Development of IL-17A-associated autoimmunity

Eve Hornsby

Division of Molecular Immunology
National Institute for Medical Research
The Ridgeway, Mill Hill,
London
NW7 1AA

A thesis presented in partial requirement for the degree of Doctor of Philosophy of University College London

2010

Statement of declaration

I, Eve Hornsby, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Acknowledgements

I would like to begin by thanking my supervisor, Gitta for initiating this project and for providing me with consistent support and guidance throughout.

I would also like to thank all the members of labs 312 and 326 who have provided a source of technical advice, scientific discussions and encouragement. Thanks especially go to Ying for re-cloning the IL-17A_Cre construct and for generating the reporter mice and to Keiji for his friendliness, patience and knowledge. Thanks also to Georgina for helping me out with the EAE model and to Thea – who I can't thank enough for helping me to prepare for presentations, for reading a large amount of this thesis and for helping me to keep things in perspective.

The members of my thesis committee, Ben and George, a huge thank you for constructive criticism, practical advice and ideas, and particularly to Ben for welcoming me in the lab this last year.

I would also like to express my gratitude to Alexandre and his lab for my first experience of cloning and to Anna for giving me a solid introduction into the world of targeting ES cells. Thanks also go to everyone in MI and ICB for continued enthusiasm and interest in what I am doing.

A big thank you also goes to those I have shared the PhD adventure with – Claire, Athina, Tim, Alan, Fatima, Becky, Becki, Vicky, Bob, Chris and Josquin, and also to my family - thanks for persevering!

Abstract

Autoimmunity results from a breakdown in tolerance to self-antigens. Interleukin-17A (IL-17A) is a cytokine that has been implicated in the development of certain autoimmune disorders, notably multiple sclerosis and its mouse model experimental autoimmune encephalomyelitis (EAE). In order to further understand mechanisms that lead to the development of autoimmunity, the objectives of this study were to investigate the sequence of immunological events that lead to the development of an autoimmune response and to generate and characterise a reporter mouse for IL-17A.

EAE is a well-established model of an autoimmune response directed against self-antigens in the central nervous system and mimics many aspects of the human disease multiple sclerosis. EAE is a CD4 T cell-mediated disease, in that these cells can be used to transfer disease to naïve recipient mice. Following EAE induction, IL-17A-expressing cells were increased in frequency within the CD4 and γδ T cell populations in the draining lymph nodes, with a simultaneous increase in the number of these cell populations in the blood. Disease development was associated with the appearance of IL-17A and IFN-γ-expressing CD4 T cells, as well as IL-17A-expressing γδ T cells in the spinal cord. EAE induction requires the systemic administration of pertussis toxin for disease development. It was found that pertussis toxin enhanced antigen-specific IL-17A and IFN-γ production in the periphery.

An IL-17A reporter mouse was generated in which activation of the IL-17A promoter is reported by expression of Enhanced Yellow Fluorescence Protein (EYFP). In order to generate the mouse, a strain was first generated in which Cre recombinase expression is driven by the IL-17A promoter. This mouse was then crossed with a ROSA-26_EYFP strain in which expression of EYFP in the ubiquitously expressed ROSA-26 locus is usually inhibited by the presence of a LoxP-flanked-transcriptional stop sequence. Expression of Cre recombinase would

remove the transcriptional stop sequence, leading to irreversible expression of EYFP in all cells that had activated IL-17A and their progeny.

The results from this study suggest that pertussis toxin can amplify antigen-specific cytokine responses in EAE, an effect which could be attributed to enhancing disease pathogenesis. The IL-17A reporter mouse will be an invaluable tool to investigate the generation, lifespan and function of IL-17A-expressing cells in the development of immune responses.

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°C	degrees Celsius
%	percentage
7AAD	7-aminoactinomycin D
AB-IMDM	air-buffered Iscove's modified Dulbecco's medium
ACK	Ammonium chloride potassium carbonate solution
AHR	aryl hydrocarbon receptor
AIRE	autoimmune regulator protein
APC	antigen presenting cell
BAC	bacterial artificial chromosome
BBB	blood-brain barrier
BCR	B cell receptor
BCSFB	blood-cerebrospinal fluid barrier
Bp	Base pair
BSA	bovine serum albumin
CCL	chemokine (C-C motif) ligand
CCR	chemokine (C-C motif) receptor
CD	cluster of differentiation
cDNA	complementary DNA
CFA	complete Freund's adjuvant
CIA	collagen-induced arthritis
CNS	central nervous system
CO_2	carbon dioxide
CSF	cerebrospinal fluid
CTLA	cytotoxic T lymphocyte antigen
CXCL	chemokine (C-X-C motif) ligand
CXCR	chemokines (C-X-C motif) receptor
dATP	deoxyadenosine triphosphate
DC	dendritic cell
dCTP	deoxycytidine triphosphate

dGTP	deoxyguanosine triphosphate
dLN	draining lymph node
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
D-PBS	Dulbecco's phosphate buffered saline
dTTP	deoxythymidine triphosphate
EAE	experimental autoimmune encephalomyelitis
EAU	experimental autoimmune uveoretinitis
(E)GFP	(enhanced) green fluorescent protein
ES	embryonic stem
(E)YFP	(enhanced) yellow fluorescent protein
FACS	fluorescence activated cell sorting
FCS	foetal calf serum
FICZ	6-formylindolo [3,2-b] carbazole
Foxp3	forkhead box P3
FSC-A	forward scatter-area
FSC-H	forward scatter-height
FSC-W	forward scatter-width
g	gramme
GCSF	granulocyte colony stimulating factor
GMCSF	granulocyte macrophage colony stimulating factor
GWAS	genome-wide association study
HBS	hepes buffered saline
HEL	hen egg lysosyme
iCre	modified Cre recombinase
ICAM-1	Intra-cellular adhesion molecule 1
IDDM	insulin-dependent diabetes mellitus
IFA	incomplete Freund's adjuvant
IFN	interferon
Ig	immunoglobulin

IL	interleukin
IL-17AR	IL-17A receptor
IMDM	Iscove's modified Dulbecco's medium
IRES	internal ribosomal entry site
Kbp	Kilobase pair
KIR	killer inhibitory receptor
L	litre
LB	Lura Bertani
LFA-1	Leukocyte function-associated antigen-1
LIF	leukaemia inhibitory factor
LPS	lipopolysaccharide
M	molar
MBq	megabequerel
MBP	myelin basic protein
μg	microgramme
mg	milligramme
MHC	major histocompatibility complex
μl	microlitre
mL	millilitre
μm	micrometre
mM	millimolar
MOG	myelin oligodendrocyte glycoprotein
mRNA	messenger RNA
MS	multiple sclerosis
Mtb	*Mycobacterium tuberculosis*
Neo	neomycin resistance gene
NFκB	nuclear factor kappa-light chain enhancer of activated B cells
ng	nanogramme
NK	natural killer
NOD	nucleotide-binding oligomerization domain
ORF	open reading frame

OVA	chicken ovalbumin
PAMP	pathogen associated molecular pattern
PBS	phosphate buffered saline
PdBu	phorbol 12,13 dibutyrate
pg	picogram
PKG	phosphoglycerate kinase
PKR	protein kinase receptor
PLP	proteolipid peptide
PMNL	polymorphonuclear leukocyte
PCR	polymerase chain reaction
PRR	pattern recognition receptor
PTX	pertussis toxin
RA	rheumatoid arthritis
RAG	recombination activating gene
RORγt	RA-related orphan receptor gamma t
RPMI	Roswell Park Memorial Institute-1640
S-1-P	sphingosine-1-phosphate
SAP	shrimp alkaline phosphatase
SDS	sodium dodecyl sulfate
SLE	systemic lupus erythematosus
SPF	specific pathogen free
SSC-A	side scatter-area
STAT	signal transducer and activator of transcription
SV40	simian virus 40
T bet	T box expressed in T cells
TCR	T cell receptor
TGFβ	transforming growth factor beta
Th	T helper cell
TLR	toll-like receptor
TNF	tumour necrosis factor
TRA	tissue-restricted antigen

T-reg	regulatory CD4 T cell

Chapter 1: Introduction

1.1 Overview of the immune system

The immune system is comprised of an integrated network of cells and molecules that functions to protect organisms from infection.

Contact with potentially disease-causing agents can occur through internal or external epithelial surfaces, including the respiratory tract, gastrointestinal tract, urogenital tract and the skin. Pathogen entry can be prevented at these sites by a number of constitutive defense mechanisms. These include the presence of epithelial cell tight junctions which physically restrict pathogen entry, the secretion of anti-microbial agents and mucus by epithelial cells which can prevent microorganisms from adhering to the epithelium, and the presence of commensal microflora which out-compete pathogens for essential nutrients and attachment sites. Despite these defences, pathogenic microorganisms such as bacteria, viruses, parasites and fungi have developed ways to invade and establish infection in host tissue.

Invading pathogens trigger the activation of a wide range of host receptors, referred to as Pathogen Recognition Receptors (PRRs). PRRs encompass secreted receptors, produced by the liver, such as mannan-binding lectin, C-reactive protein and serum amyloid protein; cell-surface receptors such as macrophage mannose receptor and toll-like receptors (TLRs); and intra-cellular receptors such as the protein kinase receptor (PKR) and nucleotide-binding oligomerization domain (NOD) proteins. The molecules that are responsible for binding and activating these PRRs are conserved products of microbial pathogens known as Pathogen-Associated Molecular Patterns (PAMPs) and include lipopolysaccharide (LPS), which is a cell wall component of gram negative bacteria; unmethylated CpG DNA motifs found at a high frequency in microbial DNA sequences; bacterial flagellin; double stranded

RNA produced during viral infection; and zymosan, a component of yeast cell walls (Janeway 1989; Janeway and Medzhitov 2002). The recognition of exogenous pathogenic agents by host receptors initiates a cascade of events, culminating in the recruitment and activation of a number of short-lived effector cells to the site of infection, with the primary goal of eliminating the pathogen. This process is described as inflammation and can result in clearance of the pathogen and repair of damaged host tissues. However, in the event that the pathogenic agent persists, clonal expansion of lymphocytes with receptors that specifically recognize antigens within the invading pathogenic organism is initiated. The expansion and activation of these clones of cells and the effector mechanisms associated with them generates a highly specific immune response directed at the pathogenic insult. The long-term survival of small numbers of these antigen-specific clones allows for the development of immunological memory, which provides immediate immunity upon re-infection with the same pathogen.

The immune system is broadly categorized into innate and adaptive immunity. Innate immunity is the non-specific response induced by any invading pathogen and generally consists of all the cells and molecules involved in inflammation. Adaptive immunity is the clonal expansion of pathogen-specific lymphocytes. Despite this characterization, the two systems are closely interlinked and cells of the innate and adaptive immune systems can interact via the secretion of soluble molecules such as interleukins (ILs), chemokines and immunoglobulins.

1.2 The main cellular components of the immune system

1.2.1 Granulocytes and mast cells

Granulocytes or polymorphonuclear leukocytes (PMNLs) include neutrophils, basophils and eosinophils. These cells are short-lived, circulating in the blood of healthy individuals. During an immune response, they are released in increased

numbers from their progenitors in the bone marrow and extravasate into infected tissue, where they contribute to pathogen eradication. Neutrophils represent the major component of blood granulocytes, and are involved in the immune response against a wide range of bacterial infections. They are one of the first types of haematopoietic cell an invading pathogen will encounter and their primary function is to phagocytose and kill invading pathogens through their ability de-granulate, a process which releases anti-microbial molecules into the infected site (Nathan 2006). Eosinophils and basophils are smaller populations of blood leukocytes and are involved in the immune response against parasites and some viruses. Degranulation of these cells results in the release of pro-inflammatory molecules including histamine from basophils and neurotoxin, eosinophil cationic proteins and major basic protein from eosinophils (Stone, Prussin et al. 2010).

Mast cells are tissue resident cells, which are predominantly associated with the immune response in parasitic infections. They reside mainly near small blood vessels and release substances such as histamine, prostaglandins and leukotrienes upon degranulation which affect vascular permeability and recruit other cells such as eosinophils and basophils to the site of infection (Stone, Prussin et al. 2010).

1.2.2 Mononuclear phagocytes

Mononuclear phagocytes are a broad characterisation of immune cells, which reside in the blood and in tissues during steady state and inflammatory conditions and encompass monocytes, macrophages and dendritic cells (DCs).

Monocytes are a heterogenous population of cells that circulate in the blood, bone marrow and spleen and can produce cytokines such as tumour necrosis factor (TNF)α, IL-1 and IL-10 in inflammatory conditions (Auffray, Sieweke et al. 2009). In an inflammatory environment they also have the capacity to migrate from the

blood into tissues and to differentiate into inflammatory DCs and macrophages (Serbina, Jia et al. 2008).

Macrophages are tissue resident phagocytic cells, are present in lymphoid and non-lymphoid tissues and play a role in steady-state tissue homeostasis via the clearance of apoptotic cells and the production of growth factors (Geissmann, Manz et al. 2010). Macrophages have surface PRRs, which allow them to become directly activated upon interaction with an infectious pathogen. This induces the production of a wide range of inflammatory cytokines, induces their killing mechanisms, increases antigen presentation and allows them to directly activate antigen-specific T lymphocytes (Gordon 2002). They are a heterogenous population of cells and encompass microglia in the central nervous system (CNS), dermal macrophages in the skin, and splenic and metallophilic macrophages in the spleen, all with different phenotypes and functions (Geissmann, Manz et al. 2010). Although many macrophage subsets are renewed solely from blood progenitors, there are exceptions, such as the microglia, which have self- renewal capacity (Ajami, Bennett et al. 2007).

Dendritic cells can be broadly divided into classical and plasmacytoid DCs. Classical DCs are specialised antigen processing and presenting cells. They are the primary cell type able to activate naïve T lymphocytes and therefore trigger clonal expansion of antigen-specific clones (Banchereau and Steinman 1998). As immature cells, the classical dendritic cells are highly migratory, in that they can move in and out of lymphoid and non-lymphoid tissues. Like the macrophages, they possess PRRs on their surface, which allow them to be activated by an invading pathogen. Upon activation, the classical DCs can be induced to express a range of cytokines and to migrate to lymphoid tissue where they can activate naïve T cell clones (Geissmann, Manz et al. 2010). Classical DCs are derived from blood-borne precursors, distinct from the monocytic cell precursors of macrophages (Merad and Manz 2009). One exception to this is are the Langerhans cells in the skin which are thought to be derived from a blood-borne monocytic precursor (Ginhoux, Tacke et

al. 2006) and which also display self-renewal properties (Merad, Manz et al. 2002). Plasmacytoid DCs are longer lived cells than classical DCs and are present in the bone marrow and peripheral organs. They respond to viral infection by producing Type I Interferons and can also act as antigen presenting cells to activate T cell responses (Colonna, Trinchieri et al. 2004).

1.2.3 Natural killer (NK) cells

NK cells are large granular lymphocytes which are present in lymphoid and non-lymphoid tissue (Gregoire, Chasson et al. 2007). They have the capability to kill virus-infected cells as well as tumour cells via the release of perforin and granzyme from their cytoplasmic granules, which initiates death of target cells by apoptosis. They are also a source of pro-inflammatory cytokines such as TNF-α and interferon (IFN)-γ (Vivier, Tomasello et al. 2008). Their activation is controlled by activatory and inhibitory receptors. In the mouse, inhibitory receptors are the Ly49 receptors and in humans the analogous set of receptors are the Killer Inhibitory Receptors (KIRs). These receptors are involved in the recognition of Major Histocompatibility Complex (MHC) class I molecules which are expressed on the surface of most host cells. Many viruses and intracellular bacteria have developed ways of down-regulating or completely shutting off MHC class I expression by host cells, however this down regulation of MHC class I is a trigger for the activation of NK cells (Raulet and Vance 2006). NK cells can also be triggered to divide and up-regulate killing activity through Toll-like receptor (TLR) ligands (Sivori, Falco et al. 2004), through detection of stress induced self-ligands (Lanier 2005) and through the actions of cytokines like Type I interferons produced by virus-infected cells, IL-12, IL-18 and IL-15 (Walzer, Dalod et al. 2005).

1.2.4 Lymphocytes

The T and B lymphocytes of the adaptive immune system are derived from the common lymphoid progenitor in the bone marrow (Kondo, Weissman et al. 1997) and subsequently develop in the primary lymphoid organs: the bone marrow and thymus. The lymphocytes that develop in the bone marrow are the B lymphocytes and the lymphocytes that develop in the thymus are the T lymphocytes.

The B and T cell populations have an extraordinarily diverse set of receptors on their cell surface, with each cell having a unique specificity of receptor. These B and T cell populations are collectively capable of recognizing virtually any molecular structure and each cell has the capacity to clonally expand upon an appropriate activation stimulus. The B cell receptor (BCR) is a membrane-bound immunoglobulin (Ig) protein composed of two heavy and two light polypeptide chains (Schroeder and Cavacini 2010). The T cell receptor (TCR) is composed of an α polypeptide chain and a β polypeptide chain which have structural similarities to the heavy and light polypeptide chains of the immunoglobulin receptor (Bentley and Mariuzza 1996). Each receptor has a variable region, which is the external part of the receptor and is capable of interacting and binding to pathogen molecules. Although the B and T cells have structurally similar antigen recognition receptors, their ability to recognise antigen is different. B cells recognise intact antigen directly through their BCR and T cells recognise fragments, which have been processed and presented on MHC and MHC-like molecules on the surface of antigen presenting cells (APCs). The diverse repertoire of receptors on the B and T lymphocytes is generated from a relatively limited set of genes through the process of recombination, which leads to the development of a unique antigen receptor on each individual lymphocyte. This process is dependent upon the recombination-activating genes *RAG1* and *RAG2* (Schatz, Oettinger et al. 1989) and occurs in the bone marrow for the development of B cells and in the thymus for the development of T cells.

In the thymus the majority of developing T lymphocytes (thymocytes) differentiate into to the αβ T cell lineage. αβ T lymphocytes can be further divided into cells expressing the CD4 receptor: the CD4 T cells, and T lymphocytes expressing the CD8 receptor: the CD8 T cells. CD4 T cells are termed T helper cells as they were first defined on their ability to activate B cells to produce antibody. The CD8 T cells are activated in response to intracellular infections and are termed cytotoxic cells as they are involved in the direct killing of infected cells via the release of toxic substances. CD4 T helper cells recognise peptide expressed by MHC class II molecules on the surface of APCs such as dendritic cells, macrophages and B cells. CD8 cytotoxic T cells recognise peptide expressed by MHC class I presented on the surface of most host cells.

A small proportion of αβ T cells also express the cell-surface NK1.1 marker and are classified as NK T cells. These cells express a very limited diversity of T cell receptors in comparison to the conventional T and B cells and recognise lipids and glycolipids presented by CD1 molecules rather than peptides presented by MHC (Godfrey, Pellicci et al. 2010). A small population of developing thymocytes also rearrange the γ and δ chains of the TCR rather than the α and β chains. These γδ T cells differ from the αβ T cells in the more restricted range of antigen specificities they recognise and in their anatomical distribution in the periphery (Chien and Hampl 2000).

B cells recognise intact antigen directly, which does not have to be presented by a specialised cell or molecule. They internalise the antigen through their BCR and can process and present peptides from the antigen on MHC class II molecules, which are recognised by previously primed CD4 T helper cells. This interaction between the B cell and the CD4 T helper cell is important for the activation and clonal proliferation of the B cell, by providing signalling through the CD40 receptor (Noelle, Roy et al. 1992) but also via the action of cytokines such as IL-4 (Valle, Zuber et al. 1989) which can drive the proliferation and differentiation of the B cell into an antibody-secreting plasma cell. Antibodies secreted by activated B cells circulate in the blood

and extra-cellular fluid and have various effector functions (Schroeder and Cavacini 2010). The variable, antigen-binding region of the antibody molecule determines the specificity of the antibody and the constant region determines the isotype of the antibody. Antibodies can bind directly to pathogens or toxic products, which can inhibit the actions of the pathogen or toxic product, therefore having a neutralising effect. The binding of antibodies to pathogens can also enhance the recognition of pathogens by phagocytes such as macrophages and neutrophils, which have receptors on their surface (Fc receptors) for the recognition of constant regions of antibody, in a process called opsonisation. The formation of antibody-antigen complexes can also activate plasma proteins called complement proteins. The activation of complement results in an inflammatory cascade involving the recruitment and activation of many of the effector cells described above.

1.3 Tolerance and autoimmunity

Generation of the diverse range of lymphocyte receptors in the primary lymphoid organs has the potential to generate a large number of clones that are capable of binding to self-antigens with high affinity. It is therefore important that self-reactive clones are regulated in order to prevent induction of an immune response directed against self-antigens in the periphery (an autoimmune response).

Mechanisms by which tolerance against self-reactivity is achieved are classically divided into central and peripheral tolerance mechanisms. Central tolerance consists of the deletion of potentially self-reactive lymphocytes, as well as the positive selection of regulatory lymphocyte populations in the primary lymphoid organs and peripheral tolerance consists of the mechanisms by which self-reactive lymphocytes, which have escaped central tolerance can be inhibited from causing pathology in the periphery.

1.3.1 Central tolerance

TCR-expressing thymocytes are positively selected in the thymus for their ability to recognize self-MHC in context with self-antigen, thereby allowing mature T cells the capability to respond to foreign peptide presented by a self-MHC molecule in the periphery. This concept was originally proposed after experiments in which the MHC genotype of thymic grafts determined the MHC genotype to which T cells would respond in the periphery (Fink and Bevan 1978). The use of transgenic models has subsequently shown that the inability to recognize self-MHC in context with self-antigen expressed by the thymic epithelium results in the death of T cells by apoptosis, also called death by neglect (Huesmann, Scott et al. 1991). However, not all thymocytes that recognize self-peptide/self-MHC complexes enter into the periphery as this would lead to the generation of a peripheral T cell repertoire that was highly auto-reactive. Indeed, there is also a process by which potentially auto-reactive T cells are negatively selected in the thymus before release into the periphery. This theory was originally proposed by Burnet (Burnet 1976) and a classical example of this concept of negative selection is in mice expressing a transgenic TCR specific for the male H-Y antigen. These transgenic cells are positively selected in female mice but negatively selected in male mice. (Kisielow, Bluthmann et al. 1988). Negative selection of thymocytes can be mediated by several different cell types, including bone marrow-derived dendritic cells and macrophages, as well as thymic epithelial cells, in particular medullary epithelial cells (Gao, Lo et al. 1990; Webb and Sprent 1990; Sprent, Kosaka et al. 1992) The extent of negative selection is specified by the diversity of self-antigens that are presented within the thymus (Kyewski and Klein 2006). Furthermore, mice resistant to myelin basic protein (MBP)-induced experimental autoimmune encephalomyelitis (EAE), an animal model for multiple sclerosis, have increased thymic expression of MBP (Liu, MacKenzie-Graham et al. 2001), suggesting that in these mice negative selection to this auto-antigen has been more thorough, leading to enhanced tolerance to disease development. In addition, the thymic expression of occular auto-antigens also correlates with resistance to experimental autoimmune uveoretinitis (EAU), an

animal model for human uevitis (Egwuagu, Charukamnoetkanok et al. 1997), and levels of insulin expression in the thymus of mice modulated peripheral reactivity to insulin (Chentoufi and Polychronakos 2002). The mechanisms by which peripheral self antigens are expressed in the thymus for negative selection can include access via the blood circulation. For example, complement protein C5 can access the thymus via the blood circulation and can induce clonal deletion of C5-specific T cells (Volkmann, Zal et al. 1997). In addition, migratory DCs from the periphery have been shown to induce clonal deletion in the thymus (Bonasio, Scimone et al. 2006). However, for the majority of peripheral antigens that are not blood-borne or are not presented by migratory DCs, negative selection is mediated by expression of these tissue-restricted antigens (TRAs) in the thymus (Jolicoeur, Hanahan et al. 1994). Regulation of the expression of ectopic TRAs in the thymus is controlled in part by the transcription factor AIRE (autoimmune regulator protein). Indeed, AIRE is expressed in mouse and human medullary epithelial cells and mutations in the AIRE gene are responsible for the human autoimmune syndrome, autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (Pitkanen and Peterson 2003). Mice lacking AIRE also develop organ-specific autoimmune disease (Anderson, Venanzi et al. 2002).

The thymus is also an important location for the generation of regulatory CD4 T cells (T-regs), capable of inhibiting potentially auto-reactive immune responses in the periphery. Early experiments showed that mice thymectomized early in life developed organ-specific autoimmunity and that transfer of adult peripheral cells could prevent this development of disease (Nishizuka and Sakakura 1969). It was subsequently shown that $CD25^+$ cells in the adult peripheral T cells were responsible for this suppression (Sakaguchi, Sakaguchi et al. 1995; Asano, Toda et al. 1996) and that these $CD25^+$ T cells could mediate suppression of CD4 T cell responses *in vitro* (Itoh, Takahashi et al. 1999). Development of T-regs has been implicated to involve interactions with self-peptide and MHC class II in the thymus. This has been suggested after observations in transgenic mice where development of T-regs is dependent upon expression of endogenously re-arranged TCRs. Furthermore, when

the transgenic mice were crossed onto a RAG-deficient background and therefore solely expressed the transgenic TCR, T-reg development was abrogated (Itoh, Takahashi et al. 1999). The implications of this have directly been shown in mice expressing a transgenic TCR specific for MBP. These mice developed autoimmune disease against the specific antigen but only when the transgenic strain was crossed onto a RAG-deficient background (Hori, Haury et al. 2002). In addition, retroviral expression of T-reg TCRs into $CD25^-$ $CD4^+$ cells showed that the T-reg TCRs had more efficient interactions with MHC class II-bound self peptides from the periphery than $CD25^-$ cells (Hsieh, Liang et al. 2004) The importance of T-regs in the tolerance against self has been implicated in mice that either lack these cells or that have mutations in genes involved in their development. One such example is mutations within the Forkhead box p3 (Foxp3) gene which is a transcription factor found to be expressed at a high levels in $CD25^+$ T regs (Fontenot, Gavin et al. 2003). Foxp3 mutations result in immune dysregulation in both humans (called IPEX syndrome) and in mice, characterized by excessive lymphoproliferation, multi-organ infiltrates and wasting disease (Bennett, Christie et al. 2001; Brunkow, Jeffery et al. 2001).

Immature B cells in the bone marrow expressing an auto-reactive receptor also undergo a selection process, which has been studied using transgenic mouse models. Upon encountering self-antigen in the bone marrow, self- reactive B cells have been shown to be deleted if the antigen is in high abundance and is multivalent such as multiple copies of MHC molecules on a cell surface (Nemazee and Burki 1989). B cells have also been shown to enter a state of unresponsiveness, called anergy, induced by interaction with soluble antigen in the bone marrow. These B cells leave the bone marrow but have a defect in signal transduction so they cannot be activated by antigen in the periphery (Cornall, Goodnow et al. 1995). Self-reactive B cells also have the option to generate another BCR by process of rearranging genes of the light chain of the receptor (Tiegs, Russell et al. 1993).

1.3.2 Peripheral tolerance

Self-reactive lymphocytes can escape the process of negative selection in the primary lymphoid organs and are present in the circulation of healthy individuals. For example, in mice with a transgenic TCR specific for MBP, CD4 T cells expressing the receptor are positively selected and enter the periphery, however a spontaneous autoimmune reaction against MBP does not occur (Liu, Fairchild et al. 1995). This study went on to show that the transgenic CD4 MBP-specific T cells found in the periphery were of low affinity for peptide and infusion of the mice with an MBP with high affinity for the transgenic TCR led to negative selection of thymocytes in the thymus whereas MBP with a low affinity for the transgenic TCR did not. Thus, the authors concluded that potentially auto-reactive T cells with a low affinity for self-peptide/MHC must rely on peripheral tolerance mechanisms in order to remain non-pathogenic. A similar study using double-transgenic mice engineered to express a TCR specific for chicken ovalbumin (OVA) as well as OVA peptide in the thymus medullary epithelial cells and pancreas showed that these mice developed low affinity OVA-specific T cells in the periphery, that did not induce an auto-reactive response against OVA in the pancreas under normal conditions (Zehn and Bevan 2006), which again showed that low affinity cells could escape central tolerance and enter the periphery.

Studies in CD8 T cells have shown that peripheral tolerance of auto-reactive clones in the periphery to their self-antigen can depend on the level of self-antigen expressed. Moreover, even if an auto-reactive T cell is quite capable of making an antigen-specific response, the level of self-antigen expressed in the periphery might be so low that the T cell is ignorant to the presence of the auto-antigen (Kurts, Sutherland et al. 1999). Anatomical location can also make a contribution to the ignorance of auto-reactive T cells to the presence of auto-antigen. Furthermore, mature naïve T cells normally circulate from blood to the secondary lymphoid organs, to efferent lymph and then back to blood (Lammermann and Sixt 2008) and

are usually excluded from peripheral tissues where the likelihood of coming into contact with an APCs expressing high levels of self antigen is high.

However, ignorance or deletion is not the only means by which peripheral tolerance in achieved. In order for a T cell to become activated into an effector cell capable of contributing towards an antigen-specific response it must receive specific signals from the environment. These signals include triggering of the TCR via peptide presented on MHC molecules, as well as co-stimulation through CD28 on the T cell surface and triggering of cytokine receptors (Lenschow and Bluestone 1993). It has been shown that in the absence of co-stimulatory signals, T cells can enter a state of tolerance known as anergy characterised by inhibition of proliferation and differentiation (Schwartz, Mueller et al. 1989). The cells usually responsible for delivering these activation signals to CD4 T cells are activated DCs and it has been shown that DCs can also adopt a tolerogenic phenotype whereby presentation of antigen to the T cell results in anergy. This tolerogenic phenotype has been shown to be induced by apoptotoic cells and it has been postulated that this process is important for the maintenance of self-tolerance against self-reactive T cells in the periphery (Hawiger, Inaba et al. 2001).

Self-reactive B cells are also kept in check in the periphery by the absence of CD4 T cell help, which is required in most cases for the maturation of mature B cells into plasma cells (Fulcher and Basten 1997). Self-reactive B cells can also be eliminated in the periphery, in germinal centres during immune responses. Moreover, B cells undergo a process called somatic hypermutation during clonal expansion and differentiation which allows them to modify their antigen receptor. If, in this process a strongly self-reactive receptor is generated, these cells can be deleted (Shokat and Goodnow 1995).

In addition to the generation of T-regs in the thymus, *de novo* induction of Foxp3-expressing T-regs in the periphery can also be induced upon immunisation (Apostolou and von Boehmer 2004). DCs can induce the generation of peripheral T-

regs (Mahnke and Enk 2005) *in vivo* and transforming growth factor (TGF) β has been shown to induce T-regs *in vitro*, that are capable of suppressing autoimmunity *in vivo* (Luo, Tarbell et al. 2007). The mechanisms by which T-regs can suppress effector T cell responses has been investigated mostly in *in vitro* studies and include providing competition for the T cell growth factor, IL-2 with effector cells, direct killing of effector cells via cytotoxic activity and expression of surface molecules such as galectin-1 which can induce cell cycle arrest in effector cells upon ligation (Shevach 2009).

1.3.3 Autoimmunity

Autoimmune diseases occur as a result of specific adaptive immune responses directed against self-antigens. In the case of an adaptive immune response against a foreign pathogen, the antigen can be cleared resulting in a resolution of the immune response. In autoimmunity, the persistence of the self-antigen induces a chronic, sustained reaction resulting in clinical disease. Autoimmunity can be distinguished from generalized immune pathology by the identification of a specific self-antigen against which the response is directed. Immune pathology is the result of dysregulated immunity where the antigen against which the response is directed is either exogenous or, as yet, unidentified.

