN γ or IL-4 production (Gerber et al, 1999; Hao et al, 2011; Matsuzaki et al, 2002). The limited repertoire of V γ V δ chains utilised by $\gamma\delta$ T cells and their tissue specific expression suggest that particular subsets of $\gamma\delta$ T cells are suited for recognition of local antigens.

1.12 Modes of $\gamma\delta$ T cell activation and antigen diversity

It is still unclear what antigens most of the $\gamma\delta$ T cells are specific for and many of those identified to date differ substantially from those recognised by $\alpha\beta$ T cells. $\gamma\delta$ T cells have been shown to respond to a wide range of protein as well as non-proteinaceous antigens. In contrast to conventional T cells, recognition of antigens by $\gamma\delta$ T cells is not restricted by classical MHC molecules. Furthermore, $\gamma\delta$ T cells are thought to be able to respond to native unprocessed antigens, analogous to B cells or antibody recognition of antigens. In support of this idea, mouse V γ 1.2V δ 8 T cell clone was shown to respond to soluble herpes simplex virus type 1 transmembrane glycoprotein. Antibodies directed against either the TCR or the glycoprotein inhibited $\gamma\delta$ T cell activation. Furthermore, APCs incapable of antigen processing transfected with WT glycoprotein, but not APCs transfected with a mutant version of the glycoprotein lacking the transmembrane domain, activated the V γ 1.2V δ 8 $\gamma\delta$ T cell clone (Sciammas et al, 1994). Recently, mouse and human $\gamma\delta$ T cells have been shown to directly recognise a B cell antigen, phycoerythrin, in a TCR dependent manner, independent of antigen presentation or processing (Zeng et al, 2012).

Many of the antigens recognised by the TCR of $\gamma\delta$ T cells identified to date include host molecules or products of microbial metabolism. $\gamma\delta$ T cells are able to recognise stress induced non classical MHC molecules. For example, human V δ 1 $\gamma\delta$ T cells respond to MHC class I related molecules MICA and MICB and mouse $\gamma\delta$ T cells have been shown to recognise the related molecules T10 and T22 (Adams et al, 2008). In both mice and human these are non-peptide loading molecules and act as antigens on their own right. $\gamma\delta$ T cells have also been shown to respond to CD1c and CD1d, non-polymorphic CD1 molecules, which are exclusively expressed on APCs and capable of presenting lipid and glycolipid antigens (Behar & Porcelli, 2007; Dascher & Brenner, 2003). In humans, V δ 1 $\gamma\delta$ T cells have been shown to be

activated by sulphatide (a myelin glycosphingolipid) loaded CD1d tetramers, while hybridomas expressing mouse TCR $\gamma\delta$ clones responded to cardiolipin presenting CD1d (Bai et al, 2012; Born et al, 2003). On the other hand, CD1c mediated activation of $\gamma\delta$ T cells was found to be both antigen-dependent and independent (Cui et al, 2009; Spada et al, 2000).

Human and mouse $\gamma\delta$ T cells have been shown to respond to endogenous and pathogen derived heat shock proteins (HSP). HSP are constitutively expressed molecular chaperons that become significantly upregulated under conditions of stress, such as infection, cancer, environmental stress or cell differentiation (Tsan & Gao, 2004). HSP are normally confined to the intracellular compartment, however, they can be released from necrotic cells or in response to some stressful stimuli (Basu et al, 2000; Davies et al, 2006; Hirsh et al, 2006). HSP65 isolated from mycobacterial extracts and also recombinant HSPs, including the mammalian homologue HSP60, activated mouse V γ 1 and V γ 6 $\gamma\delta$ T cell subsets (Kobayashi et al, 1994; O'Brien et al, 1992). Recognition of HSP60 was shown to be TCR dependent but independent of accessory cells and antigen processing (Fu et al, 1994). Both human and mouse $\gamma\delta$ T cells were found to respond to HSP70 stimulation, and killing of tumour cells by $\gamma\delta$ T cells was shown to be inhibited when HSP70 was silenced (Hirsh et al, 2006; Kishi et al, 2001; Zhang et al, 2005).

Another unique feature of $\gamma\delta$ T cells is the ability to respond to non-peptide antigens. Human blood V γ 9V δ 2 were shown to respond to phosphoantigens such as hydroxyl-methyl-butyl-pyrophosphate (HMBPP) or isopentenyl pyrophosphate (IPP) (Espinosa et al, 2001; Hintz et al, 2001; Tanaka et al, 1995). HMBPP is an intermediate in the non-mevalonate pathway of cholesterol synthesis used in many bacteria species and apicomplexan protozoa, but absent in vertebrates (Hintz et al, 2001). Conversely, IPP is an intermediate in the mevalonate pathway which is conserved in vertebrates and thus represents a self-antigen. Although both intermediates are capable of activating human $\gamma\delta$ T cells, HMBPP is a 10,000 times more potent stimulator and the physiological concentrations of IPP are not sufficient for activation (Eberl & Moser, 2009; Hintz et al, 2001). Human V γ 9V δ 2 $\gamma\delta$ T cell activation by phosphoantigens has been shown to be TCR-dependent and independent of MHCI, MHCII and CD1 (Bukowski et al, 1995; Morita et al, 1995). However, it was found to be greatly enhanced in the presence of human and non-human primate, but not

murine, APC (Eberl & Moser, 2009; Green et al, 2004; Wei et al, 2008). The mechanism by which APC promote phosphoantigen-induced $\gamma\delta$ T cell activation is unclear, but V γ 9V δ 2 tetramer binding to APCs diminished after protease treatment of APC suggesting involvement of a cell surface expressed protein (Wei et al, 2008).

Recently, a member of B7 family, CD277, has been implicated in $\gamma\delta$ T cell recognition of phosphoantigens (Harly et al, 2012). Interestingly, CD277 is not present in mice, which could explain the inability of murine APC to enhance phosphoantigen-induced V γ 9V δ 2 T cell activation. Consistent with the lack of CD277 expression in rodents, no phosphoantigen reactivity of $\gamma\delta$ T cells has been documented in mice thus far (Vantourout & Hayday, 2013).

Apart from the recognition of antigens via T cell receptor, $\gamma\delta$ T cells have been shown to respond to stimulation via TLR and other innate type receptors. Many of the $\gamma\delta$ T cell antigens can be recognised both by TCR and innate receptors (Tsan & Gao, 2004; Wrobel et al, 2007). This creates a situation where the same molecule can act both as signal one (stimulation via the TCR) and the costimulatory signal via the innate receptors.

Expression of various TLRs has been detected in both human and mouse $\gamma\delta$ T cells and varies among V γ V δ subsets. Certain TLRs, such as TLR2 or TLR6, were expressed in freshly isolated resting cells, while the expression of other TLRs was detectable only after activation (Beetz et al, 2008; Fang et al, 2010; Martin et al, 2009; Mokuno et al, 2000; Pietschmann et al, 2009; Schwacha & Daniel, 2008; Shimura et al, 2005; Wesch et al, 2006). Human V δ 1 but not V δ 2 $\gamma\delta$ T cells were found to produce IFN γ in response to flagellin. The amount of IFN γ secreted was enhanced by simultaneous stimulation through TCR (Pietschmann et al, 2009). In the same study, TLR2 ligand Pam₃CSK₄ was found to have a costimulatory effect on freshly isolated human $\gamma\delta$ T cells activated via TCR (Pietschmann et al, 2009). Mokuno et al. showed TLR2 mRNA expression in mouse peritoneal V γ 6V δ 1 T cells. These cells expanded in response to *E. coli* lipid A, and expansion was impaired in TLR2 deficient mice (Mokuno et al, 2000). A more recent study has shown that only CCR6⁺ IL-17-producing $\gamma\delta$ T cells in the peritoneum of C57BL/6 mice expressed TLR1, TLR2 and dectin 1 mRNA and could be directly activated by stimulation with the cognate ligands (Martin et al, 2009).

NKG2D is an activating C-type lectin receptor which recognises stress induced proteins, such as MICA and MICB in humans or H60 and RAE1 in mice (Bauer et al, 1999; Diefenbach et al, 2001). NKG2D is expressed on all mouse and human NK cells and on some other immune cells, such as CD8 cells, $\gamma\delta$ T cells and macrophages, but there are differences between mice and humans. While NKG2D is present on all human $\gamma\delta$ T cell subsets, mouse $\gamma\delta$ T cells do not show this universal expression and only 25% of $\gamma\delta$ T cells in the mouse spleen were found to be NKG2D positive (Girardi et al, 2001; Jamieson et al, 2002). NKG2D has been shown to directly activate $\gamma\delta$ T cells as well as to have a costimulatory role (Girardi et al, 2001; Nitahara et al, 2006; Rincon-Orozco et al, 2005). NKG2D triggers cytotoxicity independent of TCR signalling in mouse and human $\gamma\delta$ T cells. In addition, human V γ 9V δ 2 T cells secreted TNF- α , but not IFN γ , while mouse V γ 5V δ 1 cells produced IFN γ in response to NKG2D ligation (Nitahara et al, 2006; Rincon-Orozco et al, 2005). $\gamma\delta$ T cells have been shown to respond directly to cytokines in the absence of TCR stimulation. For example, naïve mouse $\gamma\delta$ T cells have been shown to produce IL-17 in response to IL-1 β and IL-23 or IL-18 and IL-23 stimulation, while bovine and mouse $\gamma\delta$ T cells produced IFN γ in response to IL-12 and IL-18 (Haas et al, 2009; Johnson et al, 2008; Lalor et al, 2011; Sutton et al, 2009a). In conclusion, this is evidence that $\gamma\delta$ T cells can be activated by a variety of pathogen and host derived stimuli making them perfectly suited for quick responses to infection and surveillance of cellular distress.

1.13 $\gamma\delta$ T cell subtypes

Despite the common use of V γ V δ nomenclature to discriminate different subsets of $\gamma\delta$ T cells, efforts have been made to find markers to segregate $\gamma\delta$ T cells into functional subsets based on cytokine production, similar to T helper cells. Many markers have been proposed to identify IL-17 and IFN γ producing cells in mice. Shibata et al. found that all of the mouse peritoneal V γ 6V δ 1 cells, which produced IL-17 in response to *E. coli* infection, expressed CD25 (IL-2R α chain). IFN γ producing cells, on the other hand, lacked CD25 but expressed CD122 (IL-2/IL-15R β chain). Therefore CD25 has been proposed as a marker of naturally occurring IL-17-producing $\gamma\delta$ T cells. However, CD25 expression was not detectable in the thymus and was only acquired in the periphery (Shibata et al, 2008). In addition IFN γ -

producing $\gamma\delta$ T cells were reduced in IL-15^{-/-} mice, while IL-17⁺ $\gamma\delta$ T cells were decreased but still present in IL-2- or CD25-deficient mice, suggesting a role of IL-2 for their maintenance (Shibata et al, 2008). Another group showed that, in contrast to CD25, CCR6 is expressed already on thymic $\gamma\delta$ T cells destined to become IL-17 producers, while NK1.1 marks IFN γ -producing $\gamma\delta$ T cells (Haas et al, 2009). These results agree with the data generated in the Veldhoen lab which showed that CCR6⁺ is expressed on peripheral lymph node and peritoneal IL-17 producing $\gamma\delta$ T cells (Martin et al, 2009). In addition, they found that majority of CCR6⁺ cells in the lymph node express V γ 4 chain. In a study involving β 2m-deficient mice, which fail to express the nonclassical MHC I molecules T10/T22 on their surface, $\gamma\delta$ T cells specific for those antigens were shown to develop normally (Jensen et al, 2008). However, they expressed lower levels of CD122 indicative of an antigen-naïve phenotype. When stimulated with the anti-TCR $\gamma\delta$ GL4 antibody, antigen naïve $\gamma\delta$ T cells produced IL-17 and antigen-experienced $\gamma\delta$ T cells produced IFN γ . The authors therefore proposed that encountering the antigen in the thymus does little to influence $\gamma\delta$ T cell development or antigen specificity but determines its effector function (Jensen et al, 2008). However, the expression of the above markers could not be used to discern functional subsets of $\gamma\delta$ T cells in all of the sites in the body. Ribot et al. proposed CD27 (a TNFR family member) as the universal marker distinguishing IL-17- from IFN γ -producing $\gamma\delta$ T cells. CD27 was found to be expressed on-IFN γ producing $\gamma\delta$ T cells in the spleen and lymph nodes (axillary, inguinal, bronchial and mesenteric) and in the peripheral tissues (lung or gut) but was absent from IL-17-producing $\gamma\delta$ T cells. Furthermore, CD27 accounted for all of the IFN γ -producing CD122⁺ as well as the CD122⁻ $\gamma\delta$ T cells which also produced this cytokine. The same study found that generation of IFN γ ⁺ CD27⁺ T cells was dependent on both CD27 and TCR signalling which supports the idea that IFN γ producing cells have already experienced the antigen in the thymus (Ribot et al, 2009). In addition, human IL-17 producing $\gamma\delta$ T cells polarised in the presence of IL-1 β , IL-6, IL-23 and TGF β were shown to express CCR6 and lack CD27 expression, similar to mouse $\gamma\delta$ T cells. However, CCR6 rather than CD27 expression co-related with the effector cytokine profile as IFN γ -producing cells were also found to be CD27⁻ (Caccamo et al, 2011).

1.14 $\gamma\delta$ T cell function

$\gamma\delta$ T cells perform their effector function very similar to the conventional T cells – they produce cytokines, chemokines and kill infected or malignant cells. The major difference between these two subsets of T lymphocytes is the kinetics of their response. While $\alpha\beta$ T cells require priming by professional APC and undergo affinity maturation in response to a new pathogen, $\gamma\delta$ T cells can mount a significant response without extensive expansion. This means that they become activated at the same time as the innate immune cells and can act to control the infection before the appearance of antigen-specific cells, as well as influence the type of the adaptive response that will follow.

$\gamma\delta$ T cells have been shown to be an important early source of IFN γ , TNF α and IL-17 in various disease settings, including infection, cancer and autoimmunity, with the capacity to contribute both to resolution of the disease or its pathogenicity. They have been shown to be able to rapidly produce large amounts of cytokines, sometimes exceeding those produced by T helper cells. For example, murine V δ 1 $\gamma\delta$ T cells were found to be the major source of IL-17 after i.p. injection of *Escherichia coli*. Neutralisation of IL-17 or depletion of $\gamma\delta$ T cells resulted in reduced infiltration of neutrophils into the peritoneal cavity and delayed bacterial clearance (Shibata et al, 2007). Similarly, in *Lysteria monocytogenes* infected mice, V γ 6 and V γ 4 $\gamma\delta$ T cells, but not Th17 cells, were found to be the main producers of IL-17 (Hamada et al, 2008a; Hamada et al, 2008b; Xu et al, 2010). In addition, V γ 6 T cells were also found to be an early source of IFN γ (Hamada et al, 2008a). Interestingly, IL-17⁺ but not IL-17⁻ $\gamma\delta$ T cells promoted cytotoxic T cell responses against *L. monocytogenes* by enhancing cross-presentation, thus playing an important role in the defences against both extra- and intracellular bacteria (Xu et al, 2010). Recently, early IL-17 production by hepatic $\gamma\delta$ T cells has been found to have an important role in priming of adaptive immune responses in viral hepatitis (Hou et al, 2013). Neutralisation of either IL-17 or IL-23 led to increased liver injury and decreased accumulation of CD8⁺ and Th1 cells.

However, IL-17 producing cells have also been shown to have detrimental roles in certain diseases. For example, the production of IL-17 by $\gamma\delta$ T cells was found to enhance the development of pathogenic Th17 cells in mouse models of T cell-mediated colitis and multiple sclerosis (Do et al, 2011; Sutton et al, 2009a).

Pathogenic IL-17 producing $\gamma\delta$ T cells have also been detected in the dermis of psoriatic mice (Cai et al, 2011). Interestingly, the majority of $\gamma\delta$ T cells in the dermis belonged to the $V\gamma 4^+$ subsets, while epidermal $\gamma\delta$ T cells, which failed to secrete IL-17, were almost exclusively $V\gamma 5^+$. In agreement with the mouse studies, IL-17-producing $\gamma\delta$ T cells have been found to be increased in circulation of patients with ankylosing spondylitis, as well as cerebrospinal fluid of multiple sclerosis patients and the skin of psoriasis patients (Cai et al, 2011; Kenna et al, 2012; Schirmer et al, 2013).

In a model of West Nile Virus infection, $\gamma\delta$ T cells were found to be crucial for control of the infection and prevention of mortality (Wang et al, 2003). $\gamma\delta$ T cells were shown to enhance maturation of naive DC through secretion of IFN γ , TNF α and IL-6. DC co-cultured with virus-infected $\gamma\delta$ T cells had enhanced levels of co-stimulatory molecules, MHC class II expression and IL-12 production, which directly correlated with their ability to prime naïve T helper cells (Fang et al, 2010). Furthermore, the beneficial effect of $\gamma\delta$ T cells was found to be mediated by the $V\gamma 1^+$ subset, which was the major producer of IFN γ in this disease model. $V\gamma 4^+$ T cells, on the other hand, produced IL-17 and TNF α and were found to contribute to the pathogenesis of the disease through suppression of $V\gamma 1$ cell expansion via TGF β and IL-10. Mice depleted of $V\gamma 4^+$ subset had lower viral loads in the brain and a decreased mortality due to WN encephalitis (Welte et al, 2011; Welte et al, 2008). In contrast, IFN γ production by $\gamma\delta$ T cells has been shown to increase demyelination in coronavirus infected mice (Dandekar et al, 2005). Human $V\gamma 9V\delta 2$ T cells expanded in the presense of aminobisphosphonate pamidronate were shown to efficiently kill influenza virus-infected lung alveolar epithelial cells (Li et al, 2013).

Mevalonate pathway has been found to be upregulated in some tumours leading to intracellular accumulation of IPP and thus making them susceptible to phosphoantigen-specific $V\gamma 9V\delta 2$ T cell killing (Gober et al, 2003). Mouse $\gamma\delta$ T cells, on the other hand, have been shown to have an important protective role in tumour immunity by producing IFN γ early following chemically induced tumourgenesis or B16 inoculation (Gao et al, 2003).

Although many studies focus on the pro-inflammatory roles of $\gamma\delta$ T cells, these cells have also been shown to be capable of dampening the inflammation, antigen

presentation, B cell help as well as maintenance of homeostasis. Mouse V γ 4 $\gamma\delta$ T cells have been found to have a protective role in the *L. monocytogenes* induced liver injury by regulating CD8⁺ T cell responses. This effect was found to be dependent on IL-10 production by V γ 4⁺ T cells and their TCR dependent interaction with macrophages and CD8⁺ T cells (Rhodes et al, 2008). $\gamma\delta$ T cells from immunised mice have been shown to express MHC class II and co-stimulatory molecules after activation *in vitro*. While human V γ 9V δ 2 cells were shown to be effective presentation of antigens to CD4 T cells and cross-priming of CD8⁺ T cells to tumour and microbial antigens (Brandes et al, 2009; Brandes et al, 2005). A subset of human V γ 9V δ 2 cells expressing CXCR5 has been shown to behave like follicular T helper cells after co-culture in the presence of phosphoantigen and IL-21. Upon stimulation with the antigen *in vitro* these cells secreted IL-4, IL-10 and CXCL13, and provided B-cell help for antibody production (Caccamo et al, 2006; Caccamo et al, 2012).

1.15 Other innate and innate-like lymphocytes

1.15.1 NK cells

Natural killer (NK) cells are innate lymphocytes which survey the body for infected, malignant or otherwise stressed cells and eliminate them. In addition, they produce proinflammatory cytokines such as IFN γ and TNF α . NK cells become rapidly activated upon recognition of a target cells or in response to cytokine stimulation and play an important role in macrophage activation and promotion of cytotoxic and Th1 responses. NK cells depend on IL-15 for their development and survival (Kennedy et al, 2000; Lodolce et al, 1998).

NK cells express a variety of activating and inhibitory receptors on their surface. The inhibitory receptors include the members of the Ly49 family in mice, KIR receptors in humans and CD94/NKG2A heterodimers in both species. The activating receptors include murine Ly49H, Ly49D, and NK1.1 as well as NKp46 and NKG2D which are expressed both in mice and humans (Lanier, 2008). Inhibitory NK receptors contain ITIM motifs while activating receptors associate with ITAM motif containing molecules. Engagement of the inhibitory receptors leads to the recruitment of phosphatases SHP-1 and SHP-2 which dephosphorylate the substrates of tyrosine kinases downstream of the activating NK receptors. A net balance of activating and

inhibitory signals transmitted by NK cell receptors decides the outcome of the interaction between an NK cell and the target cell. Inhibitory receptors recognise classical and non-classical MHC I molecules and interaction with a healthy cell results in transmission of the inhibitory signal and thus lack of the effector function activation (Smyth et al, 2005). However, if the sampled cell becomes in any way stressed, its MHC expression level may become altered. For example, virally infected or malignant cells downregulate MHC expression to avoid being recognised by T cells. This in turn makes these cells more vulnerable to NK cell killing. Furthermore, stressed cells upregulate the expression of stress induced molecules which engage the activating NK receptors. Examples of such molecules recognised by the NKG2D activating receptors include MIC in humans and RAE1 and H60 in mice (Bauer et al, 1999; Lanier, 2008).

Upon a positive interaction with a target cell, NK cells release cytotoxic granules containing mainly granzyme and perforin which results in lysis and death of that cell. Another means by which NK cell can induce target cell death is by upregulation of death receptor ligands, such as Fas ligand (FasL) or TRAIL, and secretion of TNF α (Eischen & Leibson, 1997; Kayagaki et al, 1999; Smyth et al, 2005). All of these molecules bind to members of the TNF receptor family. Engagement of death receptors starts a signalling cascade which culminates in activation of caspase dependent cell death (Micheau & Tschopp, 2003; Smyth et al, 2005). NK cells produce IFN γ in response to stimulation with cytokines such as IL-12, IL-18, IL-15 and IL-21

1.15.2 ILC

Innate lymphoid cells (ILC) are closely related to NK cells, lack the expression of RAG-dependent rearranged antigen receptors and are dependent on Id2 transcriptional repressor. ILC are characterised as being lineage negative, lacking B, T and myeloid cell markers, but CD45 and CD127 positive. For this reason ILC are normally identified using negative gating in flow cytometric studies. Precise identification of these cells requires use of extracellular and intracellular markers. ILCs have received much attention in recent years due to the emerging evidence showing that these cells are an important source of T helper cytokines during the

early disease stages. Similar to T helper lymphocytes, ILC can be divided into three subpopulations, ILC type 1, 2 and 3, based on their cytokine profile and transcription factor expression. These in turn can be further subdivided into populations with particular characteristics and marker expression. ILC1 express CD127, NK1.1, and Tbet and produce IFN γ upon activation. They are thought to have an important role in priming of the cellular immune responses. Human ILC1 cells have been reported to produce IFN γ in response to IL-12 or IL-12+IL-15 and to have a pathogenic role in IBD (Bernink et al, 2013). In a mouse model of *Toxoplasma gondii* infection ILC1 cells were found to be the main producers of IFN γ and TNF α in response to the parasite and transfer of ILC1 cells to alymphoid mice resulted in substantial reduction in parasite titres (Klose et al, 2014).

ILC2 produce Th2 cytokines such as IL-5 and IL-13 in response to epithelia derived IL-25 and IL-33. They are commonly identified by the expression of c-kit, Sca-1 and ST2 (IL-33R) and their development is dependent on Gata3 and ROR α (Halim et al, 2012; Hoyler et al, 2012; Spits et al, 2013). ILC2 have been shown to promote helminth expulsion by inducing goblet cell hyperplasia in an IL-13 dependent manner (Moro et al, 2010; Neill et al, 2010). Inappropriate ILC2 responses can lead to development of conditions such as allergic airway inflammation (Halim et al, 2012).

ILC3 produce IL-17 and IL-22 and require Ror γ t for their development (Eberl et al, 2004; Luci et al, 2009; Sanos et al, 2009). They can be subdivided into three populations: CD4⁺ LTi(lymphoid tissue inducer) cells, NKp46⁻ ILC3s, and NKp46⁺ ILC3s (Spits et al, 2013). LTi cells are required for the formation of lymph nodes and Payer's patches as mice lacking LTi cells fail to develop these lymphoid structures (Boos et al, 2007; Eberl et al, 2004; Sun et al, 2000). NKp46⁺ILC3 are an important source of IL-22 in the gut and can produce this cytokine in response to IL-1 β +IL-23 or IL-23 alone (Mielke et al, 2013; Sanos et al, 2009; Satoh-Takayama et al, 2008). In oral infection with *Candida albicans* ILC3 cells were shown to be the main producers of the early IL-17 which is essential for the pathogen clearance (Gladiator et al, 2013). ILC3 produced IL-22 has been shown to play protective role in the lung (Taube et al, 2011; Van Maele et al, 2014). However, ILC3 cells have also been reported to contribute to the pathology of psoriasis (Ward & Umetsu, 2014)

1.15.3 NKT cells

NKT cells are a subset of T cells which recognise lipid antigens presented by the CD1d molecule. Their name derives from the fact that they were originally described as cells expressing an $\alpha\beta$ TCR together with NK cell marker NK1.1 (or CD161 in humans)(Godfrey et al, 2004). However, not all mouse strains express NK1.1 and in those that do, not all NKT cells are NK1.1 positive (Hameg et al, 2000). Both in mouse and humans NKT cells are found at the highest frequency in the liver (Middendorp & Nieuwenhuis, 2009). NKT cells, similar to $\gamma\delta$ T cells, are an innate source of T helper cytokines. They contribute to immune responses against tumours and various classes of pathogens, but can potentially promote development of autoimmune diseases (Boyton, 2008; Juno et al, 2012; Smyth et al, 2002). For example, in a mouse model of arthritis, NKT cell deficient mice had significantly lower levels of collagen-specific IL-17 production compared to wild type mice suggesting that NKT cells contribute to autoimmune inflammation in this disease model (Yoshiga et al, 2008). However, NKT cells have also been shown to have immunomodulatory properties and to suppress the induction of autoimmune inflammation in mouse models of such immune disorders as diabetes, multiple sclerosis and non-infectious liver disease (Halder et al, 2007; Jahng et al, 2004; Subramanian et al, 2012). Mouse NKT cells can be either $CD4^+$ or $CD4^-CD8^-$ and different proportions of the two subsets are present in different tissues (Eberl et al, 1999). In humans CD8 α molecule is expressed on up to 50% of NKT cells (Gumperz et al, 2002). The importance of $CD4^+$ expression on NKT cells is unclear, but some functional differences between $CD4^+$ NKT cells and $CD4^-$ NKT cells have been observed. For example, human $CD4^+$ NKT cells produce higher levels of IL-4 and IL-13, while both $CD4^+$ and $CD4^-$ populations were shown to produce IFN γ , TNF α , IL-17 and IL-22 (Gumperz et al, 2002; Lee et al, 2002; Moreira-Teixeira et al, 2011). In mice $CD4^+$ and $CD4^-$ NKT cells have been shown to have a different impact on tumour rejection in vivo, although no apparent cytokine difference was observed (Crowe et al, 2005). It appears that cytokine production by mouse NKT cells segregates better with NK1.1 expression and generally NK1.1 $^-$ cells are the dominant producers of IL-17 and IL-4, while NK1.1 $^+$ NKT cells produce mainly IFN γ (Coquet et al, 2008; Doisne et al, 2011). Although some studies have found no difference in cytokine production between the two populations(Wondimu et al, 2010).Two subsets of NKT cells can be distinguished based on the TCR repertoire

usage: invariant or type I NKT cells and non-invariant or type II NKT cells (Brennan et al, 2013; Godfrey et al, 2004). Invariant NKT (iNKT) cells are characterised by the expression of a specific TCR V α chain (V α 14J α 18 in mice and V α 24J α 18 in humans) together with a limited number of TCR- β chains. iNKT cells recognise the marine sponge glycosphingolipid α -galactosylceramide (α GalCer) and can be detected using α GalCer loaded CD1d tetramers. Non-invariant NKT cells express various TCR $\alpha\beta$ chain combinations, do not recognise α GalCer and consequently have been less well characterised as they cannot be identified using α GalCer loaded CD1d tetramers. However, a subset of non-invariant NKT cells has been shown to recognise a self-lipid sulfatide in a CD1d restricted fashion and CD1d-sulfatide tetramers have been used to identify these cells in a number of studies (Blomqvist et al, 2009; Girardi et al, 2012; Halder et al, 2007).

1.15.4 MAIT cells

Mucosa associated invariant T (MAIT) cells are a T cell subset unique to mammals which is enriched in the mucosa or organs such as gut, liver and lung. MAIT cells express an invariant TCR consisting of a single TCRV α chain paired with a limited number of variable TCR β chains. In humans MAIT cells express the V α 7.2J α 33 mainly in combination with V β 6 and V β 20, while the mouse V α chain orthologue - V α 19J α 33 - pairs with V β 6 or V β 8. Mouse MAIT cell are CD4CD8 double negative and are present at lower frequencies than in humans (Tilloy et al, 1999). They recognise numerous yeast and bacteria species in an MR1 restricted manner. MR1 is a ubiquitously expressed MHC-I related molecule with high degree of homology in mice and humans and is required for the thymic selection of MAIT cells (Riegert et al, 1998; Seach et al, 2013). Recently MR1 has been shown to activate MAIT cells via presentation of bacteria derived riboflavin (vitamin B) metabolites (Kjer-Nielsen et al, 2012; Patel et al, 2013). These findings explain why certain types of bacteria fail to activate MAIT cells as only bacteria with an intact riboflavin pathway can generate their specific ligands (Birkinshaw et al, 2014). MAIT cells promote antimicrobial responses by production of various cytokines and by direct killing of infected cells (Le Bourhis et al, 2013; Le Bourhis et al, 2010). Similar to $\gamma\delta$ T cells, they have also been shown to produce IL-17 independent of TCR stimulation (Chiba et al, 2012). Although MAIT cells contribute to the bacterial recognition in a unique way, very few

studies documented a significant role for these cells in vivo. In mouse models of pulmonary infection MAIT cells have been shown to contribute to the antimicrobial responses by providing an early source of cytokines (Chua et al, 2012; Cowley et al, 2010; Meierovics et al, 2013). A regulatory role was suggested for these cells in the mouse model of multiple sclerosis as well as in human patients (Croxford et al, 2006; Miyazaki et al, 2011). MAIT cells were found to be increased in the mucosa of IBD patients and to produce significantly more IL-17 upon TCR stimulation in vivo (Serriari et al, 2014). The nature of MAIT antigens makes these cells an attractive target for immune therapies. However, further studies are necessary to clarify the role of MAIT cells in various disease settings.

1.16. *Bordetella pertussis*

Bordetella pertussis (*B.pertussis*) is a Gram negative bacterium which causes whooping cough (pertussis), a severe debilitating respiratory disease affecting mostly children. Despite the widespread use of vaccination, pertussis is one of the most common causes of vaccine-preventable deaths worldwide (McIntyre & Wood, 2009). Furthermore, in recent years, a resurgence of whooping cough has been observed in highly vaccinated populations. This is thought to be due to waning immunity or an inadequate protection induced by the acellular vaccine (Pa), which replaced the whole cell vaccine in developed countries in the 1990s. The Pa has become the vaccine of choice due to its reduced frequency of adverse effects such as fever or seizures, and the associated increased compliance. Natural infection with *B. pertussis* induces lifelong protection and many studies are investigating differences between the immune responses elicited by the infection and the two available vaccines in hope of improving the acellular vaccine formulation.

Humans are the only natural host of *B. pertussis*, however, mice and pigs can also be infected under experimental conditions. Apart from *B. pertussis* there are 3 other members of the the *Bordetellae* family that can infect humans and other mammals, namely *B. parapertussis*, *B. bronchiseptica* and *B. holmesii*. The first two species are very closely related to *B. pertussis* while *B. holmesii* is a more distant relative. *B. parapertussis* infects humans and sheep and causes a milder pertussis-like disease in children (Heininger et al, 1994; Porter et al, 1994). *B. bronchiseptica* is typically a veterinary pathogen. Nonetheless, it remains clinically important as it can cause a

serious disease in immunocompromised individuals (Belen et al, 2003; de la Fuente et al, 1994; Viejo et al, 2002). *B. holmesii* is the least well studied of the *Bordetellae* and to date it has been reported to cause a variety of opportunistic infections, including pertussis-like disease, especially in patients with red blood cells disorders or asplenia (Clare et al, 2010; Livovsky et al, 2012; McCavit et al, 2008; Moissenet et al, 2011; Panagopoulos et al, 2010).

B. pertussis expresses many virulence factors which enhance bacterial survival and modulate immune responses of the host. Many of these factors such as filamentous hemagglutinin (FHA), pertactin, or adenylate cyclase toxin (ACT) are common to different *Bordetella* species. FHA is the major attachment factor of *B. pertussis* and can be expressed on the cell surface or secreted into the extracellular milieu (Locht et al, 1993). Soluble FHA has been shown to inhibit IL-12 but enhance IL-10 production by macrophages and DC, which in turn leads to generation of Treg cells that suppress Th1 responses (McGuirk et al, 2002; McGuirk & Mills, 2000). Furthermore, FHA has been shown to bind to CD11b/CD18 on human neutrophils and enhance phagocytosis of *B. pertussis*, most likely as a mechanism of gaining access to the cell interior rather than unintentional opsonisation of bacteria (Mobberley-Schuman & Weiss, 2005). Recently, FHA has been shown to inhibit the induction of IL-17 responses in the lung (Henderson et al, 2012). Infection of mice with FHA-deficient *B. bronchiseptica* resulted in greater inflammatory response at the early stages of infection with a steady influx of IL-17⁺ neutrophils and macrophages.

ACT, similar to FHA, is able to bind to CD11b/CD18 integrin expressed by macrophages and neutrophils. However, it is also able to intoxicate CD11b/CD18 negative cells (Eby et al, 2012). ACT is a bipartite toxin with adenylase cyclase and pore forming activities (Benz et al, 1994). Enzymatic activity of the toxin leads to deregulation of cellular signalling through simulation of uncontrolled ATP to cAMP conversion, while its pore forming ability induces innate IL-1 β production in macrophages and DCs (Dunne et al, 2010). The adenylate cyclase activity of ACT has been shown to inhibit activation of APCs and induce their apoptosis (Kamanova et al, 2008; Khelef & Guiso, 1995). Furthermore, it was found to inhibit IL-12 production by DC but enhance LPS induced IL-10 secretion by these cells thus promoting induction of Tr1 cells and inhibiting generation of Th1 responses (Boyd et al, 2005; Hickey et al, 2008; Ross et al, 2004).

Pertactin (PRN) is a 69kDa autotransporter protein and has been implicated in mediating adherence of *B. pertussis* to mammalian cells by studies of Leininger and colleagues (Leininger et al, 1991). However, other groups have failed to confirm this role and PRN-deficient *B. pertussis* strains showed no impairment of colonisation of murine respiratory tract compared to wild type bacteria (Everest et al, 1996; Khelef et al, 1994; Roberts et al, 1991; van den Berg et al, 1999). Nonetheless, high anti-PRN antibody titres were found to correlate with protection against *B. pertussis* (Storsaeter et al, 1998). In addition, PRN has been shown to be required for *B. bronchiseptica* to resist neutrophil mediated clearance (Inatsuka et al, 2010).

Pertussis toxin (PT) is expressed exclusively by *B. pertussis* (Parkhill et al, 2003). It is a secreted ADP-ribosylating toxin consisting of 1 active subunit and 5 binding subunits (AB₅ toxin). It binds to glycosylated molecules and thus can potentially intoxicate any mammalian cell (Witvliet et al, 1989). Upon entering the cytosol, PT interferes with G protein coupled receptor signalling (Katada et al, 1983). Purified PT has been shown to enhance bacterial colonisation when administered intranasally even 14 days prior to infection, suggesting that it acts mainly on lung resident and not recruited cells (Carbonetti et al, 2003). PT has been shown to impair the function of airway macrophages and to interfere with neutrophil recruitment through inhibition of chemokine production (Andreasen & Carbonetti, 2008; Carbonetti et al, 2007). Although PT functions mainly to inhibit host immune responses, it was also found to promote IL-17 production at the later stages of the infection (Andreasen & Carbonetti, 2008; Carbonetti, 2010).

Rationale and objectives for the study:

A majority of studies investigating the role of $\gamma\delta$ T cells focus on cells present in the spleen and lymph nodes. While this gives us insight into the overall response during infectious or autoimmune diseases, it tells us very little about what happens in tissues during the initiation phases of the disease. Given that cells present in tissues are likely to be the first to encounter the triggering factor, they are in a position to influence the systemic responses and as a consequence have a great role in deciding the outcome of the infection. Furthermore, in any given disease, the role of resident cells may be quite different from that of their peripheral counterparts. Understanding how tissue resident innate cell populations respond to pro-inflammatory stimuli may help development of therapies that would be able to skew or dampen the immune responses at the source. Identification of stimuli promoting specific $\gamma\delta$ T cell responses may be explored in vaccine design to induce better protection.

The overall objective of the study was to investigate the activation and function of mouse mucosal $\gamma\delta$ T cells. In order to do that the following specific objectives were set out:

- 1) The first objective of the current study was to examine the effects of cytokines on expansion of $\gamma\delta$ T cell *in vitro* in order to facilitate studying tissue resident $\gamma\delta$ T cell populations.
- 2) Next, the characteristics and activation requirements of gut and lung $\gamma\delta$ T cells were compared to those of cells resident in secondary lymphoid organs.
- 3) Finally, the role of $\gamma\delta$ T cells in *B. pertussis* infection was investigated.

Chapter 2: Materials and Methods

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2.1 Materials

2.1.1 Cell culture medium

Roswell Park Memorial institute (RPMI)-1640 medium (Sigma) was supplemented with 10% heat inactivated foetal calf serum (FCS), 100mM L-Glutamine (Gibco) and 100ug/mL penicillin/streptomycin (Biowest). Complete RPMI (cRPMI) was used to culture immortalised bone marrow derived macrophages while cRPMI supplemented with β -mercaptoethanol (2-ME 50 μ M) was used to culture T cells, and lymph node cells ex vivo. cRPMI lacking penicillin/streptomycin was used to culture bacteria infected cells.

2.1.2 Phosphate buffered saline (PBS) 20X

320g Sodium Chloride (NaCl, 1.4M)

46g Sodium hydrogen phosphate (Na_2HPO_4 , 0.08M)

8g Potassium di-hydrogen phosphate (KH_2PO_4 , 0.01M)

8g Potassium chloride (KCl, 0.03M)

Dissolved in 2L of dH_2O and adjusted to pH 7.0

2.1.3 Red blood cell (RBC) lysis buffer

4.35 g Ammonium Chloride NH_4Cl

Dissolved in 500 mls ddH_2O

2.1.4 ELISA reagents

2.1.4.1 ELISA wash buffer (PBS-Tween)

500mL 20X PBS

9.5L dH₂O

5mL Tween 20

2.1.4.2 Phosphate citrate buffer

20.38g Citric acid (C₆H₈O₇)

73.8g di-Sodium hydrogen orthophosphate dodecahydrate (Na₂HPO₄•12H₂O)

Made up to 2L with dH₂O, pH 5.0

2.1.4.3 ELISA substrate solution

1 10mg o-Phenylenediamine dihydrochloride (OPD) tablet (Sigma)

25mL Phosphate Citrate Buffer

7μL H₂O₂

2.1.4.4 ELISA stop solution (1M H₂SO₄)

26.74mL 18M H₂SO₄

473.26 mL dH₂O

2.1.4.5 ELISA kits

Cytokine	Supplier	Working concentrations range
IL-6	BD Biosciences	0-5000 pg/mL
IL-5	BD Biosciences	0-2500 pg/mL
IL-4	BD Biosciences	0-2500 pg/mL
IL-18	BD Biosciences	0-4000 pg/mL
IFN γ	R&D Systems	0-10 ng/mL
IL-17A	R&D Systems	0-1000 pg/mL
IL-17F	R&D Systems	0-1500 pg/mL
IL-22	R&D Systems	0-2000 pg/mL
IL-10	R&D Systems	0-2000 pg/mL
TGF β	R&D Systems	0-2000 pg/mL
IL-23	R&D Systems	0-2500 pg/mL
IL-1 β	R&D Systems	0-1000 pg/mL
IL-1 α	R&D Systems	0-1000 pg/mL
TNF α	R&D Systems	0-2000 pg/mL
IL-12p70	R&D Systems	0-2500 pg/mL
IL-12p40	R&D Systems	0-5000 pg/mL
IL-27	R&D Systems	0-2000 pg/mL
IL-13	R&D Systems	0-4000 pg/mL

2.1.5 FACS reagents

2.1.5.1 FACS buffer

10 mL FCS

500mL 1xPBS

2.1.5.2 FACS antibodies

Antigen specificity	Fluorochrome	Clone	Isotype	Supplier
CD11b	A780	M1/70	Rat IgG2b, kappa	eBioscience
CD11b	APC	M1/70	Rat IgG2b, kappa	eBioscience
CD11c	PE	HL3	Armenian hamster IgG1, lambda 2	BD
CD11c	PE-Cy7	N418	Armenian hamster IgG	eBioscience
CD19	FITC	1D3	Rat IgG2a, kappa	eBioscience
CD19	PE-Cy7	1D3	Rat IgG2a, kappa	eBioscience
CD27	PE-Cy7	LG.7F9	Armenian hamster IgG	eBioscience
CD3	A700	17A2	Rat IgG2b, kappa	eBioscience
CD3	A780	17A2	Rat IgG2b, kappa	eBioscience
CD3	FITC	145-2C11	Armenian hamster IgG	eBioscience
CD3	PE-Cy5	145-2C11	Armenian hamster IgG	eBioscience
CD3	PE-Cy7	145-2C11	Armenian hamster IgG	eBioscience
CD3	PE-Cy7	145-2C11	Armenian hamster IgG	eBioscience
CD3	PerCP-Cy5.5	145-2C11	Armenian hamster IgG	eBioscience
CD3	V450	145-2C11	Armenian hamster IgG	eBioscience
CD3	PE	145-2C11	Armenian hamster IgG	eBioscience
CD4	A780	RM4-5	Rat IgG2a, kappa	eBioscience
CD4	APC-Cy7	GK1.5	Rat IgG2b, kappa	BD
CD4	PE-Cy5	GK1.5	Rat IgG2b, kappa	eBioscience
CD4	PE-Cy7	GK1.5	Rat IgG2b, kappa	eBioscience
CD44	eFluor® 605NC	IM7	Rat gG2b, kappa	eBioscience
CD45	PerCP-Cy5.5	30-F11	Rat IgG2b, kappa	eBioscience
CD62L	PerCP-Cy5.5	MEL-14	Rat IgG2a, kappa	eBioscience
CD8α	A780	53-6.7	Rat IgG2a, kappa	eBioscience
CD8α	FITC	53-6.7	Rat IgG2a, kappa	eBioscience
CD8α	PE	53-6.7	Rat IgG2a, kappa	eBioscience
CD8α	PE-Cy7	53-6.7	Rat IgG2a, kappa	eBioscience
CD49b	PE	DX5	Rat IgM, kappa	eBioscience
CD49b	V450	DX5	Rat IgM, kappa	eBioscience
F4/80	PE-Cy5	BM8	Rat IgG2a, kappa	eBioscience
F4/80	PE-Cy7	BM8	Rat IgG2a, kappa	eBioscience
γδTCR	APC	GL3	Armenian hamster IgG	eBioscience
γδTCR	FITC	GL3	Armenian hamster IgG	eBioscience
γδTCR	PerCP-e710	GL3	Armenian hamster IgG	eBioscience
IFNγ	FITC	XMG1.2	Rat IgG1, kappa	eBioscience
IFNγ	PE-Cy7	XMG1.2	Rat IgG1, kappa	eBioscience
IL-1α	FITC	ALF-161	Armenian Hamster, IgG	eBioscience
IL-12/23p40	PerCP-Cy5.5	C17.8	Rat IgG2a, kappa	eBioscience
IL-17a	FITC	17B7	Rat IgG2a, kappa	eBioscience

IL-17a	PE	17B7	Rat IgG2a, kappa	eBioscience
IL-17a	V450	TC11-18H10	Rat IgG1, kappa	BD
IL-22	A647	Poly5164	Polyclonal Goat IgG	Biolegend
IL-22	PE	1H8PWSR	Rat IgG1, kappa	eBioscience
IL-22	PerCP-e710	1H8PWSR	Rat IgG1, kappa	eBioscience
Ly6C	PerCP-Cy5.5	HK1.4	Rat IgG2c, kappa	eBioscience
IFNγ	PE-Cy7	XMG1.2	Rat IgG1, kappa	eBioscience
IFNγ	PE-CF594	XMG1.2	Rat IgG1, kappa	Biolegend
Ly6G	Pacific Blue	1A8	Rat IgG2a, kappa	Biolegend
NK1.1	PerCP-Cy5.5	PK136	Mouse IgG2a, kappa	eBioscience
NK1.1	V450	PK136	Mouse IgG2a, kappa	BD
NKp46	FITC	29A1.4	Rat IgG2a, kappa	eBioscience
NKp46	PE-Cy7	29A1.4	Rat IgG2a, kappa	eBioscience
proIL-1β	APC	NJTEN3	Rat IgG1, kappa	eBioscience
Siglec-F	PE	E50-2440	Rat IgG2a, kappa	BD
Siglec-F	PE-CF594	E50-2440	Rat IgG2a, kappa	BD
TCRβ	APC	H57-597	Armenian Hamster IgG	eBioscience
TNFα	PE-Cy7	MP6-XT22	Rat IgG1, kappa	eBioscience
Vγ1.1	PE	2.11	Armenian hamster IgG	Biolegend
Vγ4	FITC	UC3-10A6	Armenian hamster IgG	BD

2.1.5.3 Other reagents used for FACS

Reagents	Description	Supplier	Concentration
CFSE	Cell membrane dye (FL-1)	Invitrogen	2.5 μ g/ml
Aqua Live/Dead	Viability dye (FL-8)	Invitrogen	1/600
PMA	Mitogen	Sigma	10 ng/ml
Ionomycin	Ionophore	Sigma	500 ng/ml
BFA	Golgi inhibitor	Sigma	5 μ g/ml

2.1.6 MACS buffer

500 mL PBS

0.5% FCS

2mM EDTA

2.1.7 FACS sorting buffer

- 500 mL PBS
- 2 mM EDTA
- 25 mM HEPES pH 7.0
- 1% FCS
- 100 µg/mL penicillin/streptomycin (Biowest)

2.1.8 RT-PCR primers

Gene	Protein	Product Code	Supplier
18S rRNA	18S rRNA	4319413E	ABI
gata3	Gata3	Mm00484683_m1	ABI
lfng	IFN-γ	Mm00801778_m1	ABI
il-17a	IL-17a	Mm00439618_m1	ABI
il-17f	IL-17f	Mm00521423_m1	ABI
il-22	IL-22	Mm00444241_m1	ABI
il-1b	IL-1β	Mm00434228_m1	ABI
il-23 ^a	IL-23	Mm01160011_g1	ABI
il-6	IL-6	Mm00446190_m1	ABI
iL-10	IL-10	Mm00439616_m1	ABI
il1r1	IL-1R1	Mm00434237_m1	ABI
il-12rb1	IL-12Rβ1	Mm00434189_m1	ABI
il-23r	IL-23R	Mm00519942_m1	ABI
il18r	IL-18R	Mm00515180_m1	ABI
cd27	CD27	Mm01185212_g1	ABI
reg3g	RegIIIγ	Mm00441127_m1	ABI
Rorc	Roryt	Mm01261022_m1	ABI
defb3	mBD3	Mm04214158_s1	ABI
defb1	mBD1	Mm00432803_m1	ABI
Camp	CAMP	Mm00438285_m1	ABI
tbx21	Tbet	Mm00430960_m1	ABI
tgfb1	TGFβ	Mm00441724_m1	ABI
Tnf	TNFα	Mm00443258_m1	ABI

2.1.9 Recombinant cytokines used *in vitro*

Cytokine	Supplier	Concentration used in vitro
IL-1 β	R&D	10 ng/mL
IL-23	R&D	10 ng/mL
IL-18	R&D	10 ng/mL
IL-2	R&D	20 or 50 U/mL
IL-7	R&D	10 ng/mL
IL-15	R&D	10 or 20 ng/mL

2.1.10 Small molecules and antibodies used in vitro

Molecule	Type	Concentration used	Supplier
6-Formylindolo(3,2-b)carbazole (FICZ)	Ahr agonist	1 μ M	Enzo
All trans retinoic acid (RA)	Rar agonist	100nM	Enzo
YVAD-fmk	Caspase-1 inhibitor	50 μ M	Calbiochem
α CD3	CD3 stimulating antibody	1 μ g/mL	eBioscience
α IL-23	IL-23 neutralising antibody	5 μ g/mL	eBioscience

2.1.11 Antibodies used in vivo

Antibody	Type	Dose used	route	Supplier
α IL-17	IL-17 neutralising antibody	200 μ g/100 μ L	i.p.	BioXcell
IgG	Isotype control antibody	200 μ g/100 μ L	i.p.	Louise Boon

2.1.12 TLR ligands used in vitro

PRR ligand	PRR	Concentration used	Supplier
LPS	TLR4	100ng/mL	Enzo
Pam3CSK4	TLR1 and TLR2	100ng/mL	Invivogen
Zymosan	TLR2 and Dectin-1	10µg/mL	Invivogen
CpG	TLR9	1µg/mL	Sigma-genosys

2.1.13 *B. pertussis* molecules used for antigen recall

Molecule	Concentration used	Supplier
FHA	4µg/mL	Kaketsuken
PT	2µg/mL	List Biological Laboratories

2.1.14 Stock Isotonic percoll

Percoll is made isotonic by diluting stock Percoll (GE Healthcare) solution 9:1 with 10x PBS. Different stock Percoll solutions were prepared by diluting stock isotonic Percoll with 1xPBS (Sigma), for example:

For 40% Percoll 4 parts of stock isotonic Percoll were mixed with 6 parts of 1xPBS

For 70% Percoll 7 parts of stock isotonic Percoll were mixed with 3 parts of 1xPBS

For 30% Percoll 3 parts of stock isotonic Percoll were mixed with 7 parts of 1xPBS

2.1.15 Stainer-Scholte liquid medium (pH 7.3-7.4)

L-glutamic acid (monosodium salt)	10.72g	21.44g
L-proline	0.24g	0.48g
NaCl	2.5g	5.0g
KH ₂ PO ₄	0.5g	1.0g
KCl	0.2g	0.4g
MgCl ₂ •6H ₂ O	0.1g	0.2g
CaCl ₂ •2H ₂ O	0.02g	0.04g
Tris (Trizma base C ₁₄ H ₁₁ NO ₃)	1.525g	3.030g
	1L	2L

Made up to appropriate volume with ddH₂O and sterilised by autoclaving

2.1.16 Supplement (200x)

L-cystine	0.4g (dissolved in 1mL 37% HCl)
FeSO ₄ •7H ₂ O	0.1g
L-Ascorbic Acid C ₆ H ₈ O ₆	0.2g
Nicotinic Acid C ₆ H ₅ NO ₂	0.04g
L-Glutathione(reduced)	1.0g

Made up to 50mL with baxter water and filter sterilised.

2.1.17 1% Casein salt solution

NaCl 6.0g

BactoCasamino acids 10.0g

Made up to 1L with ddH₂O

pH 7.0-7.2

filter sterilised

2.1.18 Bordet Gengou Blood agar plates

250mL ddH₂O

2.5mL glycerol

7.5g BG Agar

Made up to 250mL with ddH₂O and autoclaved at 121°C for 20 minutes.

1mL Cephalexin (10mg/mL)

100mL Horse blood (pre-warmed to 37°C)

2.1.19 Immortalised bone marrow derived macrophages (IBMM)

IBMM from wild-type C57BL/6, generated using J2 transforming retroviruses, were a gift from Prof. D. Golenbock (University of Massachusetts Medical Center).

2.2 Methods

2.2.1 Ethics & Animal License Details

All mice were maintained according to European Union regulations and the Health Products Regulatory Authority (HPRA). Experiments were performed under HPRA license with approval from the Trinity College Dublin BioResources Ethics Committee. Individual HPRA licence number: AE19136/I100.

2.2.2 Mice

C57BL/6 mice were purchased from Harlan, UK. IL-1RI^{-/-}, TCR δ ^{-/-} and IL-22^{-/-} mice were bred in-house on a C57BL/6 background. Age matched wild type mice were bred in-house as a control for any transgenic mice used. Mice were sacrificed by cervical dislocation or by asphyxiation with CO₂. All animals used were age matched females 8-12 weeks old at the initiation of experiments.

2.2.3 Cell counts

Viable cell numbers were determined by diluting cells 1:10 in 50% trypan blue (Sigma)/50% PBS (Biowest). A disposable haemocytometer (Hycor Biomedical, UK) was then filled with 10 μ L volume of the cell suspension. Viable cells which exclude the stain and therefore appear white under a light microscope were then counted.

2.2.4 Immortalised bone marrow derived macrophages (IBMM)

IBMM were maintained in RPMI medium containing 10% FCS 100 mM L-Glutamine (Gibco) and 100 μ g/mL penicillin/streptomycin. The cells were grown in T75 cell culture flasks at 37°C, 5% CO₂ and 95% humidity. When cells were approximately 75% confluent, they were removed from the flask using a cell scraper. They were then split 1:10 by transferring 2mL of the cells into a fresh flask containing 18mL of media. Before returning to the incubator the flask was laid down and gently agitated to ensure even distribution of the cells on the bottom of the flask. In order to minimise the number of dead cells in the culture, the media was changed completely once a week by removing media and scraping off the adherent cells and centrifuging

them in a 50 mL falcon tube at 1300 rpm for 5 minutes. The cells were then resuspended in 20 mL of fresh cRPMI and split 1:10 as before. For use in assays the cells were resuspended at 1×10^6 cells/mL and allowed to adhere for 3 hours before stimulating.

2.2.5 Preparation of single cell suspension of lymph node and spleen cells

Pooled lymph nodes (inguinal, auxiliary, brachial, cervical and mesenteric) or spleens were forced through a 40 μ m cell strainer. They were then centrifuged at 1300 rpm for 5 minutes and resuspended in 20 mL cRPMI. When isolating spleen cells, red blood cells were then lysed by incubating in RBC lysis buffer (2.1.3) for 2 minutes followed by a wash with cRPMI. The cell number was determined by trypan blue method (2.2.3) and cells were used directly or specific populations were enriched by MACS or FACS sorting.

2.2.6 Optimisation of the CFSE proliferation assay protocol

Lymph node cells were forced through 40 μ m cell strainers. The cells were counted and an aliquot of unlabelled cells was taken as control. The remaining cells were washed with PBS. Stock CFSE was diluted to give 10mL stocks at concentration of 1 μ M, 0.5 μ M and 0.25 μ M. The washed cells were then resuspended in 30 mL warm PBS and split into 3 tubes. They were then mixed at a 1:1 ratio with previously prepared 10 mL CFSE stocks. This resulted in final labelling CFSE concentration of 0.5 μ M, 0.25 μ M and 0.125 μ M. The cells were mixed for 2 minutes by constant inverting. 2 mL FCS were added to each tube to quench the reaction. The cells were centrifuged for 5 minutes at 1300rpm. They were then washed twice in cRPMI and counted. The cells were then resuspended at 1×10^6 cells/mL and seeded onto 24 well plates with 1mL of cells per well. The cells were stimulated with IL-2+IL-7+IL-18, anti-CD3 or media as control for 24, 48 and 72 hours. At each time point the samples were prepared for FACS analysis of CFSE dilution: the cells were transferred into polystyrene FACS tubes. The tubes were spun for 5 minutes at 1300 rpm. The supernatants were poured off and the tubes gently flicked to resuspend the pellets. The experimental samples were then surface stained for 15 minutes in 50 μ L FACS

buffer containing appropriate antibodies (as indicated in figure legends) and the FCy block. Excess antibody was then removed by washing each cell sample with 3 mL FACS buffer, followed by centrifugation at 1300 rpm for 5 minutes. The samples were analysed on a CyAn ADP (Beckman Coulter) machine and the results analysed on FlowJo software.

2.2.7 Detection of $\gamma\delta$ T cell proliferation using carboxyfluorescein diacetate succinimidyl ester (CFSE) proliferation assay

Cells to be labelled were washed in 1x PBS (Sigma). They were then resuspended in 10mL pre-warmed PBS. An equal volume of 1 μ M CFSE was added to give a staining concentration of 0.5 μ M. The cells were incubated for 10-15 minutes at 37°C. The staining was quenched by addition of 2mL of FCS and incubation on ice for 5 minutes. The cells were then washed in cRPMI and counted. The labelled cells were resuspended at 1×10^6 cells/mL, seeded on 24 well plates at 1mL/well and stimulated with recombinant IL-1 β , IL-23, IL-2, IL-7, IL-15 and IL-18 and their combinations. They were then incubated at 37°C for 72 hours. The extent of cell proliferation was determined by FACS analysis of CFSE dilution in dividing labelled cells compared to unstimulated labelled control.

2.2.8 Detection of T cell responses *in vitro*

Isolated lymph nodes from C57BL/6 mice were forced through 40 micron filters (Nunc) onto a petri dish using a 5mL syringe barrel. The filters were flushed with cRPMI to maximise the number of cells recovered. The cell suspension was then transferred into 15mL centrifuge tubes and the cells were spun at 1250rpm for 5 minutes. The cell pellet was then resuspended in 1-2 mL of cRPMI and cell numbers and viability determined using the trypan blue method (2.2.3). The cells were then brought to concentration of 1×10^6 cells/mL, transferred onto 24 well plates (Greiner) and stimulated with recombinant IL-1 β , IL-23, IL-2, IL-7, IL-15 and IL-18 and their combinations. Supernatants were removed 72 hrs post stimulation and analysed for IFN- γ and IL-17 concentrations.

2.2.9 Isolation of Intestinal intraepithelial lymphocyte (IEL)

Small intestines were excised and placed on Petri dishes containing complete HBSS (cHBSS). Any remaining mesentery and fat were removed. The intestines were then flushed with cHBSS using a syringe with a 27G needle attached. Peyer's Patches were removed and the intestines were cut longitudinally. They were then washed with cHBSS two times, transferring onto a fresh Petri dish between each wash. The intestines were then cut into ~5mm pieces and transferred into a 50mL tube. The tissue was incubated in 20 mL of Ca^{2+} Mg^{2+} free cHBSS containing 2mM EDTA for 30min at 37°C with shaking at 200rpm. The digested tissue was passed through a 70 μm cell strainer washed in cRPMI and the cell number was determined by the Trypan blue method (2.2.3). The cells were then pelleted and resuspended in 5mL of 40% (v/v) Percoll (GE Healthcare) and overlaid over 5mL of 70% (v/v) Percoll. They were then centrifuged at room temperature for 20 minutes at 1500 rpm without break. The IELs were collected from the interphase with a Pasteur pipette, washed with cRPMI and resuspended in 1mL cRPMI. The viable cells were counted using the trypan blue method.

2.2.10 Preparation of single cell suspension of lung lymphocytes

Lungs were exised and cut into small pieces using a scalpel. They were then transferred into 1.5 mL eppendorf tubes and digested in 1mL HBSS without Mg^{2+} or Ca^{2+} ions (Sigma) in the presence of 1mg/mL collagenase D (Roche) and DNase I (20 U/mL) for 1 hour at 37°C with rotating. For experiments requiring non-enzymatic isolation of lung lymphocytes, whole lungs were placed in 2mL eppendorf tubes containing stainless steel beads (Qiagen). The tissue was then disrupted using Tissue Lyser (Qiagen) at a frequency of 30/s for 2.5 minutes. The digested or disrupted lungs were then forced through 40 μm cell strainers. The cells were washed followed by red blood cell lysis. They were then resuspended in 30% Percoll and centrifuged at RT for 20 minutes at 1500rpm without break. The cell number was determined and the cell either used directly or specific populations purified by MACS or FACS sorting.

2.2.11 $\gamma\delta$ T cell FACS sorting

Intestinal intraepithelial lymphocytes, lung lymphocytes or lymph node cells were prepared as before (2.2.5, 2.2.9, 2.2.10). The number of viable cells was established through the Trypan Blue method (2.2.3.). The cells were then centrifuged at 1300rpm for 5 minutes and resuspended at 50×10^6 cells/mL in cRPMI. Antibodies against CD3 and TCR δ (GL3 clone) as well as Fcy block were added using the optimal antibody concentration per 100 μ L to calculate the amount needed for the sort stain. When sorting $\gamma\delta$ T cell subsets, V γ 1 (clone 2.11) and V γ 4 (clone UC3-10A6) stain was included as well. The cells were then incubated at room temperature for 30 minutes in the dark. They were then washed in cRPMI and resuspended in 0.5-1mL cRPMI. The cells were then passed through a 30 μ m cell strainer into a sterile FACS tube and sorted on a MoFlo sorter (Beckman Coulter). Cellular purity of separated populations was determined by flow cytometric analysis and was routinely >90%. $\gamma\delta$ T cells were seeded on a 96 well plate at $0.2-0.5 \times 10^6$ cells/mL (or 0.02 cells/mL for $\gamma\delta$ T cell subsets) and stimulated as required.

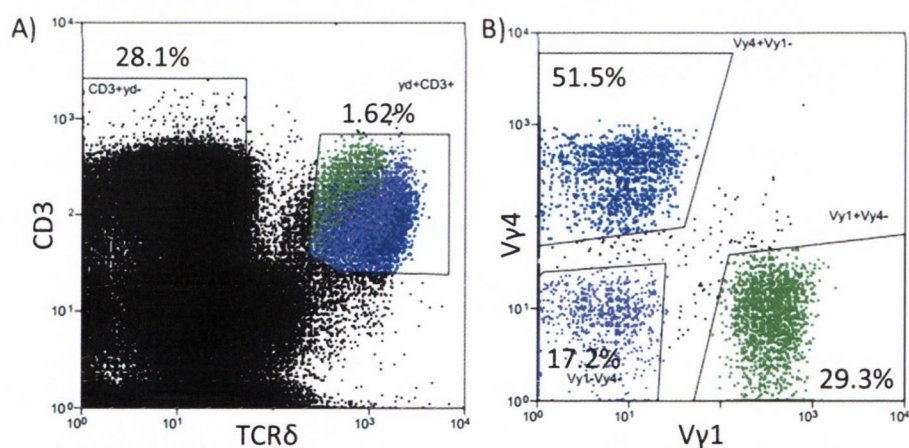


Figure 2.1 Gating strategy for $\gamma\delta$ T cell FACS sorting

In order to sort pure populations of $\gamma\delta$ T cells, sequential gating on single cells, lymphocytes and CD3 $^+$ TCR δ^+ ($\gamma\delta$ T cells) was used. When sorting $\gamma\delta$ T cell subsets, further three gates were set based on V γ 1 and V γ 4 expression. The plots show percentages of CD3 $^+$ TCR δ^+ and CD3 $^+$ TCR δ^- cells in the total lymphocyte gate (A) and percentages of the $\gamma\delta$ T cell subsets in the CD3 $^+$ TCR δ^+ gate (B).

2.2.12 Lung and spleen APC sorting

Lung and spleen cell suspension was prepared as before (2.2.5). The number of viable cells was established through the Trypan Blue method (2.2.3.). The cells were then centrifuged at 1300rpm for 5 minutes and resuspended at 50×10^6 cells/mL in cRPMI. The cells were stained with fluorescent labelled antibodies against CD3, CD19, CD11c and CD11b for 30 minutes at room temperature in the dark. The amount of the antibody required for labelling was calculated using optimal concentrations per 100 μ L. Next the cells were washed and resuspended in 0.5-1 mL of FACS sorting buffer and filtered through a 30 μ m cell strainer. The cells were then purified on a MoFlo sorter.

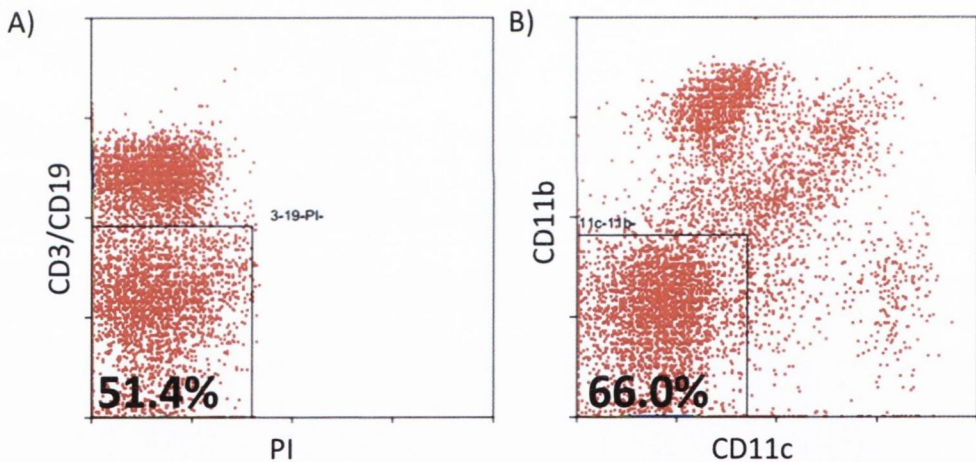


Figure 2.2 Gating strategy for FACS sorting of lung and spleen APC

In order to sort pure populations of lung and spleen APCs, sequential gating was employed. First a live and single cell gate were set. APCs were then sorted by gating on non T non B cells and then excluding CD11c⁺CD11b⁻ cells from that gate. The plots show percentages of cells in the total live gate (A) and the CD3⁺CD19⁺ gate (B).

2.2.13 $\gamma\delta$ T cell autoMACS sorting

$\gamma\delta$ T cell were purified from pooled lymph node cells by two step MACS sorting using $\gamma\delta$ T cell isolation kit (Miltenyi Biotec) as follows: first non-T cells were directly

labelled with MicroBeads conjugated to monoclonal antibodies against mouse CD45R (B220; isotype: rat IgG2a), and CD11b (Mac-1 α -chain; isotype: rat IgG2b) while $\gamma\delta$ T cells were labelled with biotin-conjugated monoclonal antibody against mouse TCR $\gamma\delta$ (isotype: hamster IgG2). Non-T cells were depleted on an autoMACS machine using the “depl05” program. $\gamma\delta$ T cells were then positively selected from the enriched T cell fraction by indirect labelling with Anti-Biotin MicroBeads. The “posseld2” program, suited for enriching of rare cell populations, was selected on the autoMACS machine. Cellular purity of separated populations was determined by flow cytometric analysis and was routinely >90%. $\gamma\delta$ T cells were seeded at $0.2-0.5 \times 10^6$ cells/mL and stimulated as required.

2.2.14 Detection of IEL activation and cytokine production *in vitro*

Isolated IELs were seeded on 24 well plates (Greiner) and stimulated with IL-1 β and IL-23, IL-18 and IL-23, IL-2 and IL-15, IL-2 and IL-18 or with cRPMI alone as control. The cells were incubated for 72 hours at 37°C, 5% CO₂. The supernatants were then removed and analysed for the presence of IFN γ , IL-17, TNF α , IL-22, IL-17f by ELISA.

2.2.15 Detection of cytokine and cytokine receptor expression by intestinal intraepithelial $\gamma\delta$ T cells

Sorted $\gamma\delta$ T cells were plated at 0.2×10^6 cells/mL on 96 well U-bottom plates. They were then stimulated with IL-1 β +IL-23, α CD3 or media as control and incubated for 72 hours at 37°C, 5% CO₂. The supernatants were then removed and transferred onto fresh 96 plates and tested for cytokine concentration by ELISA. The cells were lysed in a total of 350 μ L RLT buffer (Qiagen; (1% β -mercaptoethanol V/V)) per stimulation, transferred into 1.5mL RNase/DNase free eppendorf tubes and frozen at -80°C or processed immediately. RNA was then extracted using RNeasy kit (Qiagen) and gene expression analysed by RT-PCR.

2.2.16 Isolation of RNA using RNeasy kit (Qiagen)

Samples in 350µL of RLT buffer (1% β-mercaptoethanol V/V) were mixed with an equal volume of 70% ethanol. They were then transferred into RNeasy Mini spin columns placed in 2mL collection tubes and centrifuged at 8000 x g for 15s. The flow through was discarded and 700µL of Buffer RW1 added to each spin column. The samples were then centrifuged as before and the flow through discarded. This was repeated with 500µL of Buffer RPE. Then another 500µL of Buffer RPE were added and the samples spun for 2 minutes at 8000xg. The columns were then placed in fresh spin tubes and centrifuged for further 1 minute at full speed to dry the membrane. The columns were then placed in labelled collection tubes and 30µL of RNase free water were added directly to the spin column membrane. The samples were then centrifuged for 1min at 8000xg to elute RNA. Nucleic acid concentration was measured using a Nanodrop device.

2.2.17 RNA isolation from lung tissue

Lung samples from each animal were homogenised in 1mL Trizol Reagent® (Invitrogen) and transferred into 1.5mL RNase/DNase free eppendorf tubes. The samples were then frozen until further use. On the day of isolation the samples were defrosted on ice. 200µL chloroform were added per 1mL Trizol. The tubes were vigorously shaken for 15 seconds and incubated at room for 5 minutes followed by centrifugation at 12000xg, 4°C for 15 minutes. The aqueous layer was then transferred into fresh labelled tubes and the organic layer discarded. 500µL isopropanol were added to each tube. The tubes were then shaken for 15 seconds and incubated at room temperature for 10 minutes. They were then spun at 12000xg, 4°C for 10 minutes. The supernatants were then poured off and 1mL of 75% ethanol was added to each tube. The tubes were vortexed and then centrifuged for 10 minutes at 7500g, 4°C. The alcohol was poured off and the pellets left to air dry. 50µL of DNase/RNase free H₂O were added to each tube to dissolve the RNA.

2.2.18 Reverse transcription with High Capacity cDNA Reverse Transcription Kit (Applied Biosystems)

$\gamma\delta$ T cell and lung tissue (2.2.16-2.2.17) mRNA samples and kit components were thawed on ice. Appropriate amount of RNase free H₂O was added to each sample in order to equalise the RNA concentration between the samples. The total concentration of mRNA used for reverse transcription was ≤ 1.5 μ g RNA/reaction in the volume of 10 μ L. Reverse transcription master mix was prepared by mixing 2 μ L RT buffer, 0.8 μ L dNTP, 1 μ L reverse transcriptase, 2 μ L primers and 4.2 μ L RNase free H₂O per sample. 10 μ L MM were then placed in labelled 0.5 mL RNase free eppendorf tubes (Applied Biosystems). 10 μ L of mRNA samples were added to the tubes already containing MM and samples spun to ensure thorough mixing. The samples were incubated at 25°C for 10 minutes followed by 120 minute incubation at 37°C for 2 h. Next the samples were heated to 85°C for 5 minutes and then cooled to 4°C. The cDNA samples were then diluted with 80-150 μ L of RNase free water (depending on the starting RNA concentration) and the samples were frozen at -20°C until use.

2.2.19 RT-PCR with Taq polymerase

cDNA samples from sorted $\gamma\delta$ T cells or lung tissue (2.2.19), primers (Table 2.1.9) and TaqMan polymerase master mix (Applied BioSystems) were defrosted on ice. Appropriate amounts of the master mix and primers were mixed together in order to have 5.5 μ L of polymerase and primers per well with primers each constituting 0.5 μ L (no more than two primers per well) of the total volume and the master mix making up the rest of the 5.5 μ L. 18s rRNA was used as an endogenous control and was included on each plate to be analysed. 5.5 μ L of master mix with primers were added onto 96 well microamp plates (Applied Biosystems) followed by 4.5 μ L of cDNA sample. The plates were then sealed and spun for 30 seconds to ensure that all of the components were at the bottom of the wells. The plates were then placed in the RT-PCR machine which was set for 40 cycles of cDNA amplification. The data was analysed using 7500 Fast System Software. The fold increase in cytokine mRNA expression was analysed relative to unstimulated cells or untreated samples and data normalised relative to 18s rRNA.

2.2.20 Optimisation of *B. pertussis* culturing protocol

Stocks of *B. pertussis* were thawed and 100 μ L aliquots lawned onto 2 (method I) or onto 4 (method II) Bordet Gengou blood agar plates. For both methods the bacteria were grown on blood agar plates for 3-4 days. After that, liquid cultures were set up by inoculating conical flasks containing 100 mL Stainer&Sholte media (S&S) + supplement with loopfulls of bacteria. 2 flasks of liquid culture were set up for method I and 8 flasks for method II. For method I, the liquid cultures were incubated at 37°C with shaking for 48 hours. After that 8 flasks were set up with 90mL S&S media and 10mL of the 48 hour culture and incubated for further 24 hours. For method II, 8 flasks were inoculated with loopfulls of bacteria and cultured for 24 hours only. After the liquid culture the bacteria were centrifuged at 10500 rpm at 4-20°C for 20 minutes. The pellets were resuspended in 100mL 1% casein salts. 1 in 50 and 1 in 20 dilutions of the bacterial solutions were prepared in plastic cuvettes and the amount of bacteria determined by measuring the OD600 of diluted bacterial samples. The corresponding CFU values were then read of a previously generated standard curve. The results were multiplied by the dilution factor and the numbers were then averaged to give the *B. pertussis* concentration. The concentrations of the bacterial suspensions were then adjusted to 2×10^{10} CFU/mL and the suspensions were then used to challenge the animals.

2.2.21 Culturing of *B. pertussis*

Stocks of *B. pertussis* (strain Tohama I, 338) were thawed and 100 μ L aliquots lawned onto 4 Bordet-Gengou blood agar plates. These were then incubated at 37 degrees for 3-4 days. The solid media cultures were then used to set up overnight liquid cultures by inoculating 8 conical flasks with vented caps containing 100mL Stainer-Scholte broth plus supplement (2.1.13-2.1.14) with a generous loopfull of bacteria. The liquid cultures were incubated at 37°C with shaking (200 rpm) for 24hours. They were then spun at 10500 rpm at 4-20C for 20 minutes. The pellets were resuspended in 100mL 1% casein salts (2.1.14). *B. pertussis* concentration was then determined by measuring the OD600 of a 1/20 and 1/50 dilution of the suspension and reading the corresponding CFU values of the standard curve. The numbers were then averaged to give the *B. pertussis* concentration. The

concentration of the bacterial suspension was then adjusted to 2×10^{10} CFU/mL and the suspension was then used to challenge the animals. After switching to a new nebuliser the infecting concentration was lowered to 1×10^9 CFU/mL due to higher nebuliser efficiency.

2.2.22 *B. pertussis* aerosol challenge

C57Bl/6 mice were exposed to aerosol containing 2×10^{10} CFU/mL *B. pertussis* in a perplex chamber for 15 minutes. This was followed by a 15 minute rest period. The course of the infection was monitored by taking lungs from the infected and naive mice at various time points post infection. The lungs were divided into two or three parts and analysed for bacterial load (CFU counts), cytokine production and gene expression.

