The Role of T cell Subsets in Experimental Autoimmune Encephalomyelitis

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Declaration of Authorship

This report represents the sole work of the author except where otherwise stated and has not been submitted in whole or in part to any other university or institution as part of a degree or other qualification.

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Abstract

Multiple Sclerosis (MS) is a chronic, demyelinating disease which affects the central nervous system (CNS) resulting in progressive cognitive decline and physical disability. Experimental autoimmune encephalomyelitis (EAE) is an animal model of MS used to understand the cellular and molecular mechanisms underlying the disease. A greater understanding of disease pathogenesis can aid in the discovery of novel disease targets and more effective therapies for MS.

Infection with viral or bacterial pathogens has been linked with the development of MS, while infection with helminth parasites has been associated with protection against the symptoms of MS and other autoimmune diseases. Here, the murine model of MS, EAE was used to examine the effect of infection with the respiratory pathogen *Bordetella pertussis* on development of CNS inflammation. The data demonstrate that infection of mice with *B. pertussis* significantly attenuates the clinical course of EAE induced by active immunization or cell transfer. This was reflected in a significant reduction in VLA-4 and LFA-1 expression on T cells and infiltration of IL-17^{+}, IFN-γ^{+} and IFN-γ^{+}IL-17^{+} CD4 T cells into the CNS. Infection with *B. pertussis* induced IL-10 production from dendritic cells and enhanced the frequency of IL-10-producing CD25^{-}Foxp3$^{+/-}$CD4^{+} T cells *in vivo*. Furthermore, the suppressive effects of *B. pertussis* infection on EAE were lost in IL-10$^{-/-}$ mice. The findings of the present study demonstrate that a bacterial infection of the respiratory tract can attenuate EAE by promoting production of the anti-inflammatory cytokine IL-10 that may suppress licensing of autoaggressive T cells in the lungs, thereby preventing their migration into the CNS.

IL-17-producing T cells play a key pathogenic role in many autoimmune disorders, including MS and psoriasis, and are now major drug targets for these diseases. γδ T cells and CD4 T cells are the main sources of IL-17 during inflammation and host immunity. The current study and others have shown that IL-17-secreting Vγ4^{+} γδ T cells are found at a high frequency in the CNS of mice with EAE. This thesis describes a novel subtype of T cells distinct from conventional αβ or γδ T cells that co-express Vγ4, TCRα and TCRβ, but not TCRδ, and play a critical pathogenic role in CNS autoimmunity. TCRβ-expressing Vγ4^{+} cells co-express CD4 and CD8 and constitute around 8-10% of Vγ4^{+} cells in the spleen, lymph nodes, lungs and liver. These cells are present in WT and TCRδ$^{-/-}$ mice but not in TCRα$^{-/-}$ or TCRβ$^{-/-}$ mice. Co-

expression of α, β and γ chains was confirmed at the molecular level, revealing a restricted Vγ repertoire with more heterogeneous Vα and Vβ usage. αβVγ4 T cells express IL-1R1, IL-23R, CD4, CD73, ICOS, CD44, CCR6 and α4β1. Furthermore, these cells secrete IL-17A and IL-22, following stimulation with IL-1β and IL-23 and respond to autoantigens when purified from mice with EAE. Depletion of $V\gamma4^{+}$ cells from either WT or $TCR\delta^{-/-}$ mice dramatically attenuated EAE, and this effect was associated with a significant reduction in MOG-specific IL-17, IFN-γ, GM-CSF and IL-22 and CNS-infiltrating conventional Th17 cells. The data demonstrate that αβVγ4 T cells infiltrate the CNS as early as day 3 during EAE. These findings identify a novel population of T cells that express Vγ4 and αβ TCRs which play a critical role in autoimmunity through the activation of Th17 cells.

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Publications

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Abbreviations

APC	Antigen presenting cell
BBB	Blood brain barrier
BP	*Bordetella pertussis (B. pertussis)*
CD	Cluster of differentiations
CFA	Complete Freund's adjuvant
CFSE	Carboxyfluorescein succinimidyl ester
CIA	Collagen induced arthritis
CNS	Central Nervous System
CPL	Cecal puncture ligation
CSF	Cerebrospinal fluid
CTL	Cytotoxic T cell
CTLA4	Cytotoxic T-lymphocyte-associated protein 4
CTV	Cell trace violet
DAMP	Danger associated molecular pattern
DC	Dendritic cells
DN	Double negative ($CD4^-CD8^-$)
DNA	Deoxyribonucleic acid
DP	Double positive ($CD4^+CD8^+$)
EAE	Experimental autoimmune encephalomyelitis
EAU	Experimental autoimmune uveitis

EB/AO	Ethidium bromide/Acridine orange
EBV	Epstein Barr virus
ELISA	Enzyme-linked immunoabsorbent assay
FACS	Fluorescent-activated cell sorting
FCS	Foetal calf serum
FHA	Filamentous hemagglutinin
FMO	Fluorescence minus one
GM-CSF	Granulocyte-macrophage colony stimulating factor
HIV	Human immunodeficiency virus
IBD	Inflammatory bowel disorder
IFN	Interferon
IL	Interleukin
iTreg	Inducible Treg
LAP	Latency associated peptide
LFA-1	Lymphocyte function-associated antigen 1
LN	Lymph node
LPS	Lipopolysaccharide
MACS	Magnetic activated cell sorting
MBP	Myelin basic protein
MCAM	Melanoma cell adhesion molecule
MHC	Major histocompatibility
MMP	Matrix metalloproteinases

MOG	Myelin oligodendrocyte glycoprotein
MS	Multiple sclerosis
Mtb	*Mycobacterium tuberculosis*
mTEC	Medullary thymic epithelial cells
Mφ	Macrophage
NK	Natural killer
NLR	NOD like receptors
NOD	Nucleotide-binding oligomerisation domain
nTreg	Natural Treg
nTh17	Natural nTh17
PAMP	Pathogen associated molecular pattern
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PLP	Proteolipid protein
PMA	Phorbol myristate acetate
PML	Progressive multifocal leukoencephalopathy
PPMS	Primary progressive MS
PRR	Pathogen recognition receptor
PT	Pertussis toxin
RA	Retinoic acid
RNA	Ribonucleic acid

ROR	Retinoic acid receptor-related orphan receptor
RRMS	Relapsing remitting MS
S1P1	Sphingosine-1-phosphate receptor
SIP	Standard isotonic percoll
SOCS	Suppressor of cytokine signaling
SPMS	Secondary progressive MS
STAT	Signal transduced and activator of transcription
Tbet	T box expressed in T cells
TCR	T cell receptor
TFH	T follicular helper
TGF	Transforming growth factor
Th	T helper
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TRA	Tissue restricted antigens
Treg	Retinoic acid receptor-related orphan receptor
VLA-4	Very late antigen antigen-4

Table of Contents

List of Figures

3.7: *B. pertussis* infection enhances MOG-specific IL-17-expressing and IFN-γ in the spleen and inguinal lymph node of mice with EAE.

3.8: Infection of mice with *B. pertussis* significantly attenuates the clinical course of EAE induced by cell transfer.

3.9: Infection of mice with *B. pertussis* significantly reduces the infiltration of effector T cells into the brain during passive EAE.

3.10: *B. pertussis* induces the production of IL-10 from T cells and DCs.

3.11: Neutralisation of IL-10 enhances IL-17A and IL-17F production by T cells.

3.12: T cells from IL-$10^{-/-}$ mice cells produce significantly more IL-17A and IL-17F.

3.13: Infection with B. pertussis significantly increases IL-10 produucing T cells in active and passive EAE.

3.14: Attenuation of EAE by *B. pertussis* is reversed in IL-$10^{-/-}$ mice.

3.15: Reduction in the frequency of IL-17 and IFN-γ producing $CD4^+$ T cells infiltrating the brain of *B. pertussis* infected C57BL/6 mice with EAE.

3.16: IL-10 downregulates the expression of VLA-4 and LFA-1 integrins on T cells.

3.17: *B. pertussis* infection downregulates the expression of VLA-4 and LFA-1 integrins on T cells in the lung during EAE.

Chapter 4: αβVγ4 T cells, a novel IL-17 producing T cell subset

4.1: Gating strategy for analysing $V\gamma4^+CD3^+$ T cells.

4.2: Significant IL-17A and IL-17F production by $V\gamma4^+$ T cells in response to IL-1 and IL-23

4.3: IL-1β and IL-23 induce IL-17A and IFN-γ production by Vγ4 T cells via induction of STAT3.

4.4: TCRβ expressing Vγ4 T cells are present in various tissues

4.5: TCRδ expressing Vγ4 T cells, and not TCRβ are present in skin tissue.

4.6: Vγ1 T cells can express the TCRβ chain.

4.7: Co-localised surface expression of Vγ4 and TCRβ on T cells.

4.8: αβVγ4 T cells develop independently of Vγ4δ T cells in the thymus between day 14-16 of gestation.

4.9: Identification of $V\gamma4\beta^{+}$ T cells in $TCR\delta^{-/-}$ mice.

4.10: TCRβ expressing Vγ4 T cells that are absent in $TCR\alpha^{-/-}$ and $TCR\beta^{-/-}$ mice.

4.11: αβγ T cells express a skewed TCR repertoire.

4.12: TCRβ expressing Vγ4 T cells express variable TCRα chains.

4.13: αβVγ4 T cells express markers of the natural Th17 lineage.

4.14: αβVγ4 T cells express IL-23R and IL-1RI.

4.15: Vγ4β T cells express classical γδ T cell lineage markers, CD27 and CD73.

4.16: αβVγ4 T cells T cells from $TCR\delta^{-/-}$ and WT mice and respond to IL-1β and IL-23 stimulation to produce IL-17A, IL-22 but not IL-17F.

4.17: Vγ4β T cells respond to stimulation with IL-1β and IL-23 in the presence of MOG to augment IL-17A production

4.18: Vγ4β T cells proliferate in response to IL-1β, IL-23 and MOG stimulation.

Chapter 5: A novel T cell subset, αβVγ4 T cells play a critical role in IL-17 mediated CNS autoimmunity.

5.1: Vγ4 T cells infiltrate the CNS and produce IL-17 during EAE.

5.2: $TCR\delta^{-/-}$ mice have only marginal reduction in EAE severity compared with WT mice.

5.3: Frequency of Vγ4 T cells in the brains and spinal cord of mice with EAE at day 23.

5.4: Increased absolute number of CD4 T cells secreting IL-17 and IFN-γ in the CNS of WT mice compared to $TCR\delta^{-/-}$ mice with EAE.

5.5: Transfer of αβVγ4 T cells alone is sufficient to induce mild EAE.

5.6: Depletion of Vγ4 T cells in WT mice significantly reduces the severity of actively induced EAE.

5.7: Depletion of $V\gamma4^+$ T cells during development of EAE reduces infiltration of IL-17 and IFN-γ producing $CD4^+$ T cells into the CNS.

5.8: Increased absolute number of Vγ4 T cells secreting IL-17 and IFN-γ in the brain of EAE mice treated with PBS compared to anti Vγ4 antibody treatment.

5.9: Attenuation of EAE in WT and $TCR\delta^{-/-}$ mice with depletion of $V\gamma4^+$ cells.

5.10: Depletion of $V\gamma4^+$ cells during development of EAE in WT and $TCR\delta^{-/-}$ mice reduces infiltration of IL-17 and IFN-γ producing $CD4^+$ T cells into the CNS.

5.11: Depletion of $V\gamma4^+$ cells from lymph node and spleen cells from WT and $TCR\delta^{-/-}$ mice significantly impair the ability to transfer EAE.

5.12: Depletion of Vγ4 T cells from both WT and $TCR\delta^{-/-}$ cells significantly affects the ability of MOG-specific cells to secrete IL-17, IFN-γ, GM-CSF and IL-22.

5.13: Depletion of $V\gamma4^+$ cells from lymph node and spleen cells from $TCR\delta^{-/-}$ mice reduces infiltration of IL-17 and IFN-γ producing $CD4^+$ T cells into the CNS.

5.14: Vγ4β and Vγ4δ T cells infiltrate the CNS as early as 3 days post induction of EAE.

"*Music has no effect on research work, but both are born of the same source and complement each other through the satisfaction they bestow*."

A. Einstein

Chapter 1.

General Introduction

1.1 Innate and adaptive immunity

The performance of the immune system is a fine balance between immunity to infection and the onset of autoimmunity. The immune system is a collective system of biological processes, infrastructure, tissues and cells within an organism that function to protect against infection and tumours. In order to protect against pathogens, the immune system must detect a wide variety of microbes, including bacteria, viruses and parasites. However uncontrolled immune responses to pathogens can result in immunopathology and it is crucial that the immune system can distinguish pathogen antigens from self antigens. The immune system utilizes innate and the adaptive responses against infections.

1.1.1 Innate immunity

The innate immune system acts as the first line of host defence. Pattern recognition receptors (PRRs) are expressed on the cells of the innate immune system, such as dendritic cells (DCs), macrophages, polymorphonucelar leukocytes and natural killer (NK) cells (1). Small conserved molecules derived from microorganisms called pathogen associated molecular patterns (PAMPS) bind to pattern recognition receptors (PRRs), such as toll like receptors (TLRs) and nod like receptor (NLRs), on the surface of innate cells thereby activating innate immune responses (2). Damaged host cells are capable of releasing damage-associated molecular pattern (DAMPS) in times of stress, injury or damage following infection or injury and these DAMPS also activate innate immune cells by binding to PRRs (3). PRRs activate downstream signalling pathways leading to cellular activation and the production of cytokines and chemokines, which help to recruit both innate and adaptive immune cells to the site of the insult. One of the key roles of the innate immune system is the presentation of antigen to T cells by antigen presenting cells (APCs). The innate immune system serves to non specifically respond to pathogens but it also directs the differentiation of T cells through cytokine secretion (4). APCs, such as DCs, respond to a pathogen by phagocytosing the pathogen and internally processing the antigen. Following activation through PRRs, DCs undergo maturation and migrate to the lymph nodes, where they can present discrete peptide antigens on major histocompatability complex (MHC) molecules at the cell surface to the T cell receptor of naive T cells (5).

1.1.2 Adaptive immunity

The adaptive immune system is comprised of T and B lymphocytes. TCR and B cell receptors (BCR) are expressed on the surface of the T and B cells, respecitively, enabling them to respond to a wide array of potential antigens. Each individual TCR undergoes somatic rearrangement to result in a diverse repertoire of T cells that can respond to a wide diversity of foreign antigens (6). This interaction between MHC-bound peptide antigen presented by the APC to the TCR provides one of three signals necessary for T cell activation (7, 8). The second signal is generated by the engagement of T cell surface expressed co-stimulatory molecules, such as CD28, CD40L and CTLA4 with their co-stimulatory molecules on APCs, CD80 and CD86 (9). The final signal required for T cell activation is provided by cytokines secreted by innate immune cells which direct the differentiation of naive T cells into the appropriate T cell subset (10). The newly activated T cell can then clonally expand and elicit effector function against the invading pathogen. Activated T helper cells, primarily follicular helper T cells can then promote B cell activation and differentiation into antibody producing plasma cells (11).

1.2 Differentiation of T cell subsets

Haematopoietic T cell progenitors are first produced in the bone marrow followed by migration to the thymus to undergo maturation (12). During maturation, thymocytes migrate from the cortex to the medulla region of the thymus, maturing from $CD4^-$ $CD8^-$ double negative (DN) cells into $CD4^+CD8^+$ double positve (DP) cells and finally to single positive for CD4 or CD8 (13). However, T cells have the potential to become αβ or γδ T cells, where β or δ TCR chain selection is determined by recombination of the gene encoding the TCR chain (14). A recombined TCRβ chain gene results in a pre-TCR (TCRβ chain/pre-TCRα) complex and it is associated with a collection of proteins (the CD3/TCRζ complex) involved in signal transduction and eventual activation of the T cell (15). As cells progress from DN to DP stage, T cells express αβTCR on their cell surface and these DP αβ T cells undergo both positive and negative selection (16, 17). During positive selection, only immature T cells capable of binding antigen in combination with self MHC-encoded molecules are selected for maturation, whereas self-responsive T cells are eradicated from the repertoire in a negative selection process (17, 18). The selected T cells migrate to the peripheral lymphoid organs

from the medulla of the thymus as naive, single positive $CD4^+$ or $CD8^+$ T cells (19). CD4 cells are termed helper T cells (Th) and CD8 cells are termed cytotoxic T lymphocytes (CTL). In the lymph nodes, naive CD4 T cells are activated by mature DCs expressing co-stimulatory molecules, their cognate antigen coupled to MHC class II and the cytokine environment secreted by the DC. This promotes the differentiation of naïve helper T cells into one of five T helper subsets, Th1, Th2, Th17, T follicular helper (TFH) and Treg (Figure 1.1).

Each subset elicits distinct functions in response to infection, stress or pathology and is capable of secreting cytokines or upregulating molecules to mediate effector or regulatory functions. Th1 cells produce the cytokine IFN-γ and play a key role in macrophage activation, tumour surveillance and host defence against intracellular pathogens, such as *Mycobacteria tuberculosis* (20). Th2 cells are involved in humoral immunity and eosinophil recruitment through secretion of cytokines IL-4, IL-5 IL-10 and IL-13 (21). Th17 cells are named after their signature cytokine IL-17 and are primarily involved in neutrophil recruitment and immunity to extracellular pathogens (22). Treg cells are key regulators of the immune system and can suppress immune responses of other cells by cell-cell contact or secretion of anti-inflammatory cytokines, such as IL-10, TGF-β and IL-35 (23).

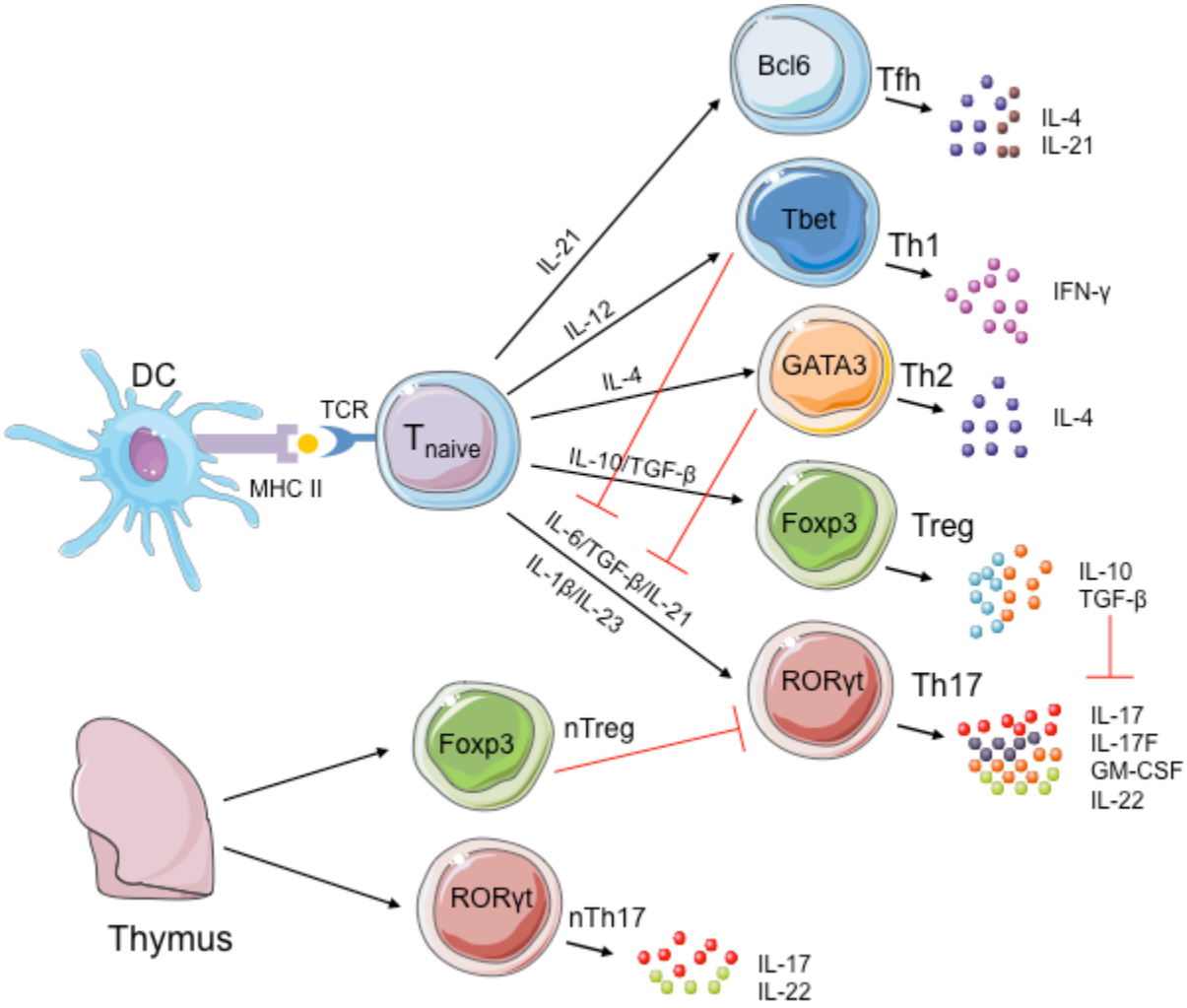


Figure 1.1: Differentiation and development of helper T cell subsets. Activated DCs, present antigenic peptide to T cells through MHC class II molecules bound to TCRs and through co-stimulatory signals. The activated APCs and other innate immune cells secrete cytokines, including IL-4, TGF-β, IL-1β, IL-6, IL-23 and IL-12. The differentiation of naïve T cells into effector cells is driven by these polarizing cytokines, where IL-21 promotes the differentiation of T follicular helper (TFh) cells, which secrete IL-4 and IL-21 and assist B cell differentiation into antibody producing plasma cells. IL-12 promotes the induction of Th1 cells, which are primarily IFN-γ producers that mediate immunity to tumours and intracellular pathogens. IL-4 increases the expression of GATA-3 promoting the development of Th2 cells, which secrete IL-4 as well as IL-5, IL-10 and IL-13. Th2 cells promote immunity to extracellular pathogens, however, they are also implicated in allergy. Inducible Treg cells (iTregs) are differentiated by TGF-β and IL-10; iTregs may or may not express the master transcription factor Foxp3, however natural Tregs (nTregs) emerge as $Foxp3^+$ from the thymus. Tregs are crucial in maintaining tolerance and preventing immunopathology through regulation of effector T cells. IL-1β, IL-6 and IL-23 promote the differentiation and expansion of Th17 cells, which secrete IL-17 (and IL-22, GM-CSF, IL-21). Th17 cells and natural Th17 cells (nTh17), which emerge from the thymus express the master transcription factor RORγt and both mediate protection to extracellular pathogens such as fungi and are heavily implicated in the pathology of many autoimmune diseases.

1.3 T helper cell subsets

The differentiation of a naive $CD4^+$ T cell into distinct helper T cell lineages of T helper type 1 (Th1), Th2, Th17 or an inducible Treg (iTreg) cell is influenced by the cytokine environment. IL-12 has been shown to enhance STAT4 and T-bet expression thereby promoting the development of IFN-γ secreting Th1 cells. IL-4 enhances expression of STAT6 and GATA-3 to induce the development of IL-4, IL-5, IL-10 and IL-13 secreting Th2 cells. IL-1, IL-23, TGF-β, IL-6 or IL-21 have been shown to induce expression of STAT3 and retinoic acid receptor-related orphan receptor gamma t (RORγt), the transcription factor necessary for IL-17 production and thus the development of functional Th17 cells (24). The classical anti-inflammatory cytokines IL-10 and TGF-β promote the development of inducible Treg (iTreg) cells, which may become $Foxp3^+$ or remain Foxp3- (25). The phenomenon of plasticity among the T helper cell subsets has been reported. Accumulating evidence suggests that Th17 and Treg cells have the flexibility to further differentiate into other T cell subsets or become dual positive cells such as IL-17- and IFN-γ-producing Th17 cells or IL-17-producing $Foxp3^+$ T cells (26). Interestingly, this transcriptional control of T cell subset differentation can be competitive, the respective Th1 and Th2 master transcription factors T-bet and GATA-3 inhibit differentiation of naive $CD4^+$ T cells into Th17 cells (27, 28). Furthermore, their associated cytokines IFN-γ and IL-4 are also capable of suppressing Th17 cell development from naïve T cells (29).

1.3.1 Treg cells

Treg cells are the master regulators of the immune system playing an indispensible role in maintaining tolerance to self antigens and preventing the development of immunopathology. They prevent the onset of autoimmunity by maintaining tolerance through suppressing self-reactive lymphocytes (30) and prevent immunopathology during infection by restricting excessive effector immune responses (31). There are two main subsets of Treg cells, inducible Treg cells (iTregs) and natural Treg cells (nTregs). nTregs constitute 1–5% of the $CD4^+$ T cell population in healthy humans; they emerge from the thymus and share common functionality with iTregs (32). Characteristically, nTregs constitutively express high levels of CD25, unlike conventional T cells which upregulate CD25 upon activation, however there is no single marker to distinguish iTregs and nTregs. The role of nTregs in protecting the

developing foetus is crucial, they serve to suppress the activation of self-reactive T cells during development (33). They also play a vital role in early life, as neonatal $CD4^+$ T cells have an intrinsic mechanism to become Treg cells in response to TCR stimulation in order to prevent immune-mediated pathology by effector T cells (34). The transcription factor Helios, was initially suggested to be an exclusive nTreg cells, however, it was later demonstrated that activated T cells and iTreg cells, under the influence of TGF-β could also express Helios (35-37).

iTregs or adaptive Tregs develop in the periphery from conventional $CD4^+$ T cells and, with TCR engagement under tolerogenic conditions they can express CD25 (38). The conversion of iTregs from naive $CD4^+$ T is influenced by IL-10, TGF-β and the vitamin A metabolite all-trans retinoic ccid (ATRA), which supports the generation of Treg cells and inhibits the differentiation of Th17 cells (39). Tregs are often characterised by expression of the master transciption factor forkhead box P3 (Foxp3), which is required for the development and function of nTregs and for the conversion and maintenance of iTregs cells (25, 40, 41). Phenotypically Tregs express the inhibitory molecule cytotoxic T-lymphocyte antigen 4 (CTLA4) and the tumour-necrosis factor (TNF)-superfamily member GITR (glucocorticoid-induced TNF receptor family related protein TNFRSF18) (42). Previously, it had been shown that Th17 and Treg share the requirement for TGF-β in their induction despite opposing functions (43, 44). However, an important study by Ghoreshi et al demonstrated that Th17 cells can develop independently of TGF-β signalling (45).

iTregs are capable of producing high levels of the anti-inflammatory cytokines IL-10 and TGF-β (46-48). TGF-β specifically inhibits Th1 responses by affecting its specific transcription factor T-bet (22). IL-10 is a potent inhibitor of inflammatory responses (49). IL-10 also inhibits Th1 and Th17 responses by directly suppressing T cell cytokine production or by inhibiting APC function through downregulating the expression of MHC class II and co-stimulatory molecules on their cell surface (50). Futhermore, IL-10 inhibits the production of polarising cytokines, such as IL-23 and IL-12 by DCs and macrophages (51). The mechanism of suppressive action of nTregs is thought to be cytokine independent; they rely on contact-mediated suppression through CTLA-4 and LFA-1 (52). The use of CTLA-4 also restricts clonal expansion of effector cells (53). nTregs can induce anergy in sub-optimally activated T cells, demonstrating their importance in immune homeostasis (54). Other studies have demonstrated that Treg cells elicit their suppressive functions on pathogenic effector T cells

through competition for shared resources in the immune system, such as IL-2, which is necessary for Treg cells signalling and for effector T cell growth (55).

IL-10 producing Tr1 cells or TGF-β producing Th3 cells are pathogen induced Treg cells which differentiate in the periphery from naive T cells under the direction of DCs. Inducible antigen-specific Tr1-type clones have been demonstrated in mice infected with *B. pertussis* and in humans infected with HCV (56, 57). Moreover, parasite specific Th3 clones have been identified in humans during chronic helminth infection (58). Th3 cells can be classified as $Foxp3^+$ or $Foxp3^-$ (59). Th3 cells express latency associated peptide (LAP) marker on their cells surface and promote TGF-β production (59). In vitro, the suppressive function of $CD4^+CD25^+LAP^+$ cells is mediated though soluble factors and cell contact suppression (59). LAP^+ Th3 cells can suppress pathogenic T cells though TGFβ mediated suppression in vivo; and this source of TGFβ is crucial in sustaining peripheral tolerance by promoting the differentiation of antigen-specific $Foxp3^+$ Treg cells (59-61). In summary, Treg cells are crucial in maintating homeostasis through the regulation of effector T cell responses during infection and dysregulation of Treg responses can lead to autoimmunity.

1.3.2 Th17 cells

Th17 cells are an inflammatory $CD4^+$ T cell subset which secrete a plethora of cytokines such as IL-17A, IL-17F, IL-21, IL-22, GM-CSF, IL-6 and TNF-α (62). With conflicting roles for TGF-β, IL-21 and IL-6 in the differentiation of Th17 cells, two seminal studies demonstrated that IL-23 was the crucial cytokine required for their development (63, 64). However, to drive the initial differentation of Th17 cells TGF-β, IL-21 and IL-6 are required (65-67). IL-6 is crucial for the activation of STAT3, which directly regulates the transcription of the genes rorc (RORγt), Il17, and Il23r, which are required for IL-17 production (24, 68, 69). Naïve T cells do not express the IL-23R, therefore IL-23 acts as a survivial signal for differentiating Th17 and is required for the maturation and pathogenic function of Th17 cells (63, 64, 70). IL-23 is composed of two subunits, p40 and p19 and is a member of the IL-12 family. The receptor for IL-23 is a composed of IL-12Rβ1 and the receptor subunit IL-23R, similarly to IL-12, IL-23 binds to the IL-12R subunit, IL-12Rβ1 but uses IL-23R for signal transduction (71). Unlike IL-12 which predominantly induces STAT4, the IL-23R predominately associates with janus kinase 2 (JAK2) in a ligand dependent manner with STAT3 (72).

STAT3 directly regulates the IL-17 and IL-21 genes but also upregulates IL-23R expression on Th17 cells, critical for the promotion of IL-17-secretion (73).

As an inducer of Th17 cells, IL-23 has been shown to contribute to chronic inflammatory processes. Transgenic mice induced to ubiquitously express the IL-23 p19 subunit develop systemic inflammation, impaired growth, and premature death (74). Furthermore, these mice were shown to have enhanced infiltration of lymphocytes and monocytes in the blood and a marked increase in the pro-inflammatory cytokines TNF-α and IL-1 in serum (74). IL-1 is another critical cytokine for Th17 effector function; IL-23 alone was demonstrated to be a poor stimulus for IL-17 production by $CD4^+$ T cells, however IL-17 production was significantly enhanced with the addition of IL-1β or IL-1α (75). Th17 cells express the IL-1R, IL-23R and there is now evidence that IL-18, like IL-1β can synergise with IL-23 to promote IL-17 production by T cells (76). Th17 cells also express aryl hydrocarbon receptor (AHR) and can be activated through a high-affinity ligand to proliferate and increase IL-17 and IL-22 production (77). Stimulation of differentiated Th17 cells with IL-1β and IL-23 promotes local tissue inflammation during infection and autoimmunity, mediated by pro-inflammtory cytokines GM-CSF, IL-22 and IL-17 (78-80).

1.3.3 Th17 cells in infection

As key secreters of IL-17A, IL-17F and IL-22, Th17 cells have an important role in immunity to extraceullular pathogens such as bacteria and fungi. IL-22 has an important role in promoting the secretion of anti-microbial peptides and maintenance of barrier function during infection (81, 82). IL-17A and IL-17F mediate their function by inducing anti-pathogenic peptide, chemokine and cytokine production by responder cells, which recruits innate cells such as neutrophils to the site of infection. IL-17R is highly expressed by epithelial cells at mucosal barriers in order to induce antimicrobial responses against bacteria such as *K. pneumoniae* (83, 84). In *Bordetella pertussis* infection, the production of IL-17 stimulated by IL-1 and IL-23, promotes neutrophil recruitment to the lung and activates macrophages to enhance killing of *B. pertussis* (85). IL-1 deficient mice fail to develop IL-17 secreting CD4+ T cells and as a result have defective clearance of bacteria from the lung post *B. pertussis* infection (86). Both IL-17A and IL-17F are required for the clearance of *C. rodentium*; early in infection IL-17A and IL-17F are equally important crucial to control bacterial burden,

whereas at later stages IL-17F is critical for clearance (87). IL-17 has recently been shown to promote protective IgA responses against the intestinal parasite Giardia, demonstrating a role for Th17 cells in mediating immunity to parasitic infections (88). An increase in mortality was observed in IL-17R$^{-/-}$ mice with *Toxoplasmosis gondii* infection due the defect in neurotrophil migration to the infection site (89). IL-17RA$^{-/-}$ mice were highly susceptible to *Candida albicans* infection (90). Furthermore, studies with human immunodeficiency virus (HIV) have shown that Th17 cells are dramatically reduced in infected patients (91, 92); which results in an increase in opportunistic infections, such as *C. albicans* in the oral epithelium of HIV patients (93).

1.3.4 Natural Th17 cells

Natural Th17 (nTh17) cells are IL-17-producing T cells which acquire effector function during IL-23 induced development in the thymus, similarly to nTregs (94). The nTh17 cells express high affinity TCRs for self antigens, evading the clonal deletion process in the thymus to enter the periphery with an activated phenotype, despite never encountering cognate antigen (95, 96). nTh17 cells are distinct from inducible Th17 cells in that they constitutively express IL-23R (IL-12Rβ1), RORγt, CD44, CCR6 and α4β1 (VLA4, CD49d/CD29) and exhibit high TCR-β clonal diversity (95, 97, 98). The IL-23/STAT3 axis appears to be involved in their development, as mice deficient in IL-23 or STAT3 had a significant reduction in IL-17 expression from nTh17 cells, whereas IL-6 appears to be indispensible (98). These cells respond rapidly during infection producing IL-17 as early as 24 hours in *C. albicans* infection rapidly recruiting neutrophils to the oral mucosa (97). nTh17 cells are a potent source of IL-17 and IL-22 and studies in animal models of psoriasis and airway hypersensitivity have sugested that they may have a pathogenic function (98, 99). Although capable of tissue remodelling and repair, their dysregulation can lead to ankylosing spondylitis (100).

1.4 γδ T cells

γδ T cells were identified over 30 years ago, however, their crucial and unique functions in immunity have only recently been appreciated. γδ T cells are a distinct subset of lymphocytes

which differ from classical T cell subtypes as they have a TCR comprised of γ and δ TCR chains, whereas classical T cell subtypes have α and β TCR chains. The γ TCR chain was first cloned in 1984, which led to the discovery of γδ T cells by Tonegawa's group in 1985 (101, 102). γδ T cells generate this unique TCR through RAG gene reaarangement of V(D)J genes in the thymus during foetal development (103). γδ T cells are a small minority of the overall T cell pool, representing just 2- 5% of T cells in secondary lymphoid organs and peripheral blood, and represent almost 50% of the intraepithelial lymphocytes (IELs) in the gut (104). γδ T cells reside predominantly in mucosal areas, such as the skin, reproductive tract and gut mucosa. Here they have the ability to recognise both self and benign antigenic peptides which is consistent with their role as a bridge between innate and adaptive immunity (104). γδ T cells play a crucial role in protective immunity early in life, as they can respond to pathogens through innate-derived cytokines (105). In mice the tissue localised compartments of γδ T cells reflect the schedule of their development. The first wave of γδ T cells home to the epidermis, the second wave to the genital tract and following that waves homing to the gut, lungs and finally the peripheral lymphoid organs (106).

γδ T cells are polyfunctional as they have the ability to produce an array of cytokines including IL-17A, IL-17F, IFN-γ, IL-22, IL-21, GM-CSF and TNF-α (107-111). IL-17-producing γδ T cells share many functional features of $CD4^+$ Th17 cells, with expression of IL-1βR, IL-23R, RORγt and CCR6 (112). γδ T cells can display plasticity like their CD4 T cell counterparts, mediating the switching of IL-17, IL-22, IFN-γ and even IL-10 in response to the upregulation of master transcription factors RORγt, Tbet and Foxp3, respectively (113-115). The primary role of γδ T cells is their rapid "innate-like" response to infection even in the absence of TCR engagement, unlike conventional αβ T cells. Despite having a fully functional TCR, γδ T cells have innate-like properties to produce IL-17, among other cytokines in response to cytokine stimulation in the absence of antigen (116). Unlike Th17 cells, which require antigen presentation to their TCR as well as CD3 and CD28 engagement and cytokine stimulation, γδ T cells are capable of producing IL-17 through stimulation with IL-1β and IL-23 alone (116). γδ T cells can also produce IFN-γ in response to stimulation with IL-1β and IL-12 or with TCR engagement (108, 117). Murine γδ T cells can be divided into subclasses depending on the expression of a unique TCRγ chain such as Vγ1-Vγ7 (Table 1.1).

Table 1.1 Classification of murine γδ T cell subsets

Heilig/Tonegawa nomenclature system	Garman nomenclature system	Location
Vγ1	Vγ1.1	Peripheral lymphoid tissue
Vγ2	Vγ1.2	Peripheral lymphoid tissue
Vγ3	Vγ1.3	Peripheral lymphoid tissue
Vγ4	Vγ2	Peripheral lymphoid tissue, lung, blood
Vγ5	Vγ3	Skin (Epidermis)
Vγ6	Vγ4	Tongue, uterus, lung, liver
Vγ7	Vγ5	Gut

The Heilig/Tonegawa nomenclature is used in this thesis.

1.4.1 Stratification of IL-17/IFN-γ producing γδ T cells

Cell surface receptors can molecularly govern γδ T cell activation by instructing downstream intracellular signals to regulate γδ T-cell cytokine secretion. This segregation of IL-17 or IFN-γ producing γδ T cells appears to begin as early as foetal development (118). It was observed that thymic maturation of γδ T cells involves the expression of the TNF receptor family member CD27, which is known to co-stimulate conventional CD4 and CD8 αβ T cells upon interaction with CD70 (119). Ribot et al revealed that CD27-expressing γδ T cells are IFN-γ-producing, whereas $CD27^-$ γδ T cells are predominantly IL-17-producing (119, 120). The signature cytokines and phenotype of the $CD27^+$ or $CD27^-$ γδ T cells remains stable even during infection (120, 121). NK1.1 has also been described as a marker of IFN-γ producing γδ T cells, while IL-17-producing γδ T cells are $NK1.1^-$ (117). Similarly to human Th17 cells, IL-17-producing γδ T cells express CCR6 (122). IL-17 producing human γδ T cells express CCR6 in response to IL-1, IL-23, IL-6 and TGF-β stimulation, however IFN-γ producing γδ T cells appear to be strictly $CD27^-$ (123). CD25 was identified as a marker of IL-17 producing Vγ6Vδ1 T cells during *E.coli* infection, whereas CD25 expression is absent in the thymus, suggesting that CD25 expression is acquired in the periphery before IL-17 producing capability (124). Shibata et al also demonstrated that IFN-γ producing γδ T cells express CD122 in the absence of CD25 (124).

Cytokine production can also be augmented through strong antigen TCR engagement, resulting in the loss of SOX13 expression and upregulation of the transcription factors nuclear factor κB (NFκB), nuclear factor of activated T cells (NFAT), T-bet and CD27, resulting in the generation of IFN-γ secreting γδ T cells (125). In summary, IL-17-expressing γδ T cells exhibit a $CD27^{-}CD25^{+}CCR6^{+}$ phenotype, while IFN-γ producing γδ T cells express NK1.1, CD122 and CD27. Interestingly, subsets of γδ T cells also appear to adhere to stratification of IL-17 or IFN-γ production. Vγ6 and Vγ4 T cells are predominantly IL-17-producing γδ T cells and Vγ1 T cells are mainly IFN-γ and IL-4 producers (126-129). The Vγ4 subset, which adopts an IL-17-producing fate, express high levels of the classical Th17 transcription factor RORγt, whereas IFN-γ "biased" $V\gamma5^{+}$ T cells expressed elevated levels of the transcription factor Egr3 (125). Further studies documented the genetic network of transcription factors required for the generation of $V\gamma4^{+}$ IL-17^{+} γδ T cells where they require a network of Sox13, Sox4, Tcf1, and Lef1 expression for their generation (130).

1.4.2 Human γδ T cells

The majority of human γδ T cells can be subclassed into Vδ1 and Vδ2 γδ T cells, based on TCR expression assigned during thymic development (131). Murine γδ T cell subsets are largely characterised by TCRγ expression while human γδ T cell subests are characterised by TCRδ expression. Moreover, human and murine γδ T cell subsets have a completley different TCR composition, where γδ T cells do not develop in CD3γ-deficient mice, whereas patients lacking CD3γ have abundant circulating γδ T cells (132). The majority (>70%) of human peripheral blood γδ T cells are characterised by the expression of TCRVγ9Vδ2, whereas Vδ1, and to a lesser extent Vδ3 γδ T cells are abundant in the gut, skin, epithelia and uterus (133-135). Vγ9Vδ2 T cells mainly respond to non-peptidic phosphoantigens, natural metabolites of isoprenoid biosynthesis in host cells. These phophoantigens can also be produced by stressed cells, edible plants or microbes (136). Phosphoantigens can be presented to γδ T cells through expression of CD1d and other unknown non-MHC molecules by DCs, B cells, T cells and monocytes (137-140). Human $V\gamma9V\delta2^{+}$ T cells can also act as antigen presenting cells in their ability to present foreign peptides to conventional $CD4^{+}$ and $CD8^{+}$ αβ T cells (141). Moreover, activated $V\gamma9V\delta2^{+}$ T cells, like professional APCs can effectively phagocytose opsonized target cells and uptake soluble antigens (142, 143).

Human γδ T cells have classically been viewed as predominantly Th1-*like* innate effector cells through the potent release of IFN-γ, TNF-α and granzymes during infection. The IFN-γ-secreting γδ T cell compartment can account for up to 50% of $CD3^+$ T cells during certain infections, as seen during ehrlichiosis, a tickborne bacterial infection (144). Furthermore, in malaria infected children in Papua New Guinea, γδ T cells are the predominant source of IFN-γ during disease (145). IFN-γ-secreting γδ T cells also play a role in the resolution of viral infection as expansion of γδ T cells is strongly associated with resolution of CMV infection in transplant recipients. Moreover, γδ T cell clones derived from the CMV-infected patients secrete IFN-γ, TNF-α in response to both virally infected cells and tumour cells (146). Recent studies have also described a role for human IL-17-secreting T cells during viral, fungal and bacterial infection. An important study by Caccamo and colleagues demonstrated that during bacterial meningitis the number of IL-17-secreting Vγ9Vδ2T cells dramatically increases in both the peripheral blood and in the cerebral spinal fluid of infected children, and this effect is reversed with antibiotic treatment (123). Furthermore, in patients with active pulmonary tuberculosis an increased frequency of IL-17-producing γδ T cells was observed in peripheral blood, however, TCR usage and the effect of IL-17-secreting γδ T cells on clinical severity were not determined (147). Notably, Vδ1 T cells from HIV infected individuals are markedly expanded and produce large amounts of IL-17 and IFN-γ, unlike Vδ1 cells from healthy controls (148). This expansion of IL-17 and IFN-γ-secreting γδ T cells in HIV infected patients may represent a compensatory mechanism for the progressive decline of $CD4^+$ T cells.

It is unclear if human IL-17-producing γδ T cells, like murine innate-like γδ T cells, are exclusively derived from fetal progenitors (106). However, the development of both human and murine IL-17-producing γδ T cells is selectively promoted by IL-7 (149). The expression of IL-7 is greatest in newborns, and this may explain why IL-17 is readily evoked from cord blood γδ T cells but not as easily from γδ T cells taken from the peripheral blood of healthy adults (149, 150). There is an extremely low frequency of IL-17-producing circulating Vγ9Vδ2 T cells in healthy individuals (151). However, the expansion of IL-17-secreting Vγ9Vδ2 T cells in the periphery and CSF during menigitis demonstrates their rapid effector capability and may be a result of cellular reprogramming through the cytokine milieu induced by the infection. The phenotype of $IL\text{-}17^+$ Vγ9Vδ2 T cells observed in children with bacterial meningitis mirrored that of in vitro differentiated $IL\text{-}17^+$-secreting Vγ9Vδ2 T cells with TGF-

β, IL-1β, IL-23, IL-2 and IL-6, demonstrating for the first time the ability to induce human IL-17-secreting γδ T cells in vitro (123).

1.4.3 γδ T cells in infection

The positioning of γδ T cells at mucosal sites, areas which interact with an array of environmental pathogens is critical for rapid immunity to infection. γδ T cells are potent producers of IL-17, they can surpass Th17 production of IL-17 even in smaller numbers. Lockharte et al elegantly demonstrated that although γδ T cells represent a small frequency of the lymphocytes in the lung, they are a more potent source of IL-17 when compared with Th17 cells, classically considered to be the major source of IL-17 (107). Innate activation of γδ T cells renders them highly primed to produce IL-17, which is responsible for neutrophil recruitment and activation in response to bacteria, such as *E.coli* and *Mycobacterium tuberculosis* (152, 153). IL-17-producing γδ T cells have been shown to be increased in patients with active pulmonary tuberculosis (154). Furthermore, γδ T cells have an important role in mediating BCG vaccine-induced immunity to tuberculosis by stimulating effector T cells and early IFN-γ production (155).

Some γδ T cells appear to display antigen specificity, in particular responding to stress antigens (113). Furthermore, the non classical MHC class I molecules T10 and T22 were found to be natural ligands for >2% of murine γδ T cells; however identification of other γδ antigens remains obstinate (156, 157). γδ T cells reactive to a glycoprotein from herpes simplex virus were obtained from infected mice demonstrating that γδ T cells can specifically respond to viral antigens (158). Equally, both Th17 cells and IL-17 producing γδ T cells are crucial for immunity to extracellular pathogens such as *C.albicans,* and the rapid IL-17 production by γδ T cells may be due to their selective expansion in response to fungal products (97, 159). Interestingly, the anatomical localization of γδ T cells has implications for their antigen specificity. Clonal expansion of αβ T cells with diverse antigen specificity, is supported by their migration to the T cell zones of the spleen and lymph nodes, sites which sustain their encounter with DCs presenting a variety of antigens (160). Splenic γδ T cells are not confined to the lymphoid area of the spleen but are found throughout the red pulp and at a variety of mucosal sites throughout the body, unlike αβ T cells (161). This is reflected in the limited TCR diversity of tissue-resident γδ T cells which are in essence monclonal in the

murine epithelia of the vagina, uterus and tongue (115). This would indicate that these tissue resident γδ T cells recognize either predictable pathogen antigens at certain tissue sites or self antigen molecules indicative of damage or a dysregulated state (113).

1.4.4 Memory γδ T cells

γδ T cells can display both an innate and memory-like phenotype. A population of γδ T cells appears to expand throughout life; Vγ9Vδ2^{+} T cells comprise a small proportion of the total T cells in newborns to constitute 80% of the total γδ in adult periperhal blood (162). Furthermore, these Vγ9Vδ2^{+} T cells express CD45RO^{hi} suggesting prior activation (162). Studies involving mycobacterial infection reveal that infection or vaccination induces memory-like mycobacterium-specific γδ T cells in cattle and macaques (114, 163). Mucosal memory γδ T cells are generated after oral infection with *Listeria monocytogenes* and these memory γδ T cells are capable of simultaneously producing IL-17A and IFN-γ in the intestinal mucosa (164). Importantly, rechallenge with oral *L. monocytogenes*, but not intravenous *L. monocytogenes* or oral *Salmonella*, induced rapid expansion of memory γδ T cells, resulting in enhanced bacterial clearance upon reinfection (164). Furthermore, a population of CD44^{+}CD27^{-} memory γδ T cells, primarily Vγ4^{+} were expanded upon reinfection of mice with *S. aureus*, producing high levels of IL-17 and mediating enhanced bacterial clearance (165). A recent study reported that γδ T cell may have autoantigen specificity; in imiquimod induced psoriasis, IL-17–producing γδ T cells, primarily Vγ4^{+}, expand in the lymph nodes and home to the skin where they persist as memory-like cells capable of rapid activation, enhanced proliferation and IL-17 production resulting in enhanced skin inflammation upon rechallenge with imiquimod (166). Memory γδ T cells play a critical role in rapid immunity to reinfection which highlights the intrinsic role of γδ T cells at mucosal surfaces. Overall, these studies demonstrate that the γδ T cell immune response during infection can be innate-like or antigen specific analogous to αβ T cells responses.

1.4.5 The γδ T cell receptor and lineage fate

Despite extensive studies regarding functional differences between αβ and γδ T cells, it is still unclear how these T cell subsets develop independently in the thymus (Table 1.2). The

complexities of TCR development and lineage commitment are still not fully understood (167-171). γδ T cells rearrange Vγ and Jγ genes which join the γ constant gene, giving rise to a full TCRγ chain, the rearrangement of Vδ, Dδ, and Jδ genes join the δ constant gene to form the full TCRδ chain (170). Given the potenty of γδ T cells in controlling infection (108, 127, 165) and cancer (121, 172), there has been a great deal of effort invested in understanding the ontogeny of these innate-like T cells. γδ T cells are derived from fetal liver progenitor cells with maturation occuring in the thymus along with conventional αβ T cells (173). Maturation begins with the phenotypic expression of $CD44^{+}CD25^{-}CD4^{-}CD8^{-}$, characterising the double negative 1 (DN1) stage in T cell development. The genes encoding the TCRβ, γ, and δ chains of the T cell receptor are rearranged and assigned at this stage, where expression of the γδ chains supports the expression of Sry-box 13(SOX13) which drives the cell into the DN4 stage of development (125).

Table 1.2 Comparison of αβ and γδ T cell characteristics

Characteristic	αβ T cells	γδ T cells
Antigen receptor configuration	CD3 complex + αβ TCR	CD3 complex + γδ TCR
MHC restriction	Yes	Rare
Phenotype	$CD4^{+}$ or $CD8^{+}$	Mostly $CD4^{-}$ $CD8^{-}$, IELs are $CD8^{+}$
Frequency in periphery	65-75%	1-5% (25-50% gut)
Distribution	Blood and lymphoid tissue	Blood, lymphoid tissues, mucosal and epithelial barriers
Effector capability	Antigen specific responses, CTLs (CD8s), pro and anti-inflammatory cytokine release	CTLs, inflammatory cytokine release

Unlike αβ cells, developing γδ T cells must undergo pre-programming which is dependent on TCR signalling (174). It has been proposed that the strength of TCR signalling delineates the mechanism by which γδ T cells develop, a weak TCR signal permits continued SOX13 expression with the upregulation of RORγt, allowing the cell to become an IL-17-producing γδ T cell (175). Sox13 has also been identified as the transcription factor reponsible for IL-17-

producing Vγ4$^+$ T cell commitment (175). The expression of Sox4, like Sox13 precedes TCR signaling and mediates the development of IL-17 expressing γδ T cells through induction of Rorc and Blk (176). Strong TCR signals appear to drive γδ T cells towards IFN-γ secretion. Vγ5$^+$ dendritic epidermal γδ T cell (DETC) progenitors that engage the antigen Skint1 through their TCR upregulate Nfat, Nfkb, and Egr3 to promote T-bet expression and IFN-γ secretion (125). Other studies have shown that thymocytes which fail to produce a functional γδ TCR go on to develop into αβ T cells, undergoing TCRβ selection with the assistance of NOTCH1 signalling and further rearrange the TCR genes to express TCRα at the DP stage (169, 177). Furthermore, strong TCR engagement has been proposed as a driver of αβ lineage commitment as a weak or absent TCR-ligand signal fails to drive the cell into the DP phase, thereby the cell may express γδ TCR (171, 178).