Autoimmune diseases are classified as either organ-specific or systemic, depending on the nature of the auto-antigen to which the response is elicited. Examples of organ-specific autoimmune diseases in which immune pathology is associated with a restricted organ include Hashimoto's thyroiditis, Grave's disease, Type 1 insulin-dependent diabetes mellitus (IDDM), multiple sclerosis (MS), Myasthenia Gravis and Goodpasture's syndrome. Examples of systemic autoimmune disease are systemic lupus erythematosus (SLE), Sjogren's syndrome and rheumatoid arthritis (RA). Most studies into the immunological aspects of these clinical diseases are investigated using mouse models in which antigen-specific autoimmune responses

are actively induced against an endogenous antigen, such as in EAE (Tafreshi, Mostafavi et al. 2005), collagen-induced-arthritis (CIA) (Courtenay, Dallman et al. 1980), autoimmune gastritis (Scarff, Pettitt et al. 1997) or autoimmune ovarian disease (Rhim, Millar et al. 1992). Some mouse strains are highly susceptible to spontaneous induction of autoimmune diseases such as the C3H/He, MRL/lpr and NOD mice which are particularly susceptible to autoimmune gastritis, SLE and diabetes, respectively (Alderuccio and Toh 1998; Anderson and Bluestone 2005; Blank and Shoenfeld 2005). Autoimmunity is associated with the combination of both susceptibility alleles at multiple gene loci and environmental factors. These factors lead to failure in self tolerance, resulting in an autoimmune reaction directed against self. Susceptibility to autoimmune disease has most consistently been associated with MHC genotype (Fugger 2000). However other genes in which polymorphisms have been shown to be associated with autoimmunity include cytotoxic T lymphocyte antigen (CTLA)-4 (Donner, Rau et al. 1997) IL-2 (Encinas, Wicker et al. 1999) and Fas (Fischer, Rieux-Laucat et al. 2000) as well as a number of genes with no known immunological function (Wandstrat and Wakeland 2001). Genome-wide association studies (GWAS) have also recently highlighted particular DNA polymorphisms associated with autoimmune diseases such as Crohn's disease, MS, RA, SLE, IDDM. These include genes encoding IL-23-receptor (IL-23R), IL-2RA, IL-17RA, chemokine receptor 6 (CCR6) and the transcription factor, STAT-3 (Burton, Clayton et al 2007).

Development of autoimmunity is not entirely attributable to the genetic sequence in that identical twins, which share the same genetic sequence, are often discordant for major autoimmune diseases (Bach 2005). This suggests that environmental factors also play a role in autoimmune disease development. A number of infectious organisms carry epitopes that mimic self proteins, and activation of autoreactive T cells as a result of cross reactivity during infection can induce autoimmunity that persists after pathogen clearance (Horwitz, Bradley et al. 1998; Zhao, Granucci et al. 1998; Croxford, Olson et al. 2005). Other infectious organisms may target the immune system, inducing a transient state of lymphopenia that may result in the

expansion of auto-reactive cells (Forrest, Menser et al. 1971; Kopelman and Zolla-Pazner 1988; Morse, Sakaguchi et al. 1999). In addition, some microbes can act as mitogenic stimuli, or superantigens, therefore resulting in the activation of auto-reactive T cells (Brocke, Gaur et al. 1993; Cole and Griffiths 1993). Another mechanism by which infections may induce autoimmune disease is through bystander activation of autoantigen specific T cells or epitope spreading via generation of infection-associated inflammation. For example in Theiler's disease, infection initially induces viral specific encephalomyelitis in mice, which is associated with T cell reactivity to viral proteins, however, the viral-specific response is replaced by an autoimmune response directed against the CNS proteins, MBP and proteolipid protein (PLP) (Miller, Vanderlugt et al. 1997) Similarly, infection of mice with Coxackie B3 virus induced cardiomyositis first against viral proteins and then against auto-antigens (Wolfgram, Beisel et al. 1985) and Coxackie B4 virus could induce diabetes in non-autoimmune-prone mouse strains and could accelerate diabetes onset in diabetes-prone NOD mice (Horwitz, Bradley et al. 1998).

1.4 Development of T cell responses

T cells circulate through the blood, lymphatics and lymphoid organs and constantly interact with both professional APCs such as DCs and macrophages as well as host cells expressing peptide on self-MHC molecules. In a normal, steady state environment, these interactions do not induce activation and proliferation of T cell clones but they do act as a survival signal, reinforcing the process of positive selection that occurs in the thymus. Induction of an inflammatory signal induced by injury or infection leads to the maturation of professional APCs, characterized by the up-regulation of surface MHC and of co-stimulatory molecules such as CD80 and CD86. Mature APCs directly interact with and activate naïve T cells in the local lymphoid tissue, and also produce a range of pro-inflammatory cytokines, which can

regulate the differentiation of the responder cells (Banchereau and Steinman 1998; Joffre, Nolte et al. 2009).

1.4.1 CD4 T helper subsets: Th1/Th2 cells

In the 1980s, activated CD4 T cells were distinguished into two types of T cells based on their ability to activate B cell clones and induce the secretion of different types of immunoglobulin (Ig) from B cells. They were described as Th1 cells and Th2 cells. (Mosmann, Cherwinski et al. 1986; Mosmann and Coffman 1989). Th1 cells were described on the basis of IFN-γ and IL-2 secretion. These cells could induce the production of certain types of IgG from B cells, in particular IgG2a, and were primarily involved in driving cell-mediated immune responses responsible for fighting infection against intracellular parasites. Th2 cells were described based on their ability to secrete IL-4 and IL-5 and were able to induce the production of IgE from activated B cells, an immunoglobulin associated with the immune response against helminth infections and eosinophilic inflammation (Coffman and Carty 1986). The Th1/Th2 paradigm has now been well described and it is clear that the Th1 and Th2 CD4 T cells have distinct developmental pathways dependent upon the inflammatory environment in which they are activated. IL-12, a cytokine expressed by cells of the innate immune system such as DCs and macrophages is crucial for Th1 differentiation and this occurs through the activation of Signal Transducer and Activator of Transcription (STAT)-4 and T box expressed in T cells (T-bet). This activation pathway upregulates the expression of IFN-γ and downregulates IL-4 and IL-5 expression. IL-4 has been shown to induce Th2 cell differentiation through the activation of the transcription factor GATA-3, which upregulates IL-4 and IL-5 expression but downregulates IFN-γ expression. Th1 responses are associated with the recruitment of phagocytic cells and in eradicating intracellular pathogens. Th2 responses are involved in providing B cell help and in shaping the antibody response to extracellular pathogens such as helminth infections (Rengarajan, Szabo et al. 2000; Szabo, Kim et al. 2000; Afkarian, Sedy et al. 2002).

1.4.2 CD4 T helper subsets: Th17 cells

In 2005, Th17 cells were described as a third lineage of activated effector CD4 T cell that was distinct from the Th1 and Th2 lineages (Harrington, Hatton et al. 2005; Park, Li et al. 2005). It was demonstrated in these papers that Th17 cells do not share any of the transcription factors involved in Th1 or Th2 development such as T-bet, STAT-4, STAT-1, STAT-6 c-MAF or GATA-3, and that addition of blocking antibodies to cytokines associated with the Th1 and Th2 lineages including anti-IL-4 and anti-IFNγ resulted in increased IL-17 expression.

IL-17-producing CD4 T cells were originally described by Aggarwal et al in 2003 (Aggarwal, Ghilardi et al. 2003). The authors showed that activation of CD4 T cells in the presence of IL-23 led to enhanced IL-17 production. This was followed by Cua et al who described the generation of a subset of CD4 T cells characterised by the production of IL-17A, IL-17F and TNFα (Cua, Sherlock et al. 2003; Langrish, Chen et al. 2005) which were generated in the presence of IL-23 whereas, in the presence of IL-12, CD4 T cells were polarised to an IFN-γ-producing cell subset. Despite this observation, it was known that naïve T cells do not express the IL-23 receptor (IL-23-R) (Aggarwal, Ghilardi et al. 2003) and so IL-23 was unlikely to be the cytokine responsible for driving naïve T cells to become Th17 cells. Studies showed that the combination of TGFβ and IL-6 were the factors required for the differentiation of Th17 cells from a population of naïve CD4 T cells and that IL-23 was not required for the initial differentiation of these cells (Bettelli, Carrier et al. 2006; Mangan, Harrington et al. 2006; Veldhoen, Hocking et al. 2006). Although IL-23 was not involved in the initial steps driving the development of Th17 cells from naïve T cells it was found to play an important role in the stability of Th17 cells *in vivo* and for Th17 cells to fully differentiate and exhibit effector function (McGeachy, Bak-Jensen et al. 2007). TGFβ was also known to be involved in the generation of Foxp3-expressing T-regs and culture with this cytokine alone was involved in the generation of these cells. Addition of IL-6 to the cultures suppressed the production of T-regs and supported the growth of Th17 cells suggesting that

these two T cell subtypes could arise in a mutually exclusive manner (Bettelli, Carrier et al. 2006).

Expression of the transcription factor ROR-γt (RA-related orphan receptor gamma t) was found to be necessary and sufficient to drive the differentiation of Th17 cells (Ivanov, McKenzie et al. 2006). This was shown in experiments with mice expressing green fluorescent protein (GFP) in ROR-γt-expressing cells, which established a clear association between ROR-γt and IL-17 expression. In these experiments, forced expression of ROR-γt in naïve T cells was sufficient to induce IL-17 expression. STAT-3 has also been associated with development of Th17 cells (Yang, Panopoulos et al. 2007), as well as the transcription factor aryl hydrocarbon receptor (AHR) which is a ligand-dependent transcription factor (Quintana, Basso et al. 2008; Veldhoen, Hirota et al. 2008).

1.5 Primary role for Th17 cells in the host defence against pathogens

Th17 cells have been described as pathogenic cells in many autoimmune diseases. However, Th17 cells are part of the adaptive immune response against many exogenous pathogens. It has been reported that infectious stimuli such as *Borrelia burgdorferi* and mycobacterial lysates can induce the secretion of IL-17 from T cells (Infante-Duarte, Horton et al. 2000). In another study it was shown that Th17 cells were generated in response to conditioned medium from DCs pulsed with *Klebsiella pneumoniae* and that IL-17R-deficient animals had enhanced sensitivity to *Klebsiella pneumoniae* infection, suggesting a role for IL-17 in the host defense against *Klebsiella pneumoniae* (Happel, Dubin et al. 2005). IL-17R-deficient mice infected with *Klebsiella pneumoniae* had an increased mortality rate compared to control mice and this was associated with a delay in neutrophil recruitment to the alveolar space and a more disseminated infection (Ye, Rodriguez et al. 2001). IL-17-producing T cells were also shown to be induced upon infection with *Bacteroides fragilis* (Chung, Kasper et al. 2003), and were shown to accumulate in the lung after

infection with *Mycobacterium tuberculosis* (Khader, Bell et al. 2007). In these experiments depletion of IL-17 resulted in reduced recruitment of protective IFN-γ-producing CD4 T cells to the lung. IL-17A was induced in response to systemic challenge with *Candida albicans* and IL-17AR knockout mice had reduced survival which was associated with a dramatic increase in fungal burden in the kidneys and impaired mobilization of peripheral neutrophils (Huang, Na et al. 2004).

1.6 Role of Th17 cells in autoimmunity

The role of Th17 cells in autoimmune responses has been studied in a number of autoimmune mouse models but most extensively in EAE, a mouse model of multiple sclerosis and CIA, a mouse model of rheumatoid arthritis.

EAE was initially described as an autoimmune disease initiated by Th1 cells. This was based upon experiments using T-bet-deficient mice, which were resistant to EAE development (Bettelli, Sullivan et al. 2004) and by adoptively transferring disease with myelin antigen-specific Th1 cells to naïve recipient animals (Das, Nicholson et al. 1997; Ramirez and Mason 2000). Studies using neutralising antibodies to IL-12p40 also suggested a role for Th1 in EAE (Leonard, Waldburger et al. 1995; Segal, Dwyer et al. 1998), although this was subsequently shown to be due to deletion of IL-23, which uses the same p40 subunit (Cua, Sherlock et al. 2003). In contrast, mice lacking IFN-γ (Ferber, Brocke et al. 1996) or the IFN-γ receptor (Willenborg, Fordham et al. 1996) were highly susceptible to the disease suggesting that the IFN-γ produced by Th1 cells was not required for the disease or even had a protective role.

Further investigations into the effector cell lineage important for the development of EAE studied the roles of the cytokines IL-23 and IL-12 (Langrish, Chen et al. 2005). IL-12 is a heterodimeric molecule formed by the subunits p35 and p40 and IL-23 is also a heterodimeric molecule, composed of p40 and p19. In this study the authors

showed that animals deficient in p19 and therefore IL-23, were protected from EAE, whereas mice deficient in p35 were highly susceptible to disease induction suggesting that IL-23, not IL-12 was important for disease pathology. It was also reported that in the p19-deficient mice, similar numbers of T cells were found in the spinal cord after immunisation but that in contrast to WT mice there was a lower proportion of IL-17 and TNFα- expressing CD4 T cells, and a higher proportion of IFN-γ- expressing CD4 T cells. This suggested that IL-23 was responsible for driving an IL-17-expressing CD4 T cell population that could induce EAE, and that IL-12, and consequently the Th1 pathway was dispensable for disease induction. Similarly, the absence of IL-23 was protective against CIA induction, which correlated with absence of Th17 cells, and a loss of IL-12 was found to exacerbate disease symptoms (Murphy, Langrish et al. 2003).

Further support for the role of Th17 cells in the development of EAE came from experiments with mice expressing a dominant-negative mutant TGF-β receptor II in CD4 T cells. These mice were deficient in Th17 cells and were resistant to EAE (Veldhoen, Hocking et al. 2006). In addition, RORγt-deficient animals lacked Th17 cells and were resistant to EAE induction (Ivanov, McKenzie et al. 2006) and ligation of the AHR with 6-formylindolo [3,2-b] carbazole (FICZ) was shown to increase Th17 responses and induce stronger EAE in mice (Quintana, Basso et al. 2008; Veldhoen, Hirota et al. 2008).

EAE can be induced passively by adoptive transfer of central nervous system (CNS) antigen-specific CD4 T cells, however experiments using adoptive transfer of Th1 and Th17-polarised antigen-specific cells to investigate the phenotype of the effector cells required to induce disease have shown conflicting results. In one study transfer of Th17-polarised antigen-specific cells into naïve mice resulted in disease induction, whereas Th1-polarised antigen-specific cells did not, despite the presence of both effector cell types in the spinal cord. This suggested that Th17 cells, rather than Th1 cells were pathogenic in EAE (Langrish, Chen et al. 2005). However, despite these observations, other adoptive transfer studies have shown that both Th1

and Th17-polarised cells were able to induce disease (Kroenke, Carlson et al. 2008; O'Connor, Prendergast et al. 2008). It is difficult to interpret results from these adoptive transfer experiments because the transferred cells are inevitably not a homogenous population of cells and have been polarised under different conditions in each study.

The role of IL-17 in autoimmunity has been investigated using IL-17A knockout mice (Nakae, Nambu et al. 2003; Komiyama, Nakae et al. 2006) where onset of EAE and CIA was delayed and the severity of the diseases was reduced. The same effect was seen by blocking IL-17A action with an anti-IL-17A antibody (Lubberts, Koenders et al. 2004) or administrating a soluble IL-17A receptor (Bush, Farmer et al. 2002). However, in these experiments the source of IL-17A required for autoimmunity was not addressed. IL-17A has been reported to be expressed from non-CD4 T cells such as γδ T cells (Roark, Simonian et al. 2008) , CD8 T cells (Yen, Harris et al. 2009) and NK cells (Michel, Keller et al. 2007). IL-17 secretion from any of these cell types could be attributed to development of autoimmunity. Furthermore, IL-17A-expressing γδ T cells have recently been implicated in EAE pathogenesis (Sutton, Lalor et al. 2009).

Studies in humans have also shown the presence of IL-17 and IL-17-producing T cells in patients with autoimmune disease. For example IL-17A-producing T cells have been detected in the gut of Crohn's disease patients (Annunziato, Cosmi et al. 2007), IL-17 protein has been detected in the synovium of patients with rheumatoid arthritis (Chabaud, Durand et al. 1999) and the IL-17A transcript has found to be up-regulated in the leisions of patients with MS (Lock, Hermans et al. 2002; Lock and Heller 2003).

1.7 Interleukin-17

1.7.1 The IL-17 gene family

The IL-17 family of genes has six members: IL-17A-F, which have been grouped together based on similarities in their nucleotide sequences. IL-17A and IL-17F are the most closely related of all the family members. They are located on chromosome 1 in mice and are separated by 45kbp with IL-17F downstream and in the opposite orientation to IL-17A. They share 50% nucleotide and amino acid sequence homology and have a number of conserved non-coding sequences within their locus (Akimzhanov, Yang et al. 2007). IL-17B-E have between 10% and 30% sequence homology with IL-17A and have different chromosomal locations (Gaffen, Kramer et al. 2006).

IL-17A was first cloned in 1993 from an activated mouse cytotoxic T cell/ rat lymphoma hybridoma. The nucleotide and amino acid sequence is highly conserved over many mammalian species and the amino acid sequence of murine IL-17A shows 57% homology to the amino acid sequence encoded by ORF13, an immediate-early gene of *Herpesvirus Saimiri* (Rouvier, Luciani et al. 1993). The gene encodes a 1.35kb transcript including a 16bp polyA tail and a region of AT-rich repeats in the 3'untranslated region. These AT-rich repeats are typical in a number of transiently expressed genes such as oncogenes, cytokines and growth factors and signify instability of the mRNA transcript (Shaw and Kamen 1986).

Over-expression of IL-17A *in vitro* has revealed that the protein can be expressed as a 38kDa disulphide-linked homodimeric protein in both glycosylated and non-glycosylated forms (Yao, Fanslow et al. 1995; Yao, Painter et al. 1995). Findings have also shown that IL-17A and IL-17F can form heterodimers (Chang and Dong 2007; Liang, Long et al. 2007).

1.7.2 IL-17 Receptors

The first receptor for IL-17A was identified in 1995 (IL-17AR), which bound to viral and murine IL-17A recombinant proteins *in vitro*. Engagement of IL-17R by these ligands induced the activity of NFκB and the production of IL-6 by fibroblast cells, demonstrated by the use of an IL-17R monoclonal antibody which blocked these effects (Yao, Fanslow et al. 1995). Northern blot analysis indicated that IL-17AR mRNA could be detected in most tissues including the spleen, kidney, lungs and liver with weaker signals in the brain, heart, skeletal muscle and testes (Yao, Fanslow et al. 1995). The binding affinity of the cloned human IL-17RA for IL-17A was found to be relatively low and it was postulated that an additional subunit might cooperate with IL-17AR to create a receptor complex that would be capable of eliciting the biological responses seen using low concentrations of IL-17A (Yao, Spriggs et al. 1997).

1.7.3 Actions of IL-17

The events resulting from IL-17 ligation include up-regulation of a large range of inflammatory and haematopoietic chemokines and cytokines, bone metabolism genes and transcription factors. IL-17 in the presence of TNFα has been shown to stabilize the mRNA of IL-6 (Shimada, Andoh et al. 2002) and IL-17 alone has been shown to stabilise the mRNA of G-CSF (Cai, Gommoll et al. 1998). IL-17 has also been shown to activate intracellular transcription factors such as NFκB (Yao, Fanslow et al. 1995), C/EBPβ and C/EBPδ (Ruddy, Wong et al. 2004).

Human and viral IL-17A were shown to induce the secretion of IL-6, IL-8, Prostaglandin E2 and G-CSF from primary cultures of synovial fibroblasts (Fossiez, Djossou et al. 1996). The effects of IL-17A on synovial fibroblasts could also be modulated by the use of other cytokines. For example, TNF-α and IFN-γ had an additive effect on IL-17A-induced secretion of IL-6 and while neither IL-17A or TNF-α alone had any affect on the secretion of GM-CSF, the combination of these

two cytokines induced synovial fibroblasts to produce GM-CSF (Fossiez, Djossou et al. 1996). IL-17 was also shown to up-regulate the release of a range of cytokines including IL-1β, TNF-α and IL-6 from non-stimulated adherent human monoctyes (Jovanovic, Di Battista et al. 1998) This IL-17-induced release of cytokines by cells of the innate immune system has been linked to the neutrophil recruitment seen in rat bronchoalveolar lavage after administration of recombinant IL-17A (Laan, Cui et al. 1999) and to the proliferation and differentiation of $CD34^+$ haematopoietic progenitors into neutrophils when cultured in the presence of irradiated human fibroblasts and IL-17 (Fossiez, Djossou et al. 1996). An association between IL-17A and neutrophil maturation and recruitment was also shown using an adenovirus over-expression system in which murine IL-17A cDNA was expressed using adenovirus infection and resulted in a 10-fold rise in the absolute neutrophil count (Schwarzenberger, La Russa et al. 1998). IL-17A has also been shown to be involved in the induction of osteoclast differentiation from osteoclast progenitor cells, a pathway dependent upon receptor activator of NFκB (RANK) and RANK-ligand (Kotake, Udagawa et al. 1999; Bar-Shavit 2007).

1.8 Experimental Autoimmune Encephalomyelitis (EAE): a mouse model of autoimmunity

1.8.1 History of EAE

In the 1930s, experiments using Rhesus monkeys showed that injection with rabbit brain and brain extracts could induce a mild paralysis associated with perivascular infiltrates and demyelination (Rivers, Sprunt et al. 1933). Experiments that measured the antibody response induced after intra-peritoneal injections of rabbit brain into rabbits showed a proportional relationship between antibody titre, the amount of emulsion injected and the number of animals affected by paralysis, suggesting that the demyelination may have been caused by an immune response (Schwentker and Rivers 1934). Several groups then began to try to boost this antibody production by administering brain emulsions with killed *Mycobacterium* and paraffin oil, an adjuvant strategy described by Freund and McDermott (Freund,

McDermott et al. 1942). The model was termed experimental autoimmune encephalomyelitis (EAE) and could be induced in recipient animals by multiple injections of the emulsion (Kabat, Wolf et al. 1947; McGeachy, Stephens et al. 2005). The disease was subsequently induced in a number of different species including guinea pigs, rabbits, goats, mice, rats, hamsters, dogs, sheep, marmosets and chickens. Clinical features of the disease progression in each species are characterised by paralysis although the histopathology and the disease progression varies, depending on the species used (Baxter 2007). The histopathological characteristics seen in EAE have similarities with some human demyelinating diseases such as multiple sclerosis and post-infectious encephalomyelitis. Some of the major features in common include the destruction of the myelin sheaths, nerve cells and supporting structures in the CNS, the perivascular location of the lesions in the CNS, the inflammation associated with the lesions and the presence of immunoglobulin in the CNS and in the cerebrospinal fluid (CSF) (Baxter 2007). EAE is therefore used as an animal model of neuroinflammation and immune-mediated tissue injury to study these diseases.

1.8.2 Models of EAE

Originally, whole spinal cord or brain extracts were used to elicit EAE, however specific encephalitogenic peptides are now used with increased potency. Experiments attempting to isolate the encephalitogenic agent in the brain preparations focussed on myelin, a material which forms a layer around the axon of nerves in the CNS. It was found that a specific protein of myelin, MBP could elicit EAE more effectively than whole myelin (Laatsch, Kies et al. 1962). Other commonly used peptides to elicit EAE include peptides of myelin oligodendrocyte glycoprotein (MOG) (Mendel, Kerlero de Rosbo et al. 1995) and of PLP (Tuohy, Lu et al. 1989), two other proteins found in CNS myelin, The combination of peptide and strain of animal is important in the outcome of disease. For example, Lewis Rats are responsive to a MBP peptide but not a MOG peptide, and C57BL/6 mice

are responsive to a MOG peptide but not a MBP peptide. Furthermore, the strain of animal used also affects the clinical progression and pathology of disease. For example, using SJL/J or Biozzi ABH mice induces a relapsing/ remitting disease whereas using C57BL/6 mice, induces a self-limited or chronic EAE (Krishnamoorthy and Wekerle 2009).

EAE was initially induced by active immunisation, however experiments to investigate the pathogenic components of disease required the use of passive transfer experiments where disease could be transferred by auto-antigen specific T cell lines (Ben-Nun, Wekerle et al. 1981). The Lewis rat was the animal of choice for a long time due to its high susceptibility to disease by active immunisation with MBP in complete Freunds adjuvant (CFA) (Lipton and Freund 1952). More recently, mice have been more popular due to the use of this species to generate genetic knockouts and mutants (Krishnamoorthy and Wekerle 2009) Spontaneous models of EAE have also been developed which have transgenic T cell or B cell receptors expressing CNS-antigen specificity (Goverman, Woods et al. 1993; Lafaille, Nagashima et al. 1994; Waldner, Whitters et al. 2000; Bettelli, Pagany et al. 2003; Krishnamoorthy, Lassmann et al. 2006; Pollinger, Krishnamoorthy et al. 2009).

1.8.3 Cell types involved in the pathogenesis of EAE

In the 1970s a number of experiments showed that EAE was T cell mediated by depletion of thymus derived cells and transfer of T cells into depleted recipients which restored disease. (Gonatas and Howard 1974; Bernard, Leydon et al. 1976; Ortiz-Ortiz, Nakamura et al. 1976; Stohl and Gonatas 1980). Then in the 1980s, anti-CD4 antibodies efficiently reduced EAE in rats (Brostoff and Mason 1984; Brinkman, Ter Laak et al. 1985). This suggested that CD4 T cells were responsible for EAE pathogenesis.

Although many studies have concentrated on CD4 T cells in EAE there have been reports that auto-antigen specific CD8 T cells can also transfer disease into wild type recipients and CD8 T cells have been found in MS lesions (Babbe, Roers et al. 2000; Huseby, Liggitt et al. 2001; Ford and Evavold 2005). γδ T cells have also been reported in the inflammatory infiltrate in EAE (Rajan, Asensio et al. 2000; Gao, Rajan et al. 2001). One study showed that depletion of these cells immediately prior to disease onset, reduced EAE severity (Rajan, Gao et al. 1996). γδ T cells have also been shown to be a source of pro-inflammatory cytokines and chemokines in EAE (Rajan, Klein et al. 1998; Rajan, Asensio et al. 2000). Another recent report showed that adoptive transfer of γδ T cells along with CD4 T cells was required for EAE pathogenesis (Sutton, Lalor et al. 2009).

Injection of monoclonal antibodies against MOG protein enhanced demyelination in a transfer model of EAE suggesting a role for antibody in disease pathogenesis (Schluesener, Sobel et al. 1987). In addition, depletion of B cells using a depletion antibody suggested that B cells were necessary for EAE in Lewis rats (Gausas, Paterson et al. 1982) and mice (Myers, Sprent et al. 1992). In a MOG peptide-specific transgenic model of EAE, T cells were shown to selectively expand myelin-specific B lymphocytes (Pollinger, Krishnamoorthy et al. 2009) and in a model using TCR and BCR transgenic mice, in which the majority of both the B cells and the T cells were specific for MOG, B cells were shown to present auto-antigen to T cells (Bettelli, Baeten et al. 2006; Krishnamoorthy, Lassmann et al. 2006). However, immunisation of IgM heavy chain-deficient mice with MBP resulted in EAE induction which was clinically similar to the wild type (Wolf, Dittel et al. 1996). Also, a study by Hjelmstrom et al, showed that MOG peptide-induced EAE could be induced in B cell-deficient (muMT) mice suggesting that B cells and antibodies were dispensable for primary demyelination in this model (Hjelmstrom, Juedes et al. 1998). Another study repeated these findings but also showed that if mice were immunised with whole MOG protein, rather than the 35-55 peptide used normally in the induction protocols then mice were resistant to EAE (Lyons, San et al. 1999). Depletion of B cells using an anti-CD20 antibody before MOG 35-55 peptide

immunisation exacerbated EAE symptoms and depletion after disease onset suppressed clinical disease, suggesting reciprocal roles for B cells in EAE (Matsushita, Yanaba et al. 2008).

Inflammatory infiltrates in EAE have been described as containing monocytic cells (Raine 1984; Traugott 1989; Owens and Sriram 1995). Perivascular macrophages are resident APCs of the CNS that are continuously renewed by bone marrow-derived precursors (Hickey and Kimura 1988). Microglia are also a subpopulation of resident monocytic cells in the CNS which up-regulate MHC class II expression and can present antigen in an inflammatory environment (Kreutzberg 1996; Carson, Reilly et al. 1998). Macrophages and microglia have also been shown to up-regulate B7 molecules suggesting that these cells can stimulate T cell responses (De Simone, Giampaolo et al. 1995; Gerritse, Laman et al. 1996; Li, Cuzner et al. 1996; Issazadeh, Navikas et al. 1998). Activated macrophages and microglia in EAE are sources of inflammatory and toxic mediators such as TNFα (Renno, Krakowski et al. 1995) IL-1 (Bauer, Berkenbosch et al. 1993), reactive oxygen species (Huitinga, Ruuls et al. 1995) and nitric oxide (Okuda, Nakatsuji et al. 1995) and are thought to be major players involved in the process of demyelination (Raine 1984). However, Sedgwick et al passively transferred activated CD4 T cells into recipient Lewis rats that had been irradiated and were therefore leukopenic. These rats developed severe EAE despite the few infiltrating non-T cells into the CNS suggesting these non-T cells were superfluous to disease induction (Sedgwick, Brostoff et al. 1987). Despite this finding, other studies have shown a positive correlation between activated macrophages in the CNS and disease severity (Berger, Weerth et al. 1997) and that depletion of macrophages prevented disease onset (Tran, Hoekstra et al. 1998).

Studies have also reported the presence of neutrophils or PMNLs in the CNS infiltrate of animals with EAE using both flow cytometry and immunohistochemistry methods. These include in rats with hyperacute EAE (Lampert 1967; Levine 1974), in the Biozzi AB/H mouse model (Allen, Baker et al.

1993), in Balb/C mice (Maatta, Sjoholm et al. 1998), and also in C57BL/6 mice using immunisation with MOG peptide (Bettelli, Sullivan et al. 2004; Wu, Cao et al. 2010). During infections, PMNLs release a variety of agents at the site of infection including degradative enzymes and products of the oxidative burst (Campoccia, Hunt et al. 1993). They can also produce cytokines and chemokines capable of signalling to other cells (Bazzoni, Cassatella et al. 1991). In one study using SJL/J mice immunised with MBP peptide, depletion of polymorphonuclear cells beginning at day 8 after immunisation significantly delayed, and in some cases prevented the onset of clinical EAE, however depletion of these cells from day 1 until day 7 had no effect on disease. These experiments suggested that PMNLs were acting in the effector phase of EAE in this model although the authors did not address the mechanism by which this occurred (McColl, Staykova et al. 1998).

1.8.4 Migration of cells into the central nervous system

The CNS has previously been described as an immunologically privileged site due to the reported absence of resident classical APCs, and the lack of a lymphatic drainage system (Ransohoff, Kivisakk et al. 2003). It was also shown that the brain does not reject foreign tissue grafts (Medawar 1948) highlighting that the CNS is immunologically distinct from other peripheral organs. Immune reactions however do occur in the CNS such as in viral infections, ischemia, or in inflammatory diseases such as MS (Ransohoff, Kivisakk et al. 2003) and leukocytes are found in distinct regions of the CNS in healthy individuals (Svenningsson, Andersen et al. 1995). There is also evidence to suggest that CSF drains into the cervical lymph nodes (Widner, Moller et al. 1988; Cserr and Knopf 1992).