2.2.23 Anti-IL-17 treatment

Mice were injected intraperitoneally (i.p.) with α IL-17 antibody (200 μ g/mouse) or the isotype control using a 27G needle. Antibodies were diluted to required concentration in PBS and a total volume of 100 μ L per mouse was injected. Treatment was performed one day prior and two days post infection.

2.2.24 CFU counts

For bacterial load determination CFU counts were performed on lungs from individual animals. The lungs from individual animals were homogenised in 1mL 1% casein salt solution. Three serial 1/10 dilutions of the homogenate were then prepared. The lung homogenate dilutions were plated in duplicate on Bordet-Gengou agar plates and the bacterial colonies were counted after 6 day incubation at 37°C. The counts were converted into log₁₀ CFU per lung for graphical representation of bacterial loads.

2.2.25 Preparation of heat killed *B. pertussis* for in vitro stimulation

B. pertussis was prepared as described in section 2.2.20. Its concentration was then adjusted to 1×10^{10} CFU/mL. Bacteria were aliquoted into sterile 1.5 mL screw top

eppendorf tubes and incubated at 95°C for 30 minutes. After that the bacteria were allowed to cool and then frozen at -20°C until further use. 100 µL of bacteria from one of the heat killed aliquots were plated on a blood agar plate and incubated at 37°C for 4-6 days to confirm that the bacteria have been killed.

2.2.26 Preparation of heat inactivated PT for antigen recall *in vitro*.

PT stock had to be heat inactivated prior to use as an antigen *in vitro* in order to be able to separate its toxic effects from those of being recognised as an antigen by T cells. This was achieved by incubating the PT at 90°C for 25min in a glass vial.

2.2.27 Detection of cytokine production by lung lymphocytes *ex vivo*

Lung samples were digested in 1mg/mL collagenase D (Roche) in the presence of DNase I (20 KU/mL) and Brefeldin A (5 µg/mL) for 1 hour at 37°C with rotating. The digested lungs were then forced through 40µm cell strainers. The cells were then washed, resuspended in cRPMI and counted. The cells were brought to concentration of 1×10^6 /mL and 1mL of each sample transferred into sterile capped polystyrene FACS tube. The samples were then stained for FACS as required.

2.2.28 Detection of cytokine production by lung lymphocytes *in vitro*

Cells were isolated as described in section 2.2.10. They were then transferred into sterile capped polystyrene FACS tubes (BD) and stimulated for 5 hours with cytokines as required in the presence of Brefeldin A (5 µg/mL). For experiments involving stimulation with bacteria, the samples were treated with Brefeldin A for the final 3 hours of the stimulation. The cells were then washed and stained for FACS.

2.2.29 Detection of antigen specific T cell responses to *B. pertussis*

Spleen cells were isolated from day 21 *B. pertussis* infected C57BL/6 mice and processed as described in 2.2.4. They were then seeded at 2×10^6 cells/mL on 96 well round bottom culture plates (Greiner). The cells were incubated with different doses of HKBP, purified *B. pertussis* antigens, FHA and heat inactivated PT, and PMA+αCD3 as positive control. They were then placed in a cell culture incubator at

37C, 5% CO₂. After 72 hours cytokine concentration in the spleen cell supernatants was analysed by ELISA

2.2.30 BCA Assay of lung homogenate supernatant (Thermo Scientific Kit)

BCA assay was used to measure the total concentration of protein in supernatants from tissue homogenates in order to normalise the ELISA results from these samples. Samples were diluted 1 in 5 and a total volume of 25µL and put on a 96 well ELISA high binding plates (Greiner). In parallel, albumin standards were prepared in a concentration range of 20-2000µg/mL. Working reagent (WR) was prepared by mixing 50 parts of BCA reagent A with 1 part of BCA reagent B. 200 µL of the WR were added to each well and the plate was mixed for 30 seconds on a plate shaker. The plates were then incubated at RT for 30 minutes. The absorbance was then immediately measured on a plate reader at 562nm. The concentration of protein in each sample was determined with the reference to the standard curve.

2.2.31 Detection of antigen-specific $\gamma\delta$ T cell responses

Spleen cells from naïve C57BL/6 mice were resuspended at 4×10^6 cells/mL in 20% V/V FCS/PBS and irradiated for 5 minutes with 4000 Gy in a gamma cell irradiator (Gammacell 3000). They were then centrifuged, washed and resuspended in complete RPMI. The spleen cells were then seeded on 96 well round bottomed plates (Greiner) at 100µL/well and treated with heat killed *B.pertussis* (MOI 1-100) for 2 hours at 37C, 5% CO₂ to allow for antigen uptake and processing. After that, purified $\gamma\delta$ T cell subpopulations (50×10^3 cells/mL) were added to the plate in 100µL volume. The plates were then incubated at 37C, 5% CO₂. After 72 hours the supernatants were removed and cytokine concentration analysed by ELISA (2.2.35)

2.2.32 Preparation of lung histology samples

Lung tissue sections were prepared in order to assess tissue pathology. Left lobe of each lung was excised and placed directly in 10% Formalin. The samples were then left to fix for a minimum of 3 days. They were then placed in plastic cassettes and processed to paraffin by stepwise dehydration and immersion in paraffin using an

automatic tissue processor (TP 2010, Leica). The processing was performed by immersing samples in increasing concentrations of EtOH, then in xylene to clear i.e. remove the alcohol from the tissue and then infiltrated with paraffin.

Table 2.1. Tissue processor programme settings

Reagent	Time
Alcohol 70%	1h00
Alcohol 80%	1h00
Alcohol 95%	1h00
Alcohol 100%	1h00
50% Alcohol/50% Xylene	1h00
Xylene	1h00
Xylene	1h00
Paraffin	1h00
Paraffin (vacum)	1h00

Once the run was finished, the samples were embedded in paraffin blocks. A small amount of hot paraffin (60°C) was poured into the plastic mould in order to facilitate the positioning of the tissue. The individual samples were then placed in separate moulds with the ventral surface of each sample facing up. More paraffin was then poured in order to completely cover the sample. Next, the bottom part of each cassette, which was used in the previous step, was placed on top of the mould and would later serve as the base of embedded sample. The paraffin embedded samples were then allowed to set on a cooling block for about 2-3 hours and left to rest overnight at room temperature before attempting to cut tissue sections. The samples were then removed from the moulds and excess paraffin was trimmed using a microtome (Leica) in order to expose the tissue surface. 5µm thin coronal sections of the lung tissue were cut using the microtome. The cut sections were floated in water bath at 37°C in order to facilitate mounting on microscope slides. Charged polysine microscope slides (Thermo Scientific) were used to ensure strong adhesion of tissue sections to the slide. Once mounted, slides were left to dry at 37°C overnight before moving to tissue staining.

2.2.33 Haematoxylin and Eosin (H&E) staining

In order to analyse the degree of inflammation in the lungs a basic Haematoxylin and Eosin staining protocol was employed. Haematoxylin binds to acidic structures, such as DNA and RNA, and stains them blue-purple. Therefore, cell nuclei appear blue

when stained with Haematoxylin. Eosin, on the other hand, binds to basic structures and stains pink. Structures such as cytoplasm, muscle, connective tissue, and red blood cells will appear pink-red following eosin staining. Prior to staining tissue was cleared and rehydrated by placing it for 10 sec in each of xylene, xylene, 100% EtOH, 90% EtOH, 70% EtOH, tap water. The slides were then immersed for 5 minutes in Delafield's haematoxylin solution (Sigma). Next, slides were rinsed well in running tap water in order to remove excess stain. They were then placed in eosin solution for 2 minutes and rinsed as before. The samples were then dehydrated by dipping in solutions with increasing alcohol strength, 70% EtOH, 90% EtOH and 100% EtOH. The alcohol was then cleared with xylene and the samples were permanently mounted using Sub-X mounting medium (Leica) and covered with cover slips (VWR). Micrographs of tissue sections were taken using an upright Olympus BX51 microscope and analysed as described below.

2.2.34 Lung inflammation scoring

In order to quantify the differences in lung pathology between infected mice, a basic scoring method assessing the degree of inflammation was designed. Each lung histology section was overlaid by a 10X10 grid dividing the lung into small square areas which could be scored individually. A percentage of inflammation score was assigned to each area according to the following system:

Score	Description
0	normal tissue, no inflammation
0.25	light signs of inflammation
0.5	light inflammation in most of the area or strong inflammation in part of the area
0.75	Strong inflammation in most of the area or very strong inflammation in part of the area
1	Very strong inflammation with morphological changes in most of the area

The score was assigned first to areas occupying full squares followed by areas that were occupying parts of squares. To each incomplete area another area was coupled to make up to a full square. The coupled area was then crossed out (red line in Fig. 2.1) and not counted when calculating the total number of squares. The total number of inflamed squares was calculated by treating individual scores as

percentages of a full square. Then the overall percentage of inflammation was calculated according to the equation:

$$\frac{\text{number of inflamed squares}}{\text{total number of squares}} \times 100 = \% \text{ inflammation}$$

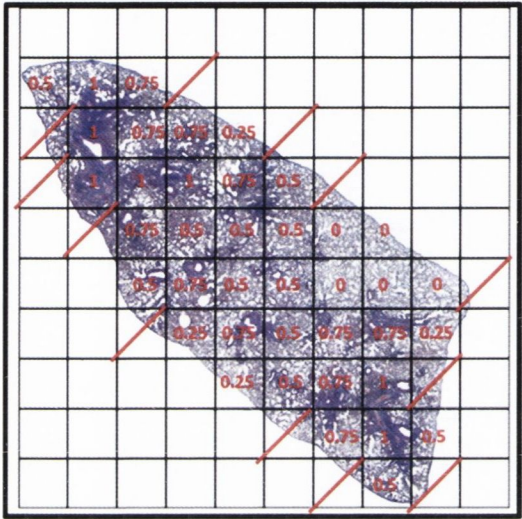


Figure 2.3 Lung inflammation scoring.

Each lung section was divided into small squares. The inflammation score was assigned to each full square followed by incomplete squares which were then coupled to other incomplete areas to make up to a full square. The coupled areas were crossed out to avoid double counting when calculating the total number of full squares.

2.2.35 ELISA

Cytokine concentrations in samples were determined using ELISA kits (IL-6, IL-5, IL-4, IL-18 and IFN γ : BD Pharmingen; IL-17a IL-17f, IL-22, IL-10, TGF β , IL-23, IL-1 β TNFa, IL-12p70, IL-27: R&D systems). High binding certified 96-well plates (Greiner Bio-one) were coated with 50 μ L/well of rat anti mouse capture antibody in PBS (all at 1 μ g/mL) and incubated overnight at 4°C. Plates were then washed in wash buffer (PBS, 0.05% Tween 20 (Sigma)) and non-specific binding sites blocked by adding 180 μ L of blocking solution (5% milk (Marvel) in PBS for BD kits and 1%BSA (Sigma) for R&D kits) for 2 h at room temperature. After the incubation, plates were washed again in the wash buffer and 50 μ L/well of test supernatants were added along with serially diluted standard recombinant protein for each cytokine. IL-12p40 standard (0-5000 pg/mL),IFN- γ standard (0-10 ng/mL), IL-18 standard (0-4000pg/mL) and IL-6 standard(1-5000pg/mL) diluted in PBS and IL-1 β standard (0-1000 pg/mL),IL-1 α standard (0-1000pg/mL), IL-17a standard (0-1000 pg/mL), IL-10 standard (0-

2000pg/mL), TNF α standard (0-2000pg/mL), IL-23 standard (0-2500pg/mL), IL-12p70 standard (0-2500pg/mL), TGF β standard (0-2000pg/mL) and IL-27 standard (0-2000 pg/mL) all diluted in 1% BSA (Sigma)/PBS. Supernatants were diluted 1:5 for detection of IFN- γ and 1:2 for detection of IL-1 α , IL-1 β , TNF α and IL-6, all dilutions made in the same buffer as standards. Neat samples were put onto the plates for detection of other cytokines. Supernatants for TGF β ELISA were heat activated at 80°C for 10 minutes. Plates were then incubated overnight at 4°C. Next, plates were washed in wash buffer followed by addition of 50 μ L/well of biotinylated goat anti-mouse detection antibody (1 μ g/mL) diluted in PBS or 1%BSA/PBS as before. Plates were then incubated for 1 h (BD Pharmingen) or 2h (R&D Systems) at room temperature in the dark and then washed in wash buffer. Plates were incubated with 50 μ L/well horseradish peroxidase (HRP)-conjugated streptavidin (BD Pharmingen; 1:1000 in PBS, R&D Systems: 1:200 in 1%BSA/PBS) at room temperature for 20 minutes. After washing in wash buffer, 50 μ L/well OPD substrate in phosphate citrate buffer were added. The colour was allowed to develop and the reaction was stopped once the standard curves for each cytokine had developed by adding 25 μ L stop solution. Plates were then read at 492 nm using a microtitre plate reader and the cytokine concentrations determined with reference to the standard curve prepared using recombinant cytokines of known concentrations.

2.2.36 Flow cytometry

2.2.36.1 Surface staining

Single cell suspensions, prepared from organs isolated from in vivo studies or obtained from in vitro cultures were added to a FACS tube at 1×10^6 cells/100 μ L/tube. The cells were then washed in FACS buffer and centrifuged at 1300rpm for 5 min. They were then incubated with 100 μ L of surface stain containing the appropriate amount of desired FACS antibody (table 2.1.5.2) and 1 μ L/test of anti-CD16/CD32 (Fc γ Block, BD Pharmingen) for 15 min at room temperature in the dark. Fluorescence minus one (FMO) and isotype controls were also stained. Single stained compensation controls were prepared using FACS comp beads (BD). The samples were then washed with 2mL/tube FACS buffer to remove the excess antibody, with centrifugation at 1500rpm for 5min. The cells were then re-suspended in 0.5 mL FACS buffer for the analysis. If the discrimination of live and dead cells

was required, the cells were washed in PBS and incubated with 1/600 diluted in PBS LIVE/DEAD® Fixable Aqua Dead Cell stain (Aqua-Live/dead, Molecular probes, Life Technologies) for 20 minutes at room temperature in the dark prior to surface staining. Aqua Live/Dead is a fluorescent dye that reacts with free amines both in the cell interior and on the cell surface, giving an intense fluorescent staining of cells with compromised membrane and a weak staining of live cells. Aqua Live/Dead fluorescence can be detected in the V500/Am-Cyan channel and is compatible with intracellular staining. Live/dead Aqua staining was not used for myeloid cell stains as many of myeloid cells are highly autofluorescent and would end up being included in the dead cell population.

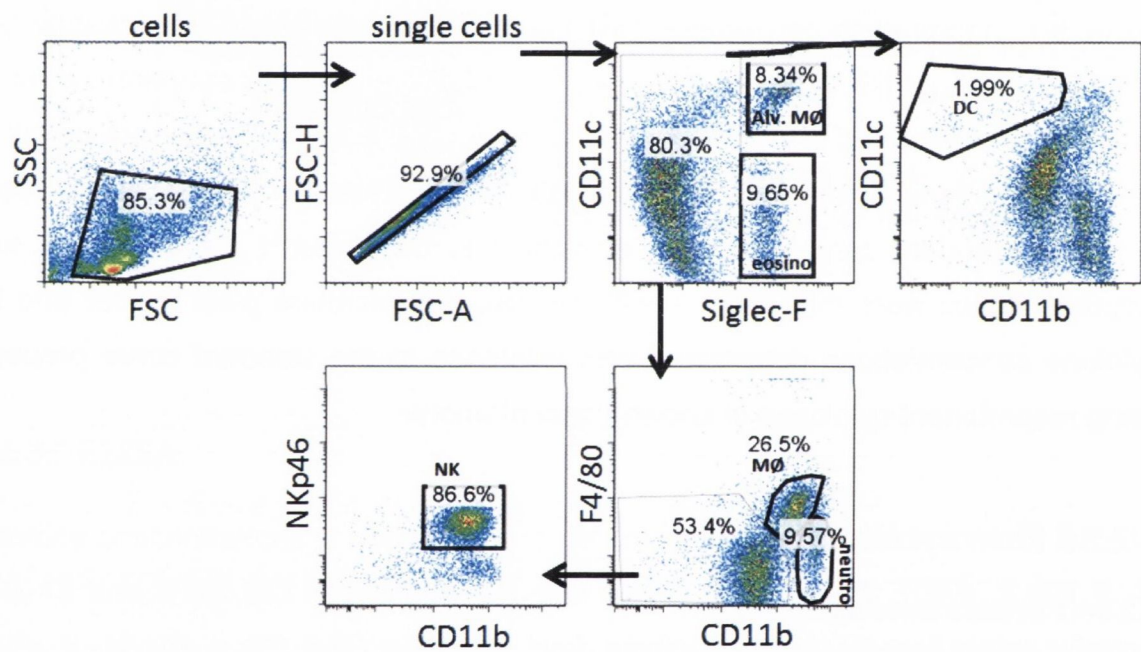


Figure 2.4 Gating strategy for myeloid cells.

Live cell gate was first applied based on forward (FSC) and side (SSC) scatter, followed by a single cell gate – forward scatter area versus height. Alveolar macrophages and eosinophils were then identified using CD11c and Siglec-F markers. All the remaining cells were analysed for CD11b and CD11c expression and DC were identified. Macrophages were identified as CD11b intermediate-high and F4/80⁺ cells. Neutrophils were identified as CD11b high cells and their lineage confirmed using the Ly6G marker. All the remaining cells were analysed for NK cell marker expression, NKp46.

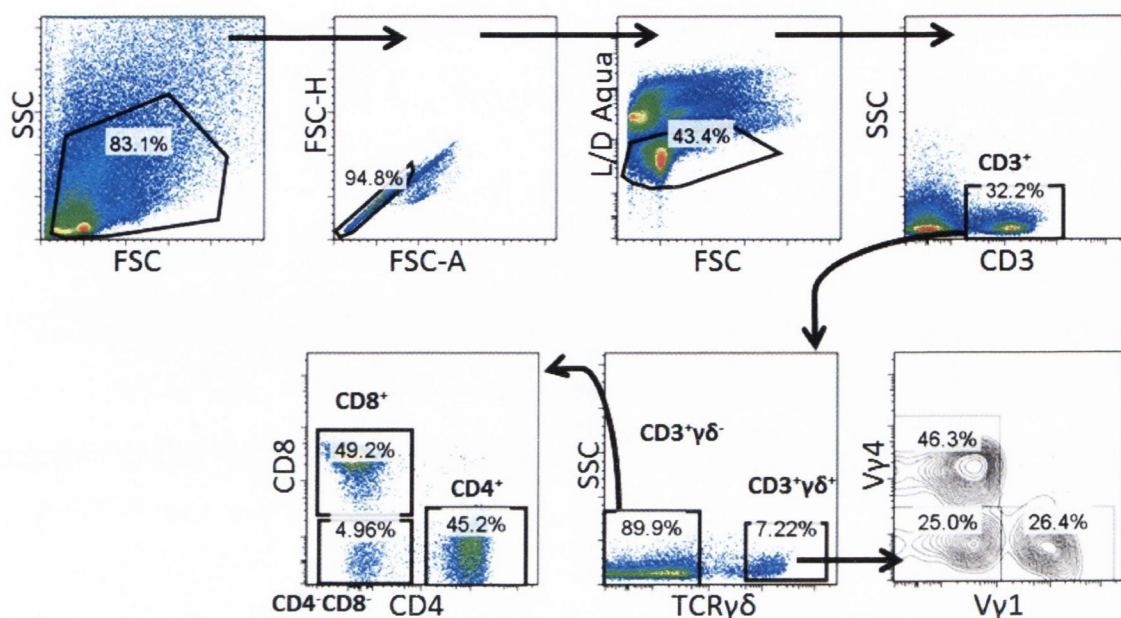


Figure 2.5 Gating strategy for T cell subsets

Live cell gate was first applied based on forward (FSC) and side (SSC) scatter, followed by a single cell gate – forward scatter area versus height. A more accurate live gate was set according to live/dead Aqua staining (live cells are live/dead Aqua negative). CD3 positive gate was then applied. Inside of CD3⁺ gate cells were separated into TCRδ positive and negative cells. Within the CD3⁺TCRδ⁺ gate 3 subpopulations of γδ T cells were found based on Vγ4 and Vγ1 expression. Also 3 populations could be found within the CD3⁺TCRδ⁻ gate based on CD8 and CD4 expression. The above gating strategy was used when looking at different T cell subsets in the lung and other tissues.

2.2.36.2 Intracellular staining

Intracellular staining was employed in order to detect soluble antigens such as cytokines. Cells were first stained with surface antibodies, as described above. After the last wash the FACS buffer was completely removed and cells were re-suspended in 50μL of 2% Paraformaldehyde (Thermo Scientific) and incubated for 20 min at room temperature in the dark. Cells were then washed 1x with FACS buffer and then 1x with 1mL permeabilisation buffer, 0.05% Saponin/PBS (Sigma). They were then stained with 100 μL of appropriate amount of fluorochrome-conjugated antibodies diluted in Permabilization buffer. After 15 min cells were

washed twice with FACS buffer and re-suspended in 0.5 mL FACS buffer for acquisition.

Stained cell suspensions were acquired using LSRFortessa (BD) or CANTO-II (BD) flow cytometers according to the manufactures instructions. The flow cytometer was calibrated using single-stained compensation beads (BD). FACS data analysis was performed using FlowJo (Treestar) software.

2.2.37 Statistical analyses

One way analysis of variance (ANOVA) was used to test for statistical significance of differences between more than two experimental groups. Two-way ANOVA was used for multiparameter analysis (e.g. differences between two or more groups on different time points). Dunnet's post-test was used for analyses comparing test groups to control group while Tukey and Bonferroni post-tests were used when comparing all experimental groups to each other. The statistical analysis was performed using Graphpad Prism (v5.0) Software.

Chapter 3: *In vitro* activation of intestinal and lung $\gamma\delta$ T cells

Chapter 3: Introduction

3.1.1 Intestinal $\gamma\delta$ T cells

In mice $\gamma\delta$ T cells constitute up to 50% of CD3⁺ cells in the intestine and their numbers tend to increase closest to the luminal part of the gut, being highest in the intraepithelial compartment (Ullrich et al, 1990). The main subset present in the mouse intestine are the V γ 7⁺ $\gamma\delta$ T cells, while in humans these are V δ 1 cells (Chowers et al, 1994; Deusch et al, 1991; Kagnoff, 1998). Most of the mouse intraepithelial intestinal $\gamma\delta$ T cells ($\gamma\delta$ IEL), and also some of the resident $\alpha\beta$ T cells, have a unique phenotype and express CD8 $\alpha\alpha$ homodimer. CD8 $\alpha\alpha$ is an isoform of the CD8 T cell co-receptor that becomes upregulated on activated T cells and can be expressed both by CD8 $\alpha\beta$ and CD4 cells (Kenny et al, 2004; Madakamutil et al, 2004). Similar to CD8 $\alpha\beta$, it interacts with downstream signalling molecules but is incapable of their activation and thus its upregulation serves as a negative feedback loop to repress the TCR signal through a dominant negative mechanism (Cheroutre & Lambolez, 2008). CD8 $\alpha\alpha$ has been shown to strongly interact with the nonclassical MHC class I molecule – thymus leukemia antigen (TL) – which is expressed by the thymic stromal cells and intestinal epithelium. Furthermore, stimulation of CD8 $\alpha\alpha$ IELs with anti-CD3 and TL increased IL-2 and IFN γ production but not cytotoxicity of these cells (Leishman et al, 2001). This expression pattern of TL is reminiscent of Skint1 and the epidermal V γ 5V δ 1 $\gamma\delta$ T cells, suggesting that CD8 $\alpha\alpha$ could have a role in selection and/or maintenance of $\gamma\delta$ IELs (Boyden et al, 2008). However, functional $\gamma\delta$ IELs have been shown to be present, although in reduced numbers, in athymic nu/nu mice indicating that gut CD8 $\alpha\alpha$ $\gamma\delta$ T lymphocytes can develop outside of thymus and consequently do not require interaction with thymically expressed antigens for their development (Emoto et al, 2004; Nonaka et al, 2005).

Not surprisingly, the proportion and absolute number of all T lymphocyte subsets was found to be normal in the gut intraepithelial compartment of TL^{-/-} mice (Leishman et al, 2001). In the same study, an increase in the proliferation of colonic CD8 $\alpha\alpha$ TCR $\alpha\beta$ and TCR $\gamma\delta$ cells was observed in the TL deficient animals. In contrast to data generated by Leishman et al., CD8 $\alpha\alpha$ T cells from TL^{-/-} mice were found to produce higher concentrations of IFN γ upon CD3 stimulation than their WT

counterparts. Furthermore, TL deficiency was shown to accelerate the onset as well as to increase the disease severity in a T cell dependent model of colitis (Olivares-Villagómez et al, 2008). The authors therefore concluded that while CD8 $\alpha\alpha$ interaction with TL is not required for the normal development of intestinal lymphocytes, it regulates their proliferation and activation. Although CD8 $\alpha\alpha^+$ $\gamma\delta$ and $\alpha\beta$ T cells have been found in human mucosa and skin, very little work has been done on the role of those cells and the significance of their CD8 $\alpha\alpha$ homodimer expression (Deusch et al, 1991; Jarry et al, 1990; Rust et al, 1992; Walker et al, 2012; Zhu et al, 2013).

3.1.2 Role of $\gamma\delta$ T cells in the intestine

The gut is inhabited by millions of commensal microorganisms, many of which are required for proper functioning of the digestive tract, the intestinal immune system must be able to tolerate those beneficial bacteria and mount a swift response against pathogenic organisms when necessary. In addition, food passing through the digestive tract daily is a source of various foreign antigens, which under normal conditions do not activate the immune system. Conditions such as food allergies, coeliac disease or inflammatory bowel disease develop when tolerance to commensal bacteria or dietary antigen is broken. Given that a great majority of intestinal intraepithelial lymphocytes, which are in the closest proximity to the gut lumen, are $\gamma\delta$ T cells, it is intuitive to expect that they will have a role to play in the maintenance of tolerance towards innocuous antigens, as well as in the immune response against gut pathogens. $\gamma\delta$ T cells have been shown to play multiple roles in the gut, ranging from maintenance of homeostasis, tissue repair, to immunity to infection.

$\gamma\delta$ T cells have been shown to protect the intestine against microbial penetration through secretion of antimicrobial peptides and cytotoxicity against infected cells (Ismail et al, 2011; Li et al, 2012). CD8 $^+$ $\gamma\delta$ IEL killing of *Salmonella* infected enterocytes was shown to be dependent on NKG2D expression (Li et al, 2012). In a nematode infection model, intestinal $\gamma\delta$ T cells have been found to help pathogen clearance and limit the infection-induced pathology. Intestinal $\gamma\delta$ T cells produce IL-13 and adoptive transfer of $\gamma\delta$ IELs or administration of recombinant IL-13 to TCR $\delta^{-/-}$ mice reduced egg production by the parasite (Inagaki-Ohara et al, 2011). Intestinal

$\gamma\delta$ T cells have also been implicated in tissue repair through secretion of keratinocyte growth factor (KGF), regulation of epithelial cell turnover and maintenance of tight junctions (Boismenu & Havran, 1994; Chen et al, 2002; Dalton et al, 2006; Komano et al, 1995).

Increased numbers of $\gamma\delta$ T cells have been observed in the small intestine of coeliac disease patients (Bhagat et al, 2008; Halstensen et al, 1989; Kutlu et al, 1993; Rust et al, 1992). However, it has been reported that percentage of intraepithelial $\alpha\beta$ T cells, but not $\gamma\delta$ T cells, correlated with the presence of gluten in their diet and with the grade of villous atrophy (Kutlu et al, 1993). Furthermore, higher percentage of TCR $\gamma\delta^+$ NKG2A $^+$ CD8 $^+$ IELs has been found in patients on gluten-free diet compared to those with active coeliac disease. These cells have been shown to suppress $\alpha\beta$ T cell secretion of IFN γ and granzyme B *in vitro* and the suppression was found to be partially dependent on TGF β produced by TCR $\gamma\delta^+$ NKG2A $^+$ CD8 $^+$ IELs (Bhagat et al, 2008). Furthermore, the percentage of $\gamma\delta$ IELs inversely correlated with the development of enteropathy-associated T-cell lymphoma in patients with refractory coeliac disease (Verbeek et al, 2008). These data suggest an immunoregulatory role for gut $\gamma\delta$ T cells in coeliac disease.

In chemical-induced colitis models, TCR $\delta^{-/-}$ animals had a more severe disease than their wild type littermates and transfer of $\gamma\delta$ T cell IELs could ameliorate the disease (Chen et al, 2002; Inagaki-Ohara et al, 2004; Kühl et al, 2007). Conversely, $\gamma\delta$ T cells were found to play a pathogenic role in a T cell mediated model of colitis by promoting pathogenic Th17 cells. TCR $\beta^{-/-}$ but not TCR $\delta^{-/-}$ mice were found to be susceptible to disease induction by adoptive CD4 T cell transfer. Moreover, TCR $\delta^{-/-}$ mice could be rendered susceptible by co-transferring $\gamma\delta$ T cells along with CD4 cells, but not by transferring $\gamma\delta$ T cells alone (Do et al, 2011). However, that study showed a pathogenic role for peripheral $\gamma\delta$ T cells and did not clarify the role of gut resident cells in the pathogenicity of colitis. Park et al. showed that $\gamma\delta$ T cells were responsible for spontaneous colitis in mice with defective Treg cells and deletion of TCR δ or transfer of wild type Treg into these animals prevented disease. The disease was also abrogated by administration of antibiotic, which eliminated commensal bacteria from the gut. Furthermore, they showed that in colitic animals $\gamma\delta$ IELs produced IL-17, which was significantly decreased in the animals that received WT Treg. The authors concluded that in steady state conditions, Tregs

function to regulate $\gamma\delta$ T cells in the gut to prevent spontaneous induction of colitis in response to normal gut flora (Park et al, 2010). In humans, increased $\gamma\delta$ T cell numbers can be detected in the circulation and mucosa of inflammatory bowel disease patients (Giacomelli et al, 1994; McVay et al, 1997; Söderström et al, 1996). However, intestinal TCR $\gamma\delta$ repertoires were similar in inflamed and non-inflamed mucosa and different $\gamma\delta$ T cell clones were present in the gut to those detected in the periphery, suggesting that intestinal $\gamma\delta$ T cell may not be involved in the pathogenesis of the disease (Holtmeier et al, 2002). It has been reported that anti-TNF α treatment leads to an increase in the number of intestinal $\gamma\delta$ T cells, however, the impact of this expansion on the local immune responses was not investigated (Kelsen et al, 2011).

3.1.3 Lung $\gamma\delta$ T cells

$\gamma\delta$ T cells account for between 5-10% of lung CD3⁺ cells (Wands et al, 2005). The main subsets of $\gamma\delta$ T cells in the lung are V γ 4, V γ 1 and V γ 6 chain expressing cells (Hayes et al, 1996; Sim et al, 1994; Wands et al, 2005). Lung V γ 6 $\gamma\delta$ T cells express a semi-invariant TCR composed of V γ 6V δ 1 chains, which is also present in the female reproductive tract and peritoneal cavity (Hayes et al, 1996; Itohara et al, 1990; Matsuzaki et al, 1999; Sim et al, 1994). Li et al. showed that in the absence of V δ 1 gene, resident $\gamma\delta$ T cell numbers are greatly reduced in newborn mice and V γ 6 chain expressing but no other V γ subsets are detectable at that age. Lung V γ gene diversity was found to increase in the older mice and the authors concluded that V γ 6V δ 1 T cells are crucial for proper expansion and recruitment of various $\gamma\delta$ T cell subsets to the lung (Li et al, 2008). Most $\gamma\delta$ T cells are found in nonalveolar regions of the lung, with the exception of the mucosa. They are located in close proximity to myeloid cells, in particular F4/80⁺ macrophages and MHC class II⁺ DC (Wands et al, 2005). The majority of studies investigating the role of lung resident lymphocytes look at the entire population of $\gamma\delta$ T cells or focus on V γ 4 and V γ 1 cells due to the lack of commercially available antibodies against the V γ 6 chain. However, Roark et al. reported that the anti V γ 5 antibody (17D1 clone) will also recognise V γ 6V δ 1 invariant TCR in the presence of anti-TCR δ , probably due to a conformational change induced upon anti- $\gamma\delta$ TCR mAb binding (Roark et al, 2004). It is, however, feasible to study V γ 6 $\gamma\delta$ T cells by negative gating on non-V γ 4 non-V γ 1 cells, as V γ 6

cells will represent the majority of $\gamma\delta$ T cells in that gate (Hayes et al, 1996; Wands et al, 2005). Limited data are available on $\gamma\delta$ T cell subsets in human lungs. Wisniewski et al reported that human lung $\gamma\delta$ T cells show a bias towards the expression of group I V γ gene segments (i.e. V γ 2, V γ 3, V γ 4, V γ 5,V γ 8), with a preferential usage of V γ 8JP2 rearrangement, as opposed to group II gene segments (i.e. V γ 9) which are most commonly expressed in the blood. Furthermore they failed to detect group III V γ gene expression (V γ 10) in either blood or lung biopsy samples (Forster et al, 1987; Wisniewski et al, 2001).

3.1.4 Role of $\gamma\delta$ T cells in the lung

The respiratory tract is constantly exposed to inhaled antigens and, similar to the intestine, has to maintain tolerance to innocuous molecules, but must be capable of mounting a protective response against viruses and bacteria. An inappropriate response may result in allergies or inflammation induced immunopathology, leading to tissue damage. It is therefore crucial to be able to contain pathogens quickly and effectively in order not to compromise the lung function due to damage caused by prolonged inflammation. Studying human lung resident $\gamma\delta$ T cells is difficult, because of access to live cell samples from lungs, and is limited to extrapolating data from mice and non-human primates.

Lung $\gamma\delta$ T cells have been shown to play an important role in defences against respiratory pathogens as well as in tissue repair. $\gamma\delta$ T cells have been shown to control the early influx of neutrophils into the lung during systemic *Candida albicans* infection. The protective effect of $\gamma\delta$ T cells was attributed to IL-17 production as impaired fungal clearance was observed in IL-17^{-/-} mice (Dejima et al, 2011). In a mouse model of *Mycobacterium tuberculosis* infection, lung $\gamma\delta$ T cells have been shown to promote protective responses by providing a non-redundant early source of IFN γ , which enhances IL-12 production by lung DCs (Caccamo et al, 2006). In contrast, in a simian model of *M. tuberculosis* infection, polyclonal V γ 9V δ 2 cells of peripheral origin, but not lung resident cells, were shown to expand in lung tissue and produce IFN γ in response to phosphoantigen stimulation *in vitro* (Huang et al, 2012). The same subset of $\gamma\delta$ T cells can play either a protective or pathogenic role depending on the disease setting. For example, Nakasone et al. showed that V γ 4⁺ $\gamma\delta$ T cells are important in the innate immune defences against *Streptococcus*

pneumoniae (Nakasone et al, 2007). $V\gamma 4^+$ T cells were found to accumulate quickly in the lungs of infected mice. The survival and bacterial clearance were severely impaired in $V\gamma 4^{-/-}$ animals and correlated with a reduced recruitment of neutrophils to the lung (Nakasone et al, 2007). On the other hand, during respiratory syncytial virus infection, pulmonary $V\gamma 4^+$ cells contributed to viral clearance, but also to the immune pathology by producing IFN γ , TNF α and RANTES at the early stages of the infection (Dodd et al, 2009). In OVA-induced airway hyperreactivity, $V\gamma 1^+$ cells were found to enhance the disease by promoting Th2 cytokines in the lung. $V\gamma 4^+$ cells, on the other hand, inhibited airway hyperreactivity and protection was shown to be dependent on IFN γ production by these cells (Hahn et al, 2004; Jin et al, 2009; Lahn et al, 2002). Simonian et al. showed that $V\gamma 6V\delta 1$ cells accumulated in lungs of mice repeatedly exposed to *Bacillus subtilis*. These cells were found to contribute to the clearance of bacteria through IL-17 production and to the protection from *B. subtilis* induced fibrosis via secretion of IL-22 (Simonian et al, 2009; Simonian et al, 2010). IL-17 producing $\gamma\delta$ T cells have been found to have a protective role in a bleomycin induced model of lung injury by promoting coordinated inflammatory response. However, the authors did not investigate IL-22 production by these cells. In TCR $\delta^{-/-}$ mice had a reduced cellular infiltration to the lung and showed a delay in the repair process (Braun et al, 2008).

Chapter 3.2: Results

3.2.1 Expression of CD27 by $\gamma\delta$ T cells is tissue specific

The majority of studies investigating the role of $\gamma\delta$ T cells focus on peripheral cells and relatively little is known about tissue resident $\gamma\delta$ T cells. This study set out to compare activation and function of murine lung and intestinal $\gamma\delta$ T cells to that of their peripheral counterparts.

CD27 is a cell surface marker that is differentially expressed on IL-17 and IFN γ producing $\gamma\delta$ T cells (Ribot et al, 2009). Expression of this surface molecule on lymphocytes isolated by non-enzymatic tissue disruption method from lymph nodes, small intestine and lung was investigated. It was found that a high proportion of $\gamma\delta$ T cells in the lymph nodes and the intestine express CD27. In contrast, lung resident $\gamma\delta$ T cells are mostly CD27⁻ (Figure 3.1).

3.2.2 $\gamma\delta$ T cells proliferate in response to stimulation with expanding cytokines.

While $\gamma\delta$ T cells constitute a higher percentage of CD3⁺ cells in tissues, functional studies of these cells are precluded by difficulty in isolating the tissue resident lymphocytes and low cell yields. In order to overcome this problem, a number of protocols were tested in an attempt to expand $\gamma\delta$ T cells *in vitro*. Lymph node cells were treated with combinations of IL-2, IL-15 and IL-18. These cytokines were chosen because of published data implicating them in driving proliferation or maintenance of lymphocytes. CFSE staining was employed in order to determine whether incubation with cytokines induced proliferation of $\gamma\delta$ T cells. Lymph node cells were stained with CFSE prior to incubation with the cytokines. CFSE is a fluorescent membrane permeable dye, which crosslinks to intracellular proteins. The intensity of CFSE fluorescence halves with every cell division, which can be detected on a flow cytometer as peaks of fluorescence in the FL-1 channel. Incubation of lymph node cells with either IL-2 or IL-15 alone induced some proliferation of $\gamma\delta$ T cells which was found to be 11.7% and 27.6%, respectively (Figure 3.2). This was increased to 43.2% when the two cytokines were used in combination, while stimulation with IL-2 and IL-18 induced 55.2% of $\gamma\delta$ T cells to proliferate. The

strongest $\gamma\delta$ T cell proliferation (68.2% of cells) was observed after stimulation with all three cytokines together. Increasing the concentration of IL-2 and IL-15 did not increase the proliferation of $\gamma\delta$ T cells.

None of the cytokine combinations tested induced IL-17 production by lymph node cells. However, all of the combinations that included IL-2 and IL-18 induced significant production of IFN γ and IL-15 was found to enhance it (Figure 3.3).

3.2.3 Purified $\gamma\delta$ T cells produce IFN γ in response to IL-2 and IL-18 stimulation.

Given that the lymph node cells were found to produce significant amounts of IFN γ in response to stimulation with IL-2 and IL-18, these studies were extended to examine whether $\gamma\delta$ T cells were a source of this cytokine. Isolated $\gamma\delta$ T cells were found to produce high levels of IFN γ in response to stimulation with IL-2+IL-18, twice as much as in response to IL-1 β +IL-23. $\gamma\delta$ T cells secreted IL-17 in response to stimulation with IL-1 β +IL-23 but not IL-2+IL-18. Addition of IL-15 to the cytokine combinations slightly elevated the amount of IFN γ and slightly decreased the amount of IL-17 secreted by the cells (Figure 3.4).

3.2.4 $\gamma\delta$ T cells are capable of producing IL-17 in response to IL-1 β +IL-23 after expansion in vitro.

In vitro expansion of $\gamma\delta$ T cells aimed at increasing cell numbers without concomitant skewing of the IL-17- to IFN γ -producing cell ratio and maintaining it as close as possible to that observed in freshly isolated $\gamma\delta$ T cells. Given that the combination of cytokines that induced strongest proliferation of $\gamma\delta$ T cells also stimulated IFN γ production by these cells, I investigated whether the cells expanded in the presence of IFN γ -promoting cytokines would be capable of producing IL-17 in response to IL-1 β +IL-23 post expansion. To address this question, lymph node cells were incubated with combinations of the expanding cytokines or with IL-1 β +IL-23 and IL-18+IL-23 to induce IL-17 production. After 72h, the supernatants were removed and the medium was changed. One set of samples was incubated for a further 72h with medium only while the other set was incubated with IL-1 β +IL-23. Significant levels of IL-17 were detected on day 3 in supernatants of lymph node cells which were incubated with IL-1 β +IL-23 and IL-18+IL-23 (Figure 3.5). Both cytokine combinations also induced low

levels of IFN γ production by these cells. IL-2+IL-18 and IL-2+IL-15+IL-18 stimulation, on the other hand, induced significant IFN γ but no IL-17 production. No IL-17 or IFN γ production was detected in the supernatants from the cells incubated with IL-2+IL-15 (Figure 3.5A). The analysis of day 6 supernatants revealed that the cells incubated with IL-1 β +IL-23 continued to produce IL-17 even after the inducing stimulus was removed, while no IL-17 was detected in the supernatants from the cells previously stimulated with IL-18+IL-23. Low levels of IFN γ were detected in the supernatants from the cells which were previously incubated with IL-1 β +IL-23, IL-2+IL-18 and IL-2+IL-15+IL-18 (Figure 3.5B). It was found that a significant amount of IL-17 was produced in response to IL-1 β +IL-23 by the cells, which were previously incubated with IL-1 β +IL-23, IL-2+IL-15 and IL-2+IL-15+IL-18. The cells which were previously incubated with IL-2+IL-18 also produced IL-17 in response to IL-1 β +IL-23 stimulation. However, the enhancement over background levels was not significant (Figure 3.5C). The concentration of IL-17 in the supernatants of IL-1 β +IL-23 stimulated lymph node cells, which were previously incubated in the presence of IL-2+IL-15, was slightly below that produced by freshly isolated lymph node cells. However, the amount of IFN γ produced by these cells was comparable to that produced by freshly isolated cells stimulated with IL-1 β +IL-23 (Figure 3.5A and 3.5A-C broken red line).

3.2.5 TCR stimulation is required for the expansion of $\gamma\delta$ T cells.

Since IL-2+IL-15 induced some proliferation of $\gamma\delta$ T cells with no concomitant cytokine production, it was tested whether this proliferation could be increased by stimulation through CD3. Furthermore, in order to investigate whether the chosen expansion protocol induced homeostatic proliferation or true expansion of cells, purified $\gamma\delta$ T cells were stimulated with IL-1 β +IL-23, IL-18+IL-23, IL-2+IL-15, IL-2+IL-18 or with IL-2+IL-7+IL-15 in the presence or absence of anti-CD3 antibody. After 72h the number of cells in each well was enumerated. It was found that incubation of $\gamma\delta$ T cells with IL-2+IL-15 led to a two fold increase in the cell number compared to that at the start of the culture. This was strongly enhanced by the addition of anti-CD3, which resulted in an overall 7 fold increase in cell numbers. The increase in cell number in response to other cytokine combinations in the absence of α CD3 was not significant and in the case of IL-1 β +IL-23 stimulated cells, the recovered cell number

post stimulation was lower than the number of cells present prior to stimulation. Addition of anti-CD3 to cells stimulated with IL-18+IL-23, IL-2+IL-18 and IL-2+IL-15+IL-18 led to a significant increase in cell number in those samples, while the IL-1 β +IL-23 stimulated cells were unaffected (Figure 3.6).

It was found that, while incubation of $\gamma\delta$ T cells with IL-2+IL-15 did not induce IL-17 or IFN γ production from $\gamma\delta$ T cells, addition of anti-CD3 lead to production of very high concentrations of IFN γ by these cells. Anti-CD3 also considerably increased the amount of IFN γ secreted by cells stimulated with other cytokine combinations. Furthermore, the IL-1 β +IL-23 and IL-18+IL-23 induced IL-17 was also increased by addition of anti-CD3 (Figure 3.7).

A higher proportion of CD27⁺ cells was present among the cells incubated with IL-18+IL-23, IL-2+IL-18, IL-2+IL-15 and IL-2+IL-7+IL-15 than among the cells incubated with IL-1 β +IL-23 or medium, all in the presence of anti-CD3 (Figure 3.8A). Furthermore, most of the responding cells were single producers of IL-17 or IFN γ , with a small proportion of double producers (Figure 3.8B).

3.2.6 Intestinal intraepithelial lymphocytes produce IL-22 in response to IL-1 β +IL-23 stimulation.

In order to investigate cytokine production by gut resident $\gamma\delta$ T cells, intestinal intraepithelial lymphocytes were incubated with different polarising cytokine combinations. Significant IL-22 production was detected in response to IL-1 β +IL-23 and IL-18+IL-23 stimulation, while no IL-17A or IL-17F was detected in response to any of the cytokine combinations tested. Furthermore, IL-18+IL-23 induced significant production of IFN γ by intestinal lymphocytes (Figure 3.9).

Similar to lymph node cells, Intestinal lymphocytes produced IFN γ in response to stimulation with IL-2+IL-18, however, the concentration of IFN γ produced was considerably lower. Furthermore, lymph node cells secreted much higher levels of IL-22 in response to stimulation with IL-1 β +IL-23 and IL-18+IL-23. Also, lymph node cells produced high amounts of IL-17 in response to stimulation with IL-1 β +IL-23 and IL-18+IL-23, while the amount of this cytokine detected in the intestinal cell supernatants was not significant (Figure 3.10).

3.2.7 FICZ decreases IL-1 β +IL-23 induced IL-17 and IL-22 production by LN cells.

Signalling through arylhydrocarbon receptor (AhR), which is a nuclear receptor involved in the toxic response has been shown to be involved in upregulation of IL-22 expression by T cells (Monteleone et al, 2011). Therefore it was examined whether an AhR agonist – 6-formylindoleo[3,2-b]carbazole (FICZ) could upregulate the production of IL-22 by IELs and LN cells. It was found that addition of FICZ to IL-1 β +IL-23 stimulated lymph node cells decreased IL-17 and IL-22 production. FICZ did not affect IL-17 or IL-22 production in response to IL-18+IL-23. No IL-17 was detected in the supernatants of cytokine stimulated IELs. Similar to LN cells, IL-22 produced by IELs was inhibited by FICZ. However the inhibition was not statistically significant. FICZ was found to enhance IL-18+IL-23 induced IFN γ both in the intestine and the lymph nodes, but the difference was not significant. FICZ did not affect the IL-1 β +IL-23 induced IL-17 secretion by lymph node cells (Figure 3.11)

3.2.8 Retinoic acid modulates the production of IL-17, IL-22 and IFN γ in the gut and lymph nodes in response to stimulation with IL-1 β +IL-23 and IL-18+23.

Retinoic acid (RA), a vitamin A metabolite, is known to modulate Th17 differentiation (Elias et al, 2008; Mucida et al, 2007; Sun et al, 2007). Therefore the effect of retinoic acid on IL-1 β +IL-23 and IL-18+IL-23 induced cytokine production by lymph node cells and IELs was examined. RA was found to inhibit both IL-1 β +IL-23 and IL-18+IL-23 induced IL-17 production by lymph node cells. In contrast, IL-22 production in response to IL-18+IL-23 was enhanced by the addition of RA. No difference was observed in the production of IL-22 by lymph node cells stimulated with IL-1 β +IL-23. The production of IL-22 by IELs in response to IL-1 β +IL-23 and IL-18+IL-23 was enhanced in the presence of RA, although the difference was not significant. RA enhanced IFN γ production by lymph node cells in response to both IL-1 β +IL-23 and IL-18+IL-23 stimulation. However, the production of IFN γ by IELs in response to IL-18+IL-23 was inhibited by the addition of RA (Figure 3.12).

3.2.9 Isolated intestinal TCR δ^+ cells fail to respond to IL-1 β +IL-23 and IL-18+23 stimulation

In order to establish whether $\gamma\delta$ T cells contribute to innate IFN γ and IL-22 production in the gut, isolated lymph node and intestinal intraepithelial $\gamma\delta$ T cells were stimulated with IL-1 β +IL-23 and IL-18+IL-23. Lymph node $\gamma\delta$ T cell produced significant amounts of IL-17, IL-22 and IFN γ in response to both cytokine combinations. In contrast, intestinal intraepithelial $\gamma\delta$ T cells failed to produce any of the cytokines tested (Figure 3.13).

Inability of intestinal $\gamma\delta$ T cells to respond to IL-1 β +IL-23 stimulation was confirmed at the mRNA level. However, despite the lack of IFN γ mRNA induction upon IL-1 β +IL-23 stimulation, the basal level of IFN γ was found to be higher in the intestinal $\gamma\delta$ T cells, when compared to lymph node $\gamma\delta$ T cells (Figure 3.14).

When the expression of IL-1RI, IL-18R and IL-23R was examined by RT-PCR, it was found that in contrast to lymph node $\gamma\delta$ T cells, the gut resident $\gamma\delta$ T cells do not express any of these receptors. Lymph node $\gamma\delta$ T cells were found to upregulate IL-1RI and IL-23R expression upon IL-1 β +IL-23 stimulation (Figure 3.15).

3.2.10 Different $\gamma\delta$ T cell subtypes respond to IL-1 β +IL-23 stimulation in the lung and lymph node

To investigate the ability of lung $\gamma\delta$ T cells to produce IL-17 and IL-22 in response to cytokine stimulation, lung and lymph node cells were incubated with medium or IL-1 β +IL-23 for 5 or 24h. Both lung and lymph node $\gamma\delta$ T cells produced IL-17 and IL-22 after 5 hours of cytokine stimulation. However, the percentage of responding cells was much higher in the lung. The percentage of cytokine positive cells was increased when the cells were stimulated for 24h (Figure 3.16). Both in lung and lymph node, the majority of IL-22 producing cells co-produced IL-17. Lung and lymph node cells were incubated with medium, IL-1 β , IL-23 or IL-1 β +IL-23 for 5h in order to examine the ability of either IL-1 β or IL-23 alone to induce IL-17 and IL-22 production by $\gamma\delta$ T cells. In the lung, substantial IL-17 production was observed in response to stimulation with either IL-1 β or IL-23 alone. This was not the case for lymph node cells. Stimulation with IL-23 alone did not induce IL-22 production from either lymph node or lung $\gamma\delta$ T cells (figure 3.17).

When IL-17 production by $\gamma\delta$ T cell subsets in the lung and lymph node was investigated, it was found that the majority of IL-17 in the lymph node samples was produced by $V\gamma 4^+$ $\gamma\delta$ T cells. In contrast, in the lung IL-17 was produced by $V\gamma 4^+$ $\gamma\delta$ T cells as well as another subset of $\gamma\delta$ T cells (Figure 3.18). $\gamma\delta$ T cells expressing the other V γ chain were also responsible for the majority of IL-17 production in response to stimulation with either IL-1 β or IL-23 alone. $V\gamma 1^+$ $\gamma\delta$ T cells were found not to contribute to IL-17 production in either lung or the lymph node (Figure 3.19).

Since IL-22 was co-produced by IL-17 producing cells both in the lung and lymph node, but more than one subset of $\gamma\delta$ T cells contributed to IL-17 production in the lung, the production of IL-22 and IL-17 by $\gamma\delta$ T cell subsets in these tissues was investigated. The cells were divided into three subsets within the TCR δ^+ gate: $V\gamma 4^+$, $V\gamma 1^+$ and $V\gamma 4^-V\gamma 1^-$ (Figure 3.20A). It was found that while in the lymph node IL-22 was produced by $V\gamma 4^+$ cells, in the lung the majority of IL-22 was produced by the resident $V\gamma 4^-V\gamma 1^-$ (other V γ) $\gamma\delta$ T cells (Figure 3.20B).

3.2.11 CD3⁺CD4⁻CD8⁻TCR β ⁺ cells contribute to IL-17 and IL-22 production in the lung and lymph node

When IL-17 production in response to IL-1 β +IL-23 by different CD3⁺ cells was analysed, it was observed that apart from $\gamma\delta$ T cells, another subset of CD3 cells was responding to these cytokines. These cells were found to be CD4⁻CD8⁻ double negative both in lung and lymph node (Figure 3.21). A significant percentage of CD3⁺CD4⁻CD8⁻TCR δ^- in lung and lymph node produced IL-22 in response to IL-1 β +IL-23. Furthermore, in the lung, a significant percentage of CD3⁺CD4⁻CD8⁻TCR δ^- cells produced IL-17 in response to IL-1 β alone (Figure 3.22). Upon closer investigation, it was found that the majority of IL-17 producing CD3⁺CD4⁻CD8⁻ TCR δ^- cells were TCR β positive (Figure 3.23).

3.2.12 Lung $\gamma\delta$ T cells produce IL-17, IL-22 and IFN γ in response to stimulation with different cytokine combinations

Given that lymph node $\gamma\delta$ T cells respond to IL-2+IL-18, IL-18+IL-23 together with published data on the role of IL-12+IL-18 in induction of IFN γ secretion by lymph node $\gamma\delta$ T cells, the ability of these cytokine combinations to activate lung $\gamma\delta$ T cell subsets was investigated. It was found that IL-18+IL-23 stimulation results in

induction of production of IL-17, IL-22 and IFN γ by lung $\gamma\delta$ T cells (Figure 3.24). However, the percentage of IL-17 producing cells is lower than that induced by IL-1 β +IL-23 stimulation. On the other hand, IL-18+IL-23 induced a greater percentage of IFN γ producing $\gamma\delta$ T cells than did IL-1 β +IL-23 stimulation. Both of these cytokine combinations induced a similar percentage of IL-17 and IL-22 double secreting cells. However, a greater percentage of IL-22 single positive cells was observed in response to IL-1 β +IL-23. IL-2+IL-18 and IL-12+IL-18 stimulation was found to induce lung $\gamma\delta$ T cells to produce predominantly IFN γ . Furthermore, the combination of IL-12+IL-18 was found to be the strongest stimulus for the induction of IFN γ secretion as 40% of lung $\gamma\delta$ T cells were found to produce IFN γ in response to this cytokine combination compared with only about 10% in response to IL-2+IL-18. In addition, IL-12+IL-18 was found to induce IL-22 production from $\gamma\delta$ T cells.

When cytokine production by the individual lung $\gamma\delta$ T cell subsets was investigated, it was found that they have differential capacity to produce IFN γ , IL-17 and IL-22. V γ 4 T cells produced mainly IL-17 and IL-22 and some IFN γ while V γ 1 $\gamma\delta$ T cells produced predominantly IFN γ and very little of any of the other two cytokines (Figure 3.24B). In contrast, V γ 6 $\gamma\delta$ T cells were found to be highly capable of producing all three cytokines tested. V γ 4 and V γ 6 accounted for a similar percentage of IL-17 producing cells in response to IL-1 β +IL-23 and IL-18+IL-23. Furthermore, a similar percentage of V γ 6 and V γ 4 cells produced IL-22 in response to IL-1 β +IL-23. However, V γ 6 cells were found to be the main source of IL-22 in response to IL-18+IL-23. In addition, a similar percentage of V γ 6 and V γ 1 cells produced IFN γ in response to IL-2+IL-18 and IL-12+IL-18.

3.2.13 CD27⁺ and CD27⁻ lung $\gamma\delta$ T cells produce IFN γ , while IL-17 is produced exclusively by CD27⁻ $\gamma\delta$ T cells.

Given that CD27 expression by $\gamma\delta$ T cells is thought to differentiate IFN γ from IL-17 producing cells, we investigated the production of these cytokines by lung CD27⁺ and CD27⁻ $\gamma\delta$ T cells. It was found that IL-1 β +IL-23 induces IL-17 and IFN γ production from CD27⁻ but not CD27⁺ $\gamma\delta$ T cells (Figure 3.25). IL-2+IL-18 on the other hand promoted some IL-17 production from CD27⁻ cells and some IFN γ from CD27⁺ cells. Non-specific stimulation with PMA and ionomycin induced IL-17

production from CD27⁻ lung $\gamma\delta$ T cells only, while IFN γ was produced by CD27⁺ and CD27⁻ cells.

3.2.14 Lung CD3⁺CD4⁻CD8⁻TCR δ ⁻ cells produce IL-17, IL-22 and IFN γ in response to cytokine stimulation.

The current study has found that CD3⁺CD4⁻CD8⁻TCR δ ⁻ cells, similar to $\gamma\delta$ T cells, are able to produce IL-17 and IL-22 in response to IL-1 β +IL-23 stimulation. Given that lung $\gamma\delta$ T cells can become activated by different cytokine combinations, the responses of lung CD3⁺CD4⁻CD8⁻TCR δ ⁻ cells to these cytokine combinations were investigated. CD3⁺CD4⁻CD8⁻TCR δ ⁻ cells were found to produce IL-17, IL-22 but no IFN γ in response to IL-1 β +IL-23 (Figure 3.26). In contrast, upon stimulation with IL-18+IL-23 CD3⁺CD4⁻CD8⁻TCR δ ⁻ cells produced all of the cytokines tested. Stimulation with IL-2+IL-18 was found to induce IFN γ and some IL-17. IL-12+IL-18 on the other hand, induced IFN γ secretion from almost 30% of lung CD3⁺CD4⁻CD8⁻TCR δ ⁻ cells.

3.2.15 Lung $\gamma\delta$ T cells secrete IL-17 in response to live and heat killed *B. pertussis* stimulation.

In order to investigate the response of $\gamma\delta$ T cells to a lung pathogen, lung, lymph node and spleen cells were incubated with medium, heat killed or live *Bordetella pertussis* at an MOI of 1 or with IL-1 β +IL-23. It was found that while cells from all three tissues responded strongly to cytokine stimulation, only lung $\gamma\delta$ T cells produced IL-17 in response to live and, to a lesser extent, heat killed bacteria (Figure 3.27). When production of IL-17 in response to bacteria by lung V γ $\gamma\delta$ T cell subsets was investigated, it was found that while both V γ 4⁺ and V γ 6⁺ respond strongly to IL-1 β +IL-23 stimulation, only V γ 6⁺ cells responded to live and heat killed bacteria (Figure 3.28)

3.2.16 Lung CD3⁺CD4⁻CD8⁻TCR β ⁺ cells produce IL-17 in response to *B. pertussis*.

Since CD3⁺CD4⁻CD8⁻TCR β ⁺ cells, similar to $\gamma\delta$ T cells, produced IL-17 in response to IL-1 β +IL-23, it was tested whether the lung CD3⁺CD4⁻CD8⁻TCR δ ⁻ cells would respond to heat killed and live *B. pertussis* stimulation. It was found that incubation of lung cells with live *B. pertussis* induced CD3⁺CD4⁻CD8⁻TCR δ ⁻ cells to produce IL-

17. Given that $CD3^+CD4^-CD8^-TCR\delta^-$ are likely to be NKT cells, the expression of NK cell markers, NK1.1 and CD49b, on these cells was investigated. It was found that, neither IL-1 β +IL-23 nor LBP responsive $CD3^+CD4^-CD8^-TCR\delta^-$ cells, expressed NK1.1 or CD49b (Figure 3.29).

3.2.17 IL-17 production by lung $\gamma\delta$ T cells and $CD3^+CD4^-CD8^-TCR\delta^-$ cells is dependent on IL-1RI signalling

In order to investigate whether *B. pertussis* induced IL-17 production by $\gamma\delta$ T cells was dependent on IL-1 β , lung cells were incubated with medium, LBP, LBP and caspase 1 inhibitor YVAD-fmk or with IL-1 β +IL-23. It was found that addition of YVAD to LBP stimulated lung cells lead to a decrease in IL-17 production by $\gamma\delta$ T cells (Figure 3.30).

When lung cells from WT and IL-1RI deficient mice were incubated with HKBP, LBP or IL-1 β +IL-23, it was found that IL-17 production by both $\gamma\delta$ and $CD3^+CD4^-CD8^-TCR\delta^-$ cells was dependent on IL-1RI signalling in response to all of the stimuli tested (Figure 3.31).

3.2.18 $V\gamma6^+$ $\gamma\delta$ T cells respond to stimulation with LBP conditioned IBMM supernatants

To determine whether lung $V\gamma6^+$ $\gamma\delta$ T cells are directly recognising *B. pertussis* or responding to soluble mediators either induced or secreted by the bacteria, lung cells were incubated with medium, HKBP, LBP, filter sterilised supernatant from LBP conditioned IBMM or with IL-1 β +IL-23. It was found that while both $V\gamma6^+$ and $V\gamma4^+$ $\gamma\delta$ T cells produced IL-17 in response to IL-1 β +IL-23, only $V\gamma6^+$ $\gamma\delta$ T cells responded strongly to stimulation with live or heat killed bacteria or to LBP conditioned medium (Figure 3.32)

3.2.19 IBMM produce proinflammatory cytokines in response to stimulation with live and heat killed *B. pertussis*.

Macrophages are one of the major antigen presenting cell types in the lung. To examine the type of response induced in macrophages by *B. pertussis*, immortalised bone marrow derived macrophages (IBMM) were incubated with medium, HKBP or LBP and their cytokine profile was analysed after 24 hours post stimulation. Both

HKBP and LBP induced IL-1 α , IL- β and IL-23 production by IBMM. However, the concentration of these cytokines was higher in response to live than heat killed bacteria. HKBP, on the other hand, induced higher levels of TGF β , IL-27, IL-10 and TNF α . Similar levels of IL-6 were detected in supernatants from HKBP and LBP treated cells. No IL-12p70, IL-18, IL-4 or IL-5 was detected in the supernatants from either HKBP or LBP stimulated IBMM (Figure 3.33).

3.2.20 Different innate cytokine profile is induced in lung lymphocytes compared to IBMM in response to in vitro stimulation with *B. pertussis*.

In order to verify whether the cytokine profile of IBMMs is representative of the cytokines induced by *B. pertussis* in the primary cells, lung cells and IBMM were incubated with medium, HKBP or LBP for 24h. Similar to IBMM, higher concentration of IL-1 β was detected in the supernatants from LBP stimulated lung cells. However, in contrast to IBMM, similar IL-1 α concentration was detected from HKBP and LBP stimulated lung cells. Furthermore, LBP and HKBP failed to induce IL-23 and IL-12p40 secretion from lung cells while high levels of both were detected in supernatants of IBMM. No significant amount of IL-12p70 was detected from either lung cells or IBMM. In contrast to IBMM, lung cells did not secrete IL-27. However, similar to IBMM, lung cells produced IL-6 and TNF α in response to both heat killed and live bacteria. In addition, high basal level of IL-6 was detected in medium incubated lung cells. Significant concentration of IL-10 was detected in supernatants of lung cells incubated with HKBP and LBP and, in contrast to IBMM, the concentration of IL-10 was not influenced by the viability of bacteria. Lung cells secreted significant amount of IL-5 in response to live *B. pertussis*. However, no significant IL-4 or IL-13 was detected in supernatants of bacteria stimulated IBMM and lung cells (Figure 3.34).

3.2.21 Heat killed and live *B. pertussis* differently activate lung neutrophils and macrophages.

Macrophages and neutrophils are the two main cell types capable of responding directly to *B. pertussis* and thus likely to be important for activation of $\gamma\delta$ T cells via cytokine production and possibly through other mechanisms. They are also the cell types known to be specifically targeted by *B. pertussis* as means of avoiding host

immune responses. The induction of proinflammatory cytokines, IL-12/23p40, pro-IL-1 β , IL-1 α and TNF α , in neutrophils and macrophages in response to HKBP and LBP was investigated. It was found that both HKBP and LBP induced proIL-1 β in macrophages, although LBP was a much stronger stimulus with about 80% macrophages being positive for this cytokine (Figure 3.35). Some proIL-1 β was induced in neutrophils in response to HKBP and about five times as much in response to LBP. However, the percentage of proIL-1 β positive neutrophils was much lower than that of pro-IL-1 β positive macrophages. No IL-12/23p40 was detected in neutrophils and macrophages upon either HKBP or LBP stimulation. A high percentage of neutrophils was found to be positive for IL-1 α in the absence of stimulus. This was enhanced upon stimulation with HKBP or LBP. A small percentage of TNF α producing neutrophils was induced upon HKBP and, to a lesser extent, LBP stimulation. In contrast to neutrophils, only a small percentage of IL-1 α positive macrophages was detected in the absence of stimulation. HKBP and LBP were found to activate macrophages to secrete IL-1 α and TNF β . However, HKBP was found to be a much greater stimulus for TNF α and IL-1 α production. Furthermore, a higher percentage of TNF α /IL-1 α double positive cells was induced in response to HKBP than in response to LBP.

3.2.22 Purified lung and spleen antigen presenting cells secrete IL-1 β but not IL-23 in response to live *B. pertussis*.

In order to investigate whether the differences in responses of lung and peripheral $\gamma\delta$ T cells were due to the quality of the pro-IL-17 signal or the number of antigen presenting cells present in those tissues, CD11b $^{+}$ and CD11c $^{+}$ cells from lung and spleen were purified on a flow cytometer (>97% pure). Cellular composition of each sorted sample was determined by flow cytometry. Both lung and spleen cells consisted of a similar percentage of macrophages/monocytes (17.9% and 13.5%, respectively), NK cells (33.2% and 30.6%, respectively) and eosinophils (7.6% and 5.1%, respectively). A higher percentage of neutrophils and DC was present in the sorted cell population from the spleen, while purified lung CD11c $^{+}$ and CD11b $^{+}$ cells contained alveolar macrophages which were absent from the spleen (Figure 3.36). These purified CD11b $^{+}$ and CD11c $^{+}$ populations will be referred to as lung and spleen antigen presenting cells hereafter. Lung and spleen APC were incubated with medium or with live *B. pertussis* for 24 hours. Similar concentrations of IL-1 β were

detected in the supernatants from lung and spleen APC. However, no IL-23 was detected in either of the supernatants. Furthermore, lung and spleen APC failed to produce IL-1 α in response to LBP stimulation (Figure 3.37).

The induction of IL-1 β , IL-23, IL-6 and TGF β was investigated at the RNA level. LBP was found to strongly induce both IL-1 β and IL-6 gene expression in lung and spleen APC (Figure 3.38). IL-23 gene induction was also detected. No significant upregulation of TGF β gene was detected in response to LBP stimulation.

3.2.23 Isolated lung but not lymph node $\gamma\delta$ T cells strongly respond to stimulation with LBP conditioned supernatants.

Given that lung and spleen APC were found to respond in a similar fashion to stimulation with LBP, the ability of their supernatants to induce IL-17 production from lung and peripheral (lymph node) $\gamma\delta$ T cells was investigated. It was found that supernatants from LBP conditioned lung and spleen APC were able to induce IL-17 production from lung $\gamma\delta$ T cells (Figure 3.39). The APC supernatants were found to induce similar percentage of IL-17 producing lung $\gamma\delta$ cells to that induced by IL-1 β +IL-23 stimulation. V γ 6 cells were found to be the main IL-17-producing lung $\gamma\delta$ T cell subset in response to lung and spleen APC supernatant treatment. V γ 4 $\gamma\delta$ T cells accounted for about a sixth of IL-17-producing lung $\gamma\delta$ T cells in response to APC supernatants. However, spleen APC supernatant was found to induce a greater percentage of IL-17⁺ V γ 4 $\gamma\delta$ T cells than did the supernatant from lung APC. Lung APC supernatant failed to induce IL-17 production from lymph node $\gamma\delta$ T cells. Spleen APC supernatants, on the other hand, induced similar percentage of IL-17⁺ lymph node V γ 4 $\gamma\delta$ T cells to that induced by IL-1 β +IL-23.

3.2.24 Expression of IL-1, IL-23 and IL-18 receptors and T helper lineage transcription factors by lung $\gamma\delta$ T cell subsets.

Lung $\gamma\delta$ T cell subsets were found to have a differential capacity to respond to LBP and LBP conditioned supernatants, as well as to stimulation with different cytokine combinations. The expression of IL-1, IL-23, IL-18 and IL-12 receptor by V γ 4, V γ 6 and V γ 1 $\gamma\delta$ T cells was therefore investigated. It was found that lung V γ 6 $\gamma\delta$ T cells express significantly higher levels of IL-1RI than do the other two V γ subsets in the lung (Figure 3.40). V γ 6 and V γ 4 cells were found to express much higher levels of

IL-23 receptor than did V γ 1 cells. Furthermore, lung V γ 6 $\gamma\delta$ T cells were found to express significantly higher levels of IL-23R than V γ 4 $\gamma\delta$ T cells, although the difference was not as pronounced as that relative to V γ 1 $\gamma\delta$ T cells. Lung V γ 6 $\gamma\delta$ T cells were found express significantly more IL-18R than did any of the other two subsets of lung $\gamma\delta$ T cells. IL-12R β was found to be highly expressed by lung V γ 6 and V γ 1, but not V γ 4 $\gamma\delta$ T cells. However, the differences were not significant.

T helper cells can be divided into subsets based on the predominant production of IL-17 (Th17), IFN γ (Th1) or IL-5 (Th2). Each subset is also expresses characteristic transcription factors, namely Ror γ t, Tbet and Gata3. AhR has been implicated in promoting IL-22 production by T cells. The expression of these transcription factors by lung $\gamma\delta$ T cell subsets was analysed. It was found that lung V γ 6 $\gamma\delta$ T cells expressed significantly higher levels of Ror γ t than did V γ 4 and V γ 1 $\gamma\delta$ T cells (Figure 3.41). V γ 6 $\gamma\delta$ T cells were also found to express the highest levels of Tbet followed by V γ 1 $\gamma\delta$ T cells. No significant difference in Gata3 or AhR expression was detected between lung $\gamma\delta$ T cell subsets.

The expression of IL-1RI, IL-23R and IL-18R as well as T helper transcription factors by lymph node and lung $\gamma\delta$ T cell subsets was then compared. Lung V γ 6 $\gamma\delta$ T cells were found to express significantly higher levels of IL-1R than lung or lymph node V γ 4 $\gamma\delta$ T cells (Figure 3.42). In contrast, lymph node $\gamma\delta$ T cells were found to express higher levels of IL-23R than did any of the other subsets analysed. Similarly, lymph node $\gamma\delta$ T cells were found to express the highest levels of IL-18R. Furthermore, lymph node V γ 4 $\gamma\delta$ T cells were found to express significantly higher levels of IL-18R than did lymph node V γ 1 $\gamma\delta$ T cells, although the difference was much smaller than that observed between lymph node V γ 4 $\gamma\delta$ T cells and lung $\gamma\delta$ T cell subsets.

When expression of Th transcription factors by lung and lymph node $\gamma\delta$ T cell subsets was compared, it was found that, similar to lung $\gamma\delta$ T cells, V γ 1 subset in the lymph node expresses the highest level of Tbet (Figure 3.43). Furthermore, the level of expression of Tbet in lymph node V γ 1 cells was higher, albeit not significantly, than that in lung V γ 1 $\gamma\delta$ T cells. Similarly, lymph node V γ 4 $\gamma\delta$ T cells were found to express higher levels of Ror γ t than did lung V γ 4 and V γ 6 $\gamma\delta$ T cells, although the

difference was not significant. Gata3 was most strongly expressed by lymph node V γ 1 $\gamma\delta$ T cells.

3.2.25 The role of IL-23 in the LBP induced IL-17 production.

No IL-23 protein was detected in supernatants of lung lymphocytes and purified lung APC. However, IL-23p19 gene induction was detected in lung APC. In order to verify whether IL-23 has a role in LBP induced IL-17 production, IL-23 neutralising antibody approach was used. It was found that incubation of lung lymphocytes with LBP in the presence of anti-IL-23 antibody resulted in decreased percentages of IL-17-producing V γ 6 and V γ 4 $\gamma\delta$ T cells (Figure 3.44). However, isotype control antibody was also found to reduce the percentage of IL-17 positive cells. Anti-IL-23 antibody was found to inhibit IL-1 β +IL-23 induced IL-17 production by lung V γ 4 and V γ 6 $\gamma\delta$ T cells. In contrast to LBP-induced IL-17 production, incubation of IL-1 β +IL-23 with isotype control antibody did not result in a reduction in the percentage of IL-17 positive cells.

In order to further investigate the role of IL-23 in LBP induced IL-17 production, isolated lung $\gamma\delta$ T cells were incubated with LBP conditioned APC supernatants in the presence or absence of anti-IL-23 antibody. IL-1 β +IL-23 stimulation was used as a control for cell responsiveness and efficiency of anti-IL-23 inhibition. FACS data revealed that anti-IL-23 did not reduce the percentage of IL-17 producing lung $\gamma\delta$ T cells induced in response to stimulation with sterile LBP conditioned APC supernatants (Figure 3.45A). IL-1 β +IL-23 induced IL-17 production, on the other hand, was completely removed when anti-IL-23 was added to the culture. Similarly, reduced concentration of IL-17 was detected by ELISA in the supernatants of lung $\gamma\delta$ T cells stimulated with IL-1 β +IL-23 in the presence of IL-23 neutralising antibody (Figure 3.45B). However, in contrast to the FACS data, IL-17 was found to be reduced in the supernatants of $\gamma\delta$ T cells incubated with LBP conditioned APC supernatants and anti-IL-23, but not with the isotype control. Although the difference between anti-IL-23 or IgG treated samples was not significant.

3.2.26 TLR ligands and LBP fail to induce cytokine production from purified $\gamma\delta$ T cells either alone or in combination with IL-1 β .

The current study has shown that LBP induced IL-17 production is dependent on IL-1RI signalling. Given the published data on TLR expression by $\gamma\delta$ T cells, the possibility that TLR agonist together with IL-1 β can activate lung $\gamma\delta$ T cells to produce IL-17 was investigated. It was found that none of the TLR agonists tested was able to induce IL-17A, IL-17F, IL-22 or IFN γ production from purified lung $\gamma\delta$ T cells, either alone or in combination with IL-1 β (Figure 3.46). Furthermore, IL-1 β alone failed to induce IL-17 from purified lung $\gamma\delta$ T cells. IL-1 β +IL-23, on the other hand, induced significant production of all of the cytokines tested. In addition, CpG+IL-1 β stimulation was found to induce some IFN γ from CD3⁺TCR δ ⁻ cells.

Since *B. pertussis* expresses many other molecules that can potentially activate lung $\gamma\delta$ T cells, the induction of $\gamma\delta$ T cell cytokine production by intact live *Bordetella*, either alone or in combination with IL-1 β , was investigated. It was found that IL-1 β and LBP either alone or in combination were not able to stimulate IL-17 production by lung $\gamma\delta$ T cell subsets or CD3⁺TCR δ ⁻ cells (Figure 3.47). Interestingly, V γ 6 were found to produce the greatest concentrations of IL-17, IL-17F, IL-22 and IFN γ in response to IL-1 β +IL-23 compared to all other lung V γ subsets.