Two models had previously been proposed in an attempt to explain αβ or γδ T cell lineage commitment and they differ based on the role of the TCR in determining lineage fate. The stochastic model describes a minor role for the TCR in lineage fate, where T cell commitment is assigned prior to TCR expression and only precursor T cells whose cell fate matches the expressed TCR will survive and develop into mature αβ or γδ T cells (168, 179-182). The instructive model advocates that the TCR plays a primary role in determining lineage fate, where a precursor T thymocyte expressing the γδTCR will be directed towards the γδ lineage, and preTCR expression instructs αβ lineage commitment (168, 179-182). However, inconsistincies in TCR transgenic studies questioned these models as fully explanatory of the T cell fate decision. Studies in αβTCR transgenic mice demonstrated that a population of DN αβTCR$^+$ cells could display functional and phenotypic characteristics of γδ T cells, indicating that the αβTCR could direct thymic precursor cells towards the γδ lineage (183, 184). Furthermore, it was demonstrated that DP thymocytes, classified as αβ lineage cells, could be detected in significant numbers TCRβ$^{-/-}$ mice, despite these cells remaining incapability of expressing the pre-TCR or the αβTCR (185). This would suggest that the γδTCR may direct cells to the αβ lineage. A seminal study by Hayes and colleagues then proposed a new model based on strength of the TCR signal on lineage commitment (178). Through genetic manipulation of γδTCR surface expression or by modulating the γδTCR signaling response, they demonstrated that strong γδTCR signals instruct thymocytes towards the γδ lineage, while a weak signals favoured αβ lineage development. This led to the widely accepted signal strenth model, where the the strength of the TCR signal is the critical determinant in the lineage fate decision.

The cytokine environment in the thymus also confounds the complexity of TCR development. Analysis of precursor αβ or γδ T cells in the thymus demonstrated that IL-7R^{lo} DN2 cells were more likely to differentiate into αβ T cells, while high expression of IL-7R on cells was indicative of γδ lineage commitment (186). IL-7 is crucial in the rearrangement of the γ TCR gene (187, 188), whereas it is only an additional factor to IL-2 and stem cell factor (CSF) for the the rearrangement of TCRβ and δ genes (189). Moreover, IL-7 has also been shown to selectively promote IL-17–competent human and mouse γδ thymocytes (149). IL-2 is required for the development of T cells, however, IL-4 synergises with IL-2 to enhance the proliferation of γδ^{+} expressing thymocytes over αβ^{+} thymocytes (190, 191). Importantly, IFN-γ can inhibit the IL-4-mediated prolifertaion of γδ^{+} thymocytes (192). Recently, Sox13 has recently been identified as a distinct marker of γδ lineage commitment. The transcription factor Sox13 has been classified as a marker of both γδ lineage commitment and of the IL-17-producing Vγ4^{+} T cell subset (193-195). Expression of the TCR inducible ligand CD73 also appears to be a γδ lineage marker, as over 90% of peripheral γδ T cells express CD73 upon TCR stimulation (196). Furthermore, γδ CD73^{+} T cell progenitors cannot undergo fate switching to the αβ T cell fate, while CD73^{-} γδ T cell progenitors can (196).

Populations of T cells expressing βδ TCR heterodimers (103), TCRβ with TCRα and TCRδ segments (δ/αβ cells)(197) or both αβ and γδ TCRs (198) have been described, which is at odds with the paradigm of T cell devopment. Furthermore, it has also been shown that 10-20% of γδ T cells express TCRβ proteins, mostly in the cytoplasm (199, 200). Remarkably, these cytoplasmic TCRβ chains were able to efficiently partake in αβ development (201). Despite novel conformations of TCRs little functional relevance to immunity has been described and these unconvenional TCRs remain an anamoly. Initial TCR development studies demonstrated that a precursor T cell must either first express TCRδ or TCRβ in order to develop into the corresponding γδ or αβ T cell (186, 198). However, single cell fate mapping demonstrates that there is no commitment to either the αβ or γδ lineage before TCR expression (202). If committed γδ T cells could express a functional yet inert TCRβ chain by rearrangement, this seems at odds with the development theory of αβ and γδ T cells. The development of TCRβ proteins in the cytoplasm of γδ T cells may be a result of precursor cells that lack the genes encoding TCRα, therefore the cell is unable to form a functional preTCR complex (TCRβ subunit in association with the pre-TCRα chain) (199, 200, 203). However, evidence from several laboratories report that developing T cells which express a functional γδ TCR are commonly rejected from the αβ lineage (204). αβ and γδ T cell

develop from a common precursor thymocytes in mice, and remarkably γδ T cells are enhanced in mice that are deficient in genes encoding TCRβ or TCRα which suggests they can compensate for the αβ T cell compartment (205, 206). However, in humans, sequencing studies reveal that all αβ and γδ T cells have rearranged TCRγ sequences, whereas less than 4% of γδ T cells have a rearranged TCRβ chain (207). This would suggest that TCRγ rearranges in all T cells but the rearrangement of the TCRβ appears to be restricted until after αβ T cell lineage commitment (207). There are many conflicting opinions on the correct model for TCR development and evidently the paradigm for αβ/γδ T cell development and TCR formation requires further investigation.

1.5 Autoimmunity

Thymic maturation ensures T cells are appropriately reactive to foreign pathogens but not host antigens. Medullary thymic epithelial cells (mTecs) can express tissue-restricted antigens (TRA) so that during thymic maturation T cells specific for host antigen or T cells responding to antigens that are otherwise only found in specialized peripheral tissues are deleted (208). In addition, auto-reactive T cells are deleted by lymph node–resident immature DCs that present host antigen (209, 210). However, a breakdown in immunological tolerance results in the maturation of auto-reactive T cells which migrate to the lymph nodes and spleen. Subsequent activation and clonal expansion of these auto antigen-specific T cells elicits a powerful immune response against the host, resulting in autoimmunity (211). Depending on the site of this autoreactivity, diseases such as rheumatoid arthritis, psoriasis, type 1 diabetes and multiple sclerosis can develop.

1.5.1 Multiple sclerosis

Multiple sclerosis (MS) is a progressive inflammatory, autoimmune disorder of the central nervous system (CNS) triggered by environmental factors in genetically susceptible individuals (212). The term 'sclerosis' is a Greek word meaning “hardened tissue” and MS is characterised by the presence of inflammatory plaques or scar tissue in the spinal cord and brain of patients (213). The first description of MS dates back to the 14th century, but it was Jean Charcot that correlated the clinical symptoms of MS with the observed post mortem

pathological changes in 1868 (214). MS results in gradual loss of neurological function and ascending paralysis. MS is the most common progressive and disabling neurological condition among young people. Typical onset is between 20 and 40 years of age, with average onset between 30 to 33 years. At present, it affects over 8,000 people in Ireland and approximately 2.5 million people globally (215).

The presence of the blood brain barrier (BBB) limits lymphocytes and soluble damaging factors egressing from the periphery and entering the CNS. Despite the existence of tight junctions regulating the integrity of the BBB, lymphocyte entry into the CNS is a key early event in the pathogenesis of MS (216). In MS the myelin sheath, a fatty, axon-enwrapping membrane that serves to speed up neural conduction in the CNS is damaged indirectly by auto-reactive T cells (211). These auto-reactive T cells are specific for myelin antigens, such as myelin oligodendrocyte glycoprotein (MOG), proteolipid protein (PLP*)* and myelin basic protein (MBP). Once these myelin specific T cells are reactivated in the CNS, these auto-reactive T cells mediate inflammation through pro-inflammatory cytokine and matrix metalloproteinases (MMPs) secretion, leading to the damage of myelin sheaths (211). This inflammatory pathology is progressive and as the protective sheath is destroyed, neuronal function is progressively inhibited resulting in the hallmark symptoms of MS, decline in neurological function and increased disability (212).

MS is a heterogenous disease, three of the most common forms are primary progressive (PPMS), secondary progressive (SPMS) and relapsing remitting MS (RRMS). The most prevalent form RRMS results in acute inflammatory episodes classified as a relapse, which induces a decline in neurological function (217). After a relapse RRMS patients can experience partial recovery, however, the majority will advance to a more progressive form of the disease SPMS (218). SPMS is thought to be independent of T cell mediated inflammation, but nevertheless results in a greater decline in neurological function with ascending paralysis (212). PPMS is a form of the disease where the spinal cord is greatly affected and fewer brain lesions are observed (219). However, from onset of first symptoms, the progression of PPMS is continuous from the outset and is not characterized by relapse, symptoms progressively worsen over time (220). Although neuroaxonal degeneration appears to be the basis of PPMS, the role of the autoreactive T cells in mediating disease pathogenesis is still unclear (220).

1.5.2 Experimental Autoimmune Encephalomyelitis

Due to the complex environmental and genetic factors influencing MS, the pathogenesis of this disease is still poorly understood. In order to elucidate the causes and to develop new therapies, an animal model for MS was developed, experimental autoimmune encephalomyelitis (EAE) (221, 222). There are many different forms of EAE induced in a variety of animals through immunization of any one of several CNS antigens. For active EAE methods include using spinal cord or brain homogenates or myelin as encephalitogens or CNS antigens such as MOG, PLP, MBP or synthetic peptides to mimic the amino acid sequences of these proteins. The actively induced EAE model is based on PPMS, a chronic form of MS and it is generally induced in C57BL/6 mice by immunization with the MOG35-55 antigen emulsified in complete Freund's adjuvant (CFA), which is injected sub-cutaneously (s.c.) (223). Pertussis toxin (PT) is subsequently administered by intraperitoneal (i.p.) injection on days 0 and 2 post induction. PT promotes the onset of EAE symptoms by permeabilising the BBB by promoting of inflammatory cytokine production, allowing infiltration of peripheral auto-reactive T cells into the CNS (224-226). The symptoms of actively induced EAE manifest as gradual ascending paralysis and the mice retain these symptoms as in PPMS. Investigations into RRMS are based on a different relapsing remitting model of EAE, where disease is induced in SJL/JHan strain of mice and PLP139-151 is used as the myelin peptide (227). Other models include the adoptive transfer EAE model, where EAE is induced by transferring myeling reactive T cell populations into naive mice and these mice will develop clinical symptoms; this model is useful in the determination of which cell types are important for EAE induction. The use of transgenic animals has also greatly contributed to understanding the pathogenesis of EAE. Another model utilises MBP-specific TCR-transgenic mice and administration of PT to induce the clincal symptoms of EAE (228). These variations on the standard protocol for EAE can offer further insight into the mechanisms of CNS inflammation.

EAE is a useful model for understanding the pathoegenesis of MS and it also offers an insight into other organ-specific autoimmune diseases. However, a major caveat of the model remains in the fact that the disease is centred around acute CNS inflammation and immunopathology, while MS is both an autoimmune and neurodegenerative phenomenom (229). In the later stages of secondary and primary progressive MS, brain atrophy and irreversible disability occur in the absence of new plaques of inflammatory demyelination (230, 231). In this absence of inflammation of the brain's white matter, there must be other

factors independent of the immune system contributing to neuronal degeneration; unfortunately, this cannot be explored in the acute inflammatory model that is EAE.

The latter phase of MS is poorly understood, difficult to treat and there are very limited models to study neuroprotection and remyelination (229). However, animal models which attempt to replicate the early and late phases of MS are emerging, and together with EAE, these may provide further insight into the disease. Optic neuritis is an early symptom of MS where inflammatory damage of axons in the optic nerve result in the loss of retinal ganglion cells (232). Animal models of optic neuritis have recently been developed, such as mice expressing MOG-specific TCR with a retinal ganglion cell restricted-Thy1 promoter-controlled cyan fluorescent protein (233, 234). MOG expression is higher in the optic nerve than in the spinal cord so mice develop optic neuritis without the paralysis associated with EAE. This model may be a useful tool in understanding disease pathogenesis before onset of disablity as optic nerve damage can be monitored by examining the cyan fluorescent protein expressed in the retinal ganglion cells. EAE in Biozzi ABH mice has been shown to replicate the clinical symptoms observed in the latter phases of SPMS. Moreover, the pathology induced in this EAE model includes axonal, neuronal loss, demyelination as well as remyelination, which is common in many MS lesions (235-237).

There are several important differences in the pathogenesis of EAE and MS. EAE predominantly affects the spinal cord white matter; while in MS, demyelination predominantly occurs in the cerebral and cerebellar cortex of the brain (238). MS lesions are characterised by clonally expanded $CD8^+$ infiltrating T cells, however, in EAE $CD4^+$ T cell appear to be the primary encephalitogenic T cell population and there is little understanding for the role of $CD8^+$ T cells (239). Furthermore, evidence from clinical trials suggests a role of B cells in the pathogenesis of MS but their role in EAE is largely undetermined (240, 241). Overall, the multitude of EAE models and varying results reflects its complexity and the complexity of MS. Despite the limitation of EAE it has contributed extensively to understanding of CNS inflammation and the pathogenesis of MS. Moreover, EAE models have assisted in the design, development and validation of therapeutics for the treatment of MS (242).

1.5.3 IL-17 versus IFN-γ in EAE

Prior to the discovery of Th17 cells, Th1 cells were thought to be key mediators in the pathology of EAE. This was based on the fact that they potently secreted IFN-γ in response to IL-12, which was thought to be pathogenic in autoimmune conditions (243, 244). Further evidence of a role for IFN-γ and Th1 cells in the pathogenicity of EAE came from the observation that IL-12p40$^{-/-}$ defective mice were resistant to EAE; IL-12p40 is one of the two subunits of IL-12 (245). This theory was questioned when studies demonstrated that mice defective in IL-12, IFN-γ or STAT1 were still susceptible to EAE (245-247). This enigma was resolved following the identifcation of the IL-12 cytokine family member, IL-23. IL-23 and IL-12 share a common p40 chain, which associates with a p35 chain to make IL-12 or p19 for IL-23 (72). Interestingly, the IL-12p35$^{-/-}$ mice were susceptible to EAE, while IL-23p40$^{-/-}$ and IL-23p19$^{-/-}$ mice are resistant to EAE, demonstrating the pathogenic role for IL-23 in driving EAE (245). Upon further investigation IL-23 was then shown to be the essential cytokine to induce the expansion of effector Th17 cells (78, 248, 249).

Since their discovery, Th17 cells have been implicated in a variety of autoimmune diseases, such as MS, RA, psoriasis, IBD and in the animal models of these diseases (78, 250-254). IL-23 has been shown to be a stabilising or maintaining cytokine for Th17 cells and other IL-17-producing cells; Th17 cells proved to have a critical function in EAE, as they could transfer disease into naive mice (78). Langrish et al demonstrated that IL-23 could induce a population of pathogenic IL-17 secreting T cells, whereas TGF-β and IL-6 induced a population of CD4$^+$ T cells that secrete IL-17 and IL-10 which failed to induce EAE, due to the immunoregulatory action of IL-10 (78, 255). Furthermore, IL-23 alone has also been shown to induce IL-17 production by memory CD4$^+$ T cells and in the EAE model, these memory Th17 cells induce earlier onset and increased severity of disease (256). RORγt and STAT3 are essential for IL-17 secretion and mice with T cells deficient in either of these transcripton factors fail to develop EAE (24, 257, 258). Despite clear evidence for the role of Th17 cells in EAE, the relative roles of Th1 and Th17 cells in the pathogenesis of EAE are still uncertain. There appears to be plasticity between these T cell subsets and CD4$^+$ T cells secreting both IL-17 and IFN-γ are detectable in the CNS of mice with EAE (259-261).

Until very recently, the role of Th1 cells and IFN-γ in the pathogenesis of autoimmune diseases has been controversial. Like Th17 cells, transfer of myelin antigen-specific Th1 cells can induce EAE in naïve mice (262, 263) or experimental autoimmune uveoretinitis (EAU)

(264). However, IFN-γ, the signature cytokine of Th1 and NK cells, has been shown to inhibit the function of pathogenic Th17 cells, as well as promoting development of encephalitogenic T cells during induction of EAE (28, 263, 265). Data from our laboratory demonstrated that NK cells infiltrate the murine CNS before the onset of clinical symptoms of EAE, and depletion of NK cells before the onset of disease significantly attenuates disease severity (263). Furthermore, the pathogenic effect of NK cells was accredited to early IFN-γ secretion, which polarised macrophages to an M1 phenotype leading to an enhanced expression of the integrin α4β1 of $CD4^+$ T cells (263). VLA-4 promotes encephalitogenic activity of $CD4^+$ T cells in EAE, however, neutralization of IFN-γ during disease progression results in more severe clinical symptoms, reflecting the inhibitory effect of IFN-γ on Th17 cells (28, 263). Collectively these studies demonstrate that both IL-17 and IFN-γ play important roles in the pathology of EAE, however IFN-γ can have dual protective and pathogenic roles through suppression of Th17 cells and upregulation of the integrin VLA-4.

1.6 γδ T cells in autoimmunity

γδ T cells have been shown to play a critical pathogenic role in autoimmune diseases such as EAE, collagen induced arthritis arthriris (CIA) and most recently EAU (244, 266, 267). Before the discovery of Th17 cells and their signature cytokine IL-17, it was previously assumed that early IFN-γ derived from γδ T cells was a key pathogenic cytokine in driving EAE. This was due to the enhancement of $CD4^+$ and $CD8^+$ T cell responses by γδ derived IFN-γ in anti-tumour immunity (172). In EAE, γδ T cells can secrete IL-17 in response to IL-1, IL-18 and IL-23 without TCR engagement, promoting the induction of Th17 cells and amplifying their encephalitogenic function during the development of disease (76, 116). In vitro studies have demonstrated that γδ T cells are indirectly responsible for axonal demyelination through toxic destruction of oligodendrocytes, an important step in the pathogenesis of EAE and MS (268, 269). Similarly to Th17 cells, γδ T cells can express IL-23R, IL-1R, the Th17-associated transcription factor, RORγt and CCR6 which is required for T cell trafficking during EAE (116, 122, 159, 270). Importantly, data from our laboratory and others has shown that γδ T cells infiltrate the brain and spinal cord in large numbers during the course of EAE, reflected in an enhancement of IL-17 (76, 116, 267, 271).

In EAE, the pathogenic role of γδ T cells was further solidified by studies with TCRδ$^{-/-}$ mice which revealed a reduction in the clinical severity (272). While in the relapsing remitting EAE model, a significant reduction in clinical severity was observed when mice were treated with a TCRδ depleting antibody immediately before onset of disease or during the chronic phase of disease (267). Experiments in the adoptive transfer model of EAE also demonstrated a pathogenic role for γδ T cells, as depletion of TCRδ^{+} cells resulted in reduced clinical severity and delayed onset of disease (273). γδ T cells contribute to the pool of IL-17 during EAE but they can also enhance Th17 pathogenicity. Studies from our laboratory have demonstrated that the addition of DCs enhanced the ability of IL-1 and IL-23 activated γδ T cells to promote IL-17 production by Th17 cells (116). Furthermore, DCs expressed IL-17R and secreted IL-23 in responses to IL-17, demonstrating the positive feedback loop of γδ T cell derived IL-17 on DC activation and Th17 cell effector function during EAE (116).

Flow cytometric analysis of the CNS during EAE revealed that the Vγ4^{+} T cell subset are the main IL-17-producing γδ T cell in the brain, with smaller contributions from IL-17-producing Vγ1 and Vγ6 T cells (116). Vγ4^{+} T cells have also been described as key players in other autoimmune conditions, such as myocarditis, where Vγ4^{+} T cells have also been shown to promote pathogenic CD8 T cells by enhancing Th1 cell activation through IFN-γ and CD1-dependent mechanisms (158). In a murine model of psoriasis, IL-23 responsive Vγ4^{+} T cells are the source of IL-17 in psoriasis-like skin lesions and imiquimod-induced skin pathology was significantly decreased in TCRδ$^{-/-}$ mice (274). IL-17-producing γδ T cells have been shown to be enhanced in the skin of psoriasis patients, and mice injected with anti-Vγ4 depleting antibody had significantly less IL-17 production from dermal γδ T cells; demonstrating the pathogenic role for Vγ4^{+} T cells in psoriasis (274). Furthermore, deficiency of IL-17-committed Vγ4^{+} T cells protects against dermatitis (175). Evidence for a pathogenic role of Vγ4^{+} T cells in CIA was also demonstrated, where analysis of the arthritic joint revealed that the number of IL-17-producing Vγ4 T cells in the draining lymph nodes was equivalent to the number of Th17 cells (275). Moreover, depletion of Vγ4 T cells in CIA significantly attenuated clinical disease and conversely, activation of Vγ4 T cells in EAE exacerbates disease (275, 276).

The pathogenic role of γδ T cells in EAE centres around their ability to traffic to the CNS during disease and produce early innate-like IL-17 which can amplify Th17 responses (Figure 1.2). VLA-4 is an integrin heavily implicated in the trafficking of encephalitogenic T cells to the CNS during EAE, and it has also been shown that it is the integrin responsible for γδ T-

cell adhesion to epithelium, fibroblasts, endothelium and their transmigration of these cells (277-282). However, it is not responsible for their migration to the CNS during EAE (271). LFA-1 (α4β2) integrin is expressed by encephalitogenic T cells and interestingly, γδ T cells express all four members of this integrin family (CD11a-d) (271). Expression of β2 integrins increases throughout the course of active EAE, allowing γδ T cells to infiltrate the CNS rapidly and propagate inflammation early in disease (283). Furthermore, it has been reported that deletion of three out of the four β2 adhesion molecules results in a significant decrease in disease severity, linking a role for the β2 integrins in γδ T-cell trafficking to the CNS (284-287). Controversially, another study reports that expression of β2 integrins on γδ T cells are not critical to the development of EAE, as reconstitution of TCRδ$^{-/-}$ mice with LFA-1$^{-/-}$ γδ T cells resulted in clinical symptoms comparable to that seen in mice reconstituted with WT γδ T cells (271). Integrins, such as α4β7 have been shown to play a role in γδ T cell homing to the gut, where here they can play a pathogenic role in enhancing intestinal inflammation through IL-17 secretion (115, 288). However, convincing evidence from our laboratory has described that under the influence of retinoic acid (RA), γδ T cells can be protective, rather than pathogenic in colitis through IL-22 production (289). γδ T cells also play a role in allergic pleurisy, where the effect of CCL25 was selective for α4β7^{+} IL-17 producing γδ T cells, and not other γδ or αβ T cell subsets, resulting in a specific increase of IL-17 in the pleura (290).

The pivotal role of γδ T cells in the pathogenicity of EAE is also reflected in MS where clonal expansion of γδ T cells has been observed in the cerebrospinal fluid of MS patients with recent disease onset (291). Furthermore, an increased frequency of γδ T cells was detected in the peripheral blood of patients with MS (292) and moreover, an accumulation of γδ T cells has been described in acute brain lesions (293) (294). Collectively, these results substantiate a critical role for γδ T cells in both the active stages of EAE and MS. However, there are still many unanswered questions on the activation of the IL-17-producing Vγ4 T cell subset and answers to these questions may present new and more selective therapeutic interventions in the treatment of autoimmunity.

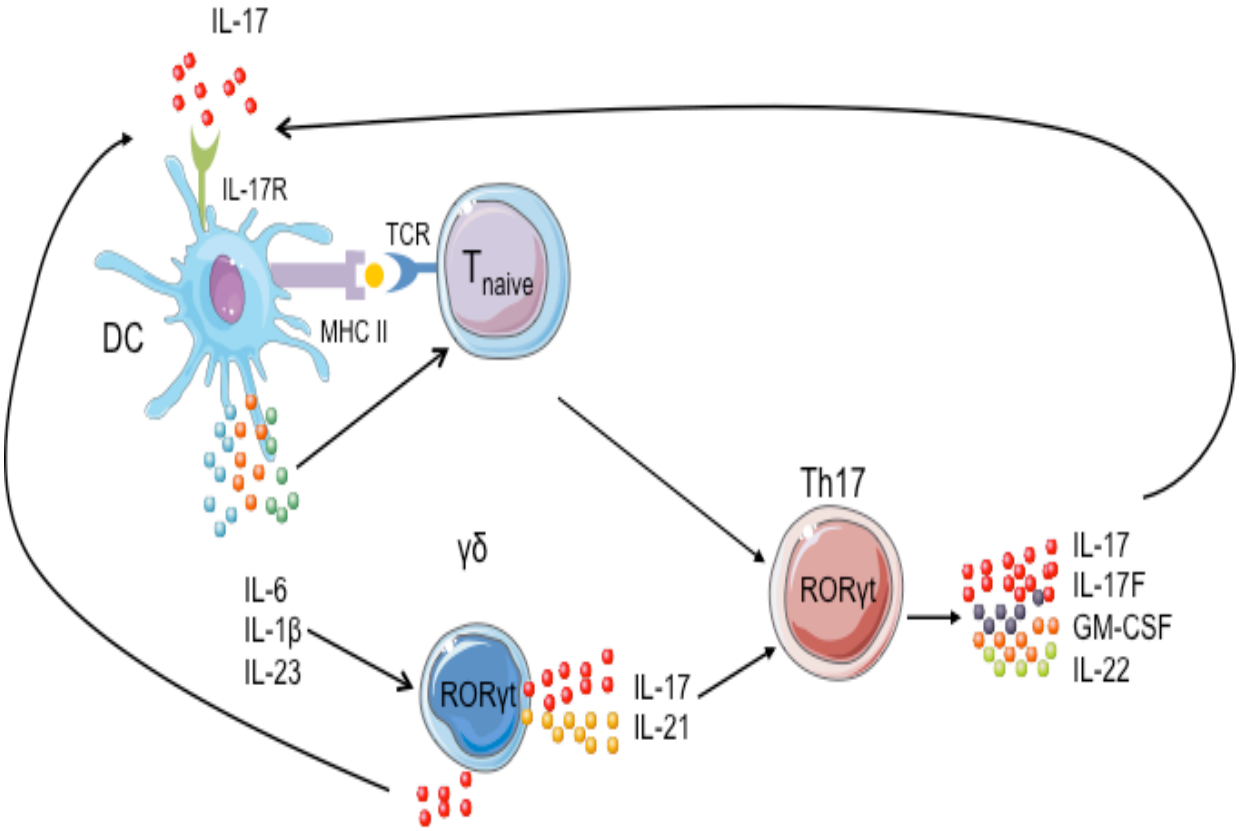


Figure 1.2: γδ T cells produce innate IL-17 to amplify effector Th17 cell responses. DCs present antigenic peptide to activate T cells through MHC class II molecules bound to TCRs and through co-stimulatory signals. The activated DCs secrete cytokines, including IL-1β, IL-6, IL-23 and IL-12. The differentiation of naïve T cells into effector cells is driven by these polarizing cytokines, where IL-1β, IL-6 and IL-23 promote the differentiation and expansion of Th17 cells, which secrete IL-17 (and IL-22, GM-CSF, IL-21). γδ T cells do not require TCR engagement to become activated, IL-1β and IL-23 stimulate γδ T cells produce IL-17 and IL-21, which act in an autocrine loop to promote further IL-17 production through DC activation promoting further Th17 cell development, amplifying their effects in the pathogenicity of EAE.

1.7 The role of infection in autoimmunity

Epidimiological evidence has revealed an association between infection and onset of autoimmunity in patients with MS, colitis and psoriasis (295-297). This breakdown in immune tolerance may be attributed to the nonspecific activation of the innate immune system during infection, or the ability of the infective pathogen to evade the immune system by using molecular mimicry based on host antigens resulting in the development of auto-reactive T cells and auto-antibodies (298). In RRMS there is strong evidence to suggest that exacerbations in symptoms occur around the time of infection, most commonly upper respiratory tract infections which can lead to greater sustained neurological damage compared with relapse in a non infected patient (299). Furthermore, there is evidence that systemic infections may trigger an increase in relapses in RRMS patients through bystander activation and sensitisation of auto-reactive myelin-specific T cells (300). The conserved molecules derived from microorganisms, or PAMPS can activate T cells directly by binding to PRRs on the surface of the T cells or indirectly by promoting innate cells to produce pro-inflammatory cytokines which activate T cells such as IL-12, IL-1β and IL-23 (298). Studies in EAE have shown that an infection with *Streptococus pneumonia* aggravates the symptoms of EAE, but this is reversible in $TLR2^{-/-}$ mice demonstrating that the exacerbation of the disease is dependent on TLR2 (301). PAMPS are potent activators of auto-reactive T cells, and peptidoglycan has been found in the tissues affected in autoimmunity, such as primate brains during EAE (302). Furthermore, peptidoglycan has been detetced in APCs in the brains of patients with MS and moreover, peptidoglycan-specific antibodies have been detected in the cerebral spinal fluid (CSF) (303). *Chlamydia pneumonia* infection is also associated with enhanced MRI spinal lesions in MS (304). The detection of both Epstein Barr virus latent protein and herpes simplex virus in the brains of patients with MS during post-mortem examinations has provided circumstantial evidence that a virus may trigger or exacerbate the onset or progression of MS (305).

Conversely, infections with certain pathogens and parasites has been associated with a reduction in autoimmunity or symptoms of disease (306). Epidemiological evidence indicates that in developing countries a higher incidence of helminth infections correlates with a reduction in the incidence of autoimmunity and allergy (307). This is supported by the fact that prevalence of MS is on the rise in western society, where there is a decrease in the incidence of infections (308). A study in South America demonstrated that MS patients with

concurrent parasitic infections had significantly lower relapse rates, regression of new lesions and a lower disability index score than uninfected MS patients (309). Recent evidence on the hygiene hypothesis suggests that anti-inflammatory cells, such as regulatory T cells induced during infection are capable of dampening the pro-inflammatory immune response that would contribute to pathology in autoimmune diseases and allergy (310). Helminths parasites are highly evolved for immunomodulation of the host through the generation of regulatory innate and adaptive cells and through the induction of anti-inflammatory cytokines such as TGF-β, IL-10 and IL-4 (311). Studies in mice have shown that infection with the helminth parasite *Fasciola hepatica* attenuates the symptoms of EAE through TGF-β-mediated suppression of Th17 and Th1 responses (312). Bacterial pathogens are also capable of immunosuppression, *Staphyloccus aureus* ameliorates EAE through an extracellular adherence protein, which has anti-inflammatory effects by binding to ICAM-1 and preventing the infiltration of myelin specific T cells across the BBB (313). In another study mice pre-exposed to *B. pertussis* or *Mycobacterium tuberculosis* through s.c. or i.p. injection were resistant to the induction of EAE through a tolergenic response to PT from *B. pertussis* and the purified protein derivative (PPD) of *M. tuberculosis* (314, 315). The regulatory responses induced by infectious pathogens may be exploitated for the development of new immunosuppressive therapeutics for human autoimmune diseases.

1.8 Immunomodulation by *B. pertussis* infection

The vaccine preventable disease pertussis, or whooping cough, is caused by *B. pertussis*, a Gram negative aerobic coccobacillus belonging to the genus Bordetella. This genus also includes *B. bronchiseptica*, which mainly infects animals, such as pigs, cats and dogs and the bacteria *B. parapertussis* which like *B. pertussis,* can infect humans (316). Whooping cough is a severe respiratory disease which primarily affects children and infants and accounts for significant childhood mortality and moribity worldwide. The World Health Organisation estimates that 195,000 children, mostly in developing countries, died from this bacterial infection in 2008 with 16 million cases of pertussis reported (317). The severity of whooping cough varies widely but the infection is persistent and the longevity of the cough typically lasts 5 weeks or more. The classic “whoop” sound of the infection occurs in patients on inhalation after a coughing fit. Aside from the severity of whooping cough in unimmunised

children, secondary respiratory infections and complications such as apnea, pneumonia, seizures, hypoxia and even encephalopathy can ensue (318). A whole cell pertussis vaccine was introduced during the late 1940s and was an extremely effective vaccine due to its ability to induce strong Th1-mediated immunity (319). However, there were also side effects associated with the whole cell pertussis vaccine so it was replaced by a safer acellular pertussis vaccine in the 1980s (320). However, the new vaccine is suboptimal as there has been a recent significant increase in the number of cases of pertussis in developed countries (321).

The pathology of the *B. pertussis* infected airways is often characterised by mucus hypersecretion and post mortem results on experimentally infected mice reveal bronchopneumonia, alveolitis, leukocyte infiltration and epithelial and ciliary damage in the lungs (322). *B. pertussis* colonises the cilia and bronchioles, bronchi and trachea of the respiratory tract. Although *B. pertussis* is considered to be an extracellular bacteria, it has been detected in resident macrophages of the lung and in epithelial cells in infected mice and children (323, 324). *B. pertussis* has a number of virulence factors, such as filamentous hemagluttinin (FHA), fimbriae, pertactin (PRN) which aids in host colonisation, immune suppression and immune evasion (325). It also produces toxins such as PT, heat liable toxin, tracheal cytotoxin (TCT) and adenylate cyclise toxin (ACT). Gram negative bacteria such as *B. pertussis* contain the endotoxin lipopolysaccharide (LPS), which is a complex glycolipid and the major constituent of the outer membrane in Gram negative bacteria. Recognition of LPS is mediated by a complex of CD14, Toll-like receptor (TLR)-4 and MD-2 (326), and studies have shown that LPS stimulation through TLR4 activates DC maturation and promotes the differentiation of Th1 cells through IL-12 (327). Furthermore, LPS activation of DCs induces innate IL-10 production during *B. pertussis* infection to promote IL-10-secreting type 1 regulatory T cells (Tr1), which inhibit Th1 cells and prevent immunopathology in the lungs (327). Effective clearance of the bacteria necessities strong cellular immunity and infection is associated with the induction of both IFN-γ-secreting Th1 cells and IL-17-producing Th17 cells (85, 328). Interestingly, *B. pertussis* induced IL-10 acts as a means of immune evasion to prolong the bacterial survival in the host by inhibiting Th1-mediated bacterial clearance (56). Conversely this inhibition of Th1 responses by innate and adaptive sources of IL-10 could limit inflammatory pathology in the infection site (85). In summary, respiratory infection with *B. pertussis* promotes the induction of *B. pertussis* specific IL-10, IFN-γ and IL-17.

1.9 Objectives of the study

MS is a hugely complex disease where genetic and environmental background influence susceptibility to disease and a variety of cells and cytokines are involved in pathogenesis. Examining the role of specific T cell subsets in the pathogenesis or attenuation of EAE can improve our understanding of the disease, in order to design more selective and improved therapies for the treatment of MS. Evidence for the hygiene hypothesis would suggest that infections with helminth and bacterial pathogens can modulate the severity of of EAE and MS through induction of regulatory cells (309, 313). This study will address the hypothesis that infections which induce regulatory immune responses can attenuate the severity of autoimmune diseases. Cells of the adaptive immune system, especially $CD4^+$ Th17 cells, are considered to be the key pathogenic lymphocytes in CNS inflammation during MS and EAE. However, in the last few years there has been increasing evidence that innate-like lymphocytes, such as γδ T cells can play an equally pathogenic role in the development of autoimmunity. γδ T cells play a central roles in immunity to infection, however dysregulation of γδ T cells can lead to severe immunopathology as γδ T cells can augment Th17 responses through innate cytokine secretion (116). γδ T cells represent up to 20-30% of the T cell infiltrate in the brains lesions of patients with MS (293, 294) and $V\gamma4^+$ T cells, a small population of IL-17 secreting T cells have been shown to accumulate in the brain during EAE (116, 329, 330). However, only a subtle difference in the severity of EAE was observed between WT and $TCR\delta^{-/-}$ mice, resulting in controversy over the role of γδ T cells in the pathogenesis of EAE. This project will address the hypothesis that IL-17-secreting $V\gamma4^+$ T cells play a central pathogenic role in EAE. The aims of this study were to:

- Examine the effect of a respiratory infection with *B. pertussis* on the outcome of EAE
- Determine the mechanism of immunomodulation by *B. pertussis* during EAE
- Examine the function of conventional γδ T cells expressing Vγ4.
- Characterise and examine the function of a novel T cell population that express αβTCRs and Vγ4.
- Examine the role of TCRVγ4 expressing T cells, such as Vγ4δ and αβVγ4 T cells in the pathogenesis of EAE.

Chapter 2.

Materials and Methods

2.1 Materials

General reagents

Cell culture medium

Roswell Park Memorial Institute (RPMI)-1640 medium (Sigma) was supplemented with 8% heat-inactivated (56°C for 30 min) foetal calf serum (FCS; Biosera), 100 mM L-Glutamine (Gibco) and 100 µg/ml penicillin/streptomycin (Gibco)

Phosphate-buffered saline (PBS; 20X)

320 g Sodium chloride (NaCl, 1.4 M)

46 g Sodium hydrogen phosphate (Na_2HPO_4, 0.08 M)

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

8 g Potassium chloride (KCl, 0.03 M)

Dissolved in 2 L of dH_2O and adjusted to pH 7.0

ELISA wash buffer

500 ml 20X PBS

9.5 L dH_2O

5 ml Tween 20

Phosphate citrate buffer

20.38 g Citric acid ($C_6H_8O_7$)

73.8 g di-Sodium hydrogen orthophosphate dodecahydrate ($Na_2HPO_4.12H_2O$)

Made up to 2 L with dH_2O, pH 5.0 (stored at 4°C)

ELISA substrate solution

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

25 ml Phosphate Citrate Buffer

7 µl H_2O_2

ELISA stop solution (1M H_2SO_4)

26.74 ml 18 M H_2SO_4

473.26 ml dH_2O

Basic FACS buffer

2% FCS

0.1% Sodium Azide ($NaNO_3$)

Made up in 1X PBS

Basic Sorting Buffer

1 x Phosphate buffered saline

1mM EDTA

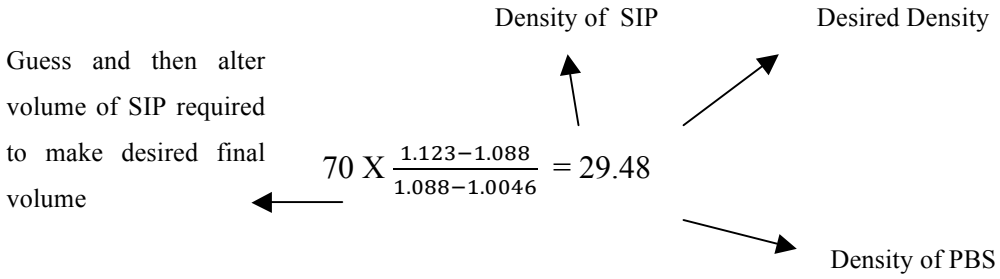
25mM HEPES pH 7.0

1% heat inactivated Fetal Bovine Serum

0.02µm filter sterilized

Stock isotonic percoll

Percoll is made isotonic by diluting 9:1 with 10X PBS (Sigma). The different density gradients can be calculated using the equations described in the percoll manual (Sigma Website). Briefly, for 1.088 g/ml: if you require ~ 100 ml,



Therefore,70 ml SIP + 29.38 ml 1X PBS = 99.38 ml 1.088 g/ml percoll.

General reagents

Reagent	Supplier
MOG	Genscript
Pertussis Toxin	Kaketsuken
CFA	Chondrex
PBS	Sigma Aldrich
EDTA	Sigma Aldrich
HEPES	Sigma Aldrich
Trizol	Invitrogen
Bovine Serum Albumin	Sigma Aldrich
Horse blood	Cruinn
LS columns	Miltenyi Biotec
rIL-1β	R&D biosystems
rIL-23	R&D biosystems
rIL-10	R&D biosystems

Reagents for restimulation of cells for intracellular staining

Reagent	Concentration in vivo	Supplier
PMA	10ng/ml	Sigma Aldrich
Ionomycin	1µg/ml	Sigma Aldrich
Brefeldin A	5µg/ml	Sigma Aldrich

Biotin antibody cocktails used for the purification of cells by negative magentic selection

Purified cell population	Targets of antibody cocktail	Supplier
CD3	CD11b, CD11c, CD19, CD45R, CD49b, CD105, MHC II, Ter-119	Miltenyi Biotec
CD4	CD8a, CD11b, CD11c, CD19, CD45R, CD49b, CD105, MHC II, Ter-119	Miltenyi Biotec

FACS antibodies

Antibody	clone	Most commonly used fluorochromes	supplier
Vγ1	2.11	PE	Biolegend
Vγ2/Vγ4	UC310A6	PeCy7, FITC	Ebioscience Biolegend
TCRβ	H57-597	APC, PeCy7	BD biosciences
TCRδ	GL3 UC713D5	BV605, BV650, BV421, PeCy7, PE	BD biosciences Biolegend
CD3	17A2 145-2C11	AF780, V450, PE, APC, BV421	BD biosciences
CD4	RM4-5 GK1.5	BV421, AF780, A700, APC	BD biosciences
CD8	53-6.7	PE	BD biosciences
CD27	LG.3A10	PE	Ebioscience
CD73	TY/23	PE	BD biosciences
Vα2	B20.1	PerCP/Cy5.5	Biolegend
Vα11	RR8-1	FITC, PE	Biolegend
Vα8.3	B21.14	Pacific blue	Miltenyi
Vα3.2	RR3-16	FITC	BD biosciences
CD44	IM7	BV605	Biolegend
ICOS	7E.17G9	PerCP	BD biosciences
CD49d	R1-2	PeCy7, PerCP-eFluor 710	BD biosciences
CCR6	140706	APC	BD biosciences
CD11a	M17/4	FITC	Ebioscience
CD25	3C7 PC61.5	Pecy7, APC	Ebioscienes BD biosciences
Foxp3	FJK-16s	APC, FITC, PE	BD biosciences
IL-17A	eBio17B7	V450, PerCP5.5, A488,	BD biosciences
IFN-γ	XMG1.2	V450, PerCP5.5, PECF594, APC	Ebiosciences BD biosciences
IL-10	JES5-16E3	PE, FITC	BD biosciences
GM-CSF	MP1-22E9	BV605	Biolegend

TNF-α	TN3-19.12	PE	BD biosciences
IL-22	1H8PWSR	PerCP-eFluor 710	BD biosciences
IL-23R	078-1208	PE	BD biosciences
IL-1R1	JAMA-147	PE	Biolegend
Ly6C	ER-MP20	APC, A780	Ebiosciene
CD11b	M1/70	PE, PeCy7	Ebiosciene
CD11c	N418	PerCP-eFluor 710	Ebiosciene
MHC II	I-A/I-E	PE, Pacific Blue	Ebioscience
Ly6G	GR1	PE, A647	Ebioscience
F4/80	BM8	PE, A647, FITC, PeCy5	Ebioscience
CD19	1D3	FITC	Ebiosciene
Isotype control Ar. Hamster	HTK888	PeCy7	Biolegend
Isotype control Ar. Hamster	eBio299Arm	APC	Ebioscience

NA/LE in vitro antibodies

Reagent	Clone	Concentration in vitro	Application	Supplier
Anti-CD3e	145-2C11	1µg/ml	Blate bound	BD pharmingen
Anti-CD28	37.51	2µg/ml	Soluble	BD pharmingen
Anti-IL-10R	1B1.3a	1µg/ml	Soluble	BD pharmingen

B. pertussis reagents

Stainer-Scholte iquid medium (1L)

L-glutamic acid (monosodium salt	10.72g
L-proline	0.24g
NaCl	2.5g
KH_2PO_4	0.5g
KCl	0.2g
$MgCl_26H_2O$	0.1g
$CaCl_22H_20$	0.02g
Tris	1.525g

Made up to 1L with ddH_2O and sterilise by autoclaving

Supplements (200x)

L-cystine	0.4g (dissolved in 1ml 37% HCl)
$FeSO_47H_20$	0.1g
L-Ascorbic acid	0.2g
Nicoinic acid	0.04g
L-Glutathione (reduced)	1.0g

Make up to 50ml in Baxter water and filter sterilise

Bordet gengou blood agar plates

250ml ddH_2O

2.5ml glycerol

7.5g BG Agar

Made up to 250ml with ddH20 and autoclaved at 121c for 20minutes

Post autoclaving add 1ml Cephalexin (10mg/ml) and 100ml of pre-warmed Horse Blood

1% Casein salt solution

NaCl	6.0g
BactoCasamnio acids	10.0g
Made up to 1L with ddH_20	pH 7.0-7.2 and filter sterilise

2.2 Methods

Mice

C57BL/6 mice were purchased from Harlan, U.K or C57BL/6 and TCRδ mutant mice, defective in TCRδ expression (*Tcrδ*$^{-/-}$), IL-1R$^{-/-}$, IL-10$^{-/-}$ on a C57BL/6 background (Jackson Laboratory), were bred under specific pathogen-free (SPF) conditions. Experiments were conducted with sex-matched mice aged 6-8 weeks. All mice were maintained according to European Union regulations, and experiments were performed under licence BI00/2412 from The Irish Medicine Board with approval from the Trinity College Dublin BioResources Ethics Committee.

B.pertussis respiratory challenge

Respiratory infection of mice was induced by aerosol challenge as described (56). Briefly, *B. pertussis* Tohoma 1 was grown on Bordet-Gengou agar plates for 4 days, and bacteria were transferred to Stainer-Scholte liquid medium for 24 hours at 37°C. Bacteria were resuspended at 1×10^9 colony forming units (CFU)/mL in physiological saline containing 1% casein, and aerosol challenge was administered over a period of 15 minutes using a nebulizer. Infection was confirmed by performing CFU counts on the lungs of mice 3 hours and 21 days post-infection as described (56).

Induction and assessment of EAE

Active EAE was induced by injecting mice subcutaneously with 100 μg of $MOG_{35–55}$ peptide (GenScript) emulsified in CFA containing 4 mg/mL (0.4 mg/mouse) of heat-killed *M. tuberculosis* (Chondrex). In addition, mice were injected intraperitoneally (i.p.) with 250 ng of pertussis toxin (Kaketsuken) on day 0 and 2. In certain experiments, mice were treated with anti-Vγ4 antibody (250 μg/mouse; BioXcell), administered i.p. on day -1, 2, 5, 7, 11, 14, 17 and 20 of EAE; control mice were administered an isotype control antibody (250 μg/mouse) at the same time points. Clinical signs of EAE were assessed according to the following score: 0; limp tail, 1; ataxic gait, 2; hind limb weakness, 3; hind limb paralysis, 4; tetra paralysis/moribund, 5.

Induction of active EAE and EAE by adoptive transfer

Active EAE was induced by injecting mice subcutaneously with 100 μg of $MOG_{35–55}$ peptide (GenScript) emulsified in CFA containing 4 mg/mL (0.4 mg/mouse) of heat-killed *M. tuberculosis* (Chondrex). In addition, mice were injected intraperitoneally with 250 ng of pertussis toxin (Kaketsuken) on day 0 and 2. In certain experiments, mice were treated with anti-Vγ4 antibody (250 μg/mouse; BioXcell) administered i.p. on day -1, 2, 5, 7, 11, 14, 17 and 20 of EAE; control mice were administered an isotype control antibody (250 μg/mouse) at the same time points. Clinical signs of EAE were assessed according to the following score: 0; limp tail, 1; ataxic gait, 2; hind limb weakness, 3; hind limb paralysis, 4; tetra-paralysis/moribund, 5. EAE was also induced by adoptive transfer of MOG-specific cells. C57BL/6 and $TCR\delta^{-/-}$ mice were immunized with MOG and CFA as described above. On day 10 post-induction, mice were sacrificed and their brachial LNs, axillary LNs, inguinal LNs and spleens were removed and prepared as single cell suspensions. For anti-Vγ4 depletion experiments ex vivo, WT or $TCR\delta^{-/-}$ cells were labeled with anti-mouse Vγ4 (0.25 μL/1 x 10^6 cells) and $V\gamma4^+$ cells were depleted by FACS. Cells were stimulated with combinations of MOG (100 μg/mL) and IL-23 (10 ng/mL) + IL-1β (10 ng/mL) for 72 hr *in vitro* in cRPMI at 10×10^6 cells/mL in 75 mL tissue culture flasks. All cytokines used were of mouse origin. After 72 hr, cells were washed and cytokine production in supernatants was measured by ELISA. 15×10^6 viable cells were injected i.p. into WT C57BL/6 recipient mice. Mice were monitored daily for signs of clinical disease and their weights were recorded. Disease severity was assessed as above for typical symptoms of EAE.

Mononuclear cell isolation from CNS tissue

Mice were sacrificed by an anaethetic overdose of 40μl of pentobarbital sodium (Euthethal) or CO_2 anaesthesia. The mice were perfused intracardially through the left ventricle with 20ml of ice cold PBS. The brain, spinal cord, spleen, inguinal, axillary and brachial lymph nodes were isolated and collected in 1ml of medium in sterile 2ml ependorfs. Tissue was lysed using the Qiagen tissue lyser (Qiagen) at 30rpm/second for 4 minutes total. Cells were then washed in PBS and resuspended in 5ml of 40% Percoll. The homogenate was overlayed onto 5ml of 70% percoll. The percoll gradients were centrifuged at 1300 x g for 20 minutes at 18c. Mononuclear cells (MNCs) were removed from the interface of the percoll gradients and washed twice with medium.

Intracellular cytokine staining

Mononuclear cells were isolated by Percoll (GE Healthcare Life Sciences) density centrifugation from the lungs, brains and spinal cords of mice perfused with PBS. Cell were stimulated with brefeldin A (5 μg/mL) alone or PMA (10 ng/mL) and ionomycin (1 μg/mL) with brefeldin A (5 μg/mL) for 5 hr. Cells were washed and stained with LIVE/DEAD Fixable Aqua Dead Cell Stain Kit (Life Technologies) and blocked with Fcγ blocker (1 μg/mL; BD Pharmingen) before extracellular staining for surface CD3, CD4, CD8, Vγ4, TCRβ and TCRδ. Cells were stained with CD11b, F4/80 and Ly-6G, and FACS analysis was performed to determine the frequency of macrophages and neutrophils. Intracellular staining for IL-17, IL-10 or IFN-γ was conducted after fixation and permeabilization (2% Paraformaldehyde, Thermo Scientific) (Saponin, Sigma Aldrich). Flow cytometric analyses were performed using either a FACSCantoII or a LSRFortessa (both BD Biosciences) with FACS Diva (BD Biosciences) or FlowJo (TreeStar Inc.) software. Gates were set on isotype or FMO controls.

Antigen-specific cytokine production

Spleen (2 x 10^6 cells/ml) were stimulated with MOG_{35-55} peptide (2–50 μg/ml) or medium only. Supernatants were recovered after 72 h, and IL-17 and IFN-γ concentrations were determined by ELISA (R&D Systems).

Dendritic cells

Mouse bone-marrow derived immature DCs were generated as described (95). Bone marrow-derived immature DC (10^6/ml) were cultured at 37°C for 24 h with heat killed (HK) or live *B. pertussis* (10^5-10^8/ml). Supernatants were removed after 24h, and the concentrations of IL-10 were quantified by ELISA (R&D Systems).

TCR Expression during ontogeny in murine thymuses

C57BL/6 were bred at the University of Birmingham. Thymuses from C57BL/6 embryos, where the appearance of a vaginal plug was designated embryonic day (E) 0, were isolated and analyzed for expression of CD3, CD4, CD8, Vγ4, TCRβ and TCRδ from E14, E16 and E18 using flow cytometry.