The movement of plasma components, red blood cells and leukocytes into the CNS is controlled by the blood-brain barrier (BBB) and the blood-cerebrospinal fluid barrier (BCSFB) (Zlokovic 2008). These are highly specialised endothelial cell barriers possessing intercellular tight junctions which line the walls of the cerebral

blood vesssels (Huber, Egleton et al. 2001). During a variety of inflammatory conditions leukocytes cross the BBB or BCSFB and can enter the CNS (Zlokovic 2008).

The process of leukocyte movement from the bloodstream into tissues is mediated by interactions between leukocytes and endothelial cells lining the blood vessel wall. Leukocytes make transient contact with the vascular endothelium, which is mediated by adhesion molecules such as α4-integrins and leukocyte function-associated antigen 1 (LFA-1) on the surface of the leukocyte and their respective ligands, such as intra-cellular adhesion molecule 1 (ICAM-1) and P-selectin on the endothelial lumen. After this initial tether, leukocytes roll along the vascular wall and bind chemotactic factors, such as chemokines immobilised on the endothelial surface. Chemokines bind G-protein coupled receptors on the leukocyte surface, a process which enhances the avidity and affinity of the interaction between integrins and their ligands and facilitates subsequent diapedesis of the leukocyte (Ransohoff, Kivisakk et al. 2003).

Chemokines are secreted chemoattractant proteins that bind to cell surface G protein-coupled receptors (Olson and Ley 2002). The interactions between chemokines and chemokine receptors are important for leukocyte trafficking in steady state and inflammatory environments. For example, the chemokine receptor, CCR7 is expressed on naïve T cells which is important for the migration of naïve T cells across high endothelial venules into the lymph nodes and then further into the T cell areas of the lymph nodes, via interaction with the chemokines CCL19 and CCL21 produced by endothelial and stromal cells of lymphoid organs (Forster, Schubel et al. 1999; Gunn, Kyuwa et al. 1999). Upon activation in the lymph nodes T cells lose their expression of CCR7 facilitating movement of activated cells out of the lymph nodes. Th1 cells express high levels of CCR5 and CXCR3, Th2 cells express high levels of CCR4 and CCR8 (Bonecchi, Bianchi et al. 1998; Loetscher, Uguccioni et al. 1998) and Th17 cells have been shown to express CCR6 (Yamazaki, Yang et al. 2008) which can influence the migration of these cells into

inflammatory sites where the respective ligands are expressed. For example, CCR5 binds the chemokines CCL3, CCL4 and CCL5 with high affinity which are expressed in the lung, CNS, skin and liver, CCR4 binds CCL17 and CCL22 which are expressed in Th2 inflammatory sites including the lung and skin and CCR6 binds CCL20 which is expressed in the intestine and the skin (Olson and Ley 2002). Chemokine expression in tissues can also be induced by inflammatory stimuli. For example, CCL20 expression is induced in the CNS during EAE (Liston, Kohler et al. 2009).

In EAE, antibodies specific for α4-integrin reduced the severity of disease (Yednock, Cannon et al. 1992) and have been used in the treatment of MS with some success (Tubridy, Behan et al. 1999; Miller, Khan et al. 2003) suggesting a central role for α4-integrin in disease pathogenesis. A role for P-selectin has also been described in trafficking of leukocytes to the CNS in EAE (Kerfoot and Kubes 2002; Piccio, Rossi et al. 2002). C-C Chemokine Receptor 2 (CCR) 2-deficient mice (Fife, Huffnagle et al. 2000) are resistant to EAE, which has been attributed to a lack of monocytes in the CNS. Two reports have also shown that CCR6-deficient mice had greatly reduced disease severity (Liston, Kohler et al. 2009; Reboldi, Coisne et al. 2009; Villares, Cadenas et al. 2009) which was attributed in one study (Reboldi, Coisne et al. 2009) to the inability of Th17 cells, which express high levels of CCR6 to access the CNS and in the other to the reduced priming of auto-reactive CD4 T cells in the draining lymph nodes (Liston, Kohler et al. 2009). However, in contrast to these findings, two other reports demonstrated that CCR6-deficient mice were susceptible to disease with enhanced disease severity over wild-type mice due to reduced T-regs infiltrating the spinal cord (Villares, Cadenas et al. 2009) and enhanced IL-17A and IFN-γ responses in the periphery (Elhofy, Depaolo et al. 2009).

1.8.5 The role of pertussis toxin in the induction of EAE

Lee and Olitsky were the first to report that pertussis vaccine administered into the peritoneum would enhance the development of encephalomyelitis in mice after administration of mouse brain antigens in CFA (Lee and Olitsky 1955). It was further noted that fractions of *Bordetella pertussis* cells (vaccine) rich in histamine-sensitizing factor were involved in increasing encephalitogenic activity of the spinal cord emulsions (Levine and Wenk 1965; Levine, Wenk et al. 1966). The histamine-sensitizing factor was also called pertussigen and is now known as pertussis toxin.

Inclusion of pertussis toxin in the immunisation protocol for EAE can enhance EAE severity in mice that are susceptible to the disease induced by CNS antigens and CFA alone, and to allow EAE development in mice that are not susceptible in this way (Munoz and Mackay 1984). Although not required in some models such as in the development of spontaneous EAE in the MBP transgenic mice (Goverman, Woods et al. 1993), administration of pertussis toxin has been shown to enhance onset and incidence of disease. In the Lewis rat, administration of pertussis toxin was not required for EAE onset but could change the clinical progression and pathology of disease (Levine and Wenk 1965; Levine, Wenk et al. 1966). MOG-induced EAE in C57BL/6 mice requires the administration of pertussis toxin (Tafreshi, Mostafavi et al. 2005). The mechanisms by which pertussis toxin influences the development of EAE are not clearly defined, however administration of pertussis toxin was shown to override genetic checkpoints important for the development of EAE (Linthicum and Frelinger 1982; Blankenhorn, Butterfield et al. 2000).

Pertussis toxin is an AB exotoxin which is secreted from the bacterium *Bordetella pertussis,* the agent that causes whooping cough (Mooi 1988). The exotoxin is composed of six subunits which are arranged in an A-B structure. The B oligomer, composed of the subunits S2-S5 (with two copies of S4), attaches to surface glycoproteins expressed on a variety of mammalian cells and allows the A oligomer,

composed of subunit S1, to enter the cell. The A oligomer is the enzymatic component of the toxin and is responsible for ADP-ribosylating G proteins inside the cell. This ADP-ribosylation, inactivates G-protein coupled receptors and therefore inhibits signalling events downstream of this receptor (Reisine 1990).

In *Bordetella pertussis* infection, pertussis toxin is released and has modulatory effects on the host immune system, presumably to allow an infection to become established. For example, the toxin has been shown to delay neutrophil recruitment to the airways in *B pertussis* infection (Carbonetti, Artamonova et al. 2003; Kirimanjeswara, Agosto et al. 2005) and depletion of airway macrophages enhanced *B pertussis* infection with a pertussis toxin-deficient strain up to wild type levels suggesting that resident airway macrophages may be the primary target for pertussis toxin in *B pertussis* infection (Carbonetti, Artamonova et al. 2007). Pertussis toxin has also been shown to suppress serum antibody responses to *B pertussis* antigens after infection (Carbonetti, Artamonova et al. 2004)to reduce MHC II molecules on the surface of human monocytes (Shumilla, Lacaille et al. 2004), to modulate surface markers on dendritic cells (Martino, Volpe et al. 2006) and to induce generalised lymphocytosis due to the failure of cells to migrate back to the peripheral lymphoid tissue (Spangrude, Braaten et al. 1984).

Pertussis toxin has been hypothesised to facilitate immune cell entry into the CNS by increasing permeability across the BBB. In the 1970s it was reported that use of pertussis toxin in Lewis rats increased vascular permeability in the central nervous system and this increase in permeability was associated with the development of paralysis when mice were induced for EAE (Bergman and Munoz 1975; Bergman, Munoz et al. 1978). Moreover, use of pertussis toxin alone has been shown to allow influx of plasma components into the central nervous system (Amiel 1976; Clifford, Zarrabi et al. 2007). Experiments using intravital microscopy of the murine microvasculature showed that injection of pertussis toxin increased the rolling and adhesion of activated lymphocytes which could be inhibited with antibodies that blocked P-selectin (Kerfoot, Long et al. 2004). In addition to an effect on vascular

permeability, pertussis toxin may facilitate EAE by acting as an adjuvant to drive T cell responses to auto-antigens. Indeed, pertussis toxin has been shown to enhance antigen specific cytokines to hen egg white lysozyme (HEL) (Shive, Hofstetter et al. 2000), to enhance Th1 and Th2 responses (Hofstetter, Shive et al. 2002; Denkinger, Denkinger et al. 2007) and also Th17 responses (Chen, Howard et al. 2007; Hofstetter, Grau et al. 2007).

1.9 Thesis aims

IL-17A is an evolutionary conserved molecule whose expression is involved in the development of immune responses. Mice lacking IL-17A expression have less efficient immune responses to pathogens such as *Klebsiella pneumoniae* and *Candida Albicans*, resulting in a reduced ability to clear the pathogens and an increase in severity of disease. In addition, reduced IL-17A expression leads to a reduction in the severity of autoimmune mouse models such as Experimental Autoimmune Encephalomyelitis (EAE) and Collagen Induced Arthritis (CIA). However, the understanding of how induction of IL-17A expression relates to either resolution of an infection or development of immune pathology is still incomplete.

In order to further understand the IL-17A response in infection and autoimmunity, to investigate the conditions in which an IL-17A-associated immune response can manifest in immune pathology, to determine which cells are responsible for expressing IL-17A and which signals are responsible for inducing its expression, the aims of this PhD were:

1) To investigate the sequence of immunological events in the lymph nodes, blood and spinal cord in the development of the IL-17A-associated autoimmune response, EAE.

2) To investigate how pertussis toxin (an agent required to be administered for disease pathology in EAE) modulates the sequence of immunological events

in EAE and to investigate whether pertussis toxin modulates IL-17A expression in EAE.

3) To generate and characterise a reporter mouse to irreversibly label all cells that have activated the IL-17A promoter and their progeny

Chapter 2: Materials and Methods

2.1 Mice

C57BL/6 female mice aged 6-8 weeks were used for all experiments. Mice were bred and housed at the NIMR facility under Specific Pathogen Free (SPF) conditions and all experiments were carried out in accordance with Home Office guidelines.

2.2 Immunisations

Buffers/solutions/media:

- Dulbecco's Phosphate Buffered Saline containing 1g/L D-Glucose, 36mg/ml Pyruvate, $CaCl_2$, $MgCl_2$ (D-PBS) (Invitrogen)

2.2.1 Preparation and injection of MOG/CFA emulsion

Desiccated, heat-killed *Mycobacterium tuberculosis* H37Ra (Difico laboratories) was prepared at 5mg/ml in incomplete Freund's adjuvant (IFA) to form complete Freund's adjuvant (CFA). MOG peptide$_{35-55}$ (MEVGWYRSPFSRVVHLYRNGK) from PepLogic was prepared at 5mg/ml in D-PBS. CFA and MOG peptide$_{35-55}$ preparations were combined in equal volumes which resulted in H37Ra and MOG peptide$_{35-55}$ at a final concentration of 2.5mg/ml. This mixture was then emulsified using an homogeniser, which was carried out on ice, homogenising approximately 3 times for 30 seconds, with a gap of approximately 30 seconds in between to prevent the emulsion from warming. 100µl of the emulsion was injected in total subcutaneously into the base of the tail using a 19 gauge needle so each mouse received 250µg of H37Ra and 250µg of MOG peptide$_{35-55}$. 50µl of the emulsion was injected either side of the tail.

2.2.2 Preparation and injection of pertussis toxin

Pertussis toxin (Calbiochem) was prepared at 2μg/ml in D-PBS and 100μl was injected into the peritoneal cavity using a 27 gauge needle so that each mouse received 200ng of pertussis toxin. As indicated in the results, pertussis toxin was either injected alone or at the same time as the MOG CFA injection, with a booster given 2 days later, or on days 1 and 3, days 3 and 5, or days 5 and 7 following the MOG CFA injection. In the experiment in which pertussis toxin was injected alone, without MOG CFA injection, 100μl of D-PBS was injected at the same time points into control mice.

2.2.3 Scoring of mice

Mice were scored for signs of clinical EAE from day 8 following the base of tail injection. Scoring was as follows: score 0- no symptoms, score 1- flaccid tail, score 2 –impaired righting reflex and/or gait, score 3 – partial hind limb paralysis, score 4- complete hind limb paralysis, score 5- complete hind limb paralysis and partial forelimb paralysis.

2.3 Isolation of cell populations

Buffers/solutions/media: (all reagents Sigma unless otherwise stated)

- Ammonium chloride potassium carbonate lysis buffer (ACK) (distilled-H_2O containing 150mM NH_4Cl, 10mM $KHCO_3$ and 0.1mM Na_2EDTA)
- D-PBS containing 1g/L D-Glucose, 36mg/ml Pyruvate, 9.01mM $CaCl_2$, 4.93mM $MgCl_2$ (Invitrogen), supplemented with 0.5% Bovine Serum Albumin (BSA).
- Phosphate buffered saline (PBS)

- Air buffered Iscove's modified Dulbecco's medium (AB-IMDM) supplemented with 0.5% BSA.

2.3.1 Blood

Mice were sacrificed by Schedule 1 Killing using a rising concentration of CO_2 and approximately 500µl of blood was collected immediately afterwards by cardiac puncture using a 27 gauge needle. The blood was added to 200µl of heparin sodium salt (reconstituted in 5ml distilled-H_2O), mixed thoroughly and stored on ice. Red blood cells were lysed by adding 4ml of ACK lysis buffer, mixing and leaving for 7 minutes at room temperature. At least 4ml of D-PBS was then added to stop the lysis reaction and the cells were collected by centrifugation (1200rpm for 4 minutes). ACK lysis was repeated, and the cells were subsequently re-suspended in D-PBS.

2.3.3 Spinal cord

A cardiac perfusion was carried out by injecting 10ml of PBS into the left ventricle of the heart. The spinal column was dissected out from the base of the tail up to the base of the brain and placed immediately into cold PBS. It was then cut into small sections and the spinal cord was carefully removed using forceps. The spinal cord was placed into AB-IMDM and the tissue was mechanically disrupted by mashing through a 70µm cell strainer. Cells were collected by centrifugation (1200rpm for 4 minutes) and were re-suspended in 10ml of 30% Percoll (GE Healthcare, prepared in D-PBS). This mixture was centrifuged for 20 minutes at 2000rpm and approximately 9.5ml of supernatant was removed from the leukocyte pellet using a Pasteur pipette. The pellet was washed and re-suspended in D-PBS.

2.3.4 Draining lymph nodes

The para-aortic and inguinal lymph nodes were removed and placed in AB-IMDM. A single cell suspension was prepared in AB-IMDM by mechanical disruption of the tissue and filtering through a 70μm cell strainer.

2.3.5 Cell counting

Leukocyte preparations for flow cytometry analysis from the lymph nodes, blood and spinal cord were counted using the Casy-1 cell counter (Sharfe system) which counts all cells between 4.8-15μM.

2.4 Flow cytometry analysis

Buffers/solutions/media: (all reagents Sigma unless otherwise stated)

- FACS buffer (PBS containing 0.5% sodium azide and 0.5% BSA)
- D-PBS containing 1g/L D-Glucose, 36mg/ml Pyruvate, $CaCl_2$, $MgCl_2$ (Invitrogen), supplemented with 0.5% BSA.
- Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% FCS (Biosera), 0.1mM β-Mercaptoethanol and Penicillin-streptomycin-glutamine (200units/ml 200μg/ml and 4mM, respectively) (Invitrogen).

2.4.1 Staining for cell-surface antigens

Cells were re-suspended at 0.5-5x10^6 cells per 200μl of FACS buffer containing a 1:10 dilution of anti-Fc receptor antibody (concentrated supernatant prepared in-house from a 2.4G2 hybridoma) for at least 15 minutes on ice. The cells were then collected by centrifugation (4 minutes at 1200rpm) and resuspended in 200μl of

antibody cocktail (**Table 2.1**) prepared in FACS buffer. 7-aminoactinomycin D (7AAD) was included in the stain at 2.5μg/ml, if required and staining was carried out for at least 30 minutes on ice in the dark. Cells were washed with FACS buffer (by centrifuging the cells at 1200rpm for 4 minutes, discarding the supernatant, resuspending the pellet in wash fluid and then collecting the cells again by centrifugation) and were fixed by resuspending in 200μl of paraformaldehyde-based intra-cellular (IC) fixation buffer (eBioscience) for 20 minutes at room temperature in the dark. The cells were then washed in FACS buffer and stored at 4°C before analysis by flow cytometry.

2.4.2 Intra-cellular IL-17A and IFN-γ staining

For staining of intra-cellular cytokines, a 4hour stimulation with Phorbol 12,13, Dibutyrate (PdBu), ionomycin and brefeldin A was required. In order to capture cell-surface molecules that could potentially be down-regulated upon stimulation with these mitogenic stimuli, cell-surface staining was carried out first. For this, cells were re-suspended at 0.5-5x10^6 cells per 200μl of D-PBS (containing no sodium azide as the cells must be kept viable for further stimulation) containing a 1:10 dilution of anti-Fc receptor antibody (concentrated supernatant prepared in-house from 2.4G2 hybridoma) for at least 15 minutes on ice. The cells were then collected by centrifugation (1200rpm, 4 minutes), resuspended in 200μl of D-PBS containing a cocktail of antibodies to extra-cellular markers (**Table 2.1**), and were left for at least 30 minutes on ice in the dark. 7AAD was not included in the cocktail at this stage. Following extra-cellular staining, the cells were washed with D-PBS, re-suspended at approximately 2x10^6 cells/ml in IMDM medium containing PdBu at 0.05μg/ml, ionomycin at 0.5μg/ml and brefeldin A at 2μg/ml and incubated at 37°C for 4 hours. Cells were then harvested by centrifugation (1200rpm, 4 minutes), washed in FACS buffer and re-suspended in FACS buffer containing 7AAD at 2.5μg/ml for 15 minutes on ice in the dark. Excess 7AAD was washed from the cells by two washes in FACS buffer and the cells were fixed by re-suspending in

200µl of para-formaldehyde-based IC fixation buffer for 20 minutes at room temperature in the dark. The cells were collected by centrifugation and were permeabilised in 100µl of the detergent, IGEPAL CA-630 (diluted 1:100 in distilled H_2O and prepared 1:10 in FACS buffer) for 4 minutes at room temperature. 200µl of FACS buffer was added to inhibit the permeabilisation, and the cells were re-suspended in 200µl of intra-cellular staining antibodies (**Table 2.2**) for at least 30 minutes at room temperature in the dark. Fluorochrome-conjugated streptavidin (Pacific Orange-conjugated streptavidin from Invitrogen) was included in the intra-cellular staining cocktail as required. The cells were then collected by centrifugation at 1200rpm for 4 minutes and were washed and resuspended in FACS buffer before flow cytometry analysis.

2.4.3 Intra-cellular Foxp3 staining

A Foxp3 staining buffer set from eBioscience was used according to the manufacturer's instructions. 0.5ml of Foxp3 Fix-Perm buffer (diluted 1:3 in Fix-Perm diluent) was added to the cells instead of IC fixative described in the intra-cellular cytokine staining protocol above, followed by 40 minutes incubation in the fridge. The cells were then collected by centrifugation (1200rpm. 4 minutes), washed with 2ml of permeabilisation buffer (diluted 1:10 in distilled H_20) and re-suspended in 100µl of permeabilisation buffer containing a cocktail of staining antibodies including anti-Foxp3 APC (**Table 2.1**) Staining was carried out at room temperature for 40 minutes and the cells were washed twice in 2ml of permeabilisation buffer before re-suspending in FACS buffer for flow cytometry analysis.

The cells were analysed by flow cytomery, collecting at least 500,000 events per sample. Flow cytometric analysis was performed using four-colour analysis on the FACSCalibur (BD) and seven-colour analysis on the FACSCanto II (BD). The data

were analysed using Flowjo software (Tree Star) as shown in **Chapter 3** for lymph nodes, blood and spinal cord (**Figure 3.1, 3.5** and **3.12,** respectively).

2.5 Bleeding of mice and Vetscan analysis

Mice were bled by cutting the end of the tail and collecting approximately 80μl of blood into a heparin-coated capillary tube. The blood was expelled from the capillary tube using a Gilson pipette and mixed in an eppendorf tube. 5μl of this was mixed with 45μl of PBS for measurement of the number and percentage of blood granulocytes, monocytes and lymphocytes on a Vetscan HM II (Abaxis).

2.6 Assessment of MOG-specific cytokine production

Buffers/solutions/media: (all reagents Sigma unless otherwise stated)

- Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% FCS (Biosera), 0.1mM β-mercaptoethanol and Penicillin-streptomycin-glutamine (200units/ml 200ug/ml and 4mM, respectively) (Invitrogen).

2.6.1 Stimulation of lymph node cells

Lymph node cells were counted as described in section **2.3.5** and cultured in U-shaped 96-well plates at $1x10^6$ cells per 200μl of IMDM medium supplemented with either MOG_{35-55} peptide (MEVGWYRSPFSRVVHLYRNGK) at a concentration of 20μg/ml or PdbU and ionomycin at concentrations of 0.05μg/ml and 0.5μg/ml, respectively. Supernatants were recovered after incubation for 24 hours at 37°C and were stored at -20°C for future analysis.

2.6.2 Flow cytometry staining

For flow cytometry analysis of the MOG_{35-55} peptide-stimulated cells (described in section **2.6.1**), brefeldin A was added to the medium at a concentration of 2μg/ml after 18 hours of culture and the cells were cultured for a further 6 hours. Cells were harvested and stained with 7AAD at 2μg/ml in FACS buffer for 15 minutes on ice in the dark. They were then washed twice with FACS buffer, followed by permeabilisation, fixation and staining with antibodies specific for intra-cellular cytokines (**Table 2.2**) as described in section **2.4.2**. Antibodies to cell-surface markers (**Table 2.1**) were this time also included in the same antibody cocktail.

2.6.3 Detection of IL-17A and IFN-γ protein in culture supernatnants

IL-17A and IFN-γ protein in supernatant samples was determined using Flowcytomix mouse Th1/Th2 10plex kit (Bender MedSystems) as directed by the manufacturer's instructions. Briefly, anti-IL-17 and anti-IFN-γ antibody-coated beads were incubated with 10μl of supernatant sample or kit standard plus biotin-conjugated detection antibodies, specific for different epitopes of IL-17 and IFN-γ. Standards were prepared by three-fold dilution using recombinant cytokines from the Flowcytomix kit with a top concentration of 20ng/ml. The bead mixture was then incubated with PE-conjugated streptavidin before analysis of the samples using FACSCalibur. The FACS data were analysed using Flowcytomix^{Tm} Pro 2.3 Software which determined IL-17 and IFN-γ protein concentrations for each sample in pg/ml.

2.7 Assessment of cell migration

Buffers/solutions/media: (all reagents Sigma unless otherwise stated)

- Roswell Park Memorial Institute-1640 (RPMI) medium (Invitrogen), supplemented with 0.5% fatty acid free BSA 0.1mM β-mercaptoethanol and Penicillin-streptomycin-glutamine (200units/ml 200ug/ml and 4mM, respectively) from Invitrogen (Assay medium)
- FACS buffer (PBS containing 0.5% sodium azide and 0.5% BSA)
- D-PBS containing 1g/L D-Glucose, 36mg/ml Pyruvate, $CaCl_2$, $MgCl_2$ (D-PBS) from Invitrogen

Transwell inserts from polycarbonate membrane 24 well transwell permeable supports (Corning CoStar) were coated with 100µl of D-PBS containing 2µg/ml fibronectin and left overnight. The following day, the fibronectin was removed and replaced with 1% fatty acid free BSA in D-PBS (blocking solution) and left for at least one hour. Peripheral lymph nodes were harvested and placed in assay medium. A single cell suspension was then prepared by mechanical disruption of the tissue and filtering through a 70µm cell strainer. For cells treated with pertussis toxin *in vitro*, cells were then adjusted to a concentration of $5x10^6$ cells/ ml using assay buffer and pertussis toxin was added to half of the cells at a concentration of 10ng/ml. The cells were then incubated at 37°C for 2 hours followed by a wash and re-suspension in assay medium at $1x10^7$ cells/ ml. For assessment of migration of cells after *in vivo* treatment with pertussis toxin, peripheral lymph node cells were removed from the mouse and re-suspended directly in assay buffer at $1x10^7$ cells/ml without any *in vitro* incubation period. CCL21 ligand (R&D Systems) was prepared in assay buffer with a top dilution of 200ng/ml and subsequent 2-fold dilutions. The blocking solution in the transwell inserts was removed and 100µl of cell suspension was added to each insert, followed by addition of 600µl of CCL21 at the appropriate concentration underneath each transwell. Input controls were included in the plate which contained 600µl of assay medium plus 100µl of cell suspension in the

absence of the transwell insert. The plates were incubated at 37°C for 4 hours and then the cell suspensions underneath the transwell inserts, as well as the input control suspensions were transferred into FACS tubes, rinsing each well thoroughly with FACS buffer. The cells were centrifuged (1200rpm, 4 minutes) and re-suspended in FACS buffer containing 7AAD (2.5μg/ml), anti-CD4 PE and anti-TCRβ FITC (**Table 2.1**) and left for at least 30 minutes on ice in the dark. Cells were then collected by centrifugation (1200rpm, 4 minutes) and re-suspended in 300μl of FACS buffer. A solution of unlabelled, calibrite beads (BD) was prepared by adding 2-3 drops to 5ml of FACS buffer. 100μl of this was then added to each sample before analysis using a FACSCalibur. Approximately 2000 beads were acquired for each sample and the percentage of migration was calculated as follows:

Number of cells per bead in sample = Number of CD4 T cells acquired / Number of beads acquired

% migration of input = Number of cells per bead in sample / Number of cells per bead in input control X 100

Table 2.1 Cell-surface antibodies used for flow cytometry

Marker	Clone	Conjugate	Supplier
TCRβ	H57-597	Biotin	eBioscience
TCRβ	H57-597	PE	eBioscience
TCRβ	H57-597	FITC	BD Pharmingen
CD4	RM4-5	eFluor 450	eBioscience
CD4	RM4-5	PE	BD Pharmingen
CD19	MB19-1	FITC	eBioscience
CD19	RM7705	APC	Invitrogen
CD8α	53-6.7	APC	eBioscience
CD8α	5H10	Pacific Orange	Caltag
Ly6G	1A8	PE	BD Pharmingen
Neutrophil (Allotypic marker)	7/4	Alexa Fluor 700	AbD Serotec
γδ TCR	eBioGL3	APC	eBioscience

Table 2.2 Intra-cellular antibodies used for flow cytometry

Marker	Clone	Conjugate	Supplier
IL-17A	TC11-18H10.1	FITC	Biolegend
IL-17A	TC11-18H10.1	PE	BD Pharmingen
IFN-γ	XMG1.2	PE	eBioscience
Foxp3	FJK-16s	APC	eBioscience

2.8 Cloning

Buffers/solutions/media: (all reagents from Sigma unless otherwise stated)

- TAE buffer (made by the NIMR media lab): 40mM Tris Acetate (pH8), 1mM EDTA
- TFB1: 30mM Potassium Acetate, 10mM $CaCl_2$, 50mM $MnCl_2$, 100mM RbCl, 15% Glycerol (adjusted to pH5.8)
- TFB 2: 10mM MOPS, 75mM $CaCl_2$, 10mM $RbCl_2$, 15% Glycerol (adjusted to pH6.5)
- STET buffer: 0.1M NaCl, 10mM Tris.Cl (pH8), 1mM EDTA, 5% Triton X-100
- LB medium (made by the NIMR media lab)

2.8.1 Phenol:Chloroform DNA extraction

An equal volume of Phenol:Chloroform:Isoamyl alcohol saturated with 10mM Tris (pH 8), 1mM EDTA was added to the bacterial or ES cell DNA solution. After briefly vortexing, the mixture was centrifuged for 30 seconds at 3000 rpm at room temperature to separate the aqueous and organic phases. The top aqueous phase was removed and transferred to a new tube containing 1 ml of 96% ethanol and 0.1M sodium acetate (pH 5.2). The tube was mixed by inversion and stored at -70ºC for at least 1 hour. The DNA pellet was collected by centrifugation for 30 minutes at 3000 rpm, washed once in 70% ethanol and dissolved in molecular biology grade water (5Prime) or TE buffer (Qiagen).

2.8.2 DNA Restriction Enzyme Digests

Restriction enzymes and buffers (which differ in salt and magnesium concentration and pH) were obtained from New England Biolabs and Roche. Digestions were

carried out using the restriction enzyme buffer diluted 1:10 in the final reaction mix and using restriction enzyme at no more than 5% of the final volume. For digestions with two different restriction enzymes, which required different buffers, two separate reactions were carried out. The DNA was purified after the first reaction with either phenol:chloroform extraction and ethanol precipitation or using a Qiagen PCR purification column as described in the manufacturers' instructions.

2.8.3 Agarose Gel Electophoresis

0.5-2% agarose gels were prepared using agarose dissolved in 1 x TAE buffer (prepared in distilled-H_20). Ethidium bromide was added at a concentration of 0.2mg/ml. 1kb and 100bp DNA ladders were used from New England Biolabs. DNA was supplemented with Orange G loading buffer at a concentration of 1:5 before loading into the gel. The gels were run for approximately 1 hour at 80-100V. DNA was visualized and photographed under short wave UV light.

2.8.4 Extracting DNA fragments from agarose gels

After running the DNA digest the appropriate DNA fragment was cut out from the agarose gel under short wave UV light using a scalpel blade. DNA was extracted from the agarose slice using a Qiagen Gel Extraction kit or GeneClean kit from Anachem according to the manufacturer's instructions.

2.8.5 De-phosphorylation of DNA fragments

Linear DNA fragments with complementary sticky ends were de-phosphorylated using Shrimp Alkaline Phosphatase (SAP, Roche Biomolecular) to remove 5' phosphate groups. This was done by adding 2 units of SAP per 1µg of DNA in the restriction enzyme digest. Buffer was used at a dilution of 1:10 in the final reaction

mix. This was incubated for 30 minutes at 37ºC, followed by 30 minutes at 56ºC and then 10 minutes at 70ºC.

2.8.6 Filling in 5' overhangs

This was carried out on restriction enzyme digested DNA by adding dNTPs (Invitrogen) at a final concentration of 200μM each and 8 units of Klenow fragment (Promega) per 1μg of DNA used in the restriction digest. Klenow is the larger fragment of DNA polymerase I which retains 5'-3' polymerase activity but has lost 5'-3' exonuclease activity, making it useful for filling in overhangs. This was incubated at room temperature for 30 minutes. The DNA was subsequently phenol:chloroform extracted and ethanol precipitated as described in section **2.8.1**.

2.8.7 Ligation reactions

Ligation of DNA insert fragments (I) into vectors (V) were carried out using T4 DNA ligase supplied from Roche. Ligations were carried out at ratios of 3:1 and 5:1 (I:V). Amounts of DNA for the ligation reactions were determined using the following formula:

$$\frac{\text{Amount of V (ng)} \times \text{Length of I (Kbp)}}{\text{Length of V (Kbp)}} \times \text{I:V} = \text{Amount of I (ng)}$$

1 unit of the enzyme was used per ligation reaction, with ligase buffer diluted 1:10 in a final volume of 10μl. The ligation reactions were carried out at 16ºC overnight.