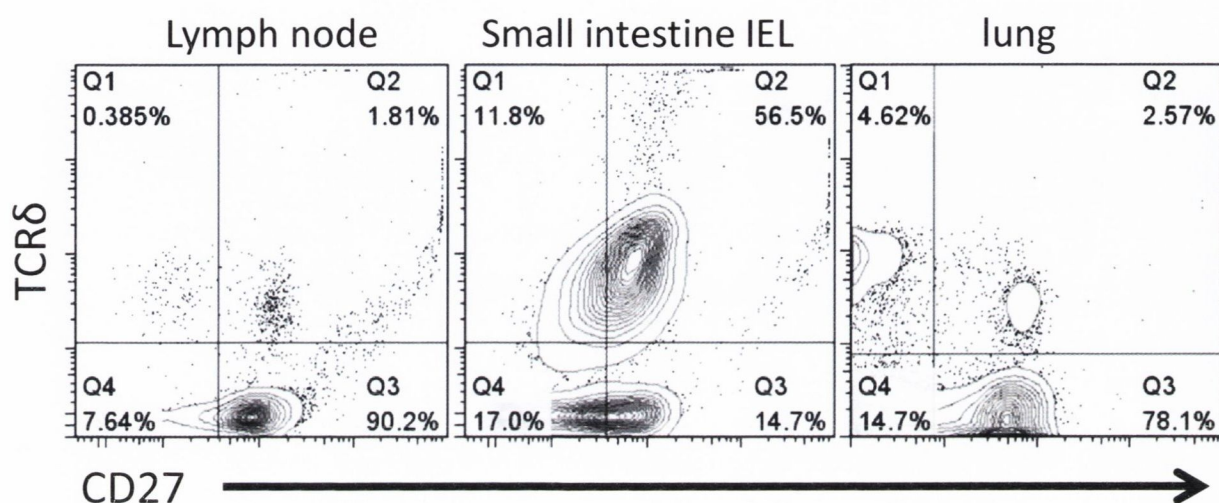


Figure 3.1 Expression of CD27 by $\gamma\delta$ T cells is tissue specific.

LN cells, Intestinal IELs and lung cells (1×10^6 cells/mL) were isolated by tissue disruption. Cells were then stained with fluorescent labelled antibodies for CD3, TCR δ and CD27. Representative plots are gated on CD3 $^+$ cells. n=1

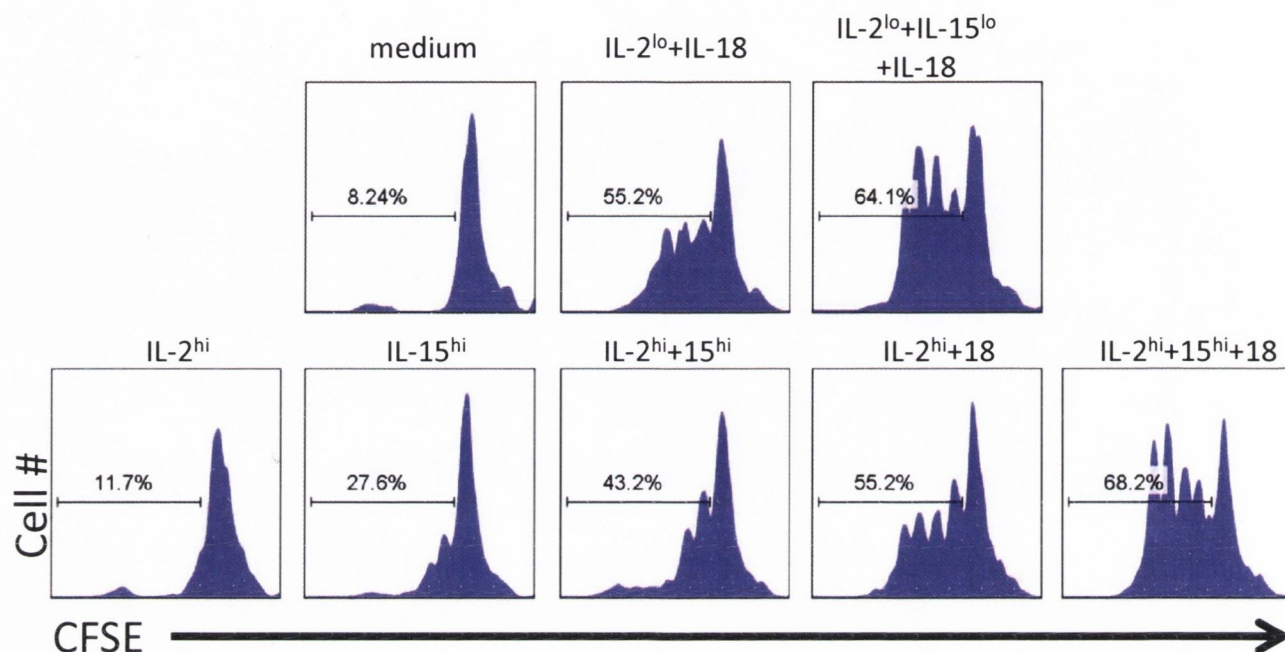


Figure 3.2 Proliferation of $\gamma\delta$ T cells in response to stimulation with expanding cytokines.

Lymph node cells ($1 \times 10^6/\text{mL}$) were labelled with CFSE ($0.5 \mu\text{M}$) and stimulated with IL-2 (IL-2^{lo}, 20 U/mL; IL-2^{hi}, 50 U/mL), IL-15 (IL-15^{lo}, 10 ng/mL; IL-15^{hi}, 20 ng/mL), and IL-18 (10 ng/mL) either alone or in combinations. After 72 hours the cells were surface stained for CD3 and TCR $\gamma\delta$ and analysed by flow cytometry. Cell divisions were observed as dilutions of the CFSE label. The plots show cells in the TCR δ^+ gate. $n \geq 3$

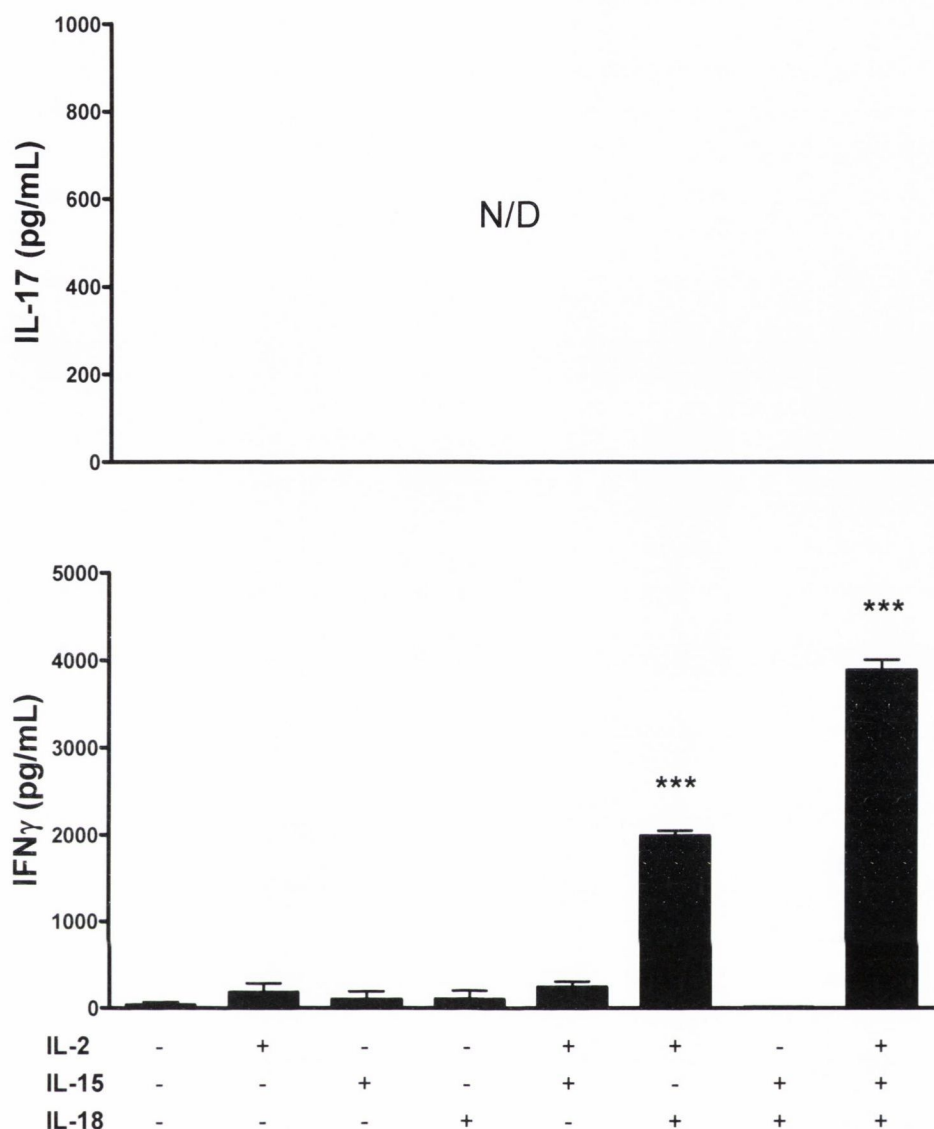


Figure 3.3 Lymph node cells produce IFN γ but not IL-17 in response to stimulation with expanding cytokines

Lymph node cells, from C57BL/6 mice, were stimulated with combinations of medium, IL-2 (20 U/mL), IL-15 (10 ng/mL), and IL-18 (10 ng/mL) as indicated. After 72h, the concentrations of IL-17 and IFN γ in the supernatants were determined by ELISA. Displayed are means ($\pm$ SD) of triplicate assays. *** $p < 0.001$ One way ANOVA with Dunnett's post test. ND: not detected. $n \geq 3$

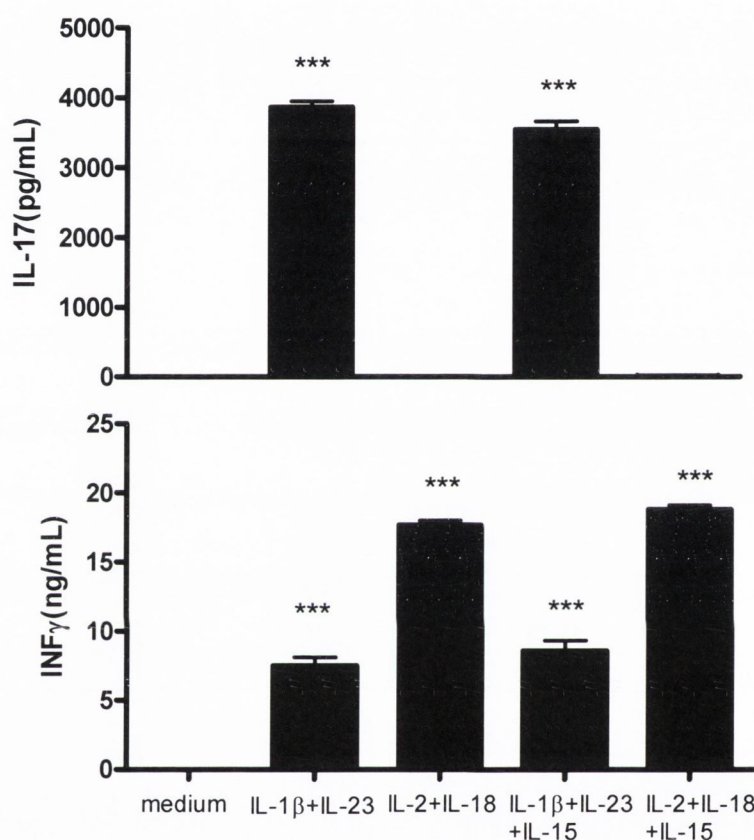


Figure 3.4 $\gamma\delta$ T cells produce IL-17 and INF γ in response to cytokine stimulation.

MACS purified lymph node $\gamma\delta$ T cells (1×10^5 cells/well) were stimulated in triplicate with combinations of IL-1 β (10 ng/mL), IL-23 (10 ng/mL), IL-2 (20 U/mL), IL-18 (10 ng/mL), and IL-15 (20 ng/mL) for 72 hours. The supernatants were then analysed for IL-17 and INF γ .***p<0.001 by one way ANOVA with Dunnett's post test. n=1

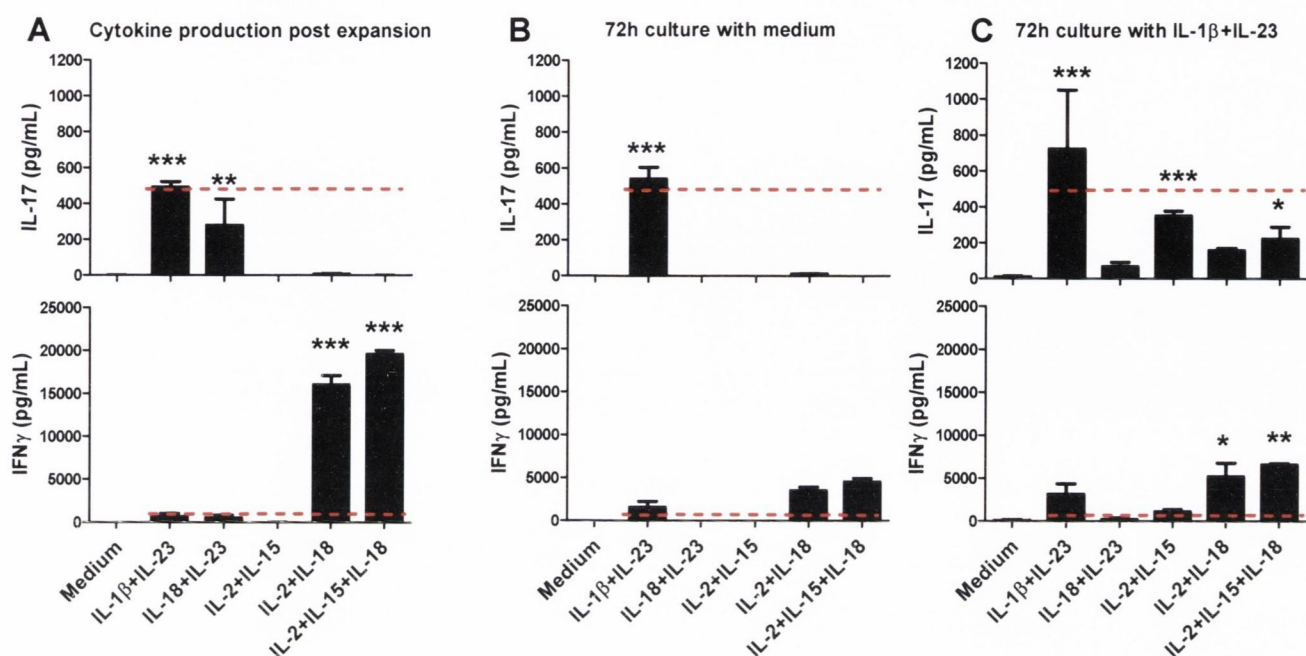


Figure 3.5 Lymph node cells produce IL-17 in response to IL-1 β and IL-23 after *in vitro* expansion. Lymph node cells (0.5×10^6 cells/mL) were stimulated with IL-1 β (10 ng/mL), IL-23 (10 ng/mL), IL-18 (10 ng/mL), IL-2 (20U/mL) and IL-15 (10 ng/mL) and IL-7 (10 ng/mL) in combinations indicated in the figure. After 72 hours supernatants were removed and fresh medium was added to the cells, with or without IL-1 β +IL-23, and cells were cultured for another 72 hours. Supernatants from day 3 (A) and day 6 (B,C) were then analysed for IL-17 and IFN γ by ELISA. The red dotted line indicates the level of IL-17 (top row) or IFN γ (bottom row) induced in freshly isolated lymph node cells upon IL-1 β +IL-23 stimulation. * $p < 0.05$ ** $p < 0.01$, *** $p < 0.001$ by one-way ANOVA with Dunnett's post test. $n=1$

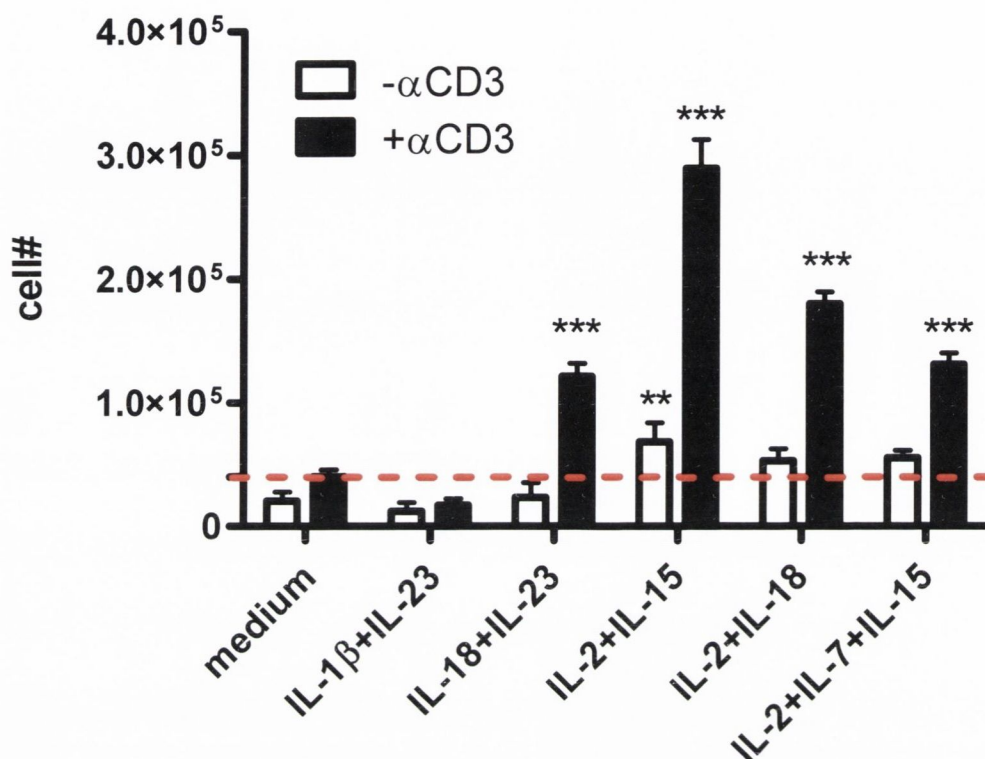


Figure 3.6 Expansion of $\gamma\delta$ T cells is enhanced by stimulation with α CD3.

Lymph node $\gamma\delta$ T cells (0.2×10^6 cells/mL) were incubated with IL-1 β (10 ng/mL), IL-23 (10 ng/mL), IL-18 (10 ng/mL), IL-2 (20 U/mL), IL-15 (20 ng/mL), and IL-7 (10 ng/mL) or medium alone, as indicated in the figure, in the presence or absence of α CD3 (1 μ g/mL) antibody. After 72 hours the number of viable cells was determined by trypan blue staining. The red hashed line indicates the number of cells in the wells prior to incubation. ** $p < 0.01$, *** $p < 0.001$ by two way ANOVA with Bonferroni post test versus initial cell number. $n=1$

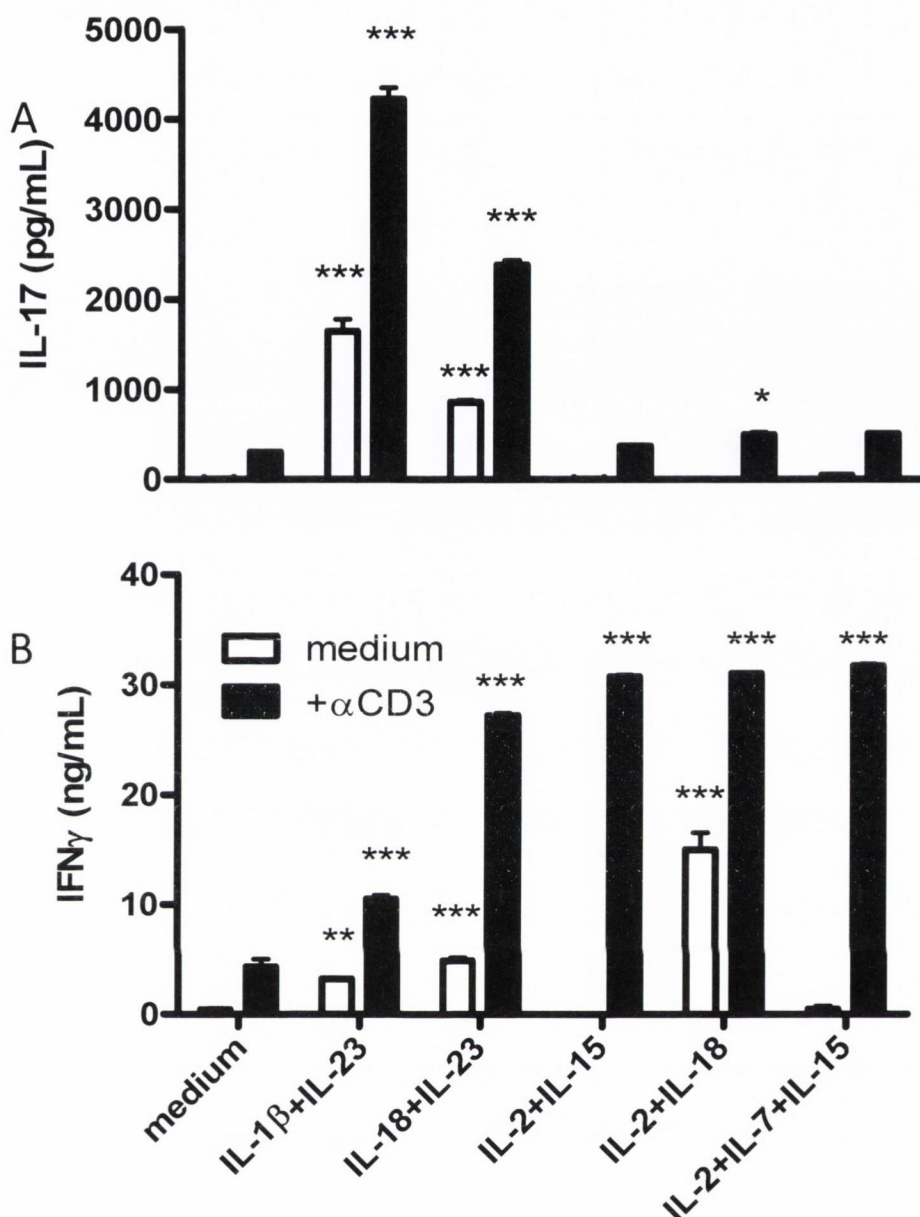


Figure 3.7 Stimulation with α CD3 enhances IL-17 and IFN γ production in response to expanding cytokines.

MACS sorted lymph node $\gamma\delta$ T cells (4×10^4 cells/well) were stimulated with IL-1 β (10 ng/mL), IL-23 (10 ng/mL), IL-18 (10 ng/mL), IL-2 (2 0U/mL), IL-15 (20 ng/mL), and IL-7 (10 ng/mL) or media alone at the combination indicated in the figure for 72 hours in the presence or absence of α CD3 (1 μ g/mL) antibody. The supernatants were then analysed for IL-17(A) and IFN γ (B) by ELISA. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by two way ANOVA with Bonferroni post test. n=1

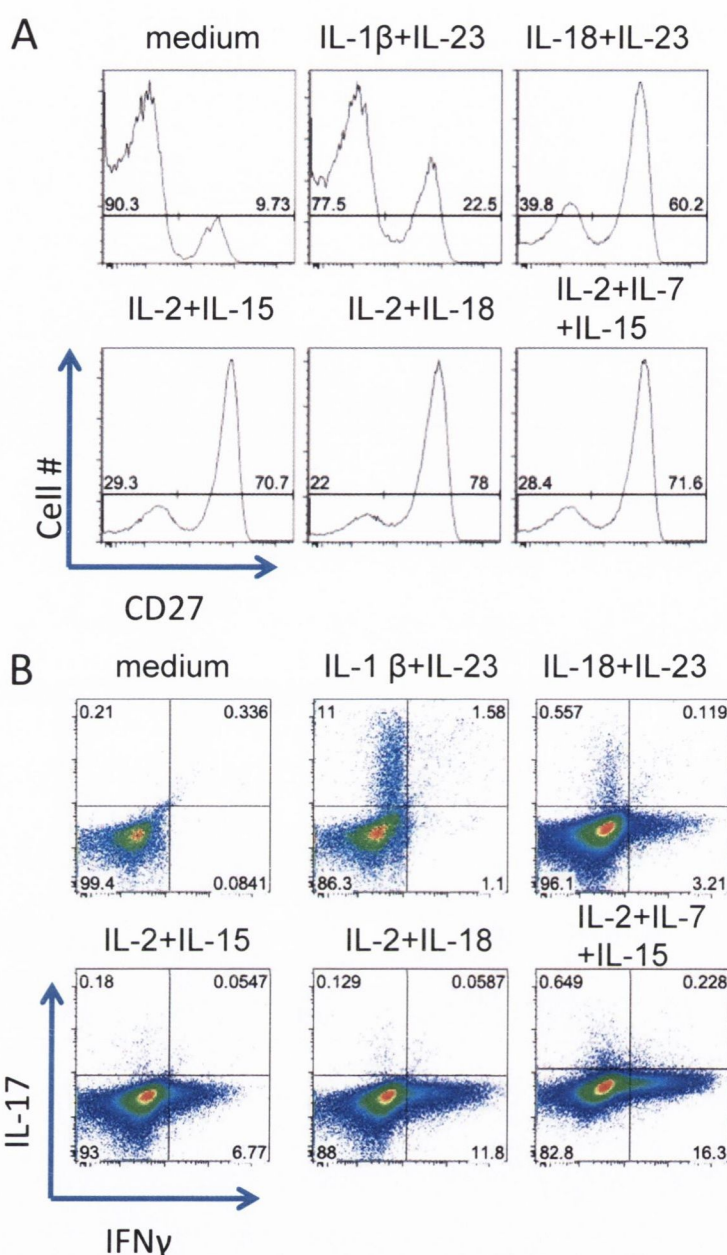


Figure 3.8 Cytokine stimulation of $\gamma\delta$ T cells in the presence of α CD3 alters the ratio of CD27⁺ to CD27⁻ cells and induces cytokine production.

Isolated lymph node $\gamma\delta$ T cells (4×10^4 cells/well) were stimulated with IL-1 β (10 ng/mL), IL-23 (10 ng/mL), IL-18 (10 ng/mL), IL-2 (20 U/mL), IL-15 (20 ng/mL), and IL-7 (10 ng/mL), at the combination indicated in the figure, in the presence of anti-CD3 antibody (1 μ g/mL) for 72 hours. BrfA (1 μ g/mL) was added for the final 5 hours of stimulation. The expression of CD27, IL-17 and IFN γ by $\gamma\delta$ T cells was then analysed by Flow cytometry. Graphs show percentages of CD27⁺ and CD27⁻ cells (A) and IFN γ and IL-17 producing cells (B) . n=1

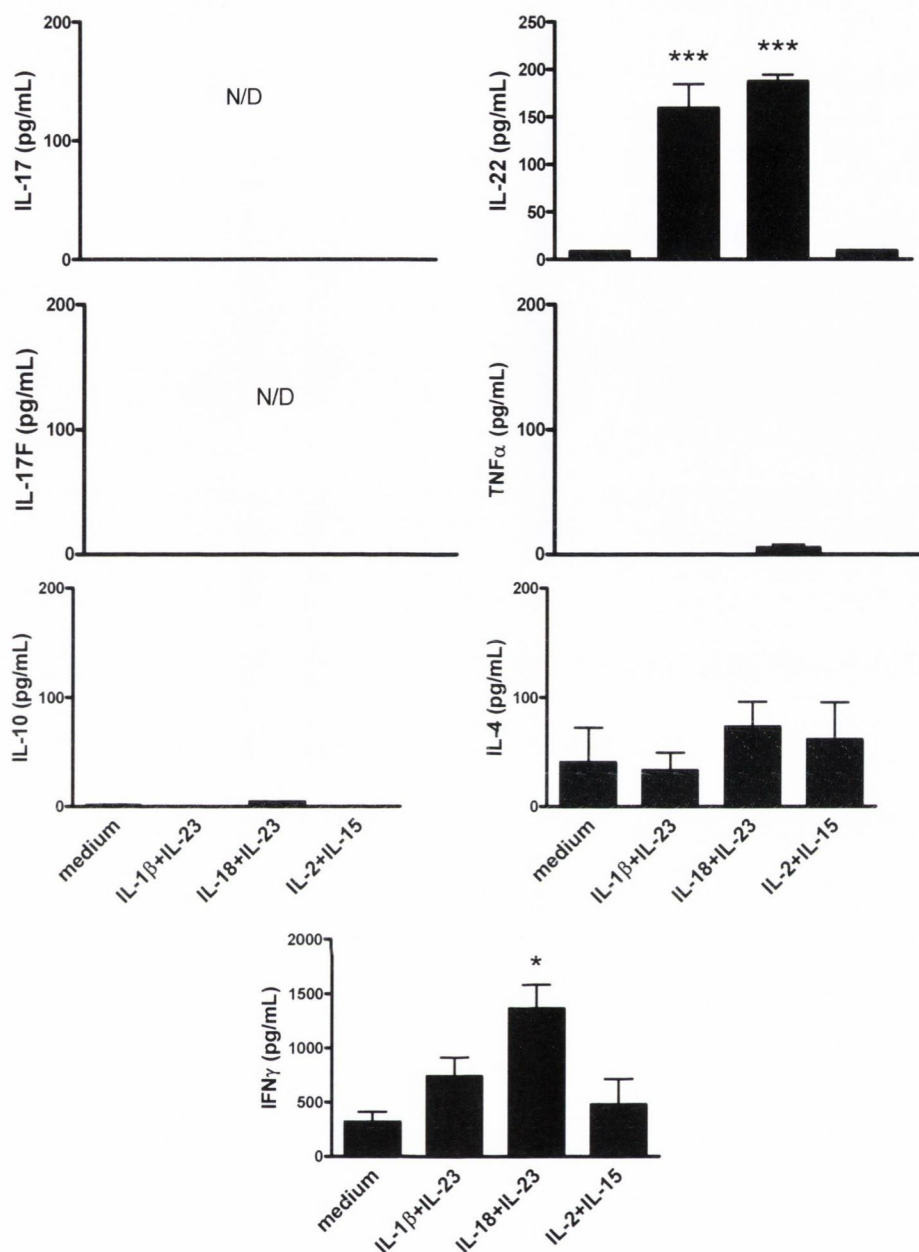


Figure 3.9 Intestinal Intrepiethelial lymphocytes produce IL-22 but not IL-17A or IL-17F in response to IL-1 β and 23 or IL-18 and IL-23.

IELs (0.5×10^6 cells/mL) from C57BL/6 mice were incubated with medium, IL-1 β (10 ng/mL) + IL-23 (10 ng/mL), IL-18 (10 ng/mL) + IL-23 (10 ng/mL), or IL-2 (20 U/mL) + IL-15 (20 ng/mL). After 72h, IL-17, IL-17F, IL-22, TNF α , IL-10, IL-4, and IFN γ concentration in the supernatants was assayed by ELISA . * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ND: not detected by one way ANOVA with Dunnett's post test. $n \geq 3$

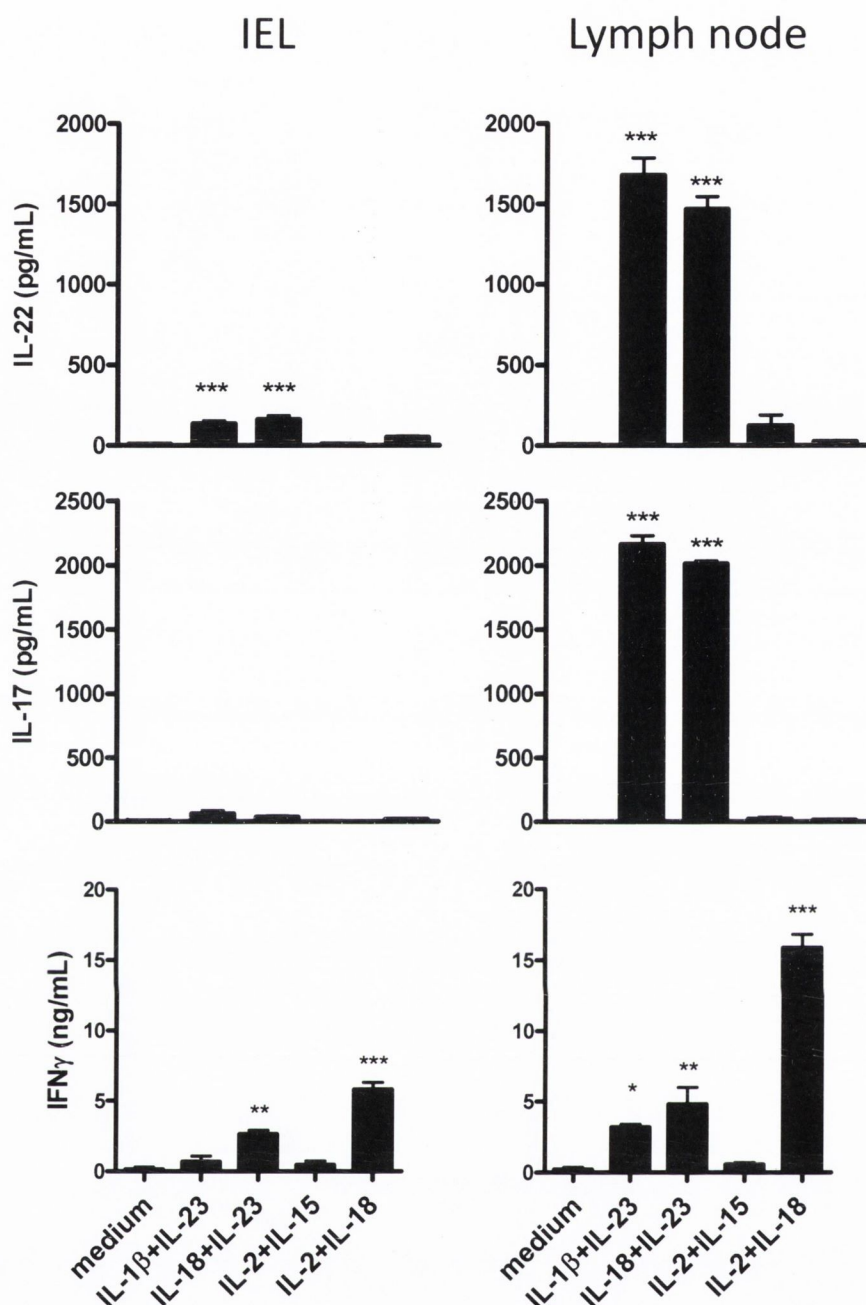


Figure 3.10 Production of proinflammatory cytokines by intestinal and lymph node lymphocytes

Intestinal Intraepithelial lymphocytes (IEL; $1 \times 10^6/\text{mL}$) and lymph node cells ($1 \times 10^6/\text{mL}$) were stimulated with media, IL-1 β (10 ng/mL) and IL-23 (10 ng/mL), IL-18 (10 ng/mL) and IL-23, IL-2 (20 U/mL) and IL-15 (20 ng/mL) or IL-2 and IL-18. The supernatants were removed 3 days post stimulation and analysed for IL-17, IL-22 and IFN γ by ELISA. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by one way ANOVA with Dunnett's post test. $n \geq 3$

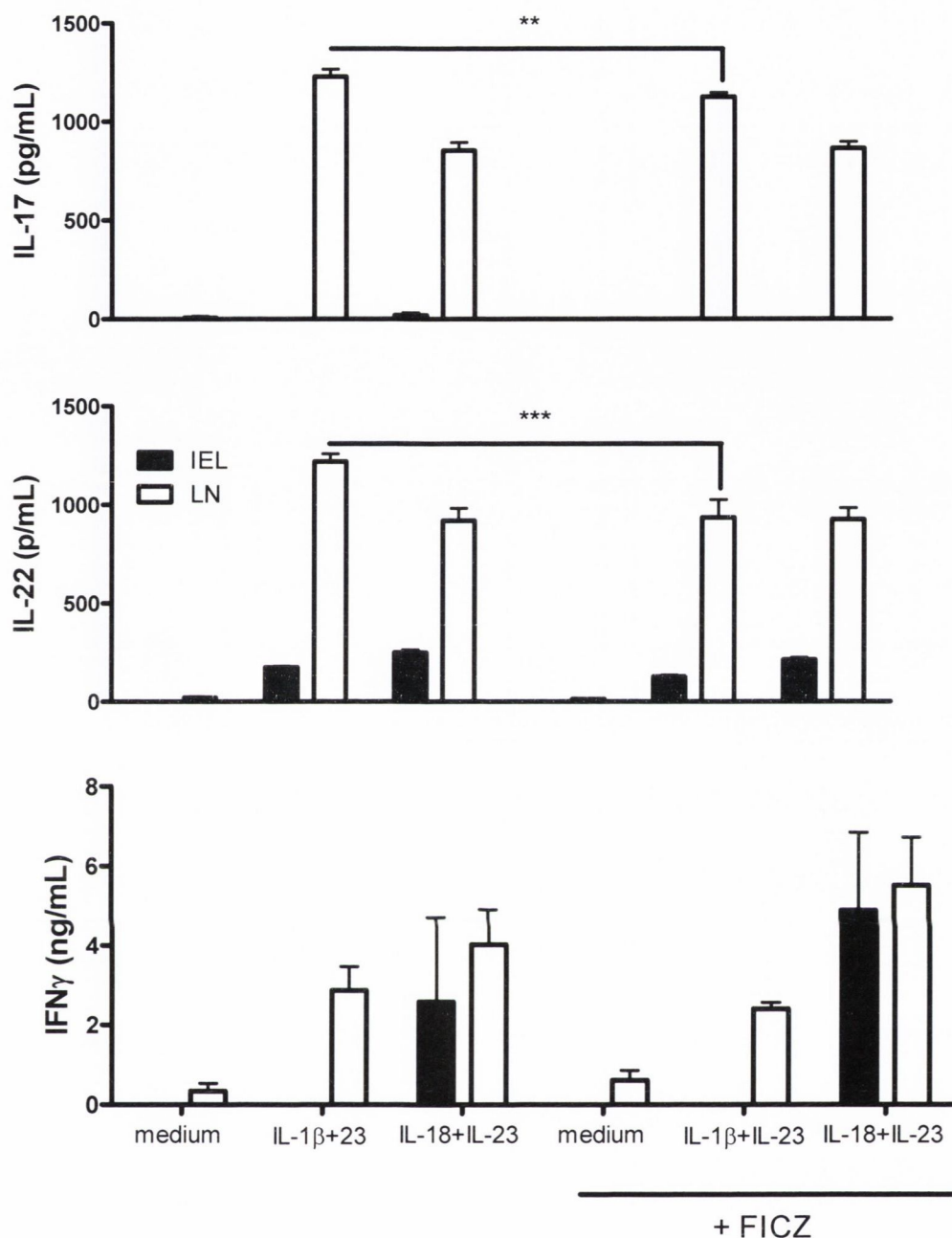


Figure 3.11 FICZ decreases IL-1β+IL-23 induced IL-17 and IL-22 production by LN cells.

Intestinal IELs and LN cells ($1 \times 10^6/\text{mL}$) were stimulated with IL-1β (10 ng/mL) and IL-23 (10 ng/mL) or IL-18 (10 ng/mL) and IL-23 (10 ng/mL) +/- FICZ (1 μM). Supernatants were removed 72 hours post stimulation and analysed for IL-17, IL-22 and IFNγ. ** $p < 0.01$, *** $p < 0.001$ by two way ANOVA with Bonferroni post test. $n=1$

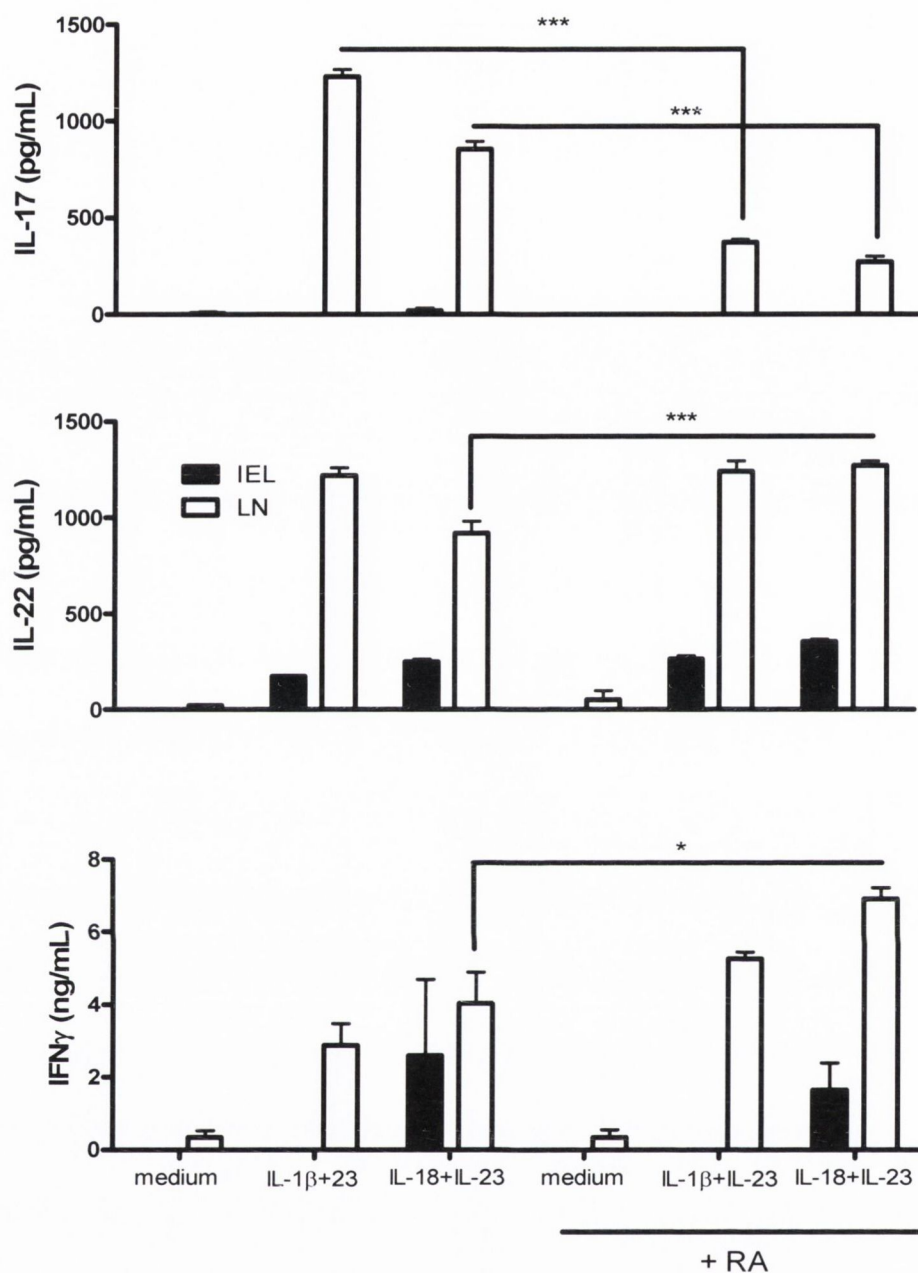


Figure 3.12 Retinoic acid suppresses IL-17, but enhances IL-22 production by IELs and LN cells in response to IL-1 β +IL-23 and IL-18+23 stimulation.

IELs and LN cells (1×10^6 /mL) were stimulated with IL-1 β (10 ng/mL) and IL-23 (10 ng/mL) or IL-18 (10 ng/mL) and IL-23 (10 ng/mL) +/- retinoic acid (RA; 100 nM). Supernatants were removed 72 hours post stimulation and analysed for IL-17, IL-22 and IFN γ by ELISA. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by two way ANOVA with Bonferrioni post test. $n=1$

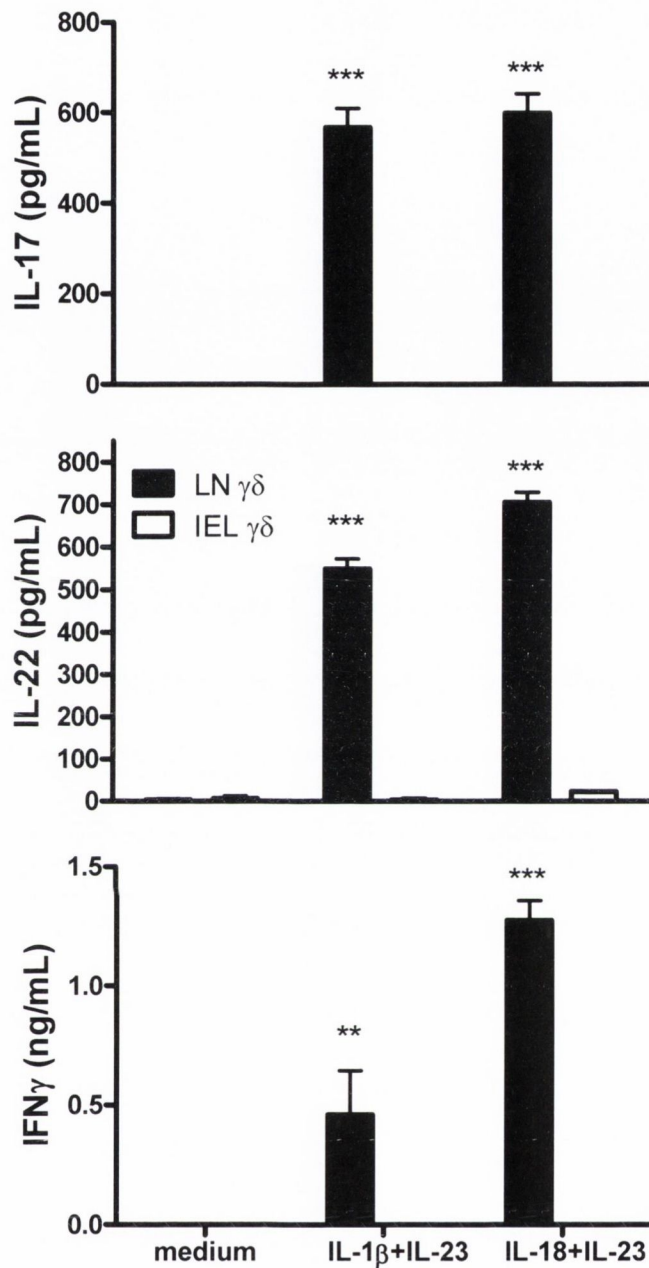


Figure 3.13 Purified intestinal $\text{TCR}\delta^+$ cells fail to respond to IL-1β+23 and IL-18+23 stimulation.

FACS sorted intestinal $\gamma\delta$ T cells and MACS sorted lymph node $\gamma\delta$ T cells ($0.2 \times 10^6/\text{mL}$) were stimulated with medium, IL-1β+IL-23 or IL-18+IL-23. After 72h. The concentration of for IL-17, IL-22 and IFNγ was analysed by ELISA. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by two way ANOVA with Bonferroni post test. $n=1$

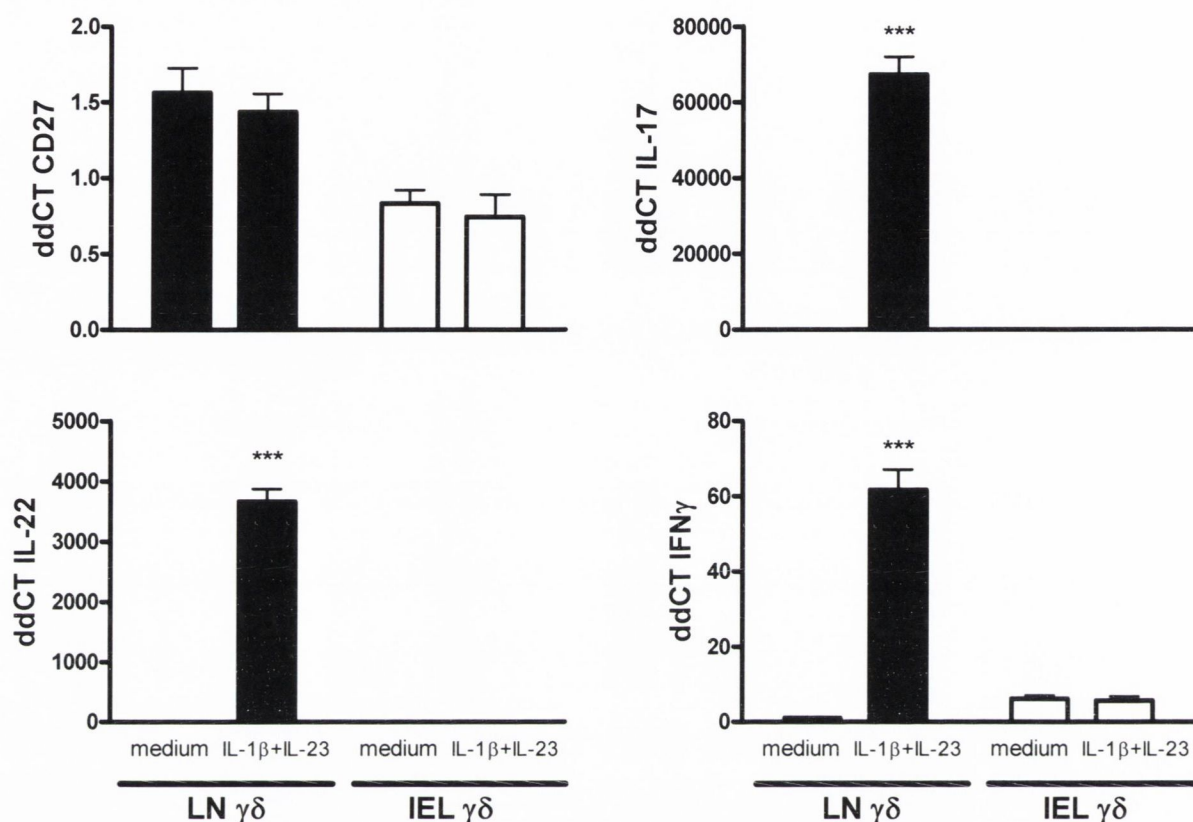


Figure 3.14 Purified intestinal $\gamma\delta$ T cells fail to upregulate the expression of IL-17, IL-22 and IFN γ in response to cytokine stimulation.

FACS purified Intestinal $\gamma\delta$ IELs (IEL $\gamma\delta$) and MACS purified lymph node $\gamma\delta$ T cells (LN $\gamma\delta$; 0.2×10^6 cells/mL) were incubated with medium or IL-1 β +IL-23 for 24 hours. The RNA was then isolated and the expression of CD27, IL-17, IL-22 and IFN γ mRNA determined by RTPCR. The data were normalised relative to 18s RNA. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by two way ANOVA with Bonferroni post test. $n=1$

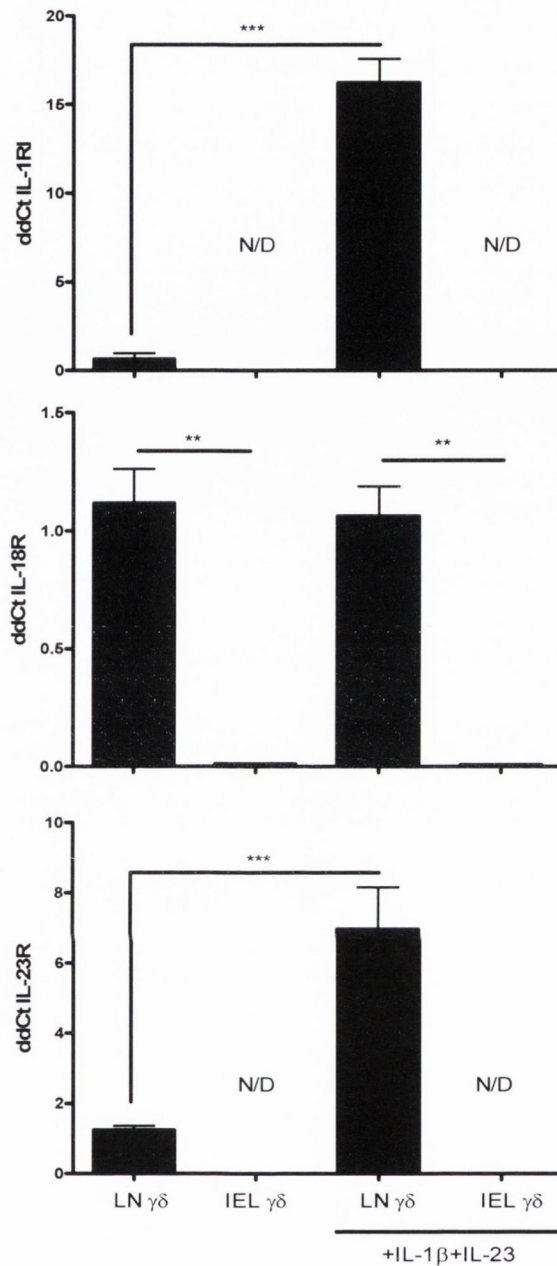


Figure 3.15 Intestinal $\gamma\delta$ T cells do not express IL-1RI, IL-18R and IL-23R.

Intestinal $\gamma\delta$ IELs purified by FACS and peripheral $\gamma\delta$ T cells purified by MACS ($0.2 \times 10^6/\text{mL}$) were incubated for 24 hours with medium or IL-1 β +IL-23. RNA was then isolated and the relative expression of IL-23R, IL-1R and IL-18R analysed by RT-PCR. The data was normalised relative to 18s RNA. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ND: not detected by one way ANOVA with Tukey post test. $n=1$

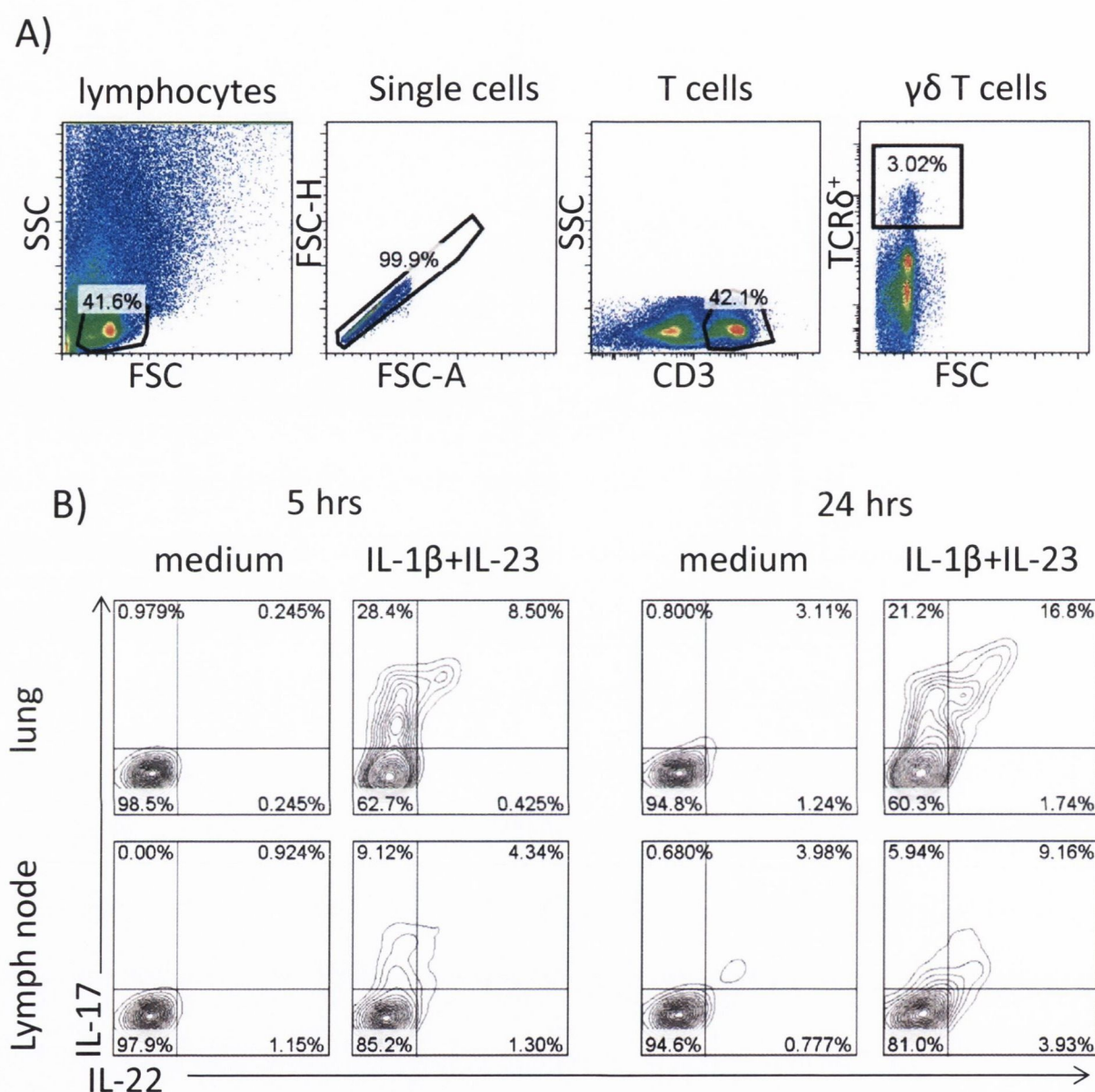


Figure 3.16 Lung $\gamma\delta$ T cells produce IL-17 and IL-22 within 5h of stimulation with IL-1 β and IL-23.

Lung and lymph node cells ($1 \times 10^6/\text{mL}$) were stimulated with medium or IL-1 β +IL-23 for 5 or 24 hours in the presence of BrfA ($5 \mu\text{g}/\text{mL}$). The expression of IL-17 and IL-22 by $\gamma\delta$ T cells was then analysed by FACS. The representative plots show A) gating strategy B) percentage of cytokine producing cells in the TCR δ^+ gate. $n \geq 3$

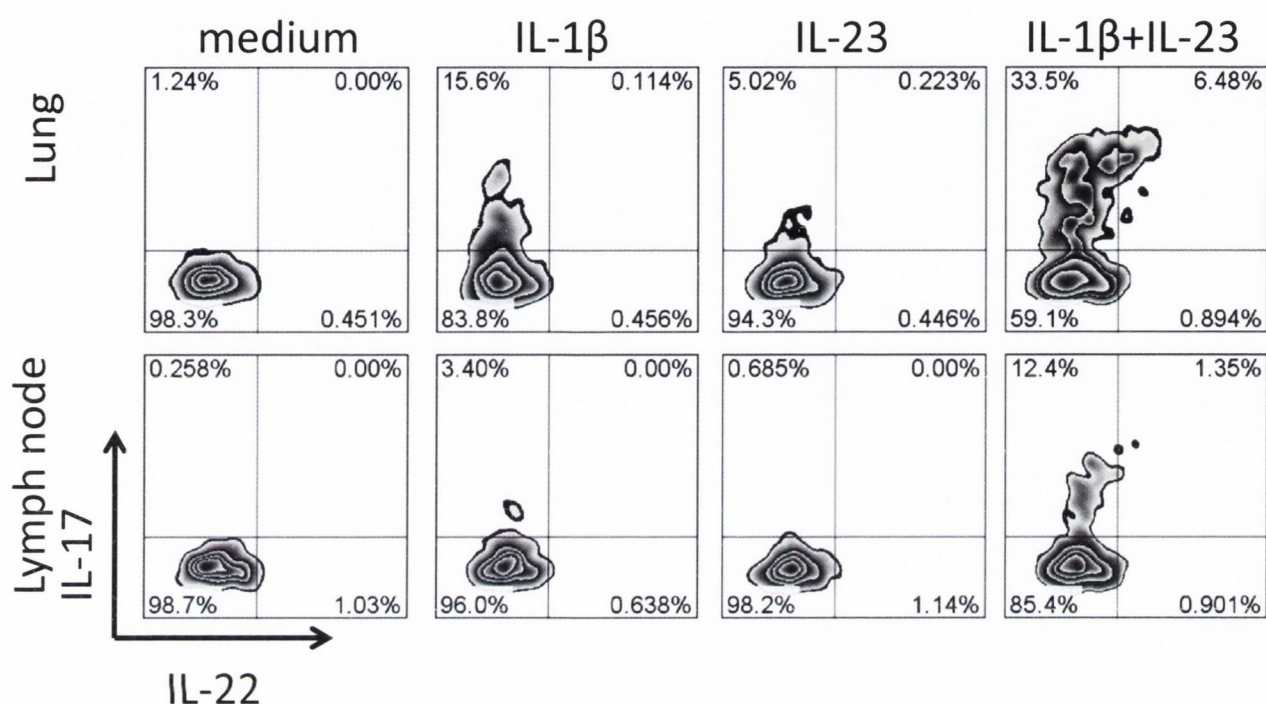


Figure 3.17 IL-17 and IL-22 production by lung $\gamma\delta$ T cells in response to stimulation with IL-1 β and IL-23 alone or in combination.

Lung and lymph node cells ($1 \times 10^6/\text{mL}$) were stimulated overnight with medium, IL-1 β (10 ng/mL), IL-23 (10 ng/mL) or IL-1 β +IL-23 in the presence of BrfA (5 $\mu\text{g/mL}$). The expression of IL-17 and IL-22 by TCR δ^+ cells was then analysed by FACS. The plots show percentages of cytokine producing cells in the TCR δ^+ gate. $n \geq 3$

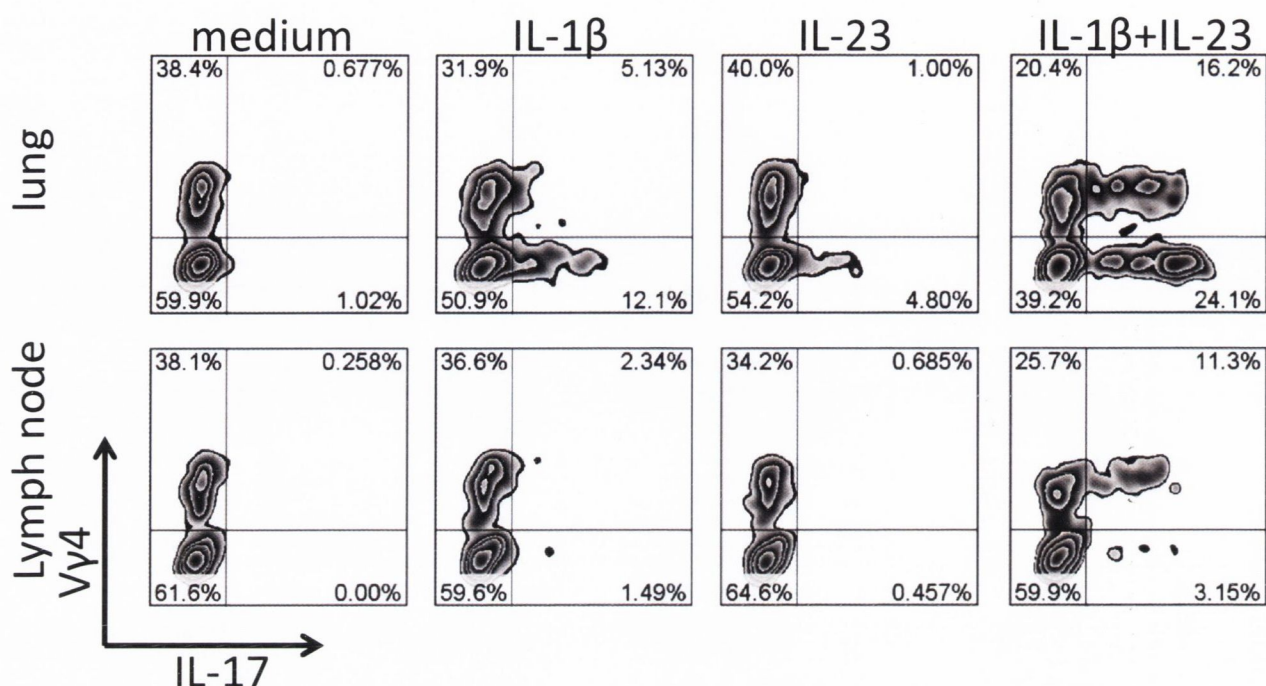


Figure 3.18 IL-1 β and IL-23 induced IL-17 is produced by both V γ 4⁺ and other V γ subset of $\gamma\delta$ T cells in the lung.

Lung and lymph node cells from C57BL/6 mice (1×10^6 /mL) were stimulated overnight with IL-1 β , IL-23 or IL-1 β +IL-23 in the presence of BrfA (5 μ g/mL). The expression of IL-17 by V γ 4⁺ $\gamma\delta$ T cells was then analysed by FACS. The plots show percentages of cells in TCR δ ⁺ gate. $n \geq 3$

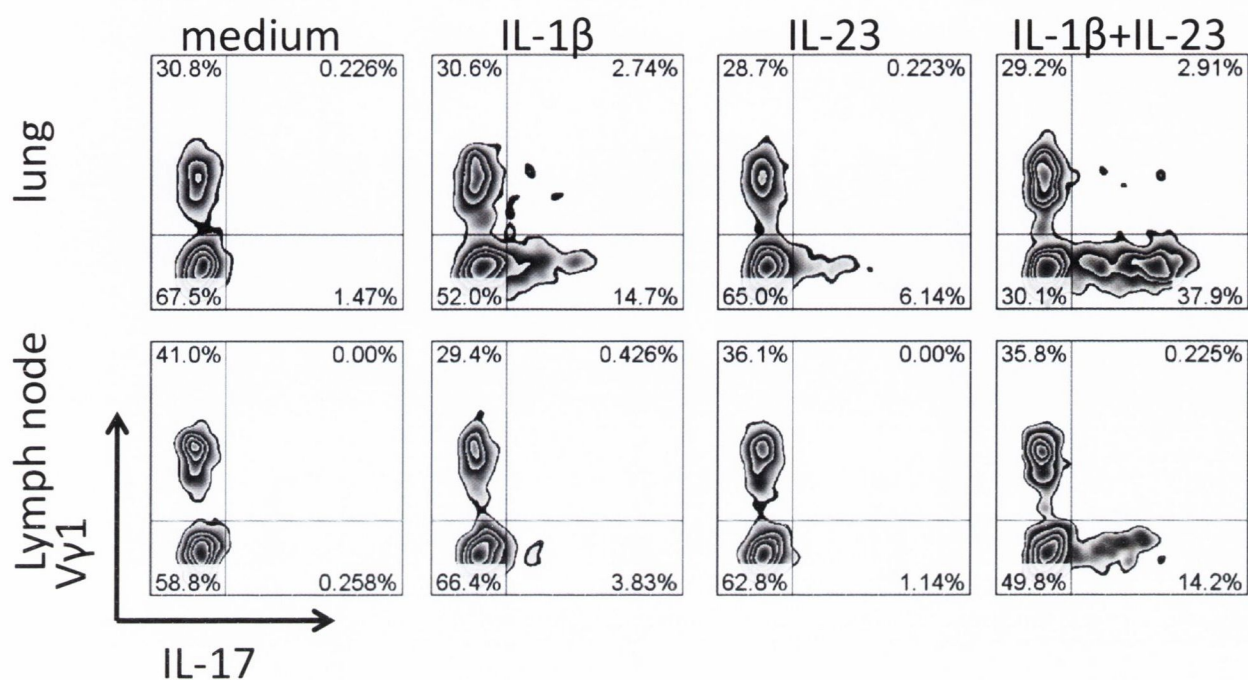


Figure 3.19 Lung Vγ1⁺ γδ T cells do not produce IL-17 in response to IL-1 β and IL-23 stimulation.

Lung and lymph node cells from C57BL/6 mice (1×10^6 /mL) were stimulated overnight with IL-1β, IL-23 or IL-1β+IL-23 in the presence of BrfA (5 μg/mL). The expression of IL-17 by Vγ1⁺ γδ T cells was then analysed by FACS. The plots show percentages of cells in TCRδ⁺ gate. $n \geq 3$

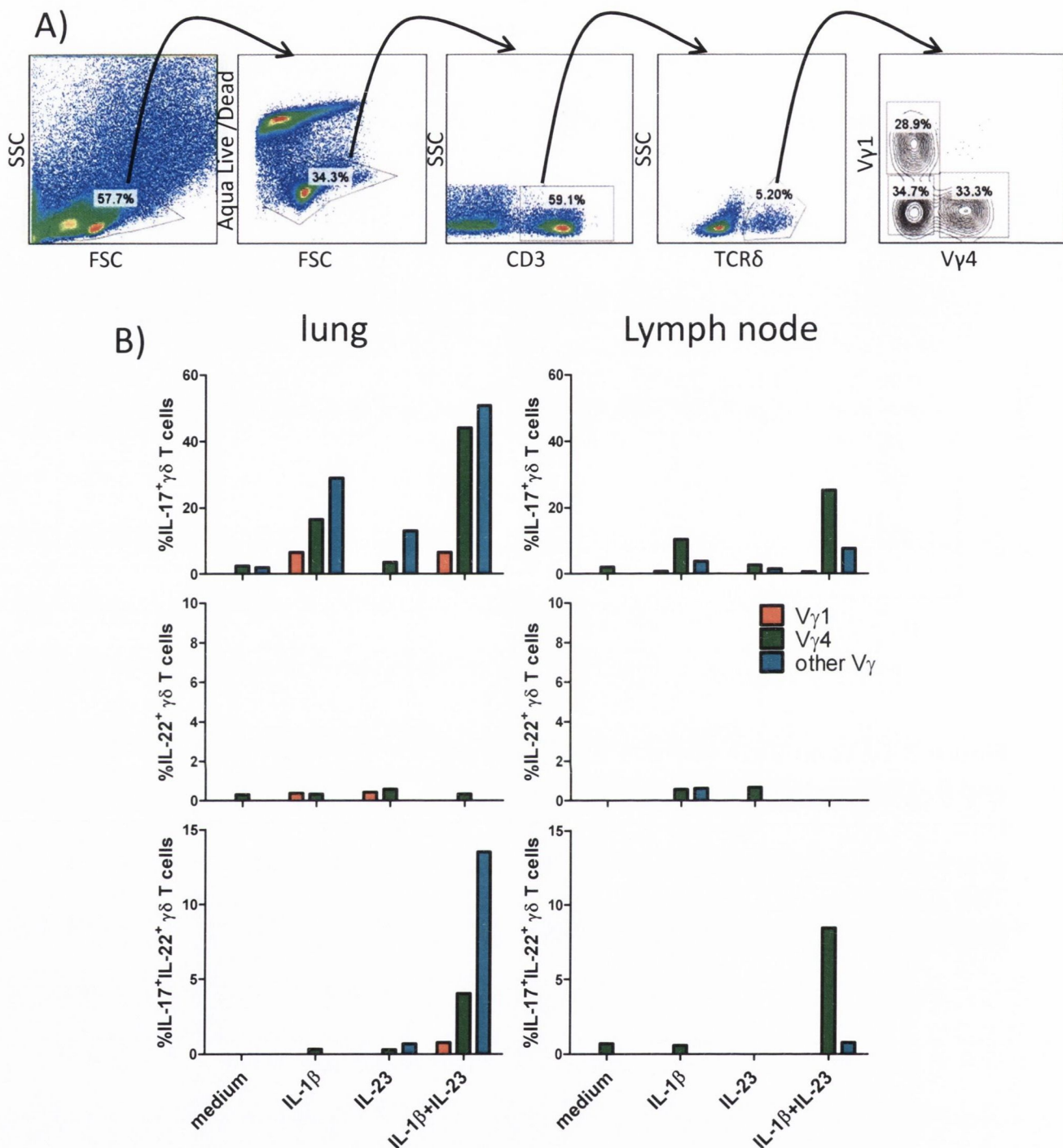


Figure 3.20 IL-17 is produced by several V γ subsets in the lung

Lung and lymph node cells from C57BL/6 mice were stimulated with medium, IL-1 β , IL-23 or IL-1 β +IL-23 in the presence of BrfA (5 μ g/mL). After 24h, the production of IL-17 and IL-22 by $\gamma\delta$ T cell subsets was analysed by flow cytometry. (A) Gating strategy. (B) Percentages of V γ subsets single or double positive for IL-17 and IL-22. n $\geq$ 3

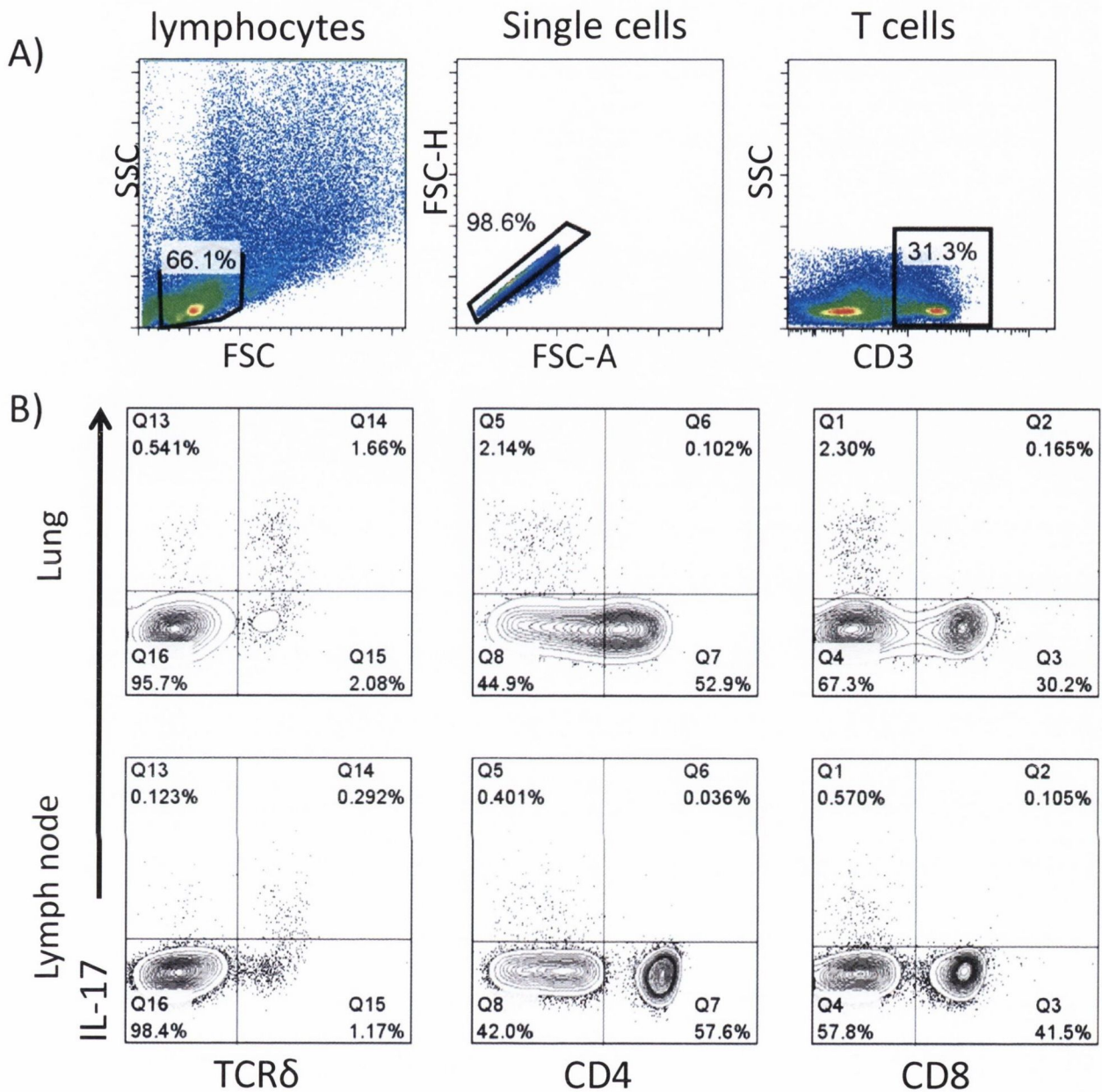


Figure 3.21 $\gamma\delta$ T cells as well as other $CD3^+CD4^-CD8^-TCR\delta^-$ cells produce IL-17 in response to IL-1 β and IL-23 stimulation.