FACS purification and culture of Vγ4 T cell subsets

WT or TCRδ$^{-/-}$ cells were purified by magnetic separation for CD3 using a Pan T cell Isolation kit (Miltenyi) and labeled with anti-mouse Vγ4 clone UC3-10A6 (0.5 μL/1 x 10^6 cells) or anti-mouse TCRβ clone H57-597 (0.5 μL/1 x 10^6 cells). Vγ4$^+$ or Vγ4$^+$TCRβ$^+$ cells were then sorted using a FACSAria Fusion (BD Biosciences) a MoFlo Legacy (Beckman Coulter). Cells were cultured with medium or various combinations of IL-1β (10 ng/mL), IL-23 (10 ng/mL), IL-2 (10 ng/mL), IL-7 (10 ng/mL) and IL-15 (10 ng/mL) in the absence or presence of anti-CD3 (1 μg/mL). Culture supernatants were harvested 24-72hr after T cell activation and measured by ELISA for secretion of IL-17, GM-CSF (R&D) and IFN-γ (BD Pharmingen). For proliferation assays, magnetically purified CD3$^+$ cells from mice with EAE were labeled with Cell Trace Violet (CTV-Life Technologies) and cultured with IL-1, IL-23 (10 ng/mL) or MOG (100 μg/mL). Proliferation was assessed by dilution of CTV on αβVγ4 T cells using flow cytometry 72hr after T cell activation.

Gating stratgey for FACS sorting T cell subsets

For FACS purification, MACS purified CD3 T cells (routinely <95% pure) Vγ4 T cells were FACS sorted on the expression of Vγ4 (Figure 2.1). αβVγ4 T cells were FACS sorted on the expresion of Vγ4 and TCRβ (Figure 2.2). A side scatter (SSc) versus forward scatter (FSc) gate was applied to cell samples to gate lymphocytes by size and exclude debris. Cells were sorted on pulse width, dead cells were excluded by PI stain and pulse width profile, forward scatter width (FSw) versus Single forward scatter and SSC versus single side scatter to exclude doublets. Live Vγ4$^+$ CD3$^+$ cells were selected on Vγ4$^+$PI$^-$ and Vγ4β$^+$ and Vγ4β$^-$ T cells were selected for TCRβ expression and gated on PI negative. FACS purified Vγ4 T cell subsets were routinely 95% pure.

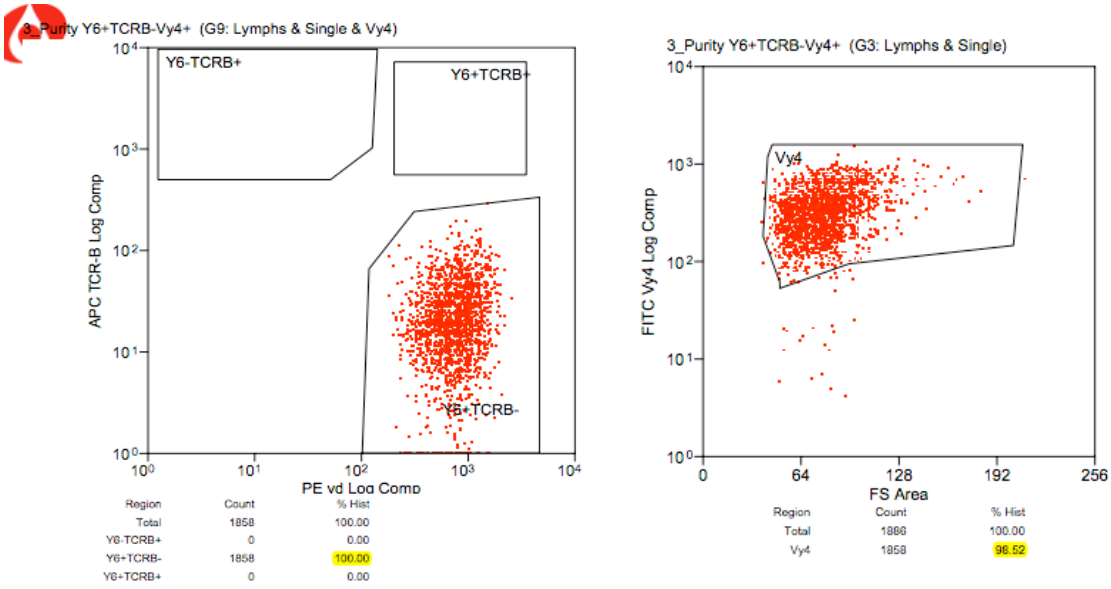


Figure 2.1: Sample puritied for FACS purified Vγ4 T cells.

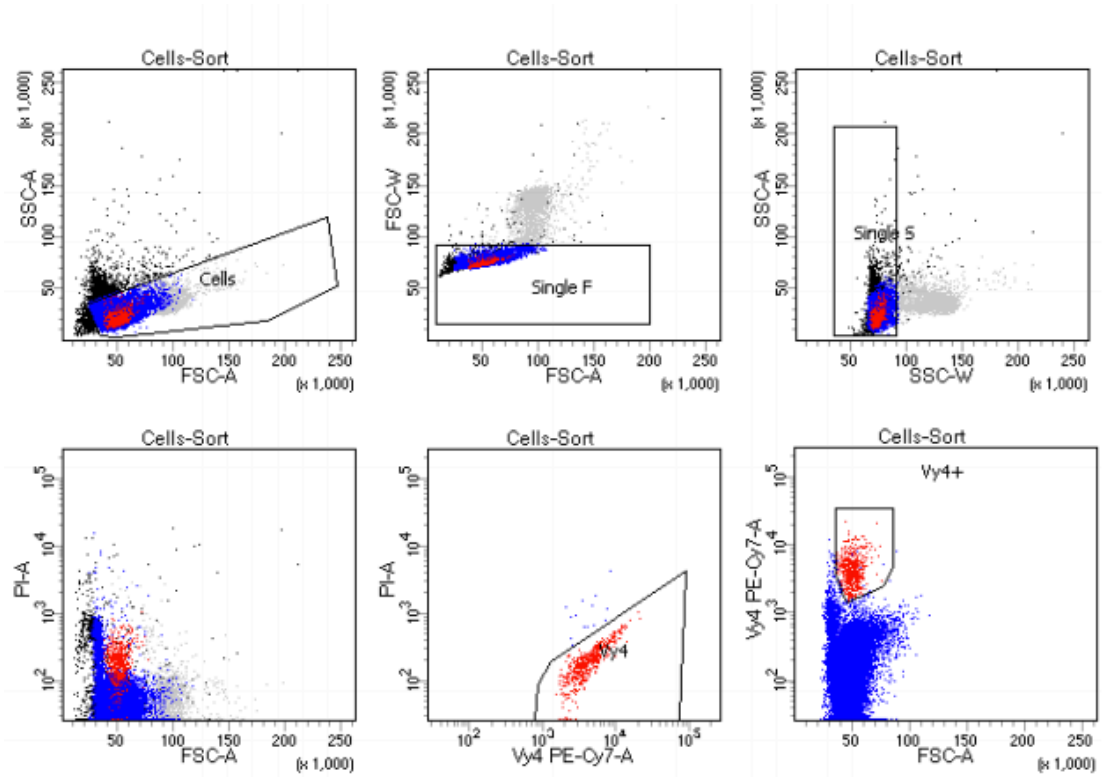


Figure 2.2: FACS purification of Vγ4 T cells.

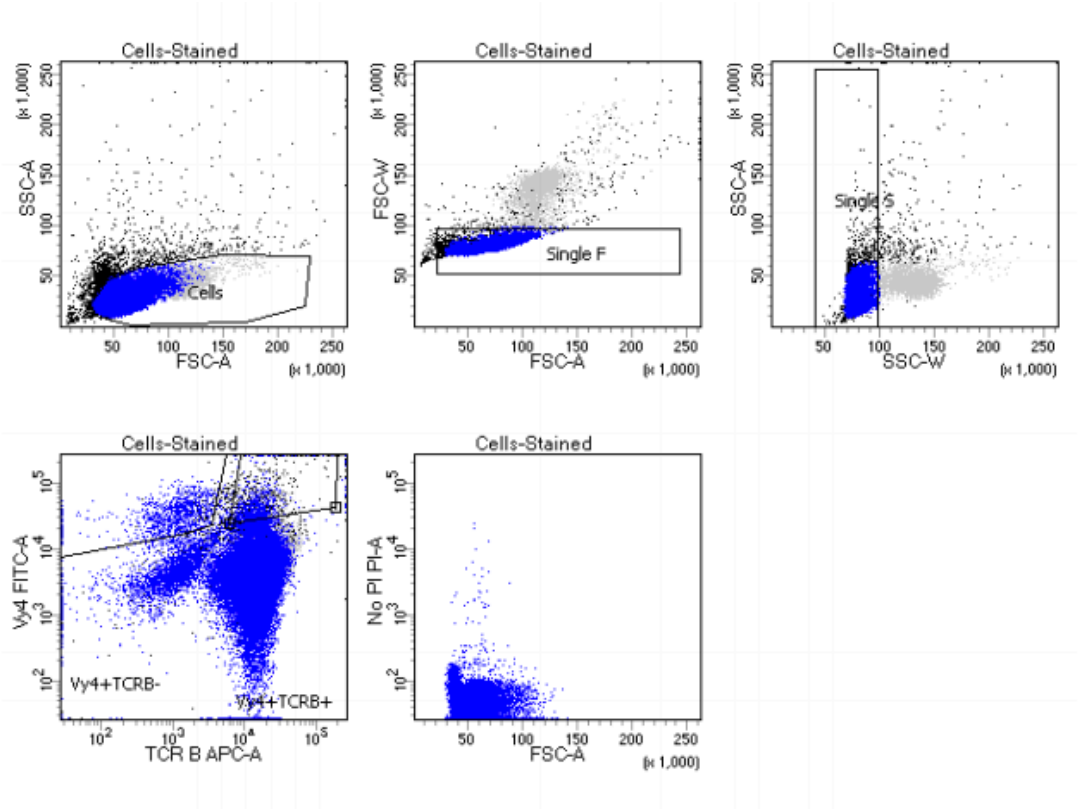


Figure 2.3: FACS purification of Vγ4β^{+} and Vγ4β^{-} T cell subsets

Acquisition and analysis of FACS data

Flow cytometric analyses were performed using either a FACSCantoII or a LSRFortessa (both BD Biosciences) with FACS Diva (BD Biosciences) or FlowJo (TreeStar Inc.) software. Cell debris was excluded by applying a FSc and SSc gate. Dead cells and doublets were excluded by their LIVE dead and pulse width profile (SSw vs forward scatter area (Fsa) and FSw VS FSa). Percentages and total number of cells were calculated on the basis of live single cells. Total T cells were selected based on CD3 expression. CD4 T cells were selected from live CD3 T cells and gated on CD4 versus FSa. Vγ4 T cells were gated from live CD3 T cells; TCRVγ4 gates were set on an isotype control versus CD3. Vγ4δ T cells were gated from live Vγ4^{+}CD3^{+} T cells and gated on TCRδ^{+}TCRβ^{-}. αβVγ4 T cells were gated from live Vγ4^{+}CD3^{+} T cells and TCRβ gates were set on an isotype control versus CD3. Other cell surface markers and cytokines were set on FMO controls.

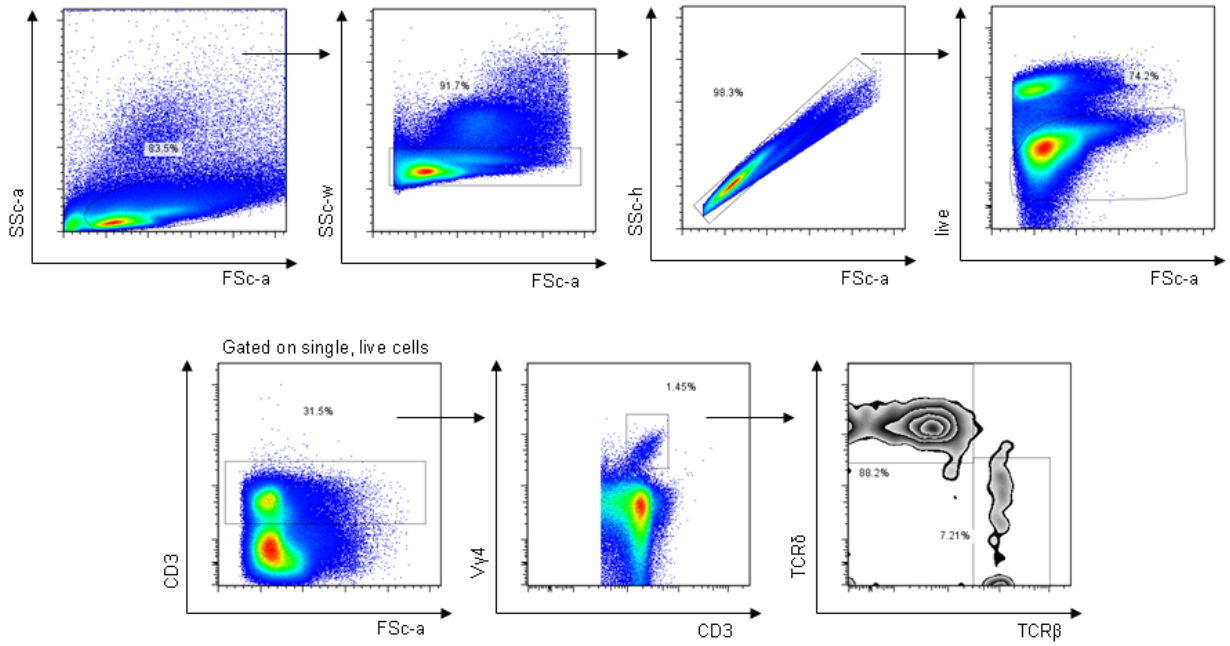


Figure 2.4: Gating strategy for Vγ4δ$^+$ and αβVγ4 T cells.

T cell purification and stimulation

Lymph nodes and spleens were isolated from naïve mice and the cells were harvested from these organs. Cell number were obtained are counted using a haemocytometer and Trypan blue at a dilution of 1:20. Cells were sorted using Magnetic Assisted Cell Sorting, MACS (Miltenyi Biotec) for CD3+, CD4+, CD8+ and γδ populations accordingly.

All cells were cultured in RPMI Medium supplemented with 10% foetal bovine serum, 100 units/ml penicillin, 100 µg/ml streptomycin, L-glutamine and 0.000014% Mercaptoethanol. Cells were cultured with with or without anti-CD3(1 µg/ml) and anti-CD28 (1 µg/ml) and in the presence of IL-1 (10 ng/ml), IL-23 (20 ng/ml), IL-6 (10 ng/ml), TGF-β (10 ng/ml), IL-12 (10 ng/ml) , IL-2 (10 ng/ml), IL-15 (10ng/ml) and IL-18 (10 ng/ml) (Immunotools). Depending on the experiment, cells were treated with anti-IL-10R antibody (10 ng/ml) (BD pharmingen). The cells were stimulated at 37c for 24-72 hours. Supernatants were stored at -20c.

Cell counting

Cells (in complete medium) were diluted in Trypan Blue and viable mononuclear cells were counted on a light microscope (Leitz) using a haemocytometer (Improved Neubauer, Hawksley). Neutrophils were excluded from human PBMC cell counts. The total number of cells was calculated from the formula:

Average cell number (in one quadrant) x 10^4 x Trypan Blue dilution factor x ml = total cell number

Enzyme Linked Immunosorbent Assay (ELISA)

ELISAs were used to quantitatively analyse various cytokines in cell culture supernatants. 200 µl of supernatants were taken from each well of the cell culture plate and the supernatants were stored at -20c. Capture antibody diluted in coating buffer (supplied with ELISA kit) was loaded onto high-binding 96-well plates (Greiner Bio-one) in 75 µl volume. Plates were incubated overnight at 4°C, capture was removed and the plates were "blocked" for non-specific binding with appropriate blocking solution for 1 hour at room temperature. After blocking, plates were washed in PBS-tween solution, and pat dried. A standard curve of serially diluted recombinant cytokine standard was also loaded onto the plates at a highest concentration of 10,000 pg/ml with a blank triplicate well of assay diluent to show the background absorbency which could be subtracted from the sample wells. The supernatant samples were loaded as triplicates into wells either neat or diluted as desired with assay diluent. Samples were incubated overnight at 4°C. After another wash step, biotinylated detection antibody was added to each well and incubated for 2 hours at room temperature. Plates were washed again and horseradish-peroxidase (HRP) conjugated to streptavidin was applied to wells for 30 minutes in the dark. Wells were thoroughly washed and soaked for 2 minutes so ensure the HRP had been thoroughly removed. The substrate OPD (+H2O2) was then loaded into all wells and the wells were covered from light while developing and stopped with the addition of 1M H_2SO_4 (35 µl/well). The absorbency of the plate was measured at 450 nm (OPD) using a microtiter plate reader. Cytokine concentrations were calculated from the standard curve and relevant dilution factors were applied.

Real time quantitative RT-PCR

Cell subsets sorted by FACS were resuspended in Trizol in sterile RNAase free ependorfs. Total ribonucleic acid (RNA) was extracted from either RNAlater fixed tissue or cells suspended in Trizol by the TRIzol/chloroform method. Fixed tissue was homogenised using a Polytron tissue homogeniser (Kinematica) in 1ml of TRIzol in sterile RNAase free ependorfs. Cell subsets were resuspened in 1ml TRIzol per 1 x 10^6 cells/ml. 200µl of chloroform was added to each ependorf, the tube was vorteced and centriguged at 12,000rpm for 15mins at 4°C. This resulted in an upper aqueous layer which was transferred to a sterile RNAase free tube. 500µl of isopropanol was added, the tubes were mixed by inversion and then stood at room temperature for 15mins, followed by centrifugation at 12,000 rpm for 15mins. Supernatants were decanted and the pellet was washed in 75% ethanol in nuclease free water at 7,600g for 5mins at 4°C. Finally, remaining ethanol wsa removed with a pipette and the ependorf tubes were inverted and allowed to air dry for 20minutes. RNA was resuspended in 40µl of nuclease free water. The concentration of RNA was determined and equalised across samples. RNA was reverse transcribed into cDNA using the Applied Biosystems High Capacity cDNA Reverse Transcription kit, according to the manufacturer's protocol, and the cDNA product was then diluted 1:8 with nuclease free H_2O. Real time PCR for the detection of mRNA was performed using pre-designed Taqman gene expression assays (Applied Biosystems). 18s ribosomal RNA was used as an endogenous control. Samples were assayed on an Applied Biosystems 7500 Fast Real Time PCR machine.

TCR clonotyping.

Viable $V\gamma4^+TCR\beta^+$ cells from WT mice were FACS-purified directly into RNAlater (Applied Biosystems). Molecular analysis of expressed *TRA*, *TRB* and *TRG* gene transcripts in FACS-purified αβVγ4 T cells was conducted using an unbiased template-switch anchored RT-PCR as described previously (331, 332). Unbiased amplification of all expressed *TRA* (5'-GGTGCTGTCCTGAGACCGAGGAT-3'), *TRB* (5'-TGGCTCAAACAAGGAGACCT-3') and *TRG* (5'-AATAGTGGGCTTGGGGGAAATGTCTGCATC-3') gene products was then conducted using a template-switch anchored reverse transcription PCR with the listed chain-specific constant region primers (332). Amplified products were ligated into pGEM-T Easy vector (Promega) and cloned by transformation of competent DH5α *E. coli*. Selected colonies were amplified by PCR using standard M13 primers and then sequenced from an insert-

specific primer using fluorescent dye terminator chemistry (Applied Biosystems). Clones were sampled, sequenced and analyzed as described previously (331).

Immunofluorescence microscopy

V$\gamma4^+$ cells were purified using a FACSAria Fusion (BD Biosciences), transferred onto poly-L-lysine-coated chamber slides and incubated for 2hr at 37 °C. Cells were then fixed by incubating with 4% paraformaldehyde in PBS for 15 min, blocked with 20% FCS for 20 min, immunostained in 5% BSA with rabbit anti-mouse TCRβ, washed and labeled with a goat anti-mouse secondary antibody conjugated to Alexa594. The slides were mounted using mounting medium (DakoCytomation) containing DAPI for DNA staining and viewed on a point-scanning confocal microscope (FV1000; Olympus). Images were obtained and analyzed using Olympus FV-10 ASW viewer software. Confocal images are representative of at least 20 captures from three independent experiments.

Statistical analysis

Statistical analyses were performed using GraphPad Prism software. Group differences were analyzed using an unpaired Student's *t*-test or a two-way ANOVA with multiple comparisons, followed by Bonferroni post-test comparisons for three or more groups. Differences between groups for clinical scores in EAE were analyzed by two-way ANOVA with repeated measures. Significance was assigned with p values ≤ 0.05.

Chapter 3.

Respiratory infection with *Bordetella pertussis* modulates the course of EAE

3.1 Introduction

Epidemiological studies have reported an association between infections with certain bacterial or viral pathogens and the development of autoimmune diseases, including MS (298). There is evidence that exacerbations of symptoms, and loss of neurological function in patients suffering from relapsing remitting MS often occur around the time of a clinical infections, most commonly upper respiratory infections (333). Furthermore, it has been suggested that systemic infections may trigger an increase in relapses in relapsing-remitting MS patients through bystander activation of auto-reactive myelin-specific T cells (300). *Chlamydia pneumonia* infection is also associated with enhanced MRI spinal lesions in MS (304). Furthermore, the detection of Epstein Barr virus latent protein and herpes simplex virus in the brains of patients with MS has provided circumstantial evidence that pathogens may trigger or exacerbate the onset or progression of MS (305).

Conversely, the prevalence of human infections, especially with helminth parasites, has been linked with a reduced incidence and severity of allergic and autoimmune diseases, including MS. This has been explained by the 'hygiene hypothesis', whereby regulatory T (Treg) cells induced by helminths and possibly other pathogens can suppress Th2 and Th17 responses that mediate allergy and autoimmunity respectively (334). Indeed it has been demonstrated that concurrent infection with helminths induced regression of new lesions and promoted remission in MS patients (309). Studies in a murine model of MS, experimental autoimmune encephalomyelitis (EAE), have shown that infection with the helminth parasite *Fasciola hepatica* attenuated symptoms of EAE through TGF-β-mediated suppression of Th1 and Th17 responses (312). The bacteria *Staphyloccus aureus* is also capable of suppressing EAE through an extracellular adherence protein, which has anti-inflammatory effects by binding to ICAM-1 and preventing the infiltration of myelin specific T cells across the blood brain barrier into the CNS (335).

B. pertussis causes whooping cough which, despite high vaccine coverage, is a re-emerging infectious disease that affects a high proportion of adults as well as children (336). Infection with *B. pertussis* is persistent even in an immunocompetent host, with the bacteria taking weeks or months to be cleared through a combination of innate and adaptive immune responses, the latter involving IFN-γ-secreting Th1 cells and IL-17-producing Th17 cells (337). However, these adaptive immune responses that eventually eliminate the pathogen are slow to develop and this reflects the various immune subversion strategies employed by *B.*

pertussis, including induction of innate IL-10 and recruitment of Treg cells to the lungs during the acute stage of disease (56).

Given the induction of both pro-inflammatory and regulatory immune reponses during *B. pertussis* infection; this study will examine the hypothesis that infectious pathogens which subvert the immune response through suppression of effector responses can modulate autoimmunity. The effects of respiratory infection with *B. pertussis* on the developmemt of autoimmunity will be investigated using the EAE model.

3.2 Results

3.2.1 Respiratory infection with *B. pertussis* attenuates EAE

To examine the effect of *B. pertussis* infection on a model of autoimmunity, EAE was induced one day after an aerosol infection with *B. pertussis* in C57BL6 mice. EAE was induced with an injection of MOG emulsified in CFA followed by administration of pertussis toxin. Mice developed EAE from day 8 post immunisation with clinical symptoms beginning with loss of tail tone (classified as score 1) and developing into hind limb weakness until reaching hind limb paralysis (classified as score 4, Figure 3.1). Infection of mice with *B. pertussis* significantly reduces both the severity of the clinical symptoms of EAE and weight loss (Figure 3.1). The mean clinical score of mice infected with *B. pertussis* was 1.75 compared with a mean clinical score of 3.97 for the uninfected group. The onset of EAE symptoms was also delayed in the infected group.

The suppressive effect was most pronounced during the period of acute infection; when mice were monitored for EAE at extended interval after *B. pertussis* challenge, when the bacteria numbers in the respiratory tract had declined (56), symptoms of EAE did appear, but the time of onset was significantly delayed compared with un-infected mice (Fig. 3.2). Furthermore, the course of EAE was not altered when it was induced in convalescent mice 36 days after challenge with *B. pertussis* around the time of bacterial clearance from the lungs (Fig. 3.3).

Mononuclear cells were isolated from the brain and spinal cords of mice by percoll density centrifugation following intracranial perfusion with PBS at day 13 post-induction of EAE Mononuclear cells were analysed by flow cytometry for the expression of CD3, CD4, CD8, γδ, IL-17 and IFN-γ following stimulation with PMA and ionomycin in the presence of brefeldin A for 5 hours. Intracellular cytokine staining of mononuclear cells from brain and spinal cord showed significantly reduced numbers of IL-17$^+$, IFN-γ$^+$ and IL-17$^+$IFN-γ$^+$ CD4$^+$ T cells in the brain and spinal cord (Fig 3.3) of *B. pertussis* infected mice with EAE when compared with uninfected mice with EAE. This was also reflected in a decrease in the number of IL-17- and IFN-γ-producing γδ$^+$ T cells in the brains of infected mice (Figure 3.4) and a significant decrease in γδ T cells in the spinal cords (Figure 3.4). To assess infiltration of innate immune cells by flow cytometry, mononuclear cells isolated from the brain were examined for the expression of CD11b, Ly6G, F4/80 and MHC II. There was a non-

significant reduction in the numbers of infiltrating neutrophils (Fig. 3.5) into the brain of infected mice with EAE. Macrophage phenotype was determined by expression of CD11b, F4/80 with M1 macrophages, MHC II^{low} and M2 macrophages, MHC II^{high}. There was a non-significant reduction in the numbers of infiltrating macrophages (Fig. 3.6) into the brain and a significant decrease in the number of M1 macrophages in the spinal cord (Figure 3.6) of infected mice with EAE.

The current study examined the possibility that *B. pertussis* modulated peripheral MOG-specific T cells responses. Spleen and lymph node cells from mice with EAE, with or without *B. pertussis* infection, produced significantly more IL-17 when stimulated with MOG compared with medium only (Fig. 3.7A). Furthermore, spleen and lymph node cells from mice with EAE, with or without *B. pertussis* infection, produced significantly greater IL-17 (Fig. 3.7A) and IFN-γ (Fig. 3.7B) when compared with *B. pertussis* infected mice without EAE. These findings suggest that *B. pertussis* infection does not inhibit induction of pathogenic T cells but may inhibit their migration to the CNS.

3.2.2 *B.pertussis* infection significantly impairs the ability to transfer EAE

In order to confirm and extend the findings that *B. pertussis* may impair the ability of encephalitogenic T cells to traffic to the CNS during EAE, the T cell transfer model was used where MOG-specific Th17-polarized cells were transferred to recipients that were then infected with *B. pertussis*. In this model, PT is not used to induce EAE, allowing us to rule out any role for *B. pertussis* infection in modulating the potentiating effects of PT on EAE. *B. pertussis* infection significantly impaired the ability of spleen and lymph nodes cells from mice immunized with MOG and CFA to transfer EAE to recipient mice (Figure. 3.8) and this was reflected in a significant difference in the weight loss between infected and uninfected recipient mice. Recipient mice that were infected with *B. pertussis* had significantly fewer IL-17 and IFN-γ-secreting $CD4^+$ T cells migrating into the brains (Figure 3.9). Collectively these findings suggest that rather than suppressing induction of autoantigen-specific Th1 and Th17 cells, *B. pertussis* infection attenuates EAE and that this is associated with a reduction of pathogenic T cell migration into the CNS.

3.2.3 Induction of innate and adaptive IL-10 secretion by *B. pertussis*

One explanation for the modulatory effect of *B. pertussis* infection on the induction of EAE was that the bacteria enhanced immunosuppressive cytokine production by innate immune cells and/or T cells. Previous studies report that a significant increase in the numbers of IL-10-producing $Foxp3^{+}CD25^{+/-}CD4^{+}$ T cells is observed in the lungs during infection with *B. pertussis*, which peaked at day 14 (31). Consistent with this, there was a significant increase in the number of IL-10-producing $CD4^{+}$ T cells from the spleens of mice 14 day post infection with *B. pertussis* compared with naive controls (Fig. 3.10A and 3.10B). It has also been previously demonstrated that *B. pertussis* induces TLR4-dependant production of IL-10 as well as the pro-inflammatory cytokines IL-23, IL-1β, IL-6, IL-12 and TNF-α (85). Here, the data demonstrates that DCs produced IL-10 at high concentrations when stimulated with live *B. pertussis* and at lower concentrations in response to killed *B. pertussis* (Figure 3.10C). Furthermore, an IL-10R blocking antibody markedly increased IL-17 and IFN-γ production by spleen cells from *B. pertussis* infected mice following in vitro re-stimulation with killed *B. pertusiss* (Fig. 3.10D). Moreover, the data demonstrates that in response to IL-1 and IL-23 stimulation, treatment of CD3+ T cells with IL-1, IL-23 in the presence of an IL-10R antibody significantly enhances IL-17A production with anti-CD3 and anti-CD28 treatment (Figure 3.11). Similarily CD3+ T cells isolated from $IL\text{-}10^{-/-}$ mice produce significantly more IL-17A and IL-17F with anti-CD3 and/or anti-CD28 treatment (Figure 3.12). These findings demonstrate that IL-10 derived from *B. pertussis* can constrain Th1 and Th17 responses. Importantly, the data demonstrates a significant increase in IL-10-producing T cells in the lungs of mice in infected with *B. pertussis* where EAE was induced by passive (Fig. 3.13A) or active immunization (Fig. 3.13B, 3.13C) and approximately 30% of these IL-10-producing $CD4^{+}$ T cells were $CD25^{-}Foxp3^{+}$ (Fig. 3.13D). These findings demonstrate that *B. pertussis* induces IL-10 producing T cells in the lungs and this constrains Th1 and Th17 responses specific for *B. pertussis*, but may also exert bystander suppression of the function or migration of Th1 and Th17 cells that are pathogenic in EAE.

3.2.4 Attenuation of EAE was reversed in IL-10$^{-/-}$ mice infected with *B. pertussis*.

IL-10 is an important regulatory cytokine produced by macrophages, DCS, Treg, Th2 and even Th1 and Th17 cells during infection (338, 339). IL-10-producing Treg cells but not Th2 cells are induced during *B. pertussis* infection of mice and these cells have a role to prevent immunopathology by suppressing Th1 and Th17 cells which are critical in clearance of the bacteria from the lungs (340). To determine if *B. pertussis* induced IL-10 mediated the modulatory effect of infection on EAE, experiments examined the effect of *B. pertussis* infection on the course of EAE in IL-10$^{-/-}$ and WT mice. Consistent with the data in Figure. 3.1, infection of WT mice with *B. pertussis* significantly attenuated EAE, but this effect was completely reversed in IL-10$^{-/-}$ mice (Figure 3.14). The infected WT mice developed a mean clinical score of 0.84 whilst WT, IL-10-/- and infected IL-10-/- developed mean clinical scores of 3.91, 3.8 and 3.45, respectively (Figure 3.14A). The protection measured in weight loss observed in the WT infected mice with EAE was also reversed in the IL-10$^{-/-}$ mice (Figure 3.14B). Mononuclear cell isolation from the CNS at peak of disease revealed that although IL-10$^{-/-}$ mice with EAE had enhanced T cell infiltration into the brain when compared with WT mice, the reduction in infiltrating IL-17- and IFN-γ-producing CD4^{+} T cells observed in the brains of *B. pertussis* infected WT mice was less pronounced in IL-10$^{-/-}$ mice (Figure 3.15). These findings suggest that IL-10 is required to attenuate EAE during *B. pertussis* infection.

3.2.5 IL-10 induced by *B. pertussis* suppresses integrin expression on encephalitogenic T cells

The data demonstrated that *B. pertussis* induces IL-10 production from innate cells such as DCs and adaptive lymphocytes such as T cells and furthermore, IL-10 induced by *B pertussis* infection ameliorates the clinical symptoms of EAE. When taken together with the observation that infection also suppresses EAE induced by T cell transfer, which was associated with suppressed migration to the CNS, this suggests that IL-10 induced by *B. pertussis* infection in the lungs may inhibit migratory activity of encephalitogenic T cells. In order to provide evidence for this conclusion, the effect of IL-10 on MOG-specific Th17 cells was examined *in vitro*. The data demonstrates that IL-10 inhibited expression of VLA-4 and

LFA-1, integrins known to be involved in migration of T cells (Fig. 3.16). CD49d, a subunit of the α4β1 integrin was reduced by 50%, with a 40% reduction of CD11a, a subunit of LFA-1 on MOG-specific CD4+ T cells (Figre 3.16). Furthermore, the data show that infection of recipient mice with *B. pertussis* suppressed VLA-4 from and LFA-1 expression on CD4 T cells in the lungs when assessed 4 days after transfer of MOG-specific T cells (using the protocol to induce passive EAE) (Figure 3.17). Collectively these findings demonstrate that IL-10 induced by the respiratory pathogen *B. pertussis* suppresses integrin expression on T cells in the lungs and this may explain the attenuating effect of the infection on migration of pathogenic Th1 and Th17 cells and the induction of CNS autoimmunity.

3.3 Discussion

The significant new findings of this study are that infection of mice with a common human bacterial pathogen can significantly reduce CNS inflammation and attenuate clinical symptoms of EAE, a mouse model of MS. There was a significant reduction in disease onset, disease score and weight loss following induction of EAE in mice infected with *B. pertussis*. This was also reflected in a reduced number of infiltrating neutrophils, macrophages, Th1, Th17 and γδ T cells in the brains and spinal cords of *B. pertussis* infected mice with EAE. However, the reduced infiltration of IL-17 and IFN-γ producing T cells into the CNS in *B. pertussis* infected mice was not reflected by a corresponding reduction in peripheral MOG-specific Th1 and Th17 cells in infected mice, suggesting that the bacterial infection may prevent the migration of encephalitogenic T cells into the CNS and thereby limiting CNS pathology associated with EAE.

The data demonstrates that EAE induced by transfer of MOG-specific T cells was, like active EAE, inhibited during infection with *B. pertussis,* and this was associated with reduced migration of the transferred T cells into the CNS. Together with the observation that the MOG-specific T cell responses induced in the spleen by active immunization with MOG and CFA was not suppressed in mice infected with *B. pertussis*, suggests that the suppressive mechanism involved inhibition of T cell migration or effector function. The studies using IL-$10^{-/-}$ mice demonstrated that the suppressive effects of *B. pertussis* infection on the development of EAE were mediated by IL-10; *B. pertussis* infection failed to suppress clinical symptoms of EAE in IL-$10^{-/-}$ mice. Furthermore, the suppression of Th1 and Th17 migration into the CNS following infection was mostly reversed in IL-$10^{-/-}$ mice. Furthermore, the current study demonstrated that expression of the integrins, VLA4 and LFA-1, which are known to be involved in cell migration into the CNS, was suppressed on MOG-specific Th17 cells in the lungs during *B. pertussis* infection or when cultured in vitro with IL-10.

Autoimmune diseases, such as MS, develop as a result of breakdown in immune tolerance resulting in the development of adaptive immune responses against self antigens. The precipitating factors are believed to include pathogen-associated molecular patterns (PAMPs) released during viral or bacterial infections, danger-associated molecular patterns (DAMPs) released from dead or dying cells during sterile inflammation or microbe-associated molecular patterns (MAMPs) released from inflammatory commensal bacteria (298). These molecules

bind to pathogen recognition receptors on innate immune cells leading to DC maturation and production of IL-1β, IL-6, IL-12 and IL-23 that direct the induction of Th1 and Th17 cells. Indeed, the development of MS and other autoimmune diseases has been strongly linked with certain viral and bacterial infections (298). Infection with EBV and *C. pneumonia* are heavily implicated in the development or progression of MS, where studies have suggested that detection of *C. pneumoniae* in patients with monosymptomatic MS would indicate that infection occurs early in the course of MS (112). Glandular fever, or infectious mononucleosis is a viral infection caused by EBV, the risk of developing MS is greatly increased soon after infectious mononucleosis and this risk persists for at least 30 years after infection (193). In RRMS, relapses which occur post upper respiratory infection lead to greater sustained damage to neurological function compared with relapses observed in a non-infection related relapse (125).

However, there is also evidence that infection with helminth or 'friendly' microbiota can reduce the symptoms of autoimmunity through induction of anti-inflammatory cytokines, including IL-10 and TGF-β, that promote induction of $FOXP3^+$ Treg or IL-10-secreting Tr1-type cells (311). One study demonstrates that concurrent parasite infection in MS reduces the clinical symptoms of MS, and also reduced the number of new sclerotic plaques as detected by MRI (309). They observed an increase in IL-10 and TGF-β, together with induction of $CD25^+$ $CD4^+$ $FOXP3^+$ T cells in infected MS patients compared with uninfected MS patients; suggesting that regulatory T cells induced during parasite infections can modify the course of MS (309). In another study, administration of *Trichuris muris* live eggs every two weeks for 24 weeks to Crohn's patients, demonstrated that 80% of patients reported a decrease in symptoms, with 72% in remission (194); and 43% had an improvement in symptoms of ulcerative colitis after ingesting the *T. muris egg* in a 12 week trial (195). Murine studies have reported that infection with the helminth parasite *Heligmosomoides polygyrus* decreases IL-17 production in the mesenteric lymph nodes (341). Studies in mouse models of autoimmunity have shown that infection with helminth parasites can attenuate autoimmunity; *Shistomsoma mansoni* antigens have been reported to prevent the onset of type 1 diabetes (342) and colonisation with H. *polygyrus* inhibits colitis, through IL-10 independent mechanisms (341). Furthermore, *H. polygyrus* induced Treg cells have been shown to suppress Th2-mediated airway allergic responses through suppression of Th1 and Th2 cells by $CD4^+$ $CD25^+$ T cells from the mesenteric lymph node (343). Furthermore, infection with *F. hepatica* attenuates the

symptoms of EAE through TGF-β-mediated suppression of Th17 and Th1 responses, demonstrating the role of Treg cells in mediating protection in CNS autoimmunity (312).

The current study has extended these findings through the demonstration that the bacteria *B. pertussis*, which promotes IL-10 production by DC and induces *B. pertussis*-specific Treg cells, suppresses the migration of pathogenic Th1 and Th17 cells to the CNS during EAE. To date the effect of bacteria on the development of autoimmunity appears to be a more contentious subject than the effect of helminth or viral infection. Studies in bacterial infection during EAE have demonstrated that an infection with *Streptococus pneumonia* aggravates clinical symptoms of disease, with greater CNS cellular infiltration (344). Another study describes that epsilon toxin emitted from *Clostridium perfringens,* has a tropism for the BBB and could facilitate lesion formation in MS by binding to oligodendrocytes and myelin (345). A recent screening of sera and CSF from MS patients and healthy controls revealed that immunoreactivity to epsilon toxin is 10 times more prevalent in MS patients than healthy controls, indicating previous exposure to this toxin in MS patients (345). Studies in patients with MS demonstrate that stimulation of innate cells with microbial peptides appears to affect the generation of monocyte derived DCs, and their ability to alter myelin reactive T cell activation patterns; this may be correlated to relapse initiation during bacterial infection (346). Internalisation of bacterial peptidoglycan in APCs has been demonstrated in the brains of MS patients, where these peptodoglycan positive APCs produce TNF-α, IL-1β, IL-6 and IL-12 thereby contributing to CNS inflammation (303). In addition, peptidoglycan specific antibodies have been detected in the CSF of MS patients (303).

Bordetella pertussis is the causative agent in pertussis infection or whooping cough, a persistent infection typically lasting 5 weeks or more and effective bacterial clearance necessitates IFN-γ-producing Th1 cells and IL-17-producing Th17 cells. Mice defective in these cytokines or cells cannot facilitate bacterial clearance, revealing that these cells and their respective cytokines are the critical mechanism in effective clearance of *B. pertussis* (286, 287, 347). Pertussis is characterised by lung immunopathology with mucus hypersecretion. Post mortem results on experimentally infected animals reveal bronchopneumonia, alveolitis, leukocyte infiltration and epithelial and ciliary damage in the lungs (100). In conjunction with the induction of pro-inflammatory T helper cells, *B. pertussis* specific IL-10 production is crucial to control the damage to lung architecture and function. *B. pertussis* induces innate IL-10 secretion by DCs, which promotes the induction of IL-10-

secreting Tr1 cells, thereby dampening IFN-γ mediated pathology by Th1 cells in response to the infection (315). *B. parapertussis,* a related bacterium of *B. pertussis,* induces the production of IL-10, which facilitates its persistence within the host by restricting protective IFN-γ production (348). *B. pertussis* possesses a plethora of virulence factors such as LPS, tracheal cytotoxin, pertactin, filamentous haemagglutinin adhesion and pertussis toxin (PTx), a secreted soluble toxin composed of several subunits. The B subunit of PTx is known to facilitate *B. pertussis* colonization and has been shown to inhibit the migration of B and T lymphocytes into splenic white pulp cords, thus preventing lymphocyte activation (349). Interestingly, repetitive PTx treatment protects mice from development of CNS autoimmune disease through induction of regulatory cytokines and expansion of Treg cells (350).

To demonstrate that the protective effect of *B. pertussis* infection on attenuating EAE is mediated through IL-10 induction, EAE studies using IL-$10^{-/-}$ mice demonstrated that clinical severity and weight loss were reversed in IL-$10^{-/-}$ mice infected with *B. pertussis*,. Furthermore, this effect was also reflected in the cellular infiltrate into the CNS of IL-$10^{-/-}$ mice infected with *B. pertussis,* while a marked reduction in IL-17, IFN-γ producing T cells was observed in WT infected mice with EAE. It has been demonstrated that autoreactive T cells can be reprogrammed in the lungs enabling these T cells to migrate to the CNS during EAE. Odoardi et al revealed that i.v. transferred encephalitogenic T cells only gain the capacity to enter the CNS from the blood after first residing within the lung (351). The findings of this study demonstrated that EAE induced by transfer of MOG-specific T cells was, like active EAE, inhibited during infection with *B. pertussis,* and this was associated with reduced migration of the transferred T cells into the CNS. Together with the observation that the MOG-specific T cell responses induced in the spleen by active immunization with MOG and CFA was not suppressed in mice infected with *B. pertussis*, suggests that the suppressive mechanism involved inhibition of T cell migration or effector function. Furthermore, it was demonstrated that expression of the integrins, VLA4 and LFA-1, which are known to be implicit in the migration of effector T cells into the CNS, were suppressed on MOG-specific Th17 cells in the lungs during *B. pertussis* infection or when cultured in vitro with IL-10. These findings are consistent with reports that overexpression of IL-10 protects against EAE disease, whereas deletion of IL-10 exacerbated disease (352, 353). In MS patients, it has been reported that serum concentrations of IL-10 are increased during disease remission (335). Furthermore, the efficacies of IFN-β and glatiramer acetate as front line therapies in the treatment of MS are partly attributed to their induction of IL-10 production

(354, 355). IL-10 is a potent inhibitor of the inflammatory process (49) and inhibits Th1 and Th17 responses either by directly suppressing T cell cytokine production or by inhibiting APC function (50). However, the current study reveals that bacteria-induced IL-10 may suppress the licensing of autoaggressive T in the lungs through the downregulation of VLA-4 and LFA-1 on encephalitogenic T cells. The findings add further weight to the hygiene hypothesis, but suggest that suppression of autoimmunity by infectious agents is not confined to parasites, but can also be mediated by a common bacterial pathogen of man.

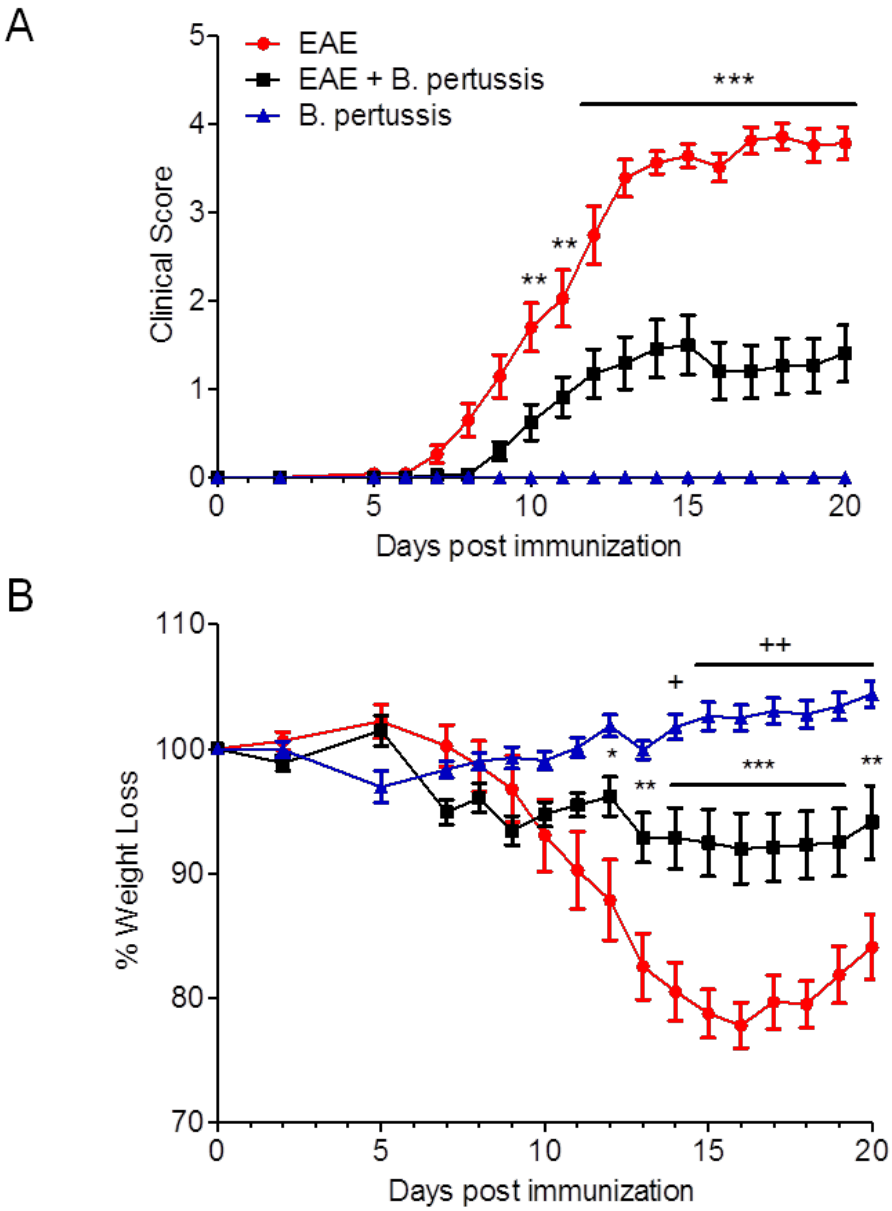


Figure 3.1: Respiratory infection with *B. pertussis* attenuates EAE. C57BL/6 mice were aerosol infected with *B. pertussis* on day -1. EAE was induced in infected or uninfected mice on day 0 by s.c injection with 200 μg of $MOG_{35–55}$ in CFA. All mice were injected i.p with PT on days 0 and 2. (A) Clinical scores were assessed daily and (B) body weights were recorded. Data are representative of three independent experiments. *P < 0.05, **P < 0.01 and ***P < 0.001 EAE Vs EAE *B. pertussis* and $^{+++}$P < 0.001 EAE Vs *B. pertussis* by two-way ANOVA and Bonferroni post hoc analysis. Results are expressed as mean ± SEM clinical score and percentage weight loss of n=6 mice/group from three independent experiments.

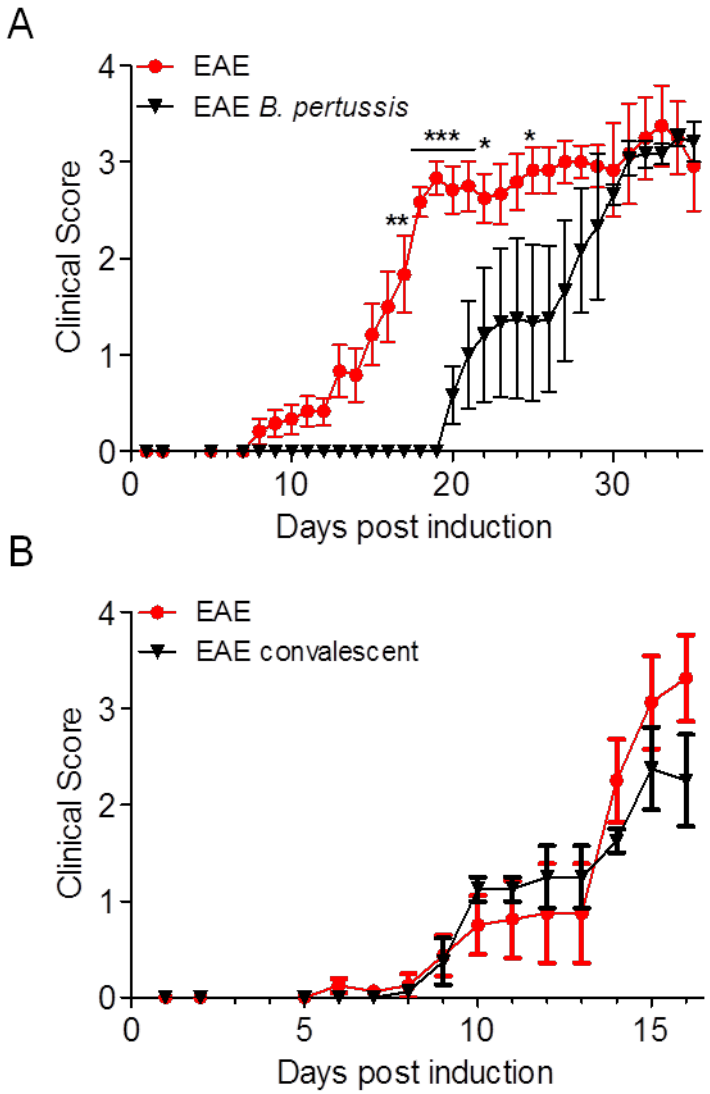


Figure 3.2: The suppressive effect of *B. pertussis* on the clinical symptoms of EAE wanes with time. C57BL/6 mice were aerosol infected with *B. pertussis* on day -1. EAE was induced in infected or uninfected mice on day 0 by s.c injection with 200 µg of MOG_{35-55} in CFA. All mice were injected i.p with PT on days 0 and 2. (A**)** Clinical scores were assessed daily. *P < 0.05, **P < 0.01 and ***P < 0.001 EAE Vs EAE *B. pertussis* and $^{+++}$P < 0.001 EAE Vs *B. pertussis* by two-way ANOVA and Bonferroni post hoc analysis. Results are expressed as mean ± SEM of clinical score for n=6 mice/group from two independent experiments. (B) EAE clinical scores in mice convalescing from *B. pertussis* (36 days post challenge) or in control naïve mice. Results are expressed as mean ± SEM clinical score for n=6 mice/group from two independent experiments.