2.8.8 Transformation of plasmid DNA and preparation of competent bacteria

One Shot INVαF' competent cells from Invitrogen were used for transformation of the targeting construct. SURE competent cells from Stratagene and competent

DH10B E coli were used for other transformations. Transformations using commercial bacteria were carried out according to the manufacturer's instructions. Transfomations using DH10B E coli was carried out as follows: Cells were defrosted on ice, removed from the freezing vial and transferred to a round-bottomed tube. 4µl of ligation reaction was added to the cells and carefully mixed. After a 30 minute incubation on ice the cells were heat-shocked at 42ºC for 40 seconds and then incubated on ice for 2 minutes. 2.5 ml of LB medium without addition of antibiotics was added and the cells were incubated for 45 minutes at 37ºC with shaking. Aliquots were plated out onto LB agar plates supplemented with an appropriate antibiotic (Ampicillin or Carbenicillin at 100µg/ml) at 37ºC overnight.

To prepare the competent DH10B E Coli cells a single colony of DH10B E Coli from an LB agar plate was inoculated in 2.5 ml LB medium and incubated overnight at 37ºC with shaking. This whole culture was then added to 250ml of LB medium containing 20mM $MgSO_4$ and the culture was grown in a 1L conical flask at 37ºC and 300rpm until the optical density absorbance at 600nm reached between 0.4 and 0.6. The cells were then pelleted by centrifugation at 3000rpm for 30 minutes at 4ºC and the pellet was re-suspended in 100 ml of ice cold TFB1. The re-suspended cells were incubated on ice for 5 minutes at 4ºC and the cells were collected as before. All subsequent steps were performed on ice, in the cold room with chilled pipettes, tubes and flasks. The cells were gently re-suspended in 10ml of ice cold TFB 2 and incubated on ice for 15-60 minutes before aliquoting 200µl of the suspension in freezing vials. The vials were snap-frozen in dry ice before storage at -70ºC.

2.8.9 Small-scale plasmid DNA preparation

A single colony was picked from an LB-agar plate and inoculated into 5ml of LB medium supplemented with the appropriate selection antibiotic (Ampicillin or Carbenicillin at 100µg/ml) for growth overnight at 37ºC, shaking at 5000 rpm. The bacterial cultures were transferred to 1.5ml eppendorfs and centrifuged at 3000 rpm for 5 minutes at room temperature. The bacterial pellet was re-suspended by vortex

in 350µl of STET buffer supplemented with 50mg/ml lysosyme. The tubes were incubated for 40 seconds at 100ºC before centrifuging at 3000 rpm for 15 minutes at room temperature. Cell lysate with genomic DNA was removed using a sterile toothpick and the plasmid DNA and RNA was precipitated by adding 420µl of room temperature isopropanol and 40µl of 3M sodium acetate (pH 5.2). This was mixed and stored at room temperature for 10 minutes before centrifuging for 3000 rpm for 15 minutes at 4ºC. The nucleic acid pellet was washed with 70% ethanol, air-dried and dissolved in 30µl molecular grade water (5 Prime).

2.8.10 Large-scale plasmid DNA preparation

A single colony was picked from an LB agar plate, inoculated into 250µl LB medium supplemented with the appropriate selection antibiotic (Chloramphenicol at 12.5µg/ml or Ampicillin or Carbenicillin at 100µg/ml) and incubated overnight for 13 hours. The bacterial culture was pelleted by centrifugation at 4000 rpm for 20minutes. Preparation of plasmid DNA was carried out using a plasmid MAXI prep kit from Qiagen and Bacterial Artificial Chromosome (BAC) DNA was prepared using a Nucleobond BAC 100 kit from Clontech, according to the manufacturer's instructions.

2.8.11 Polymerase chain reactions and site-directed mutagenesis

2.5-6.25 Units of Thermoprime Plus DNA polymerase (Thermo Scientific) was used for PCR reactions. Reactions were supplemented with 1.25µM of each primer, 3mM $MgCl_2$ and 0.5mM of each dNTP with enzyme buffer diluted 1:10 in the final reaction mix volume.

Site-directed mutagenesis of exon 2 of the IL-17A gene in the targeting plasmid was carried out using QuikChange Site-Directed Mutagenesis Kit from Stratagene using

approximately 500ng of plasmid DNA as the starting template. The upstream primer was designed incorporating the mutation in exon 2 (AT →CA).

Primers (supplied from MWG-Biotech AG) and PCR protocols:

*Amplification of the 5' long arm of homology for the IL-17A_Cre construct (Chapter 4, **Figure 4.2**):*

Sense primer (red arrow **Figure 2.1**):
5' cga gtc gac tta att acc agt ttc tca gtc tca 3'
Anti-sense primer (red arrow **Figure 2.1**):
3' tcg act agt cct gcg cgt ttg tac cgt tcg aac agg tgg tac 5'

Initial denaturation - 95°C, 1 minute
Denaturation - 95°C, 30 seconds
Annealing - 56°C, 30 seconds
Elongation - 72°C, 4 minutes
38 cycles

*Amplification of the 5' external probe for the IL-17A Southern blot (Chapter 4, **Figure 4.4**):*

Sense primer (black arrow **Figure 2.1**):
5' ctc gta ctc agg atc cag gta tta ttc tca ggg c 3'
Anti-sense primer (black arrow **Figure 2.1**):
3' gat tgg ttg gcc agg gag ctc 5'

Initial denaturation - 95°C, 2 minutes
Denaturation– 95°C, 2 minutes
Annealing- 65°C, 30 seconds
Elongation– 72°C, 25 seconds

30 cycles

*Site-directed mutagenesis of IL-17A exon 2 for the IL-17A_Cre construct (Chapter 4, **Figure 4.2**):*

Sense primer (blue arrow **Figure 2.1**):
5' ccc ttc tag tct ctg cag ctg ttg ctg ctg ctg agc 3'
Anti-sense primer (blue arrow **Figure 2.1**):
3' gct cag cag cag caa cag ctg cag aga cta gaa ggg 5'

Initial denaturation - 95°C, 30 seconds
Denaturation– 95°C, 30 seconds
Annealing- 55°C, 1 minute
Elongation– 68°C, 6 minutes
16 cycles

2.9 Blotting and hybridisation

Buffers/solutions/media: (all reagents from Sigma unless otherwise stated)

- 20x SSC : 3M NaCl, 0.3M sodium citrate.
- Hybridisation solution: 3 x SSC, 0.1% Sodium Dodecyl Sulfate (SDS), 10 x Dehardt's solution, 10% Dextran sulphate
- Denaturation solution: 0.5M NaOH, 1.5M NaCl
- Neutralising solution: 0.5M Tris pH 7.4, 1.5M NaCl
- 20% SDS (Bio Rad)

2.9.1 Southern Blot

10µg of genomic DNA, or half of the DNA generated from Embryonic Stem (ES) cell clones, was digested with the appropriate restriction enzyme in a final volume of 50µl in the presence of BSA (final concentration 100µg/ml, New England Biolabs) overnight at 37ºC. The genomic DNA digest was electrophoresed on a 0.8% agarose gel at 30V overnight. The following day the gel was photographed under short wave UV light and was washed for 40 minutes in denaturation solution followed by 40 minutes in neutralisation solution. The DNA was blotted onto a positively charged nylon transfer membrane (GE Healthcare) via upward capillary transfer using 20x SSC. This was done overnight followed by immobilisation of DNA onto the membrane by baking at 80ºC for 2 hours.

2.9.2 Radiolabelling of DNA probes

DNA probes were labelled using Ready-To-Go DNA labelling beads (Amersham Biosciences). Approximately 100ng of DNA probe was denatured at 100ºC for 5 minutes followed by immediately cooling on ice for 5 minutes. This was mixed with 1.1 MBq of [α^{32}P]-dCTP and added to the Ready-To-Go DNA labelling beads reaction mix containing dATP, dGTP, dTTP, Klenow fragment and random oligodeoxyribonucleotides. After 30 minutes at 37ºC the radiolabelled probes were extracted from the reaction mix using a NICK column (Amersham Biosciences) as per the manufacturer's instructions. The NICK column is for purification of radiolabelled DNA from unincorporated radiolabelled nucleotides using gravity flow chromatography.

2.9.3 Hybridisation of Southern Blots

Membranes were soaked in 2x SSC and pre-hybridised using 50ml hybridisation solution and 500µl denatured salmon sperm DNA (100µg/ml final concentration). This was carried out for at least 2 hours in Hybaid bottles placed in a rotating Hybaid oven at 65ºC. Radiolabelled DNA probe was mixed with 400µl of salmon sperm DNA (10mg/ml) and denatured at 100ºC for 5 minutes before adding to the pre-hybridisation mix and incubating at 65ºC overnight. The next day the membranes were washed as follows: twice using 100ml 3x SSC, 0.1% SDS then twice using 100ml 0.3x SSC, 0.1% SDS at 65ºC for 30 minutes. The intensity of signal to background was measured after each wash using a Geiger counter. The membranes were wrapped in Saran Wrap and exposed to Kodak BioMax MS film at -70ºC. The films were developed after 24 hours and re-exposed for a further 10 days if required.

2.10 ES cell targeting

Buffers/solutions/media: (all reagents from Sigma unless otherwise stated)

- EF medium: DMEM supplemented with 15% FCS (HyClone from Peribo), 2mM glutamine (Invitrogen), Penicillin/Streptomycin (50 units/ml / 50µg/ml), 0.1mM MEM non-essential amino acids (Invitrogen)
- ES medium: DMEM supplemented with 15% FCS (HyClone from Peribo), 2mM glutamine (Invitrogen), Penicillin/Streptomycin (50 units/ml / 50µg/ml), 0.1mM MEM non-essential amino acids (Invitrogen), 0.1mM B-mercaptoethanol, LIF-ESGRO (1000units/ml)
- Trypsin: 0.25% Trypsin, 1.3mM EDTA, 119mM NaCl, 837.6µM $Na_2HPO4.12H_2O$, 1.76mM KH_2PO_4, 4.96mM KCl, 5.5mM D-glucose, 24.76mM Tris, $2.5x10^{-4}$ % Phenol Red. Adjusted to pH 7.6 and sterile filtered.

- Lysis Buffer (0.1% NaCl, 1%SDS, 0.5mg/ml Proteinase K**)**
- Hepes buffered saline (HBS): 137mM NaCl, 5mM KCl, 0.7mM Na_2HPO_4, 6mM dextrose, 21mM Hepes (adjusted to pH7.1)
- D-PBS containing 1g/L D-Glucose, 36mg/ml Pyruvate, $CaCl_2$, $MgCl_2$ (Invitrogen), supplemented with 0.5% BSA.

2.10.1 Preparation of G418-resistant embryonic fibroblasts

To make G418 resistant embryonic fibroblasts a mouse in which the neomycin resistance gene is expressed (β-$2m^{-/-}$) was mated with a wild type female. The pregnant female was sacrificed at day 13 or 14 of gestation and the embryos were dissected out free of extra-embryonic membranes in sterile conditions. The embryos were killed by placing on ice for a few minutes. Each embryo was then washed in 70% ethanol followed by D-PBS and the soft organs including the liver, gut and brain were dissected out. The remaining carcass was cut into small pieces and incubated for 2-18 hours at 4ºC followed by 37ºC for 10 minutes in a 15ml falcon tube with 2ml/embryo of trypsin**.** The embryonic tissue was pipetted and an equal volume of EF medium was added. The debris was allowed to settle for 2 minutes and then the supernatant was removed and plated out into one 200ml tissue culture flask in 50ml EF medium. The flask was incubated at 37ºC for 3 days until the cells reached extreme confluency, changing the medium after the first 24 hours. The fibroblasts were then split 1:5 and passed into 5 new 200ml flasks, which was repeated 3 days later. After a further 3 days the cells from all 25 flasks were collected, γ-irradiated (3500 rads) and were frozen in EF medium containing 12% Dimethyl Sulfoxide (DMSO) at 4 million or 20 million cells/vial.

2.10.2 ES cell culture

Embryonic stem cells from 129/Sv mice were expanded by growth on a feeder layer of γ-irradiated primary embryonic fibroblasts and maintained in an undifferentiated

state by incorporation of leukaemia inhibitory factor (LIF) in the growth medium. 6 cm culture dishes were coated in 3 ml 0.1% gelatine for 30 minutes and then 2 million embryonic fibroblasts were added in 5ml of ES culture medium. Feeders were left overnight to attach and form a monolayer before addition of the embryonic stem cells. The cells were allowed to grow to confluency at 37ºC over a few days, changing the medium every day, before trypsinising and splitting onto fresh 6 cm plates. Trypsinising was carried out by washing cells first in D-PBS and then adding 0.5ml trypsin for 3 minutes at 37°C. 1 ml of ES medium was added to inhibit the reaction and the cells were pipetted vigorously to generate a single cell suspension. They were then washed by centrifugation in 10 ml of ES medium at 1200 rpm for 5 minutes at room temperature and plated onto fresh plates.

2.10.3 Electroporation of 129Sv ES cells

The targeting construct plasmid was digested with SalI restriction enzyme, phenol:chloroform extracted, ethanol precipitated and dissolved in sterile TE (Qiagen) at a concentration of 2mg/ml. Embryonic stem cells were grown to 50-100% confluency, trypsinised and re-suspended in Hepes buffered saline (HBS) with $7x10^{-4}$% 2-mercaptoethanol at a concentration of $1x10^{7}$ cells/ml. 40µg of the linearised targeting construct was added to 0.8ml of the cell suspension in a 0.4cm microcuvette (Bio Rad) and electroporated using a gene pulser at 400 volts and 25µF. The cell suspension was then added to 3.4 ml of ES cell medium and plated onto 4 x 10cm plates containing monolayers of 4 million feeder cells so that each 10 cm plate received 2 million electroporated embryonic stem cells. Selection of the neomycin resistance gene-containing clones was initiated after 24 hours by changing the medium to ES medium containing 300µg/ml G418**.** Clones were selected over a period of 10 days, changing the medium on the plates every two days. G418-resistant clones were picked in PBS, trypsinised using trypsin diluted 1:3 with D-PBS and added to 96-well plates prepared the previous day with 2 x 10^{4} feeders/ well. After a further 5 days of culture the clones were trypsinised and transferred to

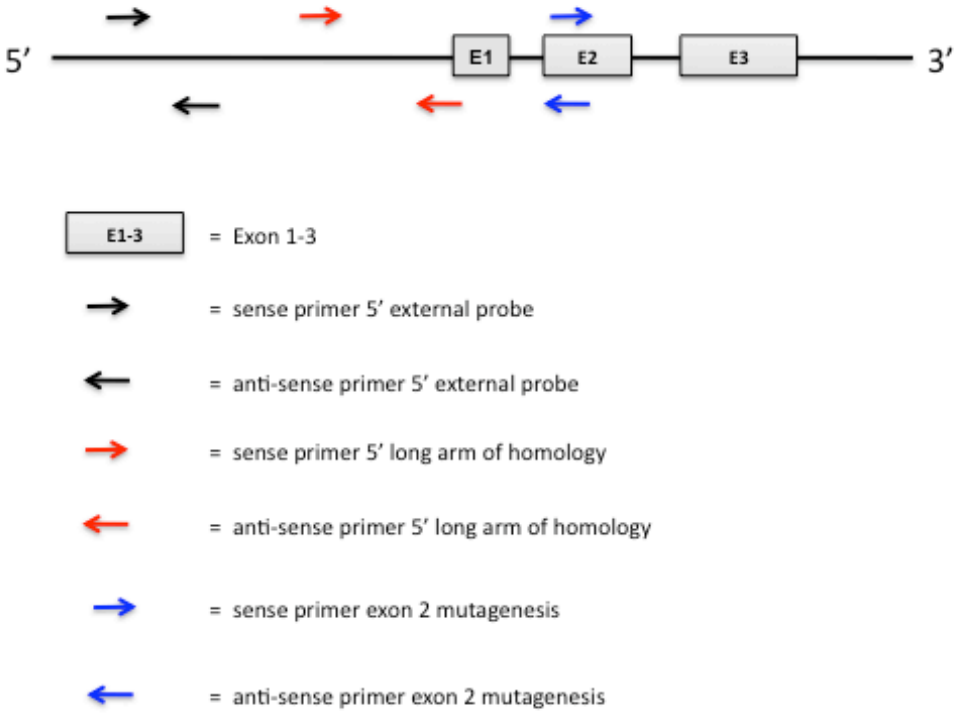
24-well plates containing 1.3 x 10^5 feeders/ well. Cells were grown for another 5 days before trypsinising and freezing half of each well in ES medium containing 12% DMSO. The other half of the cells were incubated overnight at 37ºC with 400µl of lysis buffer. DNA from the ES cell clones was then phenol:chloroform extracted and ethanol precipitated.

2.11 Statistical analysis

Statistical analysis was performed using GraphPad Prism 5.0 for Mac (GraphPad, USA). A non-parametric test was used to determine statistical significance. Comparision of two groups was performed using the Mann-Whitney U-Test, and comparison of three or more groups was performed using the Kruskal-Wallis test with Dunn's post test. In both tests, $P>0.05$ was considered not significant.

Figure 2.1 Representation of IL-17A genomic DNA and primer locations

The IL-17A locus is comprised of three exons, indicated as E1, E2 and E3. Sense and anti-sense primers are indicated by arrows pointing to the right and to the left, respectively. Black arrows represent primers to generate the 5' external probe, red arrows represent primers to generate the 5' long arm of homology and blue arrows represent primers to generate the double point mutation in exon 2.


5′
E1
E2
E3
3′
E1-3
= Exon 1-3
= sense primer 5′ external probe
= anti-sense primer 5′ external probe
= sense primer 5′ long arm of homology
= anti-sense primer 5′ long arm of homology
= sense primer exon 2 mutagenesis
= anti-sense primer exon 2 mutagenesis

Chapter 3: Characterisation of the immunological events leading to the development of an IL-17-associated autoimmune response

3.1 Introduction

EAE is a mouse model of autoimmunity in which the myelin sheath of the CNS is subject to attack by the immune system. The clinical outcome of this is progressive paralysis of the limbs. Immunisation with a myelin peptide in the presence of adjuvant, results in the activation of CD4 T cells in the peripheral lymphoid organs which when adoptively transferred into naïve recipients are capable of inducing disease (Mendel, Kerlero de Rosbo et al. 1995). The phenotype of these encephalitogenic CD4 T cells and the signals required to generate them are therefore areas of intense research.

Activation, expansion and differentiation of naïve CD4 T cells into effector T helper cells in an immune response is dependent upon activation of the TCR, delivery of a co-stimulatory signal as well as by signalling through cytokine receptors. It was for a long time believed that myelin peptide-specific Th1 effector cells, expressing IFN-γ are required for disease pathology in EAE. However, the more recent description of IL-17A-expressing Th17 effector cells has challenged this opinion and there is much evidence now suggesting that Th17 cells, not Th1 cells are important for disease pathology (Langrish, Chen et al. 2005; Veldhoen, Hocking et al. 2006).

In C57BL/6 mice, the combination of sub-cutaneous immunisation with MOG peptide emulsified in CFA and systemic administration of pertussis toxin provide the appropriate conditions for induction of encephalitogenic cells and subsequent disease development. The *Mycobacterium tuberculosis (Mtb)* component of CFA contains PAMPs which are thought to activate TLRs such as TLR1 and TLR2 on dendritic cells and macrophages (Brightbill, Libraty et al. 1999; Underhill, Ozinsky

et al. 1999; Akira, Uematsu et al. 2006), and it is this process which is believed, in part, necessary to initiate proliferation and activation of MOG-specific CD4 T cell clones. Although the specific consequences of this activation have not yet been identified, it was shown that *Mtb* can stimulate the production of TGFβ and IL-6 from bone marrow-derived dendritic cells *in vitro*, which are responsible for driving the generation of IL-17-producing CD4 T cells in mice from naïve precursors (Veldhoen, Hocking et al. 2006). Pertussis toxin is usually given systemically in the form of either an intraperitoneal or intravenous injection and has been used to facilitate EAE development since the 1970s, with the prevailing explanation that it opens up the blood brain barrier, allowing activated auto-antigen specific T cells to enter the central nervous system (CNS). Immunisation with CNS peptides in CFA was sufficient to induce EAE in early experiments with mice (Olitsky and Yager 1949) and Lewis rats (Lipton and Freund 1952) and pertussis toxin is not required in some transgenic mouse models of EAE (Goverman, Woods et al. 1993; Waldner, Whitters et al. 2000), suggesting that myelin peptide and CFA are sufficient to generate encephalitogenic T cells in the absence of pertussis toxin. Indeed, experiments in the 1970s, when it was first discovered that pertussis toxin could enhance disease severity, showed that pertussis toxin does in fact influence vascular permeability and that this could explain its role in EAE (Bergman and Munoz 1975; Bergman, Munoz et al. 1978). Moreover, pertussis toxin injected alone into mice has been shown to allow the influx of normally excluded serum proteins into the brain (Clifford, Zarrabi et al. 2007). However, in adoptive transfer models of EAE, cells are normally removed from actively immunised mice (with myelin peptide, CFA and pertussis toxin) and adoptively transferred into recipient mice without injection of pertussis toxin (Langrish, Chen et al. 2005; Kroenke, Carlson et al. 2008; O'Connor, Prendergast et al. 2008; Stromnes, Cerretti et al. 2008; Sutton, Lalor et al. 2009), suggesting that the direct effects of pertussis toxin on the blood brain barrier (Kerfoot, Long et al. 2004; Clifford, Zarrabi et al. 2007) may be dispensable for CD4 T cells to enter the spinal cord and initiate disease. In addition, it is intriguing that pertussis toxin is routinely administered at day 0 and 2, in the priming stage of disease, rather than later when cells are ready to enter the CNS.

The aims of this chapter were therefore to define the sequence of immunological events that result from immunisation with MOG CFA and pertussis toxin and result in clinical EAE, and to investigate specifically the contribution of pertussis toxin to this immune response.

3.2 Effect of pertussis toxin on the sequence of immunological events in EAE

In order to do address the role of pertussis toxin in the development of EAE we first wanted to detail the sequence of immunological events induced by MOG CFA immunisation alone, in order to establish the contribution of this component of the EAE induction protocol, an immunisation which in most cases does not lead to clinical disease. It would then be possible to investigate how administration of pertussis toxin modulates this response in order to highlight particular characteristics that are associated with EAE development. We chose to track the appearance of IL-17A and IFN-γ-expressing effector CD4 T cells (namely Th17 and Th1 cells, respectively) in the draining lymph nodes (as this is the predominant site for primary lymphocyte activation), the blood and the spinal cord where they contribute to a myelin-specific immune response.

Other cell types that may influence the ability of these effector CD4 T cells to initiate disease progression were also assessed. These included the Foxp3 regulatory CD4 T cells which have been shown to be induced to proliferate in EAE in response to MOG peptide and to have suppressive activity on peripheral MOG-specific CD4 T cell responses (Korn, Reddy et al. 2007). The importance of regulatory T cells in EAE has been shown in experiments where depletion of CD25+ cells prior to disease induction can overcome the requirement for pertussis toxin in the induction protocol (Montero, Nussbaum et al. 2004). Interestingly, pertussis toxin was also shown to reduce the number and function of Foxp3 regulatory T cells *in vivo* (Cassan, Piaggio et al. 2006). TCRδ-deficient mice develop EAE with reduced

severity and recently a role for γδ T cells in EAE has been implicated (Sutton, Lalor et al. 2009). In this paper the authors described that IL-17A-expressing γδ T cells were detected at high frequency in the spinal cords of mice with EAE and were required for MOG-specific IL-17A production. Neutrophils have been shown to infiltrate the spinal cord in mice with EAE (Wu, Cao et al. 2010), and are thought to contribute to the demyelination observed in the CNS. Indeed, a polymorphonuclear cell depletion antibody administered from day 8 after immunisation reduced disease severity and in some cases prevented the onset of disease (McColl, Staykova et al. 1998). Neutrophil accumulation in peripheral tissues has also been associated with IL-17 expression (Forlow, Schurr et al. 2001; Ye, Rodriguez et al. 2001).

3.2.1 Analysis of cell populations in the draining lymph nodes after MOG CFA immunisation

A cohort of 20 C57BL/6 female mice were immunised with MOG CFA into the base of the tail. The draining lymph nodes (inguinal and para-aortic) and blood were analysed on day 3 and day 7 following immunisation. Non-immunised, C57BL/6 female mice were analysed as controls.

The flow cytometry gating strategy used for analysis of the draining lymph nodes (dLNS) is shown in **Figure 3.1**. A gate was first drawn around cells of an approximately equivalent size to those counted by the Casy 1 cell counter (counted cells). This was followed by gating 'singlets' using FSC-W /FSC-H discrimination. Finally, live, 7AAD-negative cells were gated. (**Figure 3.1A**) 7AAD is a DNA-binding dye that can only access the nucleus following loss of membrane integrity during onset of death (Philpott, Turner et al. 1996).

In the mouse, lymph nodes are composed predominantly of T and B lymphocytes. Assessment of these lymphocyte populations showed that CD4 and CD8 T cells represented approximately 40% and 25% of lymph node cells respectively, B cells

represented approximately 15% and γδ T cells represented less than 1%. An example of these populations in a non-immunised C57BL/6 mouse is shown in **Figure 3.1B**.

As well as extra-cellular markers, T cells were also assessed for expression of intra-cellular IL-17A, IFN-γ and Foxp3 protein. This was done following a 4 hour stimulation with Phorbol 12,13 dibutyrate (PdBu), ionomycin and brefeldin A. PdBu activates Protein Kinase C, ionomycin upregulates intra-cellular calcium and brefeldin A prevents extra-cellular secretion of proteins and therefore retains protein inside the cell for detection by fluorescent antibodies. The combined effects of this stimulation resulted in the detection of a distinct population of IL-17A and IFN-γ-expressing cells. Assessment of IL-17A and IFN-γ-expressing populations in the lymph nodes of a non-immunised mouse showed that approximately 1% of CD4 T cells and 7% of CD8 T cells stained positively for IFN-γ and less than 1% of both CD4 and CD8 T cells stained positively for IL-17A (**Figure 3.1C**). 10% of γδ T cells also expressed IL-17A when measured by intra-cellular cytokine staining. (**Figure 3.1D**). Foxp3-expressing cells represented about 4% of the CD4 T cell population (**Figure 3.1E**).

Following immunisation with MOG CFA, lymphocyte populations in the dLNs of individual mice were assessed 3 days and 7 days later **(Figure 3.2)**. There was an overall increase in the number of all the cell populations measured after immunisation when compared to non-immunised control mice and statistical analysis using the Kruskal Wallis test and a Dunn's post test showed that this was significant in all cell types by day 7 and in CD4 and CD8 T cells by day 3. Expansion of any cell population in the lymph nodes could be a result of increased migration into the lymph nodes, reduced egress out of the lymph nodes, increased proliferation, increased survival, or *de novo* generation of those cells. However, the results of previous studies suggest that this non-selective expansion of lymphocyte populations in the draining lymph nodes in response to immunisation is likely to be associated with reduced egress of cell populations following antigen challenge

(Mackay, Marston et al. 1992) involving down regulation of the Sphingosine-1-Phosphate (S1P) receptor (Matloubian, Lo et al. 2004).

Activation of T cells into an effector phenotype is characterised by modification of the expression of a number of genes including the up-regulation of a number of cytokine genes. The number of IL-17A-expressing, IFN-γ-expressing and IL-17A/IFN-γ-expressing CD4 T cells increased in number following immunisation by day 7 **(Figure 3.3A).** Within the CD4 T cell population, the percentage of IL-17A-expressing and IL-17A/IFN-γ-expressing cells was also increased significantly at day 7, whereas the percentage of IFN-γ-expressing cells within the CD4 T cell population was significantly reduced by day 7 **(Figure 3.3B).** The number of IL-17A-expressing γδ T cells was also increased after immunisation which was statistically significant by day 7 when compared to the non-immunised mice **(Figure 3.4A)** and was accompanied by an increase in the percentage of IL-17A-expressing cells within the γδ T cell population. There was also an increase in the number of Foxp3-expressing CD4 T cells by day 7 following immunisation and this was accompanied by an increase in the percentage of Foxp3-expressing cells within the CD4 T cell population (**Figure 3.4B**).

In conclusion, MOG CFA immunisation alone induces an increase in the number of all cell populations measured in the lymph nodes with a relative increase in the proportion of IL-17A and Foxp3-expressing cells within the CD4 population, and IL-17A-expressing cells within the γδ T cell population.

3.2.2 Analysis of cell populations in the blood after MOG CFA immunisation

We then wanted to investigate whether activated effector cells that had been observed in the lymph nodes, characterised by their expression of IL-17A and IFN-γ could migrate to the blood in the mice immunised with MOG CFA alone. The blood of individual mice was analysed by flow cytometry for the presence of activated

CD4 T cells expressing IL-17A and IFN-γ and γδ T cells expressing IL-17A, 3 days and 7 days following immunisation with MOG CFA. A Vetsan was used to measure the number of lymphocytes in the blood, which identifies these cells based on size and granularity. Therefore, by placing a flow cytometry gate on the lymphocyte population, the number of cytokine-expressing T cell populations could be determined. An example of the flow cytometry analysis is shown in **Figure 3.5**.

IL-17A-expressing CD4 and γδ T cells were significantly increased in number in the blood by day 7 after immunisation, whereas the IFN-γ-expressing CD4 T cells showed no increase in number. **(Figure 3.6A)** This reflected the increase in the percentage of IL-17A-expressing cells in the CD4 T cell and γδ T cell populations in the lymph nodes at this time point and suggested that these effector cells had left the lymph node immediately and migrated to the blood. There was also a clear population of CD4 T cells expressing both IL-17A and IFN-γ in the blood after immunisation as illustrated in the flow cytometry plots in **Figure 3.5.** The number of CD4 T cells expressing both IL-17A and IFN-γ increased at day 7 following immunisation (**Figure 3.6A**).

Under steady state conditions, neutrophils mature from their bone marrow progenitors and are released into the peripheral circulation, a process which is dependent upon the actions of a number of cytokines and chemokines such as G-CSF and CXCL12 (von Vietinghoff and Ley 2008). In response to inflammation or injury, neutrophil mobilisation is accelerated which is important for the increased turnover of cells at the site of inflammation. The signals required for this accelerated mobilisation are likely to involve inflammatory cytokines, such as IL-17A, which has been shown to upregulate G-CSF production (Fossiez, Djossou et al. 1996). In EAE, depletion of polymorphonuclear cells in the effector stage of disease reduced the severity of symptoms suggesting these cells play a role in disease. We therefore wanted to address how MOG CFA immunisation influenced the mobilisation of neutrophils into the peripheral circulation. There was a small increase in the number of granulocytes measured using a Vetscan on day 3 following

MOG CFA immunisation and also further at day 7 when compared to non-immunised mice. The increase was not statistically significant when analysed using the Kruskal Wallis test and Dunn's post test, however the data is bi-modally distributed and clearly some mice have developed neutrophilia in response to MOG CFA immunisation **(Figure 3.6B).**

3.2.3 Effect of administration of pertussis toxin in combination with MOG CFA immunisation on clinical disease

We had established that the signals induced by MOG CFA immunisation alone were sufficient to generate IL-17A and IFN-γ- expressing effector cells in the lymph nodes which could travel to the blood. MOG CFA immunisation was also sufficient to induce mild neutrophilia in some mice. However, this immunisation protocol is not sufficient for the development of clinical EAE. Development of disease symptoms is dependent on the administration of pertussis toxin. We therefore wanted to address how pertussis toxin influenced the peripheral immune response following MOG CFA immunisation. A cohort of mice were immunised with MOG CFA, with intra-peritoneal administration of pertussis toxin and the sequence of immune events was assessed and compared to immunisation with MOG CFA alone.