Lung and lymph node cells from C57BL/6 mice (1×10^6 /mL) were stimulated with IL-1 β (10 ng/mL) and IL-23 (10 ng/mL) for 5 hours in the presence of BrfA (5 μ g/mL). The production of IL-17 by $\gamma\delta$ T cells and other $CD3^+$ cells was analysed by flow cytometry. Aqua Live/dead staining was used to gate on live cells. Representative plots show A) gating strategy, B) percentages of cells in the CD3 gate. The data is representative of at least 3 independent experiments. $n \geq 3$

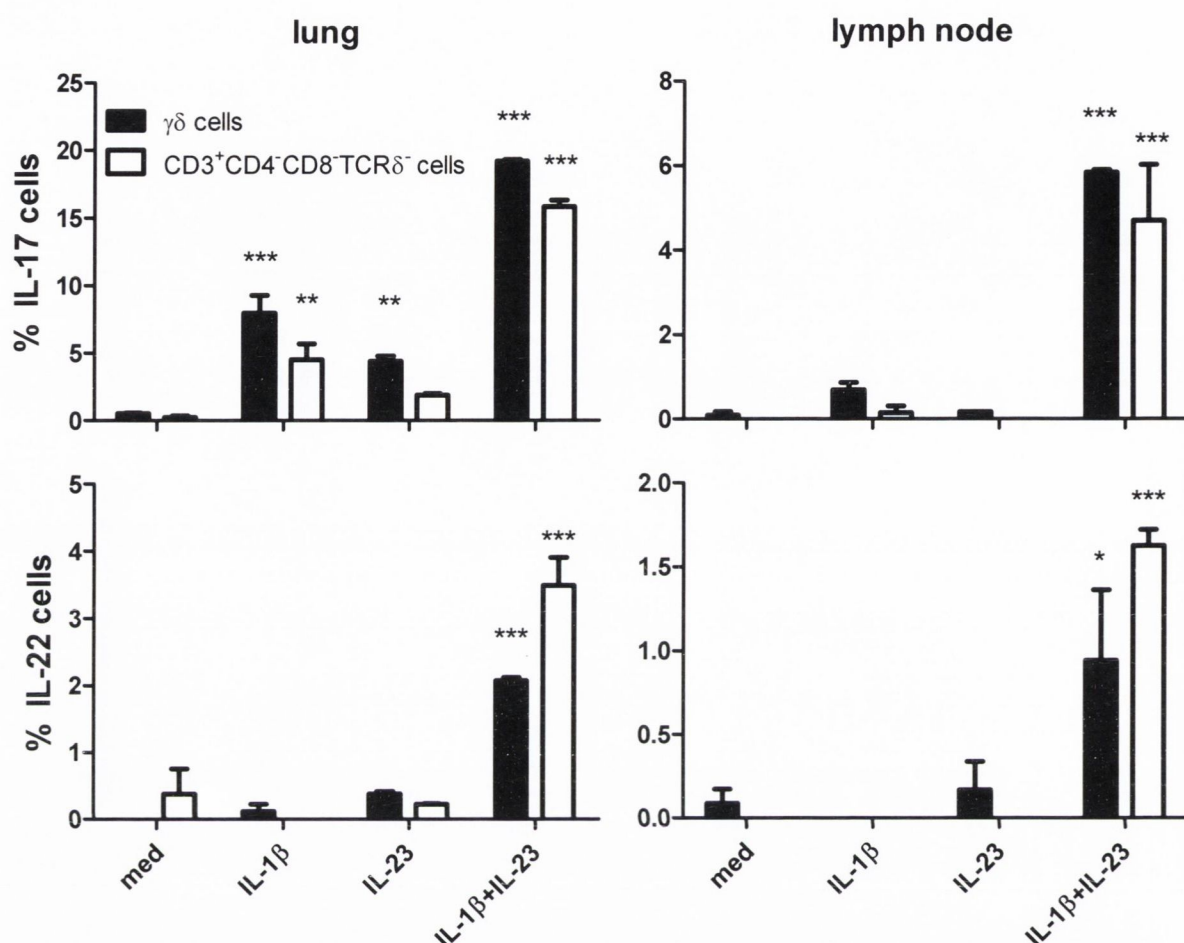


Figure 3.22 $\gamma\delta$ T cells as well as other $CD3^+CD4^-CD8^-TCR\delta^-$ cells produce IL-17 and IL-22 in response to IL-1 β and IL-23 stimulation.

Lung and lymph node cells from C57BL/6 mice (1×10^6 /mL) were incubated with medium, IL-1 β (10 ng/mL), IL-23 (10 ng/mL) and IL-1 β +IL-23 for 5 hours in the presence of BrfA (5 μ g/mL). The production of IL-17 and IL-22 by $\gamma\delta$ T cells and $CD3^+CD4^-CD8^-TCR\delta^-$ cells was analysed by flow cytometry. Aqua Live/dead staining was used to gate on the live cells. Graphs show percentages of IL-17 positive cells in the $\gamma\delta$ gate and $CD3^+CD4^-CD8^-TCR\delta^-$ gate. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by two way ANOVA with Bonferroni post test. $n \geq 3$

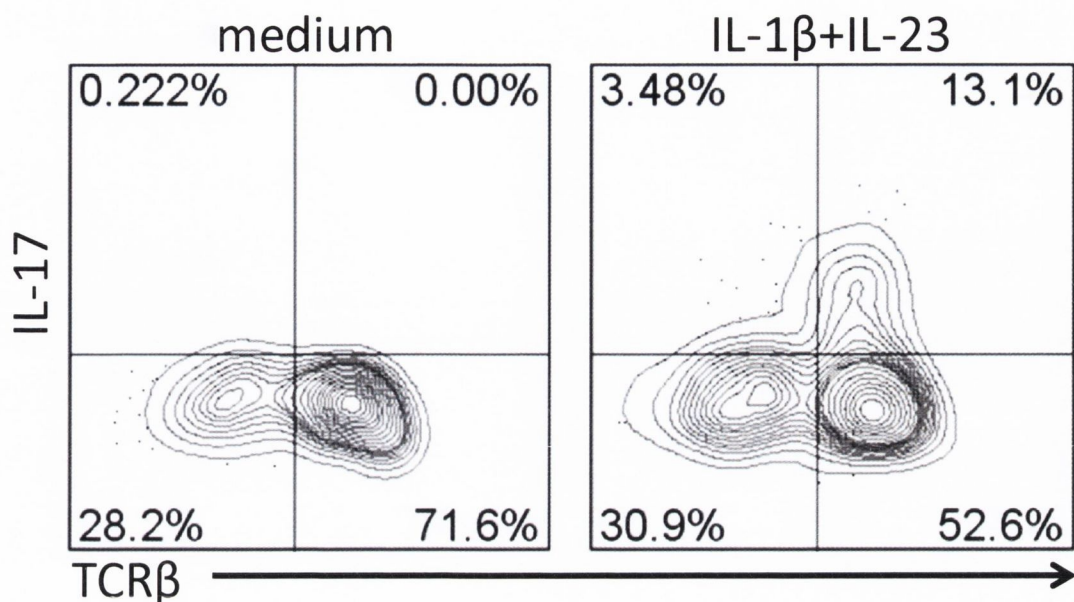


Figure 3.23 IL-17 is produced by CD3⁺CD4⁻CD8⁻TCR δ ⁻TCR β ⁺ cell population in the lung.

Lung cells from C57BL/6 mice (1×10^6 /mL) were stimulated with IL-1 β (10 ng/mL) and IL-23 (10 ng/mL) for 5 hours in the presence of BrfA (5 μ g/mL). The production of IL-17 by CD3⁺CD4⁻CD8⁻TCR δ ⁻ cells was analysed by flow cytometry. Aqua Live/dead staining was used to gate on live cells. Representative plots show percentages of cells in the CD3⁺CD4⁻CD8⁻TCR δ ⁻ gate. $n \geq 3$

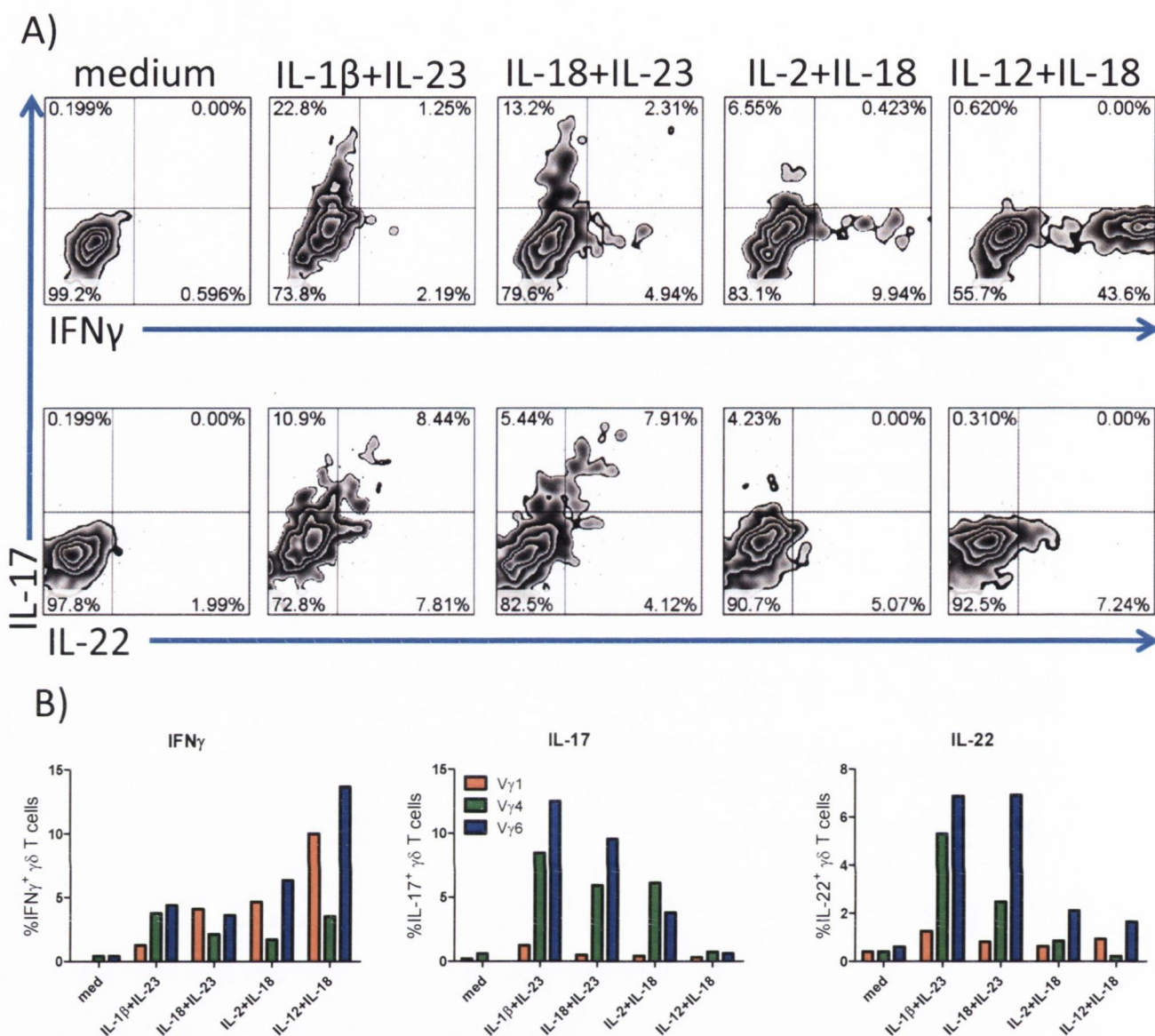


Figure 3.24 Lung $\gamma\delta$ T cells produce IL-17, IFN γ and IL-22 in response to stimulation with different cytokines combinations *in vitro*.

Lung cells from C57BL/6 mice (1×10^6 /mL) were incubated with medium, IL-1 β (10 ng/mL)+IL-23 (10 ng/mL), IL-18 (10 ng/mL)+IL-23, IL-2 (20 U/mL)+IL-18 and IL-12(10 ng/mL)+IL-18 for 5 hours in the presence of BrfA (5 μ g/mL). The production of IL-17, IFN γ and IL-22 by $\gamma\delta$ T cells was analysed by flow cytometry. Aqua Live/dead staining was used to gate on the live cells. Graphs show percentages of cytokine positive cells in the (A) $\gamma\delta$ T cell gate and (B) V γ 6, V γ 4 and V γ 1 gate. n=1

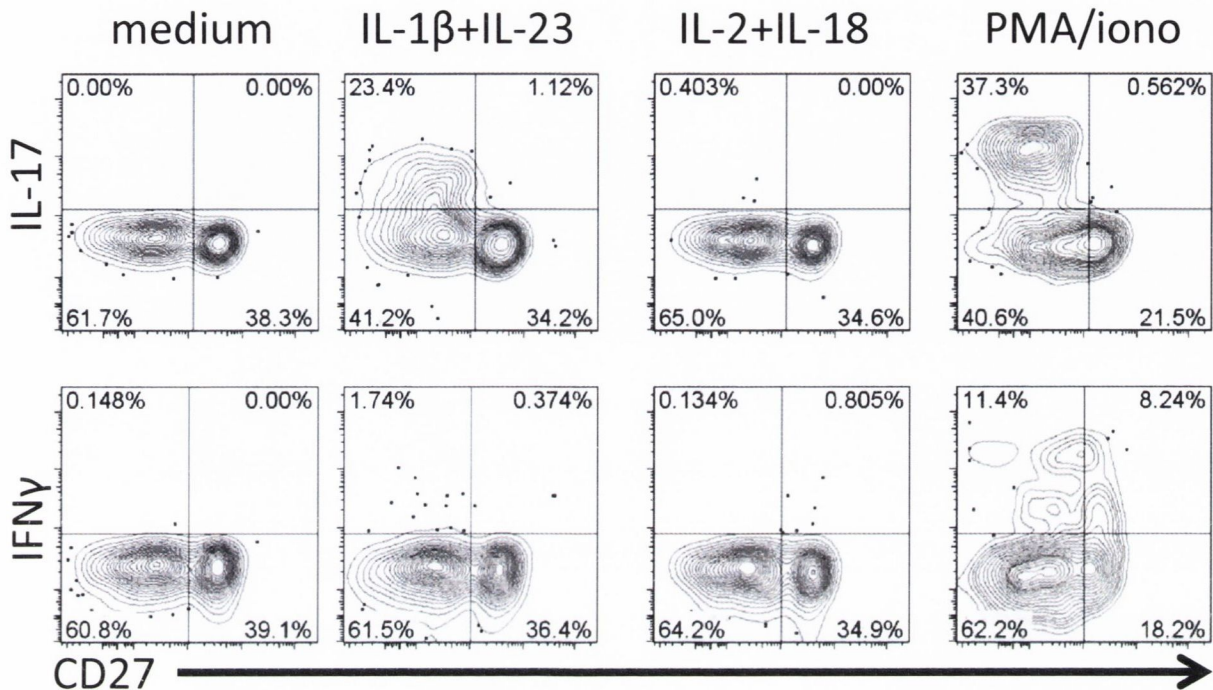


Figure 3.25 IL-17 and IFN γ production by CD27⁺ and CD27⁻ $\gamma\delta$ T cells in the lung.

Lung cells from C57BL/6 mice (1×10^6 /mL) were incubated with medium, IL-1 β (10 ng/mL)+IL-23 (10 ng/mL), IL-2 (20 U/mL) + IL-18 (10 ng/mL) or PMA (10 ng/mL) + ionomycin (500 ng/mL) for 5 hours in the presence of BrfA (5 μ g/mL). The production of IL-17 and IFN γ by $\gamma\delta$ T cells was analysed by flow cytometry. Aqua Live/dead staining was used to gate on the live cells. Graphs show percentages of cytokine positive cells in the $\gamma\delta$ T cell gate. n=1

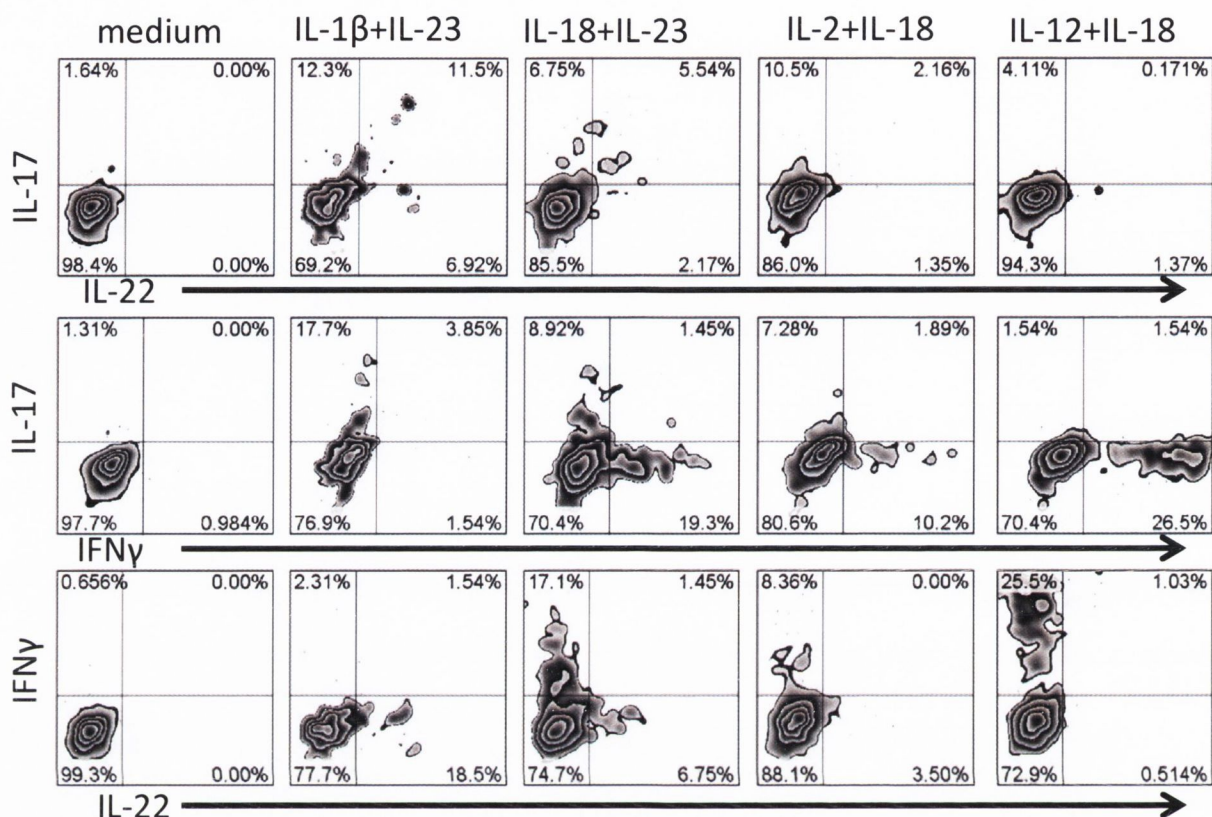


Figure 3.26 Lung CD3⁺CD4⁻CD8⁻TCR δ ⁻ cells respond to different cytokine combinations to produce IL-17, IL-22 and IFN γ

Lung cells from C57BL/6 mice (1×10^6 /mL) were incubated with medium, IL-1 β (10 ng/mL)+IL-23 (10 ng/mL), IL-18 (10 ng/mL)+IL-23, IL-2 (20 U/mL)+IL-18 and IL-12(10 ng/mL)+IL-18 for 5 hours in the presence of BrfA (5 μ g/mL). The production of IL-17, IFN γ and IL-22 by CD3⁺CD4⁻CD8⁻TCR δ ⁻ cells was analysed by flow cytometry. Aqua Live/dead staining was used to gate on the live cells. Graphs show percentages of cytokine positive cells in the CD3⁺CD4⁻CD8⁻TCR δ ⁻ gate. n=1

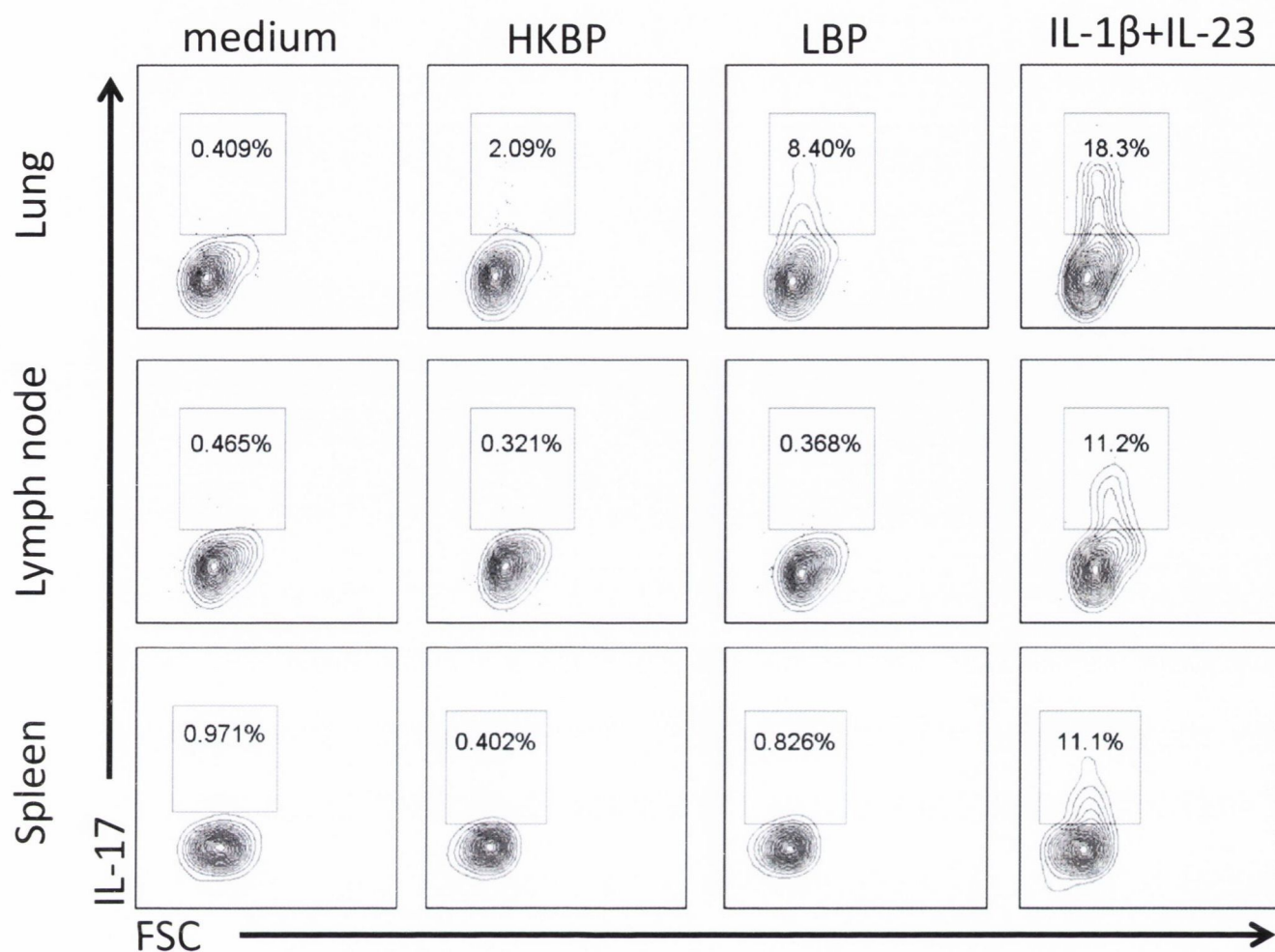


Figure 3.27 Lung but not lymph node or spleen $\gamma\delta$ T cell secrete IL-17 in response to *in vitro* stimulation with *B. pertussis*.

Lung, lymph node and spleen cells ($1 \times 10^6/\text{mL}$) from C57BL/6 mice were incubated with medium, heat killed (HKBP) or live (LBP) *B. pertussis* (MOI:1) or with IL-1 β (10 ng/mL)+ IL-23 (10 ng/mL) for 2 hours and then for additional 3 hours in the presence of BrfA (5 $\mu\text{g}/\text{mL}$). The production of IL-17 by $\gamma\delta$ T cells was then analysed by flow cytometry. Aqua Live/dead staining was used to gate on live cells. Representative plots show percentages of IL-17 secreting cells in the TCR δ^+ gate. $n \geq 3$

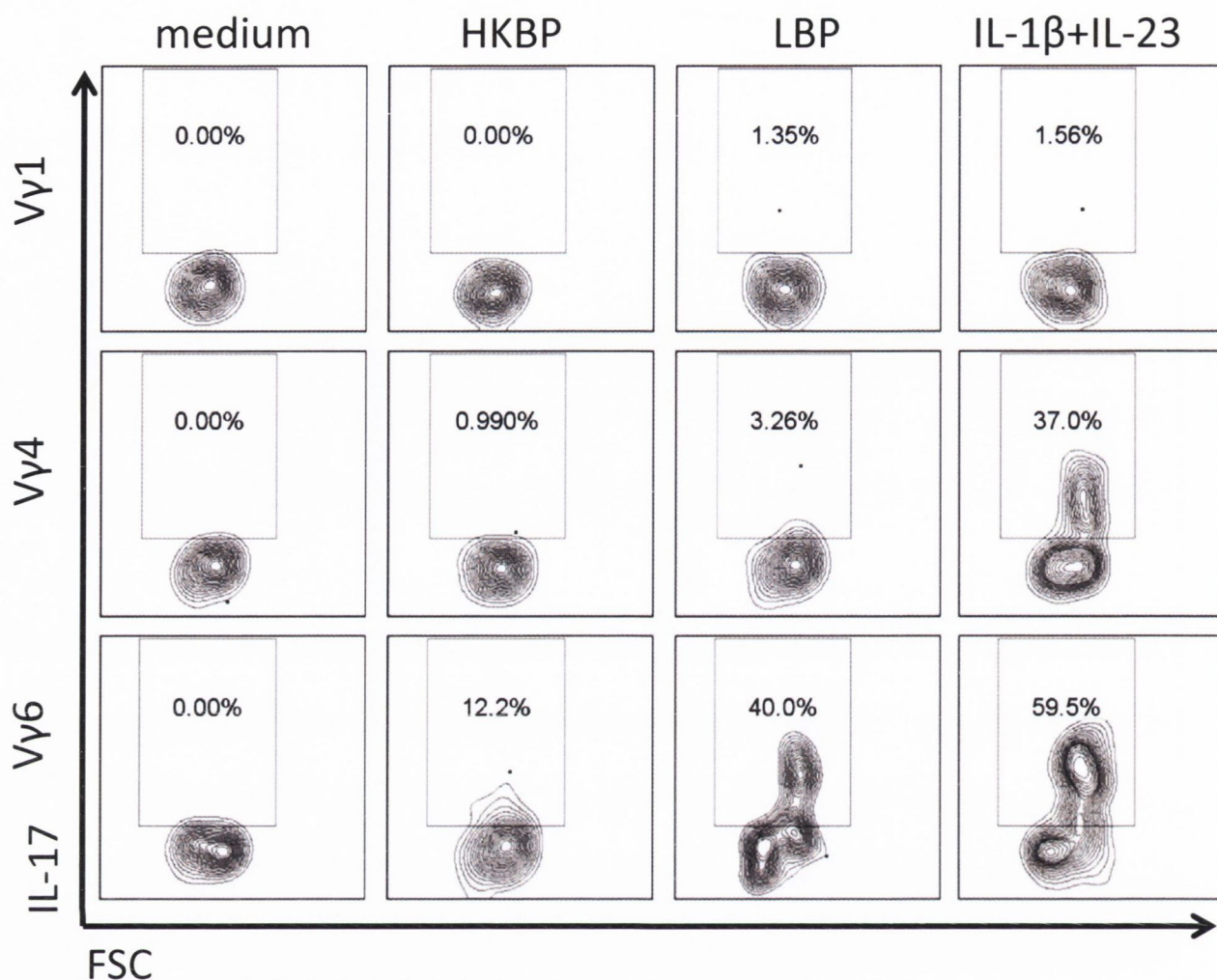


Figure 3.28 Vγ6⁺ and not Vγ4⁺ subset of lung γδ T cell responds strongly to *in vitro* stimulation with live *B. pertussis*.

Lung cells from C57BL/6 mice (1×10^6 /mL) were stimulated with medium, heat killed (HKBP) or live (LBP) *B. pertussis* (MOI:1) or with IL-1β (10 ng/mL)+IL-23(10 ng/mL) for 2 hours and then for additional 3 hours in the presence of Brefeldin A (5 μg/mL). The production of IL-17 by Vγ4, Vγ1 and Vγ6 subsets of γδ T cells was analysed by flow cytometry. Aqua Live/dead staining was used to gate on live cells. Representative plots show percentages of IL-17 secreting cells in TCRγδ⁺Vγ4⁺, TCRγδ⁺Vγ1⁺ and TCRγδ⁺Vγ6⁺ (Vγ4⁻Vγ1⁻) gates. n≥3

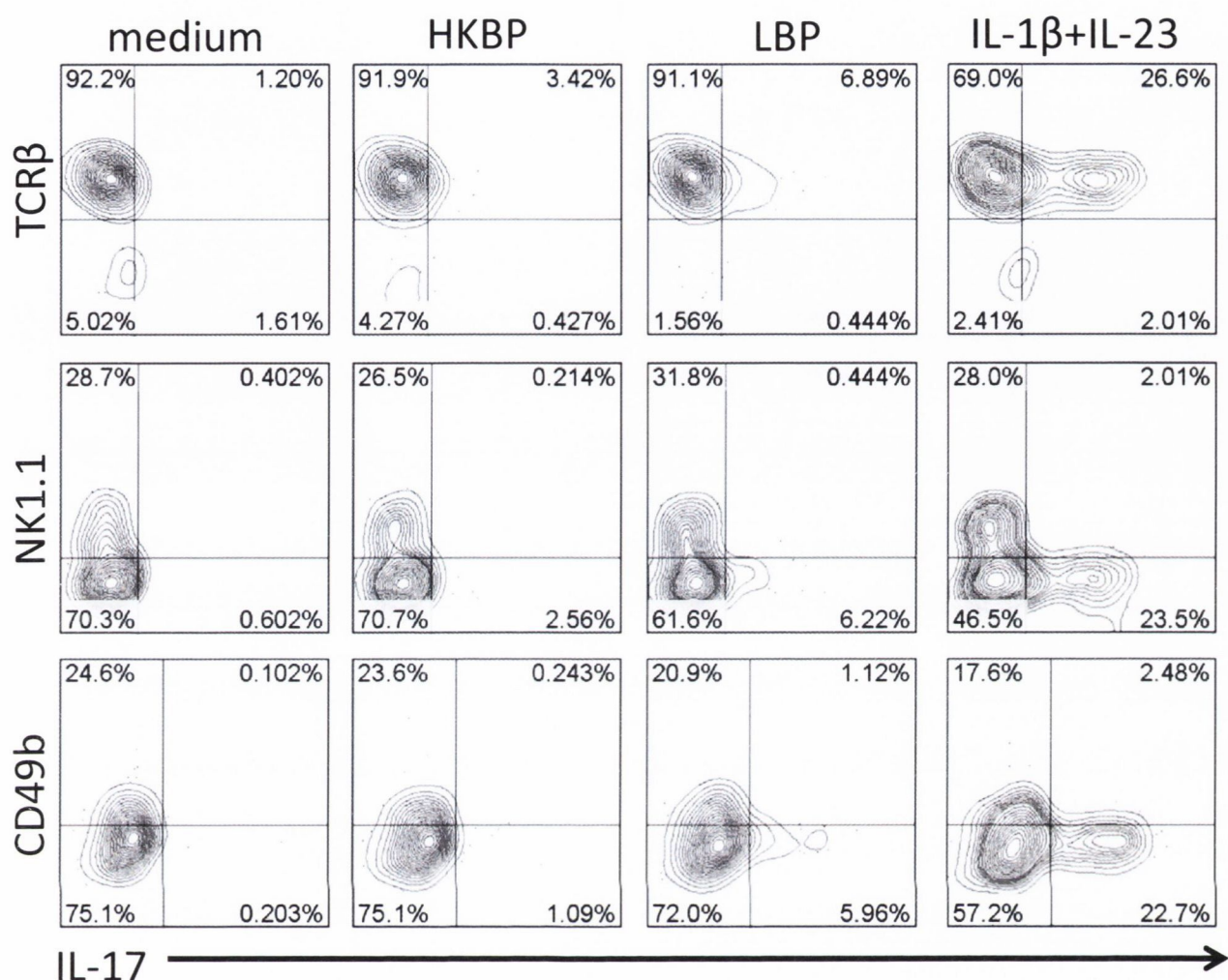


Figure 3.29 IL-17 is produced by TCRβ⁺CD4⁻CD8⁻NK1.1⁻CD49b⁻ cells in the lung. Lung cells (1x10⁶/mL) from C57BL/6 mice were incubated with medium, heat killed (HKBP) or live (LBP) *B. pertussis* (MOI:1) or with IL-1β (10 ng/mL)+IL-23 (10 ng/mL) for 2 hours and then for additional 3 hours in the presence of BrfA (5 μg/mL). The production of IL-17 by CD3⁺CD4⁻CD8⁻TCRδ⁻ cells was then analysed by flow cytometry. Aqua Live/dead staining was used to gate on the live cells. Representative plots show percentages of IL-17 secreting cells in CD3⁺CD4⁻CD8⁻TCRδ⁻ gate. n≥3

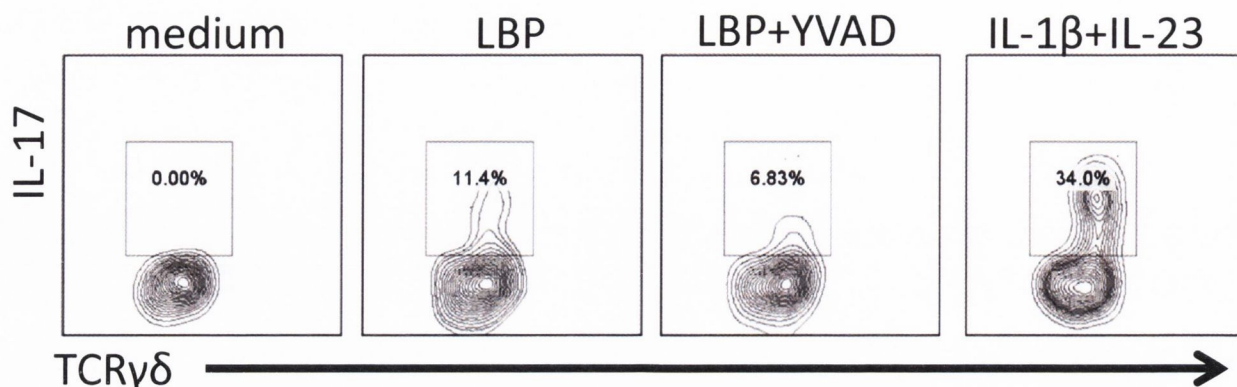


Figure 3.30 IL-17 production by lung $\gamma\delta$ T cells in response to *in vitro* stimulation with *B. pertussis* is partially dependent on caspase-1 signalling.

Lung cells from C57BL/6 mice ($1 \times 10^6/\text{mL}$) were incubated with live *B. pertussis* (LBP; MOI:1) in the presence or absence of caspase 1 inhibitor YVAD-fmk (50 μM), IL-1 β (10 ng/mL)+IL-23 (10 ng/mL) or with medium alone for 2 hours and then for additional 3 hours in the presence of BrfA (5 $\mu\text{g}/\text{mL}$). The production of IL-17 by $\gamma\delta$ T cells was analysed by flow cytometry. Aqua Live/dead staining was used to gate on live cells. Plots show percentages of IL-17 secreting cells in TCR δ^+ gate. n=1

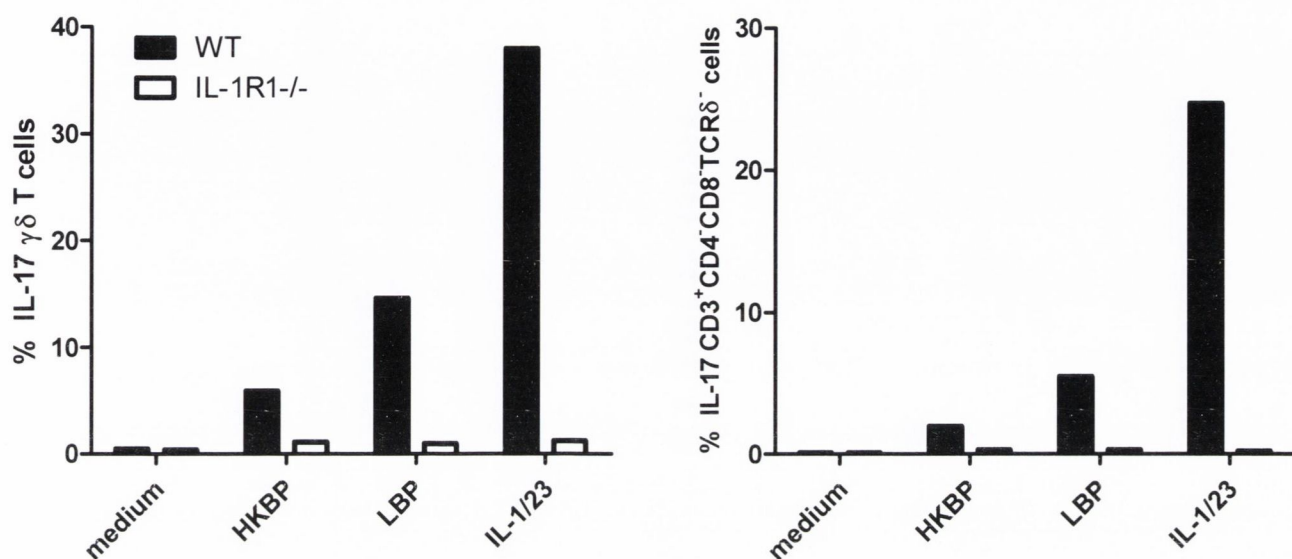


Figure 3.31 Production of IL-17 by lung $\gamma\delta$ T cells and CD3⁺CD4⁻CD8⁻TCR δ ⁻ cells in response to *in vitro* stimulation with *B. pertussis* is dependent on IL-1RI signalling.

Lung cells (1×10^6 /mL) from C57BL/6 and IL-1R1^{-/-} mice were incubated with medium, heat killed (HKBP) or live (LBP) *B. pertussis* at an MOI of 1 or with IL-1 β (10 ng/mL)+IL-23 (10 ng/mL) for 2 hours and then for additional 3 hours in the presence of Brefeldin A (5 μ g/mL). The production of IL-17 by $\gamma\delta$ T cells was analysed by flow cytometry. Aqua live/dead staining was used to gate on live cells. Graphs show percentages of IL-17 producing cells in TCR δ ⁺ and CD3⁺CD4⁻CD8⁻ gates. n=1

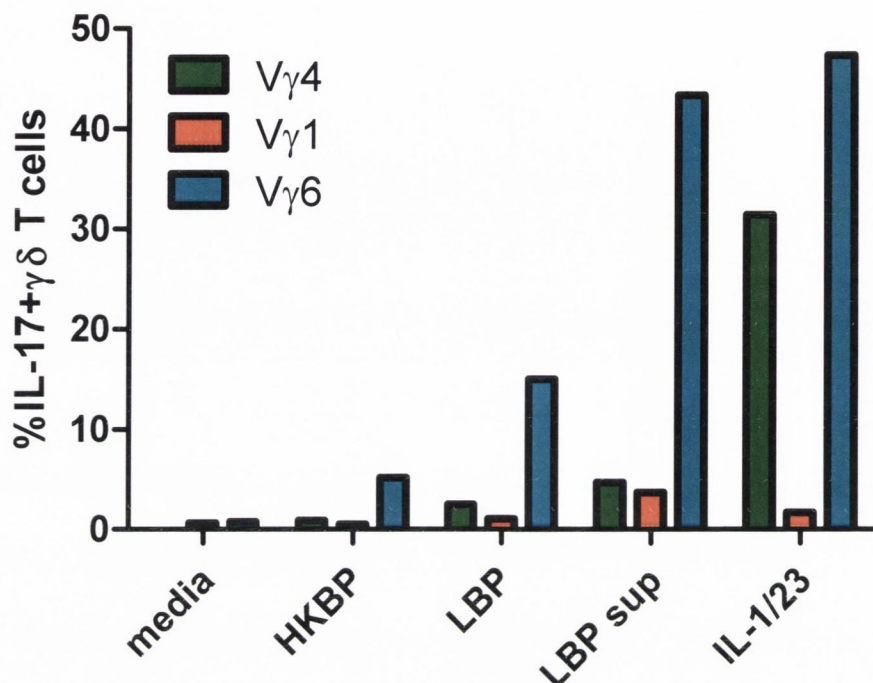


Figure 3.32 Production of IL-17 by subsets of lung $\gamma\delta$ T cells in response to *in vitro* stimulation with heat killed and live *B. pertussis* stimulation

Lung cells ($1 \times 10^6/\text{mL}$) from C57BL/6 mice were incubated with medium, heat killed (HKBP) or live (LBP) *B. pertussis* at an MOI of 1, filter sterilised supernatant from LBP stimulated immortalised bone marrow derived macrophages (IBMM; LBP sup) at a ratio of 1:1, or with IL-1 β (10 ng/mL) and IL-23 (10 ng/mL) as control for 2 hours and then for additional 3 hours in the presence of BrfA (5 $\mu\text{g}/\text{mL}$). The production of IL-17 by different $\gamma\delta$ T cell subsets was analysed by flow cytometry. Aqua Live/dead staining was used to gate on live cells. Graph shows percentages of IL-17 secreting cells in TCR δ^+ gate. n=1

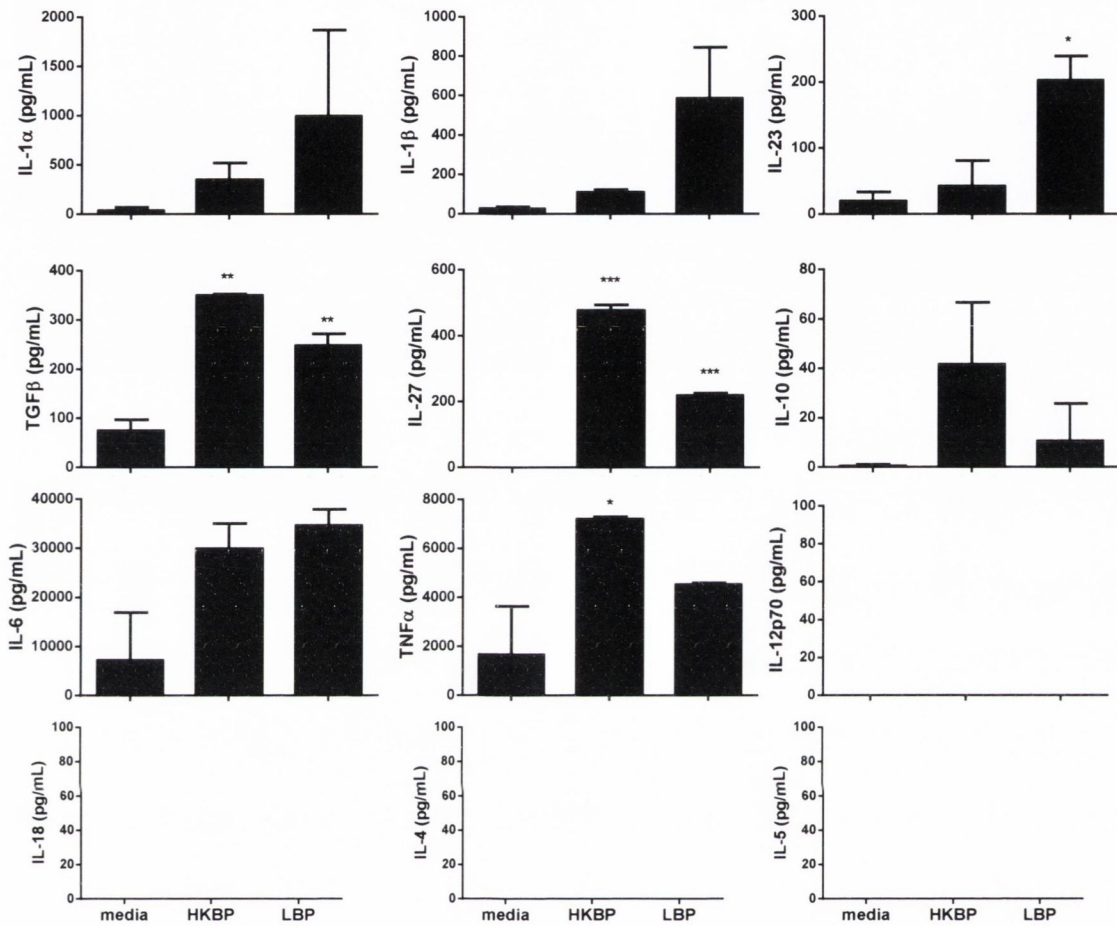


Figure 3.33 IBMM produce proinflammatory cytokines in response to stimulation with live and heat killed *B. pertussis*

Mouse immortalised bone marrow derived macrophages (IBMM; 1×10^6 cells/mL) were incubated with medium or heat killed or live *B. pertussis* (MOI:1). After 24 hours the concentration of IL-1 α , IL-1 β , IL-23, TGF β , IL-27, IL-10, IL-6, TNF α , IL-12p70, IL-18, IL-4 and IL-5 in the supernatants was analysed by ELISA. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by one way ANOVA with Dunnett's post test. $n=2$

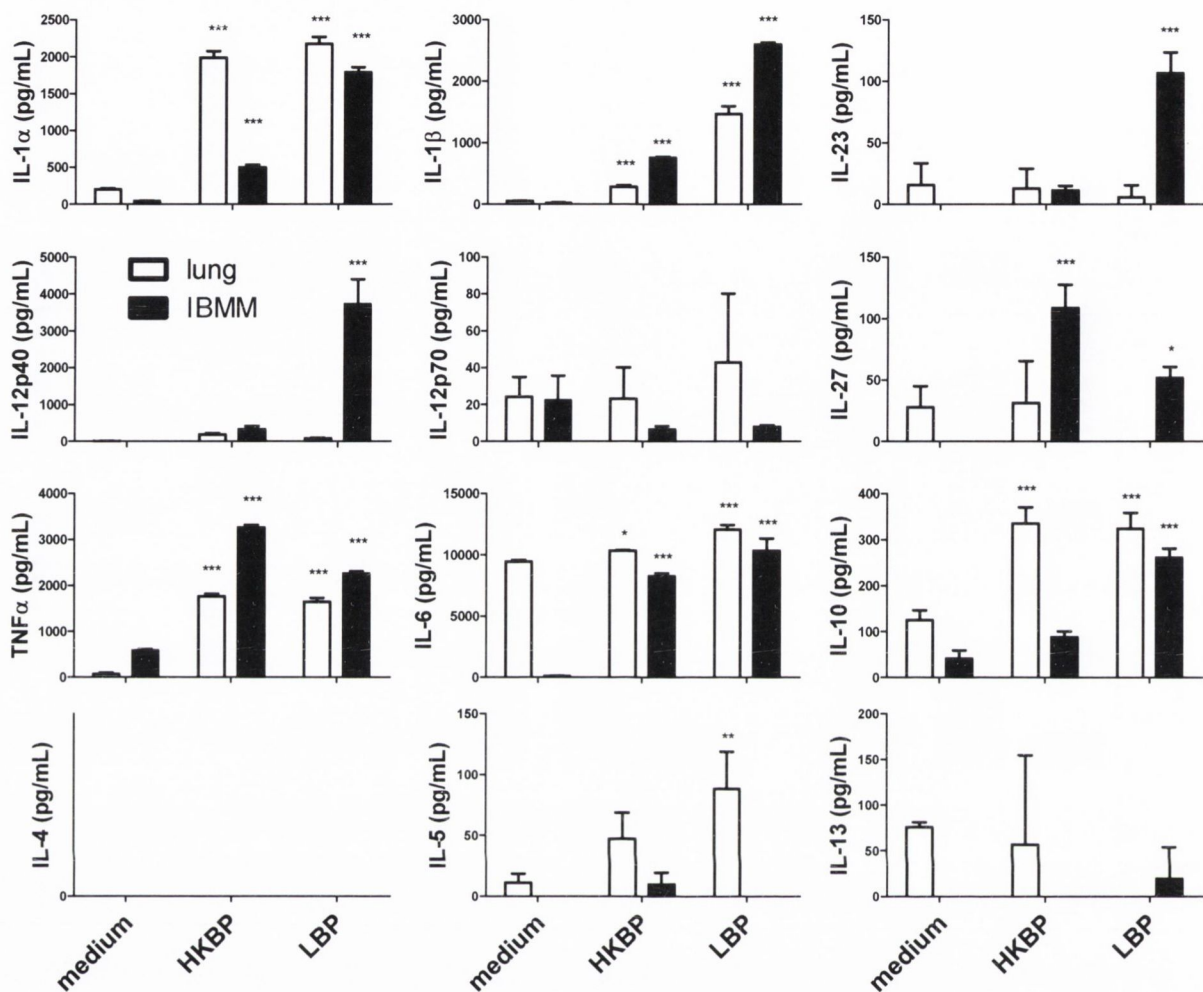


Figure 3.34 Cytokine production by lung mononuclear cells and IBMM in response to *B. pertussis* stimulation.

Lung mononuclear cells from C57BL/6 mice and immortalised bone marrow derived macrophages (IBMM; 1×10^6 cells/mL) were stimulated with heat killed or live *B. pertussis* (MOI:1) or media alone. After 24 hours the concentration of of IL-1α, IL-1β, IL-23, IL-12p40, 12p70, IL-27, IL-10, IL-6, TNFα, IL- IL-13, IL-4 and IL-5 in the supernatants was analysed by ELISA.

*p<0.05, **p<0.01, ***p<0.001 by two way ANOVA with Bonferroni post test. n=2

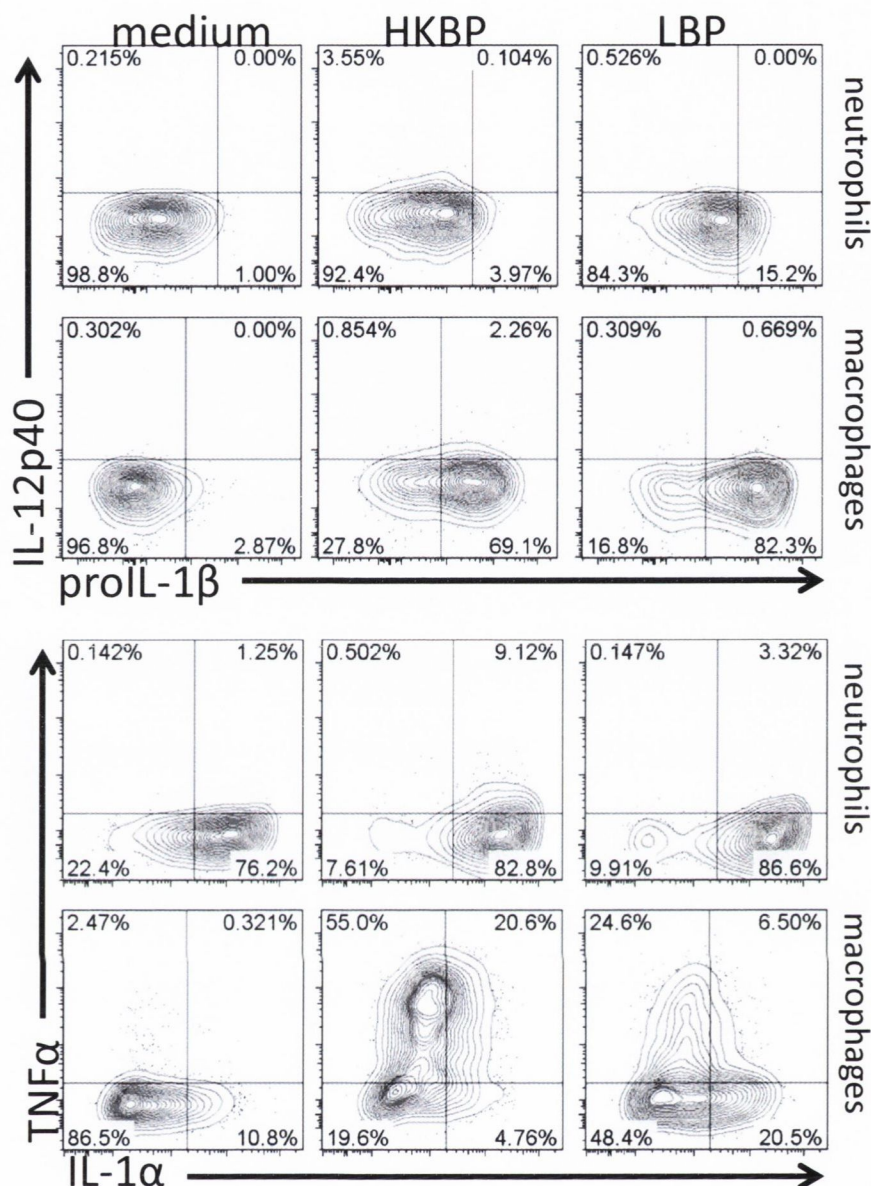
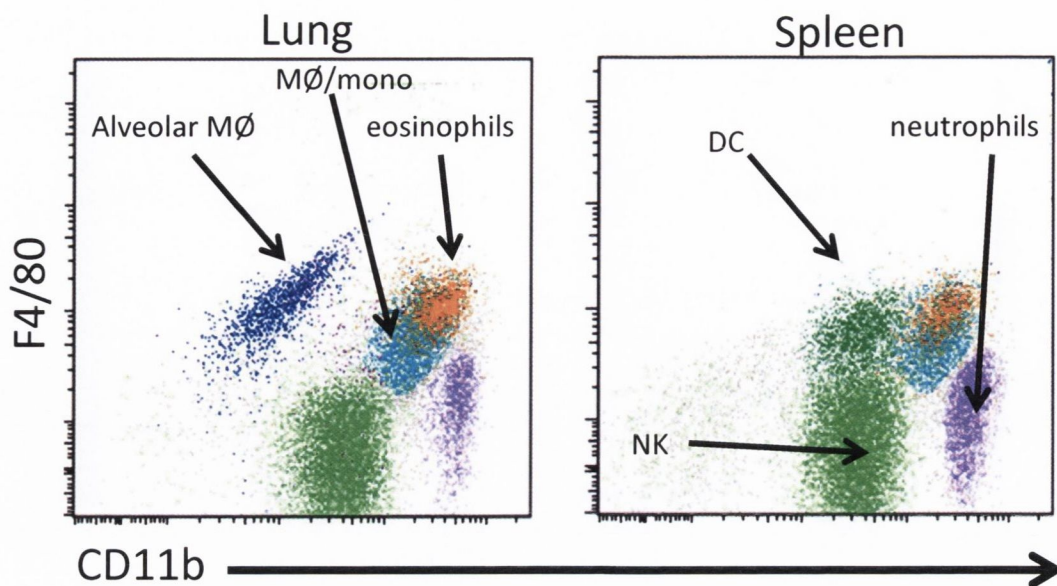


Figure 3.35 Heat killed and live *B. pertussis* differently activate lung neutrophils and macrophages.

Lung mononuclear cells from C57BL/6 mice (1×10^6 cells/mL) were stimulated with heat killed or live *B. pertussis* (MOI:1) or media alone in the presence of BrfA ($5 \mu\text{g/mL}$). After 5 hours the production of IL-1 β , IL-12p40, TNF α and IL-1 α by lung myeloid cells was analysed flow cytometry. Plots show percentages of cytokine positive cells in neutrophil (CD11b $^+$ Ly6G $^+$) and macrophage (CD11b $^+$ F4/80 $^+$) gate. n=1



Population name	Freq in the lung	Freq in the spleen
Macrophages/monocytes	17.9%	13.5%
neutrophils	6.2%	12.5%
eosinophils	7.6%	5.1%
NK cells	33.2%	30.6%
Alveolar MØ	6.6%	-
DC	0.9%	9.7%

Figure 3.36 Cellular composition of purified antigen presenting cell populations from lung and spleen.

Lung and spleen antigen presenting cells have been purified from C57BL/6 mice based on the cell surface CD11b and CD11c expression. The plots and table show percentages of various cell types in the purified populations. n=3

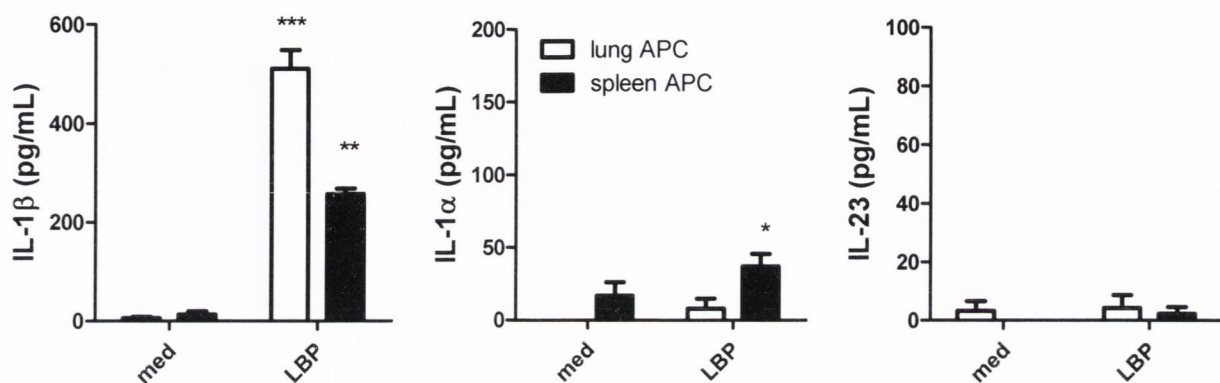


Figure 3.37 Cytokine production by lung and spleen macrophages in response to *B. pertussis* stimulation.

Purified lung and spleen antigen presenting cells (CD11b⁺ and CD11c⁺ cells; 0.5x10⁶ cells/mL) from C57BL/6 mice were incubated with medium or live *B. pertussis* (MOI:1). After 24h, the levels of IL-1 β , IL-1 α in the supernatants were analysed by ELISA. *p<0.05, **p<0.01, ***p<0.001 by two way ANOVA with Bonferroni post test. n=2

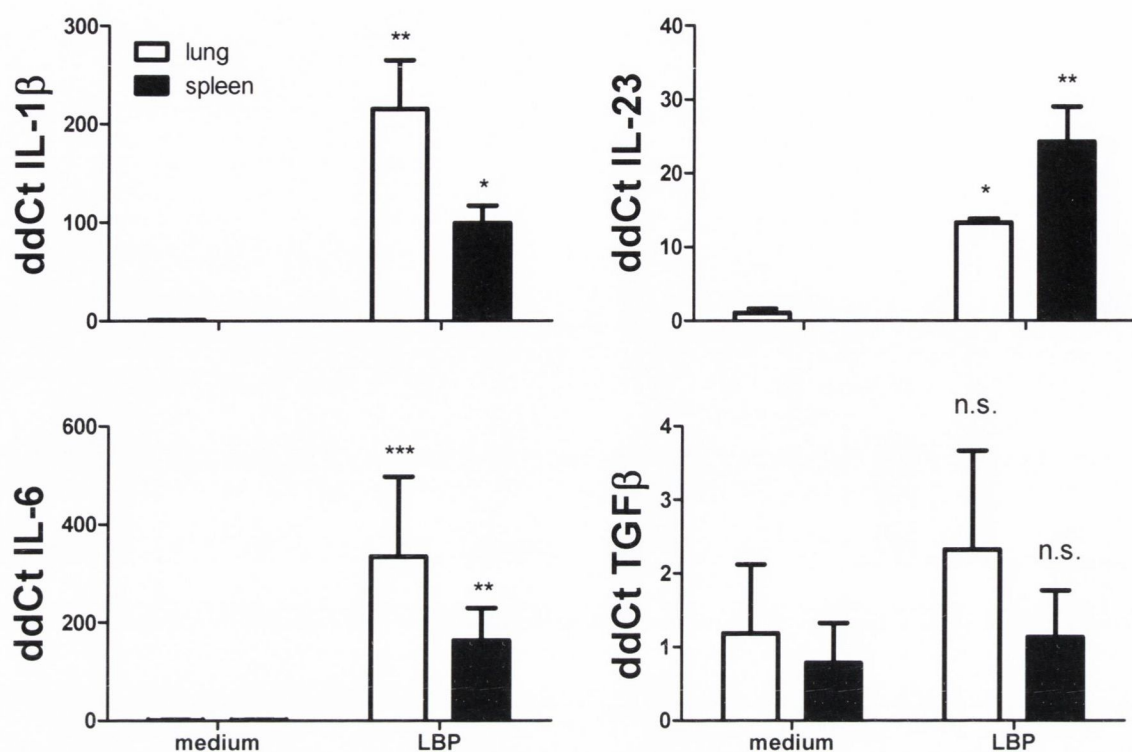


Figure 3.38 Cytokine gene expression by lung and spleen macrophages in response to *B. pertussis* stimulation.

Purified lung and spleen antigen presenting cells (CD11b⁺ and CD11c⁺ cells; 0.5x10⁶ cells/mL) from C57BL/6 mice were incubated with medium or live *B. pertussis* (MOI:1). After 24h the RNA was isolated and the fold increase in expression of IL-1 β , IL-23, IL-6 and TGF β relative to unstimulated cells was analysed by RT-PCR. *p<0.05, **p<0.01, ***p<0.001, n.s. not significant by two way ANOVA with Bonferroni post test. n=2

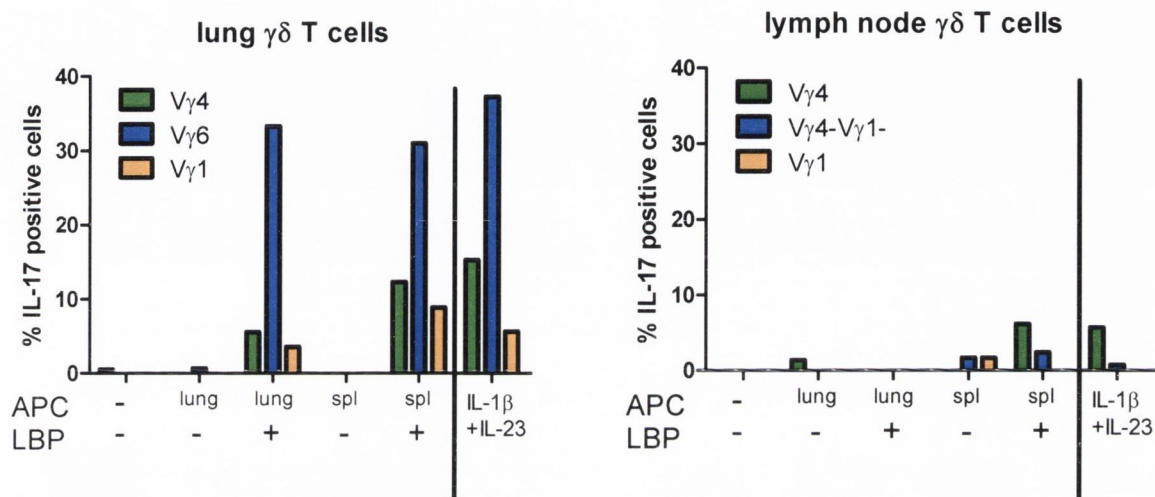


Figure 3.39 Lung $\gamma\delta$ T cells respond more strongly than lymph node $\gamma\delta$ T cells to stimulation with LBP conditioned supernatants.

MACS purified lung and lymph node $\gamma\delta$ T cells from naïve C57BL/6 mice (40×10^3 cells/mL) were incubated in the presence of BrfA ($5 \mu\text{g/mL}$) with sterile filtered supernatants from medium or live *B. pertussis* stimulated (LBP, MOI:1) antigen presenting cells (CD11b $^+$ and CD11c $^+$ cells; 0.5×10^6 cells/mL). After 12h IL-17 production by $\gamma\delta$ T cell subsets was analysed by FACS. Graphs show percentages of IL-17 producing cells in the V γ 4 $^+$, V γ 1 $^+$, and V γ 4 $^-$ V γ 1 $^-$ gate. n=1

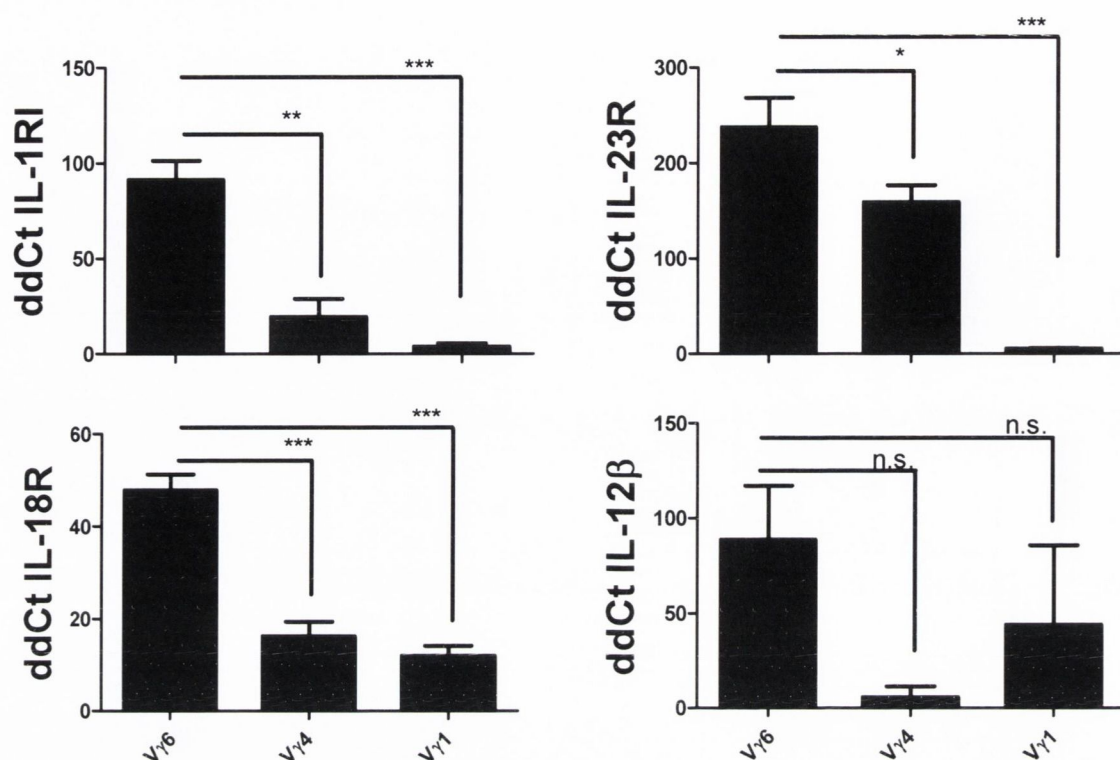


Figure 3.40 Expression of IL-1, IL-23, IL-18 and IL-12 receptor by lung $\gamma\delta$ T cell subsets.

Lung $\gamma\delta$ T cell subsets (100×10^3 cells) were purified on a FACS sorter. RNA was then isolated and the expression of IL-1RI, IL-23R, IL-18R, IL-12R β analysed by RT-PCR relative to unstimulated spleen cells and normalised to 18s RNA. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by one way ANOVA with Tukey post test. $n=1$

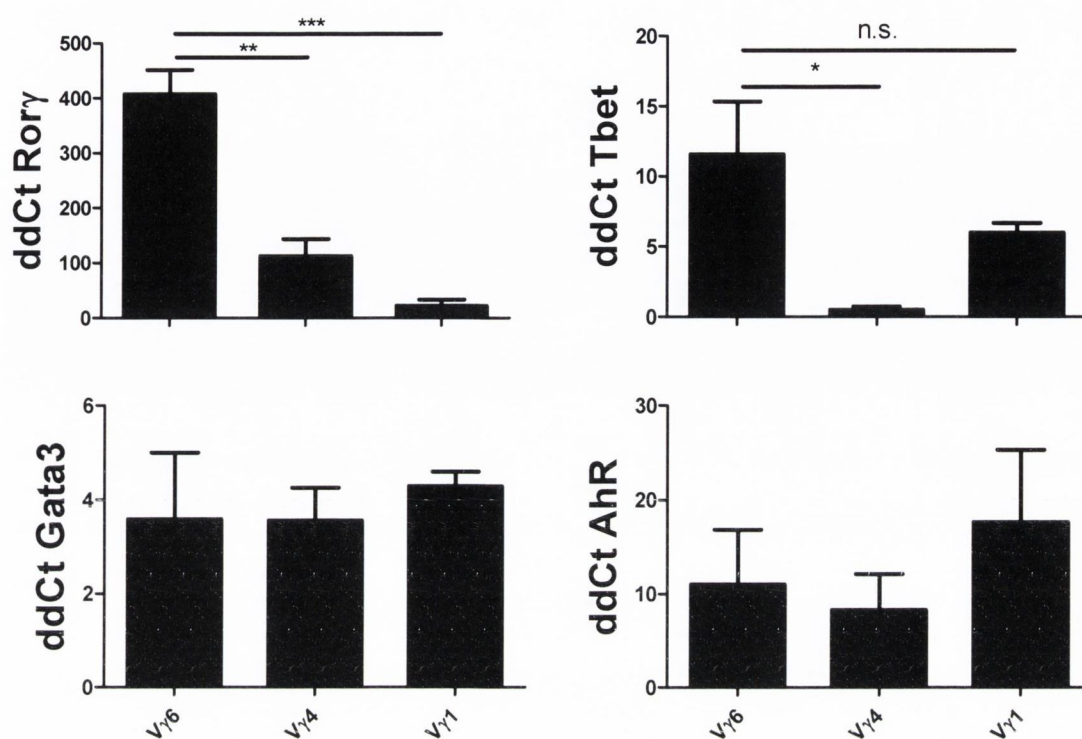


Figure 3.41 Expression of Th lineage transcription factors by lung $\gamma\delta$ T cell subsets.

Lung $\gamma\delta$ T cell subsets (100×10^3 cells) were purified on a FACS sorter. RNA was then isolated and the expression of Roryt, Tbet, Gata3 and AhR analysed by RT-PCR relative to unstimulated spleen cells and normalised to 18s RNA. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ND: not detected by one way ANOVA with Tukey post test. $n = 1$

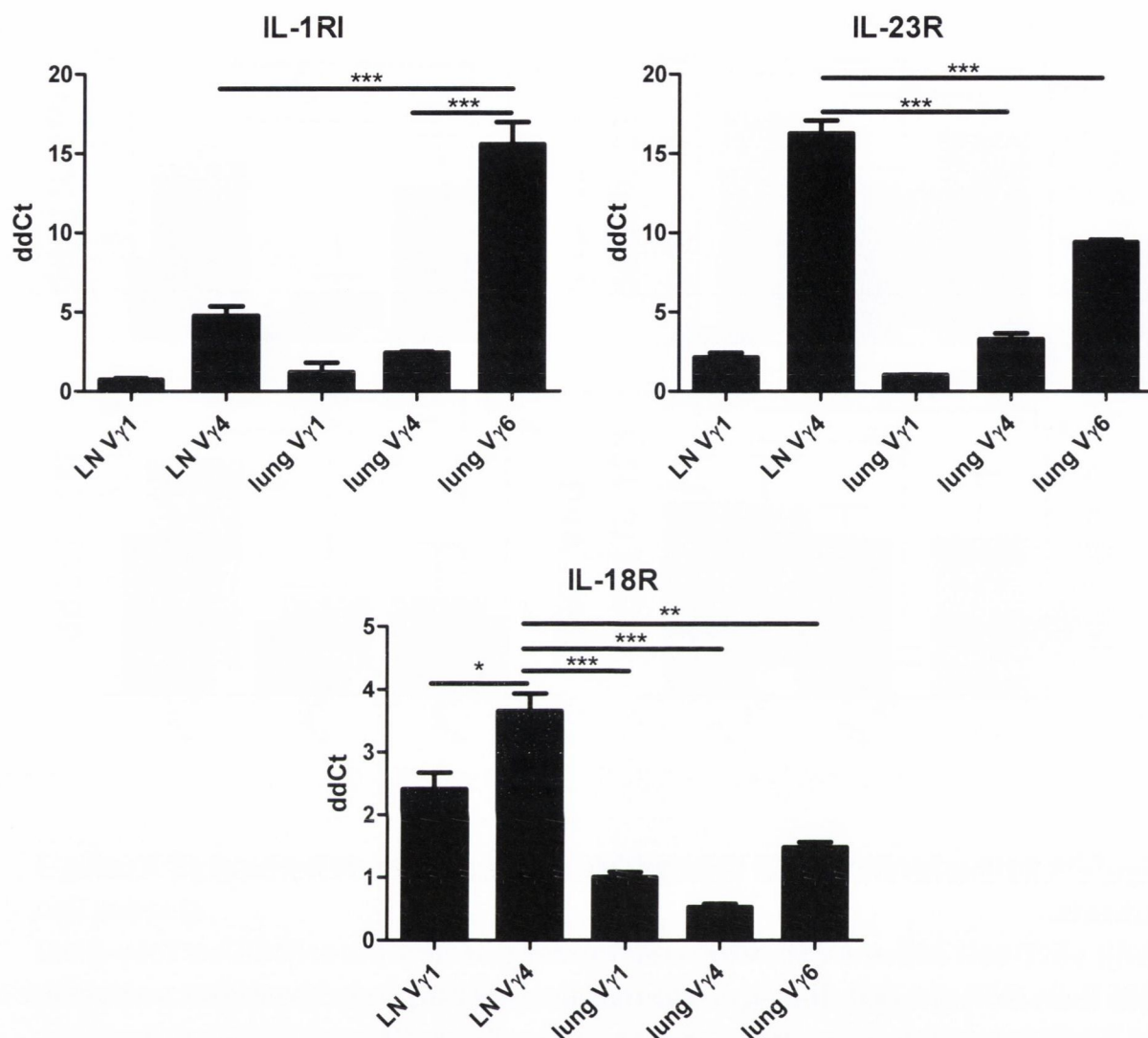


Figure 3.42 Expression of of IL-1, IL-23, and IL-18 receptor by lymph node and lung $\gamma\delta$ T cell subsets.