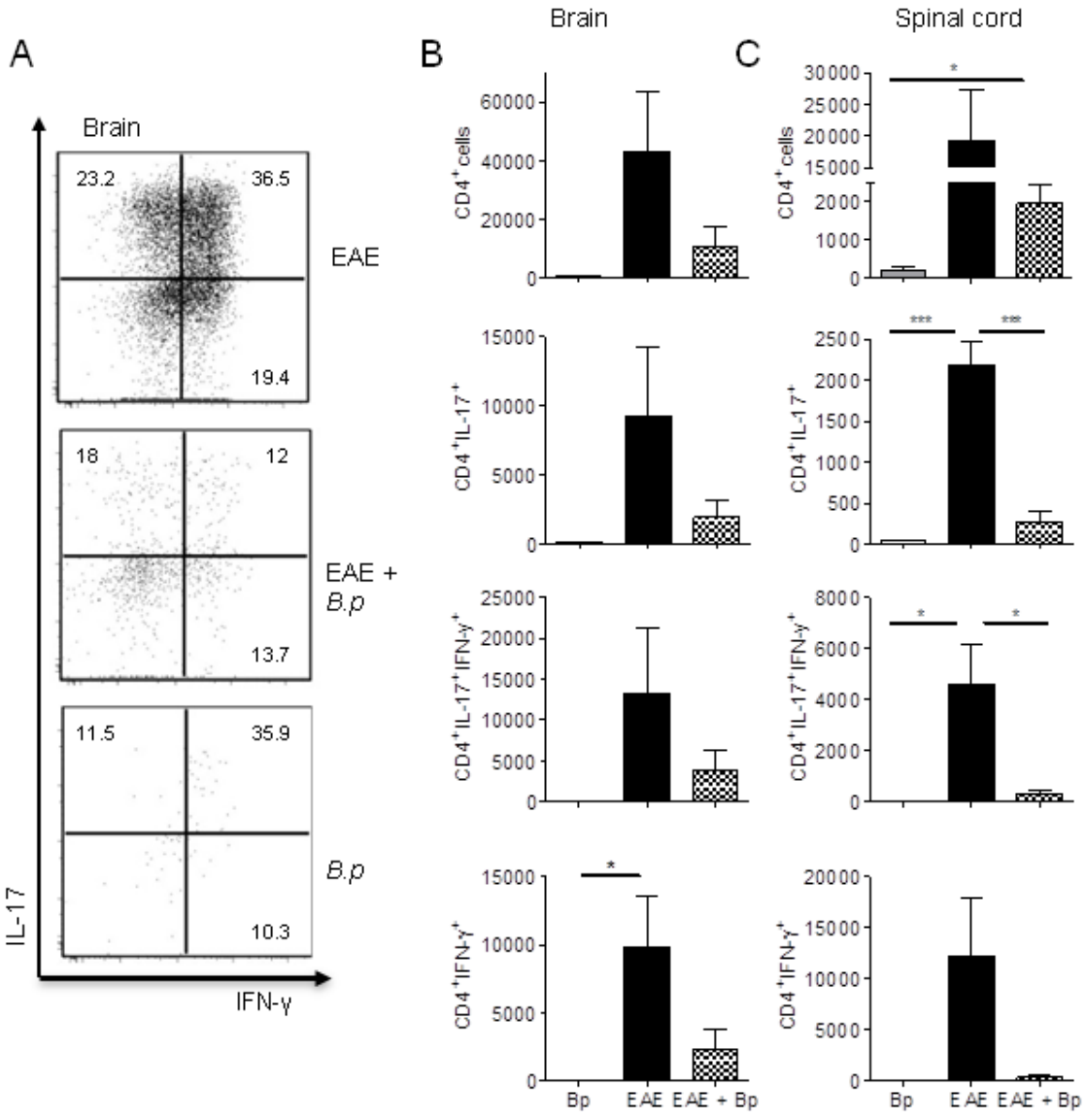


Figure 3.3: Respiratory infection with *B. pertussis* suppresses IL-17-expressing and IFN-γ-expressing CD4$^+$ T cells in the brains mice with EAE. Mononuclear cells were isolated from the brains 13 days post induction of EAE. Cells were restimulated for 5 hours with PMA, ionomycin and brefeldin A. Cells were stained with a viability dye, anti-CD4, anti-CD3, fixed and permeabilized, stained intracellularly with anti-IFN-γ and anti-IL-17 and analysed by flow cytometry. Results display representative FACS plots (A) and data are expressed as mean ± SEM of infiltrating IL-17 and IFN-γ producing CD4$^+$ T cells of n=6 mice/group in brain (B) and spinal cord (C). Data are representative of three independent experiments. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ according to *one-way ANOVA* and Bonferroni post hoc analysis.

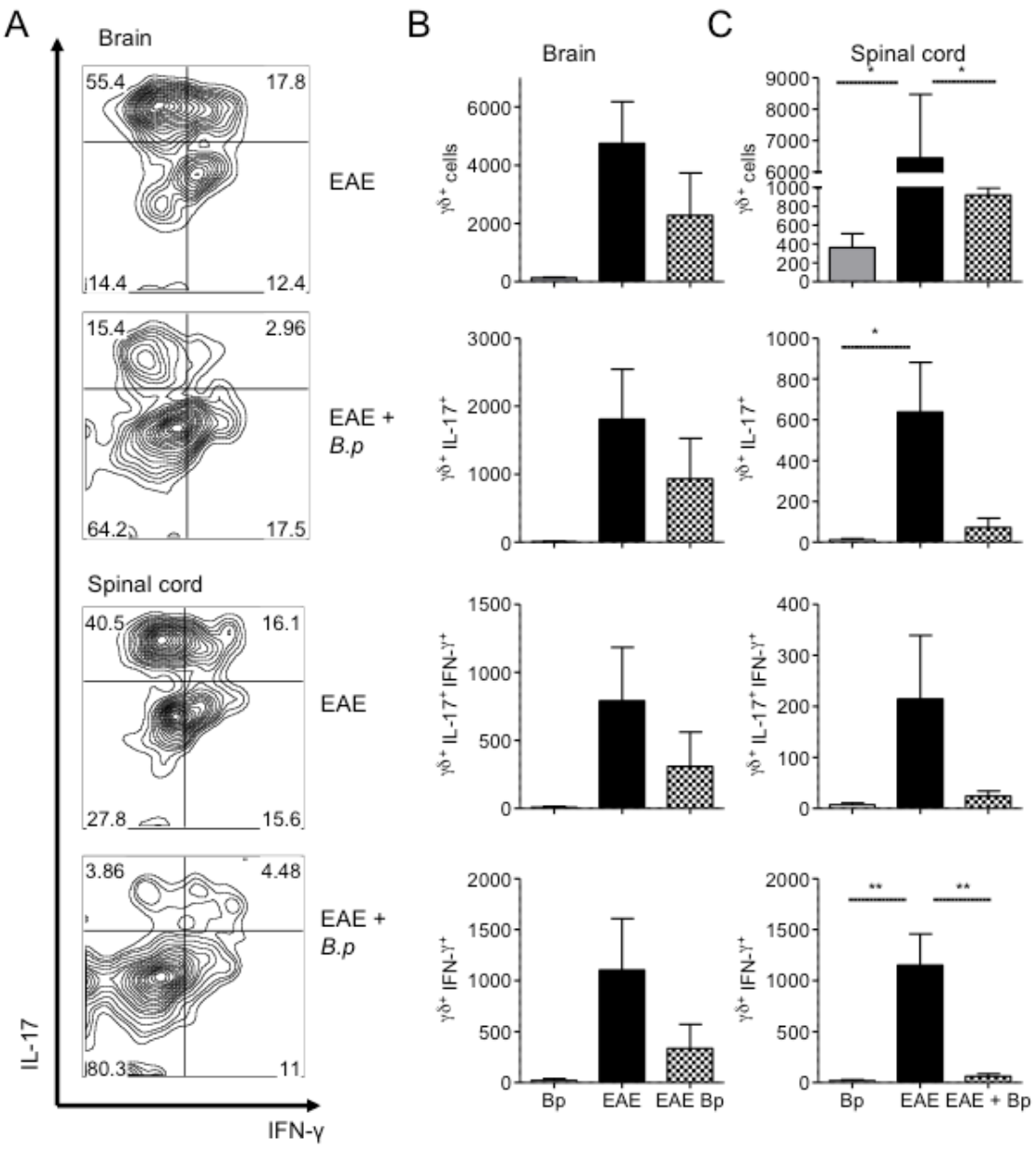


Figure 3.4: Respiratory infection with *B. pertussis* suppresses IL-17-expressing and IFN-γ-expressing γδ$^+$ T cells in the brains mice with EAE. Mononuclear cells were isolated from the brains 13 days post EAE induction. Cells were restimulated for 5 hours with PMA + ionomycin and brefeldin A. Cells were stained with anti-CD3, anti-γδ, fixed and permeabilized, stained intracellularly with anti-IFN-γ and anti-IL-17 and analysed by flow cytometry. Results are mean absolute numbers cells in the brain (A) and spinal cord (B) with representative plots of infiltrating IL-17 and IFN-γ producing γδ$^+$ CD3$^+$ cells (C). *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ according to *one-way ANOVA* and Bonferroni post hoc analysis. Results are expressed as mean ± SEM of n=6 mice/group with representative FACS plots. Data are representative of three independent experiments.

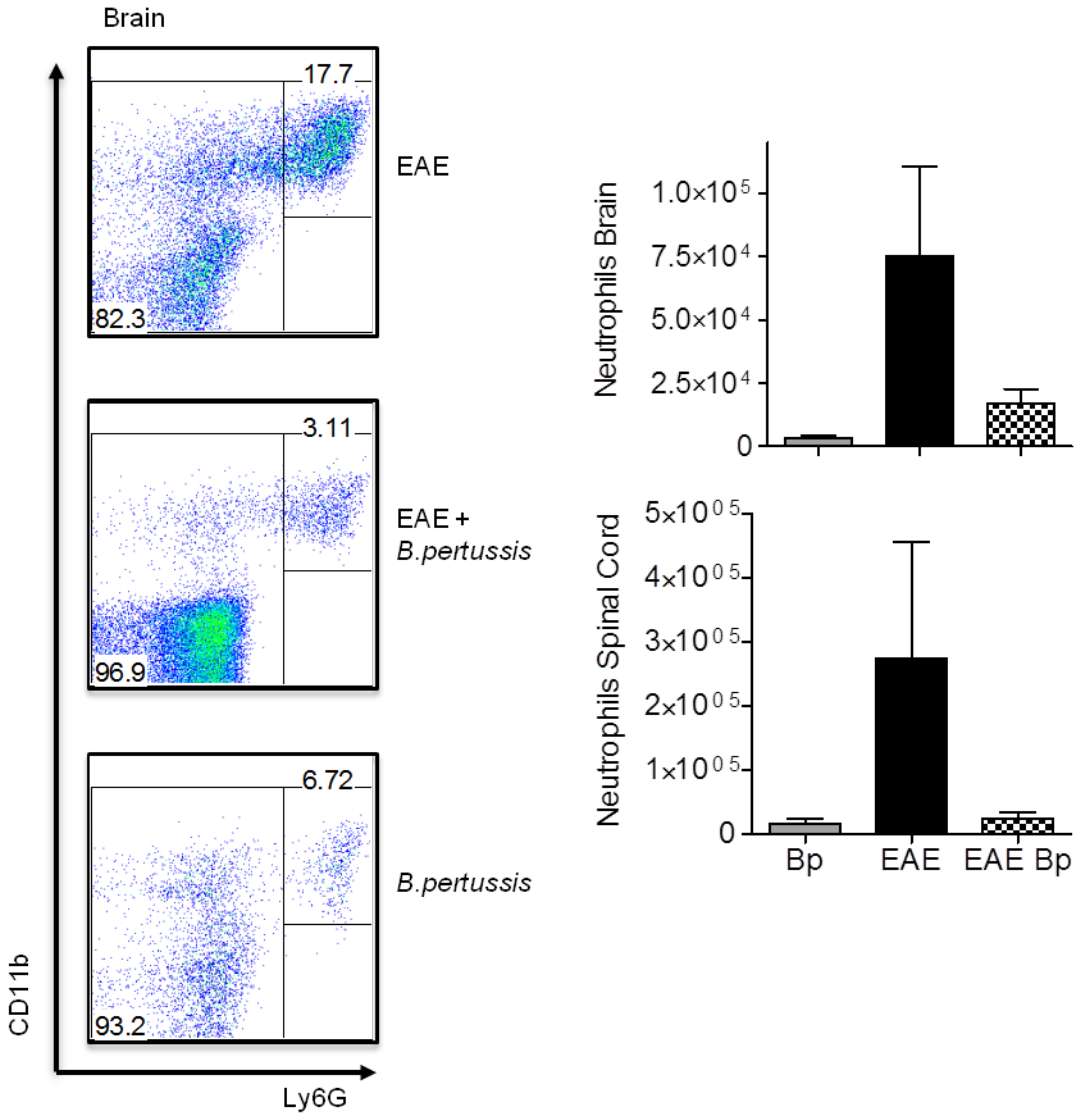


Figure 3.5: Respiratory infection with *B. pertussis* suppresses neutrophil infiltration in the brains and spinal cords of mice with EAE. Mononuclear cells were isolated from the brains 13 days post induction of EAE. Cells were stained with anti-CD11b, anti-Ly6G, fixed and analysed by flow cytometry. Results are mean absolute numbers cells with representative plots of infiltrating neutrophils in the brain of mice with EAE, infected mice with EAE and infected mice. Results are expressed as mean ± SEM of n=6 mice/group with representative FACS plots and data are representative of three independent experiments. NS according to *one-way ANOVA* and Bonferroni post hoc analysis.

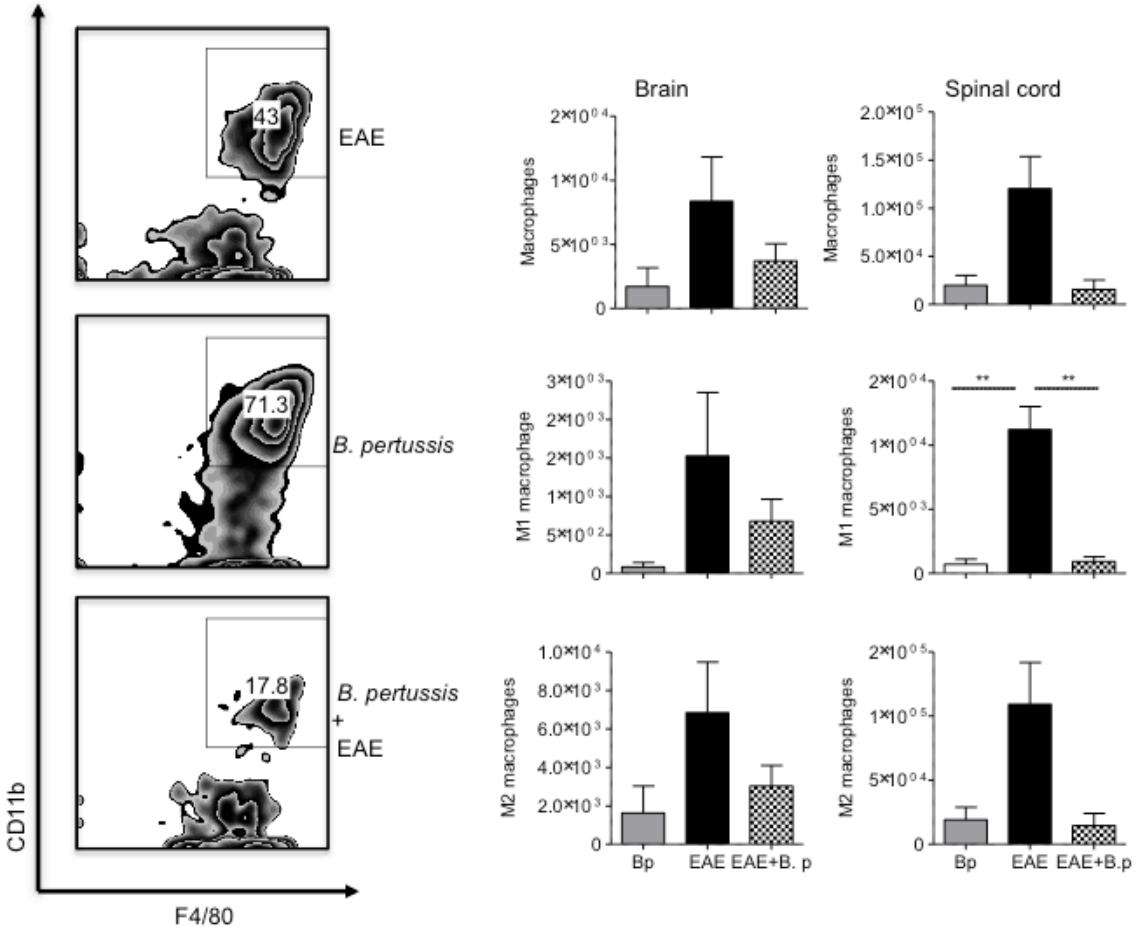


Figure 3.6: Respiratory infection with *B. pertussis* suppresses M1 and M2 infiltration in the brains and spinal cords of mice with EAE. Mononuclear cells were isolated from the brains 13 days post EAE induction. Cells were stained with anti-CD11b, anti-Ly6G, anti-F4/80 and MHC-anti II, fixed and analysed by flow cytometry. Results are mean absolute numbers cells with representative plots of infiltrating macrophages in the brain of mice with EAE, infected mice with EAE and infected mice. Results are expressed as mean ± SEM of n=6 mice/group and are representative of three independent experiments. **P* < *0.05*, ***P* < 0.01 and ****P* < 0.001 versus B pertussis infection according *one-way ANOVA* and Bonferroni post hoc analysis.

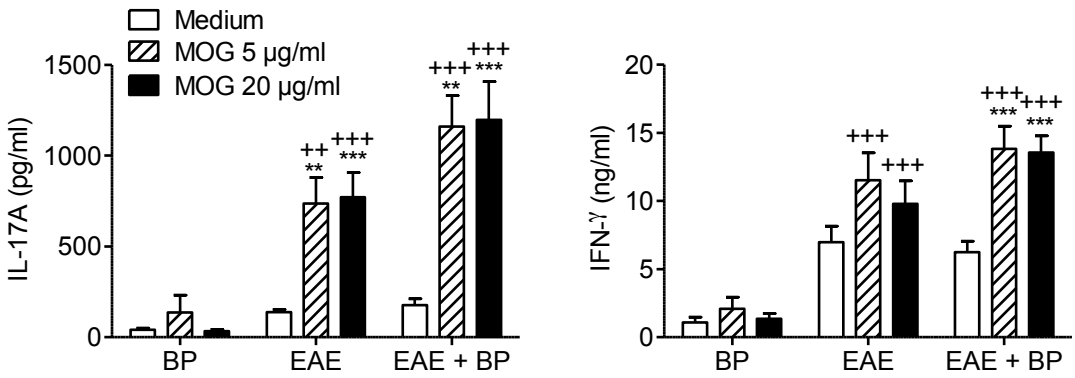


Figure 3.7: *B. pertussis* infection enhances MOG-specific IL-17 and IFN-γ in the spleen and inguinal lymph nodes of mice with EAE. *B. pertussis* infected or uninfected mice with EAE or mice infected without EAE were sacrificed day 13 post induction of EAE and lymph nodes cells and splenocytes were stimulated with MOG peptide (0-20µg/ml), medium alone. After 72 h supernatants were removed and analyzed for IL-17 and IFN-γ by ELISA. $+P < 0.05$, $++P < 0.01$ and $+++P < 0.001$ versus *B. pertussis* infection only, $**P < 0.01$, $***P < 0.001$ versus medium by ANOVA and Bonferroni post hoc analysis. Results are expressed as mean ± SD of n=5 mice/group and are representative of two independent experiments.

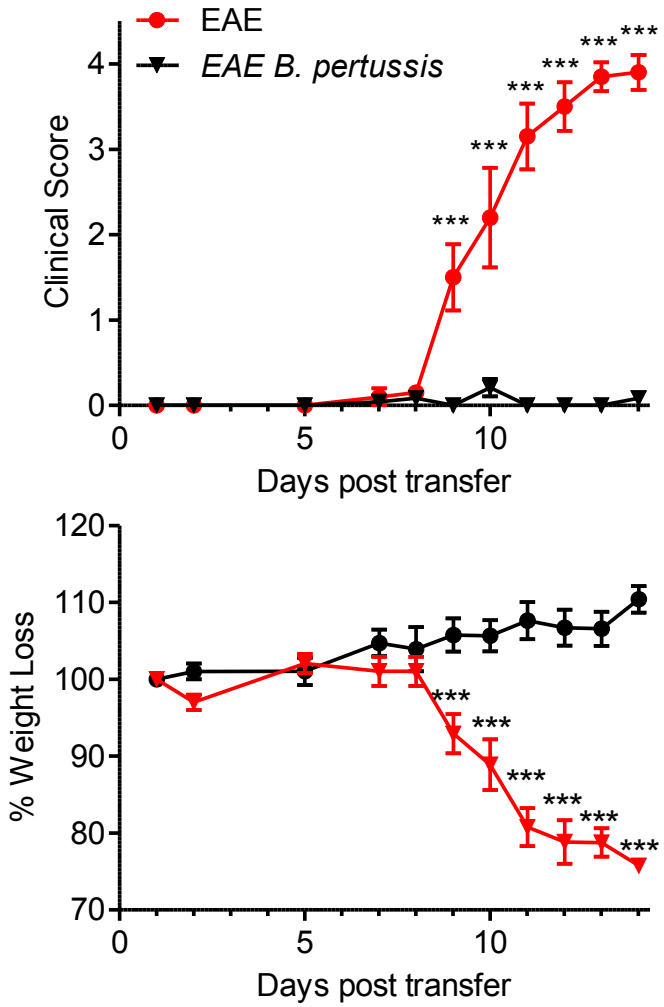


Figure 3.8: Infection of mice with *B. pertussis* significantly attenuates the clinical course of EAE induced by cell transfer. EAE was induced by passive transfer of 15 × 10^6 cells i.p. per mouse of lymph node and spleen cells (from MOG immunized mice). Cells were re-stimulated ex vivo with MOG, IL-23 and IL-1β for 72 hours and injected into naïve C57BL/6 recipient mice or mice infected with *B. pertussis* 1 day prior to transfer. (A) Clinical scores were assessed daily and (B) body weights were recorded. Results are expressed as mean ± SEM clinical score and percentage weight loss of n=6 mice/group and data are representative of two independent experiments. *P < 0.05, **P < 0.01 and ***P < 0.001 EAE Vs EAE + *B. pertussis* by two-way ANOVA and Bonferroni post hoc analysis.

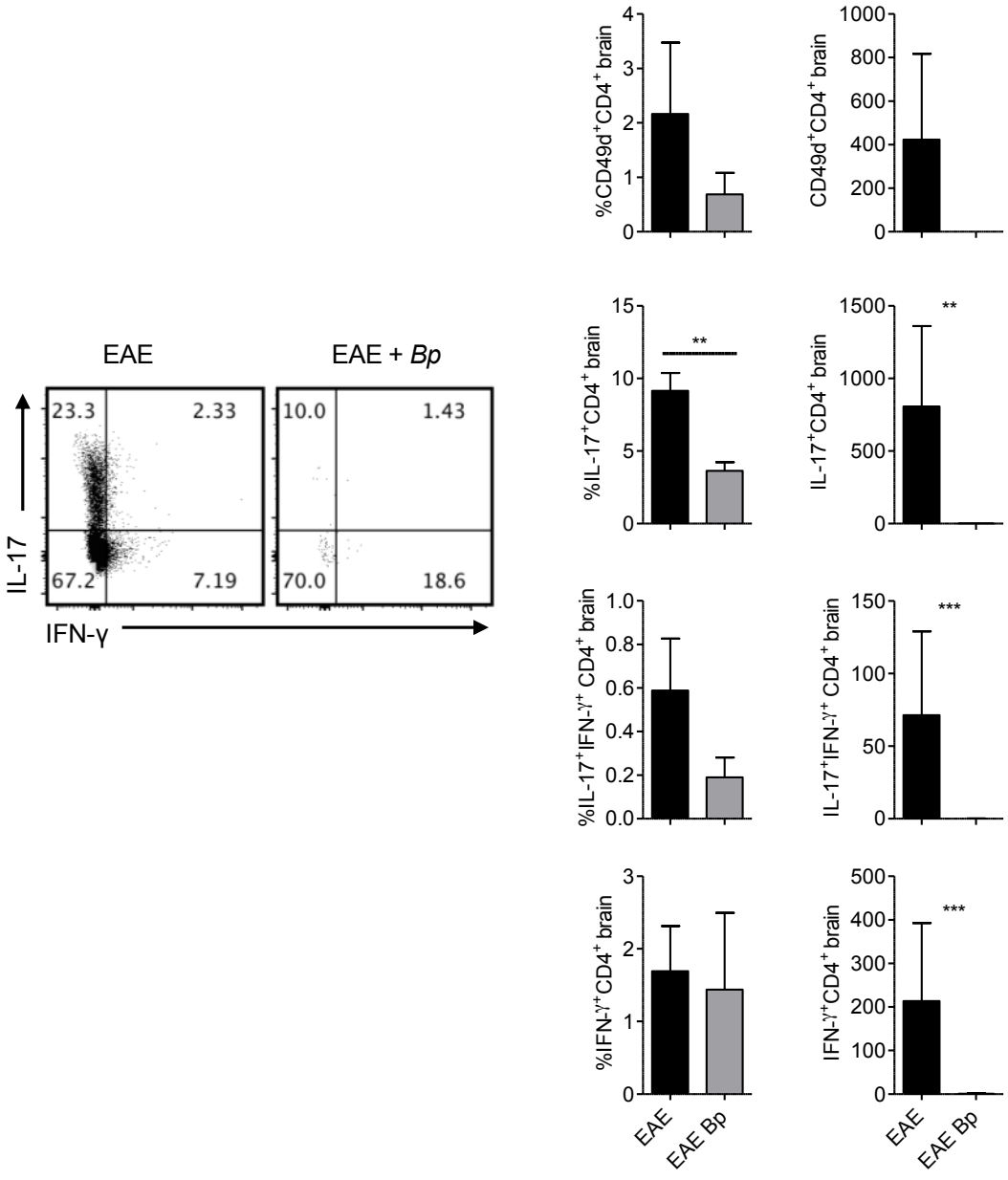


Figure 3.9: Infection of mice with *B. pertussis* significantly reduces the infiltration of effector T cells into the brain during passive EAE. Mononuclear cells were isolated from the brains on day 14 post transfer and re-stimulated for 5 hours with PMA + ionomycin and brefeldin A, and stained with anti-CD3, anti-CD4 and for intracellular IL-17 or IFN-γ and analysed by flow cytometry. Results are mean ± SEM of absolute numbers of cells for n=6 mice per group with representative dot plots (gated on CD4 T cells). Data are representative of two independent experiments. *P < 0.05, **P < 0.01 and ***P < 0.001 EAE Vs EAE + *B. pertussis* by two-way ANOVA and Bonferroni post hoc analysis.

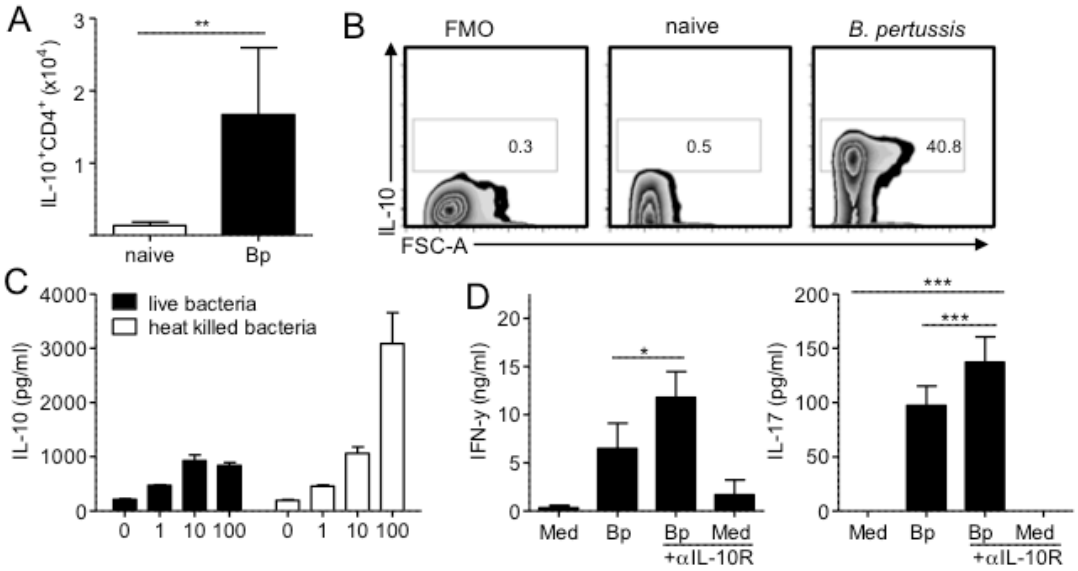


Figure 3.10: *B. pertussis* induces the production of IL-10 from T cells and DCs. Mice were infected with *B. pertussis* and spleens were isolated on day 14 from infected or from naive mice (A and B). Cells were stimulated for 5 hours with brefeldin A, and stained with anti-CD3, anti-CD4, and for intracellular IL-10 and analysed by flow cytometry. Results are mean ± SEM of absolute number of T cells from n=4 mice/group (A) with representative dot plots (B). $^{**}P < 0.01$ versus naive by unpaired student t-test. Data are representative of three independent experiments. (C) Bone marrow-derived murine DCs were stimulated with increasing concentrations of live or heat killed *B. pertussis* (1-100 x 10^6 CFU/ml or equivalent) for 24 hours. Supernatants were removed and IL-10 was quantified by ELISA. (D) Spleens were isolated from mice infected with *B. pertussis* 13 days post infection. Splenocytes were stimulated with heat killed *B. pertussis* ($1x10^6$ CFU/ml) or medium only, with/without anti-IL-10R blocking antibody (10 ng/ml) for 72h. Supernatants were removed and IFN-γ and IL-17A were quantified by ELISA. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ by unpaired student t-test.

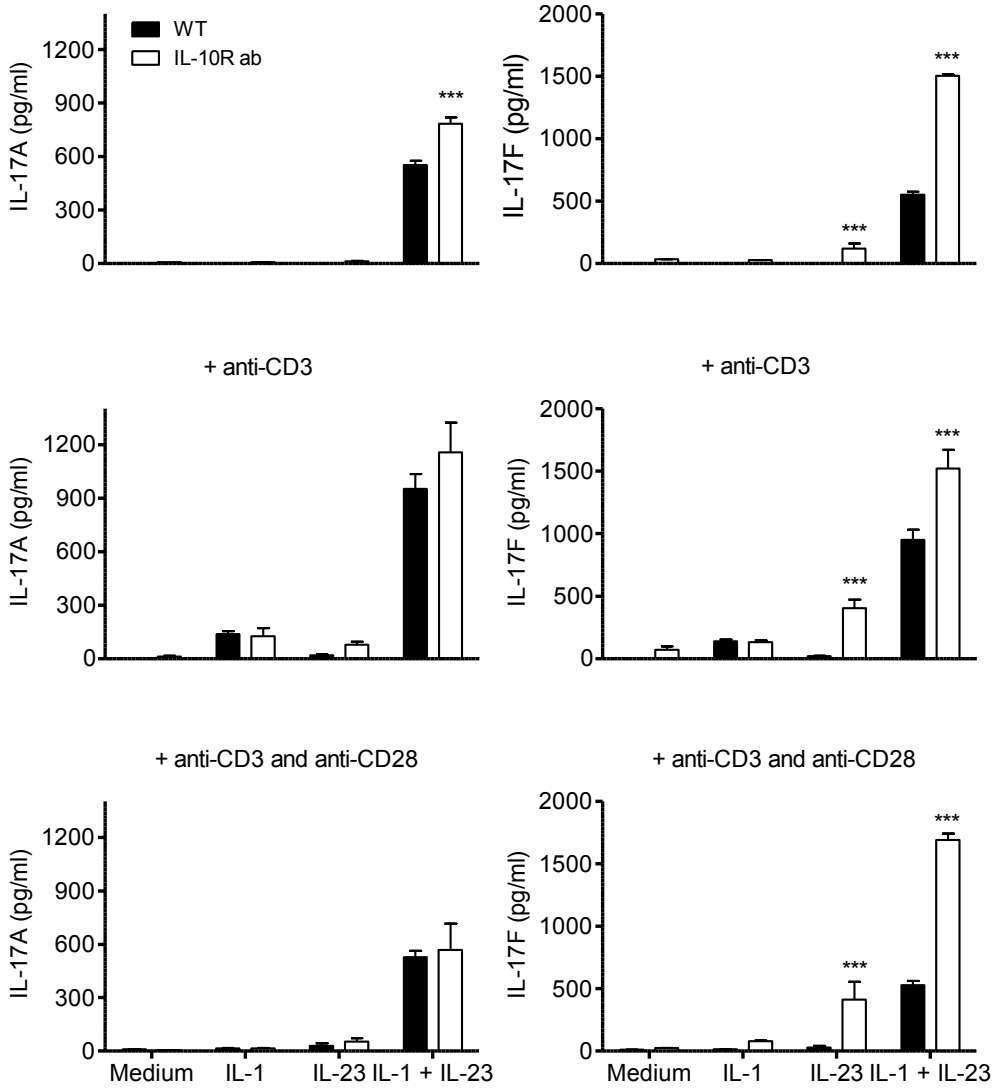


Figure 3.11: Neutralisation of IL-10 enhances IL-17A and IL-17F production by T cells. $CD3^+$ T cells were isolated by MACS purification from lymph nodes and spleens from naïve C57BL/6 mice. $CD3^+$ cells were stimulated with IL-1β (10 ng/ml), IL-23 (10 ng/ml) IL-1β with IL-23 and medium only. Cells were stimulated with and without IL-10R antibody (10 ng/ml). Cells were cultured with either medium, anti-CD3 (1 µg/ml) or anti-CD3 anti-CD28 (1µg/ml) for 72 hours. Supernatants were removed to quantify protein levels by ELISA. Results are expressed as mean concentration of triplicate wells and are representive of two-three experiments. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ by unpaired student t test.

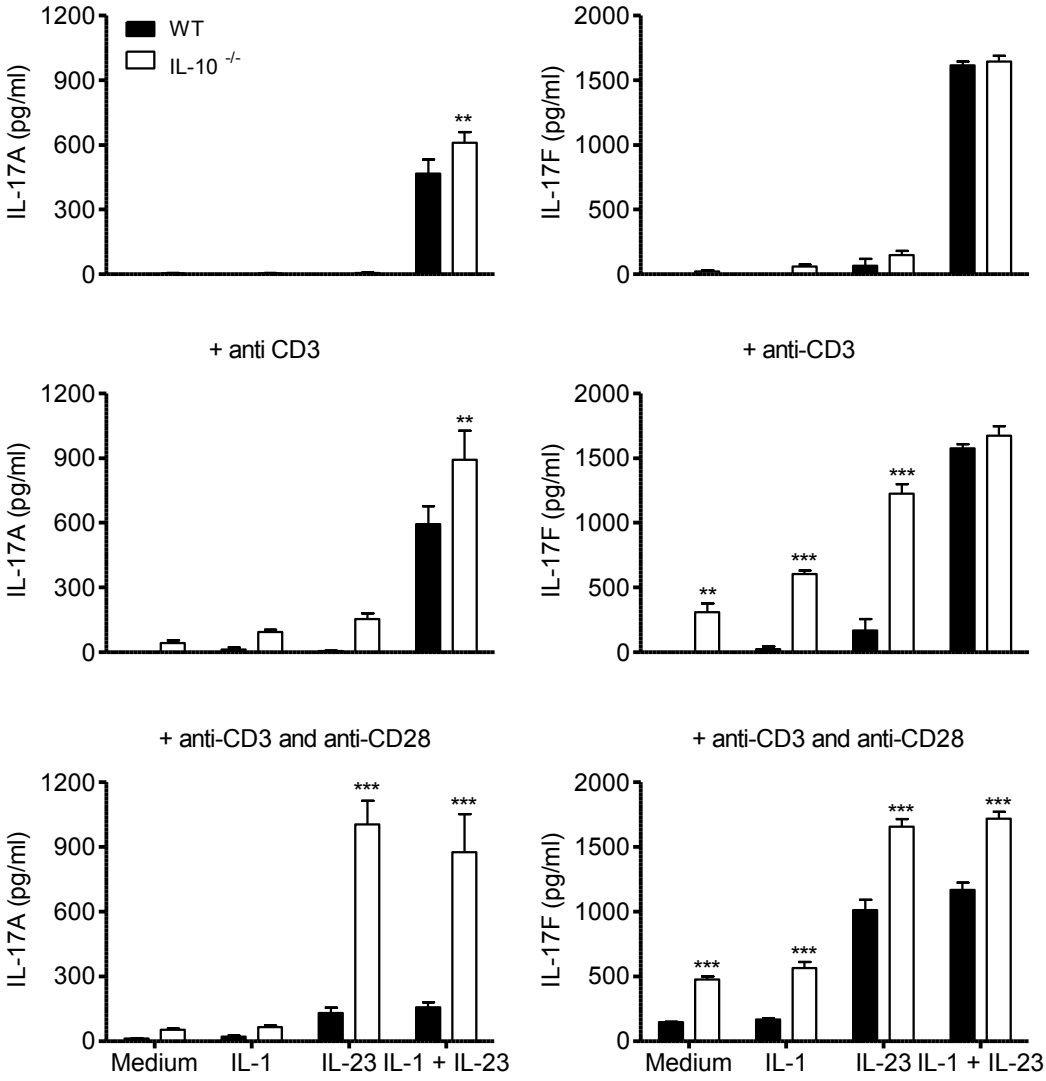


Figure 3.12: T cells from IL-10$^{-/-}$ mice cells produce significantly more IL-17A and IL-17F. CD3^{+} T cells were isolated by MACS purification from lymph nodes and spleens from naïve C57BL/6 or IL-10$^{-/-}$ mice. CD3^{+} cells were stimulated with IL-1β (10 ng/ml), IL-23 (10 ng/ml) IL-1β with IL-23 and medium only. Cells were cultured with medium, anti-CD3 (1 μg/ml) or anti-CD3 anti-CD28 (1μg/ml) for 72 hours. Supernatants were removed to quantify protein levels by ELISA. Results are expressed as mean concentration of triplicate wells and are representive of two-three experiments. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ by unpaired student t test.

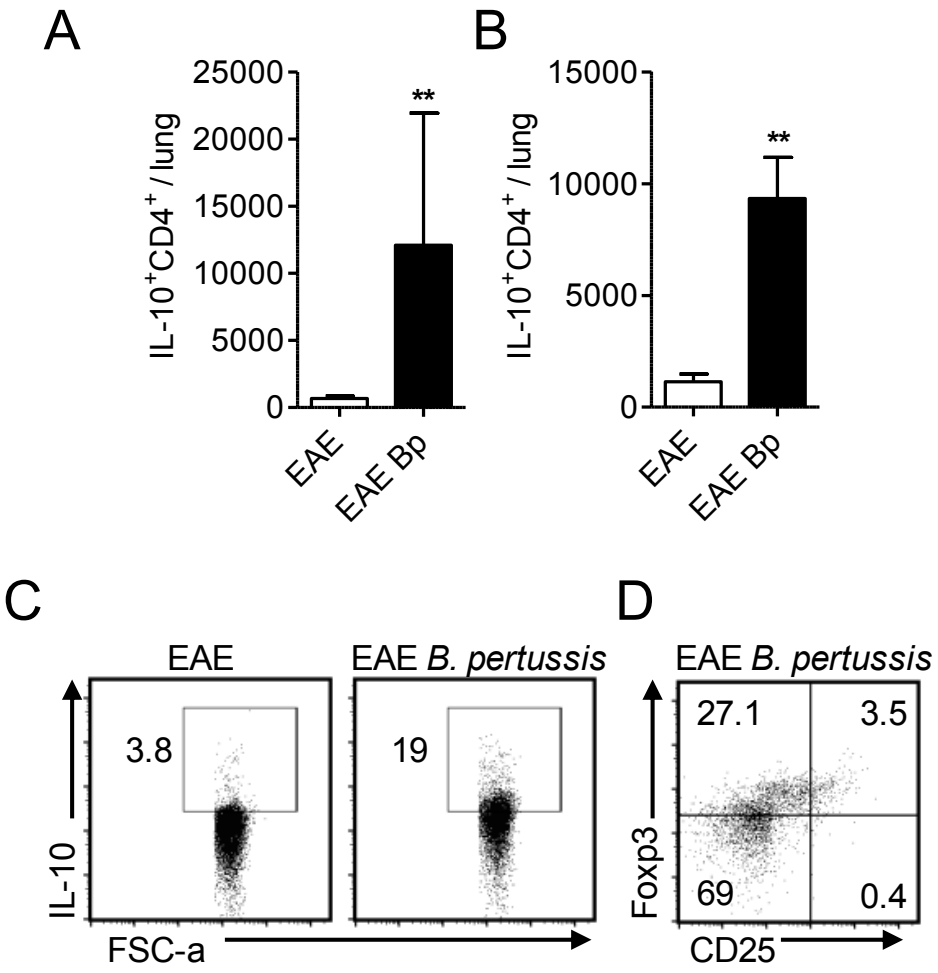


Figure 3.13: Infection with B. pertussis significantly increases IL-10 producing T cells in active and passive EAE. Mononuclear cells were isolated from the lungs of infected mice with EAE induced by passive (A) or active (B, C) immunization. Cells were stimulated for 5 hours with PMA + ionomycin and brefeldin A, and stained with anti-CD3, anti-CD4, anti-CD25 and for intracellular IL-10 and intranuclear Foxp3 and analysed by flow cytometry. Results are expressed as mean ± SEM absolute numbers of cells (A, B) from n=5 mice/group with representative dot plots for IL-10 production (C) and Foxp3 versus CD25 on IL-10^+ $CD4^+$ T cells (D). Data are representive of two independent experiments. $^{**}P < 0.01$ infected versus uninfected mice with EAE by unpaired student t-test.

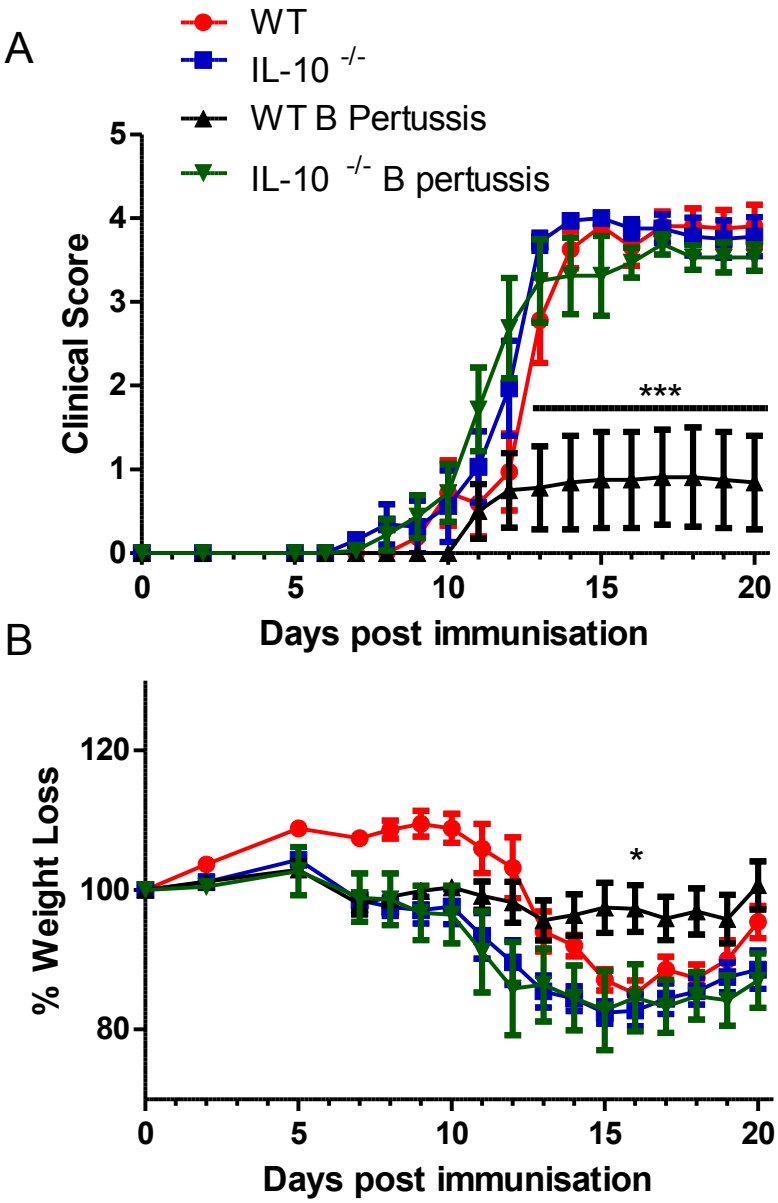


Figure 3.14: Attenuation of EAE by *B. pertussis* is reversed in IL-10$^{-/-}$ mice. C57BL/6 or IL-10$^{-/-}$ mice were aerosol infected with *B. pertussis* on day -1. EAE was induced in infected or uninfected mice on day 0 by s.c injection with 200 μg of $MOG_{35–55}$ in CFA. All mice were injected i.p with PT on days 0 and 2. (A) Clinical scores were assessed daily and (B) body weights were recorded.. *P < 0.05, **P < 0.01 and ***P < 0.001 EAE Vs EAE + *B. pertussis* by two-way ANOVA and Bonferroni post hoc analysis**.** Results are expressed as mean ± SEM clincal score and percentage weight loss for n=6 mice/group and are representive of two independent experiments. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ by two one-way *ANOVA* and Bonferroni post hoc analysis.

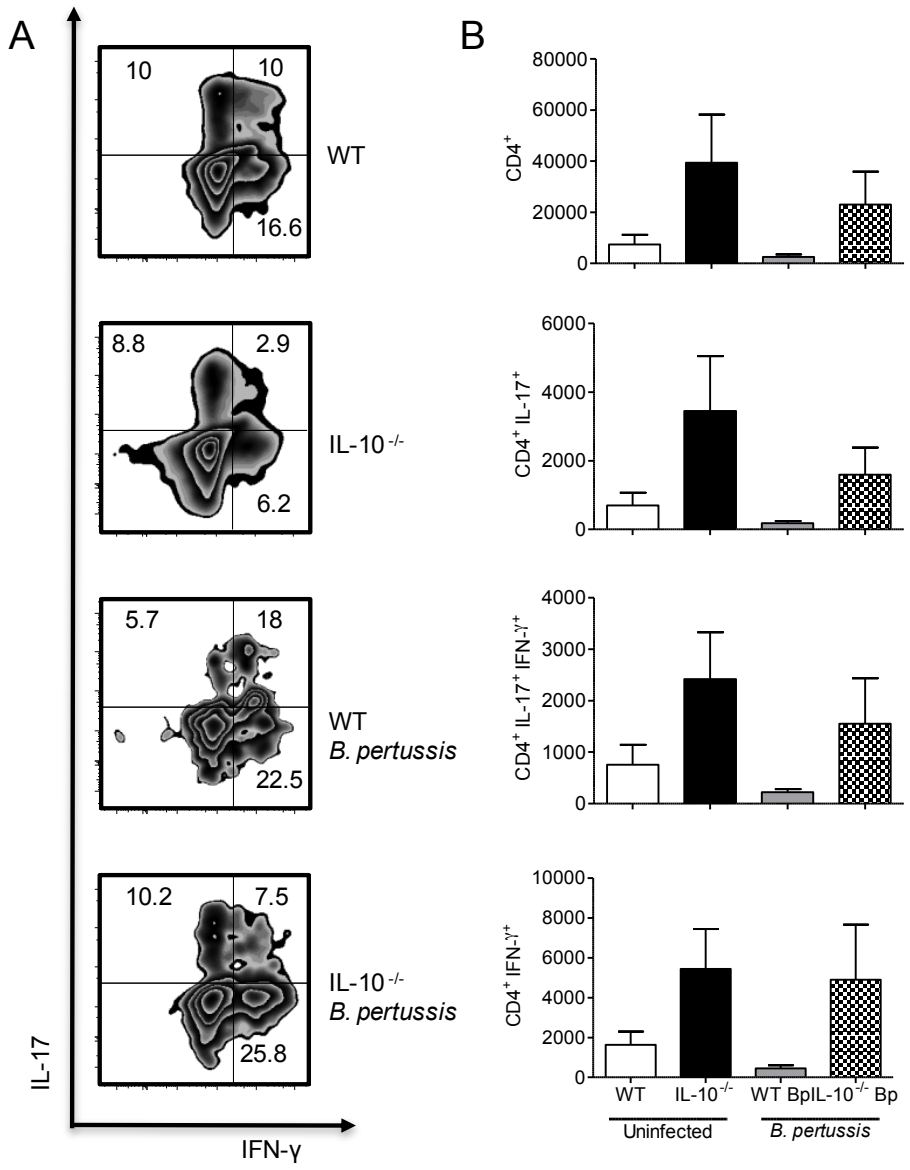


Figure 3.15: Reduction in the frequency of IL-17 and IFN-γ-producing CD4$^+$ T cells infiltrating the brain of *B. pertussis* infected C57BL/6 mice with EAE. C57BL/6 or IL-10$^{-/-}$ mice were aerosol infected with *B. pertussis* on day -1. EAE was induced in infected or uninfected mice on day 0 by s.c injection with 200 μg of $MOG_{35–55}$ in CFA. All mice were injected i.p with PT on days 0 and 2. Mononuclear cells from the brains were restimulated for 6 hours with PMA (10 ng/ml), ionomycin (1 μg/ml) and brefeldin A (5 μg/ml). Cells were washed and stained for surface CD4, CD3 and intracellular IL-17 and IFN-γ and analysed by flow cytometry. Results display representative FACS plots for IL-17 and IFN-γ–secreting CD4 T cells (A) with mean ± SEM for absolute numbers of IL-17$^+$ and IFN-γ$^+$ CD4 T cells in infected and uninfected mice with EAE (B). Results are representive of two independent experiments.