Figure 3.7 shows the clinical analysis of two groups of mice injected with either MOG CFA alone, or with MOG CFA and pertussis toxin. The pertussis toxin was administered by intraperitoneal injection on day 0 (on the same day as the MOG CFA injection) and on day 2. Administering pertussis toxin in combination with MOG CFA increased the incidence of disease from 17.8% (5 out of 28 mice) to 70.8% (17 out of 24 mice) (**Figure 3.7A**). Consequently, the mean maximum clinical score was significantly higher (using Mann Whitney U test) in the pertussis toxin-treated group (**Figure 3.7A**) and the mean clinical score of the pertussis toxin-treated mice over time was substantially higher than the mice given MOG CFA only (**Figure 3.7B**). Clinical scores for individual mice is shown at day 18 and the

difference between the two groups was highly significant (**Figure 3.7C**). In those mice that did get disease in each group, the day of onset was also earlier in the pertussis toxin-treated group, although this difference was not significant (**Figure 3.7A**).

3.2.4 Effect of administration of pertussis toxin in combination with MOG CFA immunisation on cell populations in the draining lymph nodes

The number of cell populations from the dLNs was determined for individual mice 3 days and 7 days following immunisation with MOG CFA and pertussis toxin and this was compared to the number of these cell populations in the mice immunised with MOG CFA alone.

The number of all the cell populations investigated was reduced at day 7 in the mice which received pertussis toxin compared to the pertussis toxin-untreated group, however this was only determined as significant, using the Mann Whitney U- Test, for B cells (**Figure 3.8**). Cell movement into and out of the lymph nodes is largely dependent on G-coupled receptor signalling, a signalling pathway which can be inhibited by pertussis toxin (Reisine 1990). Furthermore, this observation is likely to be a result of the effect of pertussis toxin on cell migration. It could be that pertussis toxin either reduced recruitment of lymphocytes to the lymph nodes or increased egress of lymphocyte populations out of the lymph nodes at this time point.

Although inclusion of pertussis toxin in the immunisation protocol also resulted in a marginal, non-significant reduction in the number of IL-17A-expressing, IFN-γ-expressing and IL-17A/IFN-γ-expressing CD4 T cells, at day 7 **(Figure 3.9A)**, the proportion of IL-17A-expressing, IFN-γ-expressing and IL-17A/IFN-γ-expressing cells within the CD4 T cell population did not change **(Figure 3.9B).** Similarly, although after the administration of pertussis toxin there was a small reduction in the number of IL-17A-expressing γδ T cells and Foxp3-expressing CD4 T cells, the

percentage of IL-17A-expressing cells within the γδ T cell population and the percentage of Foxp3-expressing cells within the CD4 T cell population did not change (**Figure 3.10A and B**). This suggested that pertussis toxin was not enhancing the increase of IL-17A-expressing effector cells in the lymph nodes seen after MOG CFA immunisation, although the reduction in the total number of CD4 T cells could perhaps indicate that the egress of these cells into the blood was enhanced.

3.2.5 Effect of administration of pertussis toxin in combination with MOG CFA immunisation on cell populations in the blood

Comparison of the number of effector cells in the blood after administration with MOG CFA and pertussis toxin and with MOG CFA alone showed that even if pertussis toxin was increasing the egress of lymphocytes out of the lymph nodes this was not having any significant affect on the accumulation of cytokine-expressing cells in the blood **(Figure 311A)**.

The number of granulocytes in the blood was increased in the pertussis toxin-treated group at day 3 (**Figure 3.11B**). There was also an increase in the pertussis toxin-treated group at day 7, however this was not significant. Similar to immunisation with MOG CFA alone, immunisation with MOG CFA and pertussis toxin lead to bi-modal distribution of granulocyte numbers indicating that pertussis toxin enhanced the neutrophilia observed in response to MOG CFA immunisation.

3.2.6 Effect of administration of pertussis toxin in combination with MOG CFA immunisation on cell populations in the spinal cord prior to the onset of disease

It had so far been demonstrated that pertussis toxin was not having any striking effect on the immune response induced by MOG CFA with regard to the activation of IL-17A and IFN-γ-expressing cells in the lymph nodes and the appearance of these cells in the blood prior to the onset of disease. In attempt to further investigate

the mechanism by which pertussis toxin was facilitating disease, we wanted to assess how pertussis toxin was influencing the sequential appearance of leukocytes in the spinal cord leading to clinical disease.

Cells from the spinal cord of non-immunised mice, MOG CFA-immunised mice and MOG CFA-immunised mice with injection of pertussis toxin at day 0 and 2 were analysed sequentially at day 3, day 7 and day 10 after immunisation to determine how pertussis toxin alters the influx of cells into the spinal cord before the onset of disease.

Leukocytes including lymphocytes, monocytes and granulocytes were isolated from the spinal cord by percoll separation, counted and analysed by flow cytometry. Due to low cell recovery ($<1x10^6$ cells per mouse in mice that did not show signs of EAE), the spinal cords of 3 or 4 mice from each group were pooled and an average cell count per mouse was determined. For flow cytometry analysis, a gate was first drawn around cells of an approximately equivalent size to those counted by the leukocyte counter (counted cells). This was followed by gating on 'singlets' using FSC-W /FSC-H discrimination. Dead cells were excluded by gating on the 7AAD-negative cells. For lymphocyte staining, an additional lymphocyte gate was included (**Figure 3.12A**). Absolute numbers of cell populations were calculated by determining the percentage of the cell population of the total, counted cells. Neutrophils were defined based on their extra-cellular expression of Ly6G and an epitope recognised by 7/4 antibody, and monocytes were defined based on their expression an epitope recognised by 7/4 antibody but the absence of Ly6G. B cells were defined based on the expression of CD19, CD4 T cells on the expression of the TCRβ receptor and the CD4 co-receptor and γδ T cells on the expression of the γδ TCR (**Figure 3.12B**).

The number of Ly6G-expressing neutrophils in the spinal cord increased at day 7 following immunisation with MOG CFA alone and these numbers were maintained at day 10. Administering pertussis toxin did not substantially alter the influx of

neutrophils into the spinal cord up to the onset of disease **(Figure 3.13A).** The number of 7/4+ monocytes increased at day 3 following immunisation with MOG CFA alone and further at day 7 and day 10. Pertussis toxin-treated mice had fewer 7/4+ monocytes at day 10, prior to the onset of disease **(Figure 3.13A).** The value shown is the mean of three results from separate EAE experiments where three spinal cords were pooled in each experiment. To confirm this result, more mice would need to be analysed. Nevertheless, a down regulation in the number of 7/4+ monocytes in the pertussis toxin-treated group could be the result of fewer cells being recruited to the spinal cord or it could be due to down regulation of the epitope recognised by 7/4 antibody as the monocytes differentiate into antigen-presenting cells (Henderson, Hobbs et al. 2003).

The number of B cells, CD4 T cells and γδ T cells in the spinal cord is shown over time following immunisation in **Figure 3.13B.** The number of B cells in the spinal cord was reduced after immunisation both in the presence and absence of pertussis toxin when compared to non-immunised mice. CD4 T and γδ T cells were increased only in the pertussis toxin-treated group at day 10 following immunisation when compared to non-immunised mice.

In conclusion, the signals induced by MOG CFA immunisation alone were sufficient to recruit neutrophils and monocytes to the spinal cord prior to the onset of disease, but CD4 and γδ T cells were only observed to increase in the spinal cord in the mice that had received pertussis toxin.

Effector CD4 T cells have been shown to enter the spinal cord before the onset of clinical EAE, therefore day 10 was chosen as a time point immediately before the onset of EAE to analyse IL-17A, IFN-γ and Foxp3-expressing cells. **Figure 3.14** shows examples of cytokine staining in CD4 (**A**) and γδ T cells (**B**) at day 10. In the pertussis toxin-treated mice, clear populations of CD4 T cells expressing IL-17A, IFN-γ or both IL-17A and IFN-γ could be detected, as well as γδ T cells expressing IL-17A. The percentage of CD4 and γδ T cells in the pertussis toxin-untreated

group was lower but some cytokine-expressing cells within this population could still be detected. In the non-immunised group detection of cytokine-expressing cells was less convincing due to such a low percentage of CD4 T cells. Nevertheless, non-immunised mice were still analysed in this way as a baseline control **(Figure 3.14).**

The profile of effector cells detected in the spinal cord at day 10 following immunisation is shown in **Figure 3.15**. The number of CD4 T cells expressing IL-17A, IFN-γ and both IL-17A and IFN-γ together (**Figure 3.15A)** as well as the number of IL-17A-expressing γδ T cells (**Figure 3.15B**) increased slightly in the mice immunised with MOG CFA alone over that detected in non-immunised mice. This suggested that the cells activated to express IL-17A and IFN-γ following MOG CFA immunisation could, to some extent, access the spinal cord in the absence of pertussis toxin before the onset of disease. The number of these effector cells in the pertussis toxin treated mice however was clearly higher still, indicating that these cells either had an enhanced ability to enter the spinal cord or that they were proliferating more in the target organ.

The number of Foxp3 expressing CD4 T cells was also increased in the spinal cord 10 days following immunisation with MOG CFA and pertussis toxin when compared to non-immunised mice and mice immunised with MOG CFA alone **(Figure 3.15C),** suggesting again that these cells were not recruited or were not proliferating in mice without the administration of pertussis toxin.

3.2.7 Effect of administration of pertussis toxin in combination with MOG CFA immunisation on cell populations in the spinal cord at the peak of disease

Spinal cords were also analysed during disease symptoms at day 18 to investigate how the inflammatory reaction progresses in mice immunised with MOG CFA alone

compared to mice immunised with MOG CFA and pertussis toxin. The inflammatory infiltrate of MOG CFA and pertussis toxin-immunised mice with EAE symptoms (mice with disease represented the vast majority of this group) was compared to MOG CFA-immunised mice without EAE symptoms (mice without disease represented the vast majority of this group). The inflammatory infiltrate of the spinal cords of mice within the MOG CFA-immunised group that did get disease was also included for comparison. All groups were also compared to non-immunised mice. Mice with disease were analysed individually, however the mice without disease were pooled in groups of three and an average taken for calculation of absolute numbers.

Ly6G-expressing neutrophils and 7/4+ monocytes were increased in number in the mice with disease irrespective of pertussis toxin treatment when compared to non-immunised mice and mice lacking disease but immunised with MOG CFA in the absence of pertussis toxin (**Figure 3.16A**). CD4 and γδ T cells followed a similar trend, in that they were increased in numbers in the mice with disease irrespective of pertussis toxin treatment compared to the two groups of mice lacking disease. Numbers of B cells were lower in all immunised mice, compared to non-immunised mice irrespective of disease or pertussis toxin treatment (**Figure 3.16B**). This indicated that the presence of monocytes, neutrophils, CD4 and γδ T cells are associated with disease development rather than pertussis toxin treatment and it could be that the effects of pertussis toxin are exerted at an earlier time point in disease induction.

IL-17A, IFN-γ and Foxp3-expressing CD4 T cells as well as IL-17A-expressing γδ T cells were all increased in number in the diseased mice irrespective of pertussis toxin treatment when compared to immunised mice without EAE or non-immunised controls (**Figure 3.17A-C**). This indicated that these cells were either proliferating more readily or were being recruited more efficiently in the mice with disease. Again, this result may be a consequence of disease development suggesting that the effect of pertussis toxin may be important at an earlier time point.

3.2.8 Summary

From these experiments it was established that the signals induced by MOG CFA immunisation alone were sufficient to induce IL-17A and IFNγ -expressing CD4 cells as well as IL-17A-expressing γδ T cells in the lymph nodes, which could migrate to the blood and could access the spinal cord but no more than the basal level seen in non-immunised mice. These signals were also sufficient to allow neutrophil and monocyte infiltration into the spinal cord before the onset of disease, however their persistence in the spinal cord was associated with disease development. The signals from pertussis toxin were additionally required to allow the accumulation of cytokine-expressing T cell populations in the spinal cord which could be the result of enhanced recruitment or enhanced proliferation of these activated cells in the target organ.

Figure 3.1 Flow cytometry gating strategy used for analysis of cells in draining lymph nodes.

Para-aortic and inguinal lymph nodes from a non-immunised mouse were harvested and the cells were analysed by flow cytometry. For analysis of flow cytometry data a gate was first drawn to exclude cell debris (counted cells), singlets were gated by FSC-H/FSC-W discrimination and live cells were gated based upon exclusion of 7AAD (A). B cells were defined by expression of cell-surface CD19, T cells were defined based upon expression of the CD4 and CD8 co-receptors and γδ T cells were defined based upon expression of the γδ TCR (B). Lymph node cells were stimulated for four hours with PdBu, ionomycin and brefeldin A and stained for intra-cellular IL-17A, IFN-γ and Foxp3. Intra-cellular IL-17A and IFN-γ expression is shown in CD4 and CD8 T cells (C), IL-17A expression is shown in γδ T cells (D) and intra-cellular Foxp-3 expression is shown in CD4 T cells (E).

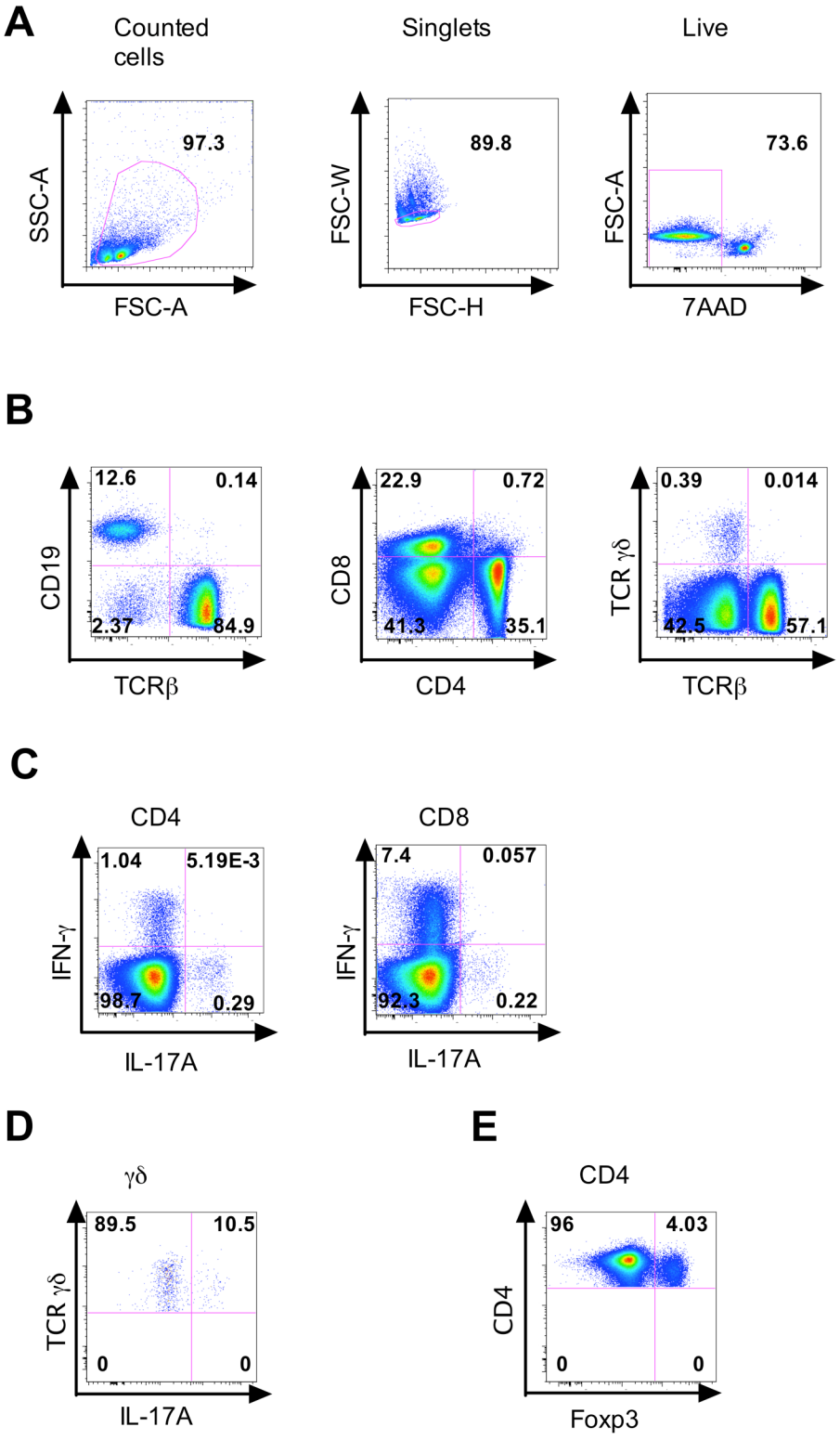

Draining lymph nodes
A
Counted cells
Singlets
Live
SSC-A
FSC-A
97.3
FSC-W
FSC-H
89.8
FSC-A
7AAD
73.6
B
CD19
TCRβ
12.6
0.14
2.37
84.9
CD8
CD4
22.9
0.72
41.3
35.1
TCR γδ
TCRβ
0.39
0.014
42.5
57.1
C
CD4
CD8
IFN-γ
IL-17A
1.04
5.19E-3
98.7
0.29
7.4
0.057
92.3
0.22
D
γδ
TCR γδ
IL-17A
89.5
10.5
0
0
E
CD4
Foxp3
96
4.03
0
0

Figure 3.2 Non-selective expansion of lymphocytes in the draining lymph nodes following immunisation with MOG CFA

C57BL/6 mice were immunised at the base of the tail with MOG CFA emulsion and the numbers of lymphocytes in the para-aortic and inguinal lymph nodes (draining lymph nodes) were assessed three days and seven days later. Age and sex-matched, non-immunised C57BL/6 mice were analysed as controls (open circles). Cell counts were performed by a Casy-1 cell counter and assessment of different populations was carried out by flow cytometry as described in **Figure 3.1**. The dots represent individual mice and the mean for each group is shown as a horizontal line. Shown are the results of three independent experiments. Statistical analysis was performed by the Kruskal-Wallis test with a Dunn's post test. (*) indicates P-value = 0.01-0.05, (**) indicates P-value = 0.001-0.01, (***) indicates P-value= <0.001.

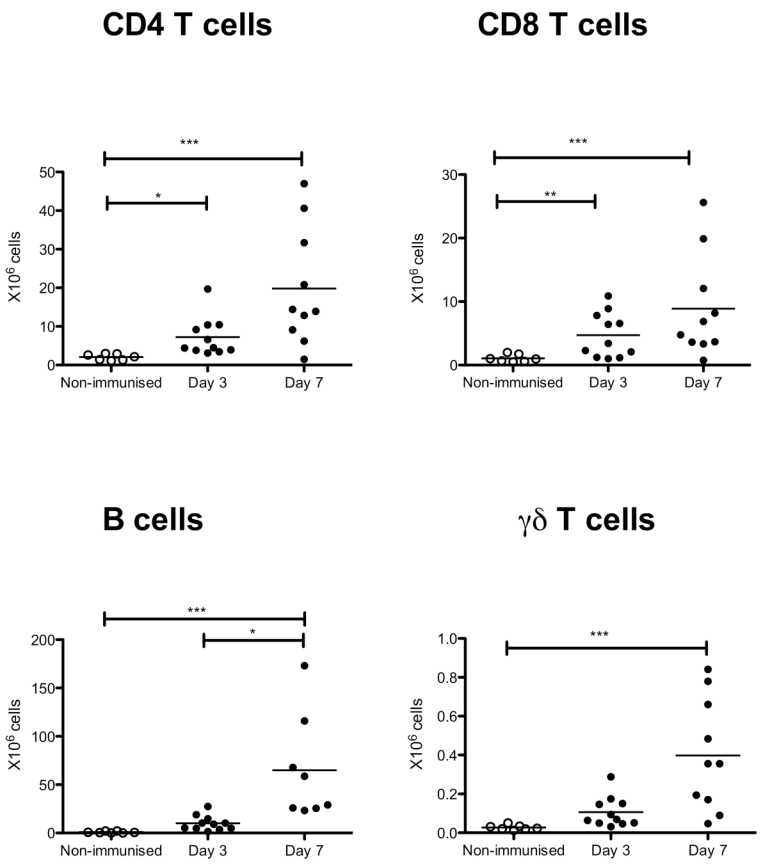

Draining lymph nodes
CD4 T cells
CD8 T cells
B cells
γδ T cells
X10^6 cells
Non-immunised
Day 3
Day 7
***
**
*

Figure 3.3 IL-17A and IFN-γ expression within the CD4 T cell population in the draining lymph nodes following immunisation with MOG CFA

C57BL/6 mice were immunised at the base of the tail with MOG CFA emulsion. The draining lymph nodes were harvested three days and seven days later and assessed for intra-cellular IL-17A and IFN-γ protein expression by flow cytometry following a four hour stimulation with PdBu, ionomycin and brefeldin A. Age and sex-matched, non-immunised C57BL/6 mice were analysed as controls (open circles). Total numbers of CD4 T cells expressing intra-cellular IL-17A and IFN-γ were determined (A). The percentage of IL-17A and IFN-γ-expressing cells within the CD4 T cell population are shown in (B). The dots represent individual mice and the mean is shown as a horizontal line. Shown are the results of three independent experiments. Statistical analysis was performed by the Kruskal-Wallis test with a Dunn's post test. (*) indicates P-value = 0.01-0.05, (**) indicates P-value = 0.001-0.01, (***) indicates P-value= <0.001.

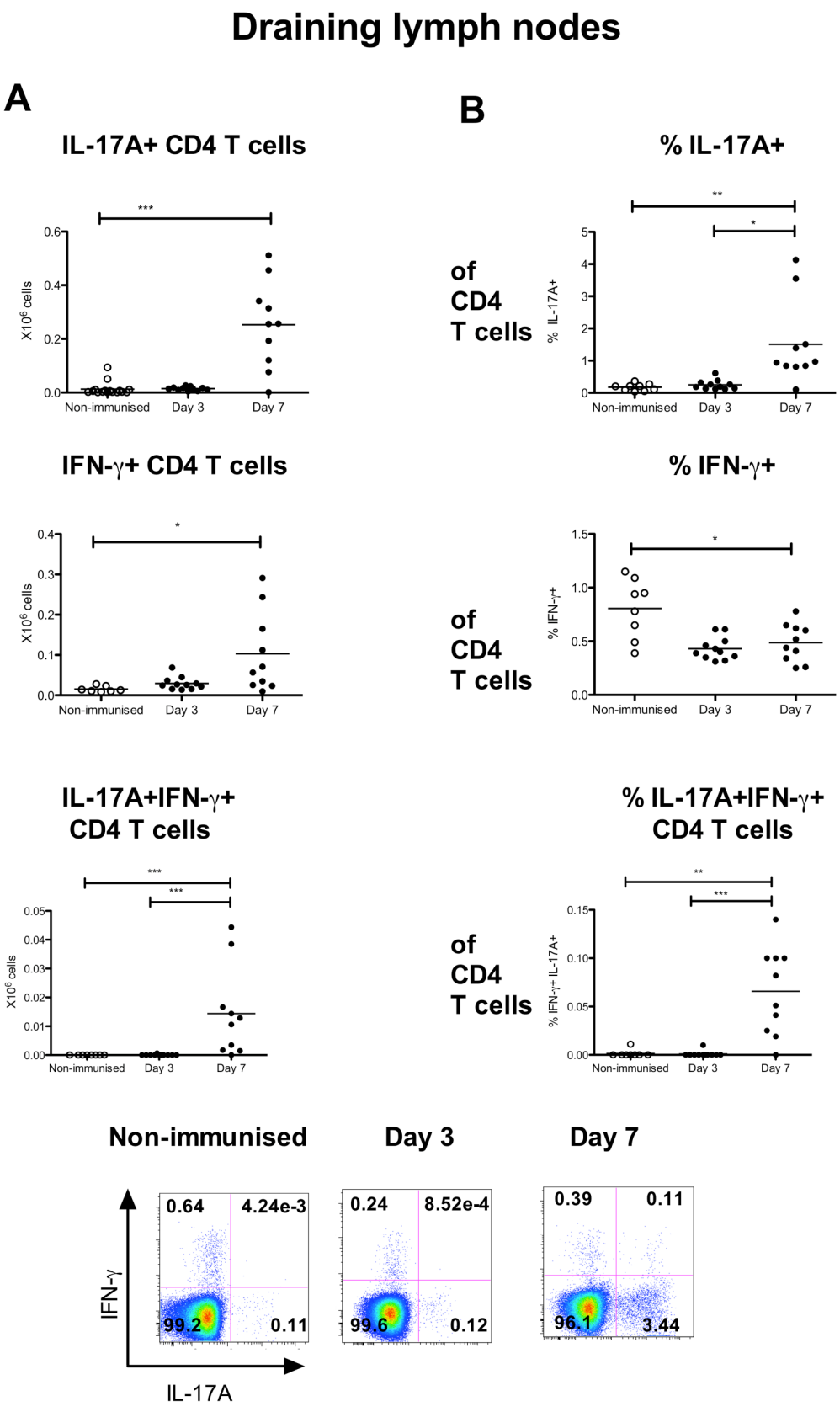

Draining lymph nodes
A
IL-17A+ CD4 T cells
X10^6 cells
Non-immunised
Day 3
Day 7
IFN-γ+ CD4 T cells
IL-17A+IFN-γ+ CD4 T cells
B
% IL-17A+
of CD4 T cells
% IL-17A+
% IFN-γ+
% IL-17A+IFN-γ+ CD4 T cells
% IFN-γ+ IL-17A+
Non-immunised
Day 3
Day 7
0.64
4.24e-3
99.2
0.11
0.24
8.52e-4
99.6
0.12
0.39
0.11
96.1
3.44
IFN-γ
IL-17A

Figure 3.4 IL-17A expression within the γδ T cell population and Foxp3 expression within the CD4 T cell population in the draining lymph nodes following immunisation with MOG CFA

C57BL/6 mice were immunised at the base of the tail with MOG CFA emulsion. The draining lymph nodes were harvested three days and seven days later and assessed for intra-cellular IL-17A and Foxp3 protein expression by flow cytometry following a four hour stimulation with PdBu, ionomycin and brefeldin A. Age and sex-matched, non-immunised C57BL/6 mice were analysed as controls (open circles). Total numbers of γδ T cells expressing intra-cellular IL-17A and the percentage of IL-17A expressing cells within the γδ T cell population are shown in (A). Total numbers of CD4 T cells expressing intra-cellular Foxp3 and the percentage of Foxp3-expressing cells within the CD4 T cell population are shown in (B). The dots represent individual mice and the mean is shown as a horizontal line. Shown are the results of three independent experiments. Statistical analysis was performed by the Kruskal-Wallis test with a Dunn's post test. (*) indicates P-value = 0.01-0.05, (**) indicates P-value = 0.001-0.01, (***) indicates P-value= <0.001

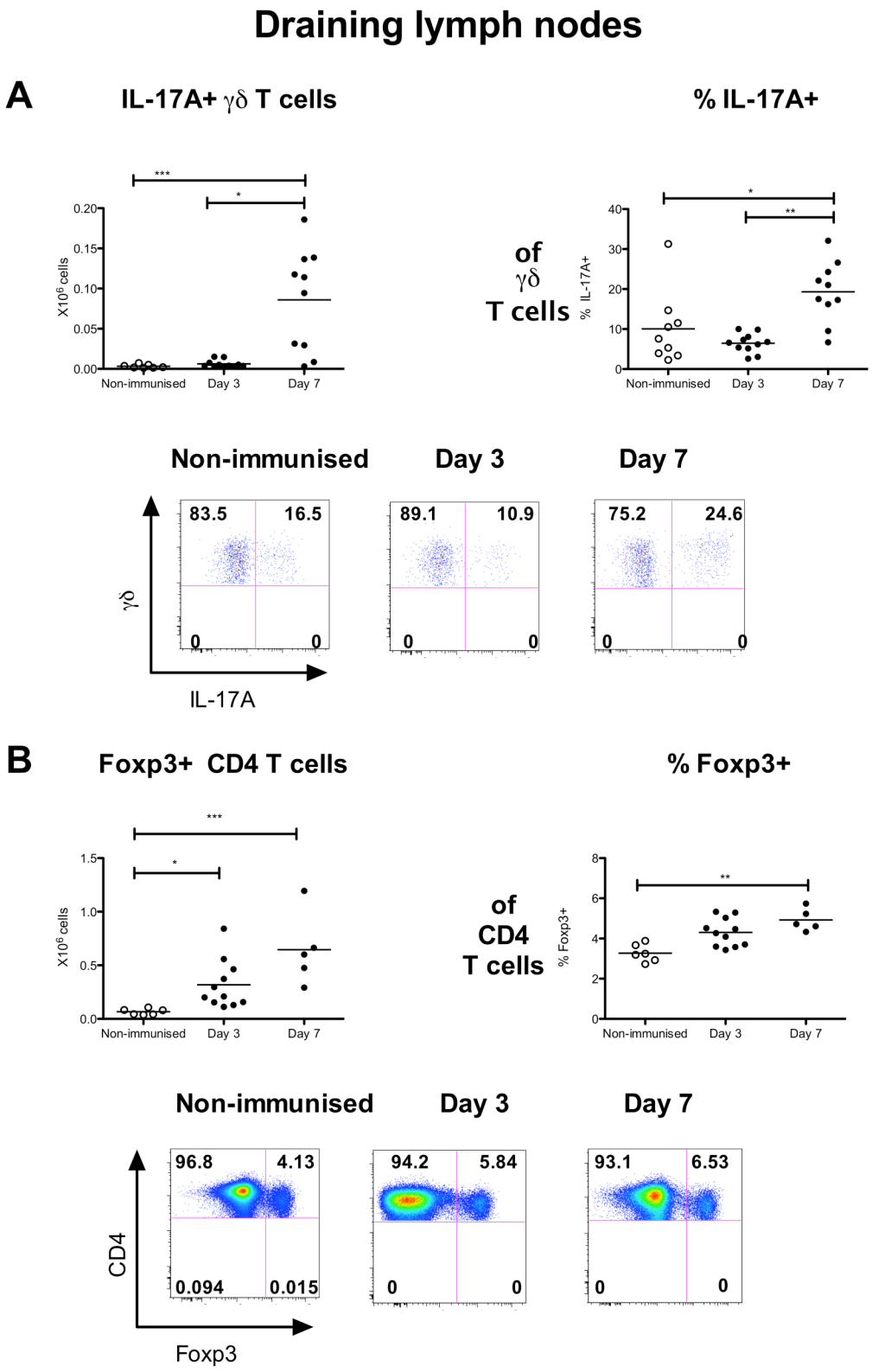

Draining lymph nodes
A
IL-17A+ γδ T cells
% IL-17A+
of γδ T cells
X10^6 cells
% IL-17A+
Non-immunised
Day 3
Day 7
Non-immunised
Day 3
Day 7
83.5
16.5
0
0
89.1
10.9
0
0
75.2
24.6
0
0
γδ
IL-17A
B
Foxp3+ CD4 T cells
% Foxp3+
of CD4 T cells
X10^6 cells
% Foxp3+
Non-immunised
Day 3
Day 7
Non-immunised
Day 3
Day 7
96.8
4.13
0.094
0.015
94.2
5.84
0
0
93.1
6.53
0
0
CD4
Foxp3

Figure 3.5 Flow cytometry gating strategy used for the analysis of CD4 and γδ T cell populations in the blood.

Blood was collected by cardiac puncture and red blood cells were lysed by ACK lysis. The remaining cells were stimulated for four hours with PdBu, ionomycin and brefeldin A and analysed by flow cytometry. For flow cytometry analysis, a gate was drawn around lymphocytes, 'singlets' were gated on by FSC-H/FSC-W discrimination and live cells were gated on based upon exclusion of 7AAD (A). IL-17A and IFN-γ-staining is shown in CD4 and γδ T cell populations from a mouse analysed on day 7 following base of tail immunisation with MOG CFA emulsion (B).