Lymph node and lung $\gamma\delta$ T cell subsets (100×10^3 cells) were purified on a FACS sorter. RNA was then isolated and the expression of IL-1RI, IL-23R, and IL-18R analysed by RT-PCR, relative to unstimulated lung Vγ1 $\gamma\delta$ T cells and normalised to 18s RNA. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by one way ANOVA with Tukey's post test. $n=1$

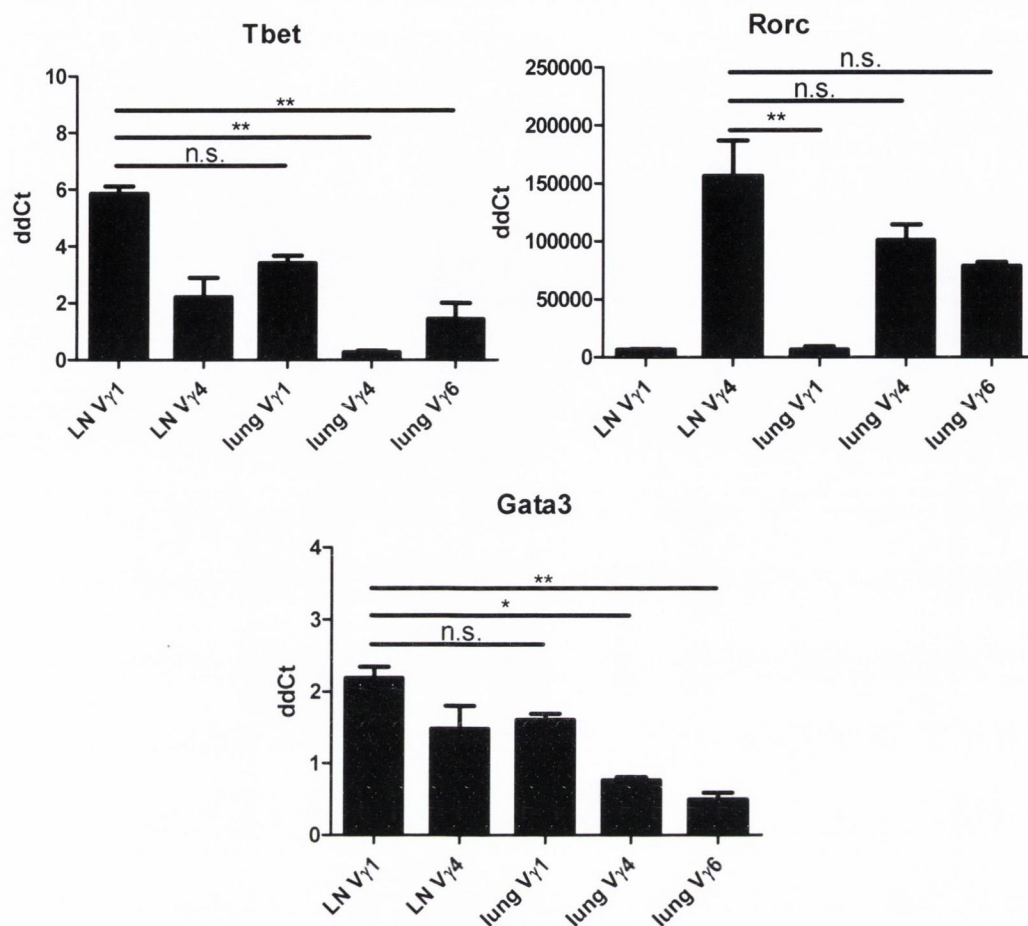


Figure 3.43 Expression of Th lineage transcription factors by lymph node and lung $\gamma\delta$ T cell subsets.

Lymph node and lung $\gamma\delta$ T cell subsets (100×10^3 cells) were purified on a FACS sorter. RNA was then isolated and the expression of Rorc, Tbet, and Gata3 analysed by RT-PCR, relative to unstimulated lung Vγ1 $\gamma\delta$ T cells. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by one way ANOVA with Tukey's post test. n=1

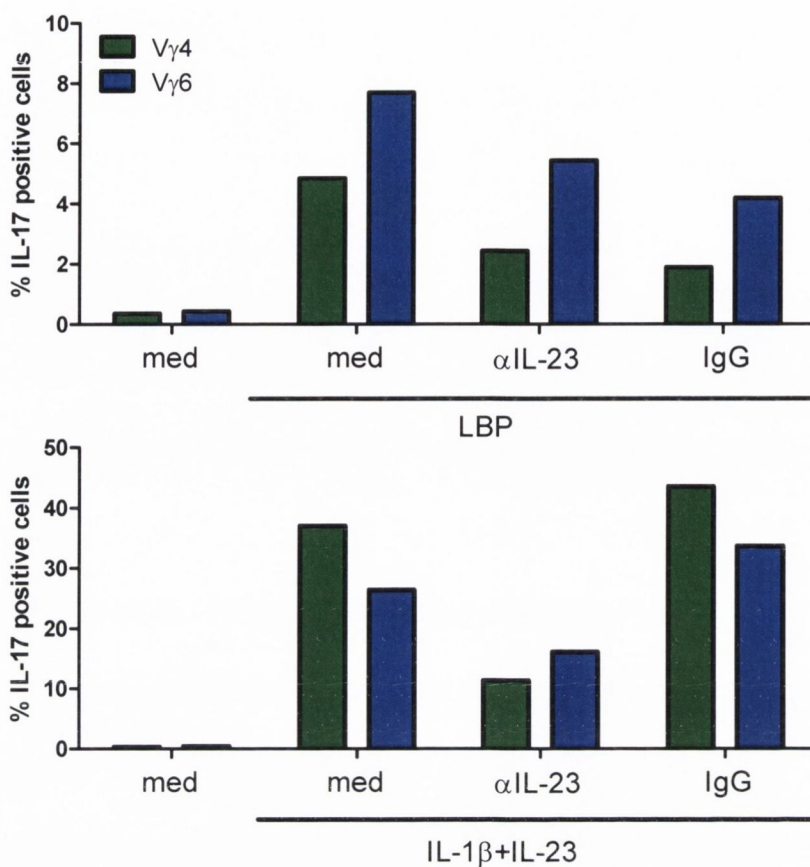


Figure 3.44 LBP-induced IL-17 is not inhibited by neutralisation of IL-23. Lung cells (1×10^6 cells/mL) from C57BL/6 mice were incubated with medium, live *B. pertussis* (LBP; MOI 1) or with IL-1 β (10 ng/mL)+IL-23 (10 ng/mL) +/- α IL-23 (5 μ g/mL), IgG (5 μ g/mL) or IL-1RA(1 μ g/mL) in the presence of BrfA (5 μ g/mL). After 5 hours the production of IL-17 by $\gamma\delta$ T cell subsets was analysed by flow cytometry. Aqua Live/dead staining was used to gate on live cells. Graphs shows percentages of IL-17 secreting cells in V γ 6 $^+$ (V γ 1 $^-$ V γ 4 $^-$) and V γ 4 $^+$ gate. n=1

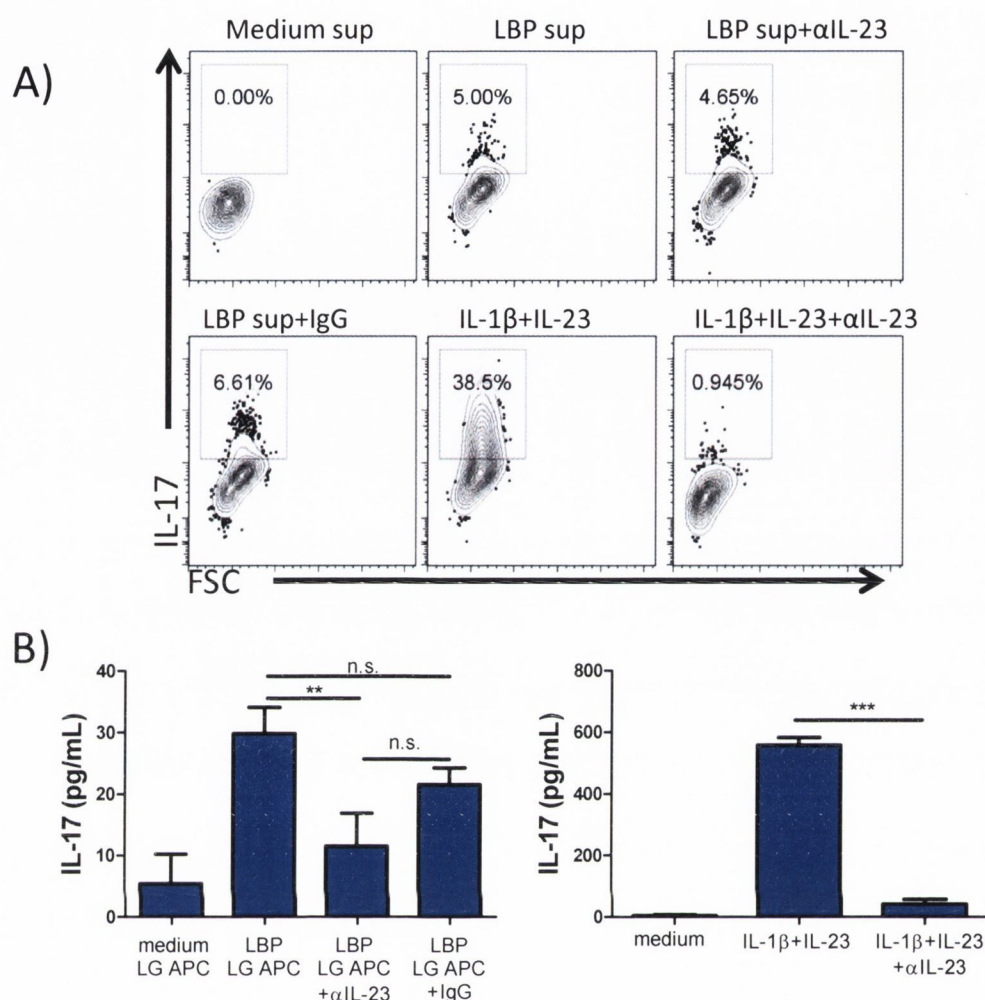


Figure 3.45 LBP supernatant induced IL-17 is not inhibited by neutralisation of IL-23.

FACS purified lung $\gamma\delta$ T cells ($75 \times 10^3/\text{mL}$) from C57BL/6 mice were incubated with filter sterilised supernatant from medium (medium sup) or live *B. pertussis* stimulated APC (LBP sup) at a ratio of 1:1, or with IL-1 β (10 ng/mL) and IL-23 (10 ng/mL) as control in the presence or absence of α IL-23 (5 $\mu\text{g}/\text{mL}$). After 12 hours BrfA (5 $\mu\text{g}/\text{mL}$) was added and the samples were incubated for additional 4 hours. The production of IL-17 by $\gamma\delta$ T cell subsets was analysed by flow cytometry. Aqua Live/dead staining was used to gate on live cells. The total IL-17 secreted protein was analysed by ELISA. Graph shows percentages of IL-17 secreting cells in the live gate (A) and concentration of IL-17 in $\gamma\delta$ T cell supernatants (B). n=1

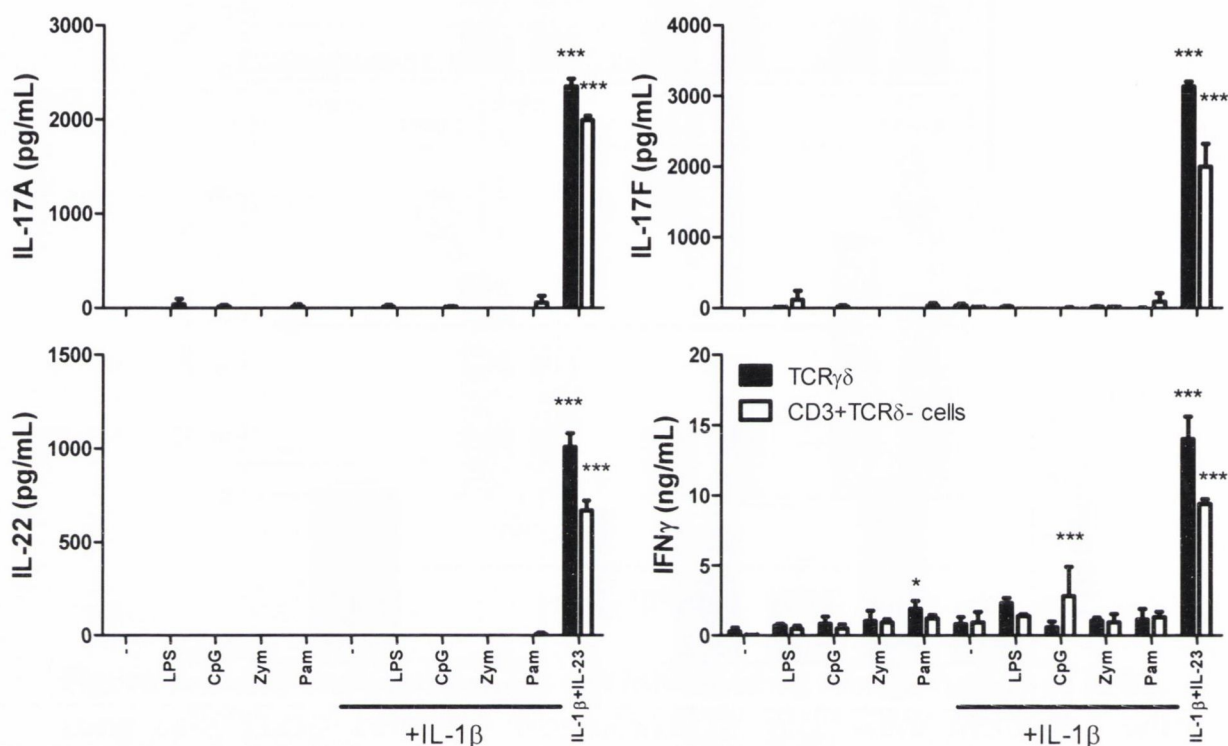


Figure 3.46 Isolated lung $\gamma\delta$ T cells fail to produce cytokines in response to stimulation with TLR agonists either alone or in combination with IL-1 β

FACS purified lung $\gamma\delta$ T cells (50×10^3 cells/mL) and CD3⁺TCR δ ⁻ cells (500×10^3 cells/mL) were stimulated with LPS (100 ng/mL), CpG (1 μ g/mL), Zymosan (10 μ g/mL), and Pam₃CSK₄ (100ng/mL) either alone or in combination with IL-1 β . After 72 hours the concentration of IL-17a, IL-17f, IL-22, and IFN γ in the supernatants was analysed by ELISA. *p<0.05, **p<0.01, ***p<0.001 by two way ANOVA with Bonferroni post test. n=1

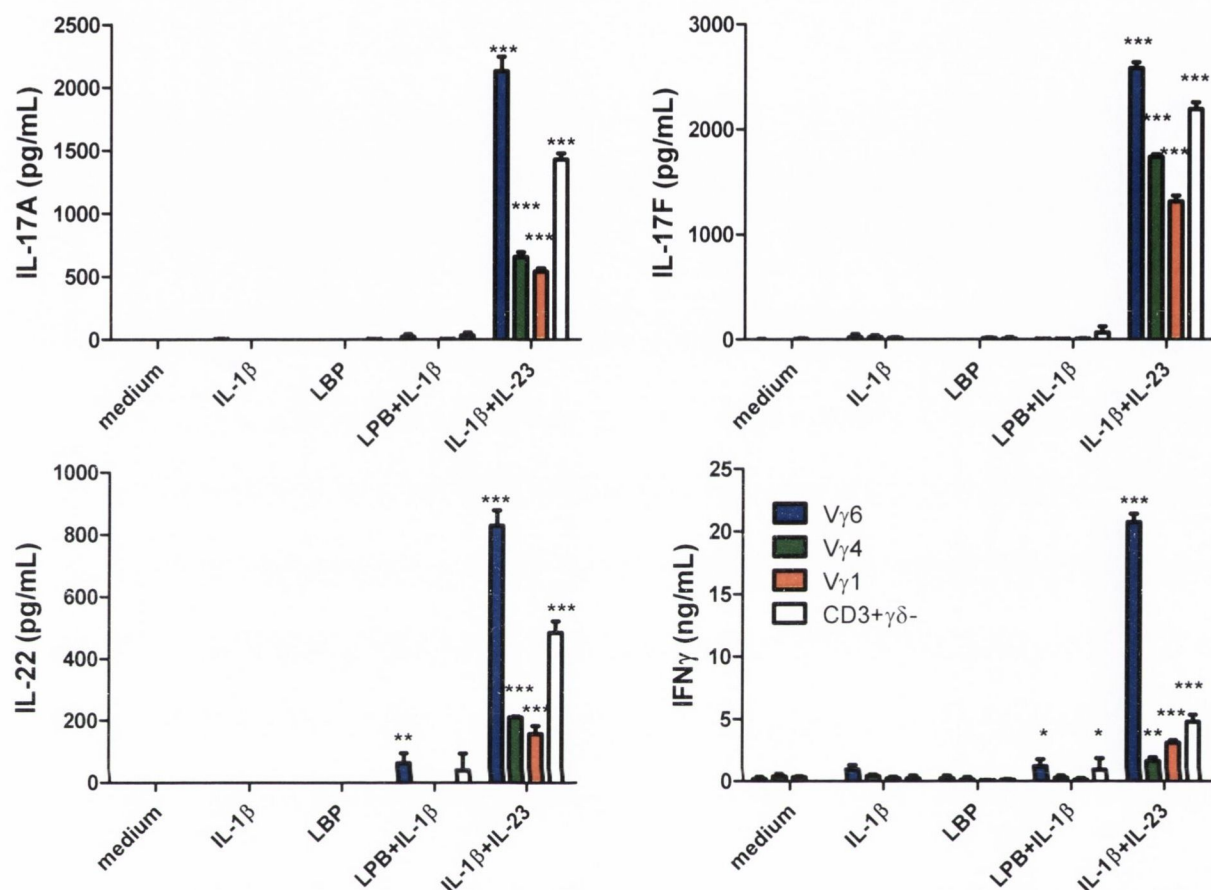


Figure 3.47 Isolated $\gamma\delta$ T cells fail to respond to LBP alone or in combination with IL-1 β .

FACS purified lung $\gamma\delta$ T cells (50×10^3 cells/mL) and CD3 $^{+}$ TCR δ^{-} cells (500×10^3 cells/mL) were stimulated with IL-1 β (10 ng/mL), LBP (MOI:1), IL-1 β +LBP or IL-1 β +IL-23 (10 ng/mL). After 72 hours the concentration of IL-17a, IL-17f, IL-22, and IFN γ in the supernatants was analysed by ELISA. *p<0.05, **p<0.01, ***p<0.001 by two way ANOVA with Bonferroni post test. n=1

Chapter 3.3: Discussion

Different subsets of $\gamma\delta$ T cells home to different sites in the body, suggesting that the TCR expressed by individual subsets may be suited for recognition of local antigens. Furthermore, cells present in particular sites encounter different quantity and quality of antigens. Intestinal $\gamma\delta$ T cells come across a vast number of antigens derived from food and commensal bacteria. Lung $\gamma\delta$ T cells, on the other hand, would receive much fewer signals on daily basis, while presence of bacteria in the circulation is a rare event associated with serious infectious disease. The current study investigated the role of cytokines in activation of intestinal and lung $\gamma\delta$ T cells. It was found that intestinal intraepithelial lymphocytes lack receptors for IL-1 β , IL-23 and IL-18 and thus are unable to mount proinflammatory responses upon stimulation with these cytokines. Lung $\gamma\delta$ T cells, on the other hand, respond strongly to various cytokine combinations to produce IL-17, IFN γ and IL-22. In addition, lung $\gamma\delta$ T cell subsets were found to have a differential capacity to respond to live *B. pertussis* derived proinflammatory stimuli and IL-1RI was found to be indispensable for their activation. However, it appears that lung $\gamma\delta$ T cells may also be activated in an IL-23 independent manner.

3.3.1 *In vitro* expansion of $\gamma\delta$ T cells

Although $\gamma\delta$ T cells constitute a greater percentage of T cells in the tissues, the absolute number of lymphocytes that can be obtained from those sites is relatively low. Furthermore, the isolation process is lengthy and harsh on the cells and results in the reduction of the number of viable cells that one can work with. Therefore, the effects of cytokines on $\gamma\delta$ T cell expansion *in vitro* were examined. A specific objective was to induce $\gamma\delta$ T cell proliferation without concomitant secretion of proinflammatory cytokines or skewing of $\gamma\delta$ T cell repertoire by favouring expansion of cells with a specific cytokine profile.

CD27 expression has been proposed as a marker to differentiate IL-17 from IFN γ producing $\gamma\delta$ T cells (Ribot et al, 2009). In contrast to other markers, which generally can only be used for cells of thymic and peritoneal origin, CD27 appears to faithfully divide $\gamma\delta$ T cells into two functional subsets both in the periphery and the tissues. The expression of CD27 by lymph node, small intestine IEL and lung $\gamma\delta$ T cells was

therefore investigated. The majority of lymph node and small intestine $\gamma\delta$ T cells were found to be CD27 positive, suggesting that they are likely to be IFN γ producers. Conversely, about 60% of lung $\gamma\delta$ T cells were found to be CD27 negative indicating they are likely to produce IL-17 upon activation. This is in agreement with the findings of Ribot et al. who also observed an increased CD27⁻ population in the lung compared to intestine and lymph node (Ribot et al, 2009).

In order to expand $\gamma\delta$ T cells in vitro, cytokines which have been implicated in stimulating proliferation of T cells and their maintenance were chosen. CD25 (IL-2R α) has been reported to be more strongly expressed by IL-17 producing $\gamma\delta$ T cells while CD122 (IL-2/15R β) expression correlated with IFN γ production by $\gamma\delta$ T cells (Jensen et al, 2008; Shibata et al, 2008). Furthermore, IL-17 and IFN γ producing $\gamma\delta$ T cells were reported to be decreased in IL-2 and IL-15 deficient mice, respectively (Shibata et al, 2008). In addition, IL-2 together with IL-15 have been shown to promote survival and proliferation of T cells (Zambricki et al, 2005). I therefore hypothesised that the combination of IL-2 and IL-15 would be ideal for expanding both CD27⁻IL-17⁺ and CD27⁺ IFN γ ⁺ $\gamma\delta$ T cells. In addition, IL-18 has been reported to enhance proliferation of IL-2, IL-7 or IL-15 stimulated human intestinal cells and therefore its capacity to promote the proliferation of mouse $\gamma\delta$ T cells has also been tested (Okazawa et al, 2004).

Concomitant stimulation of LN cells with IL-2 and IL-15 led to a strong induction of $\gamma\delta$ T cell proliferation, possibly leading to expansion of both IL-17 and IFN γ poised $\gamma\delta$ T cells. Stimulation of LN cells with IL-2+IL-18 led to an even higher proliferation and it was further enhanced by addition of IL-15. Increasing the concentration of IL-2 and IL-15 did not enhance $\gamma\delta$ T cell proliferation. Similar to the current findings, Segawa et al. observed $\gamma\delta$ T cell proliferation in response to IL-2+IL-18 stimulation. In addition, they reported that only NK1.1⁺ but not NK1.1⁻ $\gamma\delta$ T cells proliferated in response to this stimulus, favouring expansion of INF γ secreting cells (Segawa et al, 2011). Li et al. reported that IL-18 greatly enhanced expansion of IL-2 and zolendronate stimulated human $\gamma\delta$ T cells and these cells produced more GM-CSF, IFN γ , and TNF α compared to $\gamma\delta$ T cells incubated with IL-2 and zolendronate alone (Li et al, 2010).

In order to establish whether the expansion of $\gamma\delta$ T cells was accompanied by their activation, IL-17 and IFN γ concentration in the supernatants of lymph node cells was measured. Furthermore, cytokine production was also used as a read out of subtype polarisation. It was found that none of the combinations tested induced IL-17 production from lymph node cells. However, stimulation with IL-2 +IL-18 induced significant production of IFN γ by these cells. A greater amount of IFN γ was detected in cells stimulated with IL-2 and IL-18 plus either IL-15 or IL-7, suggesting that the expansion of IFN γ producing cells was favoured under these conditions. IL-18 has been shown to enhance IFN γ production and anti-tumour properties of human peripheral blood $\gamma\delta$ T cells stimulated with IL-2 and zoledronate (Li et al, 2010). The combination of IL-2+IL-18 has also been shown to stimulate IFN γ production by NK cells and $\gamma\delta$ T cells and either of these cell types could be the dominant source of IFN γ in the current setting (Matsubara et al, 2007; Okamoto et al, 2002; Segawa et al, 2011). Upon investigation of cytokine production by purified lymph node $\gamma\delta$ T cells it was found that, in agreement with the data of Segawa et al, lymph node $\gamma\delta$ T cells produce significant amounts of IFN γ in response to stimulation with IL-2+IL-18 (Segawa et al, 2011).

Therefore, although IL-2+IL-18 was found to be very good at promoting $\gamma\delta$ T cell proliferation, it also induced IFN γ production by these cells and may not represent a good protocol for expanding naïve $\gamma\delta$ T cells *in vitro*. However, given that IFN γ has been reported to inhibit, promote or be indifferent for T cell proliferation, it is possible that both IFN γ and IL-17 producing $\gamma\delta$ T cells could expand in the presence of IFN γ (MacLeod et al, 2008; Refaeli et al, 2002; Whitmire et al, 2005). It was investigated whether $\gamma\delta$ T cells expanded in the presence of IFN γ were able to produce IL-17 upon IL-1 β +IL-23 stimulation. It was found that although IL-2+IL-18 and IL-2+IL-15+IL-18 increased proliferation of $\gamma\delta$ T cells, the cells expanded using these cytokine combinations produced lower levels of IL-17 in response to IL-1 β +IL-23 than did freshly isolated $\gamma\delta$ T cells stimulated either with IL-1 β +IL-23 or IL-18+IL-23. This suggested that similar to findings of Segawa et al., IL-2+IL-18 stimulation of freshly isolated mouse $\gamma\delta$ T cells expanded IFN γ poised population (Segawa et al, 2011). The cells expanded in the presence of IL-2+IL-15 were found to secrete the highest concentration of IL-17 in response to stimulation with IL-1 β +IL-23, albeit not higher than that observed prior to expansion. Furthermore, IL-2+IL-15 expanded

cells produced similar amount of IFN γ in response to IL-1 β +IL-23 stimulation to that produced by freshly isolated LN cells. Baccala et al. reported that $\gamma\delta$ T cells required their own depletion to undergo homeostatic proliferation. They showed that $\gamma\delta$ T cells proliferated when transferred into lymphopenic host but failed to proliferate in a T cell sufficient host (Baccala et al). Therefore, it is likely that the proliferation of $\gamma\delta$ T cells observed in response to IL-2+IL-15 was also dependent on cell depletion, in this case through cell death, and served to maintain stable numbers of $\gamma\delta$ T cells in a mixed cell population.

In order to answer the question whether the incubation with the cytokines led to true expansion of $\gamma\delta$ T cells or homeostatic proliferation, purified lymph node $\gamma\delta$ T cells were stimulated with the chosen cytokine combinations, with or without α CD3, and the number of recovered $\gamma\delta$ T cell after 72 hours in culture was determined. It was found that $\gamma\delta$ T cell numbers significantly increased compared to the initial cell number per well when the cells were incubated in the presence of IL-2+IL-15. Addition of anti-CD3 antibody greatly enhanced proliferation of purified $\gamma\delta$ T cells incubated with the various cytokine combinations and, in the case of IL-2+IL-15 cytokine cocktail, the number of cells after 3 days of culture was nearly 7 fold higher compared to their initial number and represented the best expansion condition. Recently, IL-7 has been reported to selectively expand mouse and human IL-17 producing $\gamma\delta$ T cells (Michel et al, 2012). However, in the current study addition of IL-7 was found to inhibit the positive effect of IL-2+IL-15, both in the presence or absence of anti CD3.

Santos-Silva group reported that CD27⁺ $\gamma\delta$ T cells proliferated in response to anti-CD3 stimulation while CD27⁻ cells remained quiescent. Furthermore, they showed that anti-CD3 stimulation caused activation induced cell death in both CD27⁺ and CD27⁻ $\gamma\delta$ T cells, but it was greater in CD27⁻ cells. CD27⁺ $\gamma\delta$ T cells could be rescued from activation induced cell death by provision of soluble CD70, the ligand for CD27, which induced anti-apoptotic gene expression (Ribot et al, 2010). Since the incubation of $\gamma\delta$ T cells in the presence of anti-CD3 greatly enhanced their cytokine induced proliferation, CD27 expression by expanded $\gamma\delta$ T cells was investigated in order to establish whether this treatment led to a selective proliferation of one but not the other $\gamma\delta$ T cell subset. It was found that while 82% of freshly isolated lymph node $\gamma\delta$ T cells expressed CD27, after 3 days of *in vitro*

culture in the presence of anti-CD3 the majority of $\gamma\delta$ T cells were found to be CD27⁻. This suggested that, in contrast to findings of Santos-Silva and colleagues, CD27⁺ $\gamma\delta$ T cells appear to be more prone to activation induced death in the absence of co-stimulation (Ribot et al, 2010). Stimulation of $\gamma\delta$ T cells with IL-1 β +IL-23 either alone or in combination with anti-CD3 failed to induce their expansion. On the contrary, IL-1 β +IL-23 appeared to induce death of $\gamma\delta$ T cells as lower numbers of cells were recovered following stimulation. Furthermore, the CD27⁺CD27⁻ shifted towards CD27⁻ cells suggesting that a greater number of CD27⁺ cells died following stimulation with IL-1 β +IL-23. A similar observation was published in a recent report by Schmolka et al. (Schmolka et al, 2013). IL-1 β +IL-23 stimulation in the presence of anti-CD3 induced a sizeable population of IL-17 producing $\gamma\delta$ T as well as a small population of single producers of IFN γ and some double IL-17 and IFN γ producers. Interestingly, although IL-1 β +IL-23 and anti-CD3 stimulation did not increase $\gamma\delta$ T cell expansion, the appearance of IFN γ producing T cells correlated with an increased percentage and hence survival of CD27⁺ $\gamma\delta$ T cells. The CD27⁺ to CD27⁻ ratio was comparable to that in freshly isolated $\gamma\delta$ T cells when cells were incubated with anti-CD3 and all the other cytokine combinations tested. Although incubation of isolated $\gamma\delta$ T cells with IL-2+IL-15 and anti-CD3 significantly increased their numbers, it did so with concomitant induction of high IFN γ production. Despite the fact that IL-7 decreased IL-2+IL-15 induced $\gamma\delta$ T cell proliferation, it was found to greatly increase the percentage of IFN γ producing $\gamma\delta$ T cells. It would be interesting to see whether the inhibition of $\gamma\delta$ T cell expansion was due to the higher concentration of IFN γ present in the supernatant or due to a direct effect of IL-7. Importantly, $\gamma\delta$ T cells incubated with anti-CD3 alone secreted low levels of both IL-17 and IFN γ and the concentration of either of the cytokines could be increased by incubation with polarising cytokines. Expansion of $\gamma\delta$ T cells with anti-CD3 and IL-2+IL-15 was found to result in a CD27⁺ to CD27⁻ ratio similar to that in freshly isolated cells. However, the expanded cells showed increased IFN γ production upon anti-CD3 stimulation, while IL-17 remained unchanged. This suggests that although IL-2+IL-15 stimulation alone does not induce cytokine production by $\gamma\delta$ T cells, it may be priming them for IFN γ production in response to TCR stimulation. NK cells have been shown to produce IFN γ in response to stimulation with either IL-2 or IL-15 together with TLR ligands but not in response to either of the cytokines alone (Chalifour et al, 2004). It is possible that IL-2 and IL-15 have a similar IFN γ priming

role in $\gamma\delta$ T cells. In support of this idea is recent report by Ribot et al. who observed Tbet induction in human $\gamma\delta$ T cells upon stimulation with IL-2+IL-15 (Ribot et al, 2014). The same group has reported that CD28-induced autocrine IL-2 production in $\gamma\delta$ T cells, which was important for proliferation of anti-CD3 stimulated CD27⁺ and CD27⁻ $\gamma\delta$ T cells. However, they did not investigate the cytokine production by in vitro activated cells nor did they analyse the proportions of CD27⁺ to CD27⁻ $\gamma\delta$ T cells post stimulation (Ribot et al, 2012). Given their findings and the current data, it should be investigated whether incubation with IL-2+anti-CD3 or IL-15+anti-CD3 could be used as means of expanding $\gamma\delta$ T cell population with native ratio of CD27⁺ to CD27⁻ cells.

Mangan et al. used a method which involved stimulation of a purified subset of human peripheral blood $\gamma\delta$ T cells with a mitogen, Phytohaemagglutinin (PHA), followed by culture in the presence of IL-2 and irradiated feeder cells (Mangan et al, 2013). Stimulation of cells with PHA leads to crosslinking and consequent activation of numerous receptors on the cell surface, including the TCR. Therefore, the protocol used by Mangan et al. likely resulted in generation of a preactivated $\gamma\delta$ T cell population. In fact, they detected a high concentration of IL-17 in the supernatants of expanded human $\gamma\delta$ T cells in the absence of any stimulus. Furthermore, it is possible that if applied to a mixed $\gamma\delta$ T cell population, this protocol would result in skewing of the native blood $\gamma\delta$ T cell population. In the current study, anti-CD3 stimulation was found to increase IL-17 production by cells incubated with IL-1 β +IL-23 indicating that $\gamma\delta$ T cells do not produce IFN γ as a default response to TCR stimulation but that, similar to $\alpha\beta$ T cells, the cytokine milieu modulates the response of TCR activated $\gamma\delta$ T cells. Nonetheless, in contrast to conventional T cells, $\gamma\delta$ T cells are able to produce either IL-17 or IFN γ in response to cytokine stimulation alone, and therefore signals generated by the TCR may act to enhance the response induced by the innate cues.

Taken together, the current data suggest that it is not possible to induce significant proliferation of $\gamma\delta$ T cells with cytokines alone and activation via the TCR is required. However, this in turn leads to induction or enhancement of cytokine production by $\gamma\delta$ T cells. Furthermore, $\gamma\delta$ T cells were found to proliferate well in the presence of IFN γ although high concentration of IFN γ may inhibit $\gamma\delta$ T cell proliferation. It remains to be determined whether IL-2+IL-15 and aCD3 expanded $\gamma\delta$ T cells, similar to cells

incubated with IL-2+IL-15 alone, retain the ability to produce IL-17 in response to IL-1 β +IL-23 stimulation.

3.3.2 Characteristics and activation of intestinal $\gamma\delta$ T cells

High IL-1 β and IL-23 expression has been detected in the intestines of IBD patients (Casini-Raggi et al, 1995; Fuss et al, 2006; Schmidt et al, 2005). In animal models of colitis, neutralisation or deficiency of those cytokines has been associated with a reduction in clinical scores or resistance to the disease induction (Arai et al, 1998; Kwon et al, 2005; Yen et al, 2006). IL-17 production has been observed in the inflamed gut both in mice and humans and IL-17-producing $\gamma\delta$ T cells have been implicated in the pathogenesis of colitis through promotion of colitogenic Th17 cells (Do et al, 2011; Fujino et al, 2003; Hue et al, 2006; Nielsen et al, 2003). Previous work in the lab has shown that stimulation of lymph node $\gamma\delta$ T cells with IL-1 β +IL-23 or IL-18+IL-23 in the absence of TCR engagement induces IL-17 production (Lalor et al, 2011; Sutton et al, 2009). Given that $\gamma\delta$ T cells constitute a great proportion of gut lymphocytes, they are likely to be one of the first cells to sense the proinflammatory stimuli produced upon barrier breach. The responsiveness of mouse small intestinal intraepithelial lymphocytes (IEL) to cytokine stimulation was therefore investigated.

Surprisingly, in contrast to lymph node cells, IEL failed to produce IL-17A, IL-17F or TNF α in response to stimulation with IL-1 β +IL-23 or IL-18+IL-23. However, they produced significant amounts of IL-22 (Figure 3.10-3.11). Furthermore, IEL were found to produce significant amount of IFN γ in response to IL-18+IL-23 or IL-2+IL-18 stimulation (Figure 3.10-3.11). When the production of IL-22, IL-17 and IFN γ by LN and IEL was compared, it was found that LN cells were better producers of all three cytokines. However, while LN cells produced high levels of both IL-22 and IL-17, IEL were found to produce IL-22 only. IL-22 has been implicated in protection from inflammatory bowel disease in mouse studies (Sugimoto et al, 2008; Zenewicz et al, 2008). Production of IL-22 by IEL suggested that $\gamma\delta$ T cells and/or other innate like cells may be important for protecting the gut epithelium in an inflammatory environment. IL-22 production has been shown to be upregulated in the gut through stimulation of Aryl hydrocarbon receptor signalling (AhR) (Monteleone et al, 2011). AhR is a ubiquitously expressed transcription factor implicated in the toxic response through metabolism of foreign chemicals. Common AhR ligands include components

of environmental pollutants such as halogenated aromatic hydrocarbons and polycyclic aromatic hydrocarbons. However, since its discovery, natural ligands of AhR have also been discovered. The classically studied AhR ligands are tryptophan metabolites such as indolo[3,2-b]carbazole (ICZ) or 6-formylindoleo[3,2-b]carbazole (FICZ) (Busbee et al, 2013; Nguyen & Bradfield, 2008). The majority of natural AhR ligands are diet derived and the common ones include flavonoids present in tangerine and other citrus peels and indoles present in cruciferous vegetables (Busbee et al, 2013). Importantly, IEL have been shown to express high levels of AhR, and AhR signalling has been implicated in the maintenance of IEL in the gut and skin (Li et al, 2011).

RA, a vitamin A metabolite, is another diet derived molecule which, similar to IL-22, has been implicated in downregulation of inflammation in both human and mouse IBD (Bai et al, 2009). RA has been shown to inhibit Th17 differentiation and to promote generation of Treg cells (Elias et al, 2008; Mucida et al, 2007; Sun et al, 2007). Furthermore, DC resident in the intestinal lamina propria, skin and lungs as well as in their corresponding draining lymph nodes have been shown to be capable of de novo generation of RA (Guilliams et al, 2010). Given that relevance of the natural ligands of retinoic acid and aryl hydrocarbon receptor to the alimentary tract, their activation could enhance IL-22 production by IELs. The role of RA and FICZ in modulating the cytokine activation of IEL and LN cells was therefore investigated. In contrast to findings of Monteleone et al., FICZ failed to enhance IL-1 β +IL-23 or IL-18+IL-23 induced IL-22 secretion by IELs (Monteleone et al, 2011). In addition, FICZ was found to inhibit IL-17 production by LN cells and to enhance IFN γ production by LN and IELs induced by IL-1 β +IL-23. However, the difference in IFN γ was not found to be significant. The discrepancy between the results may come from the fact that Monteleone et al. looked at cytokine induction in the total intestine (including lamina propria cells) while the current study investigated the influence of AhR signalling on IEL compartment only. It is possible that FICZ exerts different effects on lamina propria and intraepithelial cells.

Consistent with the published data, RA was found to inhibit IL-17 production by LN cells. Furthermore, it was found that RA enhanced both IL-1 β +IL-23 and IL-18+IL-23 induced IFN γ production by LN cells. On the other hand, RA was found to inhibit the IL-18+IL-23 induced IFN γ production by IELs. RA enhanced IL-22 production by LN

and IEL cells, but the difference was only found to be significant for LN cells. Recently, work in the lab has shown that RA enhances IL-22 production by isolated lymph node and intestinal lamina propria $\gamma\delta$ T cells and ILC3 cells (Mielke et al, 2013). Furthermore, similar to the data on IEL presented here, RA was found to enhance IFN γ secretion by LN $\gamma\delta$ T cells stimulated with IL-1 β +IL-23 or IL-18+IL-23 (Mielke et al, 2013). RA has been shown to induce RAE-1 expression, a stress-induced non classical MHC class I molecule, in liver cells (Radaeva et al, 2007). RAE-1 is a ligand for the stimulatory NK cell receptor NKG2D also expressed by $\gamma\delta$ T cells (Bauer et al, 1999; Diefenbach et al, 2001). It is therefore possible that enhancement of IFN γ secretion by LN $\gamma\delta$ T cells is mediated through interaction with RA induced RAE-1. Furthermore, although direct recognition of RAE-1 by $\gamma\delta$ T cells has not been documented, it is possible that RAE-1 has a role in activation of $\gamma\delta$ T cells via the TCR as such recognition has been demonstrated for other non-classical MHC molecules, namely T10 and T22 (Adams et al, 2008). It remains to be verified whether RAE-1 upregulation is the mechanism by which RA enhances IFN γ production by $\gamma\delta$ T cells.

Surprisingly, while LN $\gamma\delta$ T cells produced high levels of IL-17, IL-22 and IFN γ thus mirroring the results obtained using the total LN cells, purified $\gamma\delta$ IEL failed to respond to either IL-1 β +IL-23 or IL-18+IL-23 stimulation. The lack of $\gamma\delta$ IEL responsiveness was confirmed at the mRNA level. Interestingly, higher basal IFN γ mRNA expression was detected in these cells. The lack of responsiveness to cytokine stimulation suggested that $\gamma\delta$ IEL either lack appropriate receptors or are unable to respond to cytokines in the absence of TCR engagement. Upon investigation of cytokine receptor expression by $\gamma\delta$ IELs, it was found that $\gamma\delta$ IEL do not express IL-1, IL-18 or IL-23 receptor, explaining the lack of responsiveness of these cells. Therefore, the stimulation of IEL with IL-1 β +IL-23 and IL-18+IL-23 must have activated IL-22 and IFN γ production by a cell type other than $\gamma\delta$ IEL, the most likely candidates being type 1 and type 3 ILC. Recently human intraepithelial ILC1 cells have been shown to produce IFN γ in response to IL-12 and IL-15 stimulation and to accumulate in the inflamed mucosal tissue (Bernink et al, 2013; Fuchs et al, 2013). Both mouse and human ILC1 lack the expression of IL-23R and the human ILC1 were reported not to respond to IL-18 stimulation (Fuchs et al, 2013; Spits et al, 2013). However, IL-18R expression by ILC1 cells has not been investigated and it is

possible that this subset of mouse IELs responded to stimulation with IL-2+ IL-18 to produce IFN γ in the current study (Spits et al, 2013). While the majority of intestinal IELs are T cells, NK and NKT cells have also been detected in the intraepithelial compartment and represent another possible source IFN γ (Calleja et al, 2011; Kinoshita et al, 2002; Pérez-Cano et al, 2005). Further studies are required to identify the main producers of this cytokine in response to IL-18+IL-23 and IL-2+IL-18 stimulation. Interestingly, IFN γ production has recently been shown to be associated with protection from colitis via downregulating the expression of IL-23 in murine macrophages (Sheikh et al, 2010). This gives a rationale for why molecules that confer protection in experimental colitis would enhance IFN γ production by intestinal IEL. ILC3 are likely to be the source of IL-22 in response to IL-1 β +IL-23 and IL-18+IL-23 stimulation. These cells express both IL-1RI and IL-23R and work done in the lab and by others has shown that lamina propria ILC3 produce IL-22 but not IL-17 in response to IL-1 β +IL-23 or PMA+ionomycin stimulation (Luci et al, 2009; Mielke et al, 2013; Spits et al, 2013). Furthermore, IL-22 producing NK cells have been shown to be protective in IBD and they too may contribute to cytokine induced IL-22 production by IELs (Zenewicz et al, 2008).

It has been demonstrated that lamina propria (LP) $\gamma\delta$ T cells can produce IL-17 (Asigbetse et al, 2010). Dermal but not epidermal $\gamma\delta$ T cells have been shown to be the major IL-17 producers in the skin in response to IL-23 stimulation (Cai et al, 2011). It is possible that similar to the skin, LP and IEL cells require different signals to become activated and while there is high IL-23 and IL-1 β expression in the intestine, intraepithelial lymphocytes are not the primary cell type responding to the stimulation with these cytokines. Many studies have suggested that intestinal $\gamma\delta$ T cells have protective or pathogenic roles based on experiments involving adoptive transfer of LN $\gamma\delta$ T cells, which clearly have different requirements for their activation. Furthermore, certain IBD models specifically involve adoptive transfer of LN T cells for the induction of the disease (Mizoguchi & Mizoguchi, 2010). There is no doubt that IL-17 is involved in the pathogenesis of the IBD in mice and humans. However, caution has to be exercised when drawing conclusions about the roles of gut resident $\gamma\delta$ T cells based on data from inflamed tissue and markers discerning resident from infiltrating peripheral cell should be used. Malinarich et al. showed that $\gamma\delta$ IEL readily produce IFN γ but not IL-17 in response to TCR stimulation (Malinarich

et al, 2010). The majority of gut $\gamma\delta$ T cells express CD8 α homodimer and CD27 molecule on their surface. Furthermore, the TCR on $\gamma\delta$ IELs has been reported to be constantly triggered under steady state conditions (Malinarich et al, 2010). This suggests that $\gamma\delta$ T cells may be refractory to further stimulation and require stronger signals in order to become activated. FICZ failed to upregulate IL-22 production by $\gamma\delta$ IELs in the current study and this may reflect the choice of activation protocol. However, given the high AhR expression by all IELs, it is possible that $\gamma\delta$ IELs contribute to IL-22 production in the gut when appropriately activated. It remains to be determined whether and how IL-22 production by $\gamma\delta$ IEL can be triggered.

3.3.3 Activation of lung $\gamma\delta$ T cells

The lung mucosa is in constant contact with the environment. The resident immune cells have an important role in protecting the lung against pathogens, ensuring minimal collateral damage during an immune response and promoting swift recovery of tissue after infection. Resident $\gamma\delta$ T cells can potentially play an important role those processes. It was investigated whether lung $\gamma\delta$ T cells are able to mount quick responses upon stimulation with proinflammatory cytokines. It was found that, similar to LN cells, lung $\gamma\delta$ T cells were found to produce IL-17 and IL-22 in response to stimulation with IL-1 β +IL-23. However, a greater percentage of lung $\gamma\delta$ T cells responded within 5 hours of stimulation compared with LN $\gamma\delta$ T cells and their cytokine production remained superior at 24 hours. Cytokine production in response to either IL-1 β or IL-23 alone was also investigated and it was found that lung but not lymph node $\gamma\delta$ T cells produced IL-17 in response to stimulation with either of the cytokines alone. However, both IL-1 β and IL-23 were required in order to induce IL-22 production by $\gamma\delta$ T cells.

V γ 4 T cells have been shown to be the main subset of $\gamma\delta$ T cells producing IL-17 in a number of disease settings (Roark et al, 2007; Roark et al, 2013; Welte et al, 2011; Zhao et al, 2011). However, IL-17 production by other $\gamma\delta$ T cell subsets has also been demonstrated (Hamada et al, 2008; Shibata et al, 2007; Simonian et al, 2009). It was found that in contrast to the lymph node, in the lung both V γ 4⁺ and other $\gamma\delta$ T cells produced IL-17 in response to IL-1 β +IL-23 stimulation. Furthermore, the non V γ 4⁺ cells were the major producers of IL-17 in response to either IL-1 β or IL-23 alone. While some IL-17 was detected from V γ 1 cells, they accounted for the

smallest percentage of IL-17 producing cells in the lung. The majority of IL-1 β +IL-23 responsive V γ 4⁻V γ 1⁻ $\gamma\delta$ T cells are likely to be the other main V γ subset in the lung, namely the V γ 6⁺ $\gamma\delta$ T cells. The V γ 4⁻V γ 1⁻ $\gamma\delta$ T cell population will therefore be referred to as V γ 6 cells in the context of the lung hereafter (Hayes et al, 1996; Sim et al, 1994). When production of IL-22 and IL-17 by lung and lymph node $\gamma\delta$ T cells was investigated, it was found that while the majority of IL-22/IL-17 double producers in the lungs are V γ 6⁺ cells, in the lymph node these were mostly V γ 4⁺ cells. Very few $\gamma\delta$ T cells were found to produce IL-22 and not IL-17 in lung and lymph node. IL-22 production by lung V γ 6 $\gamma\delta$ T cells has been demonstrated by Simonian and colleagues and has been associated with prevention of *B. subtilis* induced lung fibrosis. However, they observed very few IL-17 and IL-22 double producers suggesting that IL-22 production in those cells was activated via a different mechanism (Simonian et al, 2010).

The present study demonstrated that stimulation with IL-1 β +IL-23 also induced IL-17 production in other CD3⁺CD8⁻CD4⁻ cells both in the lung and lymph node. A small percentage of these double negative cells was found to produce IL-17 in the lung in response to stimulation with either IL-1 β or IL-23 alone. Furthermore, a significant proportion of double negative cells produced IL-22 in response to IL-1 β +IL-23 stimulation both in the lung and lymph node. It was found that all of the IL-17-producing cells co-expressed TCR β suggesting they belong to the NKT cell lineage.

Given that lung $\gamma\delta$ T cells were found to respond strongly to stimulation with IL-1 β +IL-23, It was investigated whether they respond to other cytokines known to activate lymph node $\gamma\delta$ T cells (Haas et al, 2009; Segawa et al, 2011). It was found that IL-18+IL-23, similar to IL-1 β +IL-23 stimulation, induced IL-17, IL-22 and IFN γ production by lung $\gamma\delta$ T cells. However, IL-18+IL-23 was found to induce a greater percentage of IFN γ producing cells compared with IL-1 β +IL-23 stimulation. In contrast, IL-2+IL-18 and IL-12+IL-18 stimulation was found to induce mainly IFN γ -producing $\gamma\delta$ T cells. V γ 4 cells were found to be good producers of IL-17 and IL-22, V γ 1 cells on the other hand produced mostly IFN γ and did not contribute much to IL-17 production. A big proportion of lung V γ 6 cells was found to be capable of producing IL-17, IL-22 and IFN γ in response to different cytokine combinations. The preferential secretion of IL-17, IFN γ and IL-22 in response to cytokine stimulation correlates with published findings of other groups. In agreement with the current

findings, peripheral V γ 1 $\gamma\delta$ T cells have been shown to produce IFN γ in response to BCG and *Listeria monocytogenes* in an IL-12 dependent manner (Dieli et al, 2004; Matsuzaki et al, 2002). Furthermore, V γ 1 $\gamma\delta$ T cell produced IFN γ was found to be important for the early immune responses against murine cytomegalovirus (Ninomiya et al, 2000). Similar to the current findings, Roark et al. failed to detect any significant IL-17 production by V γ 1 cells (Roark et al, 2008). However, V γ 1 have been reported to be the dominant source of IL-17 in the lungs of *Aspergillus fumigatus* infected mice (Romani et al, 2008). All three lung V γ subsets have been shown to produce IFN γ during *Mycobacterium tuberculosis* infection in mice, while V γ 4 and V γ 6 cells were the main source of IL-17 (Narayan et al, 2012) Lung V γ 6 $\gamma\delta$ T cells have been shown to be the major IL-22 producing population in a model of bacteria induced pulmonary fibrosis, while in the liver the V γ 6 population was found to co-produce IL-17 and IFN γ during *Listeria monocytogenes* infection (Simonian et al, 2010).

IFN γ and IL-17 production by lung $\gamma\delta$ T cell subsets based on CD27 expression was then investigated. In agreement with work of Silva-Santos and colleagues, only CD27 $^{-}$ $\gamma\delta$ T cells in the lung were found to produce IL-17 in response to stimulation with cytokines or PMA+ionomycin. IFN γ , on the other hand, was found to be produced by both CD27 $^{+}$ and CD27 $^{-}$ $\gamma\delta$ T cells. This is in agreement with the recent study by the same group showing that gene loci associated with IFN- γ production are active in both CD27 $^{+}$ and CD27 $^{-}$ $\gamma\delta$ T cells (Schmolka et al, 2013). In addition, the current study found that particular cytokine combinations induced IFN γ production by either CD27 $^{+}$ or CD27 $^{-}$ cells. This suggests that different cytokine receptors are expressed by these populations. Taken together, the *in vitro* findings of the current study show that different cytokine combinations can skew IL-17 or IFN γ production by lung $\gamma\delta$ T cells. Activation of a particular subset of $\gamma\delta$ T cell may be more or less desirable depending on the disease model.

Given that lung CD3 $^{+}$ CD4 $^{-}$ CD8 $^{-}$ TCR δ^{-} cells responded to stimulation with IL-1 β +IL-23 by producing IL-17, their responses to other cytokine combinations were investigated. Similar to $\gamma\delta$ T cells, a greater percentage of CD3 $^{+}$ CD4 $^{-}$ CD8 $^{-}$ TCR δ^{-} cells was found to produce IFN γ in response to IL-18+IL-23 stimulation compared to cells stimulated with IL-1 β +IL-23. The IL-18+IL-23 induced IL-17 $^{+}$ and IFN γ $^{+}$ populations generally did not overlap, although some double producers were detected. IL-2+IL-18 and IL-12+IL-18 stimulation was found to induce mainly IFN γ

production by CD3⁺CD4⁻CD8⁻TCR δ ⁻ cells. However, a smaller percentage of CD3⁺CD4⁻CD8⁻TCR δ ⁻ cells compared to $\gamma\delta$ T cells responded to these cytokine combinations by producing IFN γ . The combination of IL-12 and IL18 is known for its ability to synergistically induce IFN γ production by NK and NKT cells. Furthermore, NKT cells which have been treated with IL-12+IL-18 *in vitro* have been shown to promote NK cell responses when adoptively transferred into tumour bearing mice (Baxevanis et al, 2003; Lauwerys et al, 2000). In contrast to the current findings, combination of IL-2+IL-18 was reported to preferentially induce IL-13, but not IFN γ production by human cord blood NKT cells.(Fujibayashi et al, 2007). On the other hand, synergistic induction of IFN γ and cytotoxicity by IL-2+IL18 was demonstrated for human NK cells (Son et al, 2001). IL-13 production by CD3⁺CD4⁻CD8⁻TCR δ ⁻ cells was not investigated in the current study and it is possible that IL-2+IL-18 stimulated these cells to coproduce IFN γ and IL-13. Taken together, murine lung CD3⁺CD4⁻CD8⁻TCR δ ⁻ cells, similar to $\gamma\delta$ T cells, have a capacity to become rapidly activated by various cytokine combinations *in vitro*, and are likely significantly contributing to innate immune responses during infection.

Having found that lung $\gamma\delta$ T cells and CD3⁺CD4⁻CD8⁻TCR δ ⁻ cells become rapidly activated in response to cytokine stimulation *in vitro*, it was then examined whether a similar kinetics of response would be observed upon stimulation with a lung pathogen, *B. pertussis*. We found that while lung $\gamma\delta$ T cells readily responded to *B. pertussis* derived proinflammatory stimuli, the bacteria failed to elicit IL-17 production from both lymph node and spleen $\gamma\delta$ T cells. Nonetheless, $\gamma\delta$ T cells from all tissues responded strongly to IL-1 β +IL-23 stimulation indicating that lack of responsiveness was not due to an inability to produce IL-17. A possible explanation of the difference in responses of lung and lymph node $\gamma\delta$ T cells is the difference in numbers of antigen presenting cells present in each tissue. This could lead to a different level of proinflammatory cytokines induced in response to *B. pertussis*. However, similar to the lung, the spleen is rich in antigen presenting cells and thus it does not explain why spleen $\gamma\delta$ T cells failed to respond. Alternatively, the quality of the response of lung and spleen APC could be different. It is also possible that the cells in the lung were activated by *B. pertussis* induced factors other than IL-1 β and IL-23. When production of IL-17 by lung $\gamma\delta$ T cell subsets was examined, it was found that the great majority of IL-17 was produced by V γ 6 cells. However, both V γ 6 and V γ 4 cells

responded strongly to IL-1 β +IL-23 stimulation. This again suggested that factors other than IL-1 β and IL-23 may be involved in the activation of lung $\gamma\delta$ T cells by *B. pertussis*.

CD3⁺CD4⁻CD8⁻TCR δ ⁻ cells were also found to produce IL-17 in response to LBP and to a lesser extent HKBP stimulation. When the expression of NK cell markers by these cells was examined, it was found that the vast majority of the IL-17 producing cells were negative for NK1.1 and CD49b expression. Similar to the data from the current study, Michel et al. observed IL-17 production by NK1.1⁻ invariant NKT cells stimulated with α -GalCer or bacteria derived lipopolysaccharides and glycolipids. Furthermore, they showed that NK1.1⁻invariant NKT cell produced IL-17 contributed to neutrophil recruitment to the lung (Michel et al, 2007). It is possible that IL-17 producing CD3⁺CD4⁻CD8⁻TCR δ ⁻ induced in response to stimulation with *B. pertussis* and IL-1 β +IL-23 are the same NKT cell subset, although further studies are required to confirm that.

B. pertussis has been shown to promote the generation of Th17 cells through the induction of IL-1 β from murine DC in a caspase-1 and NALP3 dependent manner (Dunne et al, 2010). The contribution of *B. pertussis* induced IL-1 β to activation of IL-17 secretion by lung $\gamma\delta$ T cells was therefore investigated. It was found that in the presence of a caspase-1 inhibitor IL-17 production by $\gamma\delta$ T cells was strongly reduced. Furthermore, studies with IL-1R1 deficient mice, showed that IL-17 production by lung $\gamma\delta$ T cells and NKT cells in response to both heat killed and live bacteria was completely dependent on IL-1 β signalling. This is in agreement with findings of Dunne et al. who showed that IL-1RI signalling was required to induce protective *B. pertussis*-specific Th17 responses in the lung and spleen (Dunne et al, 2010). Similarly, IL-1 β has been shown to promote IL-17 production by $\gamma\delta$ T cells in the lung in IL-1 β induced lung fibrosis model, highlighting the importance of IL-1RI signalling in the stimulation of IL-17 production (Gasse et al, 2011; Wilson et al, 2010). However, those studies did not investigate what subset of $\gamma\delta$ T cells was the predominant source of IL-17. It is possible that the protective and pathological roles of $\gamma\delta$ T cells are activated differently and mediated by different subsets of $\gamma\delta$ T cells in the lung. In support of this, a study by Welte et al. showed that V γ 1 $\gamma\delta$ T cells promote pathogen clearance in West Nile virus infection, while V γ 4 $\gamma\delta$ T cells play a pathogenic role by promoting the virus entry into the brain (Welte et al, 2008).

Having shown that the activation of bacteria-induced IL-17 production by lung V γ 6 $\gamma\delta$ T cells is dependent on IL-1RI signalling, it was then investigated, whether the activation is dependent solely on soluble factors or requires interaction with APC. It was found that, similar to stimulation with bacteria, lung V γ 6 but not V γ 4 $\gamma\delta$ T cells responded to supernatants from LBP stimulated IBMM to produce IL-17, while both subsets responded equally well to IL-1 β +IL-23. The supernatants from LBP-stimulated IBMM contained both IL-1 β and IL-23 and their concentration was greater than that detected in supernatants of cells stimulated with HKBP. In contrast, similar concentrations of IL-6 and TGF β were detected in supernatants of IBMM stimulated with live or heat killed bacteria. IL-6 and TGF β have been implicated in promotion of Th17 cell differentiation (Bettelli et al, 2006). Therefore it is possible that these cytokines could be involved in IL-17 production by lung $\gamma\delta$ T cells. However, given the current findings it appears that IL-6 and TGF β are not important for HKBP and LBP induced IL-17 production by $\gamma\delta$ T cells. Since the percentage of IL-17 producing cells correlated with the concentrations of IL-1 β and IL-23 induced by live and heat killed bacteria, it is reasonable to assume that these cytokines were responsible for IL-17 induction in the lung cells. However, the finding that both V γ 6 and V γ 4 cells responded to exogenous IL-1 β +IL-23 while only V γ 6 cells became strongly activated by LBP or supernatants from LBP treated IBMMs suggests that these two $\gamma\delta$ T cell subsets may be activated by different factors. It is also possible that V γ 4 and V γ 6 $\gamma\delta$ T cells express different levels of IL-23 receptor and while under saturating conditions both subsets respond equally well, V γ 6 may be the predominant IL-17 producing subset when IL-23 is the limiting factor.

IBMMs are a macrophage cell line useful for studying innate immune responses. However, since these are transformed cells of bone marrow origin, they may not reflect responses induced in primary lung macrophages. The cytokine production by IBMM and lung lymphocytes was therefore compared. It was found that while LBP induced significant amount of IL-23 from IBMM, lung cells failed to produce IL-23 in response to LBP. On the other hand both lung cells and IBMM produced IL-1 β in response to heat killed and live bacteria. Given that supernatants from LBP stimulated IBMM contained IL-23 but the total lung cell supernatants did not, it appears that the activation of lung V γ 6 $\gamma\delta$ T cells may be achieved through different mechanisms, one dependent and the other independent of IL-23.

Both lung cells and IBMM secreted high levels of IL-1 α in response to heat killed and live bacteria. IL-1 α is classically thought to be a cytokine that behaves like an alarmin or a danger signal, which is passively released from dying cells. However, it has recently been demonstrated that IL-1 α secretion can be actively induced in myeloid cells and keratinocytes in both inflammasome-dependent and independent manner (Fettelschoss et al, 2011; Gross et al, 2012). Furthermore, Lukens et al. reported that IL-1 α and not IL-1 β mediated the exacerbated inflammatory responses and tissue damage in a mouse model of inflammatory skin disease (Lukens et al, 2013). Genetic ablation of *il1a* in these mice led to reduced generation of IL-17-producing cells and associated decreased recruitment of neutrophils. Since both IL-1 α and IL-1 β signal through the same receptor, it is possible that IL-17 induction in V γ 6 cells is at least partially dependent on IL-1 α signalling (Mosley et al, 1987). Nonetheless, similar concentration of IL-1 α was induced by heat killed and live bacteria, indicating that IL-1 β and not IL-1 α is the main inducer of IL-17 production from V γ 6 cells in the lung. Further investigation of cytokine secretion by myeloid cells in the lung, revealed that both macrophages and neutrophils produced IL-1 α and IL-1 β in response to HKBP and LBP. Neutrophils were found to be a much better source of IL-1 α while macrophages produced more IL-1 β . Similar percentage of IL-1 α producing macrophages was induced in response to heat killed and live bacteria, while IL-1 β was much more strongly induced upon stimulation with live *B. pertussis*. TNF α , on the other hand was found to be more strongly induced in macrophages in response to heat killed bacteria. Given that CyaA toxin from *B. pertussis* has been shown to inhibit production of TNF α and IL-12, but to promote IL-1 β secretion, the differences in macrophage activation in response to stimulation with HKBP and LBP are likely due to absence of secreted CyaA in the samples incubated with killed bacteria (Bagley et al, 2002; Dunne et al, 2010).

The results obtained using total lung cells suggest that V γ 6 and V γ 4 $\gamma\delta$ T cells respond differentially to soluble mediators induced by LBP. However, since these experiments did not involve purified cell populations it cannot be excluded that interaction with lung APC contributed to their activation. I therefore investigated whether isolated $\gamma\delta$ T cell could respond to LBP conditioned supernatants of lung and spleen APC. Furthermore, it was investigated, whether the lack of IL-17 production by lymph node $\gamma\delta$ T cells was due to the low number of APC in the lymph

nodes or the different activation requirement of lymph node $\gamma\delta$ T cells. It was found that both purified CD11b⁺ and CD11c⁺ cells from lung and spleen were composed of a similar percentage of different cell types with the exception of DC that were more abundant in the spleen and alveolar macrophages which are a lung specific cell type. For the purpose of simplicity, sorted CD11b⁺ CD11c⁺ populations from lung and spleen will be referred to as lung and spleen APC hereafter.

In agreement with the data obtained from the total lung lymphocytes, lung APC were found to produce significant amounts of IL-1 β , but not IL-23 in response to LBP stimulation. Similar cytokine profile was observed for spleen APC, although the concentration of IL-1 β was lower than detected in the supernatants of lung APC, possibly due to the lower percentage of macrophages in the spleen. Consistent with the protein data, IL-1 β gene expression was found to be strongly upregulated in lung and spleen APC stimulated with LBP. However, in contrast to data on IL-23 protein, IL-23 gene expression was found to be upregulated following stimulation with LBP. Macrophages and DC are the main producers of IL-23 and IL-23p19 expression becomes rapidly upregulated in alveolar macrophages upon bacterial stimulation *in vitro* (Rudner et al, 2007). Although IL-23 protein was not detected in APC supernatants, it is possible that IL-23 concentrations below the assay detection limit are sufficient to activate lung $\gamma\delta$ T cells.

Supernatants from LBP-conditioned spleen and lung APC induced IL-17 production by lung $\gamma\delta$ T cells. A much smaller percentage of lymph node $\gamma\delta$ T cells responded to stimulation with IL-1 β +IL-23 or the APC supernatants compared with lung $\gamma\delta$. This suggested that the differences in responses of lung and peripheral $\gamma\delta$ T cells were due to differential capacity of these cells to respond to pro-inflammatory stimuli, rather than the quality of the response mounted by APC. This could be due to different level of cytokine receptor expression by these cells. The expression of cytokine receptors by $\gamma\delta$ T cells was therefore investigated. It was found that among lung $\gamma\delta$ T cells, Vy6 cells expressed the highest levels of IL-1RI, IL-23R, IL-18R and IL-12R β . The expression of IL-1RI was several fold lower in the Vy4 subset of lung $\gamma\delta$ T cells. The expression of IL-23R was also found to be significantly lower in Vy4 cells. It was however much greater than that expressed by Vy1 cells. Lung Vy6 cells were found to express high levels of both Ror γ t and Tbet transcription factor. This is in agreement with the FACS data showing that these cells are an important source of

IL-17 and IFN γ in response to cytokine stimulation. Furthermore, V γ 1 cells were found to express high levels of Tbet. No significant difference in Gata3 and AhR expression was observed among lung $\gamma\delta$ T cell subsets. When the cytokine receptor expression of lung and lymph node $\gamma\delta$ T cell subsets was compared, it was found that lung V γ 6 cells expressed the highest levels of IL-1RI. Lymph node V γ 4 cells, on the other hand, were found to express the receptors for IL-18 and IL-23 most strongly. Lymph node V γ 1 $\gamma\delta$ T cells were found to express the highest levels of Tbet and Gata3 out of all subsets investigated. This is in agreement with the data in the literature showing preferential IFN γ and IL-4 production by these cells (Dieli et al, 2004; Hao et al, 2011; Narayan et al, 2012). In agreement with the IL-17 bias of lymph node V γ 4 T cells, they were found to express the highest levels of Ror γ t transcription factor (Pantelyushin et al, 2012; Sutton et al, 2009). The current data suggests that the cytokine profile of $\gamma\delta$ T cells and the strength of the response induced by cytokine stimulation can be predicted to a certain extent based on the transcription factor and cytokine receptor expression by these cells. The ability to produce IL-17 seems to correlate more closely with IL-1RI expression rather than IL-23R. Although the fact that lower percentage of lymph node V γ 4 $\gamma\delta$ T cells produced IL-17 in response to IL-1 β +IL-23 stimulation compared to lung V γ 4 cells, suggests that other factors may be involved.

Given the discrepancy between protein and gene expression data for IL-23 in lung APC, the role of IL-23 in LBP induced IL-17 production was investigated using a neutralising antibody against this cytokine. It was found that addition of anti-IL-23 to total lung lymphocytes reduced IL-17 production in response to LBP. However, isotype control antibody had a similar effect on IL-17 production, suggesting that the inhibition was non-specific. In contrast, IL-1 β +IL-23 induced IL-17 was strongly inhibited by anti-IL-23, but not by the isotype control. Given that concentration of exogenous IL-23 was at least several hundred fold higher than that that could potentially be produced by LBP treated lung cells, one would expect that the concentration of neutralising antibody would be enough to completely inhibit IL-23-mediated IL-17 production in LBP stimulated lung lymphocytes. Bacterial molecules have been shown to interfere with antibody binding and an antibody binding molecule is produced by *Bordetella* species, although the functional protein is missing in *B. pertussis* (Medina et al, 1999; Nordenfelt et al, 2012; Williams et al,

2008). It is possible that the decreased IL-17 production in antibody treated samples is due to sequestering of the neutralising antibodies by LBP as a mechanism of avoiding innate recognition, which in turn results in apparent inhibition of responses by anti-IL-23 and isotype antibody. To overcome these difficulties purified lung $\gamma\delta$ T cells were treated with supernatants of LBP conditioned APC in the presence or absence of anti-IL-23 or the isotype control. Co-treatment of $\gamma\delta$ T cells with LBP conditioned lung APC supernatant and anti-IL-23 did not result in a decreased percentage of IL-17-producing cells. In contrast, anti-IL-23 was found to strongly inhibit IL-1 β +IL-23 induced IL-17. However, upon investigation of IL-17 in $\gamma\delta$ T cell supernatants it was found that its concentration was significantly reduced in samples treated with anti-IL-23. However, IL-17 protein concentration was close to the assay detection limit in the samples treated with LBP conditioned supernatants. Furthermore, anti-IL-23 was found to inhibit very strongly IL-17 production induced upon stimulation with high concentrations of exogenous IL-1 β and IL-23. It is therefore difficult to draw conclusions from the current data. Given the differences in the responses of lung V γ 6 and V γ 4 T cells it is possible that IL-17 production immediately following pathogen entry is induced both in an IL-23 dependent and independent manner. Perhaps use of cells from IL-23p19^{-/-} mice could help clarify the role of IL-23 in the early IL-17 production by lung $\gamma\delta$ T cells.

$\gamma\delta$ T cells have been reported to express a number of TLRs in steady state conditions and the expression of some has been shown to become upregulated upon activation (Dejima et al, 2011; Martin et al, 2009; Mokuno et al, 2000). I therefore hypothesised that the signal synergising with IL-1 β for the induction of IL-17 in $\gamma\delta$ T cells could be induced by ligation of TLRs on their surface. None of the TLR agonists tested was able to induce IL-17 production by lung $\gamma\delta$ T cells either alone or in combination with IL-1 β . This is in contrast to findings of Martin et al. who observed IL-17 production by LN $\gamma\delta$ T cells in response TLR2 and Dectin-1 ligands in the absence of other stimuli (Martin et al, 2009). *B. pertussis* produces many molecules, including secreted virulence factors, which can potentially activate immune cells. It was therefore investigated whether whole live *B. pertussis* was capable of activating lung $\gamma\delta$ T cell subsets either alone or in combination with IL-1 β . Similar to stimulation with purified TLR agonist, no IL-17 was detected in supernatants of LBP stimulated $\gamma\delta$ T cells. Concomitant stimulation with IL-1 β + LBP

also failed to activate $\gamma\delta$ T cells to produce IL-17. Interestingly, lung V γ 6 $\gamma\delta$ T cells were found to produce the greatest amount of IL-17, IL-17F, IL-22 and IFN γ in response to IL-1 β +IL-23 suggesting that this $\gamma\delta$ T cell subset is especially suited for mounting potent responses upon stimulation with cytokines. In contrast to data obtained total lung lymphocytes, no IL-17 was observed in the supernatants of $\gamma\delta$ T cells stimulated with IL-1 β alone. This suggests that IL-1 β induced IL-17 production by lung $\gamma\delta$ T cells may be dependent on host factors induced upon cellular stress or induced by IL-1 β itself.

While *B. pertussis* induced IL-1 α is unlikely to be an important factor in the induction of IL-17 secretion by $\gamma\delta$ T cells, the concentration of this cytokine in the supernatants of lung cells stimulated with HKBP and LBP suggest that at least some of these cells may be undergoing necrotic cell death and thus release various endogenous danger associated factors (Zheng et al, 2013). Common danger associated molecular patterns (DAMPs) include components of the extracellular matrix, HSP or β -defensins and all of those molecules can be released during infection induced lung injury (Tolle & Standiford, 2013). Since $\gamma\delta$ T cells are able to recognise endogenous molecules, it is possible that some of these DAMPs may activate $\gamma\delta$ T cells in the lung. Furthermore, HSP have already been shown to activate $\gamma\delta$ T cells and both host and *B. pertussis* derived HSP may play a role in inducing IL-17 production by lung $\gamma\delta$ T cells (Kobayashi et al, 1994; O'Brien et al, 1992). $\gamma\delta$ T cells present in the whole lung preparation readily respond to IL-1 β to produce IL-17, while isolated lung $\gamma\delta$ T cells fail to respond to this stimulus. This suggests that an endogenous signal, possibly a DAMP, is involved in the induction of IL-17 production by $\gamma\delta$ T cells in the lung.

Given that live bacteria induce stronger responses in $\gamma\delta$ T cells than heat killed bacteria it is possible that stimulation of V γ 6 cells is dependent on factors secreted by *B. pertussis* or their ability to invade host cells. ACT is one of the main virulence factors secreted by *B. pertussis* and it has been shown to stimulate the induction of COX-2 expression in a PKA-CREB dependent manner in murine macrophages (Perkins et al, 2007). COX-2 is the enzyme responsible for generation of prostanoids such as PGE2 and PGI2 from arachidonic acid (Breyer et al, 2000; Smith et al, 2000). These lipid mediators, similar to ACT, can stimulate the increase in the intracellular cAMP levels and consequently some of the effects of ACT on DC could

be mimicked by PGE2 (Hickey et al, 2008; Lone & Taskén, 2013). However, PGE2 has also been implicated in promotion of Th17 cells differentiation through enhancement of IL-23 and IL-1 β production in macrophages and DCs and simultaneous downregulation of IL-12 production by these cells (Chizzolini et al, 2008; Khayrullina et al, 2008; Schnurr et al, 2005). In addition, PGI2 has been recently shown to be involved in the development of lung IL-17 producing $\gamma\delta$ T cells and a huge reduction in these cells was observed in the thymus and lungs of mice deficient in PGI2 receptor-IP. Iloprost, a stable analogue of PGI2, was found to enhance IL-17 production by splenic $\gamma\delta$ T cells stimulated with anti-TCR antibody (Jaffar et al, 2011). It would be interesting to see whether IL-17 production by lung $\gamma\delta$ T cells can be modulated by ACT induced prostanoids. Recently CD30-CD30L interaction was shown to be important for induction of IL-17 in peritoneal V γ 6 $\gamma\delta$ T cells in response to BCG (Guo et al, 2013). It is possible that this or other TNF family receptors are involved in *Bordetella* induced activation of lung $\gamma\delta$ T cells. Further studies are required in order to evaluate the contribution of host and pathogen derived signals in activation of lung resident IL-17 producing $\gamma\delta$ T cells.