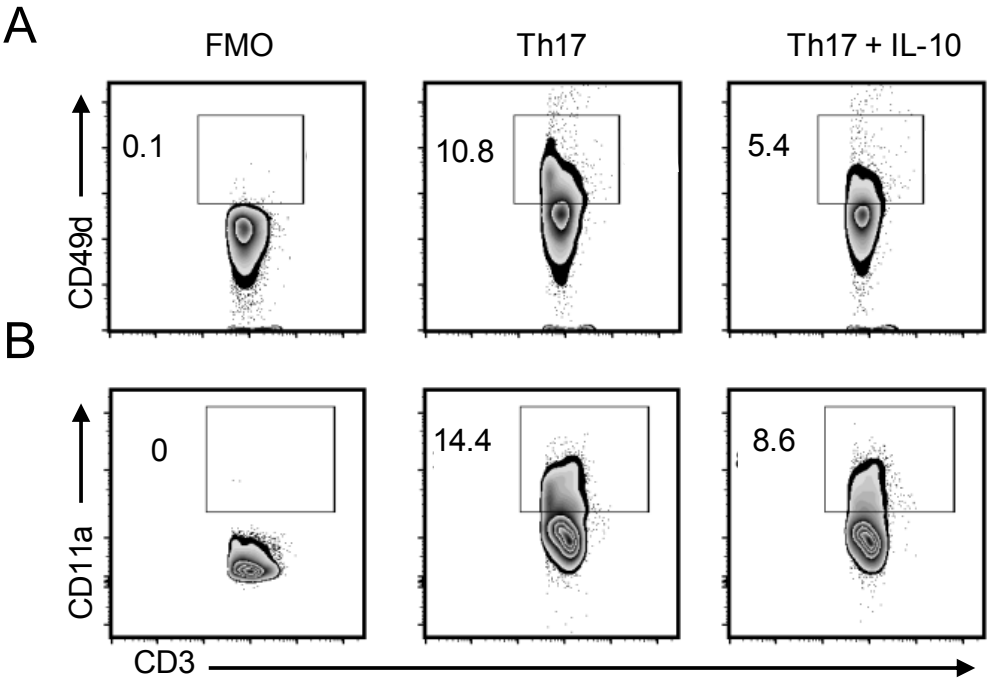


Figure 3.16: IL-10 downregulates the expression of VLA-4 and LFA-1 integrins on T cells. MOG-specific Th17 cells were expanded from mice immunized with MOG and CFA by restimulation ex vivo with MOG, IL-23 and IL-1β in the presence or absence of IL-10. After 72h, cells were stained with anti-CD3, CD4, VLA-4 (CD49d) and LFA-1 (CD11a) and analyzed by flow cytometry . Frequency of $CD4^+VLA\text{-}4^+$ cells in culture was determined by flow cytometry after gating on live single cells, CD4, and CD49d (A); frequency of $CD4^+LFA\text{-}1^+$ cells in culture was determined by flow cytomety after gating on live single cells, CD4 and CD11a (B). Representative FACS plots for n=3 mice/group from two independent experiments are shown.

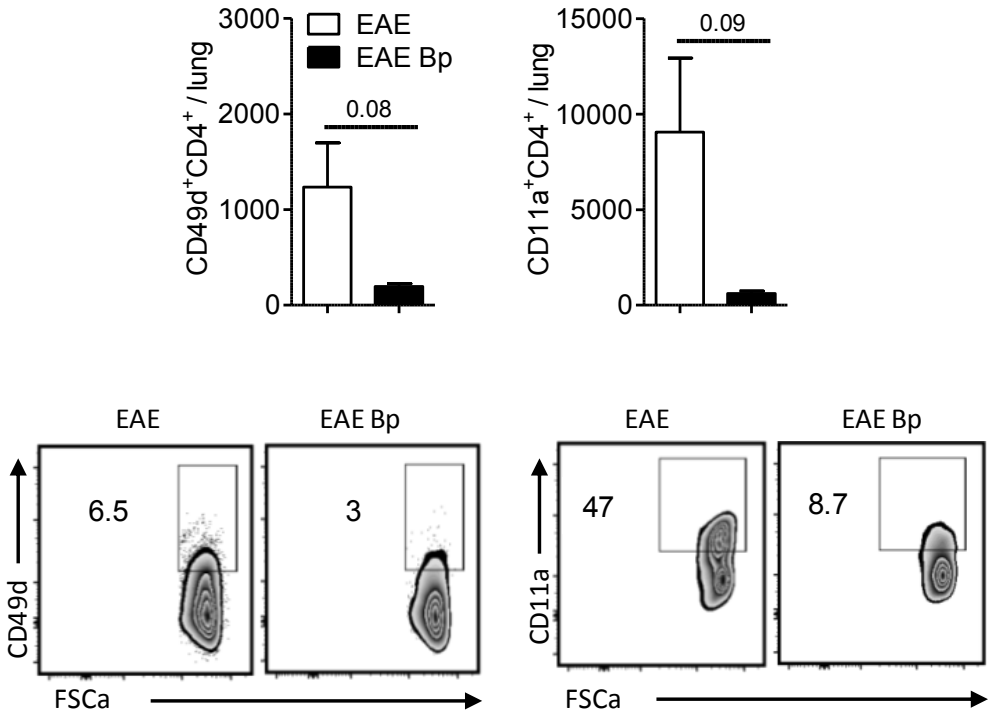


Figure 3.17: *B. pertussis* infection downregulates the expression of VLA-4 and LFA-1 integrins on T cells in the lung during EAE. Lymph node and spleen cells from MOG immunized mice and stimulated in vitro with MOG, IL-23 and IL-1β were transferred into naïve C57BL/6 recipient mice or mice infected with *B. pertussis* 1 day prior to transfer. 4 days post transfer, mononuclear cells were isolated from the lungs of infected mice and stained with anti-CD3, CD4, CD49d and CD11a. Frequency of $CD4^+VLA\text{-}4^+$ cells in culture was determined by flow cytometry after gating on live single cells, CD4, and CD49d, frequency of $CD4^+LFA\text{-}1^+$ cells in culture was determined by flow cytomety after gating on live single cells, CD4 and CD11a. Results are mean ± SEM of absolute numbers of cells for n=5 mice/group from two independent experiments. Non significant (p>0.08/0.09) according to unpaired student t-test (A) with with representative FACS plots (B).

Chapter 4.

αβVγ4 T cells, a novel IL-17 producing T cell subset

4.1 Introduction

Conventional MHC-restricted $CD4^+$ and $CD8^+$ T cells mediate adaptive immunity to pathogens and express αβ TCRs. A minor population of T cells with a distinct TCR comprised of γ and δ chains, called γδ T cells, play an important role in innate immunity at mucosal surfaces but can also display immunological memory, analogous to conventional αβ T cells (164, 165, 356). γδ T cells act as a key bridge between the innate and adaptive immune systems. They are the first lymphocytes present in the foetus and they play a role in protective immunity in young animals preceding maturation of the adaptive immune system (357). γδ T cells are key sentinel cells at mucosal and epithelial sites, such as the skin, reproductive tract and the gut, where they can account for up to half of the total intraepithelial lymphocyte population (104). γδ T cells are polyfunctional in their capability to produce an array of cytokines, including IL-17A, IL-17F, IFN-γ, IL-22, IL-21, IL-4, GM-CSF and TNF-α (107, 108, 129, 165, 277). IL-17-producing γδ T cells share many functional features with $CD4^+$ Th17 cells, including expression of IL-1R, IL-23R, RORγt and CCR6 (112). Although γδ T cells express a unique TCR, engagement of this TCR with MHC-antigen complexes is not a prerequisite for their activation (116). Unlike αβ T cells, γδ T cells respond to cytokine stimulation alone. This makes them potent early inducers of inflammation in infection and autoimmunity, such as in EAE, where they can amplify effector helper T cells responses (97, 116, 358).

The primary role of γδ T cells is to provide rapid effector responses against infection (359). Their rapid, innate-like activation allows them to carry out this function. IL-17 is important cytokine for the activation and recruitment of innate immune cells such as neutrophils (152). In particular, IL-17-producing $V\gamma4^+$ T cells in mice, according to the nomenclature of Heilig and Tonegawa (186), are thought to be key mediators of inflammation in murine models of inflammation such as Con A-induced fulminant hepatitis and in *Listeria monocytogenes* and *Candida albicans* infection (97, 126, 356). Vγ4 T cells can also prevent mortality during severe sepsis through IL-17 production in inflamed lungs (360). However, these IL-17-producing $V\gamma4^+$ T cells can also have negative effects in infection and autoimmunity. Depletion of Vγ4 T cells during infection with West Nile virus resulted in decreased viremia and mortality to WNV encephalitis; conversely, IFN-γ-producing $V\gamma1^+$ T cells play a key role in viral clearance (361).

IL-17 producing γδ T cells have also been implicated in enhancing tumour growth. In an hepatocellular carcinoma model, adoptive transfer of IL-17-producing Vγ4 T cells promoted tumour growth and their depletion resulted in a reduction in tumor burden (362). Furthermore, in response to peritoneal tumour challenge, IL-17 producing Vγ6 T cells accumulated in the peritoneal cavity where they promoted ovarian tumour growth through IL-17-mediated mobilization of small peritoneal macrophages (363). Vγ4 T cells have also been heavily implicated in the pathology of autoimmune models of disease such as EAE, CIA and psoriasis (116, 166, 274, 275, 292). Given the complex roles of IL-17-producing γδ T cells in immunity to infection and tumours and to pathology of autoimmunity, an examination of the development of these innate-like lymphocytes may facilitate a better understanding of the pathogenesis of a range of diseases.

It has been assumed that these $V\gamma4^+$ T cells exclusively express TCRδ chains. However, populations of unconventional T cells that express TCRβ with TCRα and TCRδ segments (δ/αβ cells) (197), both αβ and γδ TCRs (198) or βδ TCR heterodimers (103) have been described. This study investigated the expression of αβ and γδ TCRs by T cell subsets and the functionality of such cells. Two models had been proposed in an attempt to explain and understand αβ or γδ TCR development and T cell lineage commitment. The instructive model argued that TCR expression is the prerequisite for lineage commitment whereas the stochastic model proposed that the lineage fate is decided prior to TCR expression, with the TCR playing a secondary role. (168, 179-182). However, neither of these models could fully explain the inconsistancies observed in T cell lineage commitment. A seminal study by Hayes et all demonstrated that TCR signal alone is the critical factor which dictated T cell lineage fate and this gave rise to the signal strength model. The study demonstrated that increasing the γδTCR signal strength supported γδ lineage development, conversely weakening this γδTCR signal supported αβ lineage development (178). This study describes a novel IL-17 secreting T cell subset, αβγ T cells, which can be activated in the absence of TCR engagement. This novel T cell subset is distinct from conventional αβ or γδ T cells and co-expresses Vγ4, TCRα and TCRβ, but not TCRδ. The specific focus of this study is centred on understanding the function of conventional γδ T cells expressing Vγ4 and a novel T cell population that express αβTCRs and Vγ4.

4.2 Results

4.2.1 Vγ4 T cells produce IL-17 in response to stimulation with IL-1β and IL-23.

γδ T cells are potent producers of inflammatory cytokines, including IL-17 (24). Previous studies have shown that γδ T cells can be activated by cytokine stimulation alone, producing IL-17, IL-21 and IL-22 in response to IL-1β and IL-23 in the absence of TCR engagement (116). Studies in psoriasis have demonstrated that Vγ4δ$^+$ T cells are the main IL-17-producing cell in the dermis (274). In the EAE model, Vγ4δ T cells were identified as the main IL-17-producing γδ T cell subset in the brain (116). To examine whether Vγ4$^+$ T cells were capable of producing IL-17 in the absence of any other γδ T cells, CD3$^+$Vγ4$^+$ T cells were FACS purified from naïve C57BL/6 mice and stimulated with medium alone, IL-1β, IL-23, or a combination of both for 72 hours; the gating strategy employed is described in 2.1 and 4.1. The results demonstrated that Vγ4$^+$ T cells produce significant amounts of IL-17A and IL-17F in response to stimulation with IL-1 and IL-23 compared with cells cultured with medium only (Figure 4.2). Interestingly, stimulation with IL-23 alone also induced production of IL-17A and IL-17F by purified Vγ4$^+$ T cells.

It has previously been established that the IL-17-producing CD4$^+$ T cell subset, Th17 cells, signal through STAT3 to produce an array of inflammatory cytokines (68, 364). Sutton et al demonstrated that IL-17 producing γδ T cells express the master transcription factor RORγt, which is dependent on STAT3 activation (68, 116). The present study demonstrated that Vγ4 T cells are also potent producers of IL-17, however, the signalling cascade involved in their activation and subsequent cytokine secretion was not determined. To investigate this, Vγ4 T cells were FACS purified from the lymph nodes of naïve C57BL/6 mice and cultured with IL-1β, IL-23, a combination of both (10 ng/ml) or medium only for 24 hours. Stimulation of Vγ4 T cells with IL-1β and IL-23 induced a 30 fold increase in STAT3 mRNA expression over cells cultured with medium only (Figure 4.3A). Stimulation with IL-1β or IL-23 alone also induce STAT3 but not to the same extent as so-stimulation with IL-1β and IL-23. To investigate the impact of STAT3 signalling on pro-inflammatory cytokine secretion, Vγ4 T cells were stimulated with IL-1β, IL-23 (10ng/ml) or a combination of both in the presence of a selective STAT3 inhibitor, cucurbitican I. Stimulation of Vγ4 T cells with IL-23 or IL-23 and IL-1β induced IL-17 mRNA expression after 24 hours and this was inhibited by

cucurbitican I, suggesting that IL-17 expression was STAT3 dependent (Figure 4.3B). IFN-γ mRNA expression in response to IL-1β and IL-23 was partially reduced by STAT3 inhibition (Figure 4.3B). This suggests that IFN-γ production by Vγ4 T cells may require other signalling molecules, such as STAT1 (365). IL-1β stimulation induced comparable levels of IFN-γ mRNA expression to IL-1 and IL-23, and this was reversed by STAT3 inhibition (Figure 4.3B).

Deficiency of STAT3 has been shown to greatly decrease the expression of RORγt and RORα, transcription factors necessary for Th17 development and IL-17 production (68, 366). IL-1β or IL-23 alone failed to drive IL-17 secretion by Vγ4 T cells (Figure 4.3C). However, IL-1β alone induced a small increase in IFN-γ production (Figure 4.3C). The combination of IL-1β and IL-23 induced significant production of IL-17A and IFN-γ from $V\gamma4^+$ T cells, which was significantly reduced by inhibition of STAT3 (Figure 4.3C). This demonstrates that IL-1β and IL-23 induced IL-17 and IFN-γ production by Vγ4 T cells is controlled by STAT3 signalling (Figure 4.3C).

4.2.2 Identification of TCRβ expressing $V\gamma4^+$ T cells.

Two T cell lineages, αβ and γδ, are generated in the thymus. DP progenitor cells of the αβ lineage have been shown to regulate the development of γδ T cells (367). In humans, it has been suggested that the γδ lineage is the default fate for T cell development; if thymocyes are unable to produce a functional γδ TCR, they can then generate αβ TCR chains (368). During T cell development it has been assumed that $V\gamma4^+$ cells exclusively express TCRδ chains. However, approximately 10-20% of γδ T cells express cytoplasmic TCRβ proteins (201). Remarkably, in WT mice, surface expression of TCRβ chains on $V\gamma4^+$ cells were detected at mucosal sites, including the gut, thymus, liver and lung, as well as in peripheral lymphoid organs, including the spleen and the inguinal and mesenteric lymph nodes (Figure 4.4). These TCRβ-expressing cells represent approximately 10% of all $V\gamma4^+$ T cells. γδ T cells accounting for 50% of the total IELs in the gut (104), and the TCRβ expressing Vγ4 T cells account for around 4% of the total Vγ4 cells analysed in the gut and 3% in the lung (Figure 4.4). Interestingly, despite a high proportion of Vγ4 T cells found in the dermis of psoriatic lesions in mice (274), an examination of the dermis or epidermis of healthy skin from WT mice failed to reveal the presence of TCRβ expressing Vγ4 T cells (Figure 4.5). Vγ4-expressing γδ T cells

were not unique in their ability to express the TCRβ receptor, as the Vγ1 subset also expressed surface TCRβ chains (Figure 4.6).

Previous studies have demonstrated that αβ and γδ T cells are derived from a common precursor cell. γδ T cells have been shown to express cytoplasmic TCRβ expression (201). Furthermore, TCRδ recombining with αβTCR chains has been described (197, 200). The dual expression of surface TCRβ and TCRVγ4 was confirmed by confocal microscopy. $CD3^+$ $V\gamma4^+$ T cells were purified by FACS and analysed for surface TCRβ (Figure 4.7). Using overlay imagery, Vγ4 and TCRβ chains were found to co-localise on the surface of a small percentage of the total Vγ4 T cells (Figure 4.7A), confirming the presence of novel TCRβ expressing $V\gamma4^+$ T cells. $V\gamma4^+TCR\beta^-$ cells represent the classical Vγ4δ T cells (Figure 4.7B and C). Ontogeny studies performed on thymic cells isolated on embryonic day 14, 16 or 18 of gestation revelated that these Vγ4β cells arise in the thymus between embryonic day 14 and 16 along with conventional TCRδ-expressing $V\gamma4^+$ cells (Figure 4.8). Interestingly, FACS analysis revealed that TCRβ-expressing $V\gamma4^+$ cells are largely CD4 and CD8α double positive during thymic development despite previous studies demonstrating that TCRγ rearrangement ceases with early expression of the TCRβ chain transgene (Figure. 4.8) (170).

4.2.3 TCRβ expressing $V\gamma4^+$ T cells are present in WT and $TCR\delta^{-/-}$ mice.

$TCR\delta^{-/-}$ mice lack conventional γδ T cells, however, it is unclear whether T cells can express the TCRγ chain in the absence of TCRδ (369). The dogma of TCR development suggests that the development of αβ and γδ T cells is not an interconvertible process, with γ and δ TCR encoding genes rearranging before the genes encoding αβ TCR, as T cells expressing γδ-CD3 complex emerge from the murine thymus days before αβ-CD3 complex expressing cells (370, 371). Flow cytometry was used to investigate if $TCR\beta^+$ T cells expressing Vγ4 were present in $TCR\delta^{-/-}$ mice. The data demonstrates that TCRβ-expressing Vγ4 T cells are found in both WT and $TCR\delta^{-/-}$ mice (Figure 4.9). TCRβ expressing Vγ4 T cells account for all of the Vγ4 T cells detected in the lymph nodes and spleens of $TCR\delta^{-/-}$ mice (Figure 4.9).

Populations of T cells expressing βδ TCR heterodimers (103), TCRβ with TCRα and TCRδ segments (δ/αβ cells)(197) or both αβ and γδ TCRs(198) have been described previously. The data presented here demonstrated that Vγ4β cells are distinct from γδ T cells and are found in

WT and TCRδ$^{-/-}$ mice. FACS analysis reveals these cells are absent in TCRβ$^{-/-}$ mice (Figure 4.10). Remarkably, these cells were also absent in TCRα$^{-/-}$ mice (Figure 4.10), suggesting that T cells can express TCRα, β and γ without TCRδ chains. Interestingly, dysfunctional development of TCRγ expressing T cells has been reported in TCRβ$^{-/-}$ mice, which may be accounted for by the absence of αβγ T cells in these mice (367). Neither MHC class I nor MHC class II were required for αβγ T cell development and an accumulation of αβVγ4 T cells was observed in MHC class II$^{-/-}$ mice (Figure 4.10).

4.2.4 Molecular and phenotypic characterisation of αβγ T cells

Expression of αβγ TCRs was confirmed in purified Vγ4^{+} T cells at the molecular level by analysis of TRAV, TRBV, TRGV genes and revealed a restricted Vγ repertoire with more heterogenous Vα and Vβ usage (Figure 4.11). αβVγ4 T cells were FACS purified from four individual mice on the basis of TCRVγ4 and TCRβ expression. Reverse transcription was performed from RNA isolated from the four cell pools in order to examine TCR usage in the individual mice (HUK 1-4). Repertoire analysis revealed highly skewed expression of *TRG* gene transcripts integrating exclusive TRGV4/TRGJ1 rearrangements. Usage of TRGV2 was also apparent, solely paired with TRGJ2, suggesting conformational homology with Vγ4 at the anti-Vγ4 recognition site (Figure 4.11A). Markedly, TRGJ1 and TRGJ2 contribute an identical germline-encoded SSGFHKVF motif to the third complementarity-determining region (CDR3). The *TRA* and *TRB* gene expression profiles were more heterogeneous with no evident bias towards particular TRAV or TRBV segments (Figure 4.11B,C). These data indicate that the heterogeneous *TRA* and *TRB* gene expression by αβVγ4 T cells is not resemblant of other uncoventional T cell populations, such as invariant CD1d restricted NKT cells (372). However, the dominant TRB-defined clonotypes in two mice (HUK1 and HUK2) had some resemblances (TRBV3/CASSLGHWGDEQYF/TRBJ2-7 and TRBV3/CASSLLGGHEQYF/TRBJ2-7, respectively).

To fulfil the expression of a functional αβTCR, classical CD4^{+} and CD8^{+} T cells must express a complex of constant TCRα and TCRβ segments bound to variable (TCRVα and TCRVβ) segments. Consistent with the TCR sequencing data, flow cytometric analysis demonstrated that CD4^{+} T cells express many TCRVα chains including Vα2 (TRAV14), Vα3.2 (TRAV9), Vα8.3 (TAV12) and Vα11 (TRAV4), from herein termed αβVγ4 T cells. (Figure 4.12). An

examination of Vα chain expression on αβVγ4 T cells revealed dominant expression of Vα2 and Vα11 (Figure 4.12A). Notably, expression levels of TCRVα on the surface of the TCRβ expressing Vγ4 T cell population were similar to those of conventional αβ $CD4^+$ T cells indicating that this population may more representative of αβ T cell expressing TCRγ (Figure 4.12B).

4.2.5 αβVγ4 T cells express markers of the natural Th17 lineage

Natural Th17 cells develop independently of conventional Th17 cells, emerging from the thymus to rapidly produce IL-17 upon exposure to infection (94, 95, 97) and during the development of psoriasis in mice (99). nTh17 cells develop independently of IL-6 and STAT3 and are characterized by the constitutive expression of $TCR\beta^{lo}$, CCR6, CD44, IL-23R, RORγt and VLA4/α4β1 (CD49d/CD29) (94, 95, 98, 99). In a transgenic TCR system, self-antigen reactive nTh17 cells were shown to express α4β1 and migrate to enrich the gut, lung, and liver (95), areas that αβVγ4 T cells populate (Figure 4.4). FACS analysis demonstrated that αβVγ4 T cells expressed CD49d, a subunit of the cell adhesion molecule α4β1, very late antigen-4 (VLA-4), which is involved in the migration of encephalitogenic T cells into the CNS (103). αβVγ4 T cells constitutively expressed CCR6 (Figure 4.13), a chemokine receptor characteristic of pathogenic Th17 cells during EAE (270, 344). αβVγ4 T cells constitutively expressed CD44, ICOS, CCR6 and CD49d, an expression profile associated with natural Th17 cells. Expression of these markers was not augmented by stimulation with IL-1β and IL-23 (Figure 4.13).

Like conventional γδ T cells (116), αβVγ4 T cells express IL-23R, as well as high levels of IL-1RI (Figure 4.14). Phenotypically, αβVγ4 T cells are approximately 50% $CD27^+$ and 50% $CD27^-$ (Fig. 4.15A). CD27 is a marker used to distinguish the cytokine secreting fate of γδ T cells. $CD27^+$ γδ T cells produce IFN-γ in response to antigenic stimulation, whereas $CD27^-$ γδ T cells produce IL-17 independent of TCR activation (108, 120, 165). αβVγ4 T cells highly express CD73 (Figure 4.15B), a marker of commitment to the innate-like γδ T cell linage. Over 90% of peripheral γδ T cells express CD73 (196). Expression of CD73 is lower on αβ lineage CD4 and CD8 T cells, Th17 cells, and Treg cells (196, 373-375) and flow cytometric analysis revealed that approximately all of αβVγ4 T cells express CD73 (Figure 4.15B).

4.2.6 αβVγ4 T cells display functional characteristics of innate and adaptive immunity

The phenotype of αβVγ4 T cells shares features that are characteristic of innate γδ T cells and nTh17 cells. As γδ T cells can be activated in the absence of TCR stimulation (116, 172), experiments were designed to examine if αβVγ4 T cells could be activated by stimulation with IL-1β and IL-23. αβVγ4 T cells purified from WT mice produced significant amounts of IL-17A and IL-22 in response to stimulation with IL-1β and IL-23 in the absence of TCR engagement (Figure 4.16A). Interestingly, nTh17 cells produce IL-17A and IL-22 rapidly, however they fail to produce IL-17F (95). A similar trend was observed in purified αβVγ4 T cells which unlike $Vγ4δ^+$ T cells, produced minimal IL-17F but produced significant amounts of IL-17A and IL-22 in response to stimulation with IL-1β and IL-23 in the absence of TCR engagement (Figure 4.16A and 4.16B), albeit at lower concentrations to that produced by Vγ4δ T cells (Figure 4.16B). FACS purified $Vγ4^+$ T cells isolated from $TCRδ^{-/-}$ mice produced significant amounts of IL-17A, but not IL-17F in response to stimulation with IL-1β and IL-23 (Figure 4.16C).

The data demonstrates that like conventional γδ T cells (116), αβVγ4 T cells can respond to stimulation with IL-1β and IL-23 without TCR engagement. Since αβVγ4 T cells express lineage markers of nTh17 cells, it is possible that they may also respond to self antigen like nTh17 cells (95). Purified αβVγ4 T cells, Vγ4δ T cells and CD4 T cells were isolated from spleens of mice immunised with MOG and CFA and were restimulated with IL-1β and IL-23, MOG or combination in the presence of irradiated APCs. The data demonstrate that αβVγ4 T cells purified from MOG-immunised mice produce IL-17A and IL-22 in response to IL-1 and IL-23 (Figure 4.17). The results reveal a trend towards significance ($p>0.07$) in IL-17A production by $αβVγ4^+$ T cells in response to stimulation MOG with IL-1 and IL-23 compared with IL-1 and IL-23, suggesting that cytokine production by αβVγ4 T cells may be augmented through TCR engagement (Figure 4.17). Further evidence for this assertion that these cells may possess a functional TCR was provided by the observation that proliferation of αβVγ4 T cells from mice with EAE was modestly augmented in response to stimulation with MOG or IL-1β and IL-23 (Fig. 4.18). These data provide preliminary evidence that αβVγ4 T cells may respond via the TCR to antigen, similar to nTh17 cells and $CD27^+CD45RB^{hi}$ γδ T cells (376). These findings suggest that αβVγ4 T cells display functional properties associated with innate-like γδ T cells and nTh17 cells.

4.3 Discussion

The significant findings from the current study are the identification and characterisation of a novel T cell subtype, αβVγ4 T cells. The results revealed that Vγ4 T cells expressed TCRβ chain in place of TCRδ chain in a variety of tissues. TCRβ expressing Vγ4 T cells were absent in TCRβ$^{-/-}$ and in TCRα$^{-/-}$ mice, but notably present in both WT and TCRδ$^{-/-}$ mice, demonstrating that TCRδ is not required for their development. The data revealed that αβVγ4 T cells share phenotypic features of nTh17 and γδ T cells, such as expression of CD49d, ICOS, CCR6 and CD73. Importantly, this novel subset of T cells responded to stimulation with IL-1β and IL-23 in the absence of TCR engagement, but could proliferated when restimulated with the autoantigen MOG. This innate cytokine secretion and TCR specificity suggest that αβVγ4 T cells may play a unique role as key sentinels that sit at the interface of innate and adaptive immunity.

Populations of unconventional T cells that express αβ and γδ TCRs or βδ TCR heterodimers have previously been described (103, 198). Recently, NKT-like cells which express TCRβ with TCRα and TCRδ segments have been identified in humans (197). This novel cell type, termed δ/αβ T cells can recognize peptide and lipid-based antigens presented by HLA and CD1d, respectively. These cells represent 50% of all Vδ1^{+} human T cells (197). The current study in mice demonstrates the existence of a T cell subtype that expresses TCRαβ and Vγ4. Sequencing analysis on FACS purified αβVγ4 revealed the TR genes encoding TCRVα, β and γ, confirming the expression of these TCR chains at a molecular level and validating the phenotypic observations in the TCRδ$^{-/-}$, TCRα$^{-/-}$ and TCRβ$^{-/-}$ mice. The clonotyping analysis of αβVγ4 T cells revealed that TRV usage is restricted to two genes, while the expression profile for TRA and TRB gene were more heterogeneous and did not show bias for any particular set of genes encoding TCRα or β chains.

It is unclear how the mechanism of T cell lineage commitment can permit development of rare TCR heterodimers. An important report by the Brenner group in 1989 described a mechanism by which the deletion of the TCRδ loci can act as an exclusion mechanism in the thymic maturation of T cells. The deletion of the TCRδ loci prevents the formation of γδ TCR in place of αβ TCR receptors, but also serves to inhibit the development of βδ TCRs (103). However, Brenner and others have shown rare TCR chain associations can occur; TCRδ can substitute for TCRα to form a heterodimer with TCRβ (103). Similarly, Vδ can fuse with Jα or Cα segments, to pair with a variety of TCRβ chains (197). Additionally, TCRβ

rearrangements have been observed in γδ T cells (377). Thymocytes committed to the αβ lineage also display evidence of TCRγ and TCRδ gene rearrangements despite developing into functional αβ T cells (378, 379). Moreover, genes encoding the TCRα, β and γ chains were detected in T cell and pre-B cells isolated from peripheral blood of patients with human acute lymphoblastic leukaemias (ALL) (380). These reports of unconventional TCR development are in agreement with the findings of the present study demonstrating the expression of the TCRαβVγ4 chains expressed on the surface of a small population of murine T cells.

γδ T cells have previously been shown to express cytoplasmic TCRβ proteins (199, 200). Remarkably, cytoplasmic TCRβ chains isolated from γδ T cells can form functional αβTCR expressing T cells (201). Other reports suggest that early expression of TCRβ chains prevents γδ T cell development or represses TCRγ gene rearrangement (167, 170). The earliest precursor T cells are the $CD4^{-}CD8^{-}$DN thymocytes which develop into DP or γδ T cells (381). These DN thymocytes can be further subdivided into DN1–4 based on CD25 and CD44 expression; DN1 ($CD44^{+}CD25^{-}$), DN2 ($CD44^{+}$ $CD25^{+}$), DN3 ($CD44^{-}$ $CD25^{+}$), and DN4 ($CD44^{-}CD25^{-}$) (382). Using single cell progenitor analyses, the DN3 stage has been identified as the decision point of T cell lineage commitment, and it is also the final stage at which a DN cell can commit to αβ or γδ lineage (383, 384). Although the data presented in the embryonic thymus would suggest these cells are $CD4^{+}CD8^{+}$DP, this is at odds with the presence of TCRVγ4 on the cell surface, as the DP state of development switches off rearrangement of TCRγ (170, 381). However, this is not the first report of TCRγ expressing T cells expressing CD8 on their surface (385). It has also been previously demonstrated that αβ TCR can replace the γδ TCR in the development of γδ lineage cells and here, the expression of CD73, TCRαβ with TCRVγ4 or TCRVγ1 by this unique population indicates that it may develop from the DN stage as a result of early αβ TCR expression cell to combine with TCRγ (184).

An important study by Zarin et al concluded that if DN3 T cells were transgenetically modified to express TCRβ, γ and δ these cells would commit to the γδ lineage. These cells downregulated CD24 expression, upregulated CD73 expression and adopted a γδ T cell associated gene profile to produce IFN-γ (171). The absence of strong TCR engagement resulted in downregulation of IFN-γ and CD73 and the cells preferentially committed to the αβ lineage. Similarly, the present study demonstrates that T cells cells which concurrently express TCRβ with a TCRγ choose the γδ T-cell fate. The data demonstrates that αβVγ4 T

cells express high levels of CD73 and produce IL-17 in an innate-like fashion in response to IL-1β and IL-23. CD73 has been described as a ligand inducible marker of commitment to the γδ T lineage (196). The signal strength model has established that a strong ligand induced TCRγδ signal drives γδ T cell development (386). The use of KN6 TCR transgenic thymocytes, which adopt a γδ T cell fate in the presence of the T-10/22 ligand identified CD73 cell as a ligand induced molecular marker of γδ lineage commitment (196). CD73 is expressed by approximately 90% of peripheral γδ T cells (196). The present study showed that all αβVγ4 T cells express CD73, suggesting commitment to the innate-like γδ lineage.

Similar to γδ T cells, αβVγ4 T cells express IL-23R together with high levels of IL-1R1, suggesting that these cells are primed for cytokine production (116). The data also demonstrated that αβVγ4 T cells are approximately 50% $CD27^+$ and 50% $CD27^-$. Classically, $CD27^+$ γδ T cells are the IFN-γ-producing γδ T cell subset that respond to antigenic stimulation, whereas $CD27^-$ γδ T cells produce IL-17 independently of TCR activation, exhibiting innate-like features (119, 120, 387). The data from the present study demonstrated that αβVγ4 T cells are predominantly IL-17 secreting. However, engagement of the TCR may be required for IFN-γ production by $CD27^+$ αβVγ4 T cells. Expression of CD44 and Ly6C can also divide γδ T cells into IL-17 or IFN-γ-producing subsets. $Ly6C^+CD44^{lo}$ correspond to the IFN-γ secreting $CD27^+$ γδ T cells that rely on TCR engagement for activation and encompass memory-like T cells, whereas $Ly6C^-CD44^{hi}$ γδ T cells resemble the innate-like IL-17 producing $CD27^-$ γδ T cell subset (388). This is in agreement with the findings of the current study where αβVγ4 T cells constitutively expressed CD44 and are generally IL-17-secreting T cells.

Human γδ T cells have been shown to be highly reactive to an array of non-protein, phosphorylated antigens (136, 389, 390). As γδ T cells are not MHC restricted this enables them to recognize a vast array of antigens. The findings of the current study demonstrated that MHC class I and II are not required for the development of αβVγ4 T cells, suggesting a similar phenomenon. Furthermore, the expansion of TCRβ expressing $Vγ4^+$ T cells observed in MHC class $II^{-/-}$ mice indicates that $CD4^+$ T cells may control the balance between Vγ4β and conventional Vγ4δ thymocytes. This is in agreement with findings of the Hayday group who demonstrate that DP T cells, which develop into $CD4^+$ or $CD8^+$ T cells, regulate the development of TCRγ expressing T cells in the thymus (367). Interestingly, dysfunctional γδ T cell development has been reported in $TCRβ^{-/-}$ mice (367). αβVγ4 T cells are also absent in

TCRβ$^{-/-}$ mice and this may explain why TCRγ expressing T cell development is abrogated in the absence of TCRβ (367). Earlier studies describe that suppression of Vγ4 gene rearrangement by the TCRβ gene is necessary to prevent expression of γδ and αβ TCR complexes on the same cell (170). However, the current study has demonstrated that cells can express TCRα, β and γ chains.

IL-17-producing γδ T cells have many characteristics of IL-17-producing CD4^{+} T cell subset, Th17 cells, which have been shown to signal through STAT3 in order to produce IL-17 cells (359). The present study revealed that IL-17 and IFN-γ production by Vγ4^{+} T cells is dependent on STAT3 signalling (68, 364, 391). γδ T cells play an important role in immunity to infection however, dysregulation of innate-like lymphocytes can result in the onset of autoimmunity (75, 94, 108, 275, 392). Similarly to γδ T cells, purified αβVγ4 T cells secreted IL-17 and IL-22 in the absence of TCR engagement, suggesting that αβVγ4 T cells are rapid inducers of inflammation through innate cytokine secretion (116). Another potent producer of IL-17 are the recently identified nTh17 cells, characterised by the expression of CD4, TCRαβ, ICOS, CD44, IL-23R and the integrins CCR6 and VLA-4 (94). nTh17 cells acquire effector function during development in the thymus and can produce IL-17 and IL-22, but not IL-17F, as early as 24 post hours post infection (97). In addition to expressing the same lineage markers as nTh17 cells, the data from the current study reveals that αβVγ4 T cells produce the same cytokines (94). In autoimmune diseases such as MS and EAE, myelin-reactive Th17 and Th1 cells can express trafficking molecules such as VLA-4 and CCR6 to enter the CNS; however, there is still controversy over whether γδ T cell traffic to the CNS independently of adhesion molecules (271, 393-398). The present study demonstrates the constitutive expression of CCR6 and CD49d by αβVγ4 T cells, suggesting that αβVγ4 T cells may have the potential to traffic to sites of inflammation.

The current study demonstrates that effector function of αβVγ4 T cells may be enhanced via the TCR, as increases in IL-17 production by αβVγ4 T cells isolated from EAE mice approached significance with MOG, IL-1 and IL-23 stimulation compared with IL-1 and IL-23 stimulation alone. Moreover, profileration assays demonstrated that αβVγ4 T cells from mice with EAE proliferated in response to stimulation with MOG or IL-1β and IL-23, however it would be necessary to perform this experiment in purified Vγ4 T cells to validate this finding. γδ T cells have also been shown to respond to antigen re-challenge, when isolated from leprosy skin lesions γδ T cells proliferate in vitro specifically to mycobacterial antigens (399). Furthermore, the recently described NKT-like δ/αβ T cells, recognize

glycolipids such as α-glucosylceramide, demonstrating a novel TCR capable of antigen recognition in humans (197). Taken together, these data demonstrate that αβVγ4 T cells can be activated through a unique TCR or in an innate-like fashion, indicating that these cells may play a role in immunity to infection or autoimmunity through the activation of Th17 cells (116).

Collectively, the findings presented in this thesis identify a novel subtype of T cells with functional properties associated with both innate-like γδ T cells and adaptive $CD4^+$ T cells. These data warrant further investigations into the development of unconventional T cell subsets and the role of these cells in host immunity.

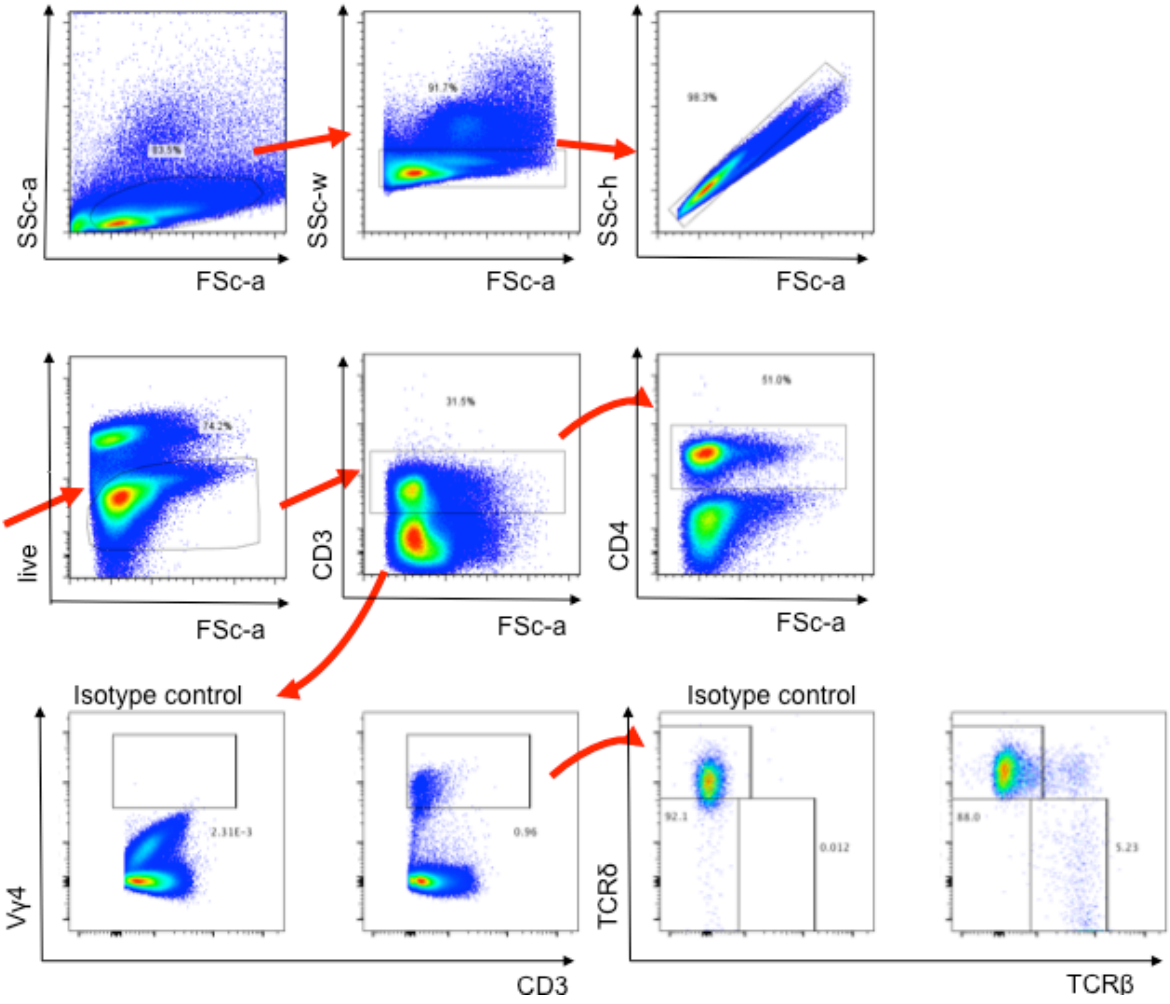


Figure 4.1: Gating strategy for analysing CD4$^+$ and Vγ4$^+$ CD3$^+$ T cells. Single cell suspensions were prepared from WT mice. Cells were stained ex-vivo with live dead, Fc blocked and stained *ex vivo* for CD3, CD4, Vγ4, TCRβ and TCRδ. Cells were gated on FSc versus SSc. T cells were gated as single, live, CD3$^+$ cells. CD4 cells were gated on CD4 versus FSc from CD3$^+$ cells. Vγ4 T cells were gated on Vγ4 versus CD3$^+$ and gates were set on an isotype control. Vγ4$^+$CD3$^+$ T cells were gated for TCRδ versus TCRβ expression where the TCRβ gate was set on an isotype control.

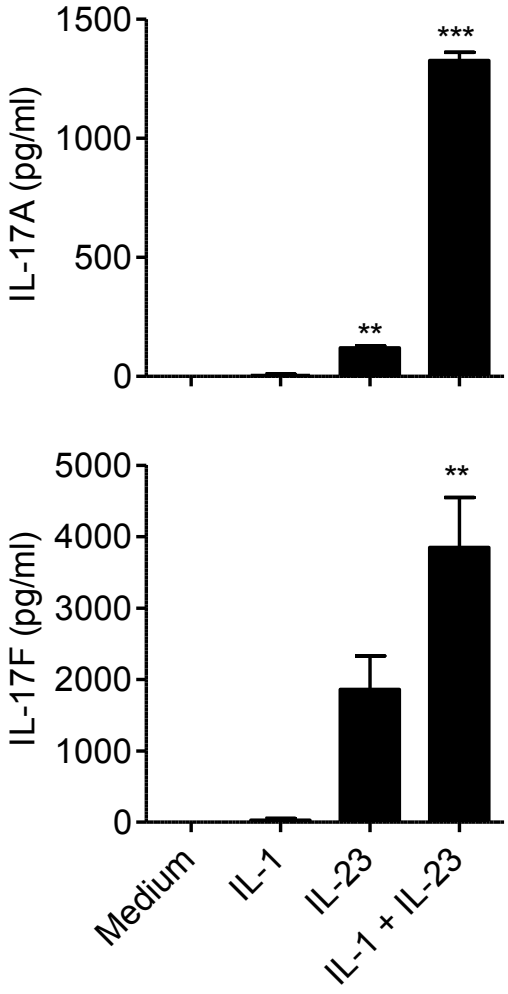


Figure 4.2: Significant IL-17A and IL-17F production by Vγ4$^+$ T cells in response to IL-1 and IL-23. Vγ4 T cells were FACS sorted from the spleens and lymph nodes of naïve C57BL/6 mice. Vγ4 cells (10,000/well) were cultured with IL-1β, IL-23, both (10 ng/ml) or medium only for 72 hours. Supernatants were removed for cytokine quantification by ELISA. Data is shown as mean concentration of triplicate wells. *p <0.05, ***p<0.001 versus medium by unpaired student t-test. Data are representive of three independent experiments.

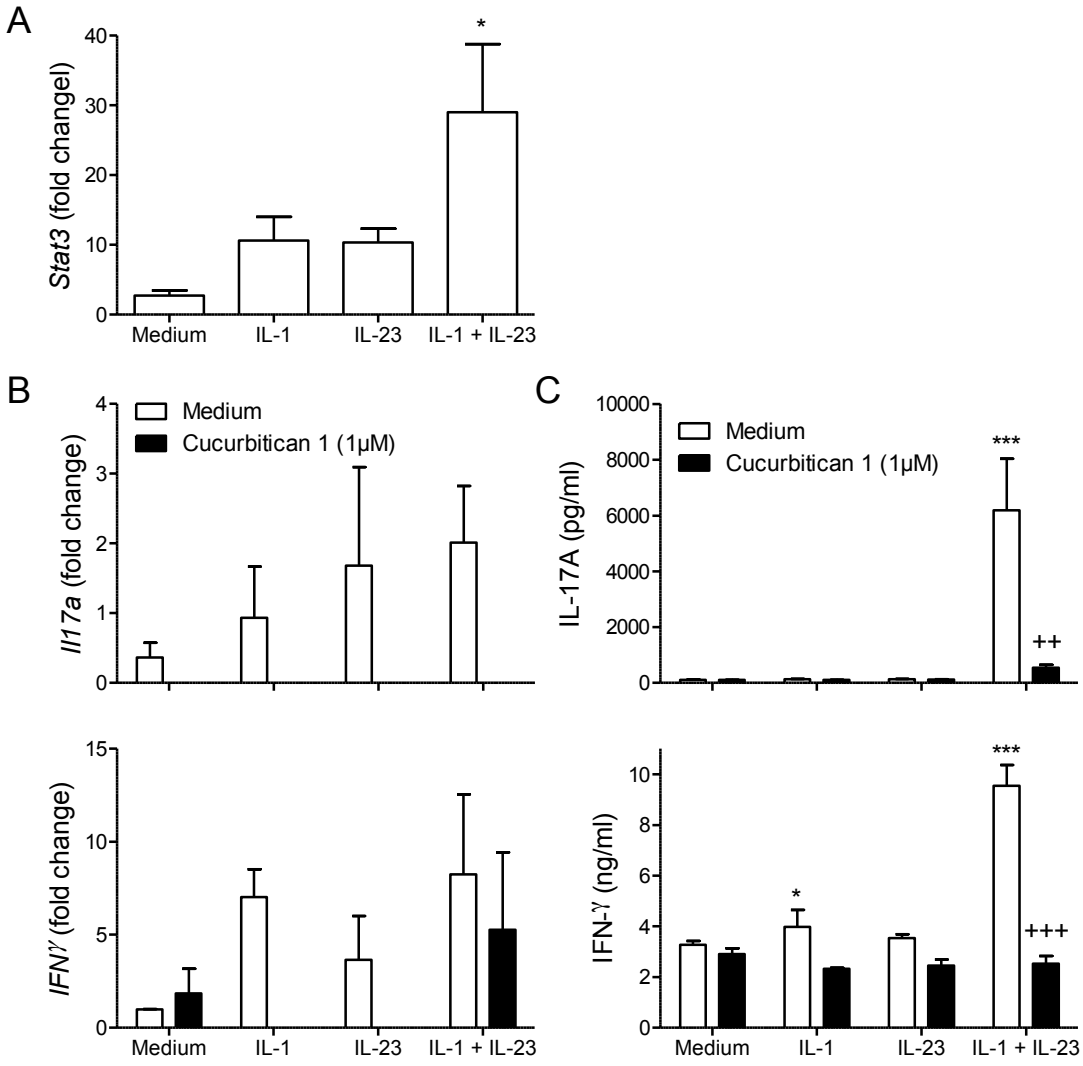


Figure 4.3: IL-1β and IL-23 induce IL-17A and IFN-γ production by Vγ4 T cells via induction of STAT3. (A) Vγ4 T cells were FACS sorted from the spleens and lymph nodes of naïve C57BL/6 mice. (B) Vγ4 cells (10,000/well) were cultured with medium, IL-1β, IL-23 or both (10 ng/ml) +/- 1 μM cucurbiticin 1, a STAT3 inhibitor. After 24 hours cells were recovered and added to Trizol. IL-17 and IFN-γ mRNA expression was quantified by real time PCR relative to medium cultured cells following normalisation by the endogenous control 18S mRNA. (C) After 72 hours supernatants were removed for cytokine quantification by ELISA. Data presented as mean ± SD fold change or concentraion for triplicate assays *p <0.05, ***p<0.001 versus medium. $^{++}$p<0.01, $^{+++}$p<0.001 versus medium by unpaired student t-test. Data are representive of two independent experiments.

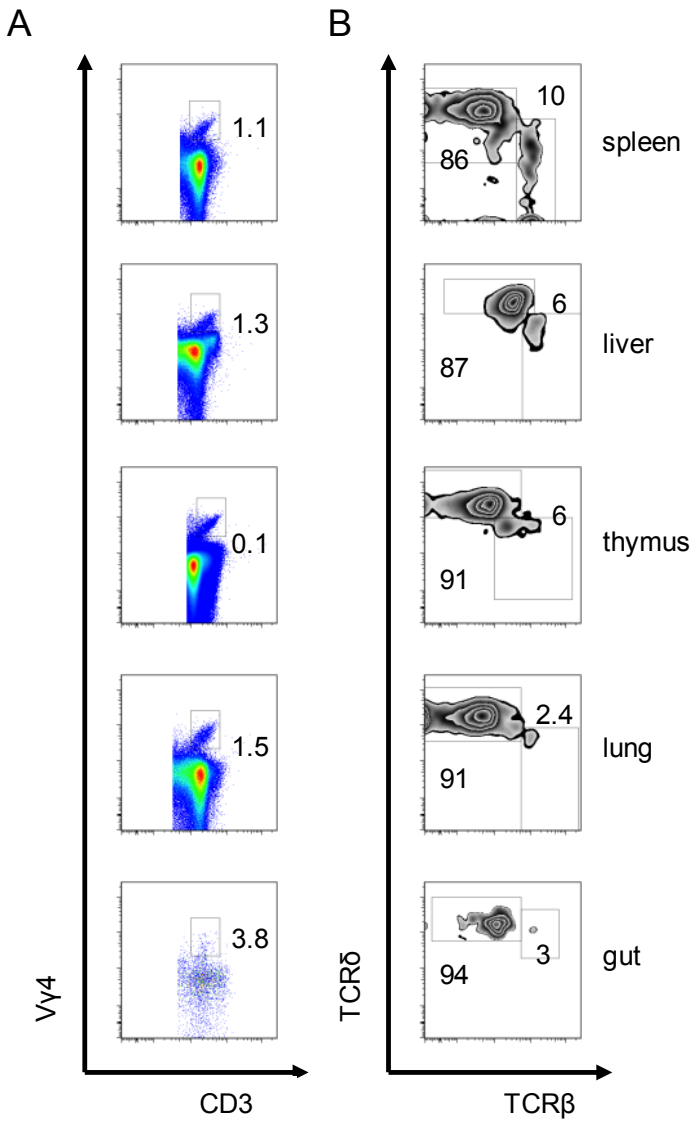


Figure 4.4: TCRβ expressing Vγ4 T cells are present in various tissues. Single cell suspensions of spleen, liver, thymus, lung and gut were isolated from WT mice. Cells were stained ex-vivo with live dead, Fc blocked and stained *ex vivo* for CD3, Vγ4, TCRβ and TCRδ expression. Gating strategy is desribed in figure 4.1. (A) Cells were gated on FSc versus SSc. T cells were gated as live, singel, $CD3^+$ cells. Vγ4 T cells were gated on Vγ4 versus $CD3^+$. (B) $V\gamma4^+CD3^+$ T cells were gated for TCRδ versus TCRβ expression. Percentages refer to $TCR\beta^+V\gamma4^+CD3^+$ cells or $TCR\delta^+V\gamma4^+CD3^+$ cells in each tissue (graphs are representative of n=3 separate mice). Data are representative of two-three independent experiments.