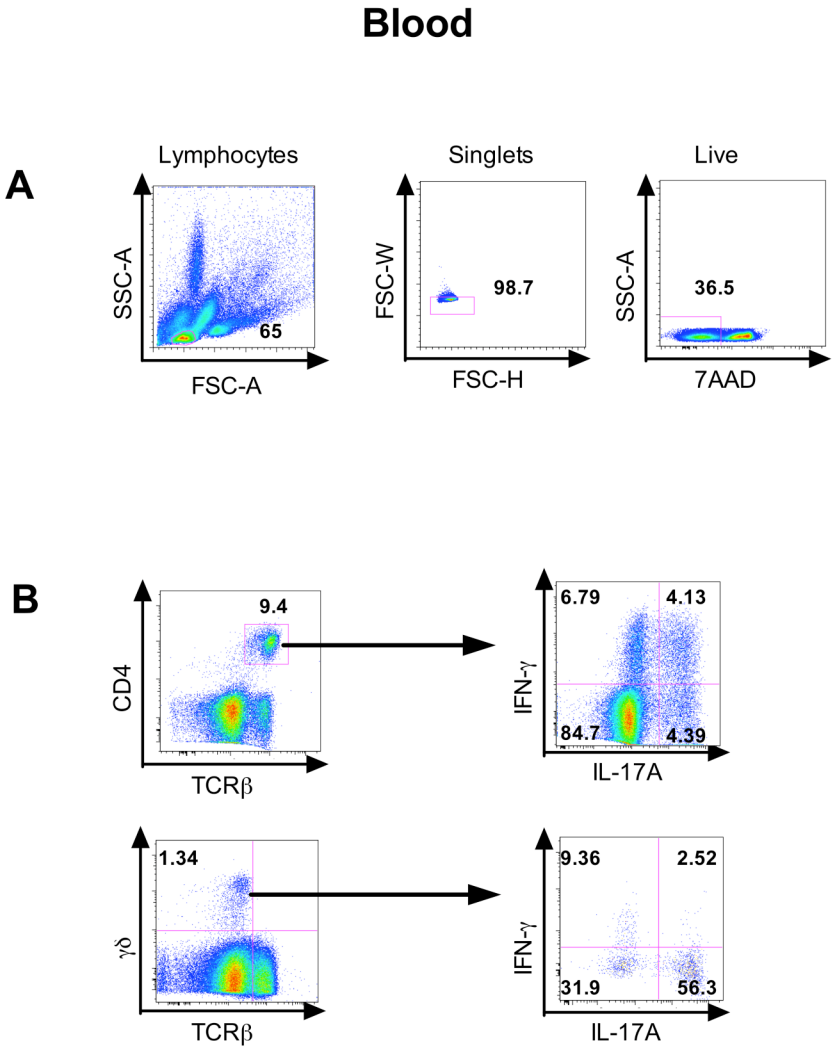
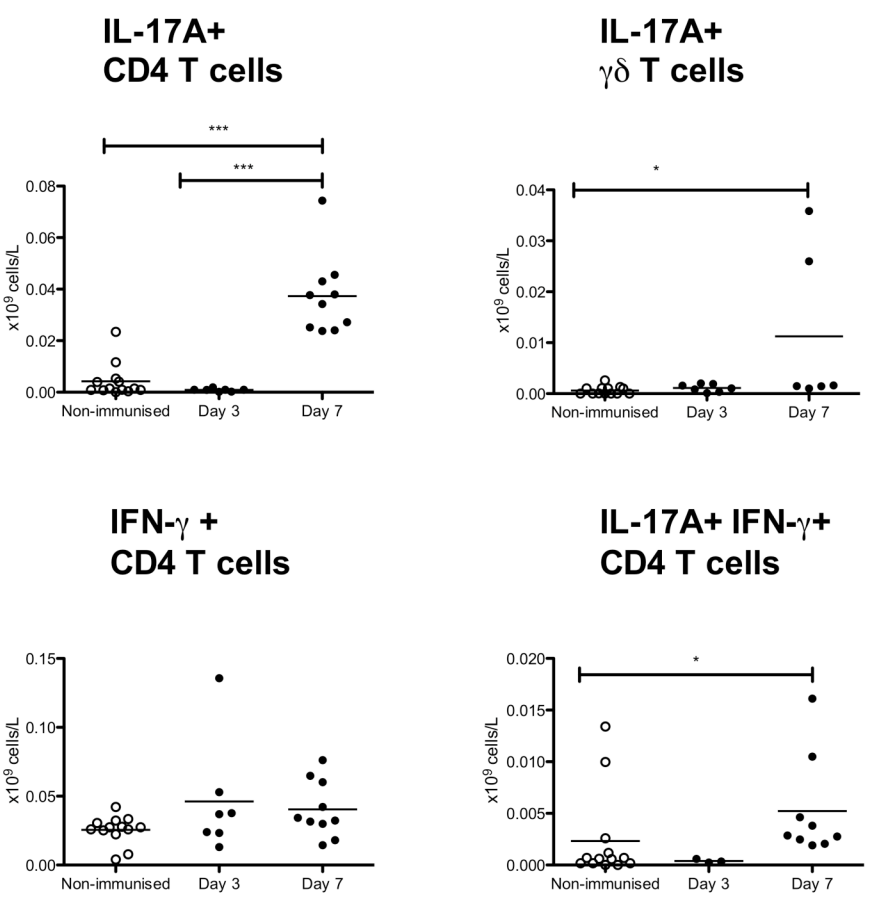

Blood
A
Lymphocytes
SSC-A
FSC-A
65
Singlets
FSC-W
FSC-H
98.7
Live
SSC-A
7AAD
36.5
B
CD4
TCRβ
9.4
IFN-γ
IL-17A
6.79
4.13
84.7
4.39
γδ
TCRβ
1.34
IFN-γ
IL-17A
9.36
2.52
31.9
56.3

Figure 3.6 IL-17A-expressing CD4 T cells, IL-17A-expressing γδ T cells and granulocytes in the blood following immunisation with MOG CFA

C57BL/6 mice were immunised at the base of the tail with MOG CFA emulsion. Blood was collected by cardiac puncture three days and seven days later and red blood cells were lysed by ACK lysis. The remaining cells were stimulated for four hours with PdBu, ionomycin and brefeldin A and analysed by flow cytometry. A sample of blood was also taken from the tail of the mice before sacrifice and analysed using a Vetscan to determine the number of lymphocytes, monocytes and granulocytes in the blood. Age and sex-matched, non-immunised C57BL/6 mice were analysed as controls (open circles). Using a combination of the number of lymphocytes determined from the Vetscan and percentages of cells from the flow cytometry analysis, the number of IL-17A$^+$ (top left), IFN-γ$^+$ (bottom left), and IL-17A$^+$IFN-γ$^+$ (bottom right) CD4 T cells as well as IL-17A$^+$ γδ T cells (top right) in the blood were determined (A). Granulocyte numbers from the Vetsan analysis are shown in (B). The dots represent individual mice and the mean for each group is shown as a horizontal line. Shown are the results from three independent experiments. Statistical analysis was performed by the Kruskal-Wallis test with a Dunn's post test. (*) indicates P-value = 0.01-0.05, (**) indicates P-value = 0.001-0.01, (***) indicates P-value= <0.001.

Blood

A


IL-17A+
CD4 T cells
***
***
0.08
0.06
0.04
0.02
0.00
x10^9 cells/L
Non-immunised
Day 3
Day 7
IL-17A+
γδ T cells
*
0.04
0.03
0.02
0.01
0.00
x10^9 cells/L
Non-immunised
Day 3
Day 7
IFN-γ +
CD4 T cells
0.15
0.10
0.05
0.00
x10^9 cells/L
Non-immunised
Day 3
Day 7
IL-17A+ IFN-γ+
CD4 T cells
*
0.020
0.015
0.010
0.005
0.000
x10^9 cells/L
Non-immunised
Day 3
Day 7

B

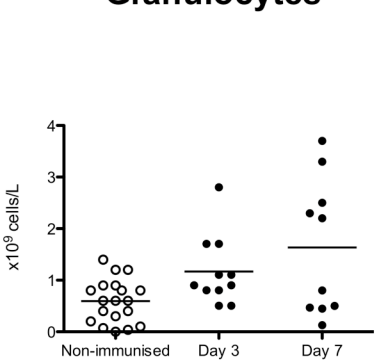

Granulocytes
4
3
2
1
0
x10^9 cells/L
Non-immunised
Day 3
Day 7

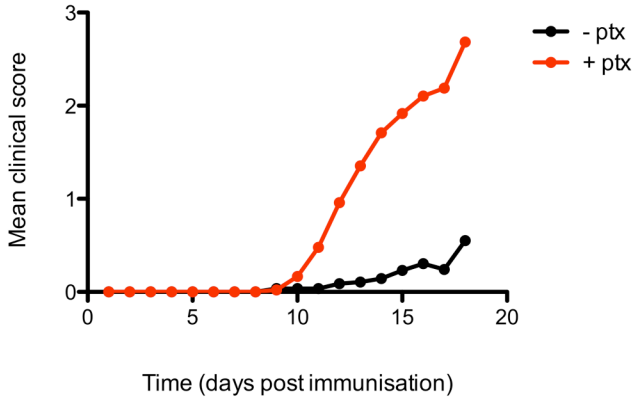
Figure 3.7 Following immunisation with MOG CFA, injection of pertussis toxin enhances the incidence of clinical disease.

C57BL/6 mice were immunised at the base of the tail with MOG CFA emulsion in the presence or absence of pertussis toxin treatment. Pertussis toxin, where given was administered by intraperitoneal injection on the same day as MOG CFA immunisation, followed by a second dose two days later. Mice were scored for symptoms of paralysis and the incidence, mean day of onset and mean maximum score were determined for each group of mice (A). The progression of the disease in the two groups of mice is illustrated by expression of mean clinical score against time following immunisation (B). The clinical scores of individual mice at day eighteen following immunisation is shown in (C) Red dots or lines indicate mice injected with pertussis toxin in combination with MOG CFA immunisation and black dots or lines indicate mice immunised with MOG CFA only. Shown are the results of three independent experiments. Statistical analysis was carried out between the two groups for the day of onset, the maximum score and the score at day eighteen using the Mann-Whitney U-Test. (***) indicates P-value= <0.001.

A

Protocol	Incidence	Day of onset (Average +/- SD)	Maximum score (Average +/- SD)	
- PTX	17.8% (5/28)	14.2 +/- 3.03	0.4 +/- 1	***
+ PTX	70.8% (17/24)	12 +/- 1.8	2.3 +/- 7	

B



C

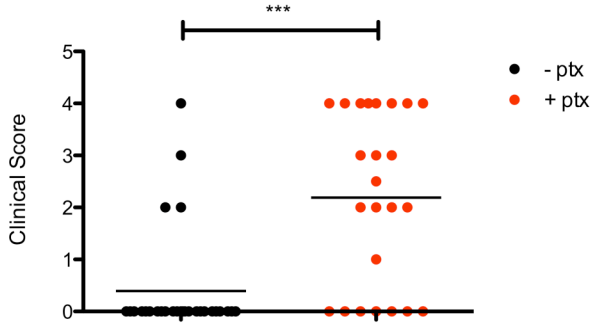


Figure 3.8 Following immunisation with MOG CFA, injection of pertussis toxin results in a non-selective reduction in the number of T cells and B cells in the draining lymph nodes

C57BL/6 mice were immunised at the base of the tail with MOG CFA emulsion in the presence or absence of pertussis toxin treatment. Pertussis toxin, where given was administered by intraperitoneal injection on the same day as MOG CFA immunisation, followed by a second dose two days later. Numbers of lymphocytes in the para-aortic and inguinal lymph nodes (draining lymph nodes) were assessed three days and seven days after MOG CFA immunisation. Cell counts were performed by a Casy-1 cell counter and assessment of different populations was carried out by flow cytometry. Red dots indicate individual mice injected with pertussis toxin in combination with MOG CFA immunisation and black dots indicate individual mice immunised with MOG CFA only. The mean for each group is shown as a horizontal line. Shown are the results of three independent experiments. Statistical analysis was carried out using the Mann-Whitney U-Test. (*) indicates P-value = 0.01-0.05.

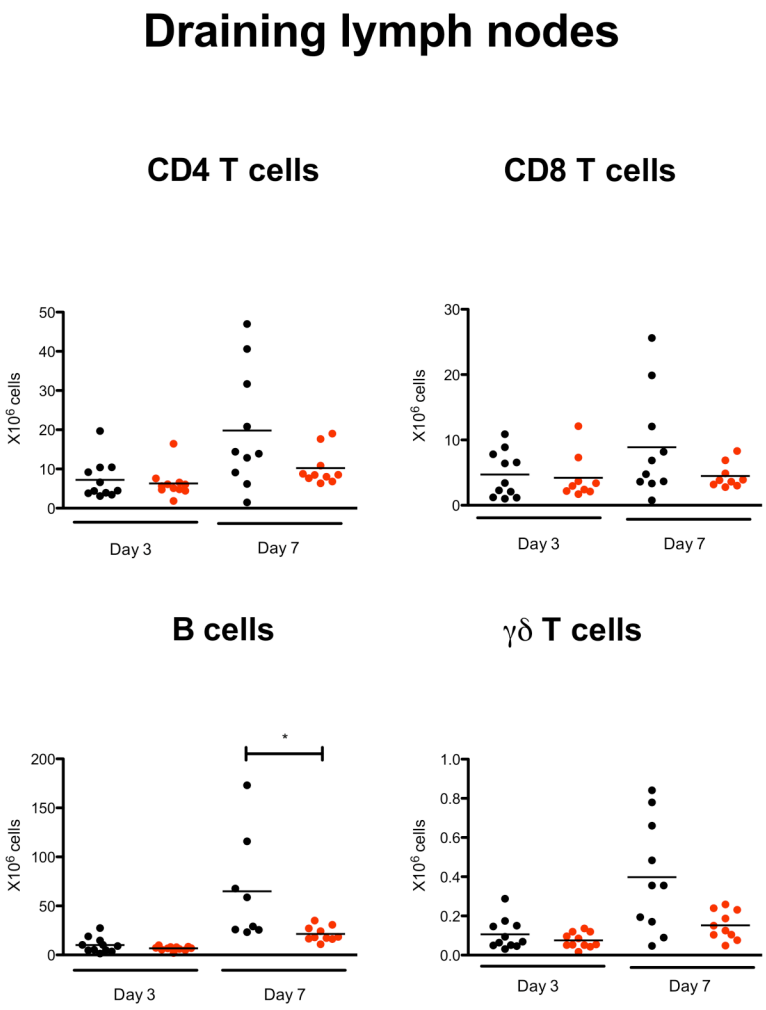

Draining lymph nodes
CD4 T cells
CD8 T cells
B cells
γδ T cells
X10^6 cells
Day 3
Day 7
*

Figure 3.9 Following immunisation with MOG CFA, injection of pertussis toxin does not significantly alter IL-17A and IFN-γ expression within the CD4 T cell population in the draining lymph nodes.

C57BL/6 mice were immunised at the base of the tail with MOG CFA emulsion in the presence or absence of pertussis toxin treatment. Pertussis toxin, where given was administered by intraperitoneal injection on the same day as MOG CFA immunisation, followed by a second dose two days later. The draining lymph nodes were harvested three days and seven days after the MOG CFA immunisation and assessed for intra-cellular IL-17A and IFN-γ protein expression by flow cytometry analysis following a four hour stimulation with PdBu, ionomycin and brefeldin A. Total numbers of CD4 T cells expressing intra-cellular IL-17A and IFN-γ protein are shown in (A). The percentage of IL-17A and IFN-γ-expressing cells within the CD4 T cell population are shown in (B). Red dots indicate individual mice given pertussis toxin in combination with MOG CFA immunisation and black dots indicate individual mice given MOG CFA immunisation only. The mean for each group is shown as a horizontal line. Shown are the results from three independent experiments.

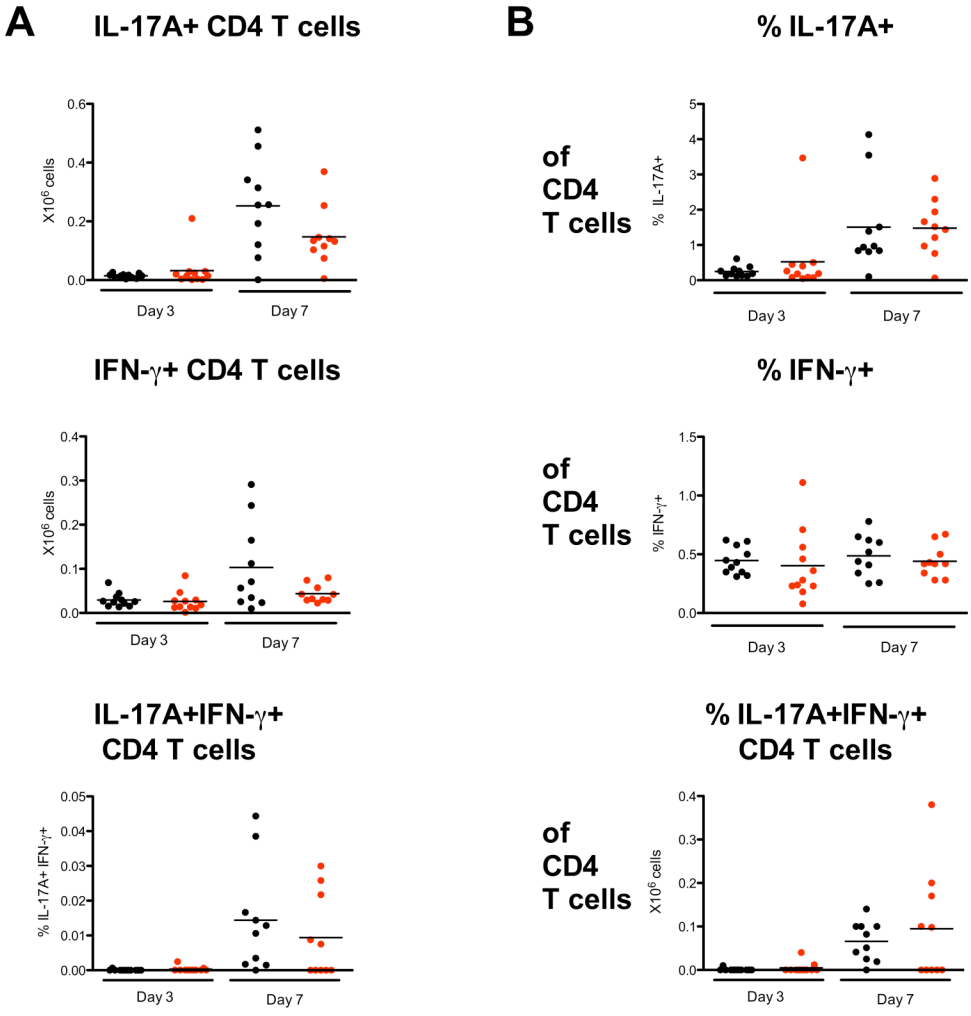

Draining lymph nodes
A
IL-17A+ CD4 T cells
X10^6 cells
0.6
0.4
0.2
0.0
Day 3
Day 7
IFN-γ+ CD4 T cells
X10^6 cells
0.4
0.3
0.2
0.1
0.0
Day 3
Day 7
IL-17A+IFN-γ+
CD4 T cells
% IL-17A+ IFN-γ+
0.05
0.04
0.03
0.02
0.01
0.00
Day 3
Day 7
B
% IL-17A+
of
CD4
T cells
% IL-17A+
5
4
3
2
1
0
Day 3
Day 7
% IFN-γ+
of
CD4
T cells
% IFN-γ+
1.5
1.0
0.5
0.0
Day 3
Day 7
% IL-17A+IFN-γ+
CD4 T cells
of
CD4
T cells
X10^6 cells
0.4
0.3
0.2
0.1
0.0
Day 3
Day 7

Figure 3.10 Following immunisation with MOG CFA, injection of pertussis toxin does not significantly alter Foxp3 expression within the CD4 T cell population or IL-17A expression within the γδ T cell population in the draining lymph nodes.

C57BL/6 mice were immunised at the base of the tail with MOG CFA emulsion in the presence or absence of pertussis toxin treatment. Pertussis toxin, where given was administered by intraperitoneal injection on the same day as MOG CFA immunisation, followed by a second dose two days later. The draining lymph nodes were harvested three days and seven days after the MOG CFA immunisation and assessed for intra-cellular IL-17A and Foxp3 protein expression by flow cytometry analysis following a four hour stimulation with PdBu, ionomycin and brefeldin A. Total numbers of γδ T cells expressing intra-cellular IL-17A protein and the percentage of IL-17A-expressing cells within the γδ T cell population are shown in (A). Total numbers of CD4 T cells expressing intra-cellular Foxp3 protein and the percentage of Foxp3-expressing cells within the CD4 T cell population are shown in (B). Red dots indicate individual mice given pertussis toxin in combination with MOG CFA immunisation and black dots indicate individual mice given MOG CFA immunisation only. The mean for each group is shown as a horizontal line. Shown are the results from three independent experiments.

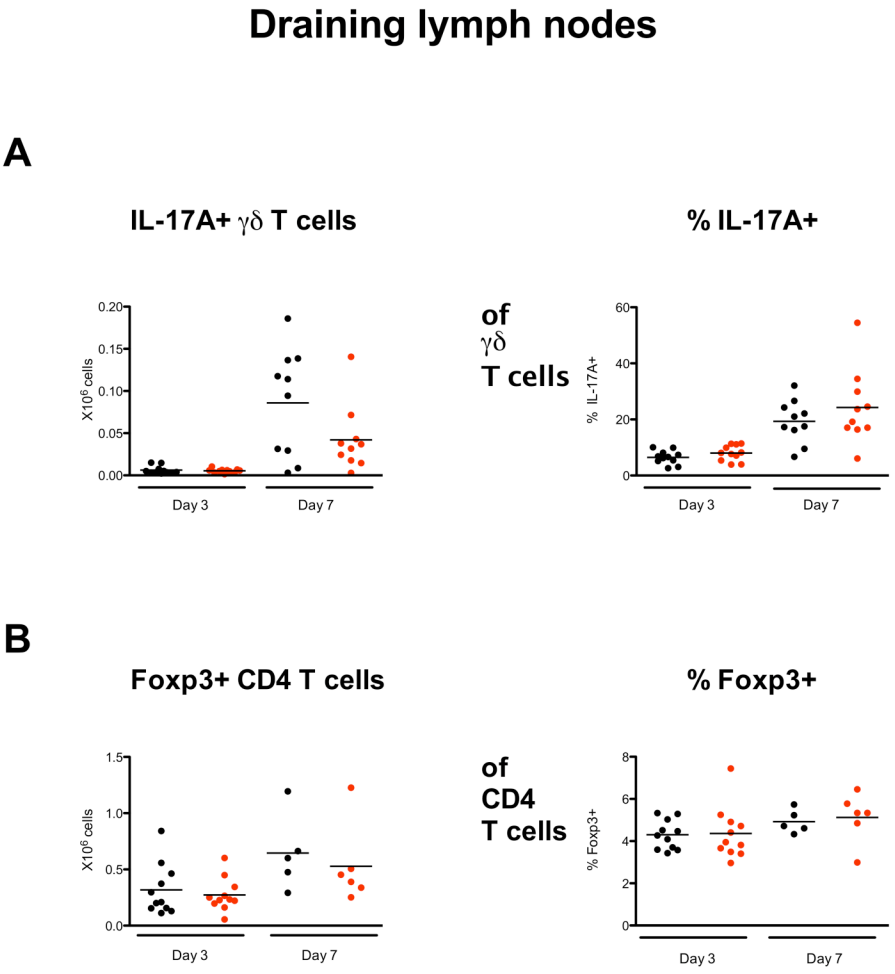
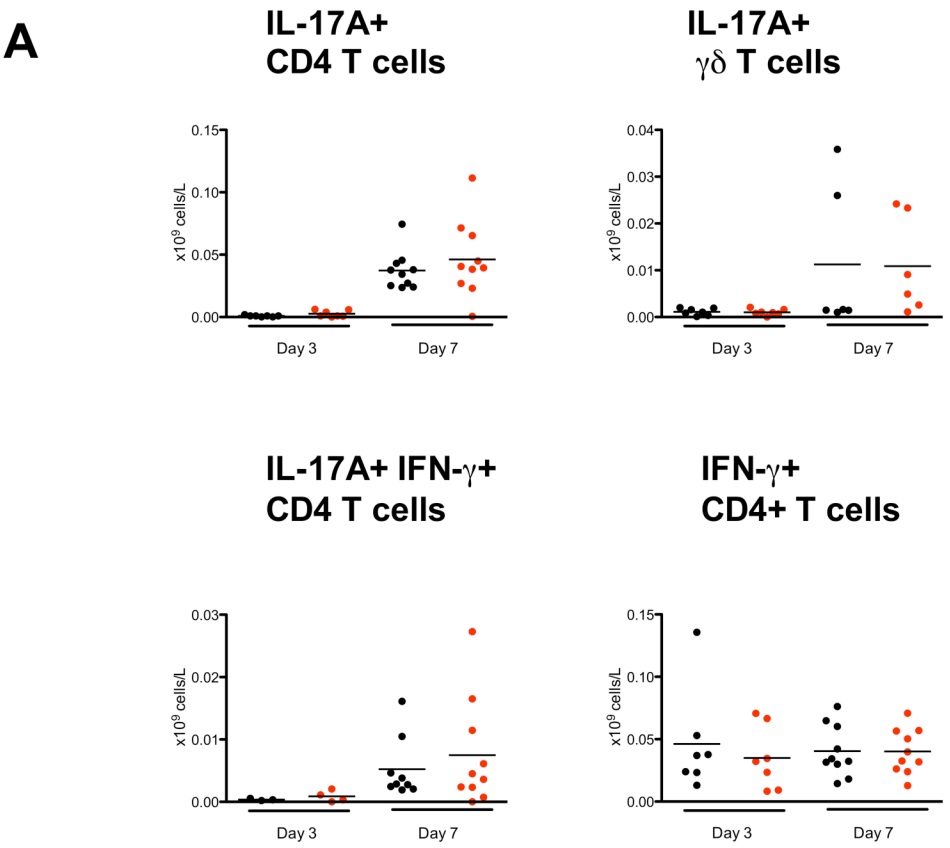

Draining lymph nodes
A
IL-17A+ γδ T cells
X10^6 cells
0.20
0.15
0.10
0.05
0.00
Day 3
Day 7
% IL-17A+
of
γδ
T cells
% IL-17A+
60
40
20
0
Day 3
Day 7
B
Foxp3+ CD4 T cells
X10^6 cells
1.5
1.0
0.5
0.0
Day 3
Day 7
% Foxp3+
of
CD4
T cells
% Foxp3+
8
6
4
2
0
Day 3
Day 7

Figure 3.11 Following immunisation with MOG CFA, injection of pertussis toxin does not significantly alter the number of IL-17A- and IFN-γ-expressing CD4 and γδ T cell populations in the blood, but results in a marginal increase in the number of granulocytes

C57BL/6 mice were immunised at the base of the tail with MOG CFA emulsion in the presence or absence of pertussis toxin treatment. Pertussis toxin, where given was administered by intraperitoneal injection on the same day as MOG CFA immunisation, followed by a second dose two days later. Blood was collected by cardiac puncture three days and seven days following MOG CFA immunisation and red blood cells were lysed by ACK lysis. The remaining cells were stimulated for four hours with PdBu, ionomycin and brefeldin A and analysed by flow cytometry. A sample of blood was also taken from the tail of the mice before sacrifice and analysed using a Vetscan to determine the number of lymphocytes, monocytes and granulocytes in the blood. Using a combination of the number of lymphocytes determined from the Vetscan and percentages of cells from the flow cytometry analysis, the number of IL-17A$^+$ (top left), IFN-γ$^+$ (bottom right) and IL-17A$^+$IFN-γ$^+$ (bottom left) CD4 T cells, as well as IL-17A$^+$ γδ T cells (top right) in the blood were determined (A). The number of granulocytes determined by the Vetscan is shown in (B). Red dots indicate individual mice injected with pertussis toxin in combination with MOG CFA immunisation and black dots indicate individual mice immunised with MOG CFA alone. The mean for each group is shown as a horizontal line. Shown are the results of three independent experiments. Statistical analysis was carried out using the Mann-Whitney U-Test. (**) indicates P-value = 0.001-0.01.

Blood

A


IL-17A+
CD4 T cells
IL-17A+
γδ T cells
IL-17A+ IFN-γ+
CD4 T cells
IFN-γ+
CD4+ T cells
x10⁹ cells/L
Day 3
Day 7

B

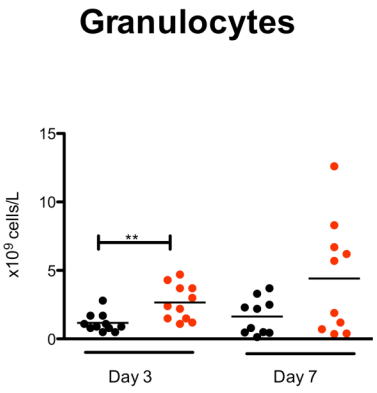

Granulocytes
x10⁹ cells/L
**
Day 3
Day 7

Figure 3.12 Flow cytometry gating strategy for analysis of spinal cord leukocytes

Leukocytes were prepared from the spinal cord as described in section **2.3.3** and assessed by flow cytometry. For analysis, a gate was first drawn to exclude cell debris (counted cells), singlets were gated by FSC-H/FSC-W discrimination, live cells were gated by 7AAD exclusion and for analysis of lymphocyte populations an additional lymphocyte gate was drawn (A). Neutrophils were defined based upon expression of Ly6G and an epitope detected by 7/4 antibody. Monocytes were defined based upon expression of an epitope detected by 7/4 antibody and the absence of Ly6G expression. B cells were detected by expression of CD19, CD4 T cells by expression of TCRβ and CD4, and γδ T cells based upon expression of γδ TCR. Ly6G, 7/4 and CD19 staining are shown in a mouse 7 days following MOG CFA and pertussis toxin immunisation and CD4, TCRβ and TCR γδ staining is shown in a mouse of disease score 4 at day 18 following MOG CFA and pertussis toxin immunisation (B).

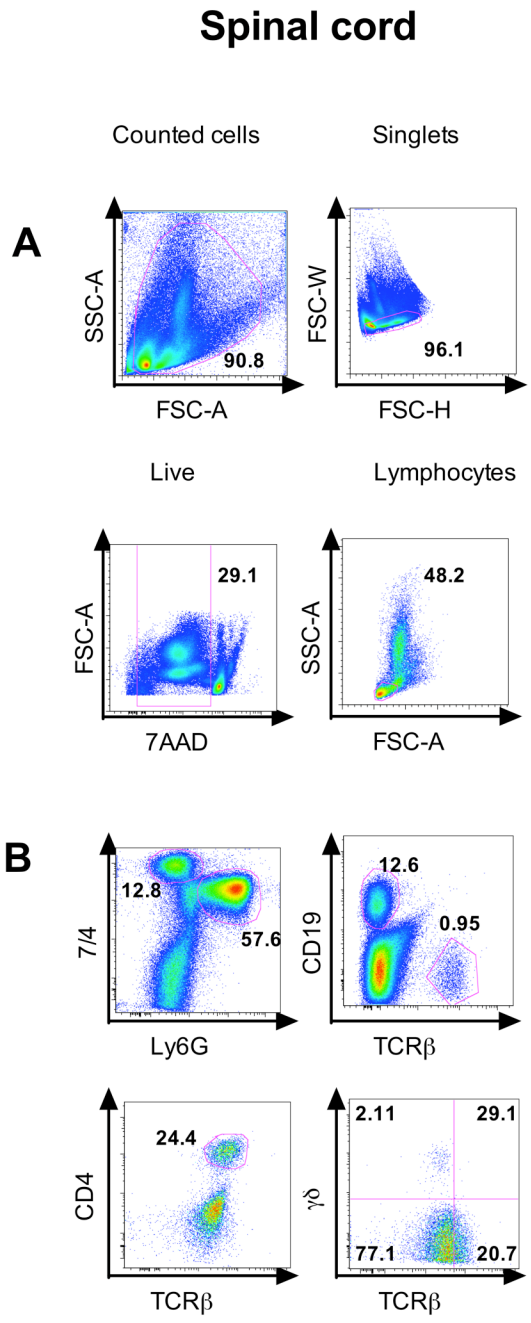

Spinal cord
A
Counted cells
SSC-A
90.8
FSC-A
Singlets
FSC-W
96.1
FSC-H
Live
FSC-A
29.1
7AAD
Lymphocytes
SSC-A
48.2
FSC-A
B
7/4
12.8
57.6
Ly6G
CD19
12.6
0.95
TCRβ
CD4
24.4
TCRβ
γδ
2.11
29.1
77.1
20.7
TCRβ

Figure 3.13 CD4 and γδ T cells accumulate in the spinal cord before the onset of clinical disease.