Taken together, the current findings show that $\gamma\delta$ T cells present in the lung, intestine and lymph node, as well as subsets within those populations, respond differently to proinflammatory stimuli. Given that $\gamma\delta$ T cells can contribute to either inflammation-induced pathology or resolution of a disease, activation of a particular subset may be of benefit in certain circumstances but not in others. Furthermore, given that cytokines produced by $\gamma\delta$ T cells early during infection can shape later immune responses, activation of particular $\gamma\delta$ T cell subsets may be exploited to promote vaccine induced protective responses. Further studies are necessary to identify molecules involved in activation of $\gamma\delta$ T cell subsets.

Chapter 4: Role of lung $\gamma\delta$ T cells in immunity to *B. pertussis*

Chapter 4.1: Introduction

4.1.1 Immune responses to *B. pertussis*

Natural *B. pertussis* infection in children and adults results in generation of pathogen-specific IFN γ -producing Th1 and CD8 T cells (Dirix et al, 2012; Hafler & Pohl-Koppe, 1998; Mascart et al, 2003; Ryan et al, 1997). The requirement of Th1 response for clearance of bacteria was confirmed in mice and a severe disseminating infection was observed in IFN γ receptor-deficient animals (Mahon et al, 1997; Mills et al, 1993). Recently a mixed Th1/Th17 response was reported in primary *B. pertussis* infection of non-human primates, with both types of *B. pertussis* specific cells detectable in circulation two years post infection (Warfel & Merkel, 2013). In addition, IL-17 has been shown to contribute to protective immunity in mice (Andreasen et al, 2009; Dunne et al, 2010). Similar to the results in macaques, mixed Th1/Th17 responses have been observed in mice vaccinated with the whole cell pertussis vaccine (Ross et al, 2013). The acellular vaccine, on the other hand, has been found to induce Th17/Th2 dominated response, and this switch from Th1 to Th2 responses has been proposed to be the reason for reduced efficacy or longevity of immune protection induced by the acellular vaccine (Ross et al, 2013). Although limited data are available on *B. pertussis*-specific Th17 responses in humans, similar to the results in mice, enhanced antigen specific Th2 responses were observed in children after administration of acellular pertussis vaccine (Dirix et al, 2009; Schure et al, 2012a; Schure et al, 2012b).

Prior to the appearance of antigen-specific T cells, *B. pertussis* infection is controlled by cells of the innate immune system and the responses of these cells shape the adaptive immune response and ultimately determine the severity and outcome of the infection (Byrne et al, 2004; Dunne et al, 2009; Higgs et al, 2012). Various innate immune cell types have been studied in the context of *B. pertussis* infection and were shown to play roles in protection as well as survival of the bacteria. Lung resident macrophages are one of the first immune cells that encounter invading pathogens. They have been shown to be the primary target of PT and, despite various killing mechanisms employed by these cells, the bacteria are able to survive within them and inhibit their function (Carbonetti et al, 2007; Friedman et al, 1992;

Lamberti et al, 2010). The mechanism by which *B. pertussis* survives in the cells includes inhibition of autophagy (C. Peral de Castro, unpublished observations). This however leads to IL-1 β production which can induce activation of other immune cell types (Peral de Castro et al, 2012). Consequently, macrophage depletion has been shown to result in increased bacterial loads in the lung highlighting the importance of these cells in the early defences against *B. pertussis* (Carbonetti et al, 2007). PT has also been shown to inhibit early neutrophil infiltration, but to stimulate IL-17 production and neutrophil recruitment to the lung at the later stages of the infection (Andreasen et al, 2009). However, depletion of neutrophils resulted in only a slight increase in the bacterial burden in the lung, suggesting that they do not play an important role in the primary *B. pertussis* infection (Andreasen & Carbonetti, 2009). NK cells are an important early source of IFN γ and they have been found to significantly contribute to innate immune responses against *B. pertussis*, as well as generation of protective immunity. Depletion of NK cells resulted in a lethal disseminating infection with diminished Th1 responses (Byrne et al, 2004). This extreme phenotype was probably due to impaired macrophage activation at the early stages as a result of decreased IFN γ as well as increased survival of *B. pertussis* infected cells in the absence of NK cell killing (Mahon & Mills, 1999).

CD8 α CD11c⁺ DC have also been implicated in the early responses against *B. pertussis*. Their numbers were found to significantly increase in the cervical lymph nodes within 4 hours post infection and in the lung at peak of bacteraemia (Dunne et al, 2009). In the lymph nodes, CD8 α ⁺ DC produced IL-4 and IL-10 and some IFN- γ , while in the lungs they produced mainly IFN- γ . Depletion of CD8 α ⁺DC during early stages of infection resulted in weaker Th1 responses and delayed bacterial clearance.

The role of $\gamma\delta$ during *B. pertussis* infection has not been well studied. However, Zachariadis et al. showed that they may act to limit the extent of the early inflammatory response during *B. pertussis* infection. TCR δ ^{-/-} mice had higher inflammation in the lungs with simultaneous increase in the neutrophil and macrophage numbers and a decrease in bacterial counts. Furthermore, they observed a shift from Th1 to Th2 profile in the antigen recall response in TCR δ ^{-/-} spleen cells stimulated with FHA (Zachariadis et al, 2006). However, cytokine production by $\gamma\delta$ T cells was not investigated in that study and their function was

deduced using reductionist approach only. Cheng and colleagues, on the other hand, found that in *Staphylococcus aureus* induced model of pneumonia, $\gamma\delta$ T cells were the major producers of IL-17 (Cheng et al, 2012). Furthermore, in contrast to Zachariadis study, they showed that mice deficient in $\gamma\delta$ T cells had reduced neutrophil recruitment to the site of infection, attenuated bacterial clearance but reduced tissue damage.

Chapter 4.2: Results

4.2.1 Optimisation of *B. pertussis* protocol for an *in vivo* aerosol infection model

In order to ensure that the bacteria used for *in vivo* experiments were in the appropriate phase of growth to effectively infect mice, two different protocols of culturing bacteria were compared. For both protocols, glycerol stocks of bacteria were lawned on BG blood agar plates and cultured for 4 days. The bacteria were then used to inoculate liquid media. For the long protocol (48h protocol) bacteria were cultured for 48h in liquid medium, then split and cultured for further 24h. For the short protocol (24h protocol) the bacteria were cultured in the liquid medium for 24h only and then used directly to infect mice. It was found that CFUs in the lungs of *B. pertussis* infected mice were highest on day 7 post infection. However, the CFUs in the lungs of mice infected with bacteria cultured using the long protocol first fell on day 3 and then increased to the peak levels observed on day 7. This initial decline in CFU counts of *B. pertussis* was not observed with the short protocol. The differences in the clearance curves of bacteria grown using the two protocols were observed only during the first 14 days of infection and the curves merged thereafter (Figure 4.2.1).

4.2.2 $\gamma\delta$ T cells produce IL-17 at the early stages of *B. pertussis* infection

Given the findings presented in chapter 3, showing that lung $\gamma\delta$ T cells rapidly produce IL-17 *in vitro* in response to stimulation with bacteria, the kinetics of IL-17 responses in the lungs and its cellular sources during the early stages of *B. pertussis* infection were investigated. It was found that $\gamma\delta$ T cells produce IL-17 as early as 2 hours post infection (Figure 4.2A). The percentage of IL-17 producing cells then declined to an undetectable level on day 3 and peaked again on day 7, when other CD3 cells became the dominant source of this cytokine. IFN γ production by $\gamma\delta$ T cells in the lung was undetectable until day 7 post infection. However, IFN γ -producing CD3⁺TCR δ ⁻ cells were present on each of the time points and peaked on day 7 post infection. The decline in the bacterial counts coincided with the increase in the percentage of cytokine producing cells in the lung (Figure 4.2A-B).

The contribution of individual cell types to IL-17 production in the lung was investigated. It was found that, apart from $\gamma\delta$ T cells, a significant percentage of $CD3^+CD4^-CD8^-TCR\delta^-$ IL-17⁺ cells were present in the lung at 2h and day 1 post infection (Figure 4.3). IL-17-producing $CD4^+$ T cells were detected on day 7, and on day 14 they were the major source of IL-17 in the lung.

Analysis of IL-17 producing lung $\gamma\delta$ T cell subsets during the early stages of *B. pertussis* infection revealed that both $V\gamma6^+$ and $V\gamma4^+$ $\gamma\delta$ T cells contribute to IL-17 production (Figure 4.4). However, a smaller percentage of $V\gamma4^+$ compared to $V\gamma6$ $\gamma\delta$ T cells secreted IL-17.

4.2.3 *B. pertussis* induces upregulation of proinflammatory cytokines in the lung

Since IL-1 β and IL-23 are known to induce IL-17 production from $\gamma\delta$ T cells, the expression of these cytokines in the lung during the first 12 hours following *B. pertussis* infection was investigated. It was found that both IL-1 β and IL-23 mRNA was strongly upregulated as early as 1 hour post infection (Figure 4.5). It then rapidly decreased and remained low. The expression of IL-1 β and IL-23 on different days during the early stages of infection was then investigated. It was found that after the early peak of expression, IL-1 β and IL-23 remained low until day 7 post infection, when the second peak of expression occurred (Figure 4.6). Although the expression levels of the two cytokines did not reach significance until day 14 post infection. The fluctuations in the level of IL-1 β and IL-23 mRNA in the lung directly correlated with the levels of IL-17a, IL-17f and IL-22. The IL-17 protein concentration in the lung was found to increase at 2h post infection, however, the amount detected was not significant (Figure 4.7). The concentration of IL-17 in the lung was elevated on day 7 and day 14 post infection, although, in contrast to the mRNA data, it was lower on day 14. Both IL-22 and IFN γ protein concentration was found to be increased on day 7 and it fell to non-significant levels on day 14.

4.2.4 Multiple cell types produce IL-22 in the lungs on day 7 post *B. pertussis* infection

Having found that IL-22 production peaked in the lungs specifically on day 7 post infection and given that $\gamma\delta$ T cells and NKT cells are known sources of IL-22, the

production of this cytokine by these and other cell types in the lung was investigated. It was found that both $\gamma\delta$ T cells and $CD3^+CD4^-CD8^-TCR\delta^-$ cells contributed to the overall production of IL-22 in the lung (Figure 4.8). $CD3^+CD4^-CD8^-TCR\delta^-$ cells were found to account for a greater percentage of IL-22 producing cells than did $\gamma\delta$ T cells. Furthermore, addition of IL-23 to BrfA stimulated cells was found to enhance the response of both $\gamma\delta$ T cells and $CD3^+CD4^-CD8^-TCR\delta^-$ cells, but did not induce IL-22 production in cells from naïve animals. IL-22 production was also detected in CD4 T cells as well as $CD3^-CD11b^-CD11c^-CD45^+NKp46^-$ cells (Figure 4.9-4.10). Given that $CD3^-CD11b^-CD11c^-CD45^+NKp46^-$ are likely to represent the ILC3 subset of innate lymphocytes, Ror γ t expression by $CD3^-CD11b^-CD45^+$ cells in the lungs was investigated. It was found that a population of $CD3^-CD11b^-CD19^-CD45^+Ror\gamma t^+$ cells is present in the lungs of naïve animals and becomes expanded in the presence of IL-1 β +IL-23 (Figure 4.11).

4.2.5 $\gamma\delta$ T cells play a redundant role in *B. pertussis* infection

Having shown that $\gamma\delta$ T cells produce IL-17 and IL-22 in the lung during the early stages of infection, it was then investigated whether these cells are essential for protective responses against *B. pertussis*. WT and $TCR\delta^{-/-}$ mice, mice lacking functional $\gamma\delta$ T cells, were infected with virulent *B. pertussis* and the bacterial burden and cytokine production in the lung as well as systemic antigen specific responses were investigated.

It was found that $TCR\delta^{-/-}$ mice do not show impaired *B.pertussis* clearance from the lung (Figure 4.12). Interestingly, it was found that in the absence of functional $\gamma\delta$ T cells, lung $CD3^+CD4^-CD8^-TCR\delta^-$ cells are expanded by about two times the percentage in WT mice (Figure 4.13). Furthermore, $CD3^+CD4^-CD8^-TCR\delta^-$ cells from $TCR\delta^{-/-}$ animals show enhanced cytokine production compared to WT mice. Consequently no impairment in neutrophil and macrophage recruitment to the lung was observed in $TCR\delta^{-/-}$ animals (Figure 4.14). When cytokine production by CD4 T cells was investigated., it was found that significantly higher percentage of IL-17-producing CD4 T cells is present in the lungs $TCR\delta^{-/-}$ mice compared to WT mice on day 14 post infection (Figure 4.15). No significant difference was observed in IFN γ production by CD4 T cells. Systemic antigen specific cytokine production on day 21 post infection was investigated. No significant differences in the production of IL-17,

IFN γ and IL-22 were observed in response to stimulation with purified *B. pertussis* antigens or HKBP (Figure 4.16). However, a significantly greater concentration of IL-5 was detected in the supernatants from non-specifically restimulated spleen cells from TCR $\delta^{-/-}$ mice.

4.2.6 Neutralisation of IL-17 at the early stages of *B. pertussis* infection leads to delayed pathogen clearance.

Given that an expanded population of CD3 $^{+}$ CD4 $^{-}$ CD8 $^{-}$ TCR δ^{-} cells is present in TCR $\delta^{-/-}$ animals, which appears to compensate for the absence of functional $\gamma\delta$ T cells, a cytokine neutralisation approach was undertaken in order to establish whether the IL-17 produced by innate-like lymphocytes ($\gamma\delta$ T cells and of CD3 $^{+}$ CD4 $^{-}$ CD8 $^{-}$ TCR δ^{-} cells) has a role in the pathogen clearance. Neutralising anti-IL-17 antibody or isotype matched control antibody were administered i.p. on day -1 and +2 post challenge. The differences in bacterial burden and in innate and adaptive immune responses were analysed at 2h and day 10 and 21 post infection. It was found that mice treated with anti-IL-17 had significantly greater numbers of bacteria in the lung at day 10 post infection (Figure 4.17). The difference was still visible on day 21, however it was no longer significant. Although there was a small decrease in the numbers of lung infiltrating neutrophils upon anti-IL-17 treatment, the difference was not significant (Figure 4.18). No differences were observed in the numbers of macrophages and alveolar macrophages in the lungs of anti-IL-17 treated mice.

The analysis of T cell responses in the lung revealed that similar percentages of IL-17 producing $\gamma\delta$ T cells were present in the lungs of anti-IL-17 and isotype control treated mice on day 10 post infection (Figure 4.19). Slightly increased percentage of IL-17 producing $\gamma\delta$ T cells was detected in the lungs of anti-IL-17 treated animals on day 21 post infection, although the difference was not significant. Smaller percentages of IFN γ producing $\gamma\delta$ T cells were present in the lungs of anti-IL-17 treated mice, however the difference was not significant. Slightly reduced percentages of IL-17 producing CD4 T cells were observed on day 10 post infection upon α IL-17 treatment. On day 21 post infection, on the other hand, anti-IL-17 treated mice had slightly more IL-17 producing cells in their lungs. IFN γ production by CD4 T cells was only detected on day 10 post infection and α IL-17 treatment was found to result in a slight reduction in the percentage of IFN γ^{+} CD4 $^{+}$ T cells. None of

the differences in the cytokine production by CD4 T cells were found to be statistically significant. Only a small percentage of CD8 T cells was found to produce IFN γ in the lungs of infected mice and no significant difference was detected upon anti-IL-17 treatment.

Upon investigation of cytokine concentration in the lung homogenate it was found that anti-IL-17 treated mice had a significantly increased concentration of IL-17 in their lungs on day 10 post infection (Figure 4.20). IL-17 was also found to be increased on day 21, however the difference was not significant IFN γ was not detected in the lung homogenates.

Given that IL-17 is known to promote the induction of anti-microbial peptides (Liang et al, 2006; Robinson et al, 2014), the induction of RegIII γ , mouse β -defensin 3 and mouse cathelicidin CAMP in the lungs of anti-IL-17 and isotype control treated mice was investigated. It was found that the expression of RegIII γ was induced in the lungs of anti-IL-17 treated and control mice (Figure 4.21). However, no difference was observed between the two groups. Mouse β -defensin 3 gene expression was also upregulated upon *B. pertussis* infection, however, similar to RegIII γ , no difference was observed between anti-IL-17 and isotype treated animals. CAMP, on the other hand, was found to be significantly reduced in the lungs of anti-IL-17 treated mice on day 10 post infection.

4.2.7 Delayed *B. pertussis* clearance and greater pulmonary inflammation in IL-22^{-/-} mice.

IL-22 is produced by $\gamma\delta$ T cells and other innate like lymphocytes at the early stages of *B. pertussis* infection. Therefore, it was investigated whether the absence of IL-22 would lead to an impaired clearance of *B. pertussis* from the lungs. While no difference between WT and IL-22^{-/-} mice was observed at the early stages of infection, the knock out mice were found to have a significantly greater bacterial burden in the lungs on day 14 and 21 post infection (Figure 4.22). However, no significant difference in the numbers of macrophages, neutrophils and alveolar macrophages was observed at any of the time points post infection (Figure 4.23).

When CD4 T cell responses in the lungs were investigated, it was found that IL-22^{-/-} mice had significantly greater percentage of IL-17 producing cells on day 14 and day

21 post infection, while IFN γ producing cells were significantly increased on day 7 (Figure 4.24). Furthermore, antigen specific responses to *B. pertussis* were also found to be greater in IL-22^{-/-} mice (Figure 4.25). Spleen cells from IL-22^{-/-} mice were found to produce significantly greater amounts of IL-17 in response to FHA, PT and HKBP, while greater amount of IFN γ was detected in response to PT and HKBP. In addition, a significantly greater IL-5 production was detected in response to polyclonal stimulation with α CD3/PMA in IL-22^{-/-} mice.

The expression of IL-10 and TGF β was investigated in order to see whether the enhanced IL-17 and IFN γ production in the lungs of IL-22^{-/-} mice was due to an impaired anti-inflammatory response in the lung. No difference was found in the expression of these two cytokines in the lungs of IL-22^{-/-} mice compared to WT controls (Figure 4.26). The expression of IL-6 and TNF α was also investigated as an indication of the overall proinflammatory status in the lung. IL-22^{-/-} mice were found to express lower levels of IL-6 and TNF α compared to WT mice on day 7 and day 14 post infection, respectively (Figure 4.27)

Given the above findings and the implication of IL-22 in mediating tissue repair, lung pathology in WT and IL-22^{-/-} mice was investigated. A basic scoring method which assessed the percentage of inflamed area was used. It was found that IL-22^{-/-} mice had a greater number of inflammatory foci and a higher overall lung inflammation on day 14 and 21 post infection, with a significantly higher inflammation score on day 14 (Figure 4.28A-C).

Similar to IL-17, IL-22 contributes to tissue protection by inducing antimicrobial peptide production (Liang et al, 2006; Steinstraesser et al, 2011; Wolk et al, 2004). However, no impairment in the antimicrobial peptide gene expression was observed in the lungs of IL-22^{-/-} mice and CAMP and RegIII γ were found to be significantly increased in the lungs of IL-22^{-/-} mice (Figure 4.29).

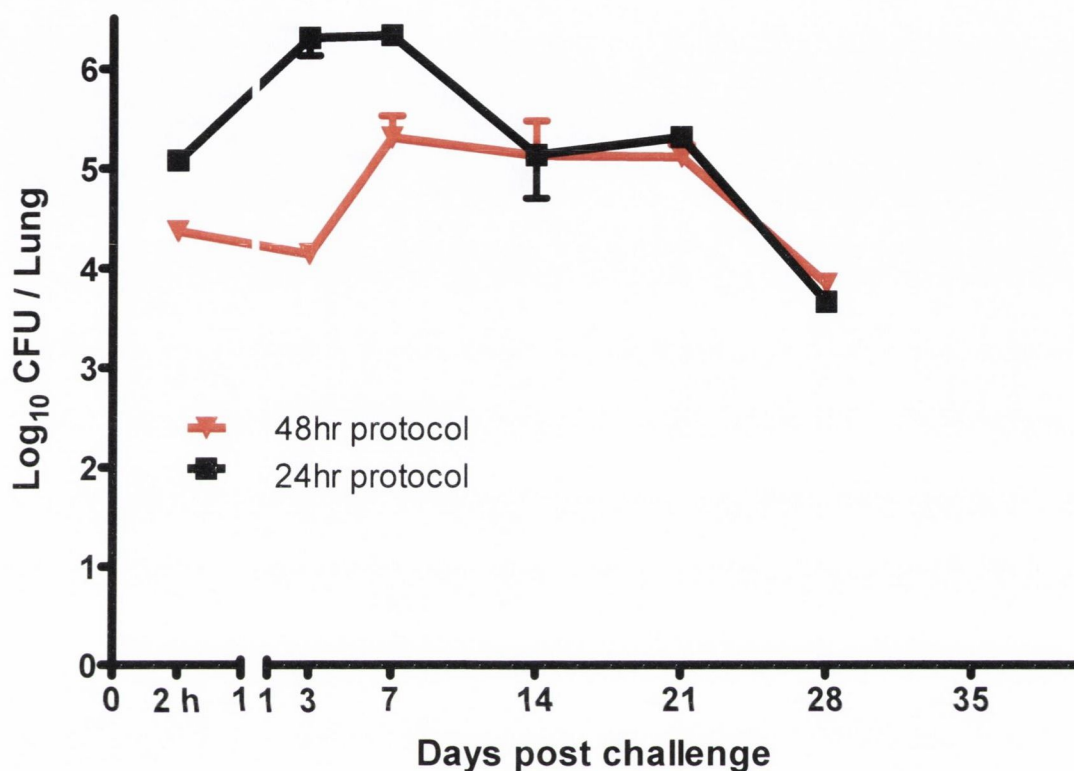


Figure 4.1 Clearance curves for *B. pertussis* grown using two different protocols.

C57BL/6 mice were infected with 2×10^{10} CFU/mL *B. pertussis* strain Bp338 grown according to two different protocols. The bacteria were grown either for 9 days (48 hours in liquid medium) or for 4 days (24 hours in liquid medium). Mice were sacrificed at time points indicated in the figure and the lung CFUs were estimated by performing colony counts of serially diluted lung homogenates from individual mice at each time point. $n \geq 3$

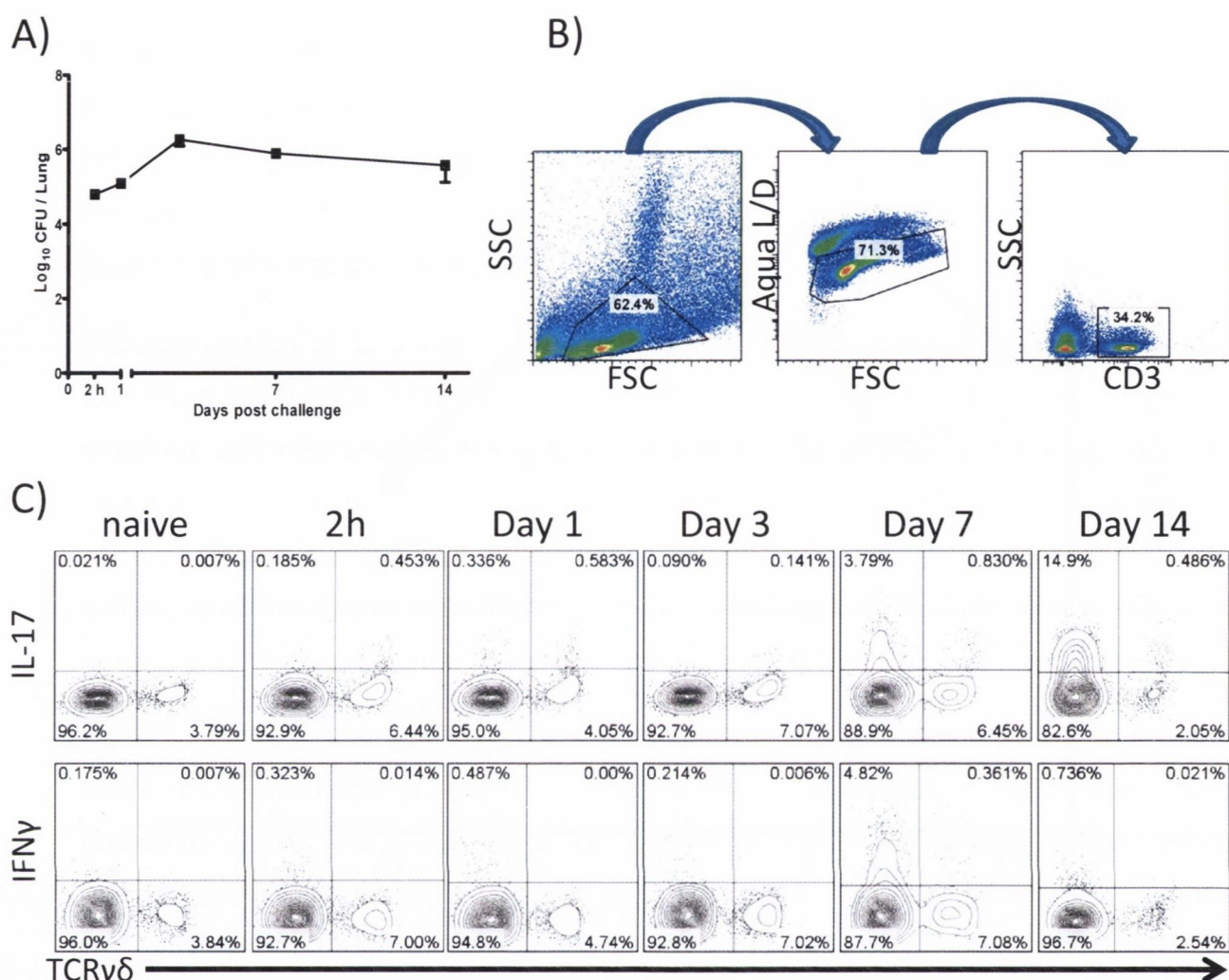


Figure 4.2 IL-17 and IFN γ production by $\gamma\delta$ T cells in the lung during the early time points of *B. pertussis* infection.

C57BL/6 mice were challenged by aerosol with virulent Bp338 strain of *B. pertussis* (2×10^{10} CFU/mL). Groups of 3 mice were sacrificed at intervals after challenge. At each time point, the production of IL-17 and IFN γ by $\gamma\delta$ T cells and other CD3 cells was analysed by flow cytometry. The number of viable bacteria was estimated by performing colony counts on serially diluted lung homogenates from individual mice. FACS plots show percentages of cells in CD3 gate from individual mice (A). Lung bacterial loads are presented as mean CFU per lung (B). $n \geq 3$

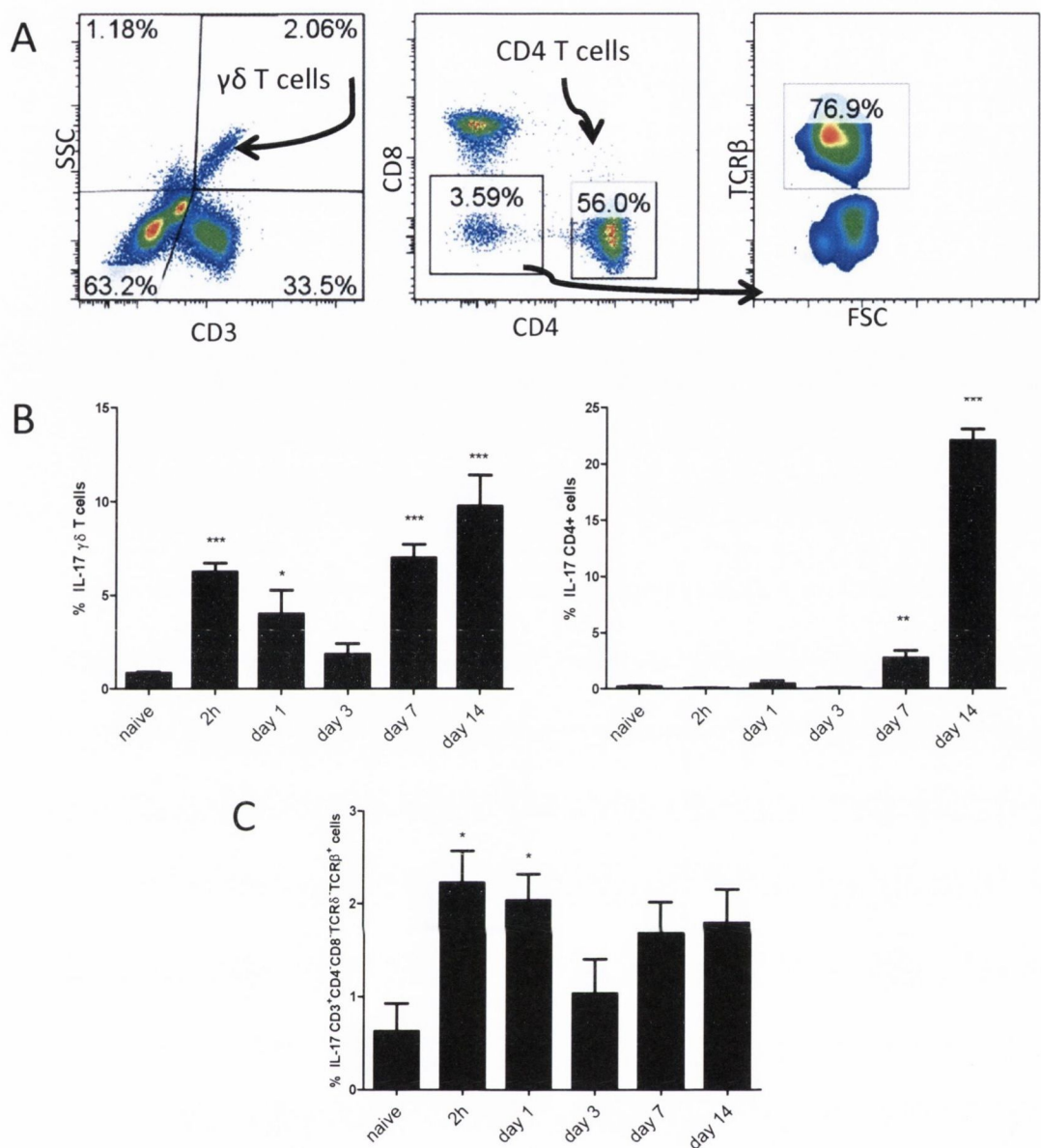
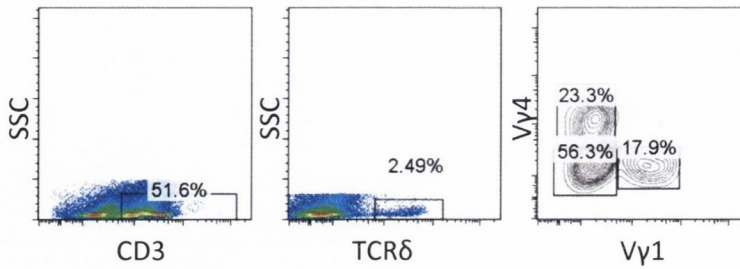


Figure 4.3 IL-17 producing $\gamma\delta$ cells are detected in the lung before the appearance of IL-17 producing CD4⁺ T cells.

C57BL/6 mice were challenged with virulent *B. pertussis* strain Bp338. They were sacrificed at different time points post infections. Lungs were digested for one hour with Collagenase IV and DNase I in the presence of BrfA (5 μ g/mL). The production of IL-17 by subsets of CD3⁺ cells was analysed by flow cytometry. The graphs show percentages of IL-17 positive cells in the $\gamma\delta$ T cell gate (A), CD4 gate (B) and CD3⁺CD4⁻CD8⁻TCR δ ⁺TCR β ⁺ gate (C). All samples were gated on single live cells. * p <0.05, ** p <0.01, *** p <0.001 by one way ANOVA with Dunnett's post test. $n \geq 3$

A)



B)

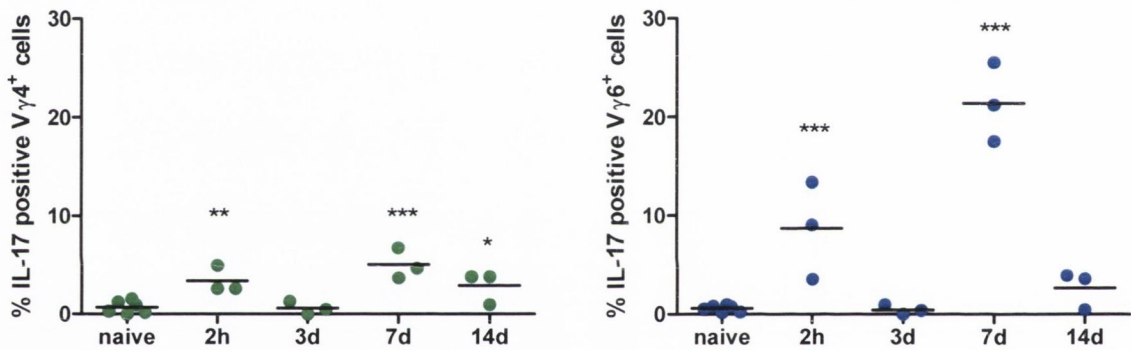


Figure 4.4 IL-17 production in the lung by $\gamma\delta$ T cell subsets.

C57BL/6 mice were challenged with virulent *B. pertussis* strain Bp338 and sacrificed at different time points post infection. Lungs were digested for one hour with Collagenase IV and DNase I in the presence of BrfA (5 μ g/mL). The production of IL-17 by $\gamma\delta$ T cell subsets was analysed by flow cytometry. The graphs show percentages of IL-17 positive cells in the V γ 4 $\gamma\delta$ T cell gate (A) , V γ 6 (V γ 4⁻V γ 1⁺) $\gamma\delta$ T cell gate (B). * p <0.05, ** p <0.01, *** p <0.001 by one way ANOVA with Dunnett's post test. $n \geq 3$

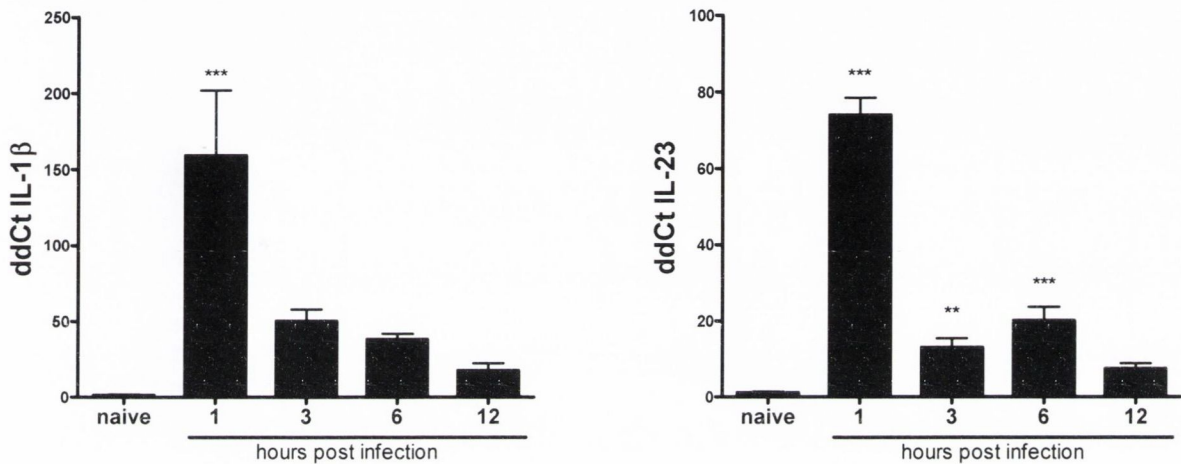


Figure 4.5 IL-1 β and IL-23 mRNA expression in the lungs of *B. pertussis* infected mice peaks at 1 hour post infection.

C57BL/6 mice were infected with virulent *B. pertussis* strain Bp338. Groups of mice were sacrificed at different intervals post infection and their lungs homogenised in Trizol. The RNA was then extracted and the expression of IL-1 β and IL-23p19 mRNA relative to naïve controls analysed by RT-PCR. The data were normalised to 18s RNA. *p<0.05, **p<0.01, ***p<0.001 by one way ANOVA with a Dunett's post test. n=1

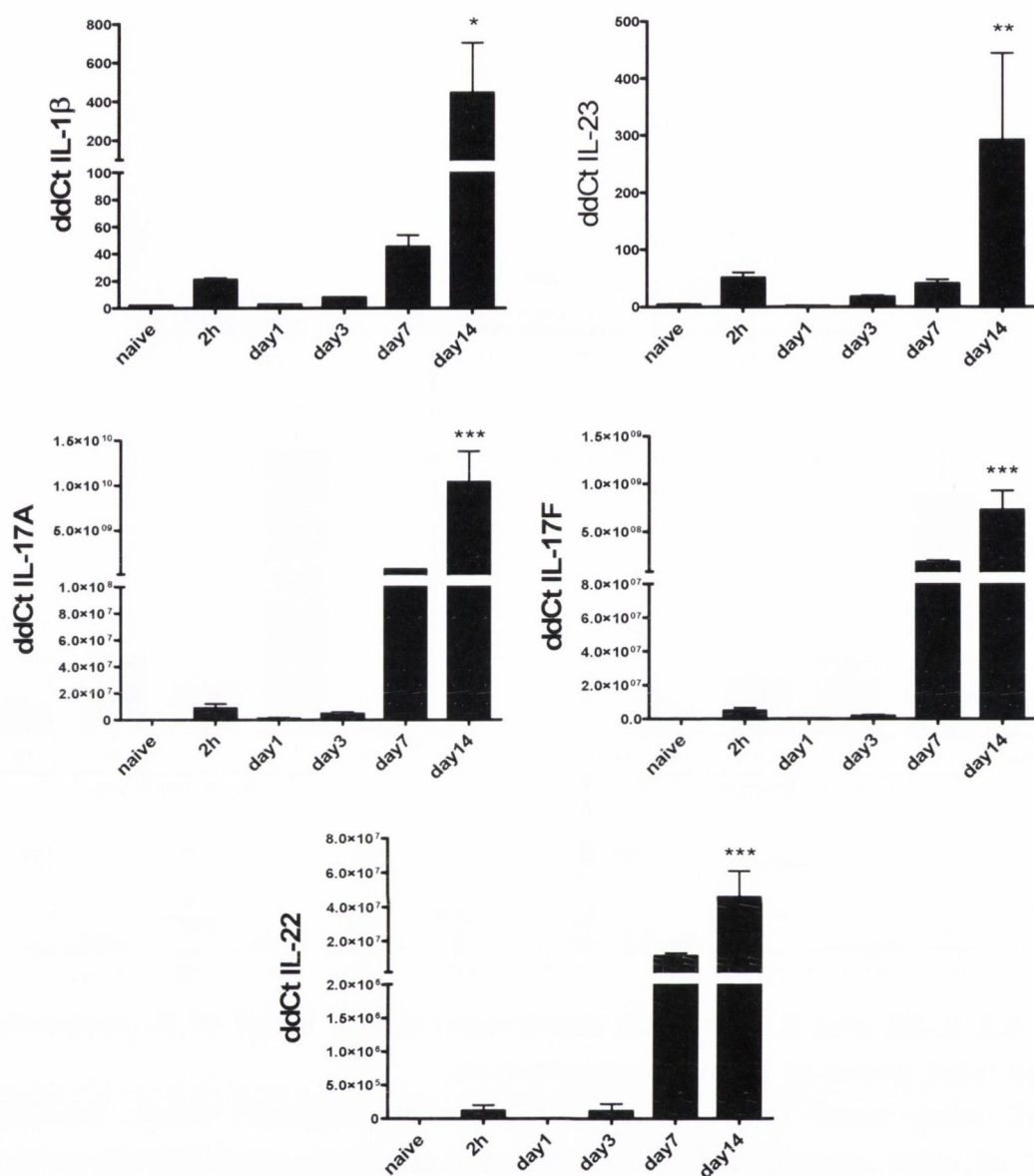


Figure 4.6 *B. pertussis* infection induces upregulation of proinflammatory cytokine genes in the lung.

C57BL/6 mice were infected with virulent *B. pertussis* strain Bp338. Groups of mice were sacrificed at different intervals post infection and their lungs homogenised in Trizol. The RNA was then extracted and the expression of IL-17A, IL-17F, IL-22, IL-1 β and IL-23p19 analysed by RT-PCR. *p<0.05, **p<0.01, ***p<0.001 by one way ANOVA with a Bartlett's post test. n $\geq$ 3

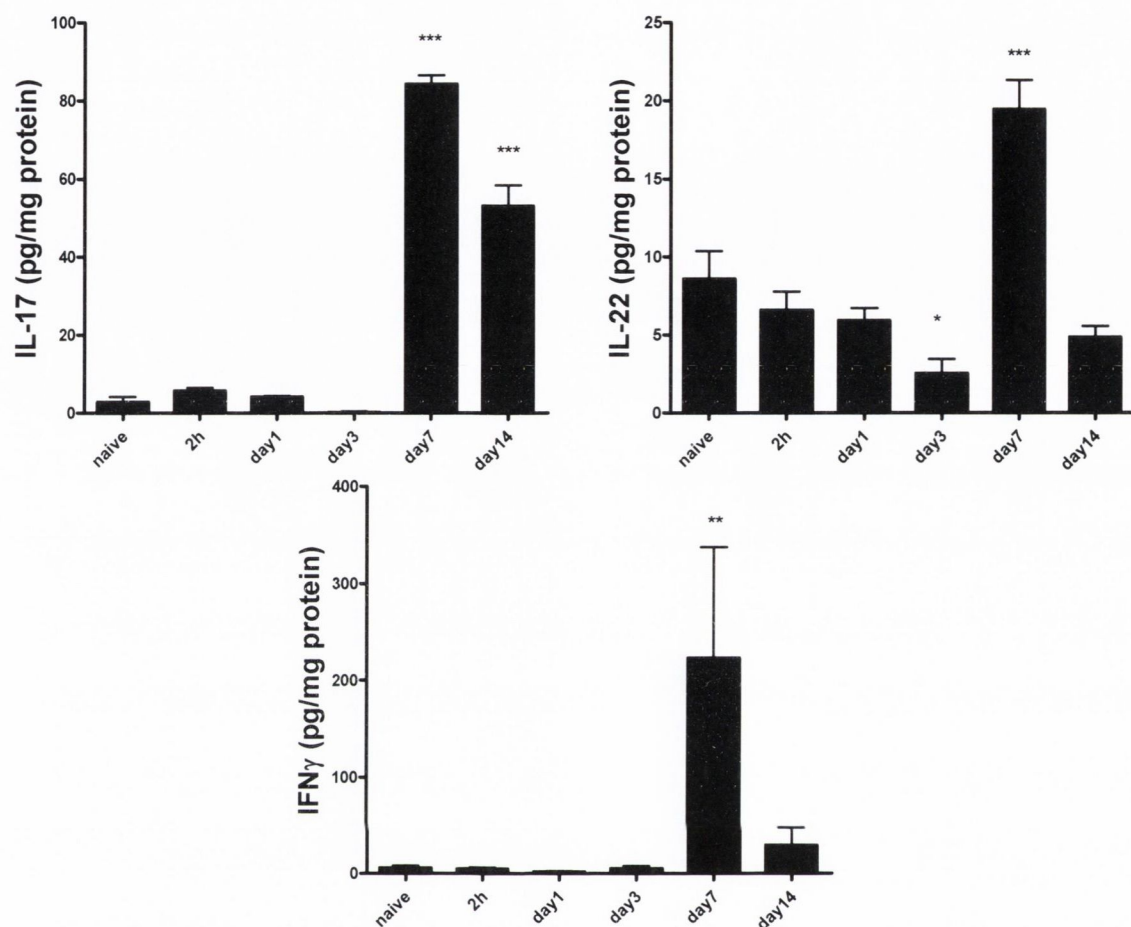


Figure 4.7 Cytokine levels in the lungs during infection with *B. pertussis*

Levels of cytokines in the lung homogenate supernatants from *Bordetella* infected C57BL/6 mice was measured by ELISA. The data was then normalised to the amount of protein in each sample measured by BCA assay. *p<0.05, **p<0.01, ***p<0.001 by one way ANOVA with Dunnett's post test. n≥3

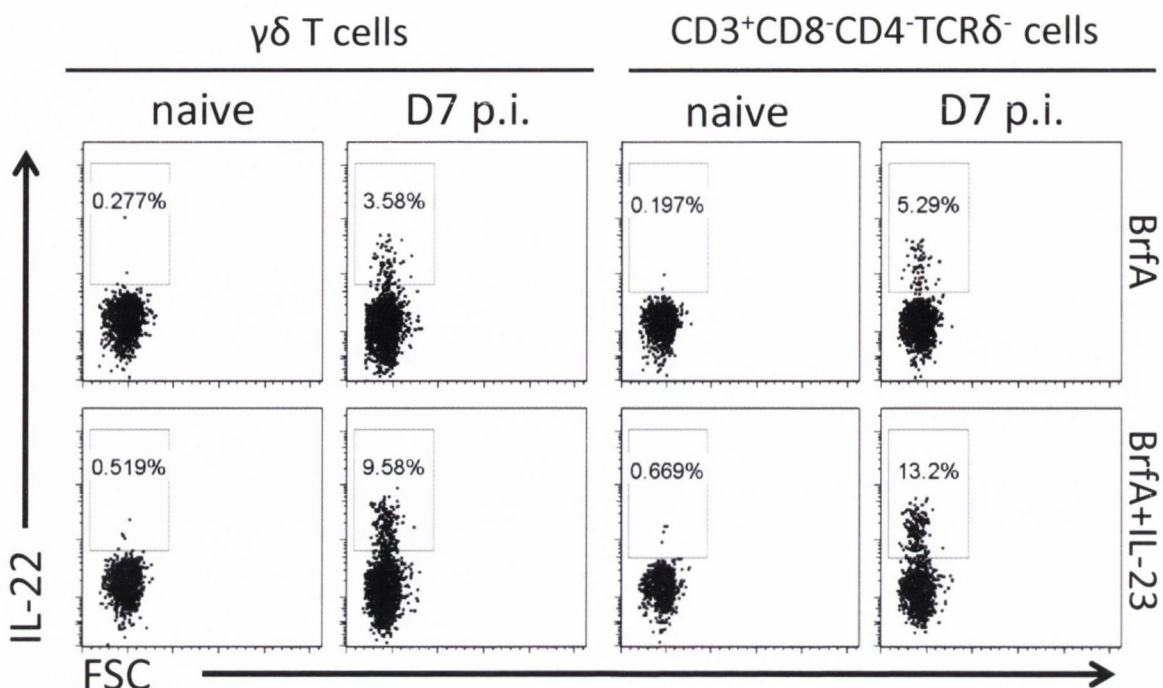


Figure 4.8 $CD3^+CD8^-CD4^-TCR\delta^-$ cells and $\gamma\delta$ T cells produce IL-22 in the lungs of *B. pertussis* infected mice

C57BL/6 mice were challenged with virulent *B. pertussis* strain Bp338. They were sacrificed at day 7 post infections. Lung lymphocytes from naïve and infected mice were then incubated for 5 hours in the presence of BrfA (5 μ g/mL) or BrfA + IL-23 (10 ng/mL). The production of IL-22 by $\gamma\delta$ T cells and NKT cells was analysed by flow cytometry. The plots show percentages of IL-22 positive cells in the $\gamma\delta$ T cell and $CD3^+CD8^-CD4^-TCR\delta^-$ cell gate. n=1

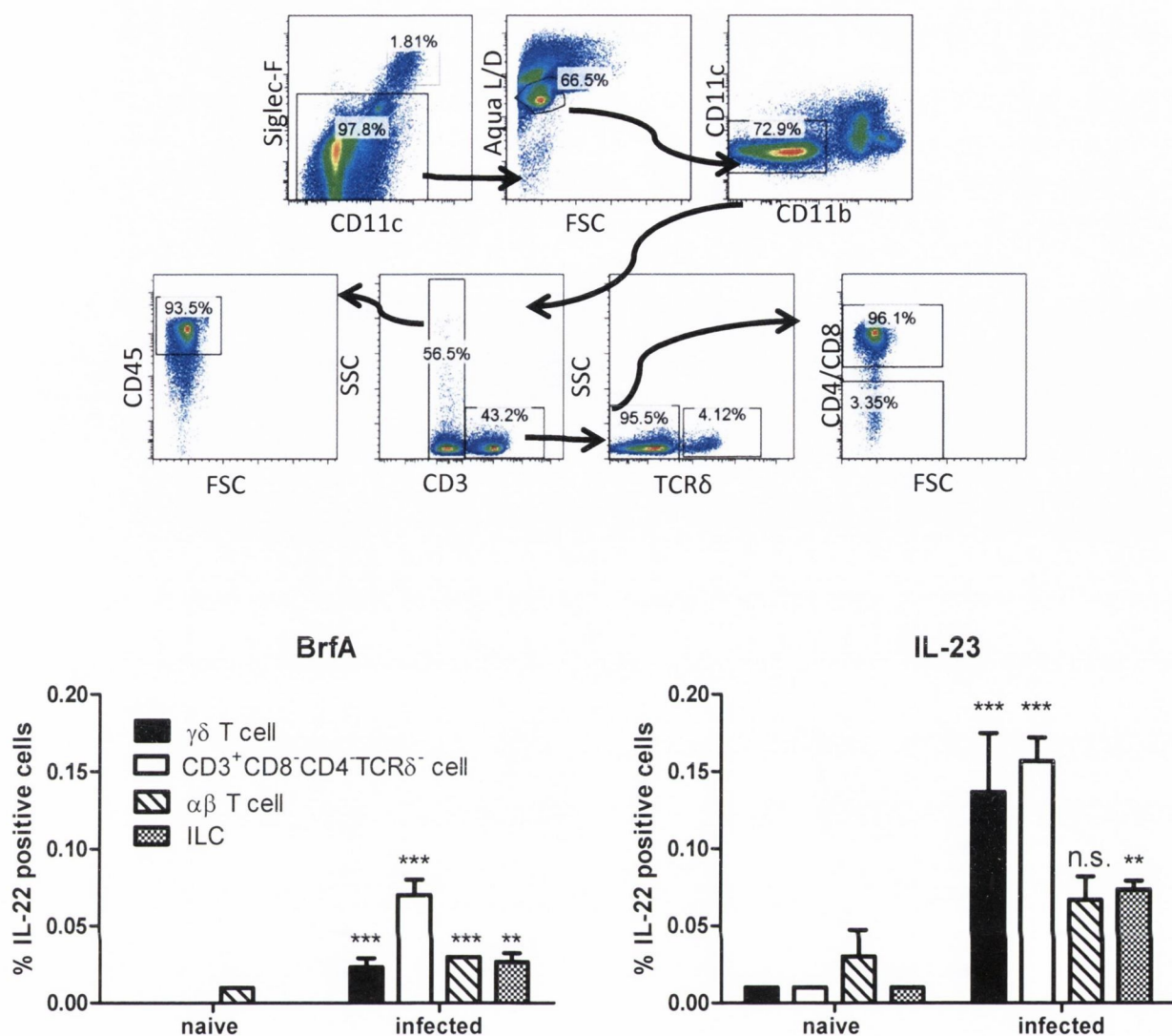


Figure 4.9 CD3⁺CD4⁻CD8⁻TCRδ⁺ cells are the dominant IL-22 producing population in the lung at day 7 post infection.

C57BL/6 mice were challenged with virulent *B. pertussis* strain Bp338 and sacrificed 7 days post infection. Lung lymphocytes from naïve and infected mice were incubated for 5 hours in the presence of BrfA (5 µg/mL) or BrfA + IL-23 (10 ng/mL). The production of IL-22 by γδ T cells and CD3⁺CD8⁻CD4⁻TCRδ⁻ cells was analysed by flow cytometry. The graphs show percentages of IL-22 positive cells in the γδ T cell, CD3⁺CD8⁻CD4⁻TCRδ⁻ cell, αβ T cell and ILC gates, expressed as percentage of total live cells. *p<0.05, **p<0.01, ***p<0.001, n.s. – not significant by Two-way Anova with Bonferroni post test. N=1

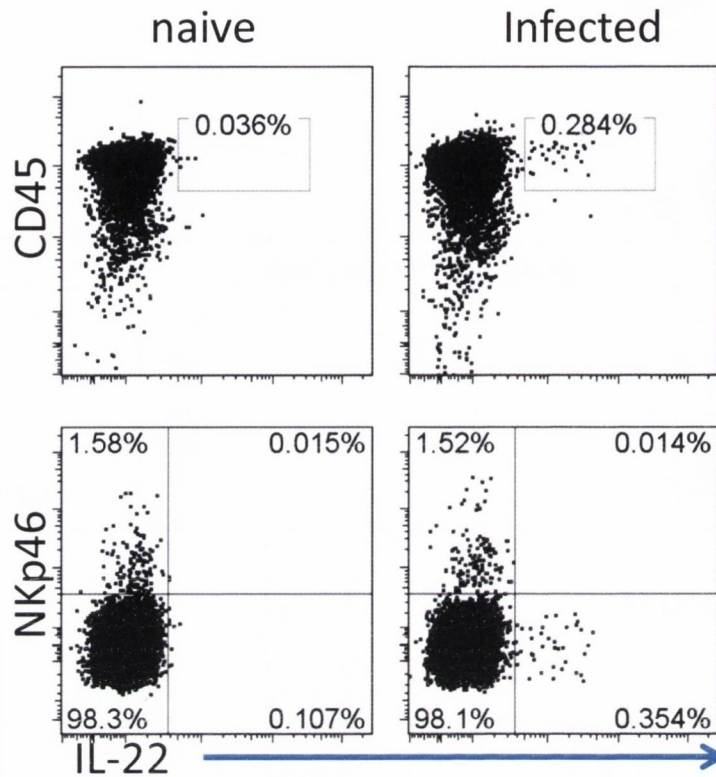


Figure 4.10 Innate lymphoid cells produce IL-22 in the lungs of *B. pertussis* infected lungs

C57BL/6 mice were challenged with virulent *B. pertussis* strain Bp338. The animals were sacrificed at 7 days post infections. Lung lymphocytes from naïve and infected mice were restimulated with IL-23 (10ng/mL) for 5 hours in the presence of BrfA (5 µg/mL). The production of IL-22 by Lin⁻ cells was analysed by flow cytometry. The graphs show percentages of IL-22 positive cells in the CD3⁻CD11b⁻CD11c⁻ (top panel) and CD3⁻CD11b⁻CD11c⁻ CD45⁺ (bottom panel) gate. N=1

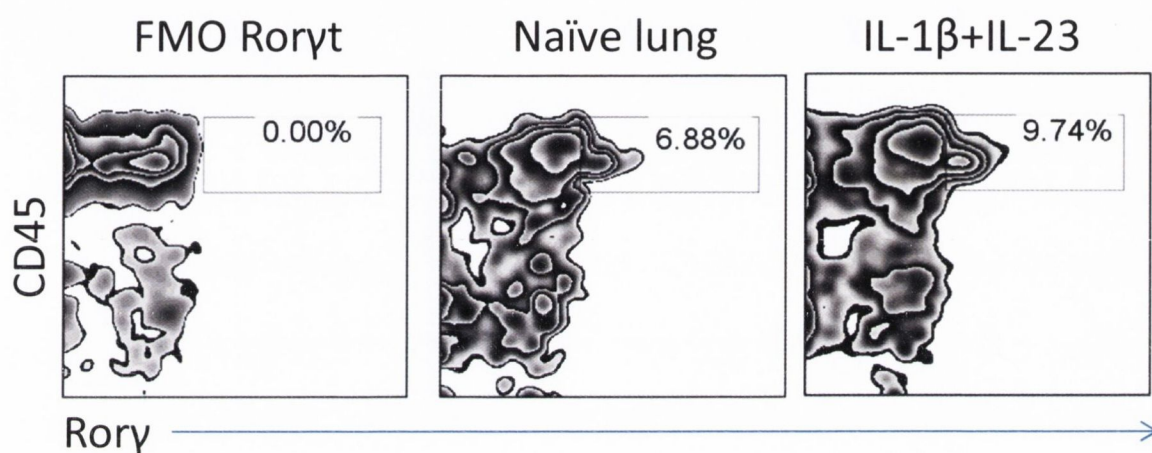


Figure 4.11 ILC3 cells are present in the lungs of naïve mice.

Lung mononuclear cells were isolated from C57BL/6 mice and the expression of ILC3 markers analysed by flow cytometry. Plots show percentages of cells in the CD3⁻CD19⁻CD11b⁻ gate. N=1

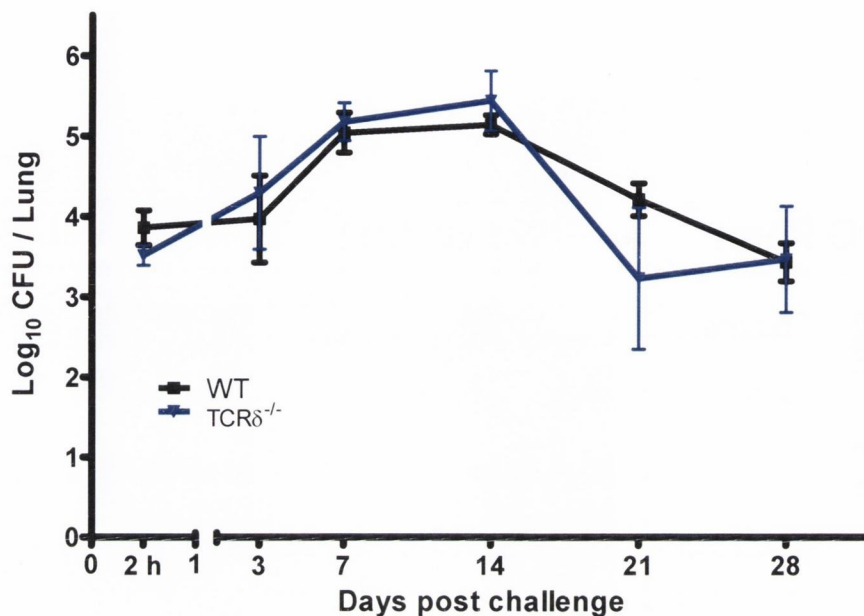


Figure 4.12 TCR $\delta^{-/-}$ do not show an impaired *B. pertussis* clearance compared to WT mice

C57BL/6 and TCR $\delta^{-/-}$ mice were challenged by aerosol with virulent Bp338 strain of *B. pertussis* (1×10^9 CFU/mL). Groups of 3 mice were sacrificed at 6 time points after challenge. The number of viable bacteria was estimated by performing CFU counts on serially diluted lung homogenates from individual mice. Results are presented as mean log₁₀ CFU per lung. N=1

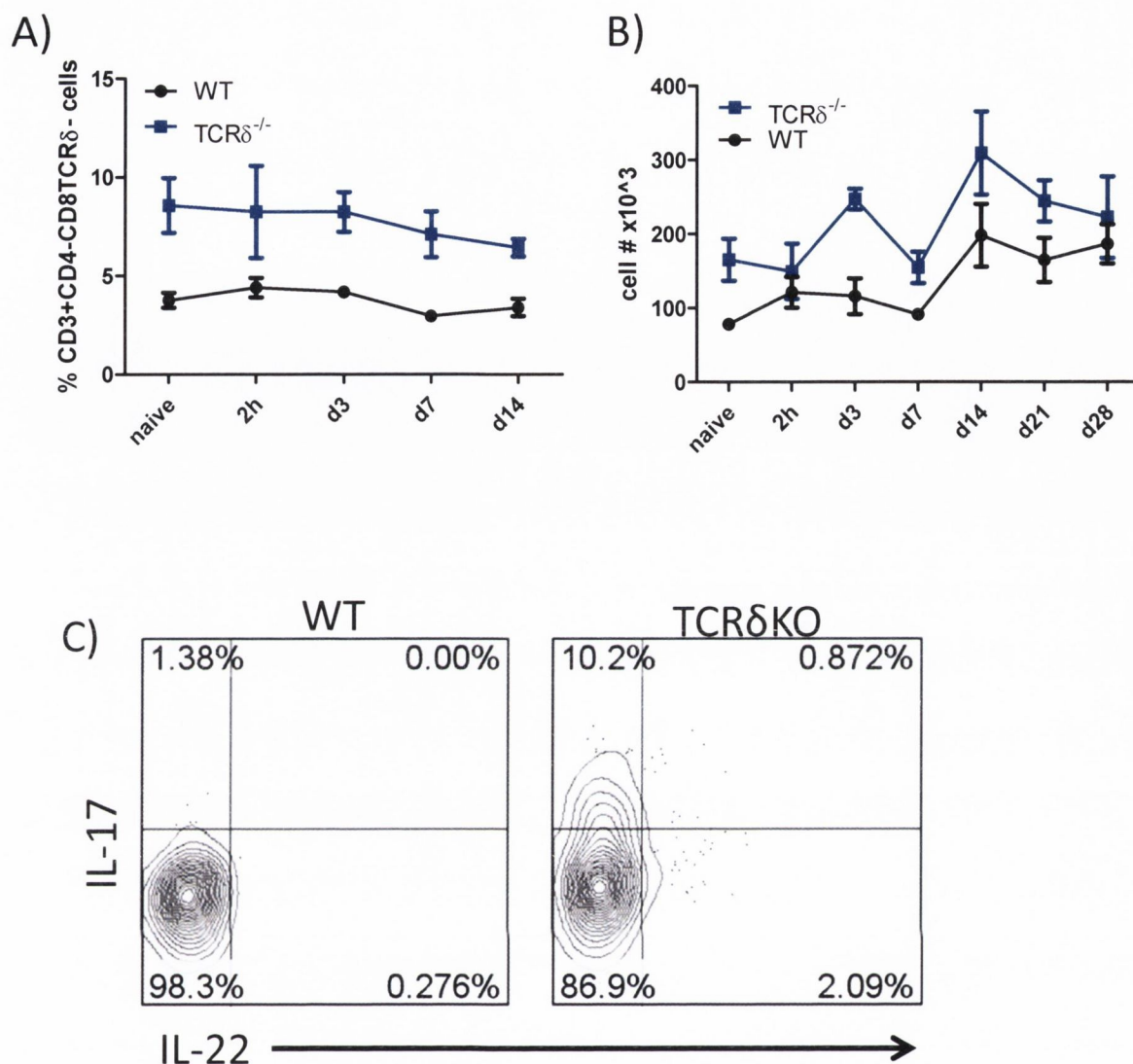


Figure 4.13 IL-17 secreting CD3⁺CD4⁺CD8⁻TCRδ⁻ cells are increased in TCRδ^{-/-} mice.

C57BL/6 and TCRδ^{-/-} mice were challenged by aerosol with virulent Bp338 strain of *B. Pertussis* (1x10⁹ CFU/mL). Groups of 3 mice were sacrificed at intervals after challenge. The lungs were digested in collagenase IV and DNase I. The expression of CD3, CD4, CD8, TCRδ, IL-17 and IL-22 was then analysed by FACS. Graphs show percentages (A), absolute numbers (B) and IL-17 and IL-22 production by CD3⁺CD4⁺CD8⁻TCRδ⁻ cells. N=1

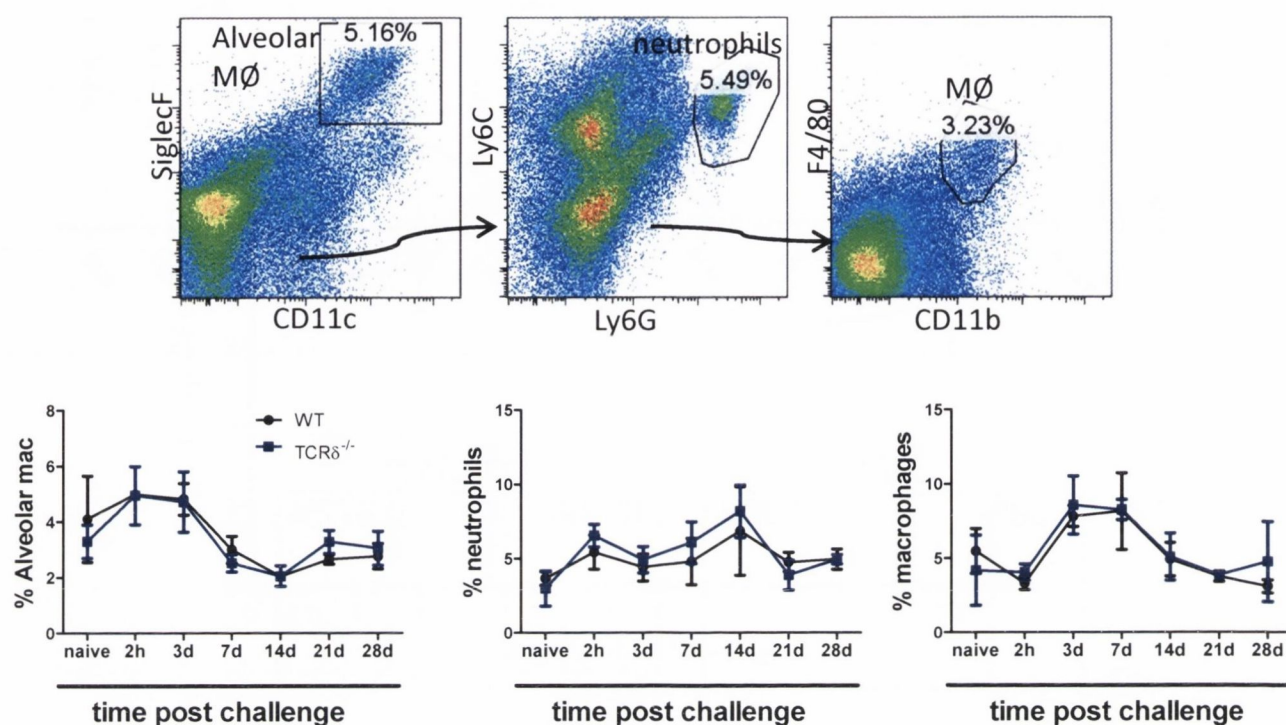


Figure 4.14 Normal numbers of neutrophils and macrophages are observed in *B. pertussis* infected TCRδ^{-/-} mice.

C57BL/6 and TCRδ^{-/-} mice were challenged by aerosol with virulent Bp338 strain of *B. pertussis* (1x10⁹ CFU/mL). Groups of 3 mice were sacrificed at intervals after challenge. The lungs were digested in collagenase IV and DNase I. The expression of neutrophil and macrophage markers was then analysed by FACS. Graphs show percentages of alveolar macrophages, neutrophils and macrophages in the live gate. N=1

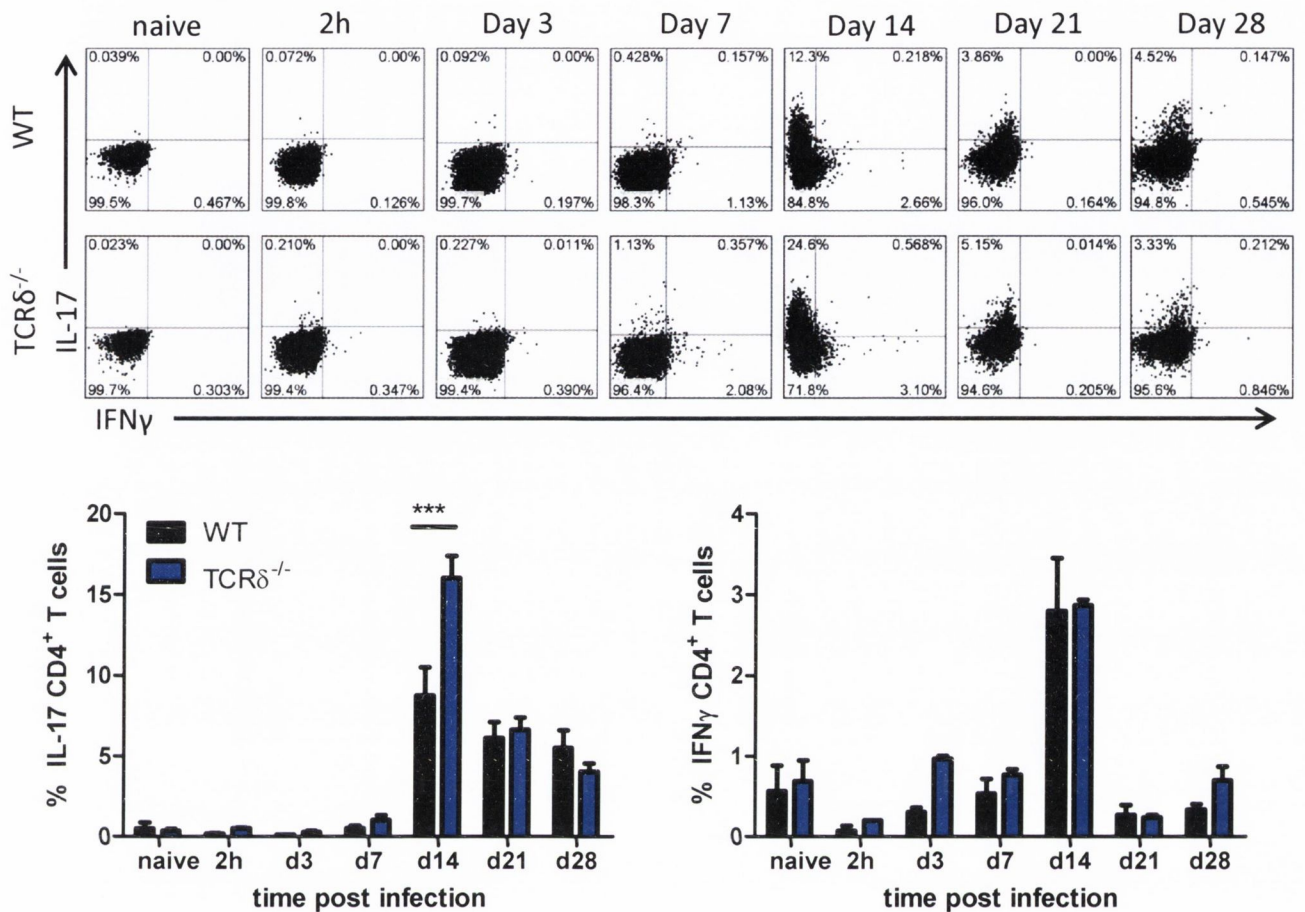


Figure 4.15 Enhanced IL-17 production by CD4 T cells in TCR $\delta^{-/-}$ mice in the lungs on day 14 post infection.

C57BL/6 and TCR $\delta^{-/-}$ mice were challenged by aerosol with virulent Bp338 strain of *B. pertussis* (1×10^9 CFU/mL). Groups of mice were sacrificed at intervals after challenge. The lymphocytes were then isolated and incubated with BrfA (5 μ g/mL) for 4 hours. The expression of IL-17 and IFN γ by CD4 T cells was then analysed by FACS. Graphs show percentages of IL-17 and IFN γ producing cells in the CD3 $^{+}$ CD4 $^{+}$ gate. *** p < 0.001 by a two way ANOVA with Bonferroni post test. N=1

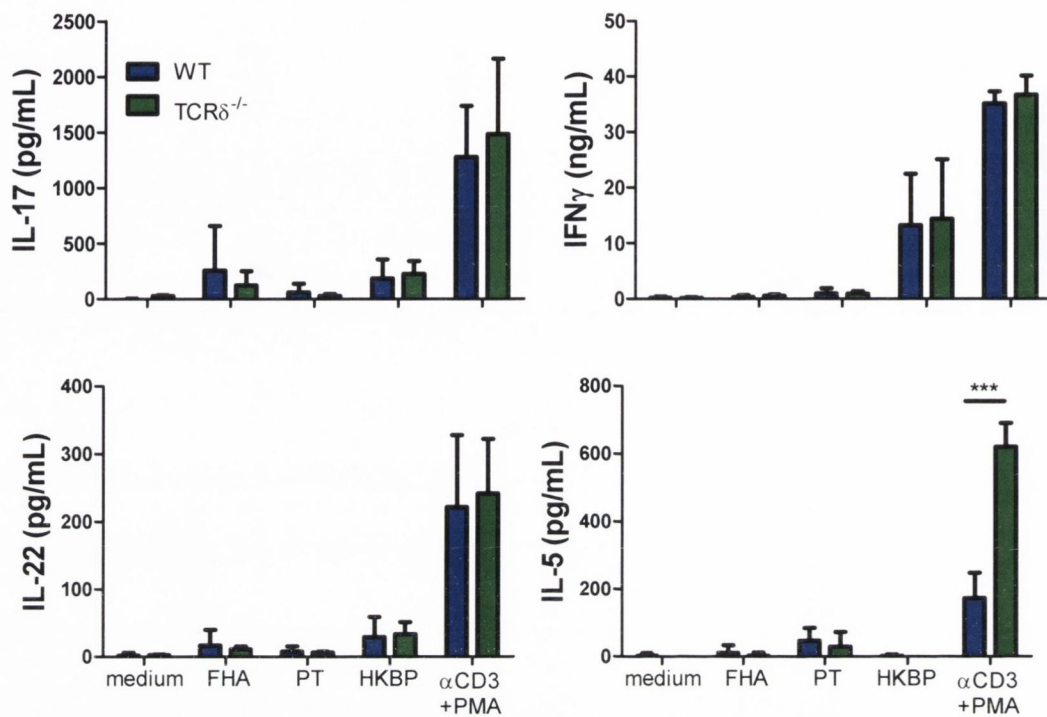


Figure 4.16 *B. pertussis* infected TCR $\delta^{-/-}$ mice do not have impaired antigen specific T cell responses .