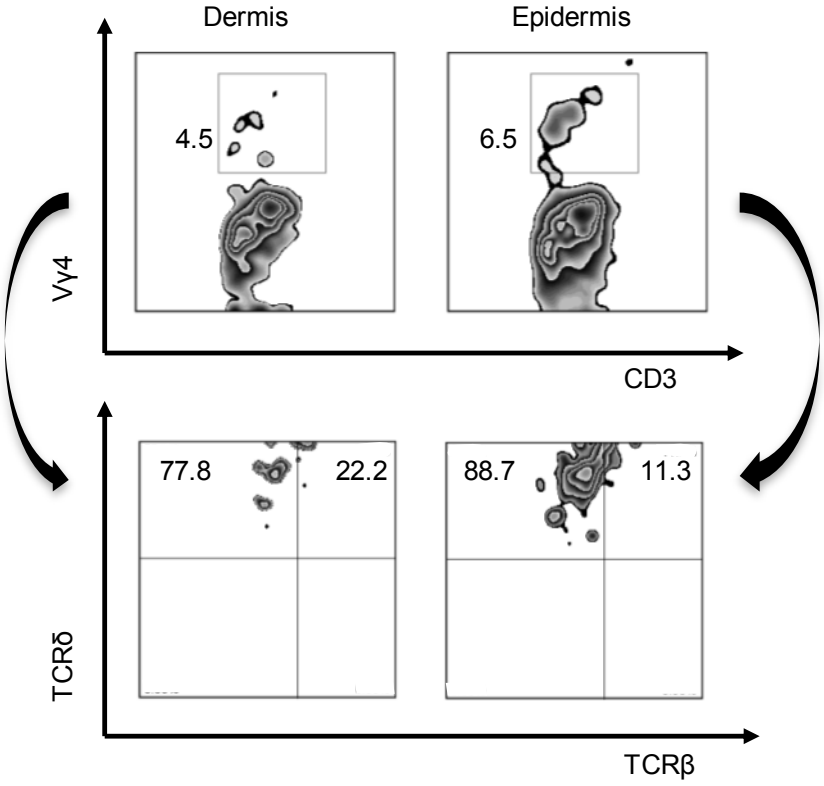


Figure 4.5: TCRδ expressing Vγ4 T cells, but not TCRβ are present in skin tissue. Single cell suspensions of dermis and epidermis were prepared from WT mice. Cells were stained ex-vivo with live dead, FC blocked and stained ex vivo CD3, Vγ4, TCRβ and TCRδ expression. Cells were gated on FSc versus SSc. T cells were gated as live, single, $CD3^+$. Vγ4 T cells were gated on Vγ4 versus $CD3^+$. $V\gamma4^+CD3^+$ T cells were gated for TCRδ versus TCRβ expression. Plots are representative of n=3 mice, and data are representative of two independent experiments.

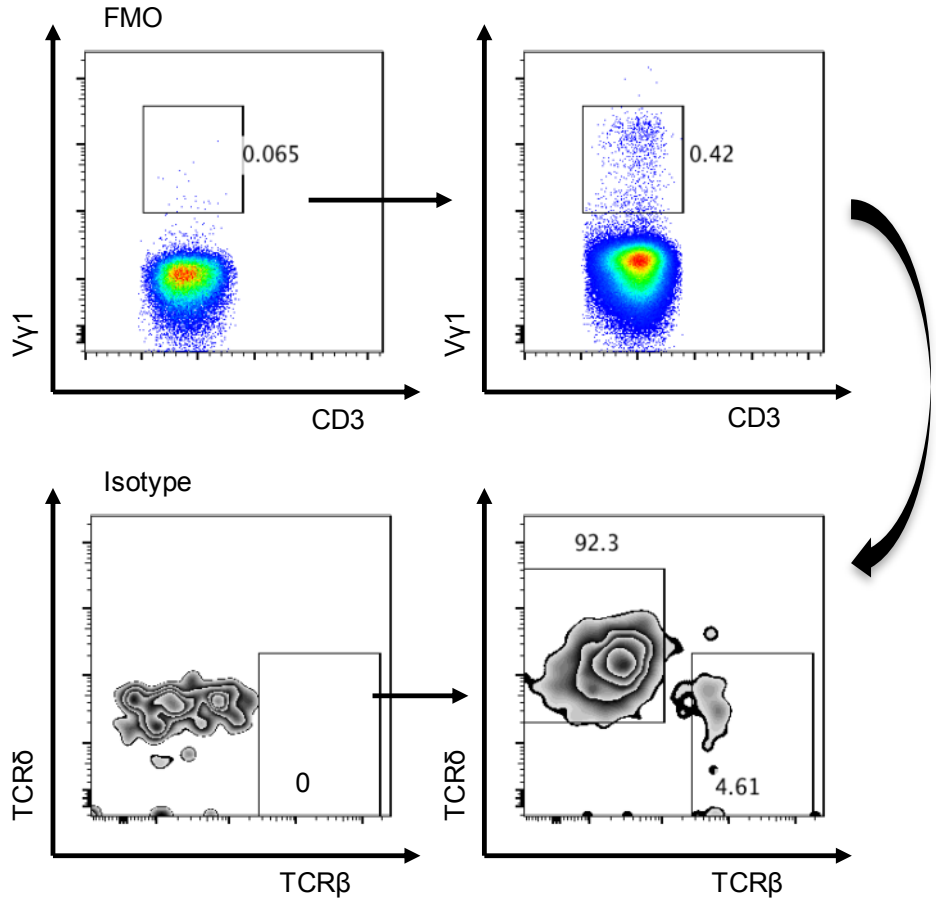


Figure 4.6: Vγ1 T cells can express the TCRβ chain. CD3 T cells were MACS isolated from spleen and lymph nodes of WT C57BL/6 mice. Cells were stained ex-vivo with live dead, FC blocked and stained for Vγ1, TCRβ and TCRδ. Cells were gated on FSc versus SSc. T cells were gated as live, single $CD3^+$. Vγ1 T cells were gated on Vγ1 versus $CD3^+$ based on FMO control. $V\gamma1^+CD3^+$ T cells were gated for TCRδ versus TCRβ expression versus isotype control. Plots are representative of n=3 separate mice and data are representative of three independent experiments.

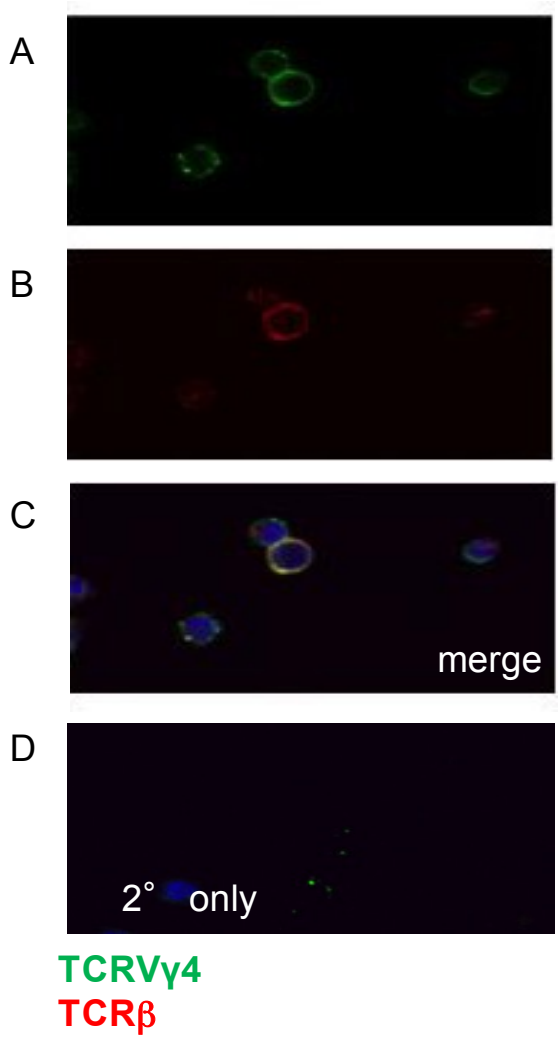


Figure 4.7: Co-localised surface expression of Vγ4 and TCRβ on T cells. FACS purified Vγ4 cells were washed and were incubated with anti-Vγ4 and anti-TCRβ primary antibodies and incubated with secondary antibodies (AlexaFluor488 for anti-Vγ4 and AlexaFluor647 for anti TCRβ). Slides were mounted with fluorescent mounting medium containing DAPI. Fluorescent microscopy was performed using 40X/60X zoom on an Olympus FV1000© confocal microscope and analysed using FV10-ASW 2.0 viewer software©. (A) DAPI stained Vγ4 sorted T cells, (B) overlay of DAPI and TCRβ staining, C) overlay of DAPI, anti Vγ4 and anti TCR β. D) Overlay of DAPI and secondary AlexaFluor647. Confocal images are representative of at least 20 captures from three independent experiments.

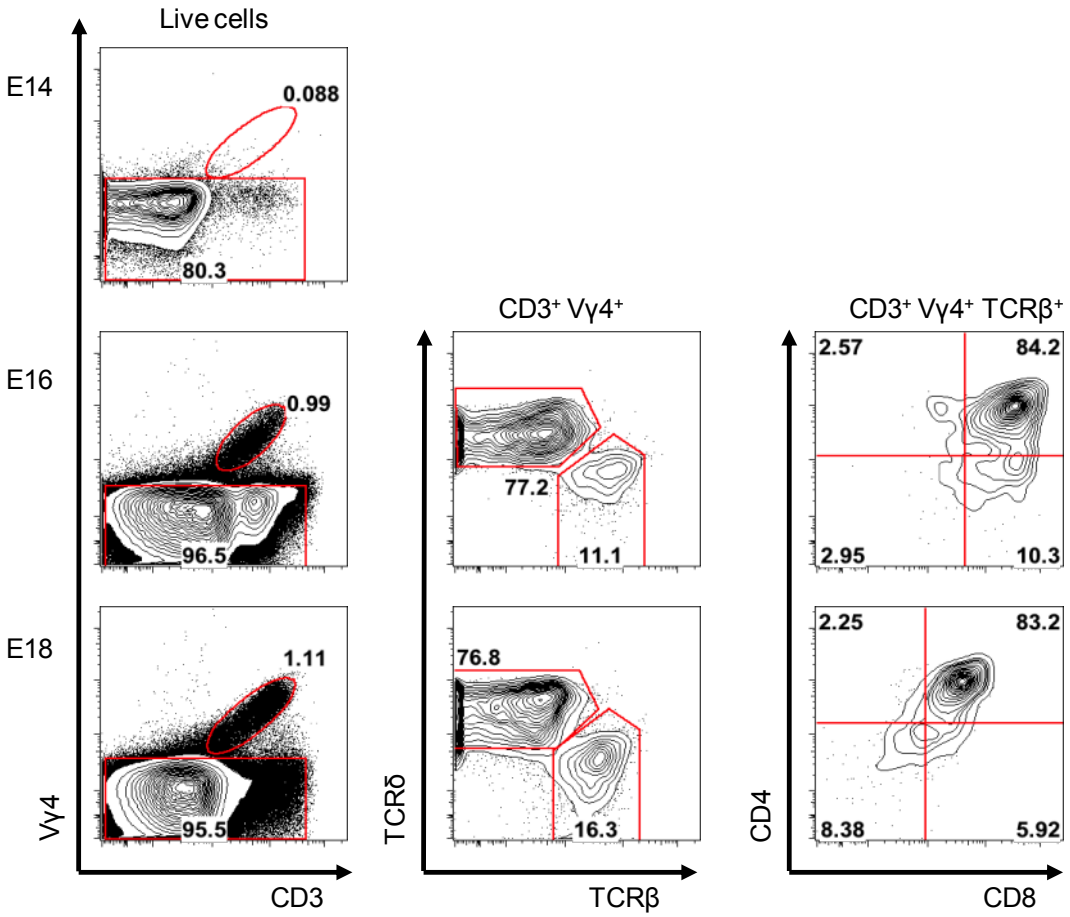


Figure 4.8: αβVγ4 T cells develop independently of Vγ4δ T cells in the thymus bweetn day 14-15 of gestation. Thymuses from C57BL/6 embryos, where the appearance of a vaginal plug was designated embryonic day (E) 0, were isolated and analyzed for expression of CD3, CD4, CD8, Vγ4, TCRβ and TCRδ from E14, E16 and E18 using flow cytometry. Cells were stained with live dead, FC blocked and stained for Vγ4, TCRβ, CD3, CD4 and CD8. Cells were gated on FSc versus SSc. Vγ4 T cells were gated on live, single, $CD3^+$ cells. Live $V\gamma4^+CD3^+$ cells were gated for TCRδ versus TCRβ based on FMO controls. $V\gamma4^+$ $TCR\beta^+$ cells were gated for CD4 versus CD8. FACS plots are representative of n=3 separate embryos and data is representative of two independent experiments. Experiments performed by Song Baik at MRC Centre for Immune Regulation, Institute for Biomedical Research, Medical School, University of Birmingham, UK.

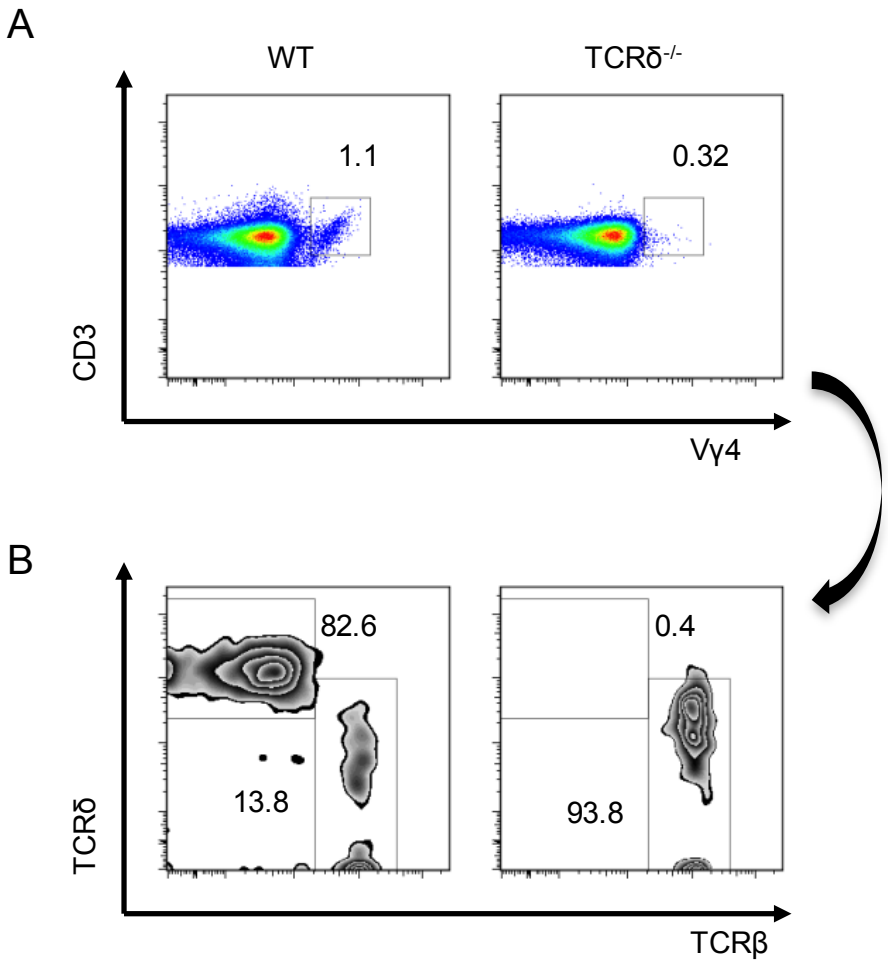


Figure 4.9: Identification of Vγ4β^{+} T cells in TCRδ$^{-/-}$ mice. CD3 T cells were purified from the lymph nodes of naïve C57BL/6 and TCRδ$^{-/-}$ mice. Cells were stimulated for 6 hours with PMA (10 ng/ml), ionomycin (1 µg/ml) and brefeldin A (5 µg/ml). Cells were washed and stained with live dead, FC blocked and for surface γδ, TCRβ, TCR Vγ4 and analysed by flow cytometry. A) Cells were gated on FSc versus SSc. Vγ4 T cells were gated on live, single, CD3^{+} cells. B) Live Vγ4^{+}CD3^{+} cells were gated for TCRδ versus TCRβ based on gate set on an isotype control. Plots are representative of n=3 separate mice and data is representative of two-three independent experiments.

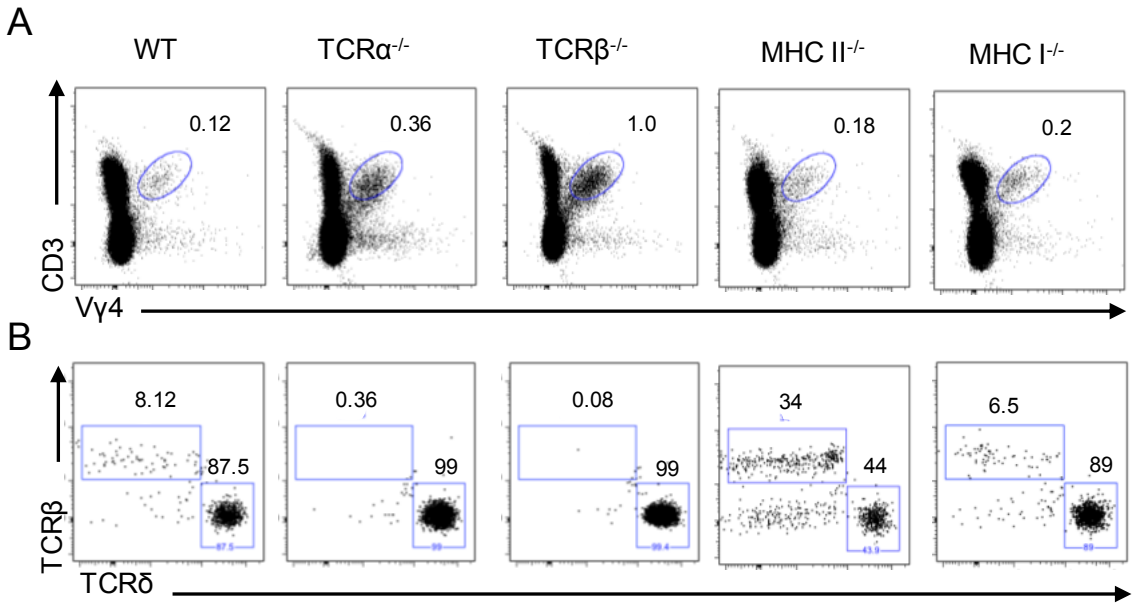


Figure 4.10: TCRβ expressing Vγ4 T cells that are absent in TCRα$^{-/-}$ and TCRβ$^{-/-}$ mice. FACS plots of lymph node cells from WT, TCRα$^{-/-}$, TCRβ$^{-/-}$, MHC II$^{-/-}$ or MHC I$^{-/-}$ mice stained for CD3, Vγ4, TCRβ and TCRδ. A) Cells were gated on FSc versus SSc. Vγ4 T cells were gated on live, single cells versus CD3. B) TCRδ versus TCRβ, gated on CD3^{+} Vγ4^{+} cells (graphs are representative of n=3 separate mice). Experiment performed by Dr. Julie. C Ribot at the Instituto de Medicina Molecular, Faculdade de Medicina, Universidade de Lisboa, Portugal.

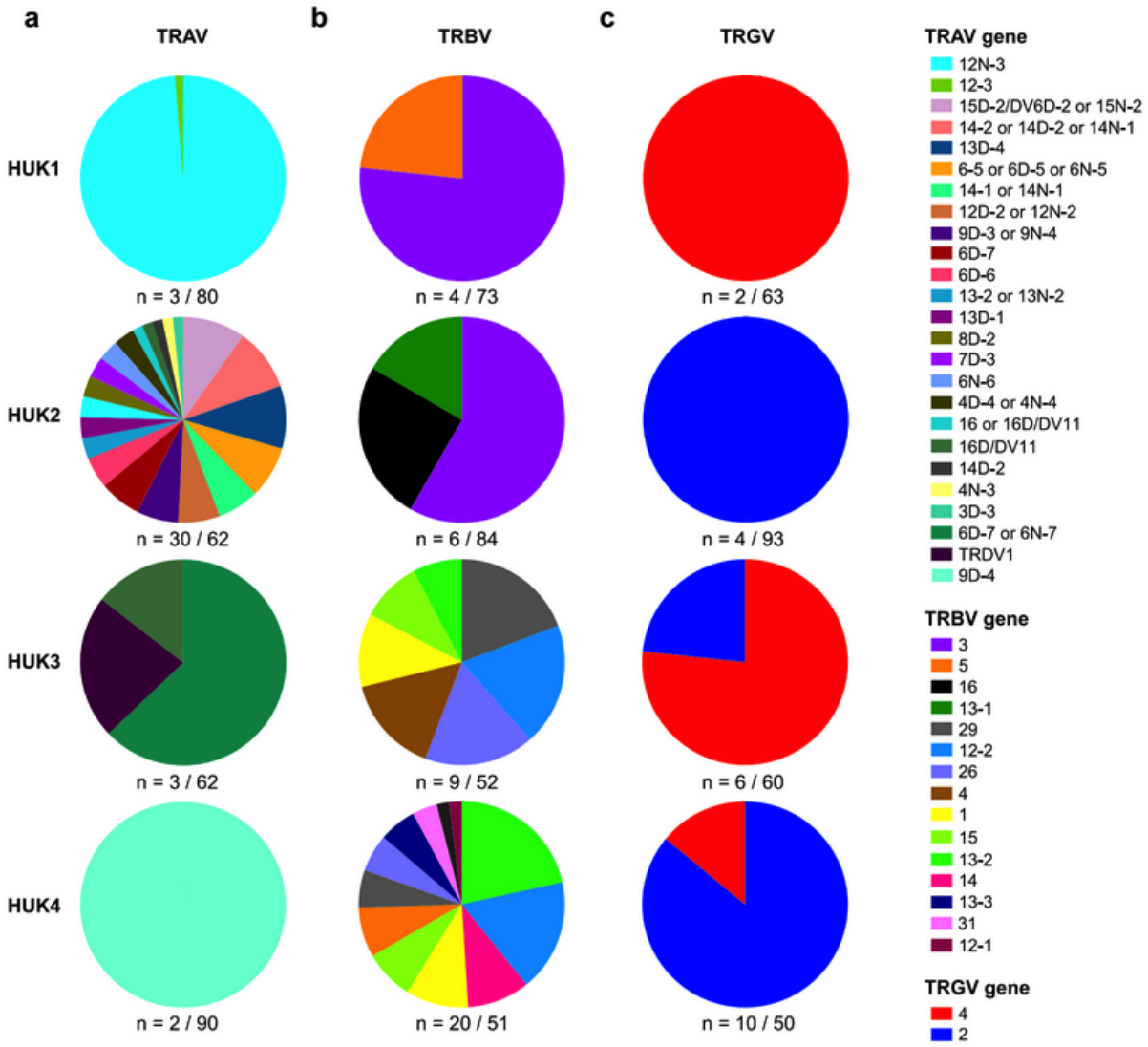


Figure 4.11: αβγ T cells express a skewed TCR repertoire. A-C, Molecular analysis of *TRGV* - TCRγ Variable (A), *TRAV* - TCRα Variable (B) and *TRBV* - TCRβ Variable (C) gene expression on FACS-purified $V\gamma4^{+}TCR\beta^{+}$ cells from WT mice using an unbiased template-switch anchored RT-PCR. Pie charts display relative frequencies for the genes depicted in the key according to IMGT nomenclature, with the total number of unique TCR sequences / analyzed TCR sequences indicated below. Data are shown from four individual mice as depicted here as Harlan UK mice 1-4 (HUK1-4). Experiments performed by Dr. James E. McLaren and Dr. Kirstin Ladell at the Institute of Infection and Immunity, Cardiff University School of Medicine, Heath Park, Cardiff, UK.

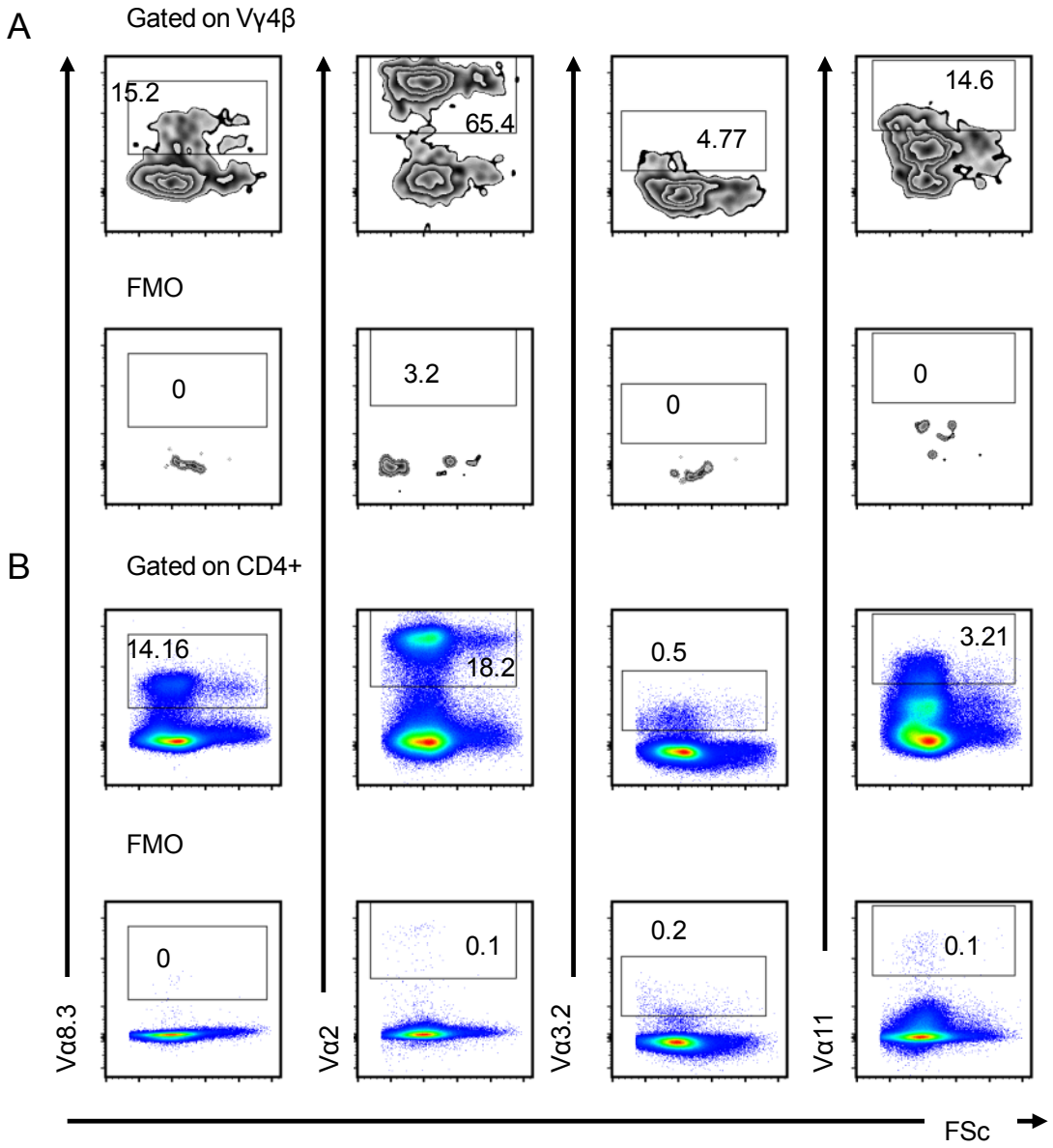


Figure 4.12: TCRβ expressing Vγ4 T cells express variable TCRα chains. CD3 T cells were purified from lymph nodes and spleens of WT C57BL/6 mice. Cells were stained with live dead, FC blocked and stained for CD4, CD3, Vγ4, TCRβ, TCRδ, TCRVα8.3, TCRVα2, TCRVα3.2 and TCRVα11. Cells were gated on FSc versus SSc. A) Vγ4 T cells were gated on live, single $CD3^+$ cells. Live $V\gamma4^+CD3^+$ cells were gated for TCRδ versus TCRβ based on isotype control. Plots represent TCRVα8.3, TCRVα2, TCRVα3.2 and TCRVα11 expression on $V\gamma4^+TCR\beta^+CD3^+$ cells versus FMO control B) CD4 T cells were gated on live, $CD3^+$ cells. Plots represent TCRVα8.3, TCRVα2, TCRVα3.2 and TCRVα11 expression on $CD4^+CD3^+$ cells versus FMO control. Plots are representative of n=3 separate mice and data are representative of two independent experiments.

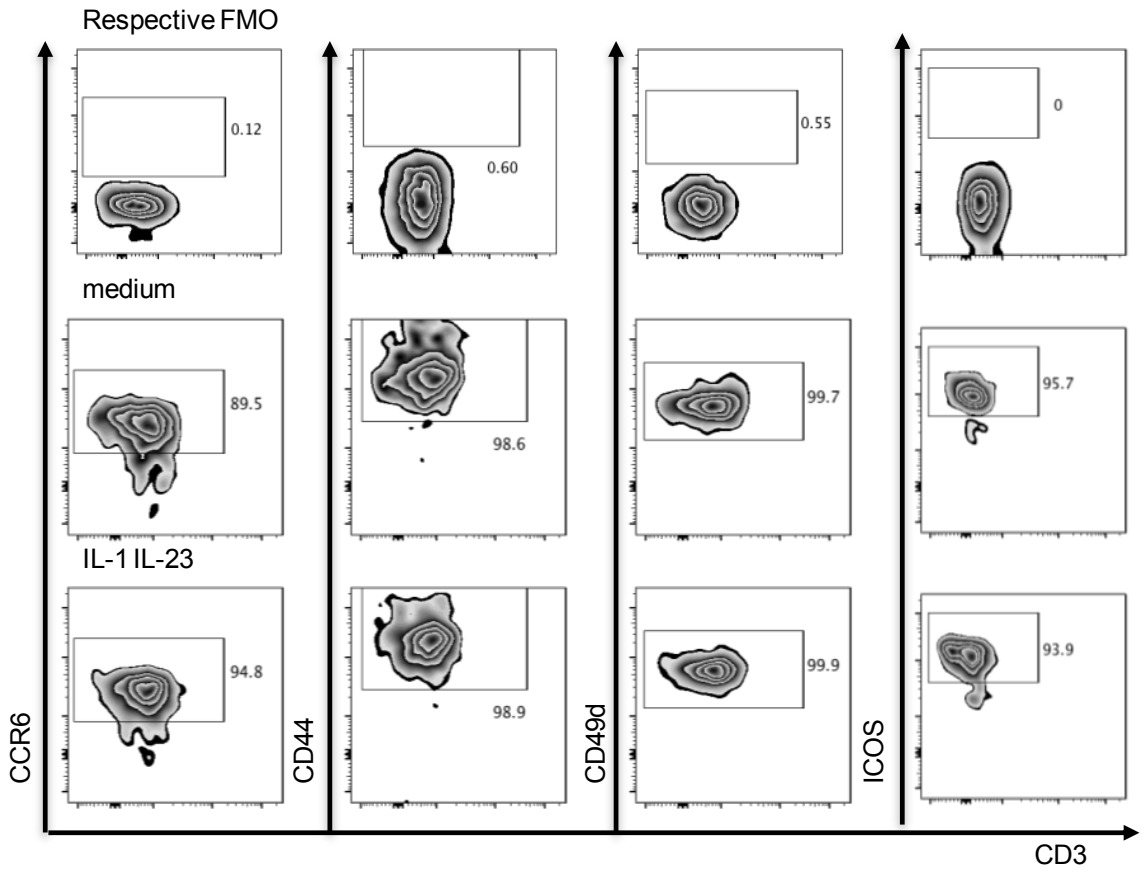


Figure 4.13: αβVγ4 T cells constitutively express markers of the natural Th17 lineage. $CD3^+$ T cells were purified from WT mice and cultured with IL-1β (10 ng/ml) and IL-23 (10 ng/ml) for 72 hours. Cells were stained with live/dead stain, Fc blocked and stained for CD3, Vγ4, TCRβ, CCR6, CD44, CD49d and ICOS. Cells were gated on FSc versus SSc. A) Vγ4 T cells were gated on live, single, $CD3^+$ cells. Live $V\gamma4^+CD3^+$ cells were gated for TCRβ based on isotype control. Plots represent CCR6, CD44, CD49d and ICOS expression on medium or IL-1β and IL-23 stimulated $V\gamma4^+TCR\beta^+CD3^+$ cells versus FMO control. Plots are representative of n=3 separate mice and data are representative of two-three independent experiments.

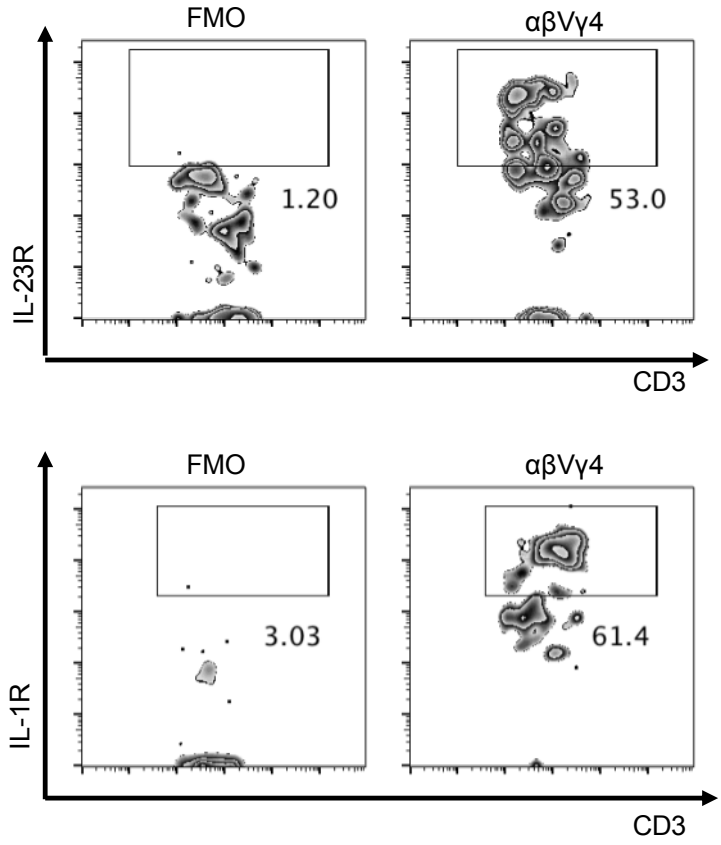


Figure 4.14: αβVγ4 T cells express IL-23R and IL-1R. $CD3^+$ T cells were purified from the lymph nodes of WT mice. Cells were stained for live/dead, Fc blocked and stained with CD3, Vγ4, TCRβ, IL-23R and IL-1R1. Cells were gated on FSc versus SSc. Vγ4 T cells were gated on live, sinlge, $CD3^+$ cells. Live $V\gamma4^+CD3^+$ cells were gated for TCRβ based on isotype control. Live, $V\gamma4^+TCR\beta^+$ cells were gated for the expression of IL-23R1 and IL-1R versus an FMO. Plots are representative of n=3 separate mice and representative of two independent experiments.

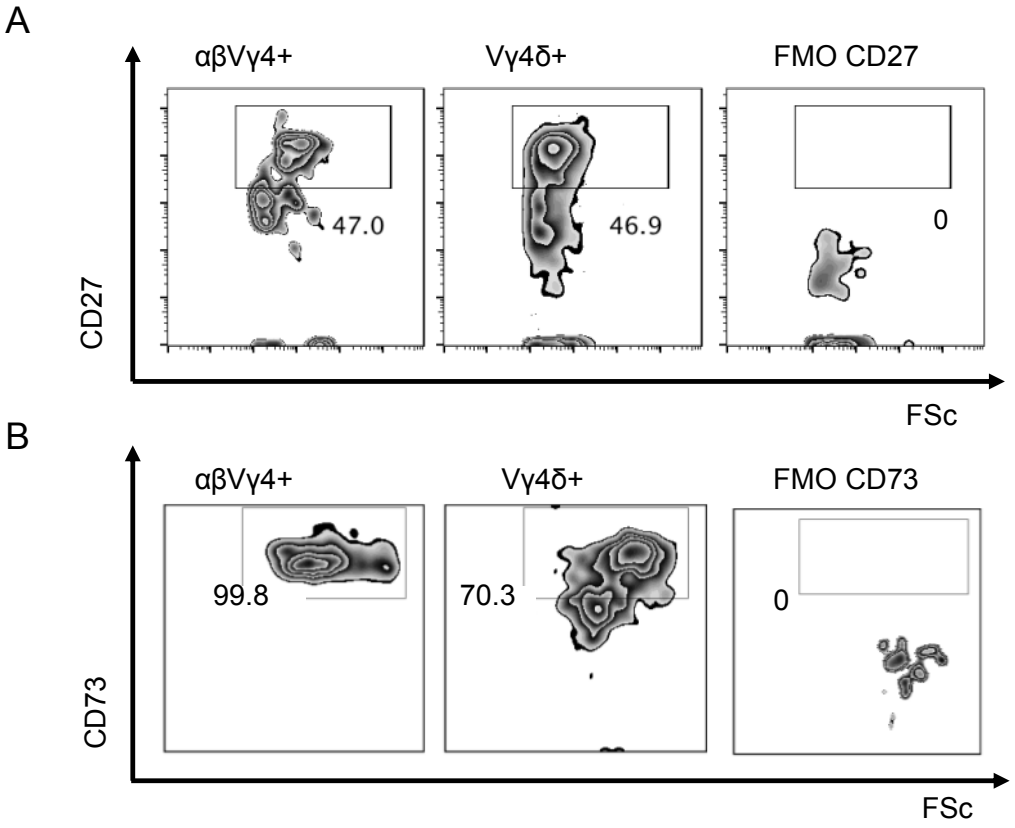


Figure 4.15: Vγ4β T cells express classical γδ T cell lineage markers, CD27 and CD73. **A)** $CD3^+$ T cells were isolated by MACS from WT mice. Cells were stained with live dead, Fc blocked and stained with CD3, Vγ4, TCRβ and CD27. **B)** WT CD3 T cells were stained for Vγ4, TCRδ, TCRβ and CD73. Cells were gated on FSc versus SSc. A) Vγ4 T cells were gated on live, single, $CD3^+$ cells. Live $V\gamma4^+CD3^+$ cells were gated for TCRδ versus TCRβ based on isotype control. Live $V\gamma4^+TCR\beta^+CD3^+$ cells and live $V\gamma4^+TCR\delta^+CD3^+$ cells were gated for CD27 and CD73 versus FMO control. Plots are representative of n=3 separate mice and data are representative of three independent experiments.

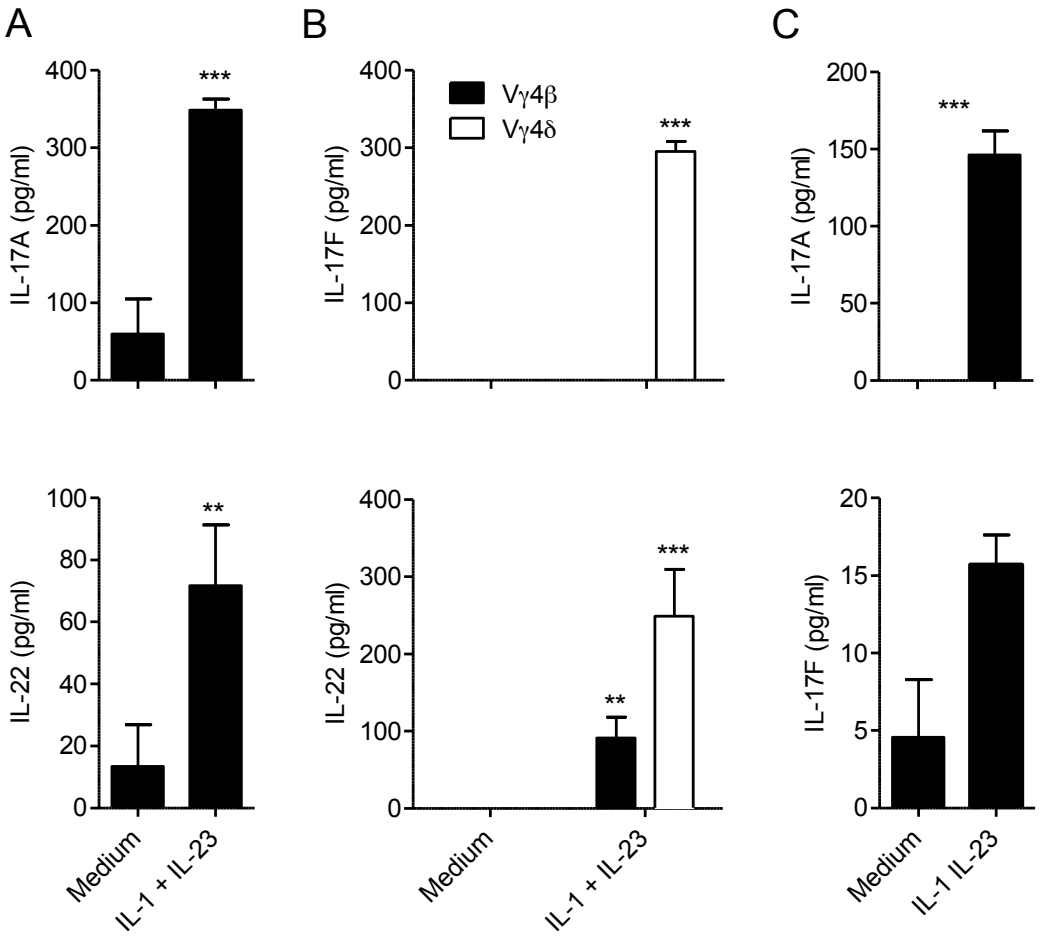


Figure 4.16: αβVγ4 T cells T cells purified from WT mice and TCRδ$^{-/-}$ mice produce IL-17A, IL-22, but not IL-17F in response to IL-1β and IL-23. A) Vγ4β T cells were FACS sorted from WT mice. Vγ4^{+}TCRβ^{+} (5,000 cells/well) were stimulated with IL-1 and IL-23 (10 ng/ml) or medium for 72h. Supernatants were removed for IL-17A and IL-22 quantification by ELISA. **B)** Vγ4^{+}TCRβ^{+} or Vγ4^{+}TCRβ^{-} cells were FACS purified from WT mice. Vγ4^{+}TCRβ^{+} or Vγ4^{+}TCRβ^{-} cells (2500 cells/well) were stimulated with IL-1 and IL-23 or medium for 72h. Supernatants were removed for IL-17F and IL-22 quantification by ELISA. **C)** Vγ4β T cells were FACS sorted from TCRδ$^{-/-}$ mice. Vγ4^{+}TCRβ^{+} T cells (3000/well) were stimulated with IL-1β and IL-23 or medium alone for 72 hours. Supernatants were removed for IL-17A and IL-17F quantification by ELISA. Data is shown as ± SEM concentration of triplicate wells. *p <0.05, **p<0.01, ***p<0.001 versus medium only. Results are representative of at least two or three independent experiments.

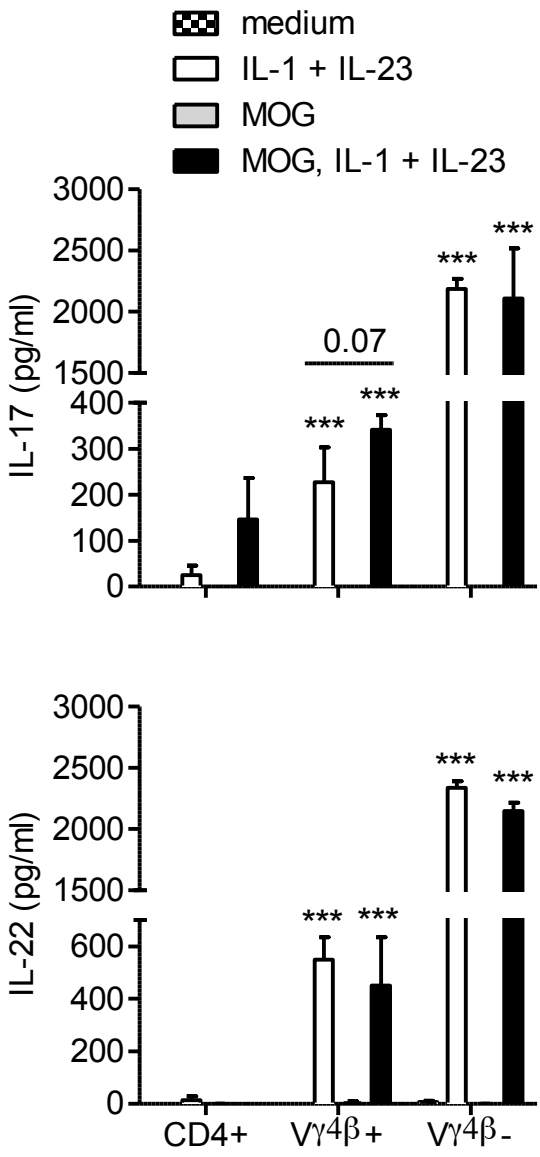


Figure 4.17: Vγ4β T cells respond to stimulation with IL-1β and IL-23 in the presence of MOG to augment IL-17A production. EAE was induced in WT mice by immunisation with MOG and CFA and 7 days later spleens and lymph nodes were harvested. CD4 T cells were isolated by MACS, $V\gamma4^{+}TCR\beta^{+}$ or $V\gamma4^{+}TCR\beta^{-}$ T cells were FACS purified from CD3 MACS purified T cells. Cells (4,000 per well) were cultured with IL-1β + IL-23 (10 ng/ml) and/or MOG (100 μg/ml) for 72 hours in the presence of irradiated APCs (2 x 10^6 cells/ml). IL-17 and IL-22 concentrations were quantified in the supernatants by ELISA. Data is shown as ± SEM concentration of triplicate wells. *p <0.05, **p<0.01, ***p<0.001 versus medium only or as indicated between columns by unpaired student t-test. Results are representative of two independent experiments.

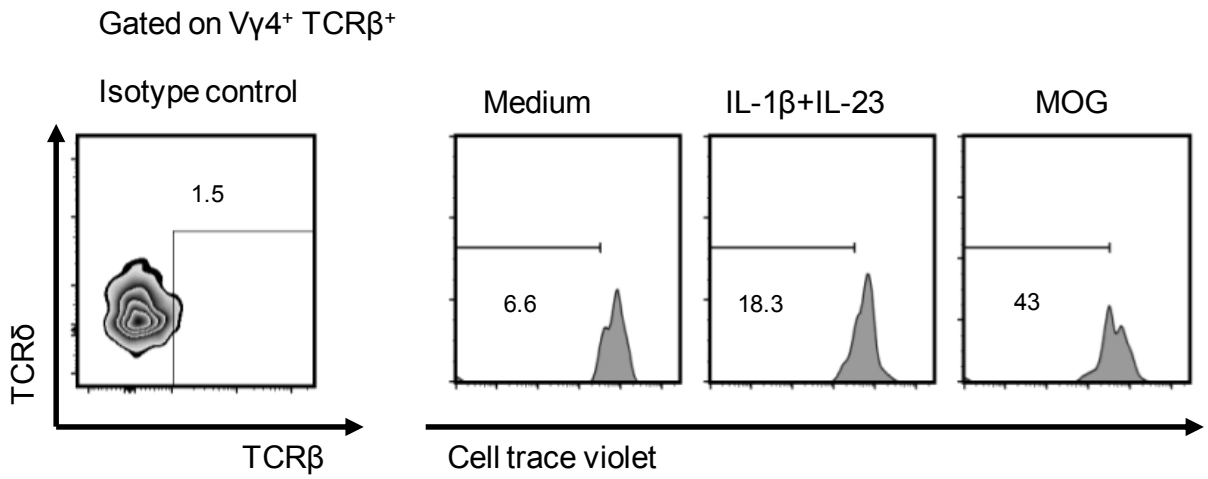


Figure 4.18: Vγ4β T cells proliferate in response to both IL-1β and IL-23 or MOG stimulation. EAE was induced in WT mice by immunisation with MOG and CFA and 10 days later spleens were harvested. Cells were MACS purified for CD3 and labeled with cell trace violet. Cells were cultured with IL-1β + IL-23 (10 ng/ml) and/or MOG (100 μg/ml) for 72 hours in the presence of irradiated APCS (2 x 10^6 cells/ml). Cells were washed and stimulated for 6 hours with PMA (10 ng/ml), ionomycin (1 μg/ml) and brefeldin A (5 μg/ml). Cells were washed and were stained with live dead, FC blocked and stained for surface TCRδ, TCRβ, Vγ4, CD4 and CD3 and and analysed by flow cytometry. Plots are representative of n=3 mice pooled for each culture flask. Results are representative of two independent experiments.

Chapter 5.

A novel T cell subset, αβVγ4 T cells play a critical role in IL-17 mediated CNS autoimmunity

5.1 Introduction

γδ T cells represent around 2-5% of peripheral lymphocytes and are known to play an important role in innate and adaptive immunity at mucosal surfaces (108, 165, 356). The IL-17-producing γδ T cells share many features with $CD4^+$ Th17 cells, including expression of RORγt, IL-1RI, IL-23R and CCR6 (112). Although γδ T cells do express a unique T cell receptor (TCR), engagement of this TCR with MHC-antigen complexes is not a prerequisite for their activation. Unlike conventional αβ T cells, cytokine stimulation alone is sufficient for activation of IL-17-secreting γδ T cells, making these cells rapid and potent mediators of inflammation (164). γδ T cells have been shown to be pathogenic in a variety of autoimmune diseases, such as EAE, collagen induced arthritis (CIA) and most recently in EAU (266, 267, 275). The pathogenic function of γδ T cells in EAE is mediated by their innate secretion of IL-17 and related cytokines, including IL-21 and GM-CSF (116). γδ T cells can secrete IL-17 in response to IL-1β, IL-18 and IL-23 without TCR engagement, promoting the induction of CD4 T cells and amplifying their encephalitogenic function during the development of EAE (76, 116, 329). Studies from our group have demonstrated that DCs can enhance the ability of IL-1 and IL-23 activated γδ T cells to promote IL-17 production by Th17 cells (116). Furthermore, DCs express IL-17R and secrete IL-23 in response to IL-17, which was enhanced by LPS and blocked by anti-IL-17R (116). These findings suggest that γδ T cell derived IL-17 may act in a positive feedback loop involving DC activation leading to enhanced Th17 cell effector function during EAE.

Importantly, data from our laboratory and others has shown that γδ T cells infiltrate the brain and spinal cord during the course of EAE, where they produce IL-17 and related cytokines (76, 116, 329). γδ T cells are also indirectly responsible for axonal demyelination through toxic destruction of oligodendrocytes, cells responsible for myelinating axons (400). Freedman et al demonstrated that oligodendrocytes cultured from surgically resected human brains were lysed in a dose-dependent manner by γδ T cells in vitro. A pathogenic role for γδ T cells in CNS autoimmunity was further substantiated in the EAE model where $TCR\delta^{-/-}$ mice displayed a milder disease phenotype (272). Studies in the relapsing-remitting EAE model demonstrate a significant reduction in clinical severity when mice were treated with a TCRδ depleting antibody immediately before disease onset or during the chronic phase of disease (267). In addition, experiments in the adoptive transfer model of EAE demonstrated that depletion of γδ T cells reduced clinical severity and delayed the onset of disease (273).