C57BL/6 mice were immunised at the base of the tail with MOG CFA emulsion in the presence or absence of pertussis toxin treatment. Pertussis toxin, where given was administered by intraperitoneal injection on the same day as MOG CFA immunisation, followed by a second dose two days later. Spinal cords were harvested three days, seven days and ten days following the MOG CFA immunisation (prior to disease onset) and leukocytes were analysed by flow cytometry. Age and sex-matched, non-immunised C57BL/6 mice were analysed as controls at the same time points as the immunised mice were analysed (blue bars). Mean and standard deviation of three independent experiments are shown, where, in each experiment three spinal cords were pooled. Numbers of monocytes and neutrophils are shown in (A). Numbers of lymphocytes are shown in (B). Red bars indicate groups of mice injected with pertussis toxin in combination with MOG CFA immunisation and black bars indicate groups of mice immunised with MOG CFA alone. ND = not determined.

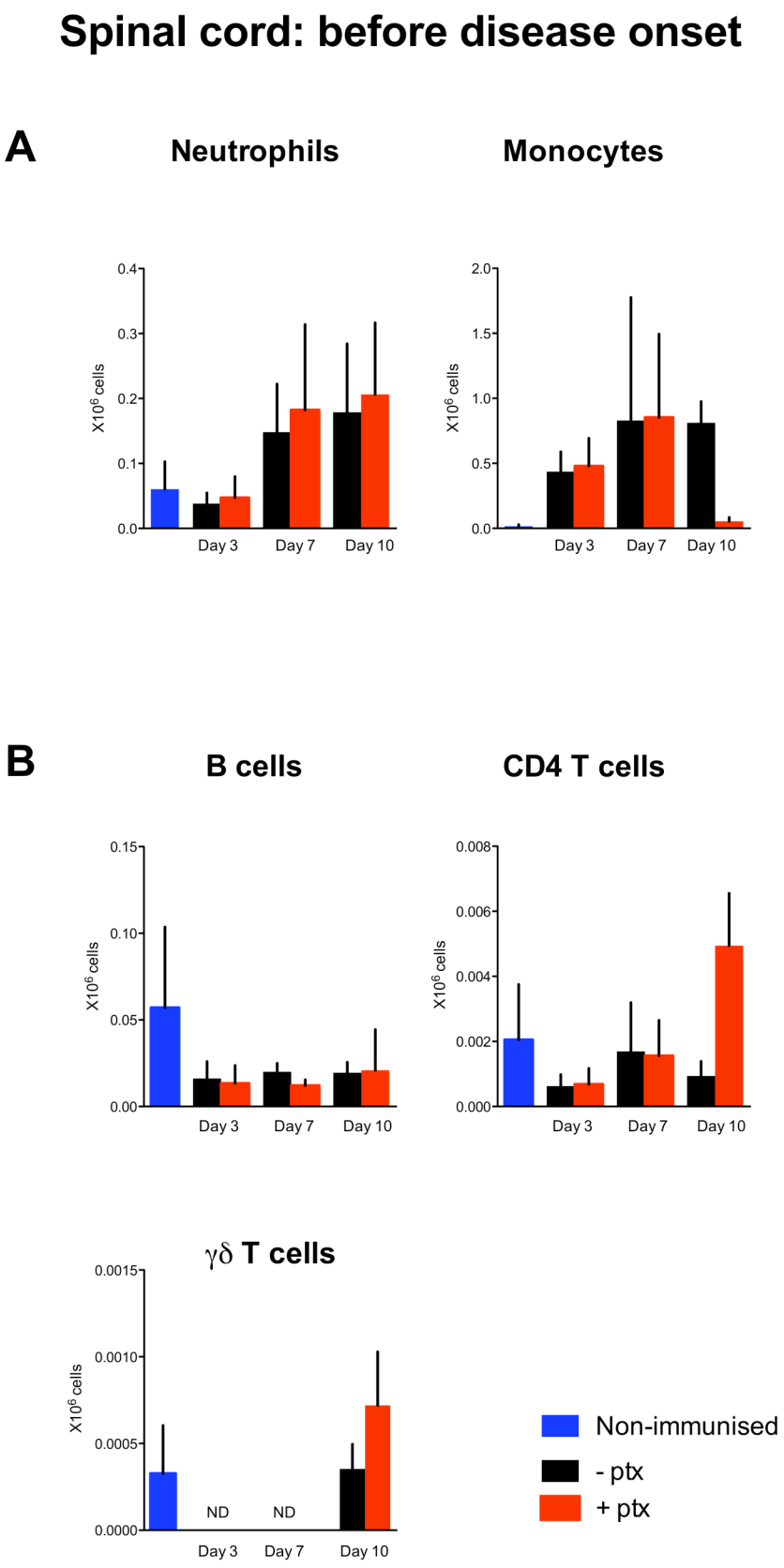

Spinal cord: before disease onset
A
Neutrophils
Monocytes
X10^6 cells
0.4
0.3
0.2
0.1
0.0
2.0
1.5
1.0
0.5
0.0
Day 3
Day 7
Day 10
B
B cells
CD4 T cells
0.15
0.10
0.05
0.00
0.008
0.006
0.004
0.002
0.000
γδ T cells
0.0015
0.0010
0.0005
0.0000
ND
ND
Non-immunised
- ptx
+ ptx

Figure 3.14 Analysis of intra-cellular IL-17A and IFN-γ protein expression in CD4 and γδ T cells in the spinal cord before the onset of clinical disease.

C57BL/6 mice were immunised at the base of the tail with MOG CFA emulsion in the presence or absence of pertussis toxin treatment. Pertussis toxin, where given was administered by intraperitoneal injection on the same day as MOG CFA immunisation, followed by a second dose two days later. Age and sex-matched, non-immunised C57BL/6 mice were analysed as controls. Leukocytes were isolated from the spinal cords ten days following MOG CFA immunisation and assessed for IL-17A and IFN-γ expression by flow cytometry following a 4 hour stimulation with PdBu, ionomycin and brefeldin A. Three spinal cords were pooled from each group of mice. Examples of the staining are shown from non-immunised mice, and MOG CFA immunised mice injected with and without pertussis toxin. IL-17A and IFN-γ staining in CD4 T cells is shown in (A) and IL-17A staining in γδ T cells is shown in (B).

Spinal cord: day 10 (before disease onset)

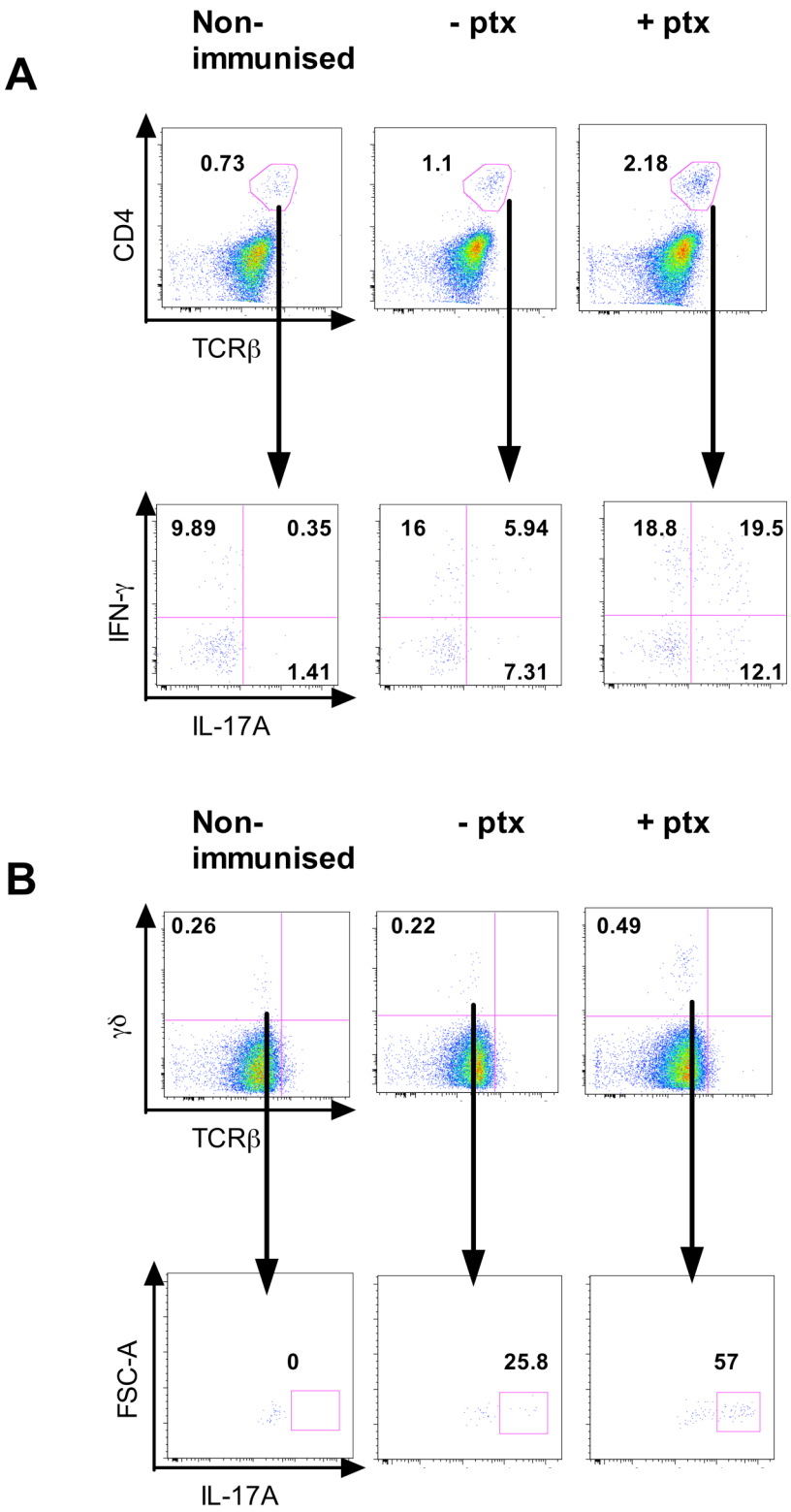


Figure 3.15 Accumulation of IL-17A-, IFN-γ- and Foxp3-expressing T cells in the spinal cord before the onset of clinical disease

C57BL/6 mice were immunised at the base of the tail with MOG CFA emulsion in the presence or absence of pertussis toxin treatment. Pertussis toxin, where given was administered by intraperitoneal injection on the same day as MOG CFA immunisation, followed by a second dose two days later. Leukocytes were isolated from the spinal cords ten days following MOG CFA immunisation and assessed for IL-17A, IFN-γ and Foxp3 expression by flow cytometry following a four hour stimulation with PdBu, ionomycin and brefeldin A. Age and sex-matched, non-immunised C57BL/6 mice were analysed as controls at the same time points as the immunised mice were analysed (blue bars). For each time point, three spinal cords were pooled from each group of mice. The mean and standard deviation from three independent experiments is illustrated in the bar charts. Numbers of IL-17A$^+$, IL-17/IFN-γ$^+$ and IFN-γ$^+$ CD4 T cells are shown in (A), numbers of IL-17A$^+$ γδ T cells are shown in (B) and numbers of Foxp3$^+$ CD4 T cells are shown in (C). Red bars indicate groups of mice injected with pertussis toxin in combination with MOG CFA immunisation and black bars indicate groups of mice immunised with MOG CFA alone.

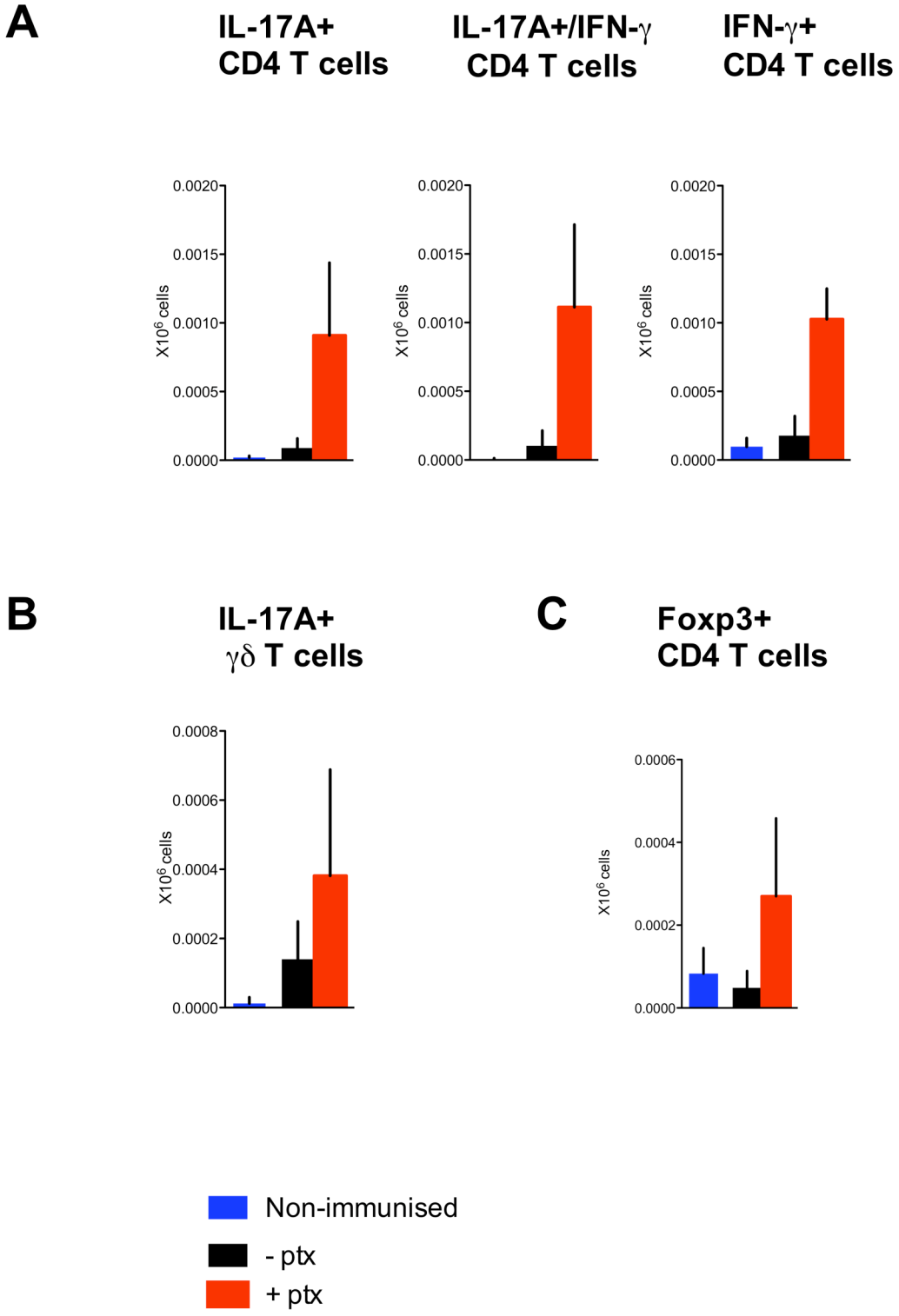

Spinal cord: day 10 (before disease onset)
A
IL-17A+
CD4 T cells
IL-17A+/IFN-γ
CD4 T cells
IFN-γ+
CD4 T cells
X10^6 cells
0.0020
0.0015
0.0010
0.0005
0.0000
B
IL-17A+
γδ T cells
0.0008
0.0006
0.0004
0.0002
0.0000
C
Foxp3+
CD4 T cells
0.0006
0.0004
0.0002
0.0000
Non-immunised
- ptx
+ ptx

Figure 3.16 Monocytes, neutrophils, CD4 and γδ T cells accumulate in the spinal cords of mice with clinical disease, irrespective of pertussis toxin treatment

C57BL/6 mice were immunised at the base of the tail with MOG CFA emulsion in the presence or absence of pertussis toxin treatment. Pertussis toxin, where given was administered by intraperitoneal injection on the same day as MOG CFA immunisation, followed by a second dose two days later. Leukocytes were isolated from the spinal cords eighteen days following MOG CFA immunisation and assessed by flow cytometry. Age and sex-matched, non-immunised C57BL/6 mice were analysed as controls at the same time points as the immunised mice were analysed (blue bars). For the non-immunised group (blue bars) and the group immunised with MOG CFA alone (black bars) that did not develop disease, three spinal cords were pooled in two independent experiments and the mean and standard deviation are shown. For the mice immunised with MOG CFA alone that developed disease, four mice were analysed individually and the mean and standard deviation are shown (black patterned bars). For the mice immunised with MOG CFA and pertussis toxin, fifteen mice were analysed individually and the mean and standard deviation are shown (red bars). Monocytes and neutrophils are shown in (A) and lymphocytes are shown in (B).

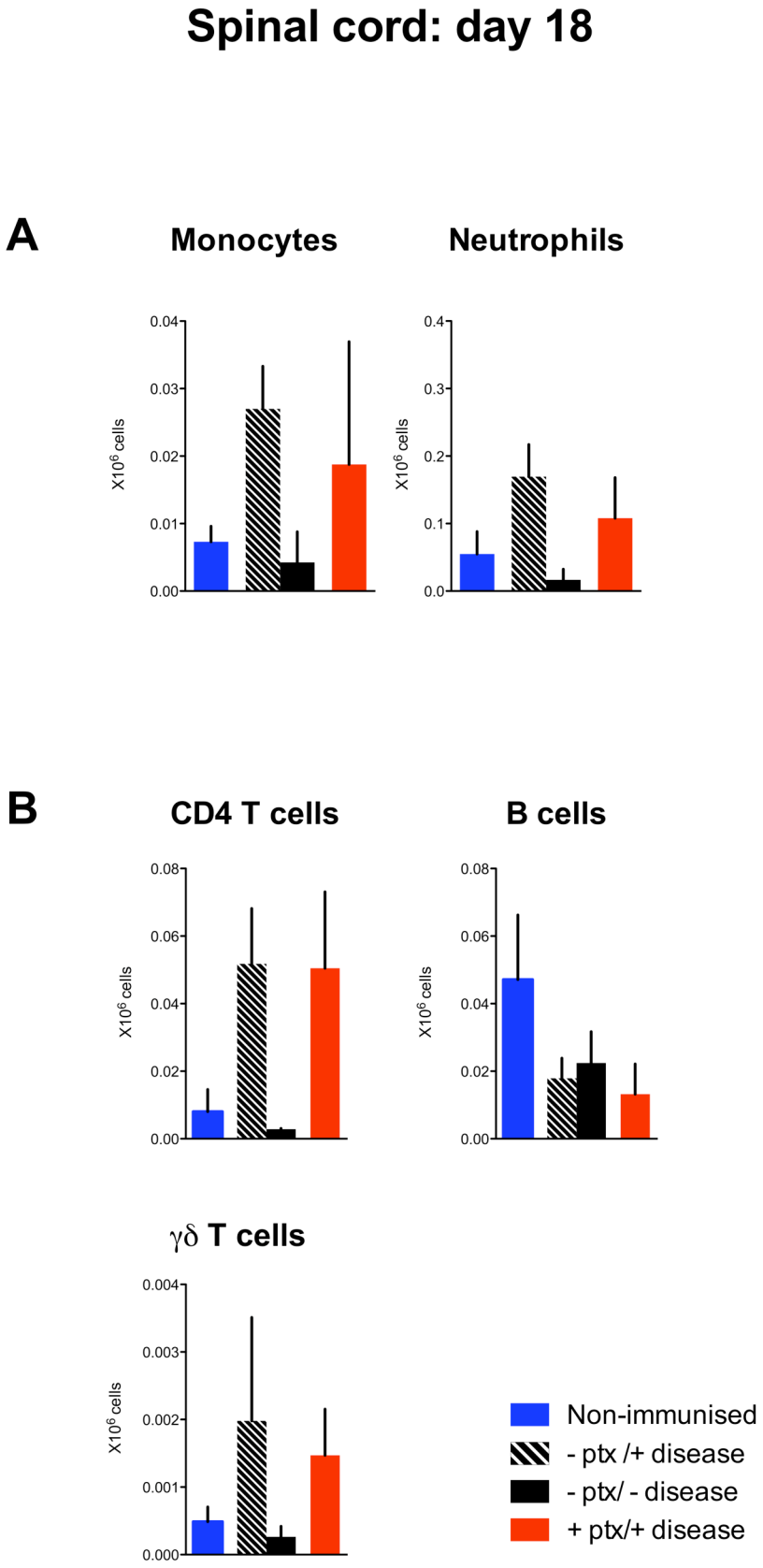
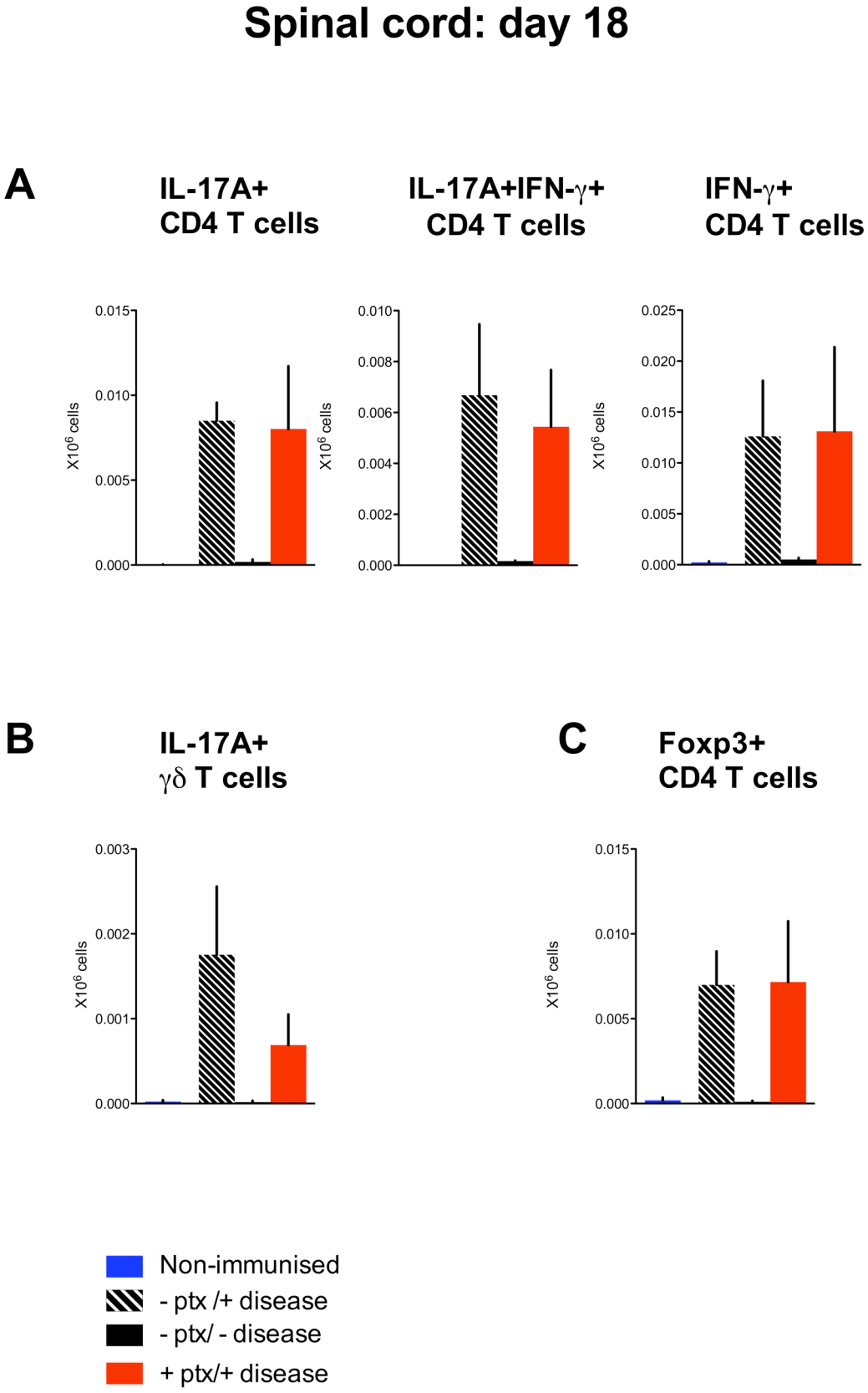

Spinal cord: day 18
A
Monocytes
Neutrophils
X10^6 cells
0.04
0.03
0.02
0.01
0.00
0.4
0.3
0.2
0.1
0.0
B
CD4 T cells
B cells
0.08
0.06
0.04
0.02
0.00
γδ T cells
0.004
0.003
0.002
0.001
0.000
Non-immunised
- ptx /+ disease
- ptx/ - disease
+ ptx/+ disease

Figure 3.17 IL-17A, IFN-γ and Foxp3-expressing CD4 and γδ T cells accumulate in the spinal cord of mice with clinical disease, irrespective of pertussis toxin treatment

C57BL/6 mice were immunised at the base of the tail with MOG CFA emulsion in the presence or absence of pertussis toxin treatment. Pertussis toxin, where given was administered by intraperitoneal injection on the same day as MOG CFA immunisation, followed by a second dose two days later. Leukocytes were isolated from the spinal cords eighteen days following MOG CFA immunisation and assessed for intra-cellular IL-17A, IFN-γ and Foxp3 expression by flow cytometry following a four hour stimulation with PdBu, ionomycin and brefeldin A. Age and sex-matched, non-immunised C57BL/6 mice were analysed as controls at the same time points as the immunised mice were analysed (blue bars). For the non-immunised group (blue bars) and the group immunised with MOG CFA alone that did not develop disease (black bars), three spinal cords were pooled in two independent experiments and the mean and standard deviation are shown. For the mice immunised with MOG CFA alone that developed disease, four mice were analysed individually and the mean and standard deviation are shown (black patterned bars). For the mice immunised with MOG CFA and pertussis toxin, fifteen mice were analysed individually and the mean and standard deviation are shown (red bars). IL-17A$^+$, IL-17A/IFN-γ$^+$ and IFN-γ$^+$ CD4 T cells are shown in (A), IL-17A$^+$ γδ T cells are shown in (B) and Foxp3$^+$ CD4 T cells are shown in (C).


Spinal cord: day 18
A
IL-17A+
CD4 T cells
IL-17A+IFN-γ+
CD4 T cells
IFN-γ+
CD4 T cells
X10^6 cells
0.015
0.010
0.005
0.000
0.010
0.008
0.006
0.004
0.002
0.000
0.025
0.020
0.015
0.010
0.005
0.000
B
IL-17A+
γδ T cells
0.003
0.002
0.001
0.000
C
Foxp3+
CD4 T cells
0.015
0.010
0.005
0.000
Non-immunised
- ptx /+ disease
- ptx/ - disease
+ ptx/+ disease

3.3 Investigating the effect of pertussis toxin in the priming phase of EAE development.

In the first part of this chapter, IL-17A and IFN-γ-expressing CD4 T cells could be detected at similar frequencies in the lymph nodes and blood of mice with and without pertussis toxin injection, but effector cells entering the spinal cord were increased in numbers in the mice treated with pertussis toxin. This immediately suggested that pertussis toxin was not having any striking affect on the ability of effector cells to be primed and enter the blood circulation. However, it seemed intriguing that in the standard protocol for EAE, pertussis toxin is administered at days 0 and 2, at the initiation stage of disease, well before effector cells begin to enter the spinal cord. The first aim of the next part was therefore to investigate the time frame in which pertussis toxin was exerting its effects in EAE. In order to investigate this we wanted to determine how long after injection of pertussis toxin, it's effects could be detected *in vivo*. In addition, we wanted to specifically address how the timing of pertussis toxin administration affected disease outcome.

The development of EAE is dependent upon the generation of MOG-specific effector cells, which are approximately 0.2% of the CD4 T cells in the lymph nodes according to MOG-tetramer staining in EAE (Korn, Reddy et al. 2007). In the first part of this chapter, effector T cells were assessed based on production of IL-17A and IFN-γ in response to PdBu and ionomycin stimulation and any differences in the ability of the MOG-specific CD4 T cells to express these cytokines may have been undetectable. We therefore wanted to assess the peripheral MOG-specific cytokine response from mice immunised with MOG CFA with or without pertussis toxin injection.

3.3.1 Pertussis toxin affects the migration capacity of CD4 T at the initiation stage and up to the onset of disease in EAE

The observation in **Figure 3.8,** that pertussis toxin-treated mice had fewer cells in the draining lymph nodes could be attributed to the effect of pertussis toxin on immune cell migration (Spangrude, Braaten et al. 1984) Indeed, pertussis toxin has ADP-ribosylase activity which can inhibit G-protein receptor signalling (Reisin 1990) such as the signalling in chemokine receptors. Chemokine receptor signalling is critical for cell trafficking including movement of cells in and out of the lymph nodes and migration of cells into peripheral tissues in steady state as well as inflammatory conditions. We therefore wanted to assess how pertussis toxin was influencing the function of chemokine receptors on CD4 T cells *in vivo* and also to assess the time frame in which it was doing this. This would give information about the timing in which pertussis toxin could potentially be acting in EAE.

We chose to investigate the effect of pertussis toxin on the migration of CD4 T cells to CCL21, a chemokine that signals through CCR7. CCR7 is expressed on naïve CD4 T cells and is involved in the recruitment of cells to the lymph nodes under steady state conditions (Forster, Davalos-Misslitz et al. 2008), however its importance in EAE has been implicated in mice that are deficient in CCR7 which are resistant to disease development (Kuwabara, Ishikawa et al. 2009).

An assay was first performed to demonstrate the effect of pertussis toxin on the migration of CD4 T cells to CCL21. This is shown in **Figure 3.18A**. Whole lymph node cells were treated with and without pertussis toxin for 2 hours prior to performing the migration assay. In the absence of pertussis toxin, CD4 T cells migrated to CCL21 in a dose-dependent manner. The cells that had been treated with pertussis toxin showed no migration capacity to CCL21. It was therefore concluded that treatment of pertussis toxin *in vitro* inhibits the migration of CD4 T cells to CCL21.

The migration capability of CD4 T cells to CCL21 was then assessed at time points following *in vivo* pertussis toxin or PBS administration. Mice were injected with either pertussis toxin or PBS on days 0 and 2, These time points represented the time points in which pertussis would be given in the standard EAE protocol. The MOG CFA injection was not necessary in this experiment as we specifically wanted to address the time frame in which pertussis toxin could be affecting CD4 T cells *in vivo*. Lymph node cells from three or four mice from each group were assessed on days 3, 6, 10, and 19 after the initial injection.