C57BL/6 and TCR $\delta^{-/-}$ mice were challenged by aerosol with virulent Bp338 strain of *B. pertussis* (1×10^9 CFU/mL). At day 21 post infection spleen cells (1×10^6 cells/mL) were incubated with FHA (4 μ g/mL), heat inactivated PT (2 μ g/mL), HKBP (1×10^6 /mL) or with PMA (25 ng/mL)+ α CD3(1 μ g/mL). After 72 hours the concentration of IL-17, IL-22, IFN γ and IL-5 in the supernatants was analysed by ELISA. *** $p < 0.001$ by a two way ANOVA with Bonferroni post test. N=1

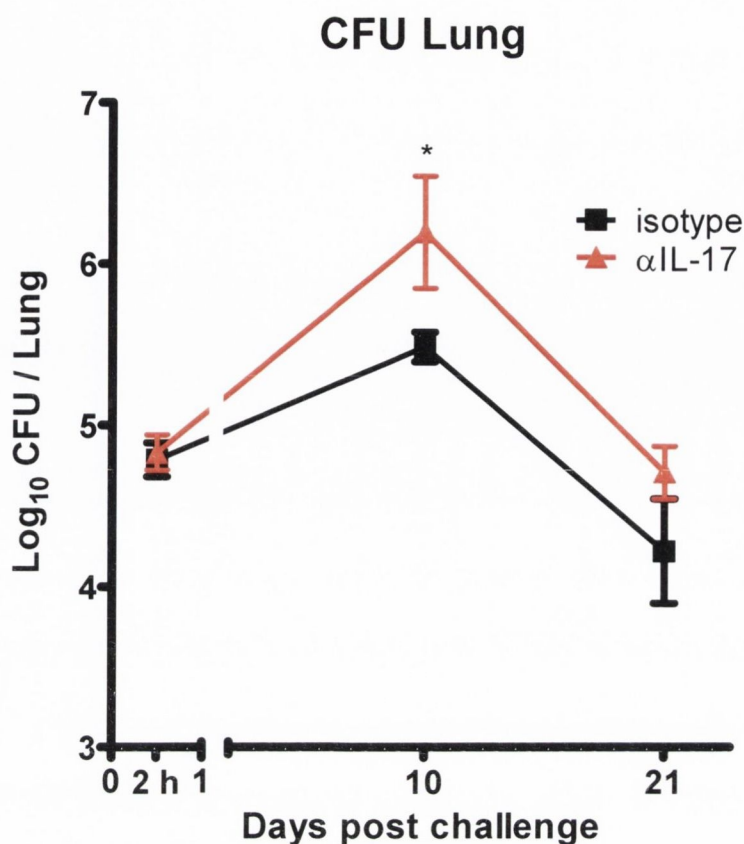


Figure 4.17 Neutralisation of IL-17 at the early stages of infection delays *B. pertussis* clearance

C57BL/6 mice were challenged by aerosol with virulent Bp338 *B. pertussis* strain (1×10^9 CFU/mL) and treated with either anti-IL-17A (α IL-17) or an isotype matched control antibody (isotype) one day prior and two days after the challenge. Groups of 3 mice were sacrificed at 2h, day 10 and day 21 after challenge. The number of viable bacteria in the lungs was estimated by performing CFU counts on serially diluted lung homogenates from individual mice. Results are presented as mean log₁₀ CFU per lung. * $p < 0.05$ by a two way ANOVA with Bonferroni post test. N=1

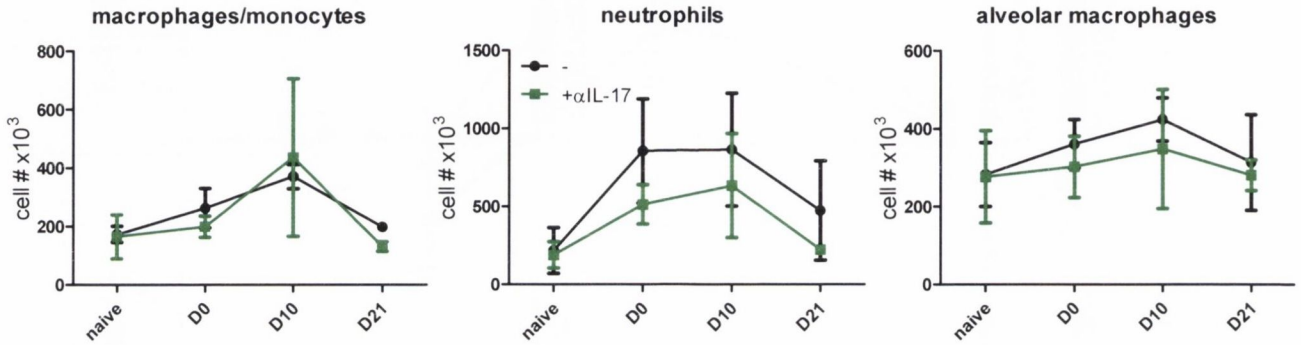


Figure 4.18 Neutralisation of IL-17 at the early stages of infection does not result in significantly impaired neutrophil infiltration to the lung

C57BL/6 mice were challenged by aerosol with virulent Bp338 *B. pertussis* strain (1×10^9 CFU/mL) and treated with either anti-IL-17A (α IL-17) or an isotype matched control antibody (isotype) one day prior and two days after the challenge. Groups of 3 mice were sacrificed at 2h, day 10 and day 21 after challenge. Lung mononuclear cells were isolated and the numbers of macrophages, neutrophils and alveolar macrophages analysed by FACS. Graphs show absolute numbers of cells in the CD11b⁺F4/80⁺ (macrophages/monocytes), CD11b⁺Ly6G⁺Ly6C⁺ (neutrophils) and CD11c⁺SiglecF⁺ (alveolar macrophages) gate. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by a two way ANOVA with Bonferroni post test. N=1

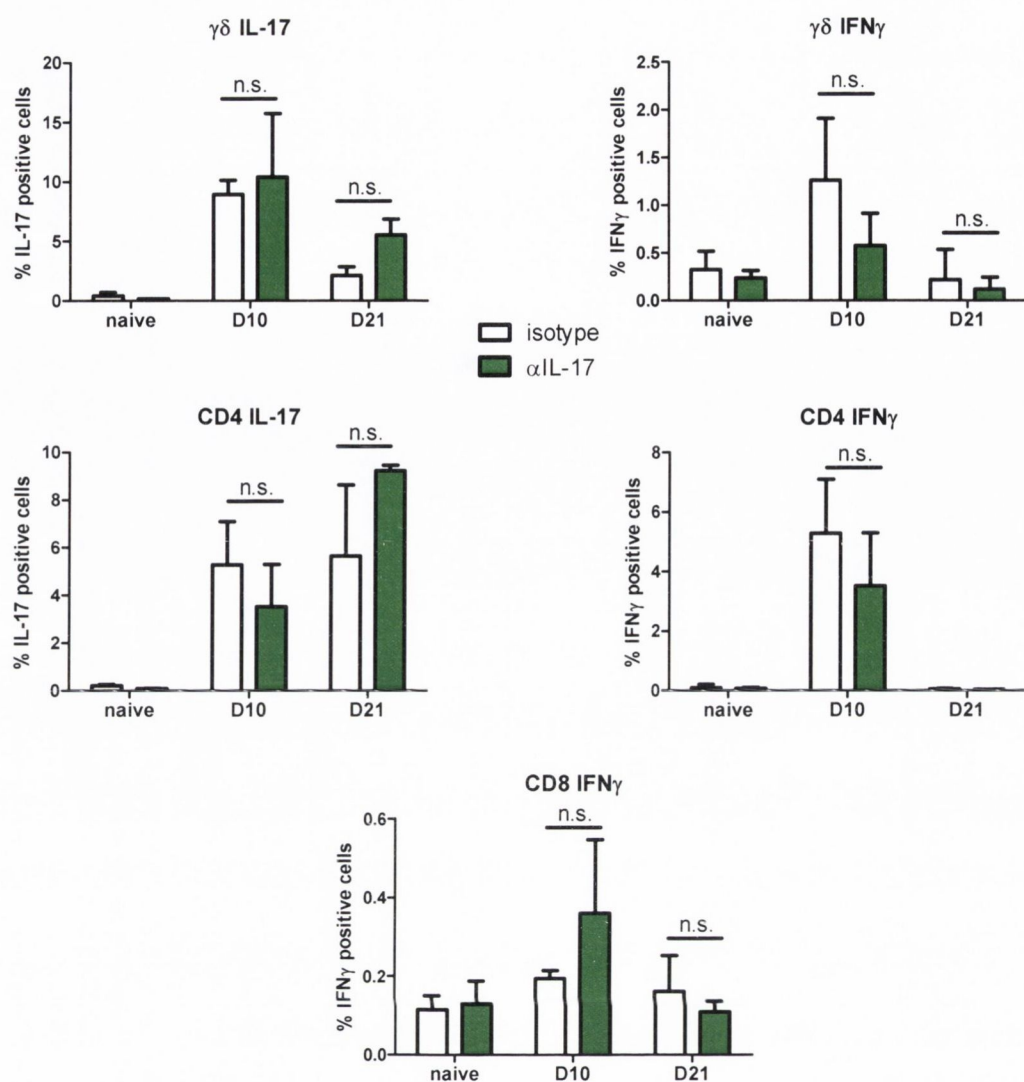


Figure 4.19 Neutralisation of innate IL-17 does not lead to a significant change in the percentage of IL-17 and IFN γ producing T cells at the later stages of the infection.

C57BL/6 mice were challenged by aerosol with virulent Bp338 *B. pertussis* strain (1×10^9 CFU/mL) and treated with either anti-IL-17A (αIL-17) or an isotype matched control antibody (isotype) one day prior and two days after the challenge. Groups of 3 mice were sacrificed at 2h, day 10 and day 21 after challenge. Lung lymphocytes were then isolated and incubated in BrfA (5ug/mL) for 4 hours). The production of IL-17 and IFN γ by αβ and γδ T cells was then examined by FACS. Graphs show percentages of cytokine positive cells in the CD3⁺CD8⁺, CD3⁺CD4⁺ and CD3⁺TCRδ⁺ gates. n.s.-not significant by two way ANOVA with a Bonferroni post test. N=1

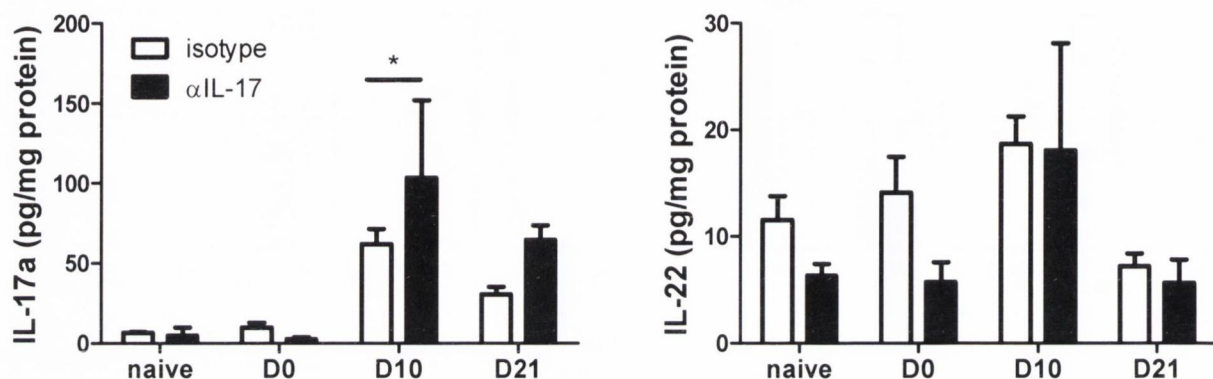


Figure 4.20 Neutralisation of innate IL-17 leads to an increase in the concentration of IL-17 in the lungs at later infection stages.

C57BL/6 mice were challenged by aerosol with virulent Bp338 *B. pertussis* strain (1×10^9 CFU/mL) and treated with either anti-IL-17A (α IL-17) or an isotype matched control antibody (isotype) one day prior and two days after the challenge. Groups of 3 mice were sacrificed at 2h, day 10 and day 21 after challenge. Concentration of IL-17a and IL-22 in the lung homogenate was then analysed by ELISA and the data normalised to the total amount of protein in the sample. * $p < 0.05$ by a two way ANOVA with a Bonferroni post test. N=1

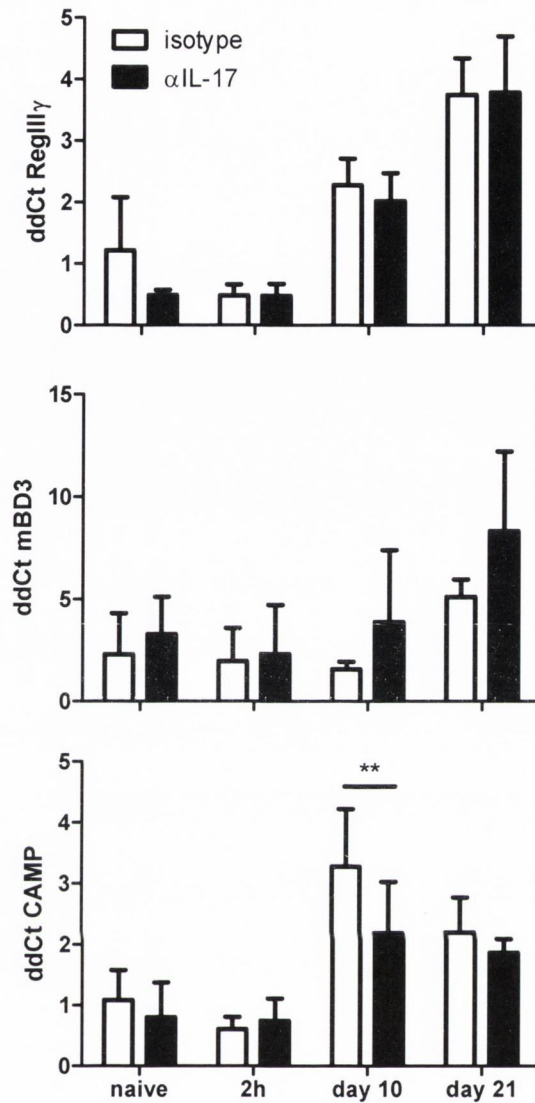


Figure 4.21 Neutralisation of innate IL-17 leads to a decreased expression of CAMP in the lungs of *B. pertussis* infected mice.

C57BL/6 mice were challenged by aerosol with virulent Bp338 *B. pertussis* strain (1×10^9 CFU/mL) and treated with either anti-IL-17A (α IL-17) or an isotype matched control antibody (isotype) one day prior and two days after the challenge. Groups of 3 mice were sacrificed at 2h, day 10 and day 21 after challenge and their lungs homogenised in Trizol. The RNA was then extracted and the expression of RegIII γ , mBD3 and CAMP analysed by RT-PCR. ** $p < 0.01$ by a two way ANOVA with Bonferroni post test. N=1

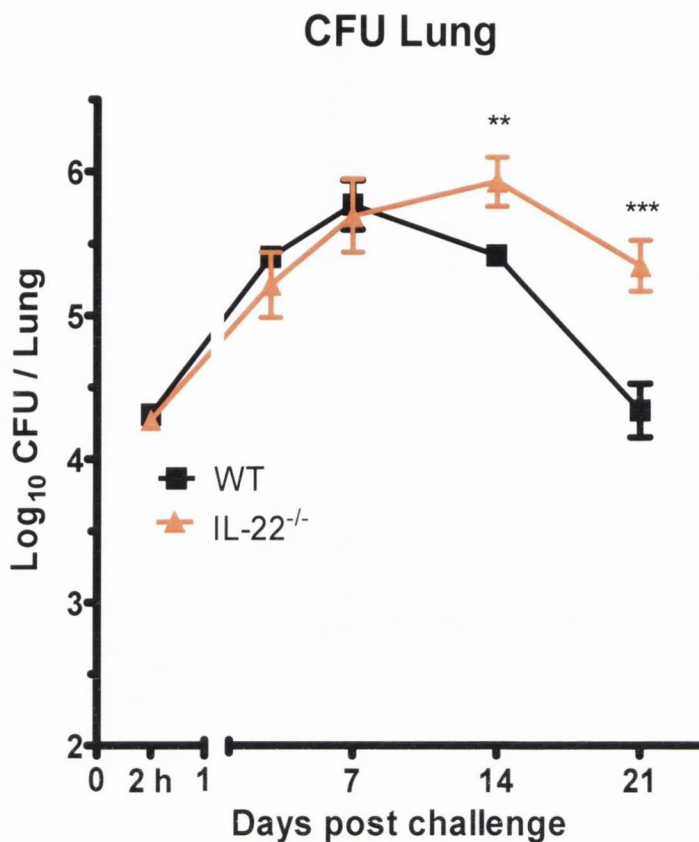


Figure 4.22 Delayed *B. pertussis* clearance in IL-22^{-/-} mice.

C57BL/6 and IL-22^{-/-} mice were challenged by aerosol with virulent Bp338 strain of *B. pertussis* (1x10⁹ CFU/mL). Groups of 4 mice were sacrificed at intervals after challenge. The number of viable bacteria was estimated by performing CFU counts on serially diluted lung homogenates from individual mice. Results are presented as mean log₁₀ CFU per lung. **p<0.01, ***p<0.001 by a two way ANOVA with Bonferroni post test. N=3

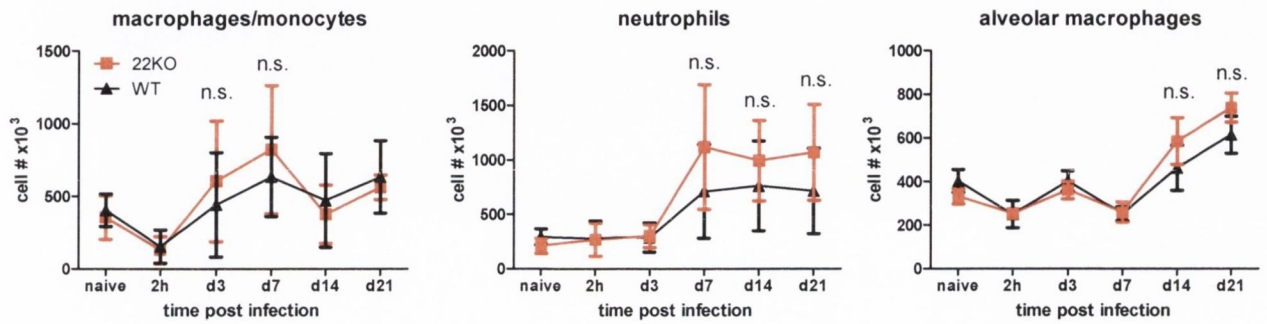


Figure 4.23 IL-22^{-/-} mice do not show impaired infiltration of macrophages and neutrophils to the lung

C57BL/6 and IL-22^{-/-} mice were challenged by aerosol with virulent Bp338 *B. pertussis* strain (1×10^9 CFU/mL). Groups of 3 mice were sacrificed at various time points after challenge. Lung mononuclear cells were isolated and the numbers of macrophages, neutrophils and alveolar macrophages analysed by FACS. Graphs show combined data of two independent experiments. absolute numbers of cells in the CD11b⁺F4/80⁺ (macrophages/monocytes), CD11b⁺Ly6G⁺Ly6C⁺ (neutrophils) and CD11c⁺SiglecF⁺ (alveolar macrophages) gate. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, n.s. – not significant, by a two way ANOVA with Bonferroni post test. N=2

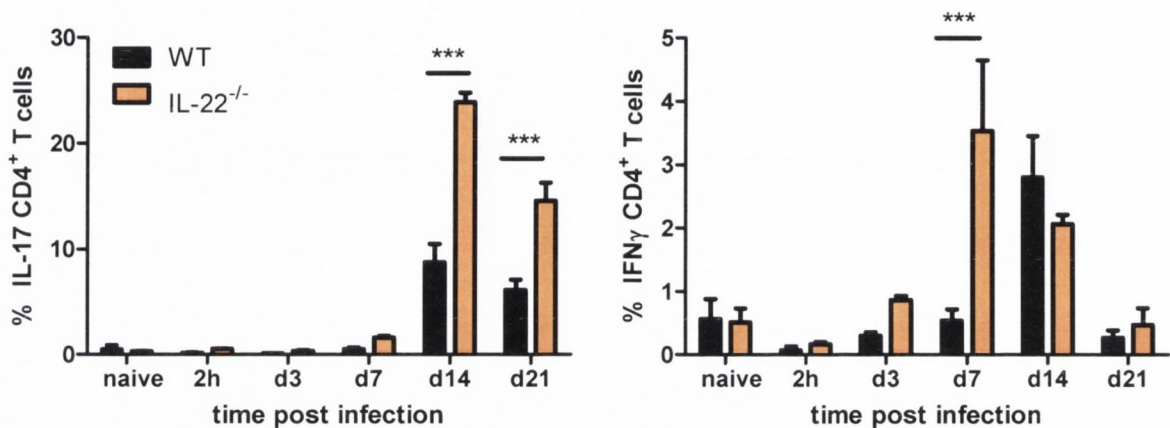


Figure 4.24 *B. pertussis* infected IL-22^{-/-} mice have increased IL-17 and IFN γ production by T cells in the lung.

C57BL/6 and IL-22^{-/-} mice were challenged by aerosol with virulent Bp338 strain of *B. pertussis* (1x10⁹ CFU/mL). Groups of 3 mice were sacrificed at time points after challenge. Lung lymphocytes were isolated and incubated with BrfA (5 μ g/mL) for 4 hours. The expression of IL-17 and IFN γ by CD4 T cells was then analysed by FACS. Graphs show percentages of cytokine positive cells in the CD3⁺CD4⁺ gate. *p<0.05, **p<0.01, ***p<0.001 by a two way ANOVA with Bonferroni post test. N=2

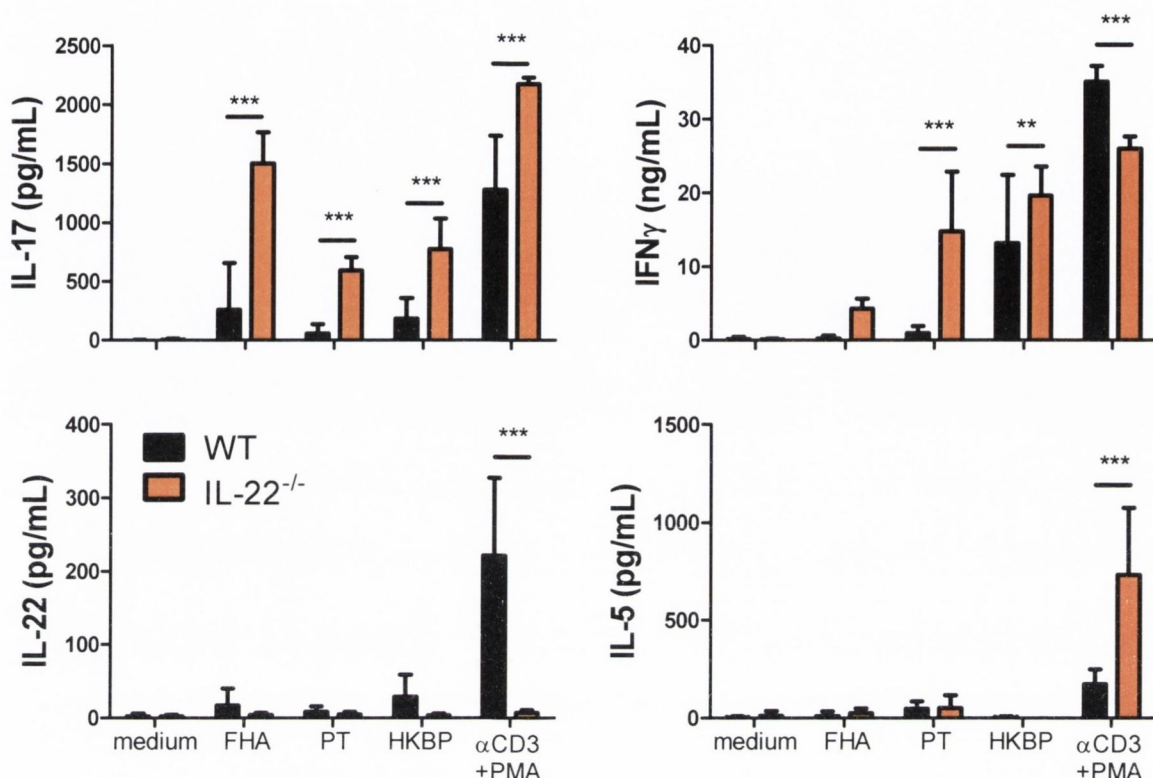


Figure 4.25 *B. pertussis* infected IL-22 deficient mice have increased IL-17 and IFN γ T cell responses to *B. pertussis* antigens.

C57BL/6 and IL-22^{-/-} mice were challenged by aerosol with virulent Bp338 strain of *B. pertussis* (1×10^9 CFU/mL). At day 21 post infection spleen cells (1×10^6 cells/mL) were incubated with FHA (4 μ g/mL), heat inactivated PT (2 μ g/mL), HKBP (1×10^6 /mL) or with PMA (25 ng/mL)+ α CD3 (1 μ g/mL). After 72 hours the concentration of IL-17, IL-22 and IFN γ in the supernatants was analysed by ELISA. **p < 0.01, ***p < 0.001 by a two way ANOVA with Bonferroni post test. N=1

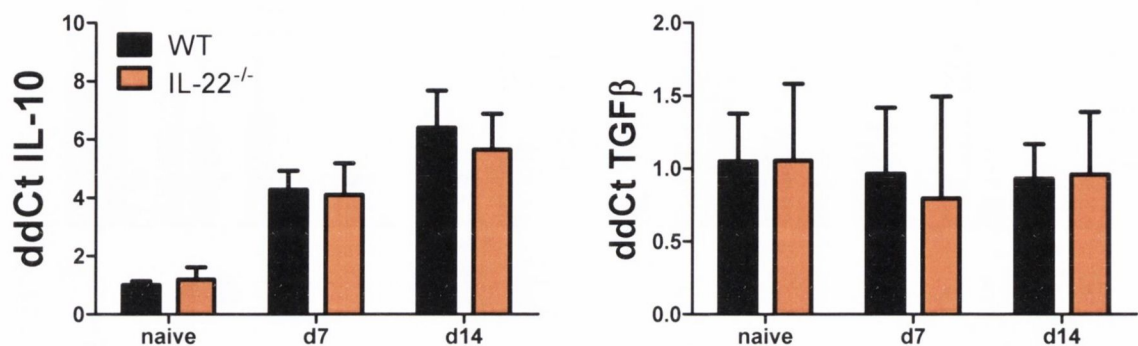


Figure 4.26 Expression of the anti-inflammatory cytokines, IL-10 and TGFβ, is not impaired in *B. pertussis* infected IL-22^{-/-} mice.

C57BL/6 and IL-22^{-/-} mice were challenged by aerosol with virulent Bp338 strain of *B. pertussis* (1x10⁹ CFU/mL). Groups of 4 mice were sacrificed at time points after challenge and their lungs homogenised in Trizol. The RNA was then extracted and the expression of IL-10 and TGFβ analysed by RT-PCR. *p<0.05, **p<0.01, ***p<0.001 by a two way ANOVA with Bonferroni post test. N=1

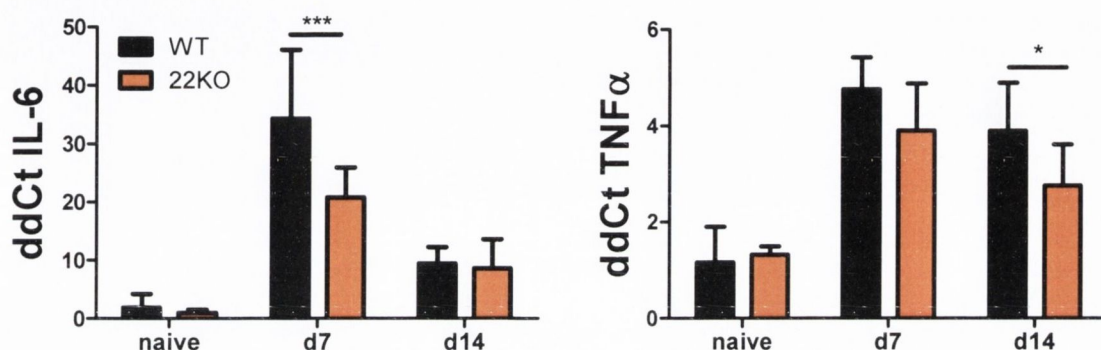


Figure 4.27 Expression of pro-inflammatory cytokines, IL-6 and TNFα, is reduced in *B. pertussis* infected IL-22^{-/-} mice.

C57BL/6 and IL-22^{-/-} mice were challenged by aerosol with virulent Bp338 strain of *B. pertussis* (1x10⁹ CFU/mL). Groups of 4 mice were sacrificed at time points after challenge and their lungs homogenised in Trizol. The RNA was then extracted and the expression of IL-6 and TNFα analysed by RT-PCR. *p<0.05, ***p<0.001 by a two way ANOVA with Bonferroni post test. N=1

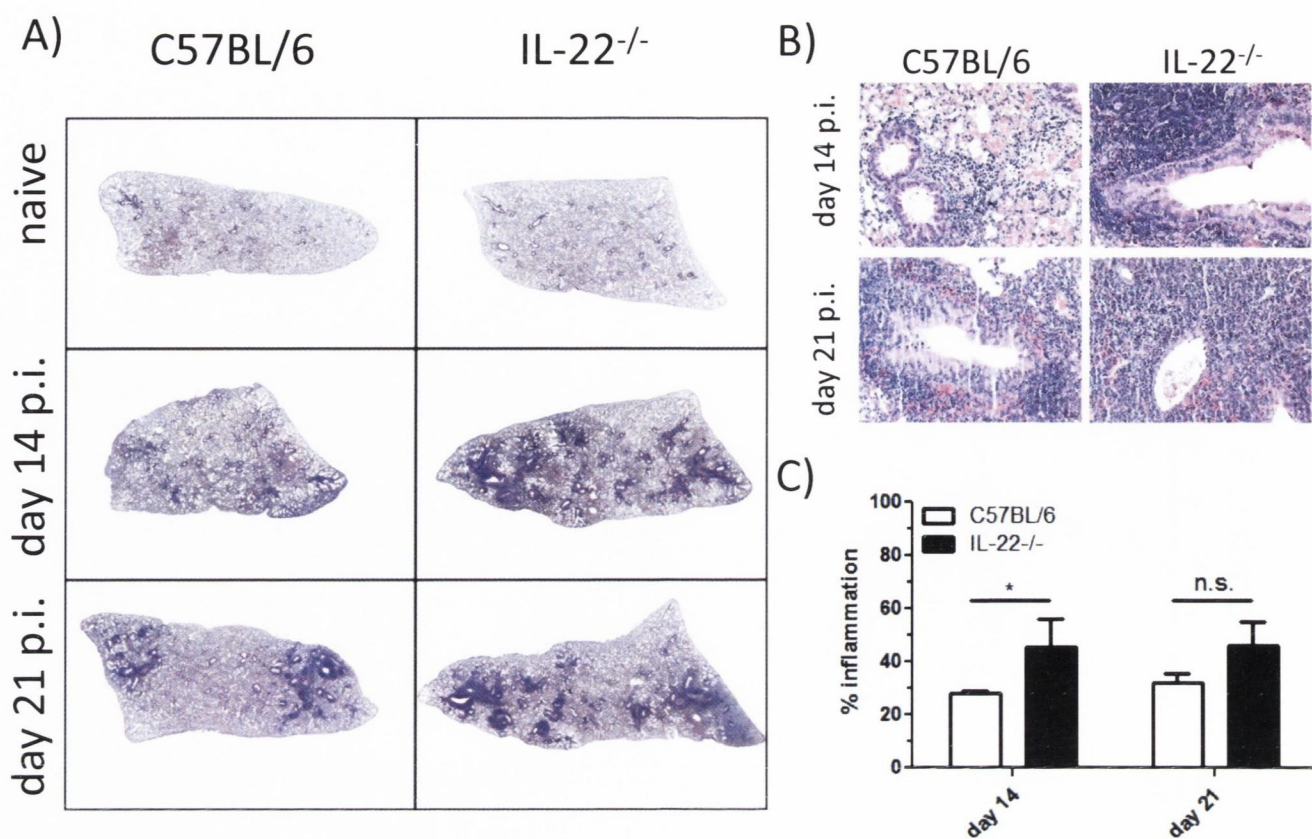


Figure 4.28 IL-22 deficient mice show more lung pathology during *B. pertussis* infection

C57BL/6 and IL-22^{-/-} mice were challenged by aerosol with virulent Bp338 strain of *B. pertussis* (1×10^9 CFU/mL). Lung tissue sections were prepared at various time points post infection and stained with H&E. A) Representative lung sections showing gross pathology, B) 20x magnification of inflamed areas, C) Percentage lung inflammation in each group. * $p < 0.05$, n.s. not significant by a two way ANOVA with Bonferroni post test. N=2

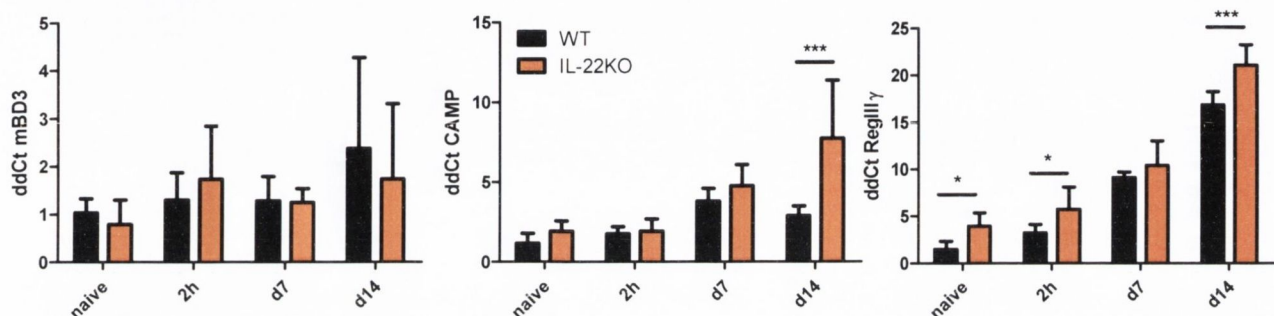


Figure 4.29 *B. pertussis* infected IL-22^{-/-} mice do not show decreased antimicrobial peptide expression.

C57BL/6 and IL-22^{-/-} mice were challenged by aerosol with virulent Bp338 strain of *B. pertussis* (1x10⁹ CFU/mL). Groups of 4 mice were sacrificed at time points after challenge and their lungs homogenised in Trizol. The RNA was then extracted and the expression of RegIIIγ, mBD3 and CAMP analysed by RT-PCR. Statistical significance of data was analysed by a two way ANOVA with Bonferroni post test. *p<0.05, **p<0.01, ***p<0.001. N=3

Chapter 4.3: Discussion

The data in chapter 3 showed that lung $\gamma\delta$ T cells become rapidly activated and secrete IL-17 *in vitro* in response to live *B. pertussis* stimulation. The relevance of these findings to a mouse model of *B. pertussis* respiratory infection was therefore investigated. It was found, that $\gamma\delta$ T cells produce IL-17 in the lung as early as 2 hours post infection. The IL-17 production gradually declined as bacteria were multiplying and was almost undetectable on day 3 post infection. Similar kinetics of IL-17 production were observed in CD3⁺CD4⁻CD8⁻TCR δ ⁻ cells, although a much lower percentage of these cells produced IL-17 compared to $\gamma\delta$ T cells. *B. pertussis* can exist in three phases: virulent, intermediate and avirulent phase. Avirulent phase is induced under conditions of stress such as low temperatures or nutrient deprivation and in the presence of high concentrations of nicotinic acid and sulphates (Nakamura et al, 2006). In the avirulent phase the expression of most of *B. pertussis* virulence factors is repressed. In the intermediate phase, on the other hand, *B. pertussis* expresses some of the early virulence factors such as FHA, while in the virulent phase the expression of late virulence factors, such as ACT and PT, is switched on (Nakamura et al, 2006; Scarlato et al, 1991). Both PT and ACT have been implicated in the inhibition neutrophil recruitment and IL-17 production (Andreasen & Carbonetti, 2008; Andreasen et al, 2009; Henderson et al, 2012). It is likely that upon entry into the airways the intermediate phase is induced in bacteria in order to allow for expression of survival factors and adaptation to the new environment. The drop in IL-17 on day 3 post infection is probably caused by increased production of ACT and PT as *B. pertussis* becomes established in the airways and exponentially increases in numbers. Although PT has been shown to repress early IL-17 production, it has been also implicated in the promotion of secretion of this cytokine at the later stages of infection and PT deficient bacteria failed to induce upregulation of IL-17 expression in the lungs on day 7 post infection (Andreasen et al, 2009). Similarly, ACT has been found to contribute to the induction of IL-17 producing cells (Dunne et al, 2010; Henderson et al, 2012). The effect of these *B. pertussis* produced toxins may depend on their local concentration in the lung as well as on the cell types they intoxicate. In agreement with findings of Andreasen et al., IL-17 production was found to increase again on day 7 post

infection, coinciding with the peak of bacteraemia, and both CD4⁺ and $\gamma\delta$ T cells were found to produce this cytokine. CD4⁺ T cells were found to be the dominant source of IL-17 on day 14 post infection. Although early IL-17 becomes quickly inhibited, IFN γ production by CD3⁺ cells could be detected at all of the time points tested. Furthermore, IFN γ production in the lung was found to peak on day 7 and then strongly decrease on day 14 post infection. This sharp pick of IFN γ production in the lung was observed both at the protein and mRNA level and coincided with the onset of clearance of bacteria.

It was found that both V γ 6 and V γ 4 cells secreted IL-17 in the lung. A higher percentage of IL-17-producing cells was detected among the V γ 6 subset at the early time points post infection. However, V γ 4 $\gamma\delta$ T cells also contributed to its production. Gene expression analysis in the lung tissue revealed that both IL-1 β and IL-23 were strongly induced at 1 hour post infection and their expression levels fluctuated similar to those of IL-17a, IL-17f and IL-22. The induction of IL-1 β and IL-23 explains why, in contrast to in vitro data, V γ 4 T cell produced IL-17 in the lungs in vivo.

V γ 6 $\gamma\delta$ T cells have been found to contribute to protective responses against bacteria through production of IL-17 in a number of disease models. For example, Simonian et al. found that lung V γ 6 $\gamma\delta$ T cells were the main IL-17 producing subset in a mouse model of bacteria induced hypersensitivity pneumonitis and contributed to bacterial clearance (Simonian et al, 2009). V γ 6 $\gamma\delta$ T cells have also been found to secrete the majority of IL-17 during early stages of bacterial infection in the peritoneal cavity and the liver (Hamada et al, 2008; Shibata et al, 2007). In addition, pulmonary V γ 6 cells were shown to produce IL-22 in the lung and prevent inflammation-induced pulmonary fibrosis (Simonian et al, 2010). In the current study IL-22 was found to peak in the lung on day 7 post infection and a number of types of lymphocytes were found to contribute to its production. In contrast to findings of Simonian et al, CD3⁻CD4⁻CD8⁻TCR δ ⁻ cells were found to be the major IL-22 source in the lungs of *B. pertussis* infected mice, although $\gamma\delta$ T cells, ILC3 and CD4 T cells were also found to contribute. Similarly, NKT cells have been reported to be the major producer of IL-22 in the lung during influenza infection and lung ILC3 cells were found to produce IL-22 during *Streptococcus pneumoniae* infection (Paget et al, 2012; Van Maele et al, 2014)

Production of IL-17, IL-22 and IFN γ was also detected in the whole lung homogenate but did not reach significance until day 7 post infection. Nonetheless it is possible that significant cytokine concentrations may be generated locally prior to reaching global significance in the tissue. Interestingly, while a high concentration of IL-17 was detected also on day 14 post infection, IL-22 and IFN γ protein concentration was found to fall dramatically after the peak on day 7. A similar observation was made by Andreasen and colleagues who also observed a sharp peak of IFN γ production in the lungs on day 7 post infection. The importance of IL-17 in the defences against *B. pertussis* has been shown in IL-17 $^{-/-}$ mice which had significantly increased bacterial burden in the lungs (Dunne et al, 2010). However, in another study intranasal administration of anti-IL-17 led to only a moderate increase in the *B. pertussis* counts (Andreasen et al, 2009). Furthermore, although neutrophil recruitment has been shown to be dependent on early IL-17 production in the lung, neutrophils have not been found to play a significant role in the primary *B. pertussis* infection (Andreasen & Carbonetti, 2008; Andreasen & Carbonetti, 2009; Andreasen et al, 2009). It therefore still remains unclear how if at all $\gamma\delta$ T cell produced IL-17 contributes to innate immune responses against *B. pertussis*.

In order to establish whether the early $\gamma\delta$ T cell responses in the lung play an important role in the immunity to *B. pertussis*, the course of *B. pertussis* infection in TCR $\delta^{-/-}$ mice was investigated. Surprisingly, no difference in clearance of bacteria was observed between TCR $\delta^{-/-}$ and WT mice. This is in stark contrast to findings of Zachariadis et al, who observed a significantly reduced bacterial burden in TCR $\delta^{-/-}$ mice (Zachariadis et al, 2006). Upon closer investigation of innate-like lymphocytes in the lung, it was found that there was an increase in the percentage and absolute number of CD3 $^{+}$ CD4 $^{-}$ CD8 $^{-}$ TCR δ^{-} cells in TCR $\delta^{-/-}$ mice. Furthermore, a greater percentage of these cells was found to produce IL-17 and IL-22 in TCR $\delta^{-/-}$ mice compared to the same population in WT mice. Therefore, it was concluded that the lack of difference in bacterial clearance may be due to a compensatory mechanism of the expanded CD3 $^{+}$ CD4 $^{-}$ CD8 $^{-}$ TCR δ^{-} population, which probably constituted mostly of NKT cells. Nonetheless, some subtle differences between WT and TCR $\delta^{-/-}$ mice were observed at the later time points during infection. For example, a significantly greater percentage of CD4 T cells was found to produce IL-17 in the lungs of TCR $\delta^{-/-}$ mice on day 14 post infection. Furthermore, a significantly increased IL-5

concentration was detected in supernatants of spleen cells from infected TCR $\delta^{-/-}$ mice in response to polyclonal stimulation with anti-CD3+PMA. This is in partial agreement with the study of Zachariadis et al. who also observed increased Th2 responses and greater inflammation in TCR $\delta^{-/-}$ mice. In contrast to the current , they also observed increased infiltration of neutrophils and macrophages to the lung at the early stages of the infection in TCR $\delta^{-/-}$ mice (Zachariadis et al, 2006). Increased macrophage numbers due to decreased apoptosis were observed in *Listeria* infected TCR $\delta^{-/-}$ mice, resulting in heightened inflammation (Egan & Carding, 2000). In the same study, $\gamma\delta$ T cells were shown to directly kill activated macrophages and their cytotoxicity was dependent on TCR signalling. Although no difference in the infiltration of neutrophils and macrophages was observed in the current study, it is possible that $\gamma\delta$ T cells have a role in limiting T cell mediated inflammation in the lung through a similar mechanism.

It is still unclear whether TCR signalling is required for $\gamma\delta$ T cell development in the thymus. Furthermore, a recent study of Koenecke and colleagues, has shown that anti-TCR δ antibody treatment does not deplete these cells but generates an “invisible” $\gamma\delta$ T cell population (Koenecke et al, 2009). It is possible that the ablation of TCR δ chain expression does not result in the absence of functional $\gamma\delta$ T cells, but merely leads to generation of TCR negative $\gamma\delta$ T cells which are still responsive to stimulation through other receptors. As a consequence studies involving TCR $\delta^{-/-}$ mice would not be looking at the role of the absence of $\gamma\delta$ T cells, but would be isolating the role of TCR $\gamma\delta$ signalling in activation of $\gamma\delta$ T cells from that of other stimuli. This possibility could be investigated by performing PCR analysis of V γ chain expression by CD3⁺CD4⁺CD8⁻TCR δ^{-} cells from TCR $\delta^{-/-}$ mice. Whether this hypothesis proves to be right or not, the current findings show that TCR $\delta^{-/-}$ model is not appropriate for assessing the role of $\gamma\delta$ T cells in the lung.

Given that CD3⁺CD4⁺CD8⁻TCR δ^{-} cells in the TCR $\delta^{-/-}$ mice appeared to compensate for the absence of functional $\gamma\delta$ T cells, the role of $\gamma\delta$ T cells was investigated by focusing on the main cytokines produced by these cells. Previous work in the lab has shown that IL-17 $^{-/-}$ mice have delayed clearance of *B. pertussis* from the lungs. However, studying the infection in complete knock outs does not separate the role of innate cells from that of antigen-specific CD4 T cells. IL-17 production by $\gamma\delta$ T cells has been reported to be important for shaping the responses of $\alpha\beta$ T cells in a

number of diseases models (Hou et al, 2013; Ma et al, 2011; Sutton et al, 2009). I therefore decided to take a cytokine neutralising approach to remove IL-17 only at the early time points of infection corresponding to the absence of IL-17 producing $\gamma\delta$ and $CD3^+CD4^-CD8^-TCR\delta^-$ cells. It was found that anti-IL-17 treated mice had significantly higher bacterial counts on day 10 post infection. This is in contrast to findings of Andreassen et al. who observed only a moderate increase in the CFU counts in antibody treated mice (Andreassen et al, 2009). However, in contrast to the protocol employed here, Andreassen and colleagues administered the first antibody dose on day 3 post infection and thus would have failed to inhibit the first peak of IL-17 production by $\gamma\delta$ T cells. Furthermore, in contrast to their findings, only a small reduction in neutrophil infiltration was observed in the current study. It therefore appears that while early IL-17 is contributing to control of bacterial growth in the lung, it is not the major factor required for the early influx of neutrophils.

Neutralisation of the early IL-17 production was found to result in a non-significant decrease in the percentage of IL-17 and IFN γ producing CD4 T cells in the lung on day 10 post infection. On day 21, on the other hand, the percentage of IL-17 producing $\gamma\delta$ and CD4 T cells was found to be increased. The total lung IL-17 protein was found to be increased both on day 10 and 21, with a significant difference on day 10. IL-17 has been shown to inhibit its own production via a negative feedback loop mechanism (Smith et al, 2008). IL-17 produced by $\gamma\delta$ T cells has also been implicated in promoting IL-17 production by CD4 T cells (Sutton et al, 2009). It is possible that the increase in IL-17 production in the lung at the later stages of infection is due to a “frustrated” IL-17 production by $\gamma\delta$ T cells in the absence of the negative feedback signal. Perhaps a conditional IL-17 knock out would be a better system for investigating the role of IL-17 produced by $\gamma\delta$ T cells.

One of the functions of IL-17 is to promote antimicrobial peptide production by epithelial cells (Liang et al, 2006; Robinson et al, 2014; Steinstraesser et al, 2011; Wolk et al, 2004). A porcine homologue of mouse β -defensin 3 (mBD3) has been shown to confer protection against *B. pertussis* in newborn piglets (Elahi et al, 2006). It is probable that this antimicrobial peptide is also important for the defences against *B. pertussis* in mice. Mouse BD1 was shown to be important for the clearance of *Haemophilus influenzae* from the lung and LL37, a human analogue of CAMP, prevented *Pseudomonas aeruginosa* invasion of lung epithelial cells by increasing

epithelial cell stiffness and decreasing transepithelial permeability (Byfield et al, 2011; Moser et al, 2002). Recently RegIII γ , an antimicrobial peptide typically associated with the gut, has also been implicated in innate immune defences in the lung (Choi et al, 2013). It was therefore investigated whether mice treated with anti-IL-17 showed impaired expression of mBD3, RegIII γ and CAMP. Although IL-17 has previously been shown to induce the expression of mBD3, IL-17 neutralisation at the early stages of *B. pertussis* infection was found not to have any impact on mBD3 gene induction in the lung (Kao et al, 2004). RegIII γ expression was also unaffected. In contrast, CAMP induction was significantly decreased in the lungs of anti-IL-17 treated mice on day 10 post infection. Robinson et al. have recently shown that TNF α induces significant expression of CAMP in the airway epithelial cells which can be enhanced by concomitant stimulation with IL-17 (Robinson et al, 2014). Therefore, it is likely, that neutralisation of IL-17 resulted in reducing CAMP expression to the levels induced by TNF α alone.

Given that IL-22 is produced by $\gamma\delta$ T cells and other innate like lymphocytes in the lungs of *B. pertussis* infected mice, it was investigated whether this cytokine had an important role in the immune responses against *B. pertussis*. IL-22^{-/-} mice were found to have increased counts and delayed clearance of bacteria from the lungs. However, no difference in the two CFU curves was observed at the early time points post infection, prior to the IL-22 peak. Similar to the current findings, IL-22 was shown to have a protective role in other Gram negative bacterial infections in the lung (Aujla et al, 2008; Peng et al, 2014) However, in *Klebsiella pneumoniae* infection a significant CFU difference was observed already at 24 hours post infection and the protective effect of IL-22 was much greater than in the current study. The course of Chlamydia induced pneumonia in anti-IL-22 treated mice, on the other hand, resembled more closely the course of *B. pertussis* infection in IL-22^{-/-} animals. In *Mycobacterium tuberculosis* infection, on the other hand, IL-22 appeared to be dispensable for protection.(Behrends et al, 2013) The differences in the contribution of IL-22 to protection against the different pulmonary pathogens are likely due to the different degree of severity of infection in mice and the life style of bacteria. It appears there is a greater protective role for IL-22 when the pathogen is strictly extracellular, as in case of *K. pneumoniae*.

The fact that absence of IL-22 signalling led to impaired pathogen clearance at the later stages of infection suggested that there was a defect in adaptive immune responses in IL-22^{-/-} mice. The cytokine production by CD4 T cells in the lungs of WT and IL-22^{-/-} mice was therefore investigated. Mice deficient in IL-22 were found to have more than two fold greater percentage of IL-17 producing CD4 T cells on day 14 and 21 post infection. The percentage of IFN γ producing cells was also significantly increased on day 7. In addition, it was found that IL-22 deficiency resulted in increased systemic antigen specific IL-17 and IFN γ responses. Interestingly, spleen cells from IL-22^{-/-} mice produced significantly greater amounts of IL-5 in response to polyclonal stimulation with α CD3 and PMA. This is in agreement with studies implicating IL-22 in limiting Th2 responses in allergic airway inflammation (Takahashi et al, 2011; Taube et al, 2011). In contrast to the current findings, IL-22 has been shown to promote IL-17 and IFN γ production in the lungs and spleen of *Chlamydia muridarum* infected mice and to exacerbate IL-17 mediated inflammation in bleomycin induced acute lung injury (Peng et al, 2014; Sonnenberg et al, 2010). Sonnenberg et al. suggested that pro-inflammatory versus tissue regenerative role of IL-22 is modulated by IL-17 with the proinflammatory function dominating in the presence of IL-17 (Sonnenberg et al, 2010). However, the findings of the current study suggest that in *B. pertussis* infection, even when high concentrations of IL-17 are present in the lungs, IL-22 has an anti-inflammatory role.

The increased inflammation in the lungs of IL-22^{-/-} mice and enhanced antigen-specific responses suggested that IL-22 may limit the extent of inflammation during *B. pertussis* infection as well as influence T cell priming. IL-22 has been shown to induce IL-10 secretion from colon epithelial cells and IL-22 mediated IL-10 production has been implicated in inhibiting of eosinophilic airway inflammation (Nagalakshmi et al, 2004; Nakagome et al, 2011). The expression of anti-inflammatory cytokines, IL-10 and TGF β , was therefore investigated in the lungs of WT and IL-22^{-/-} mice at peak of IL-22 production and on day 14 post infection. No difference in IL-10 expression was found between WT and IL-22^{-/-} mice and no significant upregulation of TGF β gene expression was detected in either of the groups. It therefore appears that the anti-inflammatory mechanism of IL-22 is independent of IL-10 and TGF β , although further studies are necessary to confirm that. Given that IL-22R is expressed by epithelial cells but not cells of the immune

system, IL-22 can only promote resolution of lung inflammation by acting on airway epithelium. This could, for example, involve induction of lipid mediator production by lung epithelial cells.

The current study also investigated, whether the increased IL-17 and IFN γ production in the lungs of IL-22^{-/-} mice correlated with a greater inflammatory damage. It was found that a significantly greater area of the lung was occupied by the inflammatory infiltrate in IL-22^{-/-} mice compared to their WT littermates. Similar to current findings, IL-22 was also found to have a role in limiting the lung inflammation during influenza infection (Ivanov et al, 2013; Kumar et al, 2013). Notably, lungs of IL-22^{-/-} mice had a greater number of inflammatory foci compared to WT mice. IL-22 has been implicated in epithelial repair and promotion of epithelial barrier function (Brand et al, 2006; Mizoguchi, 2012; Pickert et al, 2009). It is therefore possible that the more diffused inflammation seen in IL-22^{-/-} mice was due to impaired containment of bacteria caused by defective epithelial barrier.

IL-22 has been shown to induce the expression of various antimicrobial peptides, including the homologue of mBD3, from human keratinocytes alone or in combination with IL-17 (Liang et al, 2006; Wolk et al, 2004). It has also been shown to mediate immune protection to an intestinal pathogen *Citrobacter rodentium* via induction of Reg3 γ expression in the gut (Zheng et al, 2008). Recently, RegIII γ has been implicated in protective responses against MRSA pneumonia (Choi et al, 2013). I hypothesised that the increased inflammation in the lungs of IL-22^{-/-} mice could be secondary to the increased bacterial burden caused by impaired control of bacterial growth as a result of inadequate antimicrobial peptide production. The expression of mBD3, RegIII γ and CAMP in WT and IL-22^{-/-} mice early following pathogen entry, at the peak of IL-22 production on day 7 and during the clearance phase post IL-22 peak was investigated. Surprisingly, impaired antimicrobial peptide expression was not detected in the lungs of IL-22^{-/-} mice and the expression of RegIII γ and CAMP was significantly higher in the knock out animals on day 14 post infection. Given the implication of IL-17 in CAMP induction, its enhanced expression was most likely due to increased IL-17 levels on day 14. Nonetheless, other antimicrobial peptides may play a greater role in defences against *B. pertussis*. For example, lipocalin 2, an antimicrobial peptide that can be induced by IL-22 and IL-17, has been shown to be a crucial factor in immune responses against *K. pneumoniae*

(Aujla et al, 2008; Chan et al, 2009) It is possible that lipocalin 2 also plays a protective role in *B. pertussis* infection.

The current study has shown that $\gamma\delta$ T cells produce IL-17 and IL-22 in the lungs of *B. pertussis* infected mice and these cytokines contribute to the clearance of bacteria. IL-17 produced at the early stages of the infection at least partially contributed to neutrophil recruitment to the lung. IL-22^{-/-} mice show increased pulmonary inflammation and heightened Th17 and Th1 responses suggesting that IL-22 has an anti-inflammatory role during *B. pertussis* infection and may contribute to the resolution of inflammation and limiting collateral damage in the lung. However, increased inflammation in IL-22^{-/-} mice could be secondary to increased bacterial burden. Further studies are necessary to clarify whether IL-22 actively suppresses inflammatory responses in the lung during *B. pertussis* infection. It is also possible that IL-22 has a role in the recovery of lung tissue post pathogen clearance. Given the *in vitro* data generated in the current study, it is likely that different subsets of lung $\gamma\delta$ T cells contribute to different aspects of the local immune response. Dissecting these roles can help identify populations that could be preferentially targeted by immune therapies or vaccines to enhance protective responses in the lung.

Chapter 5: Memory $\gamma\delta$ T cells

Chapter 5.1: Introduction

5.1.1 Concept of immunological memory

Immunological memory is a hallmark of the adaptive immune system. Upon infection with a given pathogen, T and B cells specific for that pathogen expand exponentially. Their numbers contract to normal levels after the pathogen has been cleared. However, some of these highly specific T and B cells go on to become long-lived memory cells which persist in the body for many years. Higher frequency of antigen-specific cells allows for faster pathogen clearance, often without the infected individual experiencing any of the symptoms of the disease. Furthermore, memory cells show a greater affinity for pathogen antigens. This is achieved through affinity (B cells) and avidity (T cells) maturation of antigen-specific cells. Affinity maturation refers to the process by which high number of point mutations is introduced to the BCR gene of activated dividing B cells during the immune response. This leads to generation of B cells with varying degree of affinity to the infecting agent. B cells bearing the BCR with the highest affinity are subsequently selected and expanded to become antibody producing plasma cells or memory B cells. Avidity maturation, on the other hand, refers to the changes occurring in the activated T cells resulting in a lower signal threshold requirement by the antigen-experienced cells and their stronger response upon reinfection. Vaccination explores the memory phenomenon by inducing the expansion of protective antigen-specific T and B cells without causing the disease. Although innate immune cells do not exhibit the classical immune memory, recent studies have found that they can be trained to respond better to infections (Netea, 2013; Netea et al, 2011; Sun et al, 2014). This is known as immunological imprinting – a process by which innate immune cells become adapted via epigenetic mechanisms to respond more efficiently to infections and maintain this ability for prolonged periods of time after the pathogen clearance. It is thought that both types of immune memory are important for successful protective immunity.

5.1.2 Cell surface markers of naïve and memory T cells

Studies investigating memory T cell responses take advantage of the shift in cell surface marker expression on transition from being a naïve to a memory cell. Activated T cells change their migration pattern and modulate the expression of cytokine receptors involved in T cells survival and proliferation. Markers typically used to differentiate between T cell activation stages include CD62L, CD44, CD45RA, CCR7, CD127 (IL-7R α), and CD122.

Upon recognition of the cognate antigen in the context of an MHC molecule and with the help of co-stimulatory molecules, such as CD28 and CD27, naïve T cells become activated and start to proliferate with concomitant production of IL-2. TCR engagement leads to modulation of expression of co-stimulatory molecules and members of the TCR signalling complex. For example, there is a switch in the expression of the isoform of tyrosine phosphatase CD45. Naïve T cells mainly express the high molecular weight isoform, CD45RA, while activated T cells downregulate its expression and switch to low molecular isoform, CD45RO, which is maintained in memory T cells (Ho et al, 2013). The expression of CD28 and CD27 also changes upon activation – CD28 becomes downregulated, while CD27 upregulated. Therefore the level of expression of costimulatory molecules themselves is a marker of T cell activation (Lake et al, 1993). CD27 is also thought to be important for the generation of T cell memory partially by modulating the expression of cytokine receptors required for memory T cell survival (Dong et al, 2012; Hendriks et al, 2000; Welten et al, 2013). Many of the changes occurring in the activated T cells, such as subset polarisation and memory T cell development, are dependent on epigenetic control of gene expression (Youngblood et al, 2013). Restricting or facilitating access to particular genes modifies the ability of cells to respond to activating or inhibitory signals.

Naïve T cells express the low affinity heterodimeric IL-2 receptor (IL-2R) composed of IL-2R β (CD122) and the common γ chain, CD132. Following activation, the expression of the IL-2R α chain, CD25, is upregulated thus increasing the affinity of the receptor for its ligand (Boyman & Sprent, 2012). CD25 expression is therefore one of the markers of T cell activation. The production of IL-2, which is a potent T cell growth factor, is dependent on signals downstream of CD28, which lead to

chromatin modifications of IL-2 promoter and its consequent activation (Thomas et al, 2005). In addition to promoting T cell proliferation, IL-2 induces downregulation of CD127 (Xue et al, 2002). Thus lack of CD127 can be used to differentiate naïve T cells from activated effector T cells (Kaech et al, 2003).

Activated T cells briefly upregulate CD69 expression, often used as a marker for recent activation, which results in their retention in the lymph nodes, allowing for adequate length of interaction with the APC (Shiow et al, 2006; Ziegler et al, 1994). Upon activation T cells also downregulate the expression of lymph node homing receptors, CD62L (L-selectin) and CCR7, and concomitantly upregulate tissue homing receptors, which allows for their egress from lymph nodes and migration to tissues where they exert their effector function (Sallusto et al, 1999). After the clearance of the pathogen, the effector T cell population contracts to about 5-10% of its peak size and the surviving cells resume the expression of CD127 (Kaech et al, 2003). Therefore, re-expression of CD127 correlates with the entrance into the memory phase and has been proposed as a marker for effector cells that are destined to become long lived memory T cells (Kaech et al, 2003). However, other studies have reported that CD127 alone is not enough to predict the likelihood of a T cell to become a memory cell, and for CD4 T cells the use of markers such as Ly6C and Tbet expression was proposed, with to-be-memory cells expressing low levels of these markers (Hand et al, 2007; Lacombe et al, 2005; Marshall et al, 2011). Furthermore, both naïve and memory T cells express CD127 and are dependent on IL-7 for survival (Schluns et al, 2000; Swainson et al, 2006). Hence CD127 alone cannot be used to distinguish between these two types of T cells.

CD44 is another molecule which expression by T cells is modulated following activation and is commonly used as a marker of memory T cells (Lesley et al, 1993). CD44 is first expressed at the early T cell differentiation stages in the thymus and remains high in DN1 and DN2 cells. It then becomes downregulated and is not expressed again until antigenic stimulation (Budd et al, 1987; Godfrey et al, 1993; Wu et al, 1993). CD44 is a transmembrane protein containing a c-type lectin domain which binds to hyaluronic acid (HA) – a major component of the extracellular matrix. The interactions of CD44 with molecules expressed by endothelial cells have been shown to be important for adhesion and rolling of T cells along the endothelial vasculature and extravasation to areas of inflammation (Baaten et al, 2010; Bonder

et al, 2006). CD44, through its cytoplasmic tail, can associate with the integrin VLA-4 and the interaction of the two receptors leads to a firm adhesion and aids the T cell movement into tissues (Nandi et al, 2004). Apart from promoting adhesion, CD44 has been also implicated in T cell activation and survival (Baaten et al, 2010; Galandrini et al, 1994; Larkin et al, 2006; Shimizu et al, 1989).

CD122, the β chain of IL-2/IL-15 receptor is highly expressed by CD44⁺ CD8 but not CD4 memory T cells and has been used in studies of cytotoxic T cell memory (Judge et al, 2002; Mbitikon-Kobo et al, 2009; Zhang et al, 1998). However, it has recently been shown that CD8⁺ Treg cells are also CD122^{high} and CD44^{high} and therefore indistinguishable from memory cytotoxic lymphocytes using only these two markers (Dai et al, 2010)

Table 5.1 Cell surface markers used to identify memory T cell subsets.

CD4 T cells			
marker	Naive	CM	EM
CD62L	+	+	-
CD44	-	+	+
CD127	+	+	+/-
CCR7*	+	+	-
CD45RA*	+	-	+/-
CD45RO*	-	+	+
CD27	+	+	-
CD8 T cells			
marker	Naive	CM	EM
CD62L	+	+	-
CD44	-	+	+
CD45RA*	+	-	+/-
CD45RO*	-	+	+
CD127	+	+	+
CCR7*	+	+	-

CD27	+	+	-
CD11a**	-	+	+
CD122	-	+	+
CD132	-	+	+

CM- central memory, EM-effector memory, * Human T cells only
 ** Also CD4⁺ T cells

5.1.3 Subsets of memory T cells

In 1999 Sallusto et al. proposed division of human memory T cells into two subsets based on CD45RA and CCR7 expression: effector memory T cells (T_{EM}) and central memory T cells (T_{CM}) (Sallusto et al, 1999). They described T_{EM} cells as those that exhibit immediate protective memory responses by homing to peripheral tissues and rapidly producing effector cytokines. T_{CM}, on the other hand, are characterised by little or no effector function and secondary lymphoid organ tropism. They can, however, rapidly proliferate and differentiate into effector cells upon antigen restimulation (Sallusto et al, 2004; Sallusto et al, 1999). In agreement with their anatomical location, T_{CM} express high levels of CD62L and CCR7, while T_{EM} are low for both of these markers (Sallusto et al, 1999). Later, murine memory T cells were also found to segregate into 2 separate populations based on CCR7 expression: CCR7⁺CD62L⁺CD44^{hi} (central memory) and CCR7⁻CD62L⁻CD44^{hi} (effector memory)(Bjorkdahl et al, 2003). Subsequent studies by other groups in mice and humans failed to find a correlation between CCR7 expression and the quality of the memory response (Ravkov et al, 2003; Unsoeld et al, 2002). The nomenclature proposed by Sallusto et al. has nonetheless been widely accepted and other marker combinations, such as CD127 and CD62L or CD44 and CD62L, have been used to divide T cells into memory subpopulations based on the original subset definition (Bachmann et al, 2005; Bassett et al, 2012; Hengel et al, 2003; Moore et al, 2011). For example, T cells can be easily divided into three functional subsets based on CD62L and CD44 expression with naïve (T_N) cells being CD62L^{hi}CD44^{lo}, T_{CM} – CD62L^{hi}CD44^{hi} and T_{EM} - CD62L^{lo}CD44^{hi}. Furthermore, memory T cells show differential expression of CD27 with majority of T_{CM} expressing high levels of CD27, while T_{EM} are CD27 low or intermediate (Wherry et al, 2003).

Although none of the cell surface marker combinations can separate T cells into rigid subpopulations, they are useful for predicting the quality of protective response elicited by a given pathogen or immunisation protocol. T_{CM} cells are thought to be important for providing long lasting immunity, while T_{EM} provide immediate protection. It is thought that acute infections tend to favour generation of T_{CM} cells, while persistent infections or prime-boost immunisations with repetitive exposure to the antigen predominantly induce T_{EM} and the maintenance of these cells may be dependent on antigen persistence (Masopust et al, 2006; Shin et al, 2007; van Faassen et al, 2005). Recently, it has transpired that a population of non recirculating effector memory cells exist, dubbed tissue resident memory T cells (T_{RM}). These cells can persist in tissues long after the infection has been cleared and provide an antigen-specific immunity in the target organ of infection (Casey et al, 2012; Gebhardt et al, 2009; Gebhardt et al, 2011; Jiang et al, 2012; Liu et al, 2010; Masopust et al, 2010). Similar to T_{EM} cells, T_{RM} cells lack the expression of CD62L but in contrast they express CD69 and CD103.

5.1.4 Memory $\gamma\delta$ T cells

Kinetics of the $\gamma\delta$ T cell response are very reminiscent of a memory responses with a characteristic rapid activation of the effector function. However, $\gamma\delta$ T cells are thought to be mainly involved in innate phase of the response and to be activated in an antigen non-specific manner. Very few studies have addressed the possibility of existence of a memory $\gamma\delta$ T cell population and the role it may play in a secondary immune response (Murphy et al, 2014; Sheridan et al, 2013).

In contrast to conventional T cells, $\gamma\delta$ T cells do not appear to express CCR7 in the resting state. The expression of CCR7 can become upregulated by $\gamma\delta$ T cells upon TCR stimulation although it does not appear to be required for lymph node homing (Brandes et al, 2003; Ebert et al, 2006; Shimura et al, 2010; Vrieling et al, 2012). $\gamma\delta$ T cells express various molecules typical of memory cells. Similar to $\alpha\beta$ T cells, $\gamma\delta$ T cells segregate into subpopulations based on CD44 and CD62L expression and can be divided into CD27⁻ and CD27⁺ cells, with majority of CD27⁺ cells being also CD62L high (Haas et al, 2009; Ribot et al, 2009). Furthermore, CD27⁺ and CD27⁻ $\gamma\delta$ T cells share some characteristics of central and effector memory T cells, respectively. For example, CD27⁺ have shown a superior proliferation upon TCR

stimulation compared to CD27⁻ $\gamma\delta$ T cells (Ribot et al, 2009). In addition, subsets of CD27⁻ $\gamma\delta$ T cells were also found to express cell surface markers associated with resident effector memory cells, such as CD69 and CD103 (Antvorskov et al, 2012; Gray et al, 2011; Sumaria et al, 2011)

Nonetheless, in contrast to $\alpha\beta$ T cells, a sizeable population of CD44^{hi} $\gamma\delta$ T cells is present in naïve animals and both CD27⁺ and CD27⁻ show an immediate effector function upon TCR engagement. It is therefore unclear whether memory markers commonly used for $\alpha\beta$ T cells correlate at all with memory phenotype in the $\gamma\delta$ compartment. However, Sheridan et al. have recently reported that a population of CD27⁻CD44^{hi} $\gamma\delta$ T cells, different to the cells present in peripheral LN, expands in the gut and is capable of mounting superior protective responses upon reinfection. These responses appear to be specific for the antigen and the route of the infection as the recall response was not observed when mice were infected with an unrelated pathogen or with the same pathogen via a different route. However, the recall response was shown using a mixed cell population and thus it is unclear whether the apparent $\gamma\delta$ T cells memory response is a result of bystander activation in the presence of responding memory $\alpha\beta$ T cells. It is also unclear how closely memory markers correlate with the antigen-experienced status of “memory” $\gamma\delta$ T cells. The data of Sheridan and colleagues suggests that $\gamma\delta$ T cell may be an important player in the tissue resident memory and warrants further investigation.

Chapter 5.2: Results

5.2.1 Lung $\gamma\delta$ T cells upregulate memory markers during *B. pertussis* infection

Conventional T cells can be divided into naïve, central memory and effector memory cells using CD44 and CD62L marker expression. The expression of these markers changes during infection with progressive appearance of CD44^{hi}CD62L^{lo} cells representing activated and effector memory T cells. Given that $\gamma\delta$ T cells are known to express both of these markers, it was investigated whether $\gamma\delta$ T cells could also be divided into distinct populations using CD44 and CD62L expression and if the effector memory marker expressing cells were also increased after infection. It was found that the majority of $\alpha\beta$ T cells in the lungs of naïve mice exhibit the naïve phenotype (CD62L^{hi}, CD44^{lo}) (Figure 5.1). In contrast to conventional T cells, roughly the same percentage of $\gamma\delta$ T cells expressed the naïve and effector memory markers. On day 14 post infection the dominant population of $\alpha\beta$ T cells in the lung were effector memory cells (61% compared to only 12.6% in naïve animals). The effector memory marker expressing cells were also increased in the $\gamma\delta$ T cell compartment, albeit to a lesser extent than the conventional T cells (Figure 5.1). CD62L^{hi}CD44^{hi} cells constituted the minority of lung CD3⁺TCR δ ⁻ and CD3⁺TCR δ ⁺ T cells both in naïve and infected animals.

CD27 is a marker that distinguishes IL-17 from IFN γ producing $\gamma\delta$ T cells. Spleen CD27⁻ $\gamma\delta$ T cells have been reported to express low CD62L and high CD44 levels while CD27⁺ cells showed the opposite phenotype. It was investigated whether the putative naïve, central memory and effector memory $\gamma\delta$ T cell subsets in the lung also segregated based on CD27 expression. It was found that the majority of CD62L^{hi}CD44^{lo} cells in the lung coexpressed CD27, while CD62L^{lo}CD44^{hi} lacked expression of this marker. CD62L^{hi}CD44^{hi} were also found to be CD27 positive. Furthermore, this feature of respective subsets was stable during infection (Figure 5.2). Given that, the population of lung $\gamma\delta$ T cells that expanded during *B. pertussis* infection consisted mostly of CD27⁻ cells, it was investigated whether this expansion was more prominent in any specific subset of lung $\gamma\delta$ T cells. It was found that while the percentage of CD27⁻ cells increased among all $\gamma\delta$ T cell subsets in the lungs of

infected animals, the greatest increase in numbers of these cells was observed in the V γ 4 subset (Fig 5.3). CD27 expression has been reported to be a useful substitute for CCR7 when differentiating central memory from effector memory CD8 T cells. Given the fact that CD62L and CD27 were found to be co-expressed on $\gamma\delta$ T cells, CD27 and CD44 expression, similar to CD62L and CD44, was found to divide $\gamma\delta$ T cell into three distinct populations which could be easily related back to CD62LCD44 subsets (Figure 5.4). Consequently, the percentage of CD27⁻ CD44⁺ $\gamma\delta$ T cells was found to increase among all three lung V γ subsets following infection. The highest increase in the percentage of putative effector/effector memory cells using those markers was observed among V γ 6 $\gamma\delta$ T cells (Figure 5.4).

5.2.2 Antigen-specific $\gamma\delta$ T cell responses in lungs of infected animals

In order to establish whether increased expression of memory markers correlated with expansion of antigen-specific $\gamma\delta$ T cells *in vivo*, purified lung $\gamma\delta$ T cell subsets from naïve and *B. pertussis* infected mice were restimulated with HKBP *in vitro* in the presence of irradiated spleen cells as APC. It was found that lung $\gamma\delta$ T cells from infected, but not naïve mice, produced IL-17 in response to antigenic stimulation in a dose dependent manner (Figure 5.5). *B. pertussis* specific responses were detected from the V γ 4 subset of lung $\gamma\delta$ T cells in both experiments. Importantly, only IL-17A but not IL-17F production was detected from the HKBP restimulated cells and none of the other cytokines tested (IL-10, IFN γ , IL-22) was produced by V γ 4 cells in an antigen-specific fashion (Figure 5.5-5.7). Antigen-specific production of IL-22 and IL-17 by V γ 6 $\gamma\delta$ T cells was detected in one out of the two experiments performed. Interestingly, PMA+ionomycin induced production of IL-10 exclusively from the V γ 1 subset of lung $\gamma\delta$ T cells and V γ 1 cells produced the greatest amount of IFN γ in response to this stimulus (Figure 5.7). IL-1 β +IL-23 stimulation induced IL-17 production from V γ 4 and V γ 6 cells isolated from both naïve and infected lungs and infection did not appear to alter the ability of these subsets to respond to cytokine stimulation. V γ 1 cells were found to produce some IL-17 in response to IL-1 β +IL-23, albeit at much lower levels than those produced by either V γ 6 or V γ 4 $\gamma\delta$ T cells. Majority of cytokine induced IL-22 was produced by V γ 4 cells. No cytokine production was detected from IR spleen cells under all conditions tested (Figure 5.5-5.7).

In order to determine whether the cytokine production by “memory” V γ 4 cells was dependent on antigen presentation by APCs, purified lung V γ 4 cells isolated on day 14 post infection were incubated with varying concentrations of HKBP in the presence or absence of IR spleen cells. It was found that only cells restimulated in the presence of APC produced IL-17 while IL-1 β +IL-23 and PMA/ionomycin induced IL-17 production was unaffected by the presence or absence of APC (Figure 5.8)

5.2.3 Lung $\gamma\delta$ T cells exhibit recall responses upon reinfection

In order to investigate whether the effector memory-like phenotype was maintained in the absence of pathogen, mice were infected with *B. pertussis* and allowed to clear the infection. It was found that in all lung $\gamma\delta$ T cell subsets CD27⁻ population remained increased even after the bacteria have been cleared (Fig 5.9 top panel). All CD27⁻ $\gamma\delta$ T cells also expressed high levels of CD44⁺ and the CD27⁻CD44⁺ population accounted for the majority (89.7%) of cells in the V γ 4 $\gamma\delta$ T cell subset (Figure 5.9 bottom panel). Given that convalescent mice were found to harbour a greater number of putative effector memory cells in their lungs, it was investigated whether these cells could contribute to the quality of the secondary immune response. Unprimed and convalescent mice were infected with *B. pertussis* and their T cell responses as well as bacterial burden analysed at the early time points following the infection. It was found that while unprimed mice underwent the usual course of infection with bacterial counts increasing exponentially, the convalescent mice had cleared the infection by day 7. Furthermore, $\gamma\delta$ T cells from convalescent mice exhibited superior IL-17 responses upon reinfection compared to unprimed mice (Figure 5.10). Upon analysis of IL-17 production by different lung $\gamma\delta$ T cell subsets, it was found that the majority of IL-17⁺ $\gamma\delta$ T cells in convalescent mice bore the V γ 4 chain of T cell receptor. However, V γ 6 $\gamma\delta$ T cells also contributed to recall responses (Figure 5.11). In addition, V γ 4 cells were also the major IL-17 producing subsets in the lungs of unprimed mice 7 days post infection. Similar percentages of IL-17 producing $\gamma\delta$ T cells in the lungs of convalescent animals were detected at 2h and 3 days post infection while no IL-17 production was detected on day 7 coinciding with the clearance of bacteria (Figure 5.10 and 5.11). When the numbers of IL-17 producing cells were analysed it was found that in convalescent animals IL-17⁺ CD4 and $\gamma\delta$ T cell number peaked on day 3 post infection. In

unprimed animals the IL-17 production did not peak until day 7. No significant IL-17 production by CD4 T cells was observed at the early stages of infection in the lungs of unprimed animals (Figure 5.12)

5.2.4 Memory marker expressing $\gamma\delta$ T cells increase in the lungs of *B. pertussis* infected mice with similar kinetics of memory $\alpha\beta$ T cells

Lung $\gamma\delta$ T cells show bimodal IL-17 production during *B. pertussis* infection with an early peak just hours following pathogen entry and the second peak occurring between day 7 and 14 post infection. This is followed by a decline in the IL-17 production by $\gamma\delta$ T cells. On the other hand the peak of IL-17 production by CD4 T cells occurs later and is maintained at least until day 28 post infection. The kinetics of expansion of CD62L^{lo}CD44^{hi} $\gamma\delta$ T cells in the lung were investigated and compared to those of CD4 T cells. It was found that the numbers of CD4 T cells with an effector memory phenotype increase significantly on day 14 following infection and were further increased on day 21. The numbers of cells expressing naïve markers (CD62L^{hi}CD44^{lo}) remained constant throughout the course of infection. Similar to CD4 T cells, CD62L^{lo}CD44^{hi}, but not CD62L^{hi}CD44^{lo}, V γ 6 and V γ 4 $\gamma\delta$ T cells became expanded in the infected lungs with their numbers reaching a significant peak on day 21 post infection. In contrast to V γ 6 and V γ 4 cells, the number of CD62L^{hi}CD44^{hi} V γ 1 cells increased significantly while the number of cells expressing effector memory markers remained relatively constant (Figure 5.13). The expansion of $\gamma\delta$ T cells expressing effector memory or naïve T cell markers was mirrored by the expansion of either CD27⁻ or CD27⁺ cells, respectively, in each of the lung $\gamma\delta$ T cell subsets. Thus the increase in CD62L^{lo}CD44^{hi} V γ 4 directly correlated with the increased number of CD27⁻ cells in that subset (Figure 5.14). Taken together, similar to $\alpha\beta$ T cells, lung $\gamma\delta$ T cells bearing effector memory markers became significantly increased at the later stages of *B. pertussis* infection.