Vγ4$^+$ T cells were identified as the main IL-17-producing γδ T cell in the brains of mice with EAE, with Vγ1 and Vγ6 T cells also contributing (116). The pivotal role of γδ T cells in the pathogenicity of EAE reflects that seen in MS, where clonal expansion of γδ T cells has been observed in the cerebrospinal fluid of patients with recent disease onset (291). Furthermore, increased frequencies of γδ T cells have been detected in the peripheral blood of patients with MS (292) and an accumulation of γδ T cells has been described in acute brain lesions (294, 401). In particular, these 'innate' IL-17-producing Vγ4 T cells are thought to be key mediators of inflammation in the development of autoimmunity (402) where they infiltate the brain and spinal cords of mice with EAE (116, 403). Vγ4$^+$ T cells have also been described as key players in a variety of other inflammatory conditions. They play a critical role in promoting susceptibility to myocarditis (404), they mediate inflammation through IL-17 production in the dermis in murine model of psoriasis (274), and they accumulate in the draining lymph nodes and joint in CIA (405).

This study has demonstrated that a novel T cell subtype, the αβVγ4 T cell is a potent producer of IL-17 and IL-22, in the absence of TCR engagement, similar to γδ T cells (chapter 4). As well as having similar activation requirements to γδ T cells, the data demonstrates that this novel cell population displays features of innate-like γδ T cells such as CD73 and CD27 expression. Furthermore, the αβVγ4 T cells share some of the hallmark characteristics of the natural Th17 lineage. They constitutively express CD44, TCRβ, ICOS and the integrins CCR6 and VLA-4, which have been heavily implicated in the development of EAE and MS (97, 289, 344). Interestingly, αβVγ4 T cells demonstrate enhanced proliferation upon restimulation with MOG antigen, indicating that αβVγ4 T cells can respond to autoantigen through their TCR and also independently through cytokine stimulation. As γδ T cells have an established role in autoimmunity, we sought to assess the role of the IL-17 secreting αβVγ4 T cell subset in EAE. The aim of this study was to increase our understanding of the role of the TCRVγ4$^+$ T cells in the pathogenicity of EAE, and to investigate the role of the αβVγ4 T cells in the development of EAE.

5.2 Results

5.2.1 EAE severity is reduced in mice deficient in the TCRδ chain

It has previously been demonstrated that IL-1β and IL-23 stimulated Vγ4$^+$ T cells signal through STAT3, in the absence of TCR engagement, to produce IL-17 (chapter 4). γδ T cells have been shown to play a critical role in EAE through the production of innate IL-17, which can act to amplify pathogenic Th17 responses (116). Sutton et al demonstrated that this amplification is mediated through DCs, which express IL-17R and secrete IL-23 in response to γδ derived innate-IL-17, suggesting that innate IL-17 secreted by γδ T cells can act in a positive feedback loop to enhance Th17 cell effector function (116). Vγ4 T cells, have been shown to be the main IL-17 producing γδ T cells infiltrating the brain during EAE with assistance from Vγ1 and Vγ6 T cells (116). Here, the data demonstrate that at peak of disease, 20 days post induction of EAE, Vγ4 T cells infiltrate the brain and a significantly larger frequency produce IL-17 than CD4 (Th17) cells (Figure 5.1) The data demonstrate that Vγ4 T cells account for approximately 3% of the infiltrating T cells during EAE, with over 70% of the Vγ4 T cells producing IL-17, but not IFN-γ (Figure 5.1). CD4 T cells represent over 30% of the infiltrating T cells, however, only 8% produce IL-17 and 12% produce IFN-γ.

As γδ T cells have an established role in autoimmunity, we assessed the role of this novel IL-17 producing αβVγ4 T cells in EAE. Previous reports have suggested either key or modest pathogenic roles for γδ T cells in this disease (359), with conflicting reports on the role of Vγ1 and Vγ4 T cells in the pathogenesis of disease (116, 276). Previously, the data demonstrated that αβVγ4 T cells are still present in TCRδ$^{-/-}$ mice (chapter 4). The data demonstrate that TCRδ$^{-/-}$ mice, which lack conventional γδ T cells, had only mild, but significant reduction in clinical symptoms of EAE (Figure 5.2A) and mild weight loss (Figure 5.2B) compared with WT mice. Our findings suggest that this could reflect the presence of αβVγ4 T cells in TCRδ$^{-/-}$ mice.

Consistent with this possibility, during EAE we found that αβVγ4 T cells infiltrate the brains of TCRδ$^{-/-}$ mice while both conventional Vγ4δ T cells and αβVγ4 infiltrate the brains of WT mice (Figure 5.3). αβVγ4 T cells comprised 0.25% of total CD3$^+$ cells in the brains of WT and TCRδ$^{-/-}$ mice, whereas Vγ4δ T cells represented 0.15% of the total CD3$^+$ population in the CNS of WT mice (Figure 5.3). Overall, there was a slight decrease in the absolute numbers of IL-17-producing-CD4$^+$ T cells in the brain and spinal cord of TCRδ$^{-/-}$ mice

compared with WT mice, and this may reflect the activation of both IL-17-producing TCRδ and TCRβ expressing Vγ4$^+$ CD3$^+$ T cells (Figure 5.4). The elevated frequency of CD4 T cells in the brains was on average 2.5 x 10^4, and in the spinal cords 2 x 10^4 in WT mice. This was reduced in the TCRδ$^{-/-}$ mice with 5 x 10^3 CD4$^+$ T cells in the brains of TCRδ$^{-/-}$ mice, and 4 x 10^3 cells in the spinal cords (Figure 5.4). The difference in CD4$^+$ T cell infiltration was also reflected in cytokine producing cells in the brain and spinal cords. There was a non significant decrease in the absolute number of IL-17$^+$, IL-17$^+$IFN-γ$^+$ and IFN-γ$^+$ CD4 T cells in the brains and spinal cords of TCRδ$^{-/-}$ mice compared with WT mice. γδ T cells have been shown to accumulate in the brain during MS, EAE and during ischemia (116, 293, 406). Here, the data presented in this study demonstrate for the first time that αβVγ4 T cells infiltrate the CNS during EAE in WT and TCRδ$^{-/-}$ mice.

5.2.2 Transfer of αβVγ4 T cells is sufficient to induce mild EAE in IL-1R$^{-/-}$ mice.

Previous studies have shown that IL-1 is necessary for the induction of pathogenic Th17 cells. IL-1R$^{-/-}$ mice immunised with MOG and CFA failed to induce EAE or induces EAE with extremely late onset of disease (75, 116, 407). However, EAE can be induced in IL-1R$^{-/-}$ mice by injecting CD3 T cells from WT mice prior to immunisation with MOG and CFA. Therefore, IL-1R$^{-/-}$ mice provide a useful model to examine the precise role of distinct T cell subsets in the development of EAE (75). Having demonstrated that αβVγ4 T cells infiltrate into the CNS of WT and TCRδ$^{-/-}$ mice during the development of EAE, our findings suggest that αβVγ4$^+$ T cells have a role in mediating EAE pathogenesis. To provide further evidence for this hypothesis, IL-1R$^{-/-}$ mice were injected i.v. with CD3$^+$ T cells (5 x 10^6) from naïve TCRδ$^{-/-}$ mice, CD3$^+$Vγ4$^+$ T cells (2 x 10^4) or CD3$^+$Vγ4$^-$ T cells (2 x 10^4) from naïve TCRδ$^{-/-}$ mice one day prior to induction of EAE (Figure 5.5). EAE was induced in WT mice as a positive control. Transer of αβVγ4$^+$ T cells from TCRδ$^{-/-}$ mice into IL-1R$^{-/-}$ mice resulted in delayed onset of disease compared with WT mice, which developed severe clinical symptoms of EAE by day 10 post induction (Figure 5.5). Mild symptoms of EAE developed in IL-1R$^{-/-}$ mice which received αβVγ4$^+$ T cells from TCRδ$^{-/-}$ mice at day 24 post induction of EAE (Figure 5.5). Notably, mice which received αβVγ4$^+$ T cells developed symptoms 16 days earlier than IL-1R$^{-/-}$ mice with EAE demonstrating that αβVγ4 may play a pathogenic role in the development of EAE (Figure 5.5). Transfer of total CD3$^+$ T cells (5 x 10^6) from naïve

TCRδ$^{-/-}$ mice or CD3^{+} T cells depleted of αβVγ4 from TCRδ$^{-/-}$ mice failed to induce EAE in this model (Figure 5.5).

5.2.3 Depletion of Vγ4 T cells in WT mice significantly reduces the clinical symptoms of EAE.

To provide more definite evidence of a role for Vγ4^{+} T cells in the pathogenesis of EAE an experiment was designed to deplete these cells from mice before induction of EAE. A study in CIA demonstrated that 250μg of anti-Vγ4 antibody was effective at depleting Vγ4 T cells and reducing severity of disease (275). A preliminary study assessed the effect of 250μg anti-Vγ4 in vivo. Spleen cells were analysed by flow cytometry for the presence Vγ4^{+} T cells 3 days post i.p injection with PBS or anti-Vγ4 antibody (250 μg clone, UC310A6). The data demonstrates that a dose of 250 μg was sufficient to deplete Vγ4 expressing cells (Figure 5.6A).

To examine if depletion of Vγ4^{+} T cells could alter the course of EAE, mice were treated every five days, from one day prior to induction and on days 4, 9 and 14 with PBS or anti-Vγ4 antibody (Figure 4.11B). Undepleted mice began to show symptoms of disease 10 days after induction, manifesting as limpness of the tail on day 12 (Figure 5.6B). Clinical disease progressed to severe hind limb weakness by day 14 with an average clinical score of 3.25. The anti-Vγ4 treated mice progressed to an average clinical score of 1.25 and this was significantly less severe than the PBS-treated mice ($p<0.001$). Mice were sacrificed on day 16, mononuclear cells from the brain and spinal cord were isolated and analysed by flow cytometry for cytokine production. Treatment with the anti-Vγ4 depleting antibody every 5 days was not entirely efficient in depleting the cells from the CNS as there were a small number of Vγ4^{+} T cells detected by flow cytometry in the spinal cord (Figure 5.7). However, a significant depletion in the absolute numbers of Vγ4 T cells and a marked reduction in the absolute number of total γδ T cells was observed in the brains of mice with EAE treated with anti-Vγ4 antibody (Figure 5.7). However, the data demonstrates that anti-Vγ4 treatment significantly reduced the number of CD4 T cells infiltrating the brain and strongly reduced the number of infiltrating IL-17, IFN-γ and IL-17 and IFN-γ double-producing CD4^{+} T cells in the brain (Figure 5.8). In terms of absolute numbers, there were in excess of 6000 CD4^{+} T cells in the PBS-treated brains, with less than 1000 cells in the anti-Vγ4 treated mice (Figure

5.8). These data demonstrate that Vγ4 T cells play an important role in the activation of CD4 T cells during the development of EAE. Depletion of Vγ4 T cells strongly diminshes the infiltation of CD4 T cells into the CNS and as a result, reduces clinical severity of disease.

5.2.4 Depletion of Vγ4$^+$ T cells significantly attenuates EAE in WT and TCRδ$^{-/-}$ mice.

The findings of this study provide evidence that depletion of Vγ4 T cells attenuates clinical severity of EAE. The next aim was to assess the contribution of αβVγ4 T cells in the pathogenesis of EAE by depleting Vγ4 T cells from WT and TCRδ$^{-/-}$ mice. Treatment of WT or TCRδ$^{-/-}$ mice with anti-Vγ4 *in vivo* dramatically reduced the clinical course of EAE (Figure 5.9). The results of two independent experiments show that isotype-control treated WT and TCRδ$^{-/-}$ mice began to develop symptoms of disease 8-10 days post induction, manifesting as limpness of the tail on day 10 and progressing to severe hind limb weakness by day 13, with an average clinical score of 3.75 (Figure 5.9A). The onset of clinical symptoms in the anti-Vγ4 treated mice began on day 13. Anti-Vγ4 treated WT mice progressed to an average clinical score of 1.5 while TCRδ$^{-/-}$ mice progressed to an average clinical score of 2 and this was significantly less severe than the isotype-control treated WT and TCRδ$^{-/-}$ groups ($p<0.001$) (Figure 5.9A). The reduction in clinical severity was also reflected in a reduction in weight loss in WT and TCRδ$^{-/-}$ mice treated with anti-Vγ4 depleting antibody (Figure 5.9B). Flow cytometric analysis of the CNS at peak of disease revealed a marked reduction in the absolute number of IL-17$^+$ and IFN-γ$^+$ CD4$^+$ T cells in the brains and spinal cords WT and TCRδ$^{-/-}$ mice treated with anti-Vγ4 compared with isotype control treatment (Figure 5.10). These findings demonstrate that αβVγ4 T cells are crucial in the induction of disease, suggesting that this novel population of αβVγ4 T cells may be as pathogenic as γδ T cells in the early pathogenesis of EAE.

5.2.5 Depletion of αβVγ4 T cells from MOG-specific cells significantly impairs the ability to transfer EAE.

EAE can be induced by transfer of MOG-specific cells from donor mice immunized with MOG emulsified in CFA. Mixed spleen and lymph node cells from MOG-specific mice are

restimulated with MOG in the presence of IL-1β and IL-23 for 72 hours. This produces a highly pathogenic population of MOG-specific cells that induce EAE when injected into naïve recipient mice (408). This model was used to confirm the role of pathogenic role of TCRγ but not necessarily TCRδ T cells. FACS purification of Vγ4 T cells from WT cells removed both Vγ4δ and αβVγ4 T cells before MOG restimulation, while depletion of Vγ4 T cells from TCRδ$^{-/-}$ cells removed only αβVγ4 T cells. Depletion of Vγ4^{+} T cells from either WT or TCRδ$^{-/-}$ cell populations significantly ($p<0.001$) impaired the ability of MOG-specific spleen and lymph node cells to transfer EAE into naive WT mice (Figure 5.11). Transfer of WT or TCRδ$^{-/-}$ cells into WT recipients resulted in EAE with a mean clinical score of 4, beginning with weak tail tone from day 7 to hind limb paralysis at day 13 (Figure 5.11A). Transfer of WT or TCRδ$^{-/-}$ cells depleted of Vγ4 T cells resulted in EAE with a a mean clinical score of 1 and 0, respectively (Figure 5.11A). The reduction in clinical severity was also reflected in a significant reduction in weight loss in WT and TCRδ$^{-/-}$ mice depleted of Vγ4 T cells (Figure 5.11B). These findings suggest that depletion of αβVγ4 T cells alone significantly impairs the ability to transfer EAE into naïve recipients. Importantly, depletion of Vγ4^{+} T cells from spleen and lymph node cells of MOG-immunized WT or TCRδ$^{-/-}$ mice significantly reduced MOG-specific IL-17, IFN-γ, GM-CSF and IL-22 production in cultures (Figure 5.12). This demonstrates that αβVγ4 T cells are important amplifiers of Th17 cytokine secretion and effector function during EAE.

Overall, the clinical symptoms of mice injected with WT or TCRδ$^{-/-}$ cells depleted of Vγ4 T cells corresponds with a marked reduction in the absolute numbers of IL-17- and IFN-γ-producing CD4^{+} T cells migrating to the brains of these mice (Figure 5.13). The majority of CD4^{+} T cells in the mice injected with WT donor cells appeared to be IL-17-producing CD4^{+} T cells (Figure 5.13). In comparison with transfer of WT cells, there was a significant reduction ($p>0.05$) in number of IL-17-, IFN-γ- producing and IL-17^{+}IFN-γ^{+}CD4^{+} T cells in the brain of mice injected with TCRδ$^{-/-}$ cells depleted of Vγ4 T cells (Figure 5.13). Moreover, kinetics studies on cellular infiltration during EAE reveal that αβVγ4 T cells infiltrate the CNS of WT mice as early as 3 days post-induction of EAE (Figure 5.14). Conventional Vγ4δ T cells appear at day 7, however the number of αβVγ4 T cells peaks at day 11 while Vγ4δ T cells persist in the CNS to day 14 suggesting that αβVγ4 T cells may produce IL-17 earlier in disease and conventional γδ T cells maintain this IL-17 pool in the CNS during EAE. This data supports our hypothesis that αβVγ4 T cells serve as an early source of inflammatory cyokines that enhance the encephalitogenic function of CD4^{+} T cells.

5.3 Discussion

IL-17-producing T cells play a key role in the pathogenesis of many autoimmune disorders, including MS and psoriasis, and are now a major drug target for these diseases. IL-17-secreting Vγ4^{+} T cells are found at a high frequency in the CNS during EAE. The findings of this study have uncovered a novel T cell subtype, αβVγ4 T cells, which play a critical pathogenic role in CNS autoimmunity. The results in chapter 4 demonstrate that αβVγ4 T cells secrete IL-17A and IL-22, following stimulation with IL-1β and IL-23 in the absence of TCR engagement. αβVγ4 T cells purified from mice with EAE also respond to the autoantigen MOG. Remarkably, depletion of Vγ4^{+} T cells from either WT or TCRδ$^{-/-}$ mice dramatically attenuated active EAE, and this effect was associated with a significant reduction in CNS-infiltrating conventional αβ T cells. Moreover, the pivotal effect of cells that express TCRγ but not necessarily TCRδ in disease outcome was confirmed using the T cell transfer model of EAE. This thesis provides evidence that depletion of Vγ4^{+} T cells from either WT or TCRδ$^{-/-}$ cells significantly impairs their ability to transfer EAE, and this was reflected in a strong reduction in the cellular infiltrate in the CNS. αβVγ4 T cells traffic to the CNS as early as day 3 in EAE and secrete innate-IL-17 and IL-22 to amplify and augment the activation of Th17 cells during autoimmunity. Our findings identify a novel population of T cells that express Vγ4 and αβ TCRs and play a critical role in EAE.

The role of γδ T cells in EAE is a controversial subject, with some studies reporting an important or modest role for this subtype of T cells in disease (359). Although it has been reported that TCRδ$^{-/-}$ mice have significantly attenuated EAE, the present sudy shows that the attenuation was rather mild (272). Depletion of TCRδ T cells in both the developing and chronic phase of EAE significantly reduces the course of disease (409), however these mice still had αβVγ4 T cells. Studies in the adoptive transfer model of EAE also suggest a pathogenic role of γδ T cells, depletion of TCRδ T cells delayed the onset of EAE and significantly reduced clinical symptoms (273). In the relapsing remitting EAE model, mice treated with a TCRδ depleting antibody immediately before the onset of disease or during the chronic phase of disease had milder disease (267). However, the data presented in this study shows that while conventional γδ T cells do have a role in EAE, αβVγ4 T cells have an equally crucial role in the induction of EAE in WT and TCRδ$^{-/-}$ mice.

Vγ4 T cells have been shown to be extremely pathogenic in other models of autoimmunity such as psoriasis. Vγ4 T cells are the source of IL-17 in psoriatic plaques and mice injected

with an anti-Vγ4 depleting antibody had significantly decreased IL-17 production from dermal γδ T cells (274). Imiquimod-induced skin pathology was significantly reduced in TCRδ$^{-/-}$ mice, (which are deficient in γδ but not αβγ T cells); and interestingly, IL-17 producing γδ T cells were enhanced in the affected skin in psoriasis patients (274). Further evidence for the pathogenic role γδ T cells in autoimmunity was described in human IgA nephropathy, while αβ T cells were found in both stable and progressive IgA nephropathy, the presence of γδ T cells was only associated with progressive IgA nephropathy pathology (410). Moreover, infiltrating interstitial γδ T cells were observed in the kidneys of patients with Adriamycin-induced progressive glomerulosclerosis; where sequencing analysis revealed that the predominant infiltrating human γδ T cells in this disease used an invariant Vγ4/Vδ1 TCR (411). γδ T cells have also been shown to be the key pathogenic cell in a murine model of inflammatory cardiomyopathy, where Vγ4^{+} T cells enhance Th1 cell activation through IFN-γ to promote pathogenic CD8^{+} T cells (404). The depletion of Vγ4 T cells in an arthritis model in mice, CIA significantly reduced clinical severity of disease, and this was attributed primarily to a decrease in IL-17 in the joint and anti-collagen IgG2a antibodies (275). Analysis of the arthritic joint and the draining lymph nodes revealed that the vast majority of the Vγ4 cells produced IL-17 and moreover, the number of IL-17-producing Vγ4 T cells was equivalent to the number of Th17 cells (275). In light of the findings of the present study demonstrating that αβVγ4 T cells are pathogenic in EAE, αβVγ4 T cells may also play a role in other autoimmune diseases such as arthitis.

It has been reported that γδ T cells can have a role in recovery from EAE. CNS resident γδ T cells induce the production of IFN-γ by encephalitogenic T cells in the CNS, and IFN-γ is involved in recovery from EAE partly through the suppression of Th17 cells (412). Rajan et al observed that depletion of TCRδ cells resulted in a marked decrease in IFN-γ expression throughout the progression of disease. However, this corresponded with a reduction in the expression of IL-1β, IL-6 and TNF-α at the onset but not later in disease (409). The data presented in this study proposes that IL-17, but not IFN-γ producing-αβVγ4 and Vγ4δ T cells enhance the development and progression of EAE (172, 413). Another study in EAE demonstrated that γδ T cells can regulate disease recovery through Fas/Fas ligand-induced apoptosis of encephalitogenic CD4 T cells; however, they are responsible for mediating the initial flux of inflammation in the CNS (414). Vγ4 T cells have been shown to mediate protection during severe sepsis model through IL-17 secretion. In the cecal ligation and puncture (CLP) sepsis model Vγ4 T cells become activated earlier than αβ T cells to produce

IL-17 in the lungs, while Vγ1 and αβ T cells only produce IFN-γ (360). Remarkably, depletion of Vγ4 T cells diminished CLP induced IL-17 production in the lungs leading to exacerbated mortality from sepsis (360). Together with the results from the present study, this suggests that IL-17 secreting Vγ4 T cells can play a protective role in bacterial immunity and a pathogenic role in autoimmunity. It would be interesting to study the effect of αβVγ4 T cells in the late stages of EAE and in the resolution of sepsis given their rapid secretion of IL-17 and IL-22.

The pathogenic role of γδ T cells in EAE centres on their ability to produce early IL-17, which appears to amplify Th17 responses during disease. Data from our laboratory has identified a positive feedback loop where γδ T cell derived IL-17 amplifies Th17 effector function, and requires DCs in vitro. DCs express the IL-17R and secrete IL-23 in reponse to IL-17 (116). Vγ4δ T cells and αβVγ4 T cells contribute to the pool of IL-17 early in disease in order to promote the development of autoimmunity. In the adoptive transfer model of EAE, the removal of γδ T cells reduced the ability of encephalitogenic MOG specific T cells to transfer EAE to naïve recipients and led to reduced demyelination (273). γδ T cells promoted IL-12 production through macrophages to enhance the ability of encephalitogenic CD4 T cells to respond to MBP (273). This suggests that αβVγ4 T cells and γδ T cells may act like NK cells in the periphery, in their ability to prime encephalitogenic Th1 and Th17 cells through APCs, such as DCs and M1 macrophages (116, 263).

The attenuation of EAE in TCR$\delta^{-/-}$ mice was associated with a decreased production of MIP-1α and MCP-1, IL-1, IL-6, TNF-α, and IFN-γ (409, 415). The present study demonstrates that depletion of V$\gamma4^{+}$ cells from MOG-specific spleen and lymph node cells from WT or TCR$\delta^{-/-}$ mice significantly impaired their ability to transfer EAE to naïve recipents. FACS analysis revealed that recipient mice injected with pathogenic MOG-specific cells depleted of Vγ4 T cells from WT or TCR$\delta^{-/-}$ mice had reduced infiltration of IL-17 and IFNγ-producing T cells in the brain and the spinal cord. The data demonstrates that depletion of αβVγ4 or Vγ4δ T cells significantly reduces the concentration of MOG-specific IL-17A, IL-22, GM-CSF and IFN-γ produced in the culture prior to adoptive transfer. These results are consistent with the reports that innate-like lymphocytes contribute to the cytokine pool but also serve to augment cytkine secretion and effector function of T cells during autoimmunity, injury or inflammation (116, 406, 416).

γδ T cells have been shown to infiltrate the CNS in large numbers from as early as day 7 of EAE (116). Moreover, Vγ4 T cells are the predominant γδ T cells found in the brains of mice during EAE (116, 329). In vitro studies with human cells suggests that γδ T cells can play a role in demyelinating diseases as they are indirectly responsible for axonal demyelination through toxic destruction of oligodendrocytes (268, 269). The present study demonstrated that αβVγ4 T cells infiltrate the brain as early as day 3 in EAE, earlier than even conventional γδ T cells. This suggests that αβVγ4 may be early inducers of inflammation in both the periphery and the CNS during development of EAE. However, the accumulation of αβVγ4 T cells peaks at day 11 while Vγ4δ T cells persist until after day 14 post induction of EAE. This suggests that while αβVγ4 and γδ T cells both infiltrate the CNS during EAE, αβVγ4 may provide an earlier source of IL-17 production while conventional Vγ4δ T cells maintain IL-17 in the CNS. VLA-4 and CCR6 expression is upregulated on the surface of T cells during EAE and MS (122, 270, 271, 397, 417). The data presented in chapter 4 demonstrated that αβVγ4 T cells constitutively express VLA-4 and CCR6 (chapter 4). To our knowledge this is the first identification of VLA-4 and CCR6 as the integrins responsible for the migration of Vγ4 T cells to the CNS (271).

IL-1 is one of the key pro-inflammatory cytokines in the development of Th17 cells (75). As well as being an endogenous pyrogen, its induction has been shown to suppress regulatory cell function; excessive IL-1 signalling suppresses TGF-β induced Foxp3 expression (418). IL-1 in synergy with IL-23 can strongly induce IL-17 and IL-21 production from γδ T cells (116). Previously the data presented in chapter 4 showed that IL-1β in synergy with IL-23 drives innate-IL-17 and IL-22 production by αβVγ4 T cells. Consistent with the role of IL-1 in the induction of IL-17-producing T cells, IL-1R$^{-/-}$ mice develop extremely late onset EAE with very mild, if any, clinical signs (75, 116). Previous studies have demonstrated that removal of TCRδ^{+} cells reduced the ability of T cells from WT mice to induce EAE in IL-1R$^{-/-}$ receipient mice (75, 76, 116). The findings of this study now demonstrate that transfer of αβVγ4 T cells alone into IL-1R$^{-/-}$ mice is sufficient to develop mild clinical signs of disease. These findings are consistent with previous studies which show that innate-IL-17 derived from γδ and αβVγ4 T cells, is a critical factor in induction of EAE in IL-1R$^{-/-}$ mice, demonstrating a pathogenic role for these cells in EAE (116).

This and previous studies suggest a key role for γδ T cells in EAE to promote IL-17 production from Th17 cells by innate IL-17 production following stimulation with IL-1β and IL-23 (116). The findings presented in this study demonstrate that αβVγ4 T cells can respond

in the absence of TCR engagment to produce IL-17 and IL-22 in response to IL-1β and IL-23. IL-17 has many functions in the pathogenesis of CNS autoimmunity. IL-17 increases BBB permeability through disrupting the expression and organisation of the tight junction-associated protein ZO1 in the BBB endothelial cells (419, 420). It also downregulates occludin, a tight junction protein of the BBB (419). In MS patients IL-17R and IL-22R are expressed on BBB endothelial cells and their activation permeabilises the BBB (419). Furthermore, IL-17 and IL-22 mediate BBB disruption by increasing IL-6 CXL8 and CCL2 production by BBB endothelial cells in MS (419). IL-17-secreting Th17 cells have been shown to be cytotoxic to human neurons (419). IL-17 and IFN-γ secretion by myelin specific T cells in peripheral blood predicts EAE severity (216). Serum levels of IL-17 and its precursor cytokine IL-23, tend towards higher levels in MS patients and are associated with MS disease activity (421). In RRMS, IL-17A and IL-17F mRNA and protein are elevated in peripheral blood and are further enhanced during relapse (422, 423). The data presented in chapter 4 demonstrated that αβVγ4 T cells are an important source of innate-IL-17 and IL-22, suggesting that they may assist in disrupting BBB integrity during EAE.

Innate-like γδ T cells can amplify the host CNS immune response through the production of chemokines such as MCP-1 and MIP-1 alpha, which enhance leukocyte trafficking into the CNS during murine neurocysticercosis (424). It has been shown that γδ T cell-derived IL-17 is key in promoting cellular traffic to sites of inflammation, as seen with leukocyte recruitment during CNS inflammation (425) and with neutrophil recruitment in *Escherichia coli* infection (426). Neutrophils are capable of producing IL-17 and TNF-α, and as the first cells to traffic to inflamed sites, they represent around 5% of the CNS cellular infiltrate during EAE (427, 428). Furthermore, depletion of neutrophils reduces the severity of EAE (429). IL-17 produced by innate sources early after induction of EAE may activate neutrophils and amplify Th17 responses; this may explain how depletion of Vγ4δ or αβVγ4 T cells impacts on the development of EAE (116, 406, 430, 431). IL-17A has been shown to activate microglia, and this may represent an early event in the CNS where γδ T cells and αβVγ4 T cells promote immunopathology (432). It would be interesting to investigate the kinetics of cytokine secretion and chemokine production by αβVγ4 T cells in other models of autoimmunity and infection.

This study presents convincing evidence for a central role for IL-17-secreting αβVγ4 T cells in the pathogenesis of EAE. As a result of their capacity to promote the activation of Th17 cells and produce inflammatory cytokines, such as IL-17 and IL-22, early in the course of

disease, αβVγ4 T cells play a key role in the development of EAE. Furthermore, IL-17 and IL-22 from αβVγ4 T cells may promote the early activation of neutrophils and microglia, and enhance BBB permeability. The findings of this study demonstrate that αβVγ4 T cells infiltrate the CNS of WT and TCR$\delta^{-/-}$ mice during EAE and depletion of these cells significantly attenuates active and passive EAE. Consequently, αβVγ4 T cells may represent highly selective drug target for the treatment of MS and other autoimmune diseases.

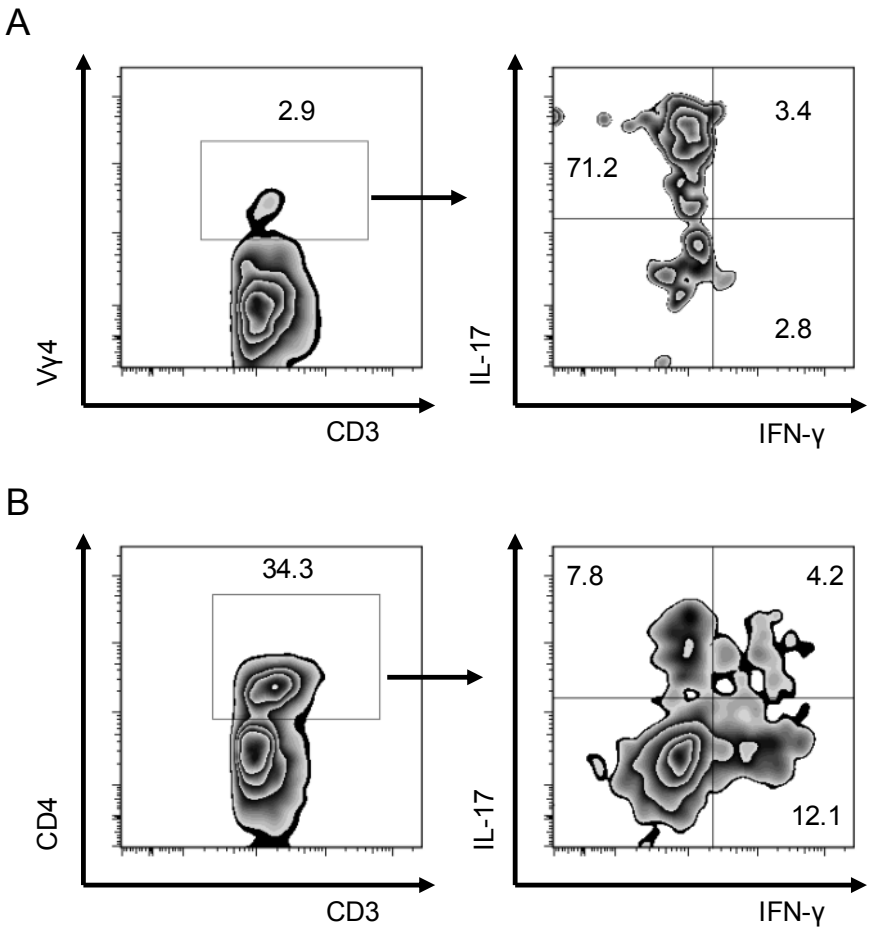


Figure 5.1: Vγ4 T cells infiltrate the CNS and produce IL-17 during EAE. EAE was induced in C57BL/6 mice. Mononuclear cells from the brains were stimulated for 6 hours with PMA (10 ng/ml), ionomycin (1 μg/ml) and brefeldin A (5 μg/ml) on day 20 post EAE induction. Cells were washed and stained for surface CD4, CD3,Vγ4 and intracellular IL-17 and IFN-γ and analysed by flow cytometry. (A) Vγ4 T cells were gated for FSc versus SSc. Vγ4 T cells gated on live, single, $CD3^+$ cells. Vγ4 T cells were analysed for IL-17 and IFN-γ production. (B) CD4 T cells were gated for FSc versus SSc. CD4 T cells gated on live, single, $CD3^+$ cells. CD4 T cells were analysed for IL-17 and IFN-γ production. FACS plots are representative of 5 independent mice (n=5) and data are representative of three independent experiments.

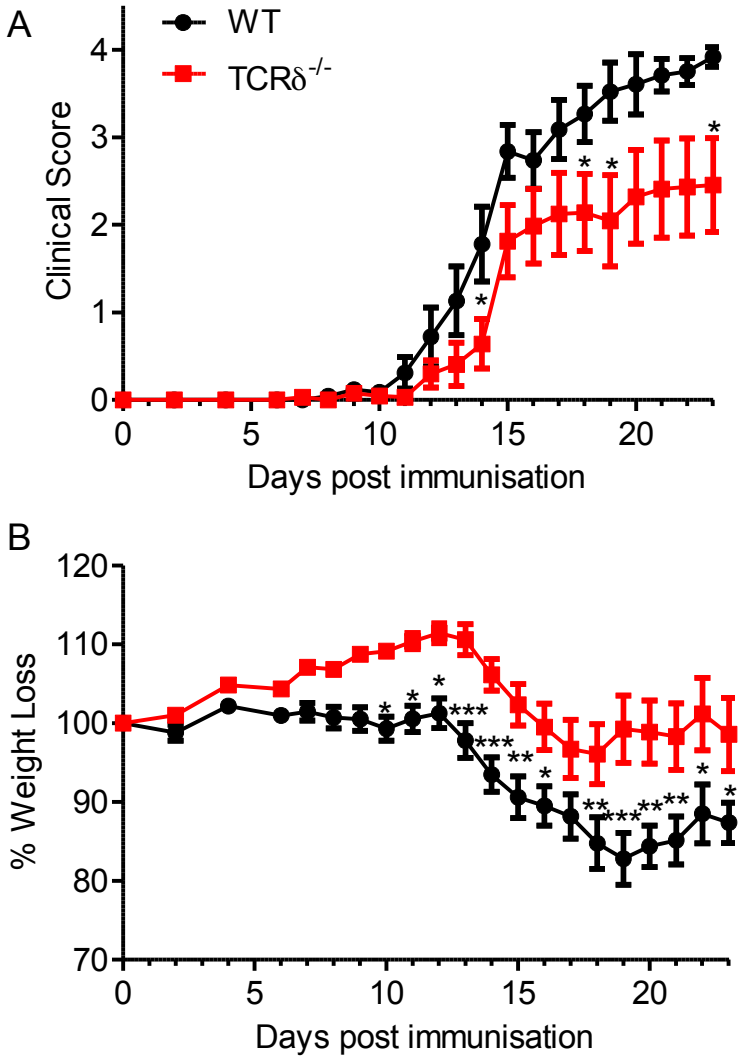


Figure 5.2: TCRδ$^{-/-}$ mice have a mild reduction in clinical symptoms of EAE compared with WT mice. EAE was induced in C57BL/6 or TCRδ$^{-/-}$ mice by immunization with MOG and CFA followed by pertussis toxin administration. Mice were observed daily for clinical symptoms of EAE (A) and weight loss (B). *p < 0.05, ** p<0.01, ***p<0.001 WT versus TCRδ$^{-/-}$. Results are expressed as mean ± SEM clinical score and percentage weight loss of n=20/24 mice/group from four combined experiments. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ by two one-way *ANOVA* and Bonferroni post hoc analysis.

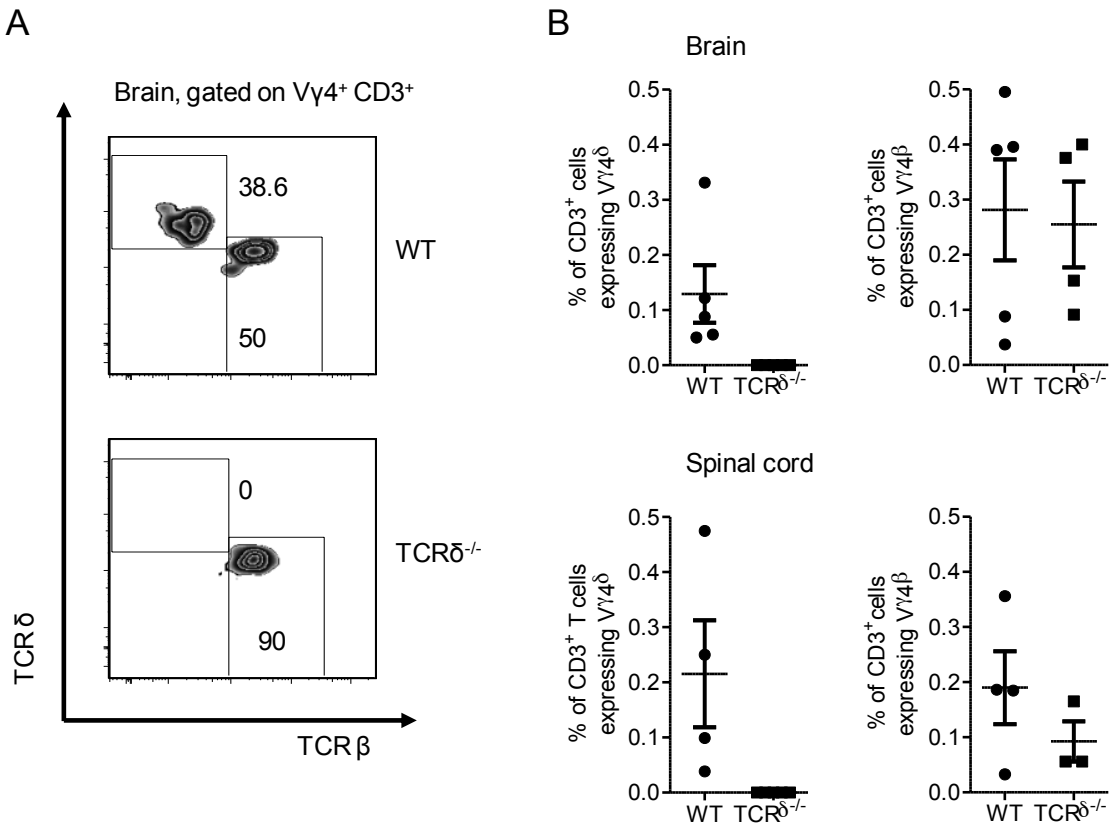


Figure 5.3: Frequency of Vγ4 T cells in the brains and spinal cord of mice with EAE at day 23. EAE was induced in C57BL6 and TCRδ$^{-/-}$ mice and at peak of disease, mice were sacrificed. Mononuclear cells from the brains and spinal cord were stimulated for 6 hours with PMA (10 ng/ml), ionomycin (1 μg/ml) and brefeldin A (5 μg/ml). Cells were washed and stained for surface CD4, CD3, TCRδ and Vγ4 and intracellular IL-17 and IFN-γ and analysed by flow cytometry. Cells were gated for FSc versus SSc. Vγ4 T cells were gated on live, single, CD3^{+} cells. Vγ4 T cells were analysed for TCRδ versus TCRβ expression. FACS plots are representative of 6 independent mice. Data represent mean ± SEM of Vγ4δ and αβVγ4 T cells for n=6 mice/group and data is representative of at least three independent experiments.

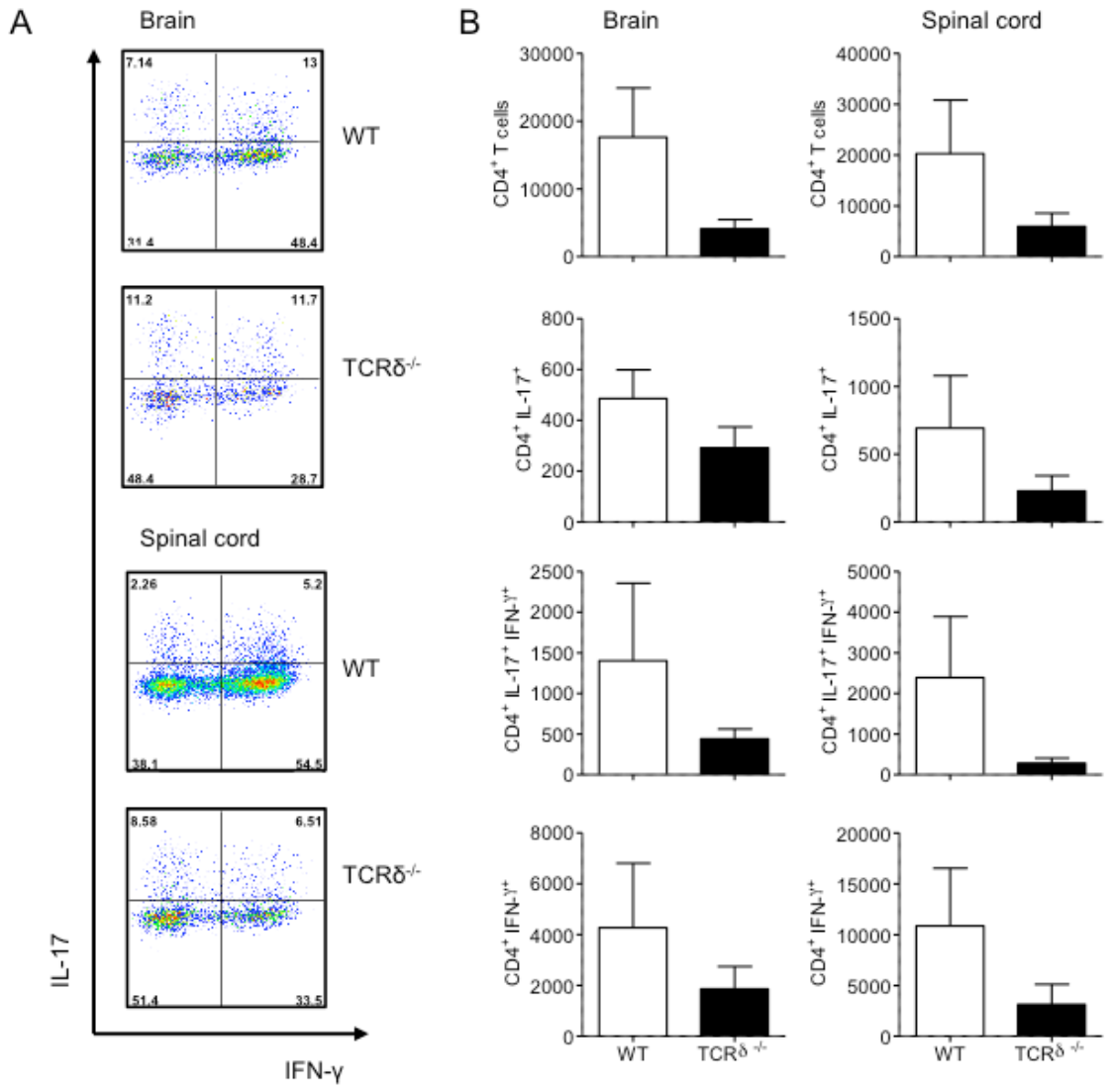


Figure 5.4: Reduced absolute number of CD4 T cells secreting IL-17 and IFN-γ in the CNS of TCRδ$^{-/-}$ mice compared to WT mice with EAE. EAE was induced in C57BL6 and TCRδ$^{-/-}$ mice and 23 days post induction of EAE animals were sacrificed. Mononuclear cells from the brains and spinal cord were restimulated for 6 hours with PMA (10 ng/ml), ionomycin (1 µg/ml) and brefeldin A (5 µg/ml). Cells were washed and stained for surface CD4, CD3 and intracellular IL-17 and IFN-γ and analysed by flow cytometry. Cells were gated for FSc versus SSc. CD4 T cells were gated on live, single CD3$^+$ cells. CD4$^+$ T cells were analysed for IL-17 versus IFN-γ production. (A) Representative FACS plots of 6 independent mice (B) Data represent mean ± SEM of IL-17$^+$, IFN-γ$^+$, IL-17$^+$IFN-γ$^+$-producing CD4 T cells for n=6 mice/group. Data is representative of at least three independent experiments.

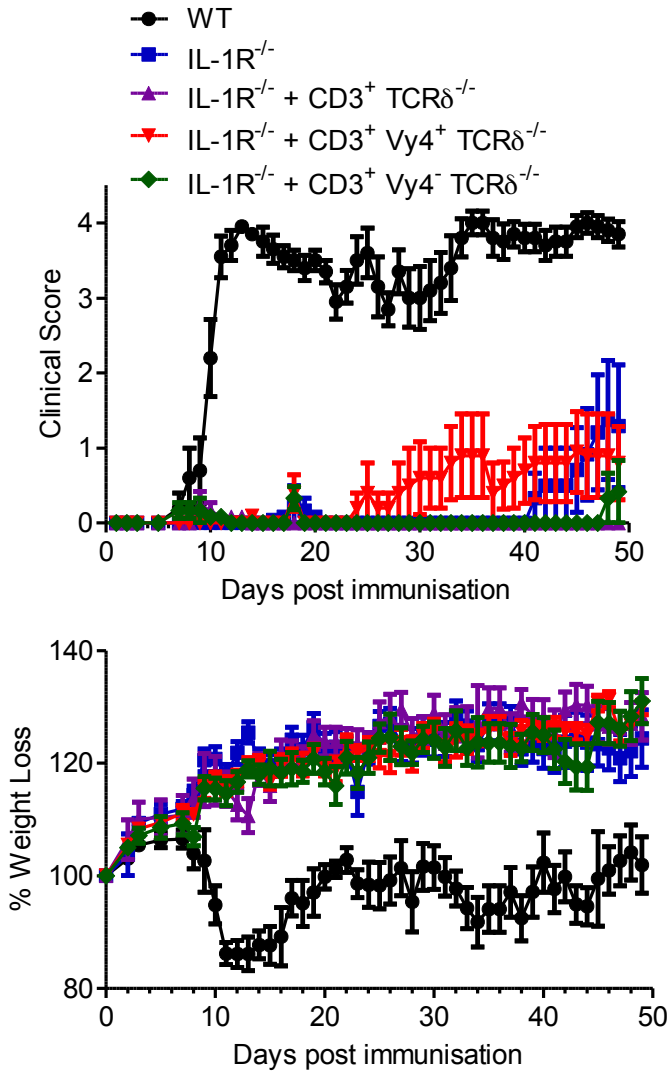


Figure 5.5: Transfer of αβVγ4 T cells alone is sufficient to induce mild EAE. IL-1R$^{-/-}$ mice were injected one day prior to induction of EAE with CD3^{+} T cells from TCRδ$^{-/-}$ mice (5 x 10^{6}) or with Vγ4^{+} cells from TCRδ$^{-/-}$ mice (2 x 10^{4}) or CD3^{+} T cells depleted of Vγ4 cells (2 x 10^{4}) from TCRδ$^{-/-}$ mice. EAE was induced in C57BL/6 mice, IL-1R$^{-/-}$ mice and IL-1R$^{-/-}$ mice that received cell transfers. Mice were observed daily for clinical signs of EAE and weight loss. Data represents mean ± SEM clinical score/weight loss for n=4 or 5 mice per group and data is representative of two independent experiments.