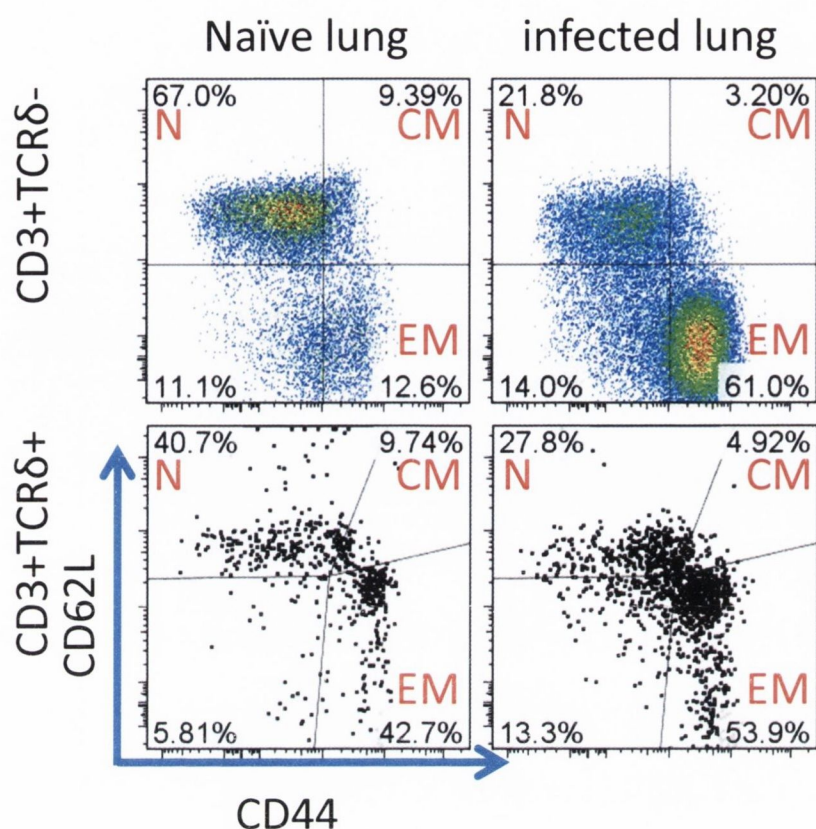


Figure 5.1 $\gamma\delta$ T cells segregate into distinct subsets based on CD62L and CD44 expression

Lung cells ($1 \times 10^6/\text{mL}$) from naïve and day 14 *B. pertussis* infected mice were purified on a percoll gradient. The expression of CD62L and CD44 by $\gamma\delta$ T cells and $\alpha\beta$ T cells was then analysed by FACS. The plots show percentages of cells in the CD3+TCR δ - (top panel) and CD3+TCR δ + gate (bottom panel). N-naïve, CM-central memory, EM-effector memory. n=2

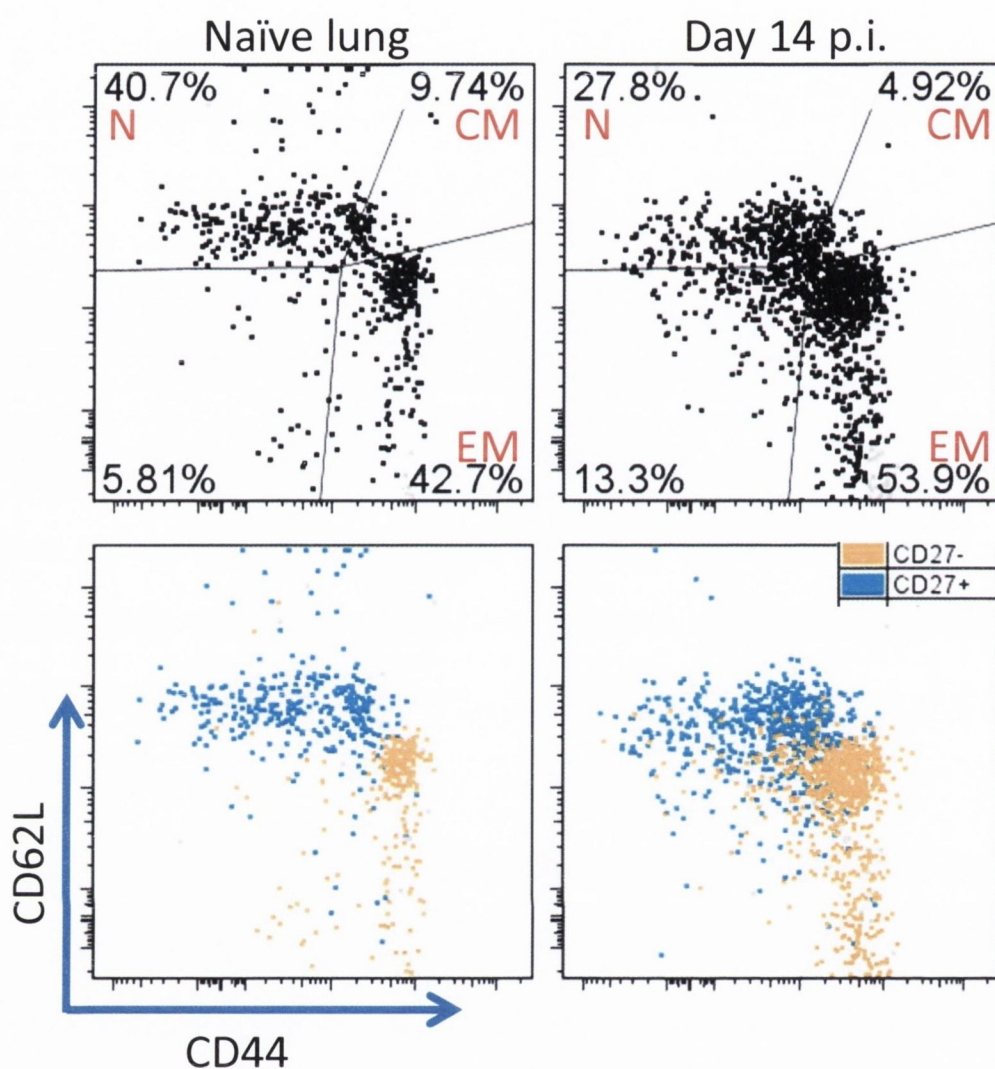


Figure 5.2 CD62L⁺CD44⁺ “effector memory” $\gamma\delta$ T cell population lacks the expression of CD27.

Lung cells ($1 \times 10^6/\text{mL}$) from naïve and day 14 *B. pertussis* infected mice were isolated using tissue lyser and purified on a percoll gradient. The expression of CD62L, CD44 and CD27 by $\gamma\delta$ T cells was then analysed by FACS. The plots show percentages of cells in the CD3⁺TCR δ ⁺. N-naïve, CM-central memory, EM-effector memory. n=1

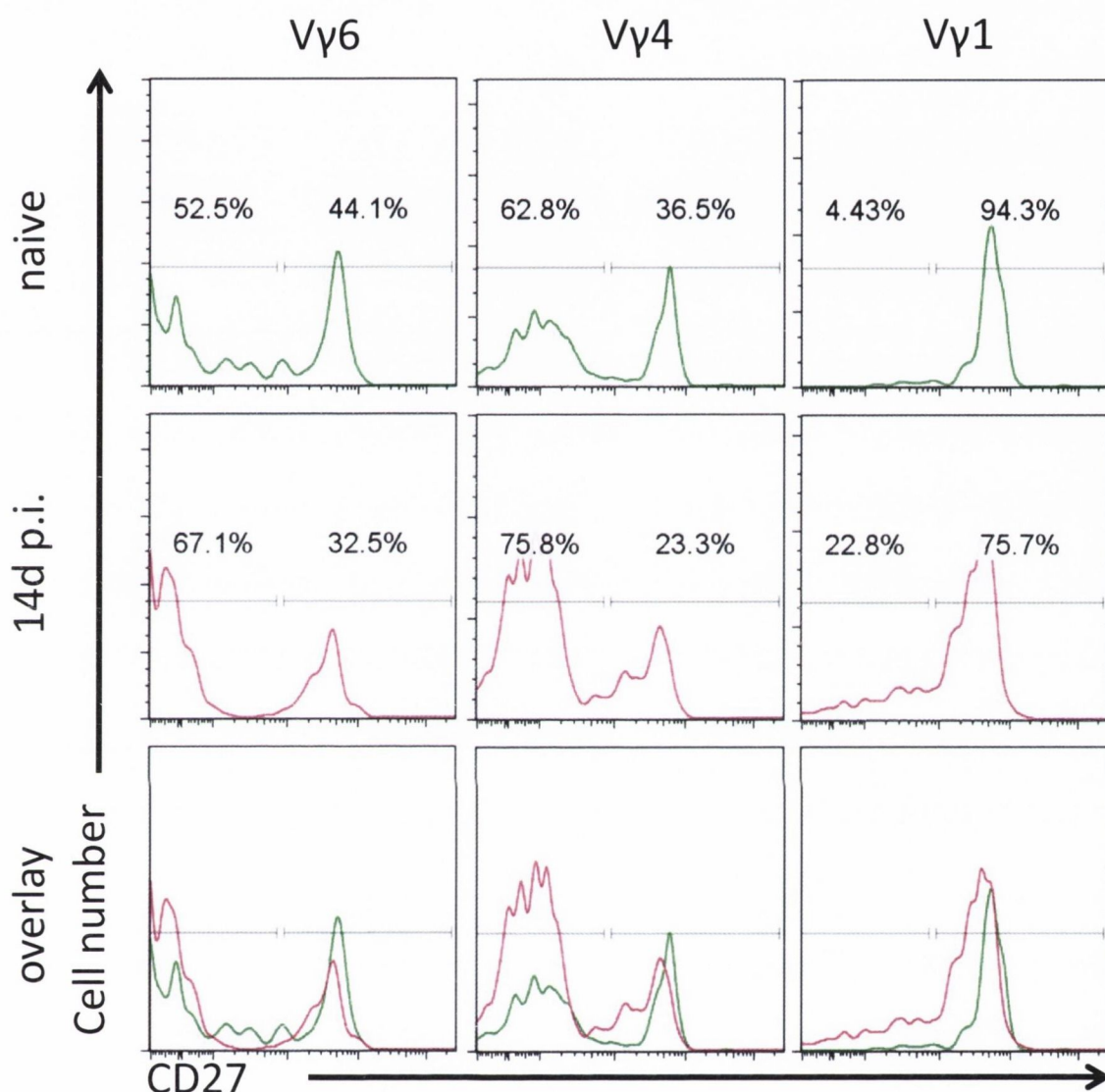


Figure 5.3 CD27⁺ $\gamma\delta$ T cells are increased in the lungs of *B. pertussis* infected mice.

Lung cells ($1 \times 10^6/\text{mL}$) from naïve and day 14 *B. pertussis* infected animals were isolated using tissue lyser and purified on a percoll gradient. The expression of CD27 by $\gamma\delta$ T cell subsets was then analysed by FACS. The plots show percentages of cells in the CD3⁺TCR δ ⁺V γ 4⁻V γ 1⁻ (V γ 6), CD3⁺TCR δ ⁺V γ 4⁺ (V γ 4) CD3⁺TCR δ ⁺V γ 1⁺ (V γ 1) gates. n=1

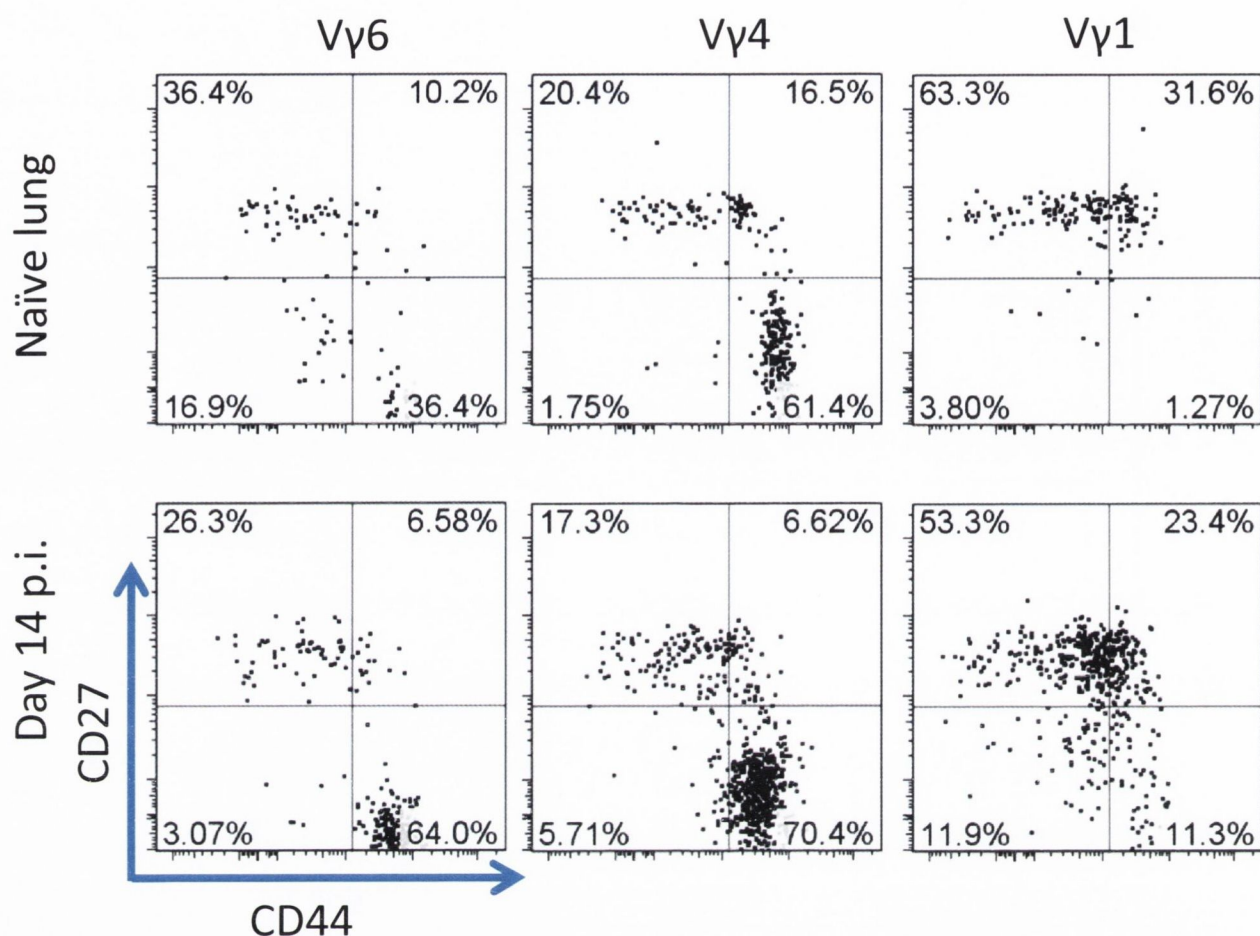


Figure 5.4 CD27⁻ CD44⁺ population is expanded in all subsets of lung $\gamma\delta$ T cells at day 14 post infection.

Lung cells ($1 \times 10^6/\text{mL}$) from naïve and day 14 *B. pertussis* infected mice were isolated using tissue lyser and purified on a percoll gradient. The expression of CD27 and CD44 by $\gamma\delta$ T cells subsets was then analysed by FACS. The plots show percentages of cells in the CD3⁺TCR δ ⁺Vγ4⁻Vγ1⁻ (Vγ6), CD3⁺TCR δ ⁺Vγ4⁺ (Vγ4) CD3⁺TCR δ ⁺Vγ1⁺ (Vγ1) gates. n=1

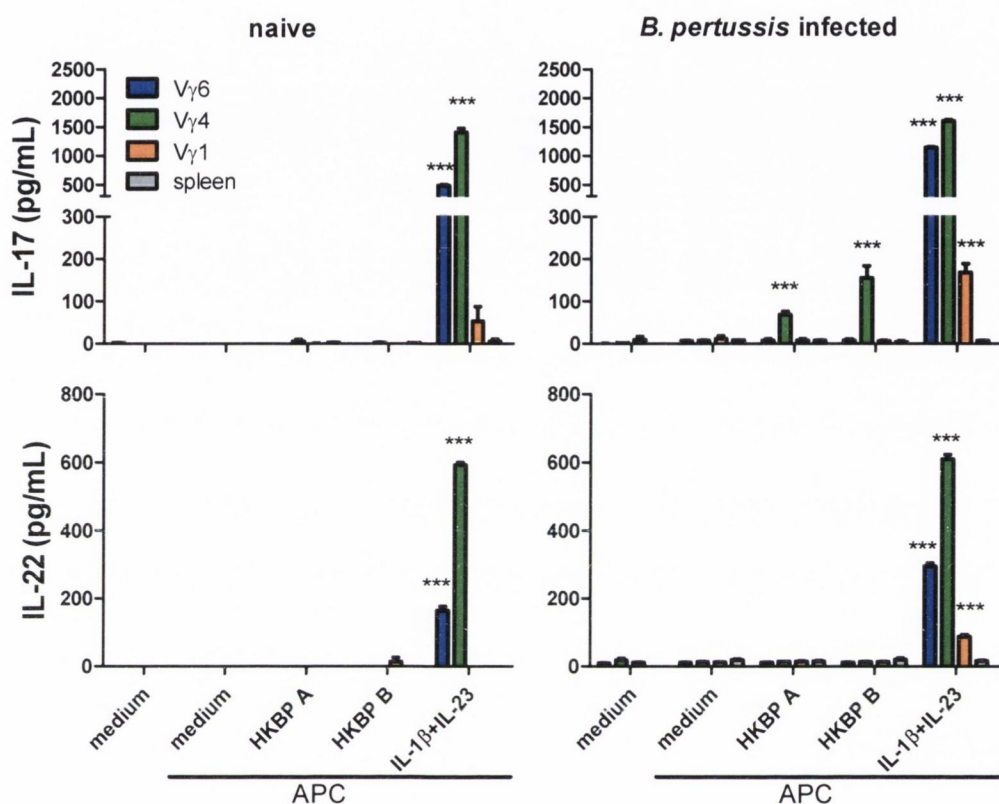


Figure 5.5 $\gamma\delta$ T cells isolated from lungs of infected, but not those of naïve animals, produce IL-17 upon restimulation with the antigen in a dose dependent fashion *in vitro*.

FACS purified $\gamma\delta$ T cells subsets from lungs of naïve and day 14 *B. pertussis* infected C57Bl/6 mice ($25 \times 10^3/\text{mL}$) were incubated with HKBP ($2 \times 10^6/\text{mL}$ - A and $2 \times 10^7/\text{mL}$ - B) in the presence of irradiated spleen cells as APC ($2 \times 10^6/\text{mL}$). After 72 hours the concentration of IL-17 and IL-22 in the supernatants was determined by ELISA. *** $p < 0.001$ by two-way ANOVA with Bonferroni post test. $n = 2$

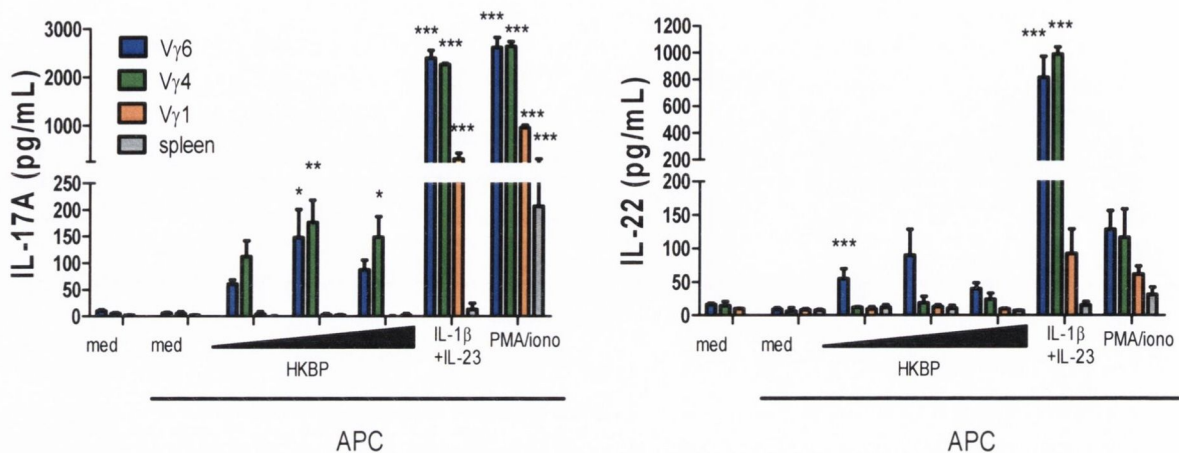


Figure 5.6 Vγ6 γδ T cells from the lungs of *B. pertussis* infected mice produce IL-17A and IL-22 upon restimulation with the antigen *in vitro*.

FACS purified γδ T cells subsets from lungs of day 14 *B. pertussis* infected C57Bl/6 mice ($25 \times 10^3/\text{mL}$) were incubated with HKBP (2×10^6 – $2 \times 10^8/\text{mL}$) in the presence of irradiated spleen cells ($2 \times 10^6/\text{mL}$) as APC. After 72 hours the concentration of IL-17 and IL-22 in the supernatants was determined by ELISA. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by two-way ANOVA with Bonferroni post test. $n=1$

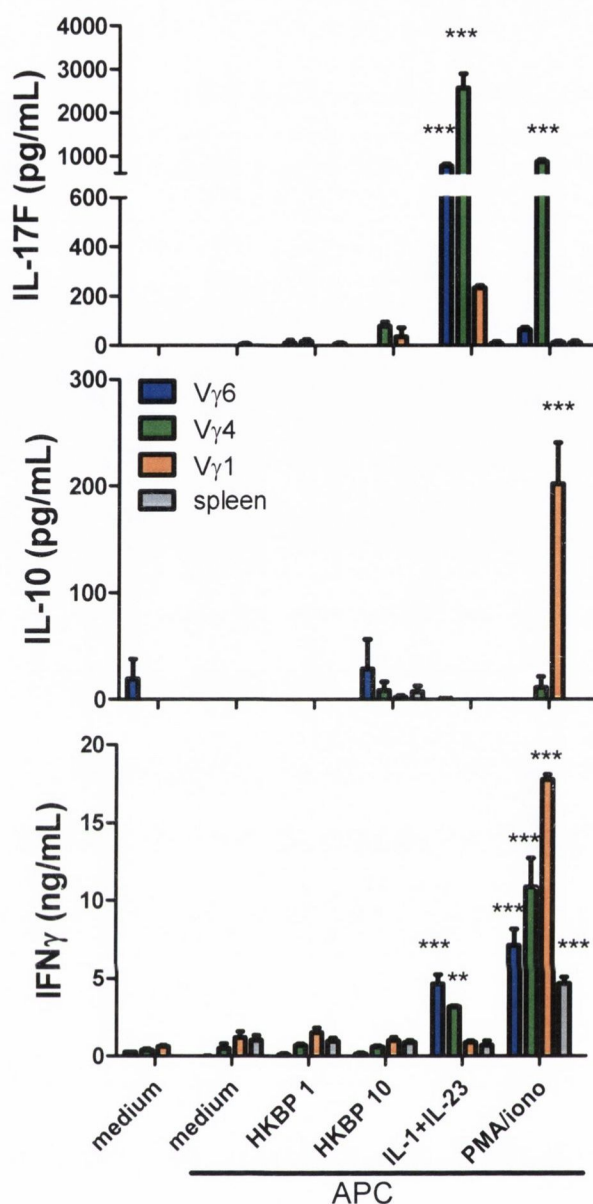


Figure 5.7 $\gamma\delta$ T cells isolated from lungs of infected animals fail to produce IL-17F, IL-10 or IFN γ upon restimulation with antigen *in vitro*.

FACS purified $\gamma\delta$ T cells subsets from lungs of day 14 *B. pertussis* infected C57Bl/6 mice ($25 \times 10^3/\text{mL}$) were incubated with HKBP (MOI 1 and 10) in the presence of irradiated spleen cells ($2 \times 10^6/\text{mL}$) as APC. After 72 hours the concentration of IL-17F, IL-10 and IFN γ in the supernatants was determined by ELISA. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by two-way ANOVA with Bonferroni post test. $n=1$

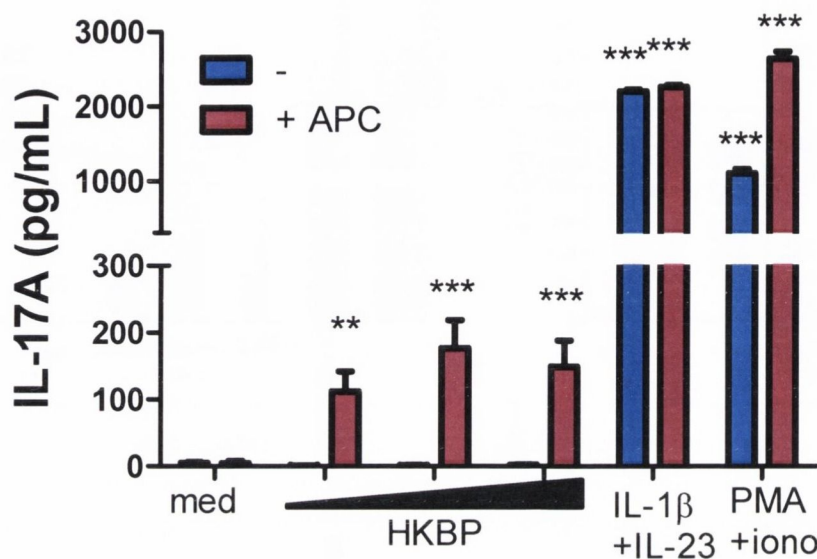


Figure 5.8 Antigen-specific IL-17 production by $\gamma\delta$ T cells is dependent on the presence of APC in the culture

FACS purified $\gamma\delta$ T cells subsets from lungs of day 14 *B. pertussis* infected C57Bl/6 mice ($25 \times 10^3/\text{mL}$) were incubated with HKBP (2×10^6 - $2 \times 10^8/\text{mL}$) in the presence or absence of irradiated spleen cells ($2 \times 10^6/\text{mL}$) as APC. After 72 hours the concentration of IL-17A in the supernatants was determined by ELISA. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by two-way ANOVA with Bonferroni post test. $n=1$

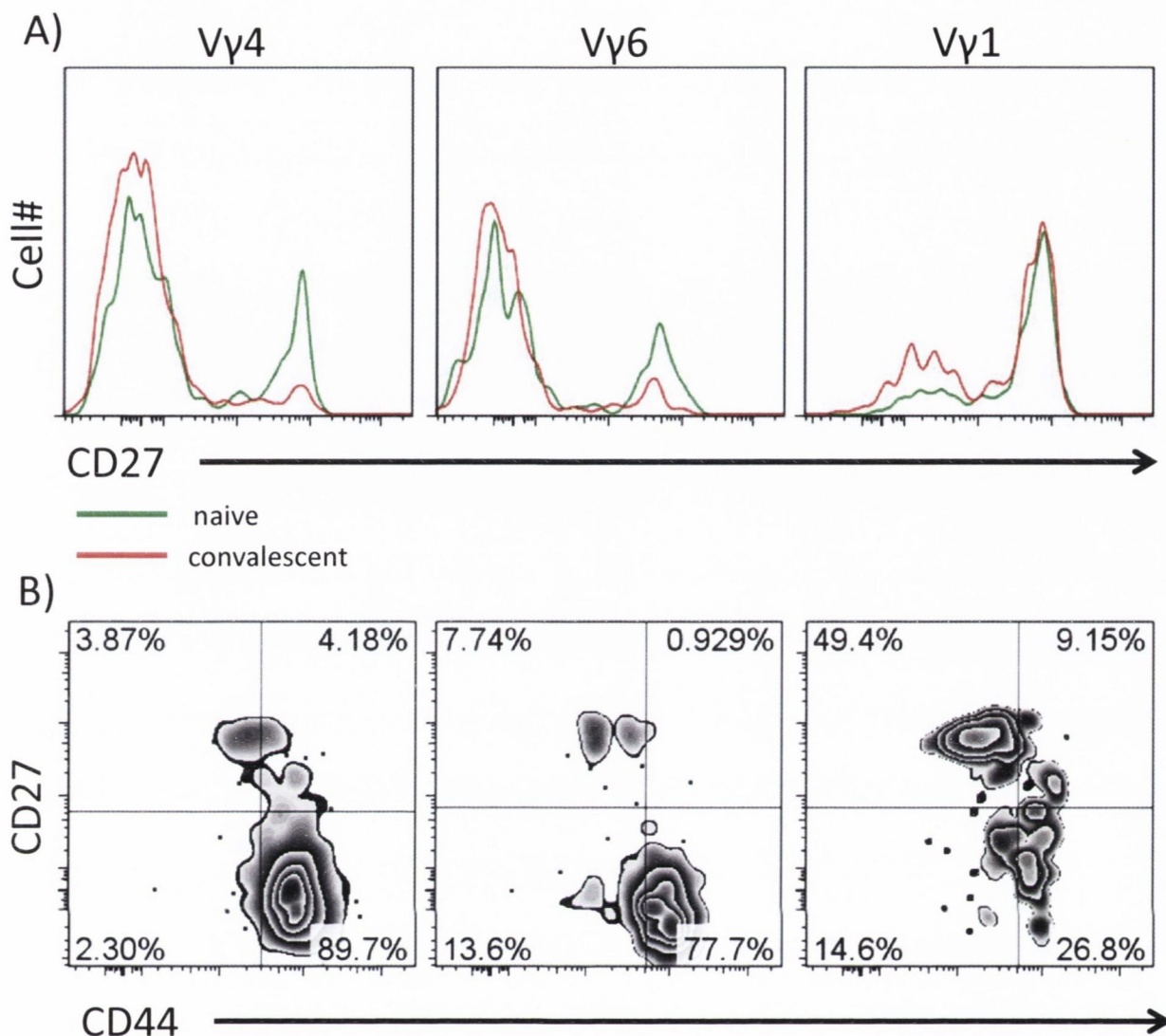


Figure 5.9 CD27⁻ $\gamma\delta$ T cell population persists after the pathogen clearance

C57BL/6 mice were challenged with virulent *B. pertussis* strain Bp338 and allowed to clear the infection. The expression of CD27 and CD44 by $\gamma\delta$ T cell subsets was then analysed by flow cytometry. The graphs show percentages of cells in the $V\gamma 4$, $V\gamma 6$ ($V\gamma 4$ - $V\gamma 1$) and $V\gamma 1$ gate in the lungs of naïve (A) and convalescent (A and B) mice. n=1

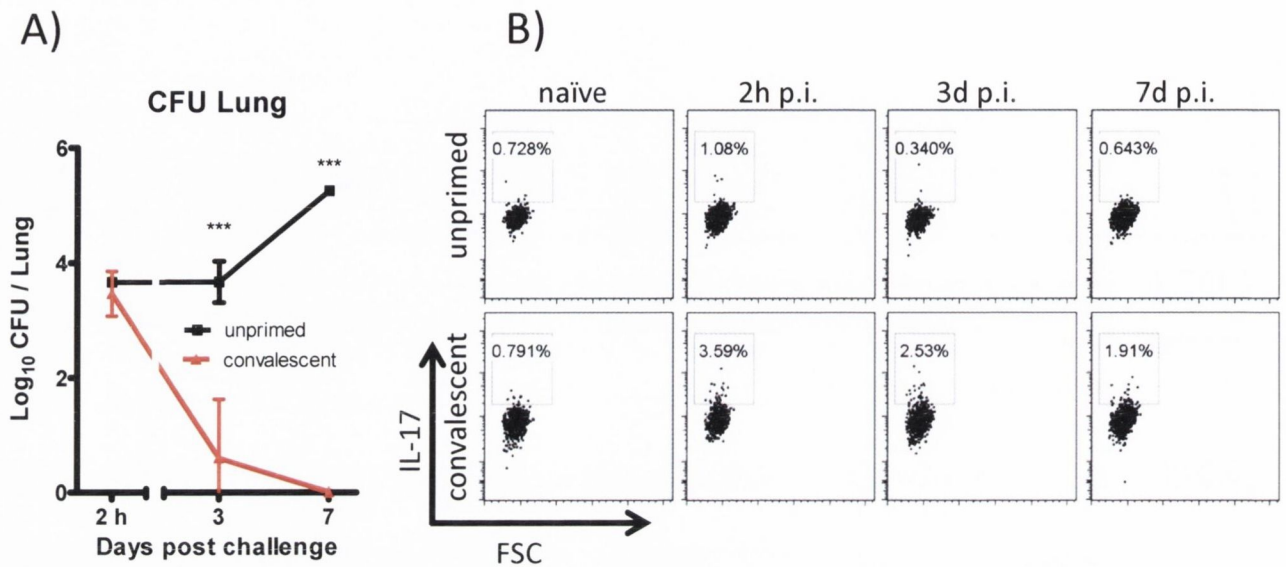


Figure 5.10 $\gamma\delta$ T cells from convalescent animals exhibit memory responses upon reinfection.

Unprimed and convalescent C57BL/6 mice were challenged with virulent *B. pertussis* strain Bp338 and sacrificed at different time points post infection. At each time point, half of the lung was used to estimate lung CFUs. Colony counts were performed on serially diluted lung homogenates from individual mice (A). Lung lymphocytes were isolated from the other half of the lung and incubated in the presence of BrfA (5 μ g/mL) for 4 hours. The production of IL-17 by $\gamma\delta$ T cells was analysed by flow cytometry (B). The plots show percentages of cells in TCR δ^+ gate from individual mice. *** $p < 0.001$ by two-way ANOVA with Bonferroni post test. $n=1$

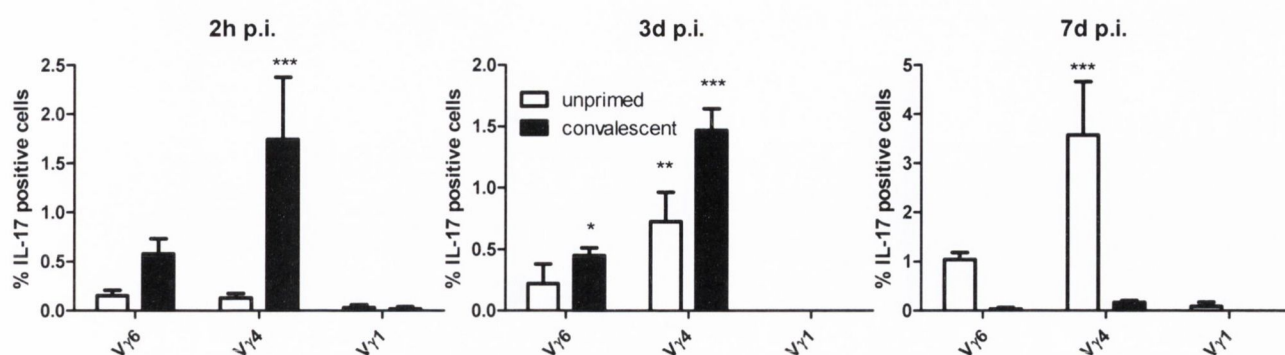


Figure 5.11 Vγ4 cells are the dominant IL-17 producing γδ T cell subset upon reinfection with *B. pertussis*

Unprimed and convalescent C57BL/6 mice were challenged with virulent *B. pertussis* strain Bp338 and sacrificed at different time points post infection. At each time point, lymphocytes were isolated from the lung and incubated in the presence of BrfA (5 µg/mL) for 4 hours. The production of IL-17 by γδ T cell subsets was analysed by flow cytometry. The graphs show percentages of IL-17 producing γδ T cell subsets expressed as percentage of total γδ T cells. n=1

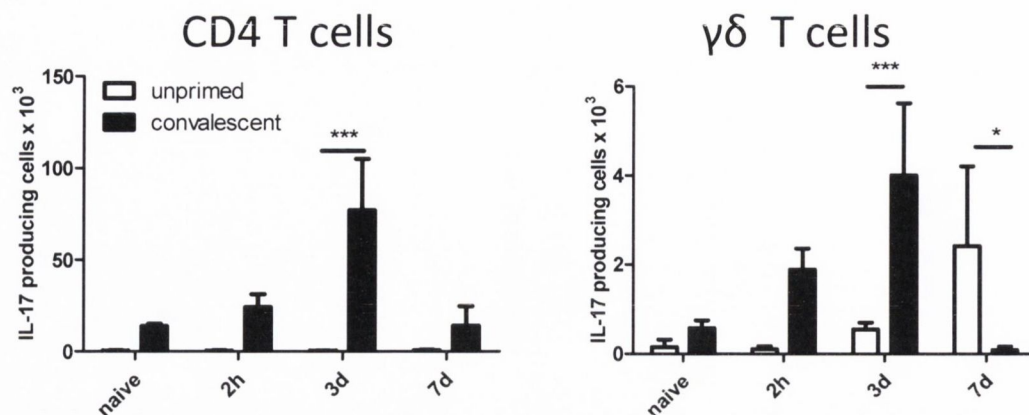
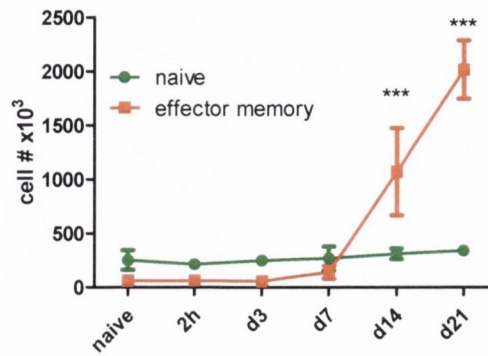


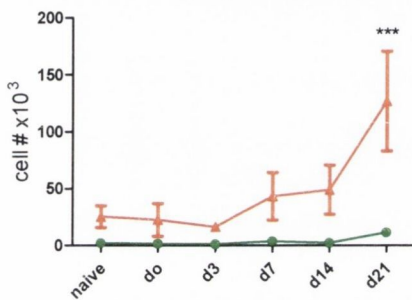
Figure 5.12 Lung CD4 and $\gamma\delta$ T cells respond rapidly to *B. pertussis* following reinfection

Unprimed and convalescent C57BL/6 mice were challenged with virulent *B. pertussis* strain Bp338 and sacrificed at different time points post infection. At each time point, lymphocytes were isolated from the lung and incubated in the presence of BrfA (5 μ g/mL) for 4 hours. The production of IL-17 by $\gamma\delta$ T cell and CD4⁺ T cells was analysed by flow cytometry. The graphs show percentages of IL-17 producing cells in the CD4 and $\gamma\delta$ T cell gate . n=1

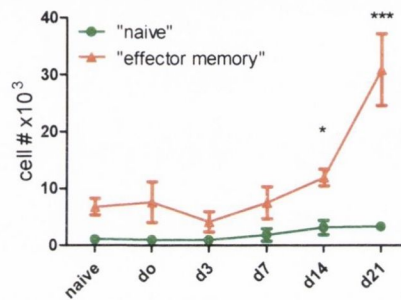
CD4 T cells



V γ 4



V γ 6



V γ 1

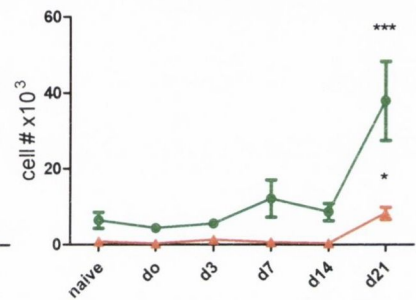


Figure 5.13 Similar to CD4 T cells, effector memory markers bearing V γ 4 and V γ 6 $\gamma\delta$ T cells accumulate in the lungs of *B. pertussis* infected mice.

C57BL/6 mice were challenged with virulent *B. pertussis* strain Bp338 and sacrificed at different time points post infection. At each time point, expression of CD62L and CD44 by $\gamma\delta$ T cell subsets and CD4 T cells was analysed by flow cytometry. The graphs show absolute numbers of cells in the CD3⁺TCR δ ⁺V γ 4⁺ (V γ 4), CD3⁺TCR δ ⁺V γ 4⁺V γ 1⁻ (V γ 6) and CD3⁺TCR δ ⁺V γ 1⁺ (V γ 1) and CD3⁺CD4⁺ gate. Statistical significance of the data was analysed by a two way ANOVA with Bonferroni post test. * p <0.05, ** p <0.01, *** p <0.001. n =1

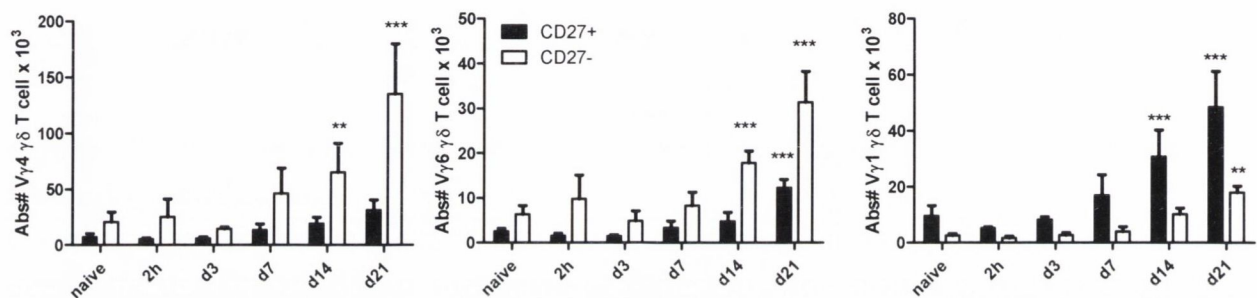


Figure 5.14 The expansion of CD27⁻ and CD27⁺ γδ T cells in the lung during the course of *B. pertussis* infection is subset specific.

C57BL/6 mice were challenged with virulent *B. pertussis* strain Bp338 and sacrificed at different time points post infection. At each time point the expression of CD27 by γδ T cell subsets was analysed by flow cytometry. The graphs show absolute numbers of CD27⁺ and CD27⁻ cells in the Vγ4, Vγ6 and Vγ1 subset in the lung of naïve and infected mice. n=1

Chapter 5.3: Discussion

$\gamma\delta$ T cells are thought to be mainly involved in the innate phase of the immune response and to be activated in an antigen non-specific manner. Robust $\gamma\delta$ T cell responses are observed in naïve animals upon stimulation with cytokines alone, without the need of TCR engagement. It is thought that many $\gamma\delta$ TCRs are specific for host derived molecules and thus serve as a sensor of cellular distress. Very few studies have addressed the possible existence of pathogen-specific memory in $\gamma\delta$ T cells or their role in immunity. Although still a matter of debate, research from different labs has generated compelling evidence suggesting that $\gamma\delta$ T cells may be capable of contributing to both the innate and antigen specific protection (Murphy et al, 2014; Pitard et al, 2008; Shen et al, 2002; Sheridan et al, 2013). Mouse $\gamma\delta$ T cells have been reported to express memory markers such as CD44 and CD62L (Do et al, 2010; Ribot et al, 2009; Sheridan et al, 2013; Zeng et al, 2012). Furthermore, similar to $\alpha\beta$ T cells, differential CD27 expression has been observed in $\gamma\delta$ T cell subsets. However, CD27 expression by $\gamma\delta$ T cells is thought to segregate with their effector function (IL-17 or IFN γ secretion) rather than their activation state (Ribot et al, 2009). In the current study it was found that a memory $\gamma\delta$ T cell population is induced in the lungs of *B. pertussis* infected mice and mounts enhanced IL-17 responses upon reinfection.

Lung $\gamma\delta$ T cells were found to segregate, similar to conventional T cells, into three subpopulations based on CD44 and CD62L expression: naïve, central memory and effector memory cells. Furthermore, the percentage of putative effector memory $\gamma\delta$ T cells was found to be increased in the lungs following *B. pertussis* infection. However, the increase in the percentage of these cells was not as pronounced as for their conventional counterparts and their percentage was already high prior to infection. This is in agreement with studies of spleen and dermal $\gamma\delta$ T cells, but in sharp contrast to the data obtained from the gut, where CD44⁺ cells constituted the minority of $\gamma\delta$ T cells in naïve animals (Ribot et al, 2009; Sheridan et al, 2013; Sumaria et al, 2011). In agreement with findings of Ribot et al. (Ribot et al, 2009), CD62L^{lo}CD44^{hi} $\gamma\delta$ T cells were found to lack CD27 expression, while the other two subsets were CD27 positive. A similar pattern of CD27 expression is observed in $\alpha\beta$ T cells: CD27 is expressed by naïve and central memory cells, but it is

downregulated on the effector memory population (Fritsch et al, 2005; Wherry et al, 2003).

Upon closer investigation of CD27 expression by subsets of lung $\gamma\delta$ T cells, it was found that CD27⁻ cells constitute a large proportion of V γ 4 and V γ 6 subset while V γ 1 cells were mostly CD27⁺. However, the percentage of CD27⁻ cells was increased in all three subsets following *B. pertussis* infection, suggesting that, similar to $\alpha\beta$ T cells, $\gamma\delta$ T cells may downregulate CD27 expression following activation (Figure 5.3). In support of these findings Zeng and colleagues showed that CD62L^{hi}CD44^{hi} $\gamma\delta$ T cells (which are also CD27⁺) expressing TCR specific for phycoerythrin become CD62L^{lo}CD44^{hi} following activation and start to produce IL-17 (Zeng et al, 2012). Given that CD62L^{hi} $\gamma\delta$ T cells co-express CD27, these two molecules can be interchanged when dividing $\gamma\delta$ T cells into memory subsets. Using CD27 and CD44 to divide $\gamma\delta$ T cells into naïve and memory populations, CD27⁻CD44⁺ effector memory cells were found to be expanded in all V γ subsets in lungs of *B. pertussis* infected mice (Figure 5.4). Similar to the findings of the current study Sheridan et al. saw a significant increase in the percentage of CD27⁻CD44^{hi} cells in the gut lamina propria of *L. monocytogenes* infected mice (Sheridan et al, 2013). They found that these cells persisted in the gut for at least 5 months and exhibited a rapid recall response upon reinfection. In addition, CD27⁻CD44^{hi} cells in the gut had a distinct phenotype from the lymph node CD27⁻CD44^{hi} $\gamma\delta$ T cells as they lacked the expression of CCR6 and CD103 (Haas et al, 2009; Sheridan et al, 2013). Similarly, human CD27 negative V δ 2⁻ $\gamma\delta$ T cells have been shown to be increased in cytomegalovirus infected patients and mount superior cytotoxic responses against infected fibroblasts compared to V δ 2⁻ cells from uninfected individuals (Pitard et al, 2008). Kirby and colleagues reported an increase in the CD44⁺CD69⁺ $\gamma\delta$ T cells in the lung following *Streptococcus pneumoniae* infection and these cells showed a lung homing capacity when transferred into either naïve or infected recipients (Kirby et al, 2007). It therefore appears that increased numbers of CD27 negative cells do correlate with protective immunity.

The use of combinations of CD62L, CD44 and CD27 does not allow a distinction between effector and effector memory T cells and thus the CD62L^{lo}CD44^{hi}CD27⁻ population in the lung would contain both of these cell types. Furthermore, it is possible that the CD27⁻CD44^{hi} $\gamma\delta$ T cells in naïve lungs represent a population of

“memory” cells specific for stress inducible self-antigens, while infection induced CD27⁺CD44^{hi} $\gamma\delta$ T cells are the true pathogen-specific memory cells. The expression of other activation markers and adhesion molecules by these cells would need to be investigated in order to determine whether there is a qualitative difference between *B. pertussis*-specific $\gamma\delta$ T cells and the CD27⁺CD44⁺ cells present in the lung of naïve animals.

The current data can be interpreted in two ways; assuming that the memory markers indeed correlate with appearance of a memory $\gamma\delta$ T cell population, then effector/effector memory cells appear to be increased in all subsets of lung $\gamma\delta$ T cells, and they can represent producers of different types of cytokines. If, however, CD27⁺CD44⁺ cell expansion correlates with increased numbers of IL-17 producing cells, then there may be greater IL-17 (and possibly Th17 related cytokine) responses among all subsets of lung $\gamma\delta$ T cells.

In order to test whether the increase in CD62L^{lo}CD44^{hi}CD27⁺ cells was associated with appearance of antigen specific memory cells, $\gamma\delta$ T cell subsets were purified from lungs of naïve and *B. pertussis* infected mice and restimulated them with HKBP in the presence of APC. It was found that IL-17 was produced in a dose dependent manner by lung Vy4 cells from infected, but not naïve mice (Figure 5.5). Furthermore this response was specific to IL-17A and IL-17F was not produced in a similar fashion (Figure 5.5 and 5.6). In addition, Vy4 cells failed to produce IL-17 in the absence of APC suggesting that the antigen must be presented to $\gamma\delta$ T cells in order to evoke the recall response (Figure 5.7). It remains to be determined whether MHC II or CD1d is involved in the presentation of the *Bordetella* antigens to the $\gamma\delta$ T cells. Given that $\gamma\delta$ T cells were isolated from lungs at the peak of *B. pertussis* infection, there was a possibility that IL-17 production would continue following isolation. However, no IL-17 was detected from Vy4 cells incubated with APC in the absence of HKBP or with medium alone. When the antigen recall experiment was repeated, it was found that both Vy4 and Vy6 $\gamma\delta$ T cells were capable of mounting antigen-specific responses and Vy6 cells were found to produce both IL-17 and IL-22 in response to HKBP. In contrast to the current data, Sheridan et al. found that the gut CD27⁺CD44⁺ cells were exclusively the Vy6 chain expressing cells (Sheridan et al, 2013). Vy6 $\gamma\delta$ T cells were also shown to significantly expand in the peritoneal cavity of *Staphylococcus aureus* infected mice and to exhibit protective antigen specific

responses upon reinfection (Murphy et al, 2014). While in the current study we have shown that a memory population of V γ 4 $\gamma\delta$ T cells is generated during *B. pertussis* infection, the presence of V γ 6 $\gamma\delta$ T cell memory is unclear given the inconsistent results. More experiments need to be performed in order to confirm whether they also play a role in the antigen specific protection in the lung.

Interestingly, although no antigen-specific cytokine production was detected from V γ 1 cells, they were found to produce the greatest amount of IFN γ and IL-10 upon PMA/ionomycin stimulation and very little IL-17 compared to other V γ subsets (Fig 5.6). These cells also contained the greatest percentage of CD27⁺ cells suggesting that CD27 expression indeed correlates with IFN γ production. Recently CD27-CD70 signalling has been shown to be associated with inhibition of IL-17 gene induction in CD4 T cells (Coquet et al, 2013a). The same group also reported that CD27-CD70 signals promote Treg development in the thymus (Coquet et al, 2013b). Furthermore, Schmolka et al. showed that while the transcription of Il17 and related genes was limited to CD27⁻ cells, IFN γ related genes were transcribed in both CD27⁺ and CD27⁻ $\gamma\delta$ T cells (Schmolka et al, 2013). This may explain why V γ 1 cells which express high levels of CD27 produce mainly IFN γ and IL-10 and very little IL-17, while V γ 4 and V γ 6 cells are able to produce both IL-17 and IFN γ . Alternative explanation could be that IFN γ is produced by naïve cells by default, while IL-17 production needs to be induced by cytokine stimulation or TCR engagement.

Knowing that antigen specific $\gamma\delta$ T cells are induced in the lungs of *B. pertussis* infected mice, I then went on to investigate whether these cells are maintained after the pathogen clearance, and if so, whether they show greater effector responses upon reinfection. It was found that CD27⁻CD44^{hi} $\gamma\delta$ T cells remained increased in the lungs of convalescent mice and were able to mount superior responses compared to unprimed animals. Furthermore, V γ 4 accounted for the majority of IL-17 producing $\gamma\delta$ T cells both in convalescent and unprimed animals. However, V γ 6 cells were also found to mount superior effector responses upon reinfection. This is in agreement with findings of other groups who also observed greater $\gamma\delta$ T cell responses in convalescent animals (Murphy et al, 2014; Sheridan et al, 2013). However, given that in the current study, the mice have been reinfected shortly after the pathogen clearance, one could argue that the superior response could be due to increased numbers of lymphocytes in the lungs of these mice and their activated status. In

order to confirm that $\gamma\delta$ T cells contribute to the long lived memory in the lung, another experiment with a longer rest period following pathogen clearance should be performed. Also, to confirm for the role of memory $\gamma\delta$ T cells in the antigen-specific protective immunity, it should be tested whether memory $\gamma\delta$ T cells are enough to confer protection when transferred into naïve animals prior to infection.

It was found that, similar to CD4 T cells, absolute numbers of effector memory V γ 4 and V γ 6 $\gamma\delta$ T cells increased in the lung during the course of *B. pertussis* infection. The number of V γ 4 and V γ 6 cells bearing the naïve phenotype, on the other hand, remained relatively constant. However, the opposite was observed for V γ 1 cells. This trend was mirrored by the increase in either CD27⁻ or CD27⁺ cells in the respective V γ subsets. This is in agreement with the data of Ribot et al. who observed an increase in numbers of both CD27⁺ and CD27⁻ cells following *Plasmodium berghei* infection. These authors suggested that CD27⁺ and CD27⁻ expression correlated solely with the effector function of $\gamma\delta$ T cells. However, in the current study, it was found that the increase in the CD62L^{lo}CD44^{hi}CD27⁻, but not CD27⁺ cell number in the lung was accompanied by appearance of antigen-specific $\gamma\delta$ T cells.

Taken together, the current data shows that both $\alpha\beta$ and $\gamma\delta$ memory T cells are induced in the lungs of *B. pertussis* infected mice. These cells persist in the lung after the pathogen has been cleared and mount superior IL-17 responses upon reinfection. It should be tested whether *Bordetella* induced memory $\gamma\delta$ T cells are confined to the lung or whether they are also present in the periphery. It would be also interesting to see whether memory $\gamma\delta$ T cells are derived from lung resident or the peripheral $\gamma\delta$ T cell pool. Furthermore, it should be confirmed that the memory $\gamma\delta$ T cells response is antigen specific using an unrelated pathogen. Although similar to $\alpha\beta$ T cells, memory $\gamma\delta$ T cells require APC for antigen-specific activation, molecules other than MHC class II may be involved in presentation of *Bordetella* antigens to $\gamma\delta$ T cells. Thus $\gamma\delta$ T cells may contribute to memory responses by recognising different types of antigens resulting in strengthening of the overall response. Further studies should investigate these and other aspects of memory $\gamma\delta$ T cell activation and their contribution to protective responses induced by infection or vaccination.

Chapter 6: General discussion

Chapter 6: General discussion

The current study has shown that $\gamma\delta$ T cells isolated from different tissues have differential capacity to respond to exogenous cytokines. Furthermore, the ability to respond to different cytokine combinations also varies between V γ subsets isolated from a given tissue and this can be in part explained by the levels of cytokine receptor expression by these cells. Only lung V γ 6 $\gamma\delta$ T cells were found to respond strongly to live *B. pertussis* induced proinflammatory stimuli, while both V γ 6 and V γ 4 cells mounted strong IL-17 responses upon IL-1 β +IL-23 stimulation. This suggested that live *B. pertussis* induced activation of V γ 6 $\gamma\delta$ T cells could be independent of either one or both of these cytokines. It was found that lung lymphocytes from IL-1RI^{-/-} mice failed to produce IL-17 in response to *B. pertussis* stimulation, showing that *B. pertussis* induced IL-17 is completely dependent on IL-1 β . It is less clear whether IL-23 is involved in *B. pertussis* mediated V γ 6 $\gamma\delta$ T cell activation. The current study has shown that $\gamma\delta$ T cells produce IL-17 early during *B. pertussis* infection and contribute to IL-22 production in the lung. It was found that both of these cytokines contribute to clearance of the bacteria. Furthermore, the current data suggests that IL-22 has a role in limiting the inflammation in the lung. It was also found that lung $\gamma\delta$ T cells are capable of antigen specific recognition of *B. pertussis* and contribute to memory responses against this pathogen.

$\gamma\delta$ T cells exhibit features of both innate and adaptive immune cells; they become rapidly activated following pathogen entry and at the same time have the potential to recognise a large number of antigens via their TCR. They have been reported to be an early source of IL-17, IFN γ and other cytokines in many disease models, which can either contribute to the disease resolution or its pathology, depending on the model in question. Knowledge of how the different effector functions of $\gamma\delta$ T cells become activated will be useful in the design of cell based therapies or to stimulate beneficial responses during disease.

Given that the cytokines produced during the innate phase of infection or induced by vaccine adjuvants are the decisive factor for the type of adaptive response that will predominate, the ability to activate particular $\gamma\delta$ T cell subsets should be considered when designing vaccine formulations.

Several differences exist between human and mouse $\gamma\delta$ T cells. Perhaps the biggest difficulty in directly comparing functions of mouse and human $\gamma\delta$ T cells comes from the fact that different subset nomenclature is in place for the two species due to the different number of variable gene sequences and their abundance. Consequently a particular subset in mice cannot be directly related to a specific subset in humans. However, similar to the mouse system, $\gamma\delta$ T cell subsets can be divided into those that are predominantly present in circulation (V δ 2 cells) and those more commonly found in tissues (V δ 1 and V δ 3 cells). Furthermore, certain features of tissue resident $\gamma\delta$ T cells are identical in mice and humans. For example, in both species $\gamma\delta$ T cells are increased in the gut intraepithelial compartment and a large percentage of them express CD8 $\alpha\alpha$. The major differences are the lack of an equivalent of mouse epidermal $\gamma\delta$ T cell population in humans and the absence of phosphoantigen reactivity in mouse $\gamma\delta$ T cells (Bos et al, 1990). Nonetheless, the similarities in homing patterns of mouse and human $\gamma\delta$ T cells suggest that these cells perform similar functions in both species.

It is becoming more appreciated that different subsets of $\gamma\delta$ T cells may play contrasting functions in a specific disease. Being able to identify signals and populations promoting beneficial responses can be instrumental in designing successful therapies for many inflammatory diseases. For example, the progression of inflammatory bowel disease is thought to be mediated by IL-17 and IFN γ and greater expression of these cytokines can be detected in serum and biopsies of patients with active disease compared to those in remission or normal controls (Fonseca-Camarillo et al, 2011; Fujino et al, 2003; Fuss et al, 1996; Kleinschek et al, 2009; Seiderer et al, 2008). Animal studies have confirmed the involvement of IFN γ and IL-23 dependent IL-17 production in the pathology of IBD (Elson et al, 2007; Hue et al, 2006; Ito et al, 2006; Yen et al, 2006; Zhang et al, 2006). Although $\gamma\delta$ T cells constitute a significant percentage of lymphocytes in the gut, very little is known about their role in IBD. Mouse studies have revealed that $\gamma\delta$ T cells have the potential to contribute to either the disease progression or its resolution (Do et al, 2011; Meehan et al, 2014). It appears that the pro-regenerative function is mediated specifically by gut resident $\gamma\delta$ IELs and not lamina propria or peripheral $\gamma\delta$ T cell subsets. The current study has found that mouse $\gamma\delta$ IEL lack the expression of IL-1 and IL-23 receptor and thus are unable to produce IL-17 in response to these

cytokines. In agreement with the present findings, Ivanov et al observed very few IL-17 producing $\alpha\beta$ and $\gamma\delta$ T cells (between 0.1%-0.2%) in the IEL compartment (Ivanov et al, 2006). In contrast, lamina propria $\gamma\delta$ T cells do produce IL-17 in response to various stimuli (Asigbetse et al, 2010; Ivanov et al, 2006). Similar to the mouse studies, IL-17 producing cells were mostly found in the lamina propria of patients with active ulcerative colitis (Fujino et al, 2003). In the present study IL-1 β +IL-23 stimulation of total intraepithelial lymphocytes resulted in induction of IL-22 production by these cells. IL-22 production in the gut has been associated with promotion of wound healing, mucus production and goblet cell restitution (Pickert et al, 2009; Sugimoto et al, 2008). The inability of intraepithelial lymphocytes to produce IL-17 upon stimulation with proinflammatory cytokines may represent a mechanism of avoiding unnecessary inflammation in the absence of barrier breach. IL-22 is known to promote antimicrobial peptide production by epithelial cells and therefore the preferential induction of this cytokine in intraepithelial lymphocytes could be a means of controlling the growth of commensal, non-invading bacteria. The presence of IL-17 producing cells in the lamina propria, on the other hand, would ensure swift pro-inflammatory response once the bacteria cross the epithelial barrier.

Retinoic acid (RA) has been implicated in the attenuation of intestinal inflammation in mice and humans (Bai et al, 2009). The data in the present study, as well as that generated by others in the lab, demonstrated that RA promotes IL-22 production by IL-1 β +IL-23 stimulated $\gamma\delta$ T cells and other innate like-lymphocytes in the gut and lymph nodes (Mielke et al, 2013). Recently, interactions between a semaphorin molecule, CD100, on the surface of $\gamma\delta$ IELs and its ligand expressed by colonic epithelial cells have been shown to promote keratinocyte growth factor 1 (KGF-1) production by $\gamma\delta$ IELs which was important for promotion of tissue repair in DSS colitis (Meehan et al, 2014). Furthermore, similar to KGF^{-/-} mice, CD100^{-/-} mice had exacerbated disease severity (Chen et al, 2002; Meehan et al, 2014). Interestingly, blocking of the CD100 ligand, plexin B2, resulted in reduced IFN γ production *in vitro*, suggesting IFN γ may be associated with protection in this disease model (Meehan et al, 2014). In addition, although $\gamma\delta$ IELs were not activated by cytokine stimulation in the current study, a higher basal IFN γ expression in these cells was observed, suggesting it may be important for mediating the tissue protective function of these

cells. IL-22 was shown to promote the proliferation of intestinal epithelial cells and to induce human β -defensin 2 (hBD2) expression by these cells (Brand et al, 2006). Recently, hBD2 has been implicated in suppression of IL-17, which was associated with increased IFN γ and IL-10 (Brand et al, 2006; Kanda et al, 2011). Furthermore, retinoic acid has been shown to promote the production of IL-22 binding protein, the natural antagonist of IL-22. Thus RA is able to both promote protective IL-22 production and at the same time limit this response. Given those observations and the findings of the current study showing that RA promotes both IL-22 and IFN γ production by IL-1 β +IL-23 stimulated IEL and LN cells, it is possible that the protective role of RA is mediated by both of these cytokines. The use of RA in conjunction with molecules stimulating activation of intraepithelial $\gamma\delta$ T cells may therefore represent a viable method for dampening the inflammation and promoting tissue repair.

Recently the pathogenicity of IL-17 and IFN γ in IBD has been questioned and their roles may not be as clearly defined as previously thought (O'Connor et al, 2009; Sheikh et al, 2010; Thelemann et al, 2014). IFN γ production has been shown to induce expression of MHC class II on intestinal epithelial cells which was associated with protection from T cell transfer induced colitis (Thelemann et al, 2014). Furthermore, IFN γ inhibited IL-23 expression during colitis in mice (Sheikh et al, 2010). This may partially explain why anti-IFN γ treatment of IBD patients was found to result in a low rate of remission and high percentage of adverse effects characterised by aggravation of the disease symptoms (Hommes et al, 2006; Reinisch et al, 2010; Reinisch et al, 2006). Similarly, conflicting data exist regarding the role of IL-17 in IBD. In a study of T cell transfer induced colitis, IL-17 $^{-/-}$ CD4 $^{+}$ T cells were found to induce a more severe disease with significant increase in IFN γ producing cells compared to WT CD4 $^{+}$ T cell transfer (O'Connor et al, 2009). In contrast, IL-17R $^{-/-}$ mice have been shown to be protected from TNBS induced colitis even though IFN γ production was increased in these animals (Zhang et al, 2006). Furthermore, anti-IL-17 treatment of IBD patients did not have a therapeutic effect and in some patients resulted in exacerbation of the disease symptoms (Hueber et al, 2012). IL-17 and IFN γ are known to regulate each other's expression and neutralisation of one cytokine may lead to abnormal increase in the expression of the other one. Furthermore, IL-17 can also downregulate its own expression. Therefore,

the failure of anti-IL-17 treatment could be either caused by exacerbated IL-17 responses in the absence of the negative feedback loop or increased IFN γ due to the lack of IL-17 mediated inhibition. Different lymphocyte populations are present in the circulation and in tissues. It is possible that the divergent roles of IFN γ and IL-17 in IBD models are in part due to the different origin of the disease initiating stimulus. Similarly, the disease is likely to be initiated in different ways in patients. It is important to dissect the roles of IL-17 and IFN γ in the local environment and in circulation. Perhaps the best IBD therapy would promote repair and shift the local cytokine balance but not remove either of the key players completely.

Very little information is available on the role of lung $\gamma\delta$ T cells during *B. pertussis* infection in humans due to difficulty in getting BAL samples from infected children. It has been reported that decreased numbers of $\gamma\delta$ T cells are present in the blood of children suffering from whooping cough compared to their healthy peers, suggesting that these cells home to the inflamed lung (Bertotto et al, 1997). The current study has found that lung $\gamma\delta$ T cells produce IL-17 in the lungs of *B. pertussis* infected mice and this cytokine was found to be important for the control of the bacterial growth at the early stages of infection. Furthermore, lung $\gamma\delta$ T cells were found to contribute to IL-22 production in the lung, which was found to have a role in limiting the degree of inflammation and to promote *B. pertussis* clearance. T cell responses induced by pertussis vaccine are influenced by the type of vaccine used. The whole cell pertussis vaccine (Pw), similar to natural infection, has been shown to result in Th1 dominated responses, while the acellular pertussis vaccine (Pa) induces a mixed Th1/Th2 response in children (Dirix et al, 2009; Ryan et al, 1997; Ryan et al, 1998). Similar antigen-specific cytokine profile is observed in mice vaccinated with Pw and Pa (Redhead et al, 1993). Studies in the lab have shown that IL-17 contributes to protective antigen specific responses induced by both Pw and Pa and appears to be in fact the main Th effector cytokine induced by Pa (Ross et al, 2013). Although data on antigen-specific Th17 responses in human patients and vaccinees is lacking, studies in non-human primates have shed some light on the nature of the protective responses likely to be induced in humans. Respiratory infection of baboons with *B. pertussis* was shown to induce mucosal IL-17 production and mixed Th1/Th17 responses in the periphery (Warfel & Merkel, 2013). Furthermore, similar to what is seen in mice, Pw vaccination resulted in a mixed Th1/Th17 response (Warfel et al,

2014). Interestingly, this group also found that PBMC from convalescent animals produced significantly greater amounts of IL-17 and IFN γ in response to HKBP compared to cells from Pw vaccinated animals. In addition, the convalescent animals were the fastest to clear the pathogen. Their findings highlight the role of tissue resident cells in the induction of protective immunity. Another interesting finding of their study is that while IL-17 was mainly produced by CD4⁺ T cells in Pw vaccinated animals, CD4⁺ and CD4⁻ T cells produced this cytokine in the peripheral blood of convalescent animals. The CD4⁻ T cell population may possibly be representing $\gamma\delta$ T cells which have emigrated from the lung to promote systemic responses.

Majority of vaccines, with a few exceptions, are delivered as intradermal or intramuscular injections. Mucosal vaccination, apart from representing a route likely to be associated with higher compliance in the general population, may represent a better way of inducing long lasting memory through activation of tissue resident cells. The current study has found that a population of $\gamma\delta$ T cells expressing memory T cell markers expands in the lungs of *B. pertussis* infected mice and persists following pathogen clearance. These cells were found to mount superior responses upon reinfection and contribute to faster clearance of bacteria. IL-17 was found to be the major effector cytokine secreted by lung memory $\gamma\delta$ T cells *in vitro* and upon reinfection with *B. pertussis*. Preliminary data suggests that IL-22 may also be produced by lung $\gamma\delta$ T cells in an antigen-specific manner. IL-17-producing $\gamma\delta$ T cells were reported to be increased in circulation of patients with active pulmonary tuberculosis (Peng et al, 2008). Resident V δ 1 $\gamma\delta$ T cells were found to be expanded in the nasal mucosa of chronic rhinitis patients (Pawankar et al, 1996). Interestingly, the majority of these V δ 1 cells, unlike the same subset in the circulation, had a memory phenotype, suggesting that the memory-like cells were generated specifically in the nasal mucosa. While the standard vaccination induces responses that are able to clear the infection before the appearance of clinical disease, induction of mucosal immunity may offer an advantage of preventing the infection itself and thus limit the pathogen transmission. General issues with mucosal vaccines are associated with lack of reactivity due to numerous tolerance mechanisms present in the mucosa. Recently, a live attenuated *B. pertussis* vaccine has been developed which was shown to be safe in adult, neonate and immunocompromised animals (Skerry et al, 2009). The vaccine strain induced

protection was comparable to that induced by infection with WT *B. pertussis* (Skerry & Mahon, 2011). Furthermore, recently the vaccine has successfully passed a phase I clinical trial involving healthy unvaccinated volunteers (Thorstensson et al, 2014). However, as with any live attenuated vaccine, there is always a small risk of the vaccine strain reverting back to virulence and generally the use of killed or subunit vaccines is favoured over vaccines containing live pathogens. Selective activation of lung $\gamma\delta$ T cells may represent a means of alerting the resident cells to the presence of pathogen molecules in the subunit vaccines and promote the recruitment of other cells types to the lung. In the current study two subsets of $\gamma\delta$ T cells were found to produce IL-17, but only one of those subsets was found to also produce IL-22. The cells co-producing the two cytokines appeared to be activated in an IL-23 dependent and independent manner. Identification of stimuli that selectively activate particular $\gamma\delta$ T cell subsets with specific cytokine profile may be useful for skewing the cytokine production in favour of the desired protective response.

The main subset of human blood $\gamma\delta$ T cells, namely the V δ 2 cells, is being explored for cancer immunotherapy due to its potent cytotoxic activity induced by phosphoantigens, which are upregulated in certain cancers. The advantage of using these cells is their quick response, which is independent of MHC mediated presentation and antigen processing. Pre-clinical studies have shown that treatment of human cancer cell lines with aminobisphosphonates, such as zoledronate, which induce accumulation of phosphoantigens, promotes $\gamma\delta$ T cell killing of these cells (Lai et al, 2012; Lertworapreecha et al, 2013; Todaro et al, 2009). Similarly, $\gamma\delta$ T cells expanded in the presence of zoledronate and IL-2 have been shown to have a greater capacity to kill transformed cells (D'Asaro et al, 2010; Lertworapreecha et al, 2013). Clinical trials are underway investigating $\gamma\delta$ T cell potential in treatment of cancer patients and have had a variable degree of success. The two main approaches involve *in vitro* expansion of autologous $\gamma\delta$ T cells or *in vivo* promotion of their expansion through infusions with zoledronate+rIL-2 (Bennouna et al, 2008; Dieli et al, 2007; Nakajima et al, 2010; Sakamoto et al, 2011). Apart from V δ 2 $\gamma\delta$ T cells, the V δ 1 subset may also have a potential for cancer immunotherapy (Devaud et al, 2013; Ebert et al, 2006). Furthermore, human phosphoantigen-reactive $\gamma\delta$ T cells have been shown to also promote antiviral immunity (Qin et al, 2009; Tu et al, 2011).

The positive results generated by human blood $\gamma\delta$ T cell studies encourage further investigations of $\gamma\delta$ T cell potential in immunotherapy. While it would not be possible to use tissue resident cells for infusions, selective activation of specific functions of these cells through treatment with $\gamma\delta$ T cell agonists could be used to promote for example tissue regeneration or vaccine-induced responses. The roles of mouse $\gamma\delta$ T cell subsets cannot be directly ascribed to particular subsets of $\gamma\delta$ T cells in humans on one to one basis. Nonetheless, mouse studies allow for identification of stimuli capable of activating specific $\gamma\delta$ T cell functions and the role of these responses in various disease settings. The observations in mice can then be validated using human cells and used to design therapies for various diseases. The fact that the majority of $\gamma\delta$ T cell antigens identified to date are small host and pathogen derived molecules, makes them an attractive alternative to antibody or cell treatment. Furthermore, *in vivo* activation of $\gamma\delta$ T cell function would represent a more universal patient nonspecific therapy than autologous cell infusions.

Conclusions and future studies

The current study has demonstrated that tissue resident $\gamma\delta$ T cells have different capacity to respond to proinflammatory stimuli. Lung $\gamma\delta$ T cells were found to contribute to both innate and antigen-specific responses against *B. pertussis* via the production of IL-17 and IL-22. Activation of specific $\gamma\delta$ T cell function may be useful for promoting protective vaccine induced responses. Future studies should investigate the nature of $\gamma\delta$ T cell antigens, the longevity of $\gamma\delta$ T cells memory responses and the contribution of these cells to infection and vaccine-induced protection. IL-22 was found to have a protective anti-inflammatory role in the lung during *B. pertussis* infection. The mechanism by which IL-22 contributes to limiting of lung pathology and inflammation as well as the possible role of other antimicrobial peptides in IL-22 mediated pathogen clearance should also be investigated.

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