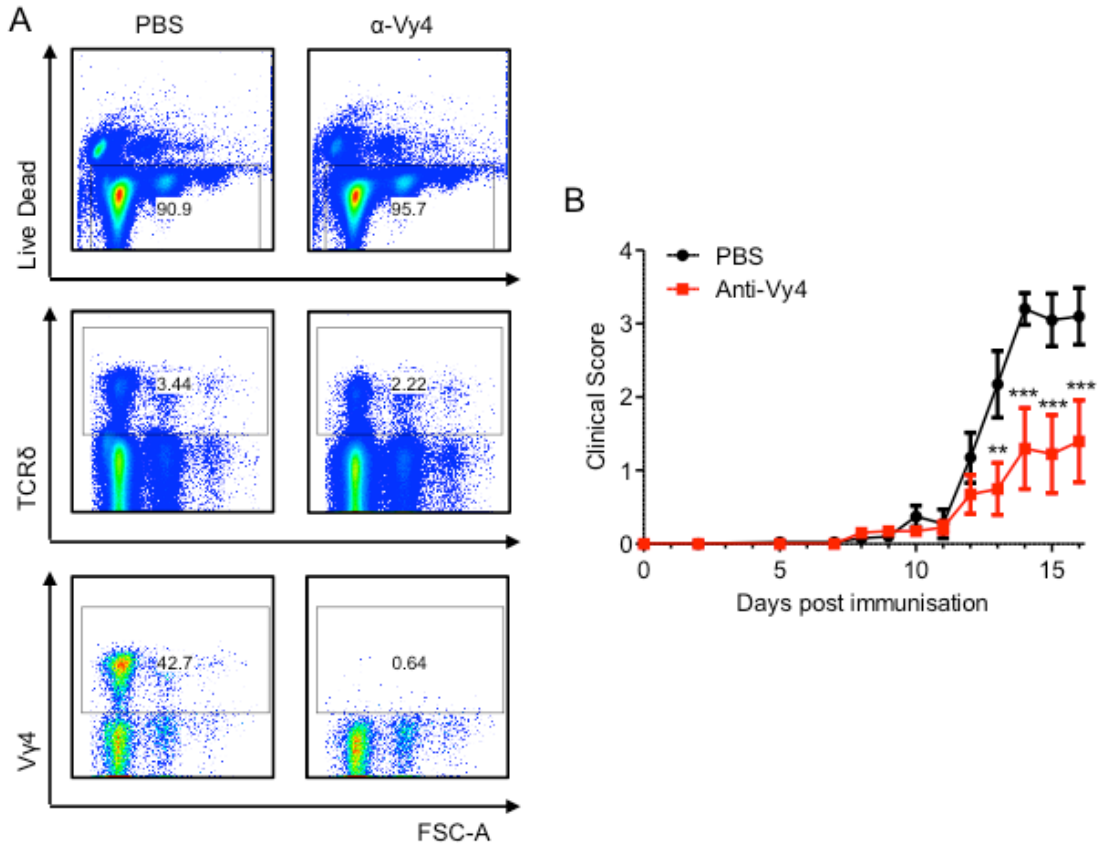


Figure 5.6: Depletion of Vγ4 T cells in WT mice significantly reduces the severity of actively induced EAE. (A) C57BL/6 mice were injected with PBS or anti-Vγ4 (UC3-10A6 250 µg). After 72 hours spleens were harvested and examined for Vγ4 expression to determine efficacy of the depletion. T cells were gated for FSc versus SSc. TCRδ T cells were gated on live, single, $CD3^+$ versus FSc. Vγ4 T cells were gated on live, $CD3^+$ cells versus FSc. FACS plots are representative of 5 independent mice (n=5). (B) EAE was induced in C57BL/6 mice by immunisation with MOG and CFA. Mice were i.p. injected with anti-Vγ4 (UC310A6 250 µg/mouse) on day -1, 4, 9 and 14 or with PBS as the vehicle control. Mice were assessed for the development of EAE by clinical score (B). *$p < 0.05$, **$p < 0.01$ versus PBS treated control by two one-way *ANOVA* and Bonferroni post hoc analysis. Results are expressed as mean ± SEM clinical score for n=5 or 6 mice/group and are representative of two independent experiments

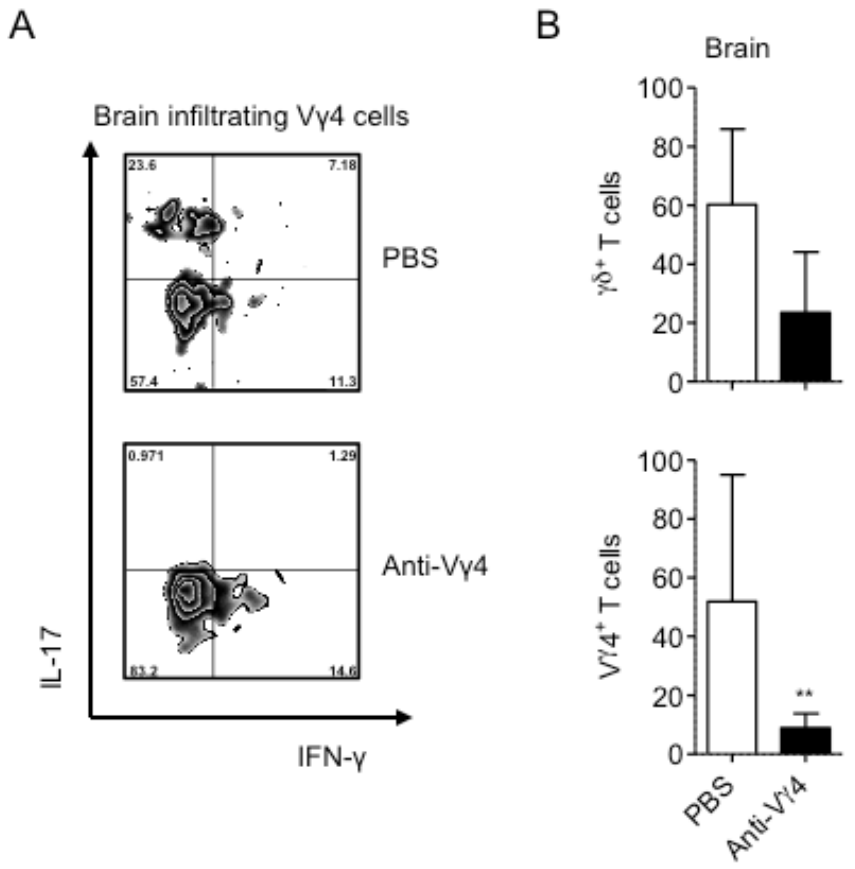


Figure 5.7: Depletion of Vγ4$^+$ T cells during development of EAE reduces infiltration of TCRδ and Vγ4 T cells into the CNS. Mice were either treated with PBS or anti-Vγ4 (UC310A6 250 μg/mouse) on day -1, 4, 9 and 14. EAE was induced in C57BL6 mice and on day 23 animals were sacrificed. Mononuclear cells from the brains were stimulated for 6 hours with PMA (10 ng/ml), ionomycin (1 μg/ml) and brefeldin A (5 μg/ml). Cells were washed and stained for surface CD4, CD3 and Vγ4 expression and intracellular IL-17 and IFN-γ and analysed by flow cytometry. T cells were gated for FSc versus SSc. TCRδ T cells were gated on live, single, CD3$^+$ cells versus FSc. Vγ4 T cells were gated on live, CD3$^+$ versus FSc. (A) FACS plots are representative of 5 independent mice. (B) Results are expressed as mean ± SEM of TCRδ expressing CD3$^+$ T cells or Vγ4 expressing CD3$^+$ T cells from n=5 mice/group and data is representative of two independent experiments.

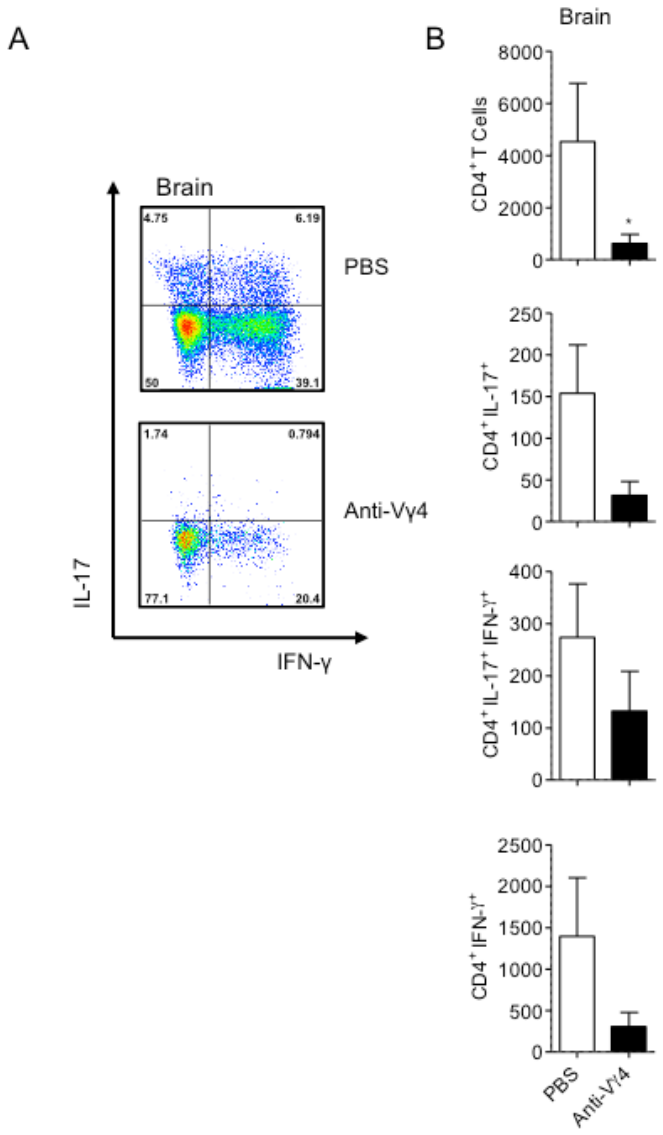


Figure 5.8: Depletion of Vγ4$^+$ T cells during development of EAE reduces infiltration of IL-17 and IFN-γ producing CD4$^+$ T cells into the CNS. Mice were either treated with PBS or anti-Vγ4 (UC310A6 250 µg/mouse) on day -1, 4, 9 and 14. EAE was induced in C57BL6 mice and 23 post induction mice were sacrificed. Mononuclear cells from the brains were stimulated for 6 hours with PMA (10 ng/ml), ionomycin (1 µg/ml) and brefeldin A (5 µg/ml). Cells were washed and stained for surface CD4, CD3 and intracellular IL-17 and IFN-γ and analysed by flow cytometry. CD4 T cells were gated for FSc versus SSc. CD4 T cells gated on live, single, CD3$^+$ cells. CD4 T cells were analysed for IL-17 and IFN-γ production. (A) FACS plots are representative of 5 independent mice (n=5). (B) Results are expressed as mean ± SEM of IL-17$^+$, IFN-γ$^+$, IL-17$^+$IFN-γ$^+$-producing CD4 T cells from n=5 mice/group and data is representative of two independent experiments.

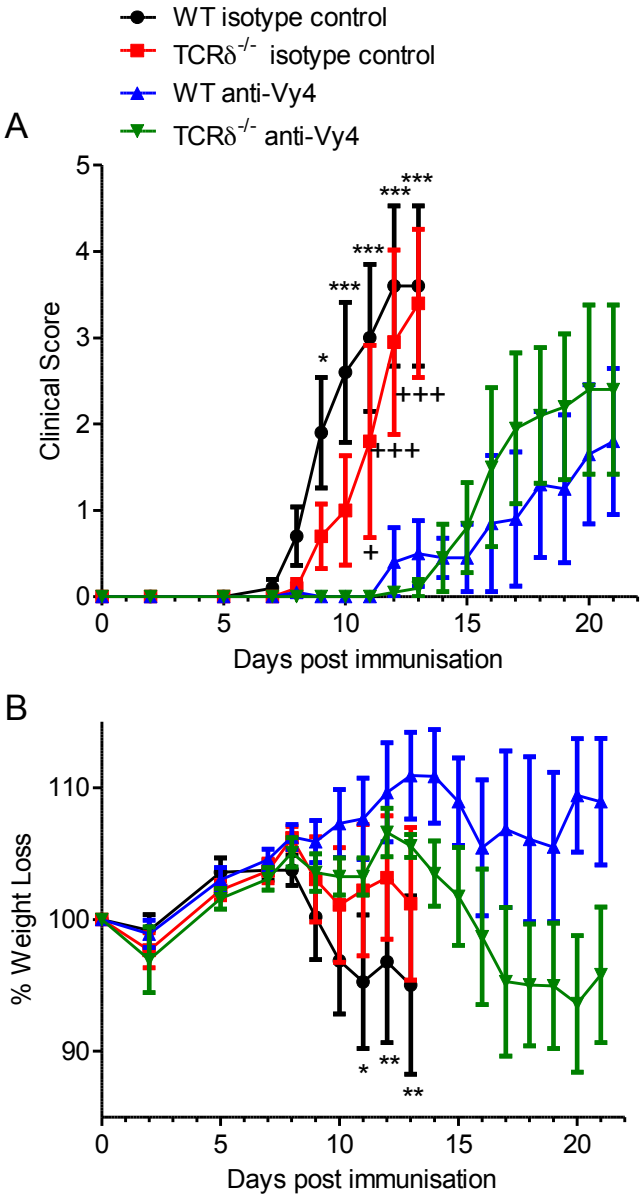


Figure 5.9: Attenuation of EAE in WT and TCRδ$^{-/-}$ mice with depletion of Vγ4^{+} cells. EAE was induced in C57BL/6 and TCRδ$^{-/-}$ mice by immunisation with MOG and CFA followed by i.p. administration of PT. Mice were treated by i.p. injection anti-Vγ4 depletion antibody UC310A6 (250 µg/mouse) on d 1, 2, 5, 8, 11, 14, 17 and 20 or with an isotype control. Mice were assessed for the development of EAE by clinical score (A) and weight loss (B). *$p < 0.05$, **$p < 0.01$ and ***p<0.001 WT PBS treated versus WT anti-Vγ4, $^{+}p < 0.05$, $^{++}p < 0.01$ and $^{+++}$p<0.001 TCRδ$^{-/-}$ PBS treated versus TCRδ$^{-/-}$ anti-Vγ4. Results are expressed as mean ± SEM clinical score or weight loss for n=5 or 6 mice/group and data is representative of two independent experiments.

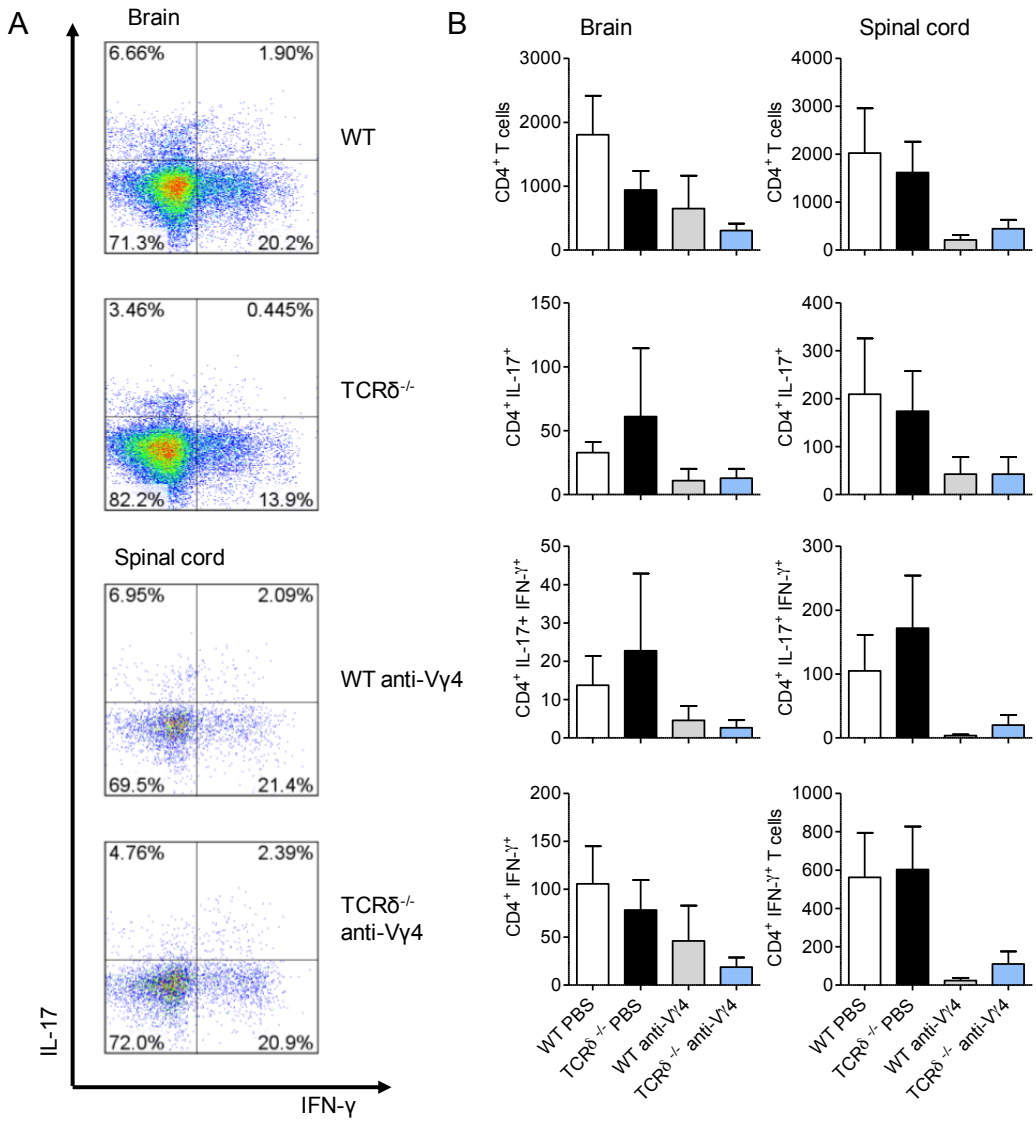


Figure 5.10: Depletion of Vγ4$^+$ cells during development of EAE in WT and TCRδ$^{-/-}$ mice reduces infiltration of IL-17 and IFN-γ producing CD4$^+$ T cells into the CNS. EAE was induced in C57BL/6 and TCRδ$^{-/-}$ mice. Mice were either treated with an isotype control or anti-Vγ4 (250µg/mouse) on day -1, 2, 5, 8, 11, 14, 17 and 20 days post induction of EAE mice were sacrificed. Mononuclear cells from brains and spinal cords were restimulated for 6 hours with PMA (10 ng/ml), ionomycin (1 µg/ml) and, brefeldin A (5 µg/ml). Cells were washed and stained for surface CD4, CD3 and intracellular IL-17 and IFN-γ and analysed by flow cytometry. CD4 T cells were gated for FSc versus SSc. CD4 T cells gated on live, single, CD3$^+$ cells. CD4 T cells were analysed for IL-17 and IFN-γ production. (A) FACS plots are representative of 5 independent mice (n=5). (B) Results are expressed as mean ± SEM of IL-17$^+$, IFN-γ$^+$, IL-17$^+$IFN-γ$^+$-producing CD4 T cells from n=5 mice/group and data is representative of two independent experiments.

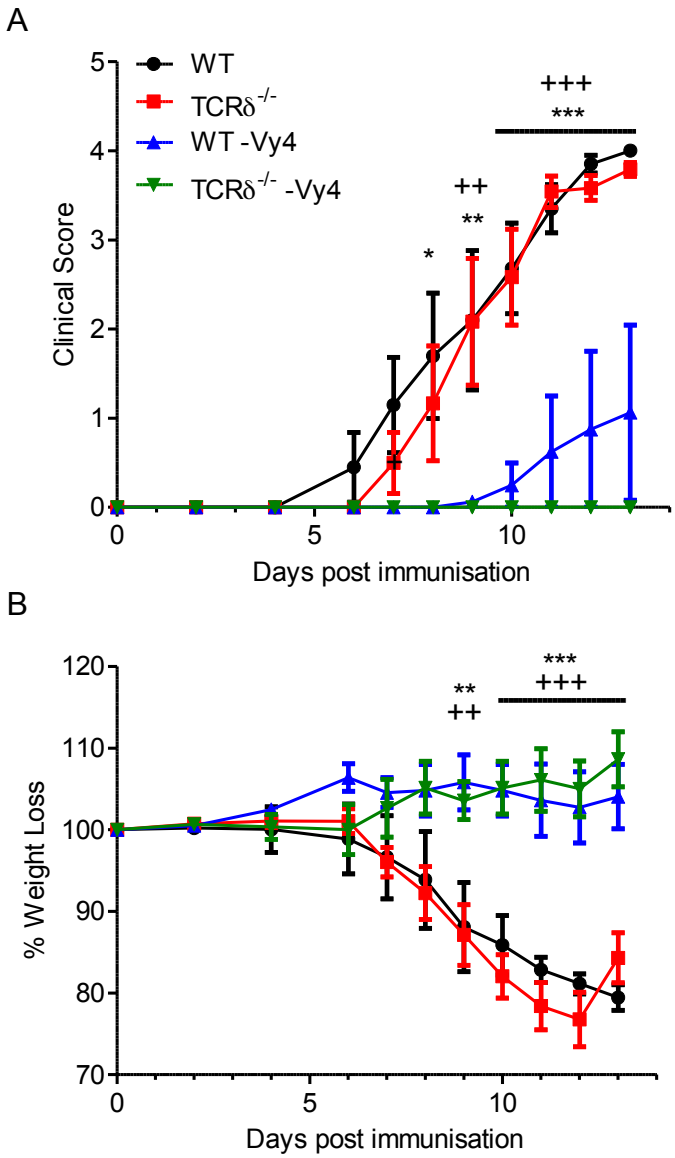


Figure 5.11: Depletion of Vγ4$^+$ cells from MOG-specific WT and TCRδ$^{-/-}$ cells significantly impair the ability to transfer EAE. EAE was induced in C57BL/6 or TCRδ$^{-/-}$ mice by immunisation with MOG and CFA. After 10 days lymph nodes and spleen cells were excised. WT cells, TCRδ$^{-/-}$ cells and TCRδ$^{-/-}$ cells FACS depleted of Vγ4$^+$ cells were cultured with IL-1 and IL-23 (10 ng/ml) with MOG (100 µg/ml). Cells (15 x 10^6) were injected i.p and mice were assessed for the development of EAE by clinical score (A) and weight loss (B). *p < 0.05, **p < 0.01, ***p<0.001 WT donor cells versus TCRδ$^{-/-}$ donor cells depleted of Vγ4$^+$ cells, ^+p < 0.05, ^{++}p < 0.01, $^{+++}$p<0.001 TCRδ$^{-/-}$ donor cells versus TCRδ$^{-/-}$ donor cells depleted of Vγ4$^+$ cells. Results are expressed as mean ± SEM of clinical score and percentage weights loss for n=5 mice/group and data is representative of two independent experiments.

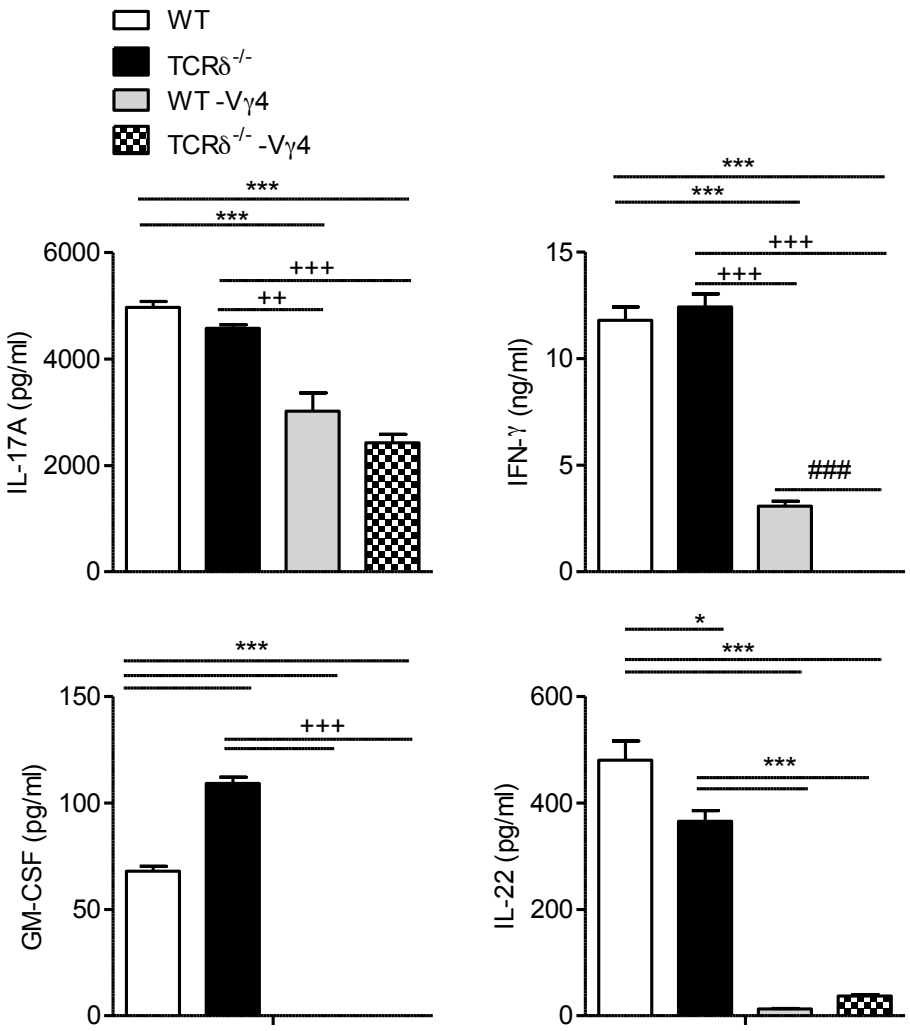


Figure 5.12: Depletion of Vγ4$^+$ cells from MOG-specific WT and TCRδ$^{-/-}$ cells significantly reduces IL-17, IFN-γ, GM-CSF and IL-22 secretion. WT or TCRδ$^{-/-}$ mice were immunized with MOG and CFA. Mice were sacrificed 10 days post-induction and their lymph node and spleen cells were untreated or depleted of Vγ4$^+$ cells by FACS and cells were stimulated in vitro with MOG (100 μg/ml) and IL-12 (10 ng/ml) or MOG, IL-23 (10 ng/ml) and IL-1β (10ng/ml). After 72h culture, IL-17A, IFN-γ, GM-CSF and IL-22 concentrations in the supernatants were quantified by ELISA. *P<0.05, **P<0.01, ***P<0.001 WT vs TCRδ$^{-/-}$, WT -Vγ4 and TCRδ$^{-/-}$ -Vγ4; ++P<0.01, +++P<0.001 TCRδ$^{-/-}$ vs WT -Vγ4 and TCRδ$^{-/-}$ -Vγ4; ###P<0.001 WT -Vγ4 vs TCRδ$^{-/-}$ -Vγ4. Results are expressed as mean ± SD concentration for tripilcate flasks and data is representative of two independent experiments.

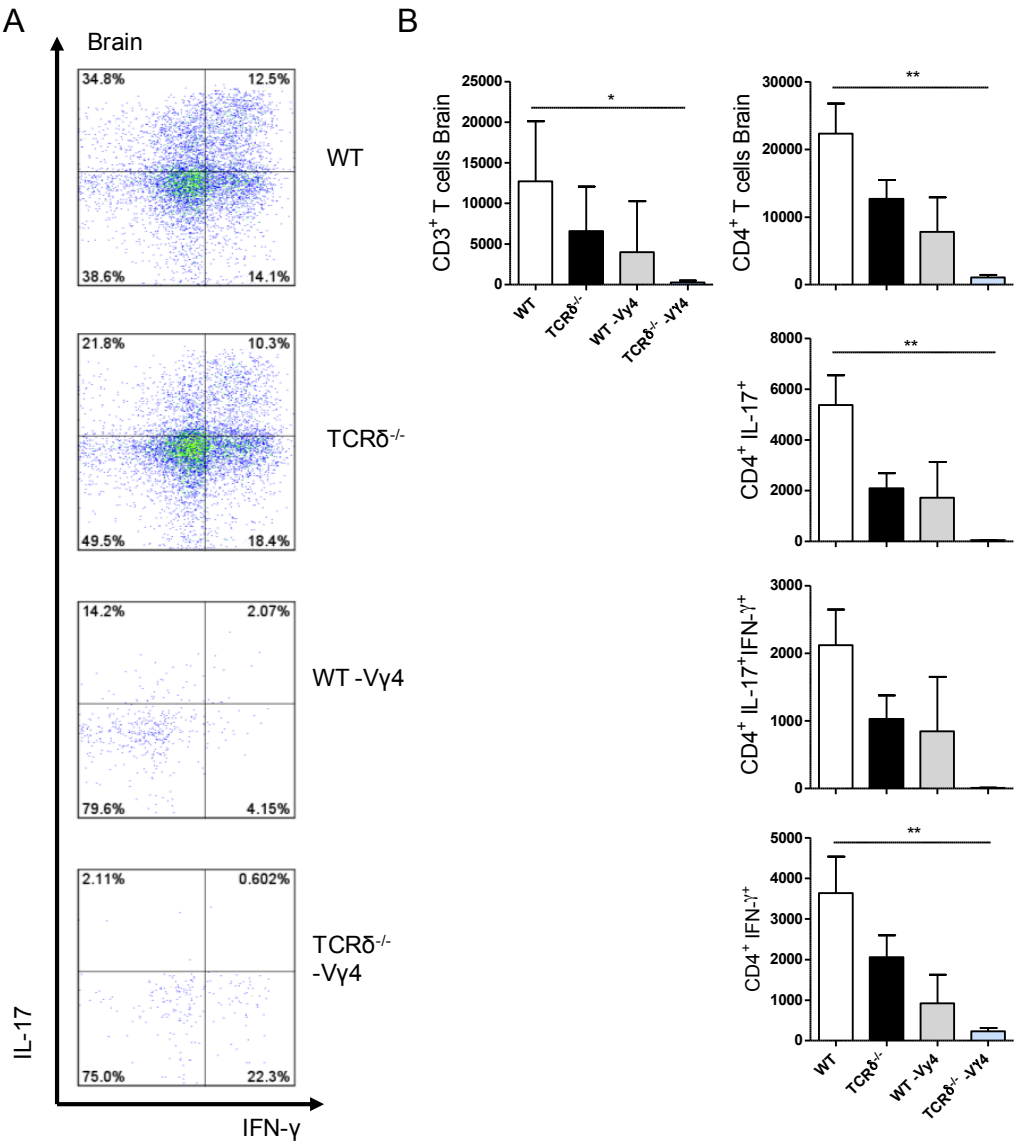


Figure 5.13: Depletion of Vγ4$^+$ cells from MOG-specific WT and TCRδ$^{-/-}$ cells reduces the infiltration of IL-17 and IFN-γ producing CD4$^+$ T cells into the CNS. MOG specific WT, TCRδ$^{-/-}$ or TCRδ$^{-/-}$ donor cells depleted of Vγ4 cells were transferred into naïve recipients to induce adoptive transfer EAE and 12 days post cell transfer animals were sacrificed. Mononuclear cells from the brains and spinal cord were stimulated for 6 hours with PMA (10 ng/ml), ionomycin (1 μg/ml) and brefeldin A (5 μg/ml). Cells were washed and stained for surface CD4, CD3 and intracellular IL-17 and IFN-γ and analysed by flow cytometry. T cells were gated for FSc versus SSc. CD4 T cells were gated on live, single, CD3$^+$ cells. CD4$^+$ T cells were analysed for IL-17 versus IFN-γ production. (A) Representative FACS plots of 5-6 independent mice. (B) Results are expressed as mean ± SEM of IL-17$^+$, IFN-γ$^+$, IL-17$^+$IFN-γ$^+$-producing CD4 T cells from n=5 mice/group and data is representative of two independent experiments.

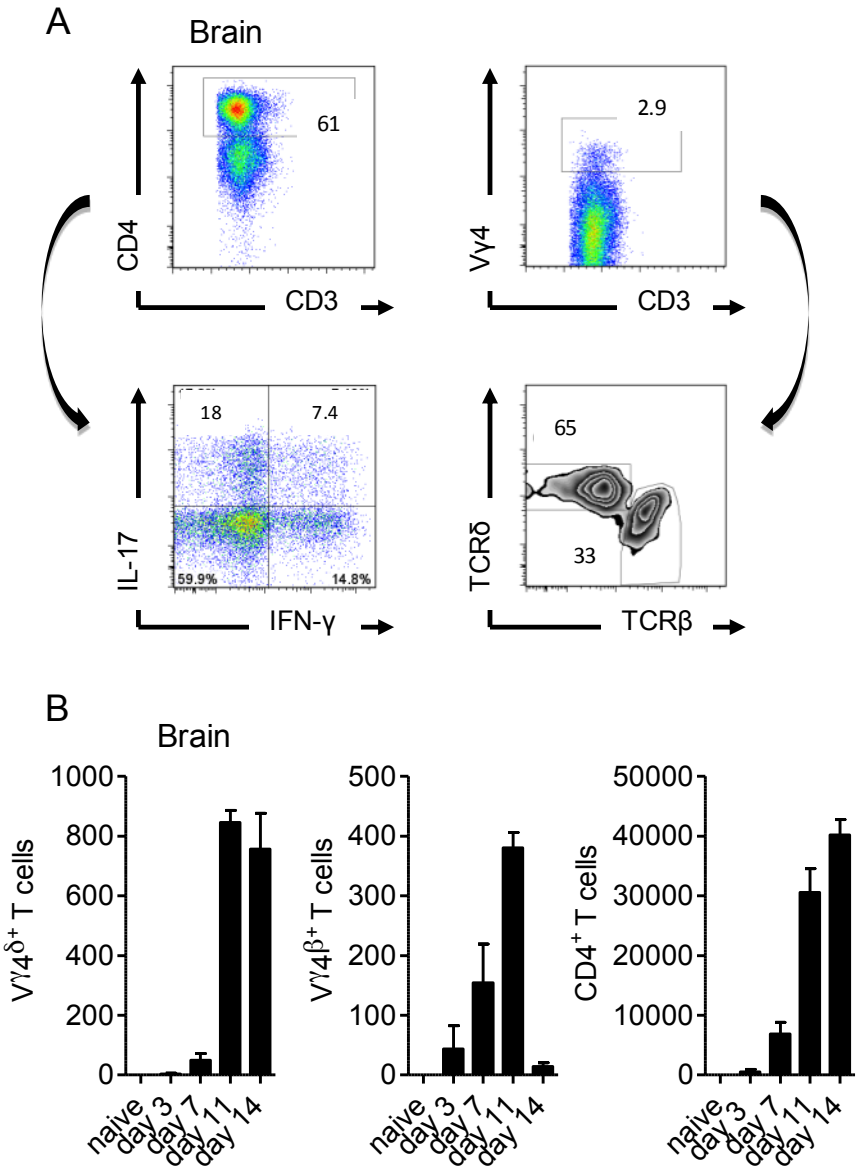


Figure 5.14: Vγ4β and Vγ4δ T cells infiltrate the CNS as early as 3 days post induction of EAE. EAE was induced in C57BL/6 mice. Mononuclear cells from the brains and spinal cord were stimulated for 6 hours with PMA (10 ng/ml), ionomycin (1 µg/ml) and brefeldin A (5 µg/ml). Cells were washed and stained for surface CD4, CD3, TCRβ, TCRδ,Vγ4 and intracellular IL-17 and IFN-γ and analysed by flow cytometry. T cells were gated for FSc versus SSc and were gated as live, single, $CD3^+$ cells. $CD4^+$ T cells gated versus CD3, Vγ4 T cells gated versus CD3. Vγ4 T cells were gated on TCRδ versus TCRβ expression. (A) Representative FACS plots of 4 independent mice. (B) Mean absolute number of $TCR\delta^+$ or $TCR\beta^+V\gamma4^+$ and $CD4^+$ $CD3^+$ T cells from the brains of mice on day 0, 3, 7, 10 and 14 days after induction of EAE. Results are expressed as mean ± SEM of cellular infiltrate from n=6 mice/group and data is representative of two independent experiments.

Chapter 6.

General Discussion

Maintaining homeostasis in the immune system is a fine balance between mediating immunity to infection and contributing to the development of autoimmune diseases. Despite their fundamental role in immunity to infection and immune regulation effector T cell subsets and their secreted cytokines have become a major target for autoimmune therapies. Deficits in peripheral or central tolerance can lead to the development of autoimmunity, where the host's immune system causes immunopathology in specific organs or tissues. Genetic and environmental factors, such as infection history have a major influence on susceptibility to autoimmune disease, such as MS (433). Animal models of autoimmunity, such as EAE can assist our understanding of how dysregulation of the immune response can lead to the development of autoimmunity (434). The data presented in this thesis demonstrates that infection of mice with a pathogen that commonly infects humans, *B. pertussis,* significantly attenuates the clinical course of EAE induced by active immunization or cell transfer. This was reflected in a significant reduction in VLA-4 and LFA-1 expression on T cells and infiltration of IL-17^{+}, IFN-γ^{+} and IFN-γ^{+}IL-17^{+} CD4 T cells into the CNS. Infection with *B. pertussis* induced IL-10 production from dendritic cells in vitro and enhanced the frequency of IL-10-producing CD25^{-}Foxp3$^{+/-}$CD4^{+} T cells *in vivo*. Furthermore, the suppressive effects of *B. pertussis* infection on EAE were lost in IL-10$^{-/-}$ mice. The findings demonstrate that a bacterial infection of the respiratory tract can attenuate EAE by promoting production of the anti-inflammatory cytokine IL-10. IL-10 suppresses licensing of autoaggressive T cells in the lungs, thereby preventing their migration to the CNS. The data presented in this thesis adds further light to the hygiene hypothesis, which proposes that incidence of infection inversely correlates with incidence of immune-mediated disorders, such as MS and asthma (435).

Previous studies have shown that infection with certain pathogens such as extracellular bacteria and helminth parasites is an effective therapeutic approach to autoimmunity in animals models and clinical trials (311, 436). Similar to the present study with *B. pertussis* infection, helminth infections elicit immunomodulatory effects on the host immune system through the induction of anti-inflammatory cytokines and regulatory immune cells, such as alternatively activated macrophages and Treg cells (437-440). The immune response induced to combat infections can in certain circumstances directly counteract the pro-inflammtory responses that drive autoimmunity, which may explain a lower prevalence of autoimmune disease in rural parts of many developing countries (308, 441). Supporting this hypothesis, infection with the helminth *S. mansoni* has been shown to be protective against colitis and type 1 diabetes animal models through the induction of Th2 and Treg cells (342, 442-445).

S. mansoni infection also significantly reduced the severity of CIA; parasite infected mice had elevated levels of IL-4 and IL-10 with a corresponding downregulation of IFN-γ, TNF-α and IL-17A (446). MS patients infected with helminth parasites have significantly lower disease score index, which corresponded with an increase in myelin-reactive IL-10 and TGF-β-producing cells compared with uninfected patients (309). Correale and Farez found an increase in the number of circulating $CD25^{+}CD4^{+}FoxP3^{+}$ T cells in infected MS patients, suggesting that infection induced Treg cells that can alter the course of disease (309). This thesis extended these findings demonstrating that a bacterial infection with *B. pertussis* can reduce the severity of EAE through systemic and local suppression of pro-inflammatory T cells.

The lung is a reservoir of myelin-reactive T cells which become programmed to traffic from the lung through the bloodstream and into the CNS during EAE (351). The results presented here suggest that the induction of $CD25^{-}Foxp3^{+/-}$ T cells and *B. pertussis*-specific IL-10 in the lungs of mice with EAE suppresses this licensing of encephalitogenic T cells, preventing their egress from the lung to the CNS. Other bacteria such as *Shigella flexneri* have been shown to modulate integrin expression on mucosal epithelial cells through effector OspE proteins in order to sustain the infection (447). The findings of this thesis are supported by other studies where IL-10 induced by bacterial or helminth infections can suppress the severity of EAE. Administration of three strains of probiotic *Lactobacillus* induced Treg cells, which suppressed the clinical symptoms of EAE in an IL-10-dependent manner (448). Moreover, *P. acidilactici* administered orally suppressed the clinical severity of EAE in both prophylactic and therapeutic settings by inducing IL-10-producing Tr1 cells (449). Infection with the helminth *T. spiralis* was found to attenuate EAE through the induction of IL-10 and Treg cells (450, 451). Interestingly, prior infection with *H. hepaticus* results in the induction of Treg cells that prevent bacteria-induced colitis in mice (452). The current study demonstrates that infection with *B. pertussis* attenuates EAE through the induction of IL-10, therefore *B. pertussis* infection may potentially modulate other models of autoimmunity.

Harnessing IL-10 to suppress effector T cell function has been utilized in the treatment of MS. The therapeutic effect of glatiramer acetate and IFN-β in MS have been associated with the induction of IL-10 (354, 355). The number of IL-10-secreting PBMCs are low in untreated MS patients, however they are augmented during and after IFN-β treatment (453). Furthermore, it has been shown that serum levels of IL-10 and $IL\text{-}10^{+}$ $CD8^{+}$ T cells are

increased during remission in MS patients (335, 454). IL-10 dampens effector T cell responses by directly suppressing cytokine secretion or inhibiting APC function (49, 50). The findings presented in this thesis are consistent with studies in EAE where deletion of IL-10 exacerbated disease and overexpression of IL-10 ameliorated clinical signs (352, 353). Conversely, Vγ1 γδ T cells have been shown to inhibit IL-10-secreting Treg cells during airway hypersensitivity and this suppression of IL-10 may in part explain the pathogenic role of γδ T cells in EAE (455). The findings of this study demonstrated that IL-10 can downregulate the expression of the trafficing molecules VLA-4 and LFA-1 on encephalitogenic T cells, demonstrating a novel mechanism of IL-10-mediated suppression and the potential of bacterial-derived products as therapeutic agents against autoimmuntity.

Unlike conventional αβ T cells, cytokine stimulation alone is sufficient for activation of γδ T cells, making these cells rapid and potent mediators of inflammation (164). γδ T cells are considered to play a key pathogenic role in the development of EAE, however, the data in TCRδ$^{-/-}$ mice, deficient in γδ T cells are not that convincing. This thesis presents the discovery, characterization and functional analysis of a novel innate-like T cell subset, termed αβγ T cells that are highly pathogenic in EAE. Through cellular and molecular studies it was determined that αβγ T cells express TCRγ, but also TCRα and TCRβ, but lack TCRδ chains. Importantly, the findings of this study reveal that Vγ4^{+} T cells are present in TCRδ$^{-/-}$ mice, and these T cells co-express TCRαβ. The perception that TCRδ$^{-/-}$ mice are devoid of all functional γTCR expressing T cells is incorrect; the findings of the present study demonstrate that Vγ4^{+} and Vγ1^{+} are present in TCRδ$^{-/-}$ mice. The use of this strain of mice for elucidating the role of γδ T cells in immunity should therefore be undertaken with caution as highly pro-inflammatory TCRγ expressing cells remain. The findings from this thesis explain the previous inconsistencies in the data on the role of γδ T cells in autoimmunity that were based on TCRδ-depletion or TCRδ$^{-/-}$ mice, often misleadingly called γδ$^{-/-}$ mice, which of course retain αβγ T cells. Moreover, these data qualify previous conclusions drawn from other studies demonstrating dysfunctional γδ T cell development in TCRβ$^{-/-}$ mice, as our data now show that highly pro-inflammatory αβVγ4 T cells cells are missing in these mice (367). αβVγ4 have many features of γδ T cells, such as innate activation in the absence of TCR engagement and expression of CD73 and CD27 (119, 196). Interestingly, αβVγ4 T cells also share phenotypic features with the IL-17-producing nTh17 lineage, with constitutive expression of CD44, ICOS, IL-23R, IL-1R1, CCR6 and CD49d, a subunit of the integrin VLA-4 (94).

The data presented in this thesis adds further light to the mechanisms of T cell development and lineage commitment. There are many contradicting theories on γδ and αβ T cell development, both γδ and αβ are derived from a common precursor thymocyte and some investigators have suggested that pre-T cell thymocytes first endeavour to become γδ T cells, and if this process fails, it then develops into an αβ T cell (200, 456). However, the findings of this study demonstrating the expression of TCRα, TCRβ and TCRγ on the same cell would argue against this theory. It has also been suggested that strength of TCR signalling decides lineage fate. The γδTCR appear to deliver stonger signals than the pre-TCR and based on these observations, TCRγδ expressing thymocytes receive strong signals to commit them to the γδ lineage and weaker signalling is associated with αβ T cell development (178). A weak or moderate TCRγδ signal may propogate αβVγ4 T cell development, as these cells straddle innate and adaptive T cell phenotypes. As DP T cells control the development of γδ T cells in the thymus, this would suggest that the αβγ T cells are DP T cells that rearrange the TCRVγ4 chain after the DP stage (367). The data from the embryonic thymus demonstrating that this novel T cell subtype are primarily $CD4^+CD8^+$ during development is consistent with this hypothesis. Unlike αβγ T cells, γδ T cells do not express CD4 or CD8 co-receptors, remaining DN during thymic development (457).

However, previous studies have demonstrated that expression of TCRβ with TCRγδ forces the cell to commit to the γδ lineage where it remains DN and upregulates CD73, consistent with the findings of this study (196). The development of this novel population may also be the result of early expression of αβTCR in DN thymocytes. Ordinarily TCRβ-preTCRα expression would shut down TCRγ rearragement, however if this population has already been driven towards the γδ lineage fate through moderate γTCR engagement this would allow TCRγ expression (178). Previous studies have described that the αβTCR can be upregulated in place of the γδTCR on cells which belong to the γδ fate and this may explain the expression of αβTCR pairing with Vγ4 or Vγ1 by this novel population (184).

Recently, VγCβ TCR expressing T cells have been described; trans-rearrangement between the Vγ2 gene and a variety of Jβ or Dβ genes results in a hybrid TCR (458). This study by Bowen et al may provide evidence for how TCRVγ4 may be incorporated into the αβTCR to form functional αβVγ4 T cells, as they demonstrate that a hybrid VγCβ TCR can assoicate with an intact TCRα. However, VγCβ are distinct from αβVγ4 T cells as they display an αβ-like T cell phenotype, commitment to either $CD4^+$ or $CD8^+$ lineage and are MHC I or II restricted. Interestingly, the increased frequency of αβVγ4 T cells observed in MHC $II^{-/-}$ mice

suggest that αβγ T cell development may be controlled by MHC II restricted T cells or as previously described by trans-conditioning in the thymus (367, 459). αβVγ4 T cells were also detectable in MHC $I^{-/-}$ indicating that αβVγ4 T cells develop in absence of MHC I and MHC II. However, it is possibile that some αβVγ4 T cells are MHC I dependent and others are MHC II dependent, therefore it would be important to examine the presence of αβVγ4 T cells in mice lacking MHC I and II. The findings of this study indicate that the paradigm of TCR development may need to be revisited. The question of TCR chain pairing is crucial for understanding the development of αβVγ4 T cells, it is currently unclear if the TCRγ heterodimerizes with TCRα or TCRβ chains or if the three TCR chains trimerize to form a functional TCR-CD3 complex. These questions will be addressed in future studies, but are complicated by the number of these rare cells required for extensive biochemical studies.

γδ T cells have previously been shown to constitutively express IL-23R and are an important source of innate IL-17, IFN-γ, IL-21 and IL-22, making them potent inducers of the inflammatory response (108, 116, 152, 460). The present study reveals that αβVγ4 T cells isolated from WT or $TCR\delta^{-/-}$ mice respond to stimulation with IL-1β and IL-23 in the absence of TCR engagement to produce IL-17A and IL-22. Furthermore, the data demonstrates that αβVγ4 T cells constitutively express IL-23R and IL-1R1, unlike inducible Th17 cells (461). Previous studies in a murine colitis model proposed that IL-23 can promote innate-IL-17 production by an unknown source (462). The current study demonstrates a novel mechanism of IL-1 and IL-23-induced innate-IL-17A and IL-22 production through αβVγ4 T cells. In order to confirm that that αβVγ4 T cells can respond to cytokine stimulation in the absence of TCR engagement, it is critical in future studies to analyze αβVγ4 T cells responses when TCR signalling is impaired through the use of TCR signalling mutant mice or inhibitors of Lck, Ras or ZAP70 (463). The proliferation of αβVγ4 T cells in response to MOG indicates that the TCR has an antigen specificity for MOG^{33-55}, to validate this finding it would be necesasary in future studies to determine if αβVγ4 T cells bind to the MOG-specific tetramer. Moreover, it would be interesting to determine if this an autoantigen specific effect by assessing αβVγ4 proliferation in an OVA-transgenic system.

The data presented in this thesis support the hypothesis that innate-like lymphocytes such as γδ and αβγ T cells can modulate CD4 T cell responses in EAE (116, 273). γδ T cells have previously been shown to modulate T cell effector function, such as IL-2 production by $CD4^+$ T cell and CTL activity (464). In a contact hypersensitivity model γδ T cells have also been shown to augment CD4 T cell-mediated immunopathology (465) and play an

immunosuppressive role in dermatitis (466). This augmentation of CD4 T cell responses by innate-like lymphocytes can impact on host defense. In WNV infection, $V\gamma4^+$ T cells suppress IFN-γ-secreting $V\gamma1^+$ cell expansion through TGF-β, which leads to enhanced viremia and brain inflammation (361). Conversely in BCG infection, TNF-α-secreting Vγ4 T cells can enhance IL-12 production by DCs to augment cytotoxic CD8 T cell responses (467). The fact that αβVγ4 T cells produce IL-17 and IL-22 in an innate-like fashion suggests that they may play a role as sentinel cells in the immune response to infection. In light of the findings of this study, IL-1β and IL-23 responsive αβVγ4 may play a role in gut and lung homeostasis through IL-17 and IL-22-mediated maintenance of barrier integrity, anti-microbial peptide generation and regulation of intestinal inflammation (81, 468, 469). IL-22 has also been shown to regulate IFN-γ-mediated inflammation in the lungs of asthmatic patients (470).

γδ T cells are found in the brains of MS patients and in EAE, a high frequency of infiltrating γδ T cells found in the CNS of mice with EAE are $V\gamma4^+$ T cells (116, 292, 293). The data presented in this thesis and previous studies in the laboratory demonstrate that a very high frequency of $V\gamma4^+$ T cells in the brains of mice with EAE secrete IL-17, a higher percentage than $CD4^+$ Th17 cells. αβVγ4 T cells can infiltrate the brain as early as day 3 post induction of EAE indicating that αβVγ4 and γδ T cells initiate IL-17 production during EAE. Activated Th17 cells then contribute and maintain this IL-17 pool later in disease (471). This study demonstrates that αβVγ4 T cells were capable of proliferating in response to restimulation with MOG, and the production of IL-17A in response to IL-1β and IL-23 is enhanced in the presence of MOG. This suggests that αβγ T cells are key pathogenic T cells in precipitating EAE by proliferating in response to IL-1β, IL-23 and MOG which drive the activation of Th17 cells. Further evidence for this assertion was provided by experiments in the passive EAE model, where depletion of αβVγ4 T cells significantly decreases the production of MOG-specific IL-17A, IL-22, GM-CSF and IFN-γ. Remarkably, depletion of αβVγ4 from $TCR\delta^{-/-}$ or WT mice in active or passive EAE significantly reduces the clinical severity of EAE, supporting the hypothesis that αβVγ4 T cells play a central role in the pathogenesis of EAE.

Previous studies have described that following induction of EAE by immunisation with MOG and CFA, a high frequency of IL-17-producing γδ T cells were detected in the draining lymph nodes days before the appearance of IL-17-producing αβ T cells (472). However, these studies utilized TCRδ antibodies to examine γδ T cells and this failed to include αβVγ4 T cells. Data from the present study indicates that this novel T cell subset may become activated

before or at least simultaneously to γδ T cells during EAE and in other inflammatory milieu. Roark et al demonstrate that Vγ4 T cells are the main IL-17-producing T cell in the draining lymph node and joint during CIA (275). In transplantation models, TCRγ expressing T cells are the main IL-17-producing cells during acute allograft rejection, as graft rejection was significantly delayed in TCRγ$^{-/-}$ mice (473). Consistent with studies in EAE, the dominant IL-17-secreting γδ T cells were Vγ4^{+} in mice with psoriasis (116, 166, 274). Interestingly, Ramirez-Valle et al reported that imiquimid-sensitized mice show more rapid skin inflammation, greater proliferation and IL-17 secretion by Vγ4^{+} T cells upon imiquimod rechallenge due to increased IL-1R1 expression. The current study demonstrates that αβVγ4 T cells express high levels of IL-1R1, CD44 and rapidly produce IL-17; it would be interesting in future studies to determine if αβVγ4 T cells display a memory-like phenotype in MOG rechallenge experiments. Overall, these data are consistent with our findings that IL-17-producing αβVγ4 T cells and γδ play a pathogenic role early in EAE and justifies further research into the role of αβVγ4 T cells in other autoimmune diseases.

Therapeutics designed to treat MS through the blockade of IL-17 have had limited success thus far, despite excellent efficacy in the treatment of psoriasis and psoriatic arthritis (474, 475). Recently it has been suggested that the role of IL-17 in MS may be opposite to that in psoriasis. GM-CSF-secreting CD4 T cells are highly pathogenic in both EAE and MS. However, in humans IL-17 has been shown to inhibit the development of GM-CSF-producing CD4 T cells, suggesting that IL-17 may be protective in MS (80, 476, 477). The current study demonstrates that depletion of αβVγ4 T cells significantly impairs MOG-specific GM-CSF secretion, indicating that αβVγ4 T cells can augment GM-CSF secreting CD4^{+} T cells in EAE. Recently it has been demonstrated that Vγ4 T cells require the expression of sphingosine 1-phosphate (S1P) receptor 1 (S1P1) in order to traffic to the brain during EAE (478). The success of FTY720 in treating MS may be attributed to inhibiting the egress of both helper T cells and Vγ4 T cells from the lymph node (479). However, treatment of MS patients with biological drugs designed to suppress the induction, migration or function of CD4^{+} T cells, such as natalizumab, although effacacious come with an increased risk of infection, in particular progressive multifocal leukoencephalopathy (PML) (417). This study highlights the contribution of a very small T cell subset, the IL-17-producing αβVγ4 T cells, in mediating and enhancing pathology during EAE and this may present a more selective target for treating MS. The findings of this thesis provide strong evidence for the central role of αβVγ4 T cells in the pathogenesis of EAE and contribute to the knowledge of T cell

function and regulation in EAE. The identification of this novel subset of T cells may have major relevance in the field of unconventional T cell biology and may represent a novel target in the treatment of autoimmune diseases.

Chapter 7.

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