Cells from pertussis toxin-treated mice had a reduced migration capacity to CCL21 on day 3, day 6 and day 10 after the first injection, which was statistically significant at day 3 according to the Mann Whitney U-test (**Figure 3.18B**), perhaps due to the fact that four mice rather than three mice were compared in this group. By day 19, migration of the pertussis toxin-treated cells was comparable to the control cells from PBS-treated mice. It was therefore concluded that pertussis toxin was affecting the migration capability of CD4 T cells up until at least the onset of disease at day 10 and so could be exerting its effects in EAE at any point in this time frame.

3.3.2 Pertussis toxin is required to be administered at the initiation stage of disease in order to initiate most severe EAE pathology

Although the effects of pertussis toxin could be influencing EAE progression up to the onset of disease, we wanted to investigate more specifically when it was required. Pertussis toxin is routinely given at days 0 and 2 to initiate EAE in C57BL/6 mice and in some protocols it is given on days 1 and 3. If pertussis toxin was acting solely by increasing vascular permeability at the blood brain barrier in EAE then administration at later time points might be sufficient to allow activated CD4 T cells to enter the CNS and to initiate disease. Studies using Evans blue dye reported an effect on vascular permeability four days after pertussis toxin injection (Clifford, Zarrabi et al. 2007).

Mice were therefore immunised mice with MOG CFA on day 0, with pertussis toxin given on days 0 and 2, 1 and 3, 3 and 5, or 5 and 7**.** **Figure 3.19A** shows the progression of the disease measured by mean clinical score against time in the different groups. Administration of pertussis toxin on days 0 and 2 induced the most severe disease, measured by the average maximum score and earliest onset (**Figure 3.19B**). Delaying the time of the pertussis toxin treatment still permited disease, however onset was later and the average maximum score was lower.

In **Figure 3.11**, mice treated with pertussis toxin in combination with MOG CFA immunisation had a marginally higher blood granulocyte count than mice immunised with MOG CFA alone. We therefore hypothesised that administration of pertussis toxin may contribute to EAE pathology by a mechanism involving the increased mobilisation of granulocytes. The experiment to investigate the timing of pertussis toxin injection on outcome of disease provided an opportunity to investigate a possible relationship between pertussis toxin administration, blood granulocyte counts and disease severity.

Figure 3.20A shows the number of blood granulocytes measured 1, 3 and 7 days after the last pertussis toxin treatment. Pertussis toxin treatment in combination with MOG CFA was associated with an increase in blood granulocytes when compared to mice immunised with MOG CFA with no pertusssis toxin treatment. However, granulocyte counts were lowest in the mice that were injected on days 0 and 2 with pertussis toxin and these mice had the most severe disease. Mice with higher granulocyte scores were from the groups injected at later time points, with lower disease severity. A comparison of peak blood granulocytes with peak disease severity in individual mice was also made which showed no direct correlation (**Figure 3.20B**). In conclusion, although a link between pertussis toxin administration in combination with MOG CFA immunisation and increased blood neutrophilia was apparent, the levels of granulocytes measured in the blood did not directly correlate with severity. However, an importance for pertussis toxin-induced

neutropilia could not be excluded, as a mere increase in blood granulocytes over a threshold level may be important for disease development.

3.3.3 Pertussis toxin amplifies the peripheral MOG-specific inflammatory cytokine response in EAE

Since pertussis toxin was required at the initiation stage of EAE to give most severe disease, this suggested an effect on the T cell priming stage of disease induction. In the first part of this chapter, the frequency of IL-17A and IFN-γ-expressing CD4 T cells in the lymph nodes and blood did not differ between the mice treated with and without pertussis toxin. The method used for detection of these cytokine-expressing cells was intra-cellular cytokine staining after a 4 hour stimulation with the mitogenic stimuli PdBu and ionomycin which would capture all cytokine-expressing cells irrespective of antigen-specificity. However, it was still a possibility that antigen-specific cytokine responses to MOG-peptide may differ between the two groups. In order to assess this, lymph node cells were activated with MOG peptide, PdBu and ionomycin or medium alone for 24 hours *in vitro* and concentrations of IFN-γ and IL-17A protein were analysed in the supernatants. In some cultures, brefeldin A was added in the last 6 hours and the cells were analysed by flow cytometry in order to assess the number of cells that were responding to the stimuli.

Inclusion of pertussis toxin with the MOG CFA immunisation increased the IL-17A and IFN-γ protein produced from lymph node cells in response to MOG peptide at day 7 and day 10 although this was only statistically significant using the Mann Whitney U Test at day 10 for IL-17A protein (**Figure 3.21A**). Stimulation with PdBu and ionomycin induced IL-17A and IFN-γ protein production from lymph nodes of both pertussis toxin-treated and untreated mice, with the pertussis toxin-untreated group producing similar, or slightly more protein at the time points measured. (**Figure 3.21B**). At day 10 some mice from the pertussis toxin-treated

group produced IL-17A and IFN-γ protein even in the presence of medium alone (**Figure 3.21C**).

The results indicated that the MOG-specific IL-17A and IFN-γ response was enhanced in the lymph nodes from mice treated with pertussis toxin, compared to those mice immunised with MOG CFA alone. In order to confirm this result, IL-17A and IFN-γ expression was investigated by intra-cellular staining in CD4 T cells stimulated with MOG peptide. This would give information about the percentage and number of CD4 T cells responding to the peptide.

The same experimental set-up was used, but this time brefeldin A was added into the cultures for the last 6 hours in order to retain protein inside the cell for detection by fluorescent antibodies. Representative flow cytometry plots are shown **(Figure 3.22A)** and the data are summarised in the bar charts **(Figure 3.22B).** Pertussis toxin administration in combination with MOG CFA immunisation increased the percentage of MOG-specific IL-17A-expressing, IFN-γ-expressing and IL-17A and IFN-γ double-expressing CD4 T cells in the lymph nodes at day 7 after immunisation as well as the corresponding absolute numbers of these cells (**Figure 3.22B**).

The results showed that CD4 T cells from the mice immunised with pertussis toxin were secreting IL-17A and IFN-γ protein in response to MOG peptide, whereas the CD4 T cells from mice immunised with MOG CFA alone were not. However in response to PdBu ionomycin stimulation the expression of IL-17A and IFN-γ protein from CD4 T cells was not different between the two groups. This suggested that the effect of pertussis toxin on the CD4 T cells *in vivo* is masked by the PdBu ionomycin stimulation but revealed when cells are stimulated with MOG peptide.

3.3.4 Summary

From these experiments, it was determined that pertussis toxin effected the migration of CD4 T cells to CCL21 for at least 10 days following *in vivo* administration and could therefore be exerting its effects in EAE up to the onset of disease, however the effects of pertussis toxin at the initiation stage of the immune response are important for most severe disease. Furthermore, pertussis toxin enhances MOG-specific CD4 T cell production of IL-17A and IFN-γ, which could be directly attributed to pathogenesis in EAE, as the ability of CD4 T cells to be re-activated by MOG peptide in the CNS is critical in the induction of a pro-inflammatory response and the progression of clinical disease (Kawakami, Lassmann et al. 2004).

Figure 3.18 Injection of pertussis toxin impairs the migration capability of CD4 T cells to CCL21 for at least 10 days following administration.

Cells from the peripheral lymph nodes of two wild type, non-immunised C57BL/6 mice were analysed for migration to different concentrations of CCL21 in a transwell assay after *in vitro* treatment with pertussis toxin. The percentage of the cells that migrated across the transwell (% of input) is shown for pertussis toxin treated cells (red line) and control cells that were pertussis toxin un-treated (black line). The mean and standard deviation of two assay replicates is shown for both groups at each concentration (A). Mice were injected with pertussis toxin (red dots) or D-PBS (black dots) on day 0, with a second dose given two days later (day 2). Peripheral lymph nodes were harvested on day three, day six, day ten and day nineteen and assessed for migration to CCL21 in a transwell assay. The peripheral lymph node cells from three or four mice were analysed individually at each time point from each group and each dot on the graph represents the mean of two replicates in the transwell assay for each mouse. The mean for all the mice analysed for each group is shown as a horizontal line. Statistical analysis was carried out between the two groups of injected mice at each time point using the Mann-Whitney U-Test. (*) indicates P-value = 0.01-0.05

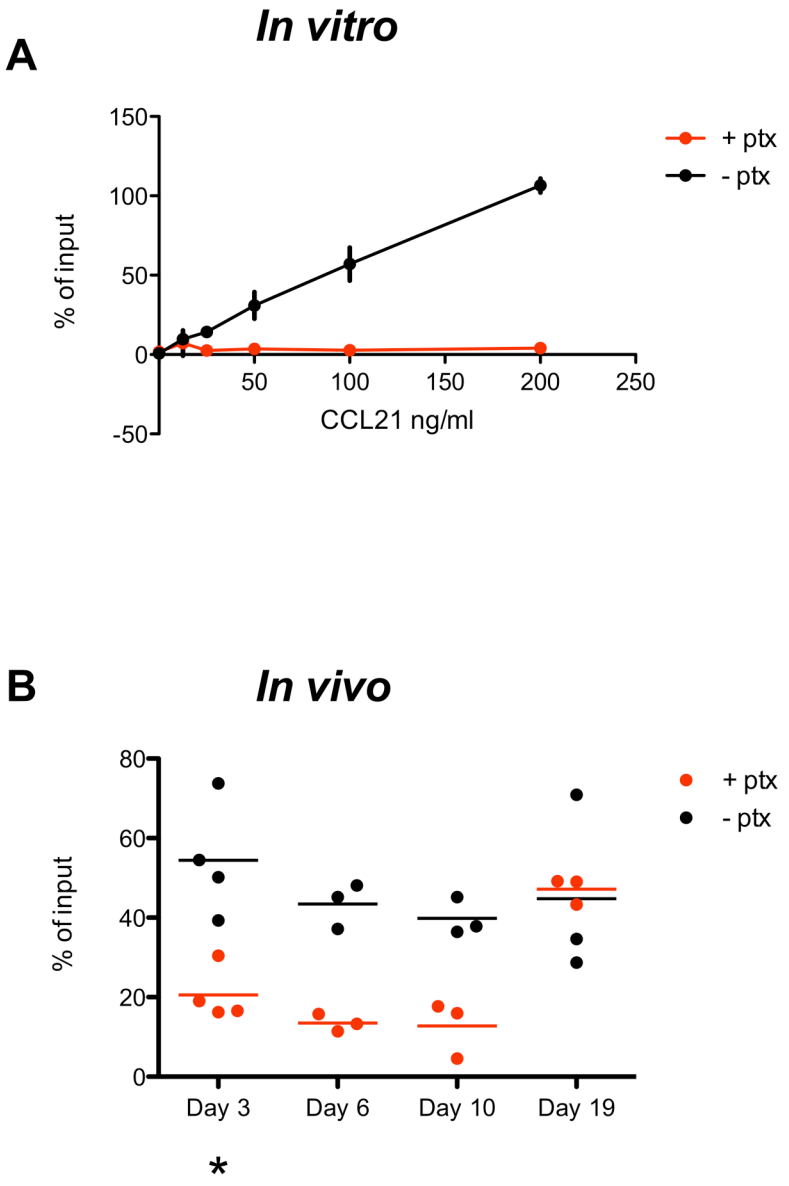
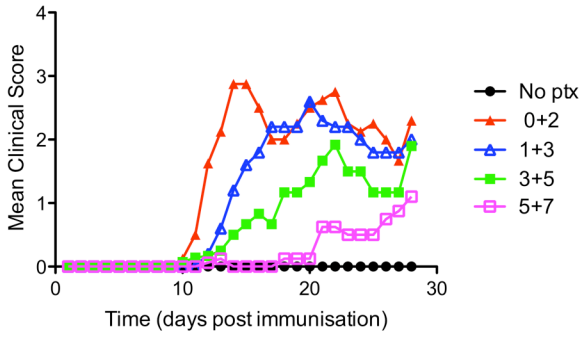

A
In vitro
150
100
50
0
-50
% of input
50
100
150
200
250
CCL21 ng/ml
+ ptx
- ptx
B
In vivo
80
60
40
20
0
% of input
Day 3
Day 6
Day 10
Day 19
*
+ ptx
- ptx

Figure 3.19 Delaying the treatment of pertussis toxin relative to MOG CFA immunisation, reduces the severity of EAE measured by onset, peak mean clinical score and incidence.

C57BL/6 mice were immunised with MOG CFA and given either no pertussis toxin (no ptx), pertussis toxin by intraperitoneal injection on the same day as the MOG CFA immunisation and also two days later (0+2), or on days one and three (1+3), days three and five (3+5) or days five and seven (5+7) following MOG CFA immunisation. Mice were assessed for symptoms of clinical paralysis and the progression of the disease is illustrated by mean clinical score for all of the groups of mice (A). The incidence, mean day of onset and mean maximum score are shown for each group of mice. The day of onset and maximum score results were analysed statistically using the Kruskal-Wallis test with a Dunn's post test. (*) indicates P-value = 0.01-0.05, (**) (B).

A

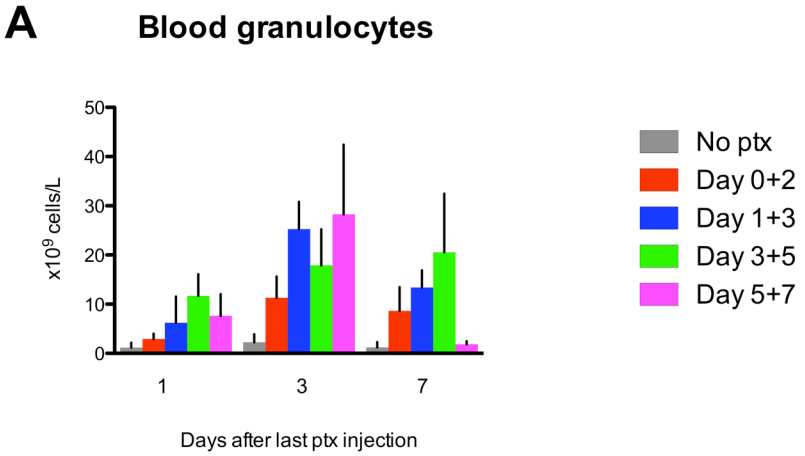


B

Protocol	Incidence	Day of onset (Average +/- SD)		Maximum score (Average +/- SD)	
No PTX	0% (0/8)	-		-	
Day 0+2	100% (4/4)	11.5 +/- 1.2	**	3.375 +/- 0.48	*
Day 1+3	100% (5/5)	13.8 +/- 1.9		2.8 +/- 0.76	
Day 3+5	100% (6/6)	16 +/- 4.3		2.08 +/- 0.8	
Day 5+7	62.5% (5/8)	22.8 +/- 4.08		1.25 +/- 1.17	

Figure 3.20 Following immunisation with MOG CFA, injection of pertussis toxin leads to an increase in the number of blood granulocytes

C57BL/6 mice were immunised with MOG CFA and given either no pertussis toxin (no ptx), pertussis toxin by intraperitoneal injection on the same day as the MOG CFA immunisation and also two days later (0+2), or on days one and three (1+3), days three and five (3+5) or days five and seven (5+7) following MOG CFA immunisation. Blood samples were taken from the tail tip, one day, three days and seven days after the last pertussis toxin injection and were analysed using a Vetscan to determine the number of granulocytes in the blood. The same mice were also assessed for symptoms of clinical paralysis. The mean number and standard deviation of blood granulocytes from between four and eight individual mice in each group is shown in (A). The peak number of granulocytes in mice injected with pertussis toxin is shown compared with the maximum clinical score of the mouse. The mean and standard deviation for between two and nine individual mice of each disease score is shown (B).


A
Blood granulocytes
x10^9 cells/L
50
40
30
20
10
0
1
3
7
Days after last ptx injection
No ptx
Day 0+2
Day 1+3
Day 3+5
Day 5+7

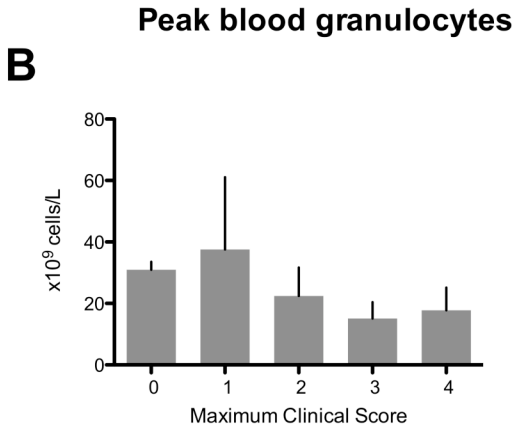

Peak blood granulocytes
B
x10^9 cells/L
80
60
40
20
0
0
1
2
3
4
Maximum Clinical Score

Figure 3.21 Following immunisation with MOG CFA, injection of pertussis toxin enhances MOG-specific IL-17A and IFN-γ protein secretion from lymph node cells

C57BL/6 mice were immunised into the base of the tail with MOG CFA emulsion in the presence or absence of pertussis toxin given by intra-peritoneal injection on the same day as the MOG CFA emulsion as well as two days later. The draining lymph nodes were harvested seven and ten days following the MOG CFA immunisation and approximately 1 million cells from each mouse were cultured with either MOG peptide$_{35-55}$, PdBu and ionomycin, or medium alone. Supernatants were harvested 24 hours later and were assessed for IL-17A and IFN-γ protein by Flowcytomix bead analysis. Concentrations of IL-17A and IFN-γ are shown after MOG stimulation (A) after PdBu and ionomycin stimulation (B) or after medium stimulation alone (C). Red dots represent the results from individual mice immunised with MOG CFA and given pertussis toxin injections and black dots represent individual mice given MOG CFA immunisation alone. The mean of each group is shown by a horizontal line. Statistical analysis was carried out between the groups of mice injected with and without pertussis toxin at each time point using the Mann-Whitney U-Test. (*) indicates P-value = 0.01-0.05.

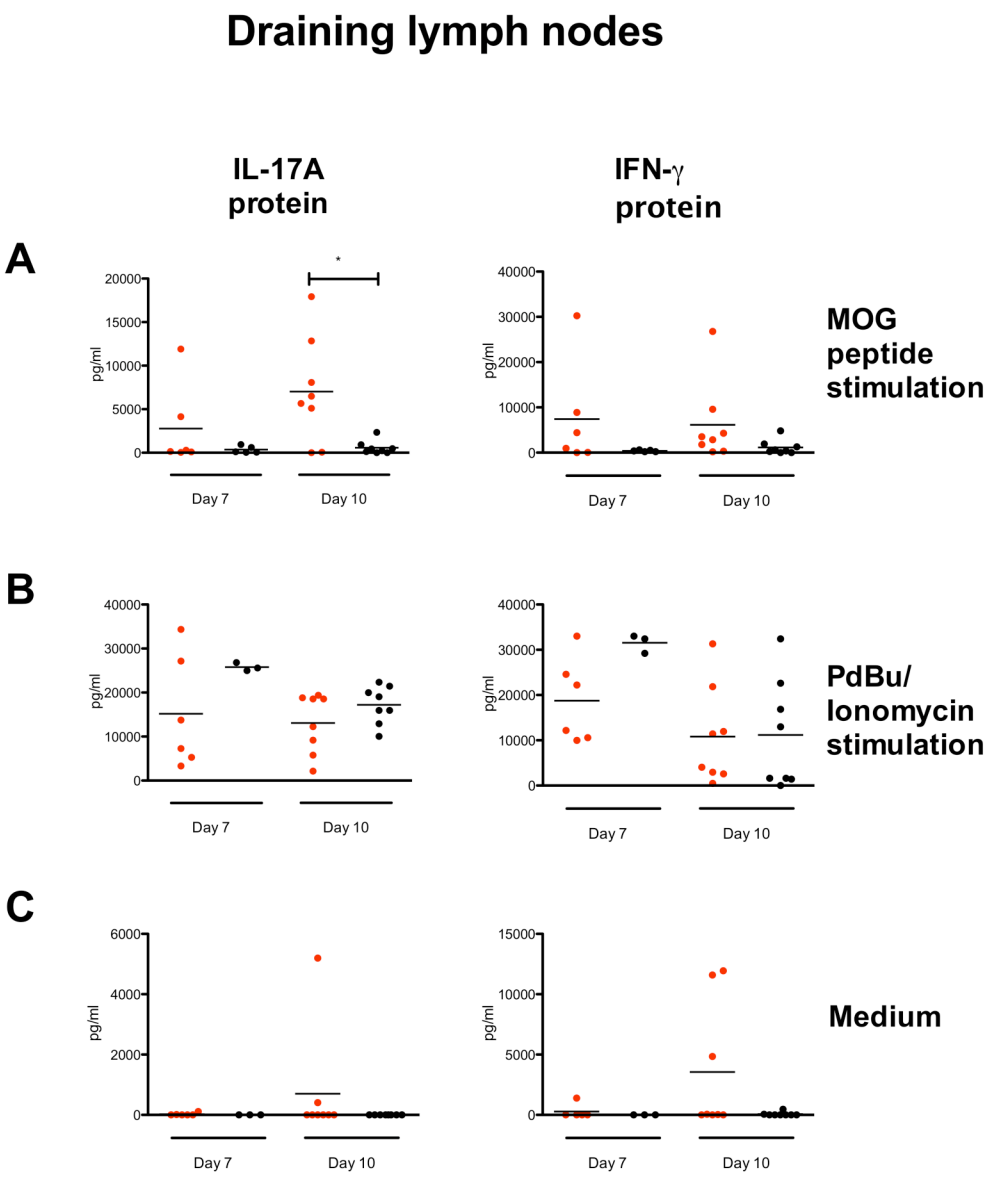
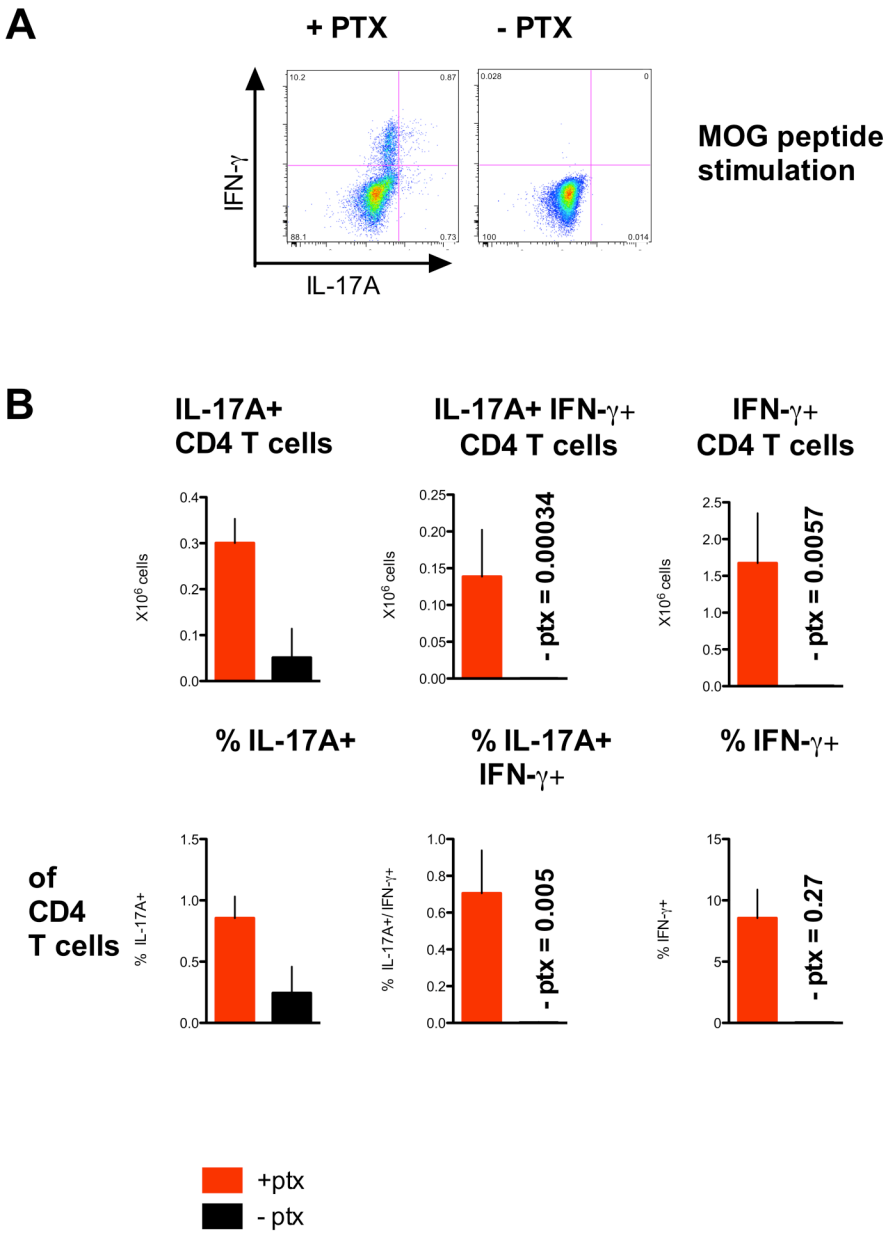

Draining lymph nodes
IL-17A protein
IFN-γ protein
A
B
C
pg/ml
Day 7
Day 10
MOG peptide stimulation
PdBu/ Ionomycin stimulation
Medium

Figure 3.22 Following immunisation with MOG CFA, injection of pertussis toxin enhances MOG-specific intra-cellular IL-17A and IFN-γ protein in CD4 T cells from the draining lymph nodes

C57BL/6 mice were immunised at the base of the tail with MOG CFA emulsion in the presence or absence of pertussis toxin treatment. Pertussis toxin, where given was administered by intraperitoneal injection on the same day as MOG CFA immunisation, followed by a second dose two days later. The draining lymph nodes were harvested seven days following MOG CFA emulsion and approximately equal numbers of cells were cultured with MOG peptide$_{35-55}$ for twentyfour hours. Brefeldin A was added to the cultures for the last six hours of culture and the cells were harvested for analysis of intra-cellular IL-17A and IFN-γ protein by intra-cellular staining. An example of flow cytometry staining in CD4 T cells from mice injected with and without pertussis toxin is shown in (A). The number of these cells in the draining lymph nodes as well as the percentage of CD4 T cells expressing IL-17A, IL-17A/ IFN-γ or IFN-γ is shown in (B). The results are shown as the mean and standard deviation of two mice from the pertussis toxin injected group and three mice from the group immunised with MOG CFA alone. Red bars indicate mice injected with pertussis toxin in combination with MOG CFA immunisation and black bars indicate mice immunised with MOG CFA alone. Where values for the group immunised without pertussis toxin cannot be seen on the graph, the mean is indicated.


Draining lymph nodes
A
+ PTX
- PTX
IFN-γ
IL-17A
MOG peptide stimulation
B
IL-17A+ CD4 T cells
IL-17A+ IFN-γ+ CD4 T cells
IFN-γ+ CD4 T cells
X10^6 cells
- ptx = 0.00034
- ptx = 0.0057
% IL-17A+
% IL-17A+ IFN-γ+
% IFN-γ+
of CD4 T cells
% IL-17A+
% IL-17A+/ IFN-γ+
% IFN-γ+
- ptx = 0.005
- ptx = 0.27
+ptx
- ptx

3.4 Discussion

The aim of the first part of this thesis was to define the sequence of immunological events leading to the development of a pathological immune response directed against a self-antigen. EAE was chosen as an animal model of autoimmunity in which immunisation with CNS peptides and adjuvant culminates in a pathological immune response directed against self-antigens in the CNS.

EAE has many similarities with the human disease multiple sclerosis including the presence of auto-reactive T cells in the CNS, the destruction of the myelin sheaths and the formation of perivascular inflammatory cell foci in the brain stem and spinal cord (Steinman and Zamvil 2006). In addition, the majority of MS patients have a course of disease that is relapsing and remitting, however some patients have a primary progressive disease (Sospedra and Martin 2005) and this is also true for different models of EAE. Factors that influence the course of disease progression in EAE include the genetic strain of animal used. For example, SJL/J and Biozzi/ABH mice typically exhibit a relapsing and remitting disease (Brown and McFarlin 1981; Baker, O'Neill et al. 1990), whereas C57BL/6 mice typically develop chronic EAE (Mendel, Kerlero de Rosbo et al. 1995). Factors such as the sex of the animal and the environment also determine disease outcome. For example, it was shown that female SJL/J mice immunised with $PLP_{139-151}$ peptide developed relapsing and remitting disease, however male SJL/J mice immunised with the same peptide did not relapse (Bebo, Vandenbark et al. 1996). In one study a cohort of SJL/J mice derived from one breeder developed chronic disease, while from another breeder they developed relapsing and remitting disease (deLuca, Pikor et al. 2010). In addition, the method of disease induction can influence disease outcome, as C57BL/6 mice immunised with sub-optimal doses of peptide, *Mycobacterium tuberculosis* and pertussis toxin developed relapsing and remitting disease (Berard, Wolak et al. 2010).

The potential of different CNS epitopes to induce disease is dependent upon the strain of mouse used. For example, myelin oligodendrocyte glycoprotein (MOG) peptide$_{35-55}$, a minor component of CNS myelin, is strongly encephalitogenic in C57BL/6 mice (Mendel, Kerlero de Rosbo et al. 1995), while in SJL/J mice MOG$_{92-106}$ (Amor, Groome et al. 1994), peptides of PLP (Tuohy, Lu et al. 1989; Greer, Kuchroo et al. 1992; Tuohy and Thomas 1995) and peptides of MBP (Amor, O'Neill et al. 1996) are more potent at inducing autoimmunity. These differences are likely to be heavily dependent upon the different MHC backgrounds of the mice as MHC molecules are responsible for presenting peptides to T cells for their activation.

Epitopes of MOG are strongly encephalitogenic in mice, rats, monkeys and marmosets (Krishnamoorthy and Wekerle 2009), and T cells reactive to MOG epitopes have been detected in MS patients (Bernard, Johns et al. 1997; Iglesias, Bauer et al. 2001). The capacity of MOG peptide to act as an auto-antigen in EAE and MS is due to breakdown in self-tolerance leading to development of autoimmunity directed against this peptide. During T cell development, auto-reactive T cells are usually deleted by negative selection, which is heavily dependent upon expression of the peptide in the thymus (Derbinski, Schulte et al. 2001). Indeed, it was shown that T-cell epitopes that were ectopically expressed in the thymus were not targets for autoimmunity, while epitopes not expressed in the thymus were targets (Klein, Klugmann et al. 2000). There is also an association between expression of CNS peptides in the thymus and susceptibility to EAE induction. For example, intra-thymic expression of the PLP peptide, DM20 is high, however the peptide PLP$_{139-151}$ is not expressed in the thymus and it has been suggested that the high occurance of PLP$_{139-151}$-reactive T cells in SJL/J mice is due to the lack of thymic expression of this peptide. (Anderson, Nicholson et al. 2000). Furthermore, BALB/C mice are usually resistant to EAE induced by MBP peptide, but if MBP-reactive cells are isolated from MBP-deficient mice and transferred into wild type recipients, EAE develops (Targoni and Lehmann 1998). These observations could be due to negative selection of these self-reactive clones in the periphery or positive selection of clones with regulatory function. There have been

some discrepancies with respect to detection of MOG expression in the thymus. Some reports have suggested MOG expression is sequestered in the CNS (Mor, Boccaccio et al. 1998; Bruno, Sabater et al. 2002), however more recent work has suggested that MOG is expressed in the medullary epithelial cells in the thymus (Derbinski, Schulte et al. 2001). Nevertheless, these discrepancies could indicate that MOG is expressed at most at low levels in the thymus. This would also correlate with the results from experiments using MOG knockout mice in which there have also been different findings. One study showed that T cells from mice lacking MOG could transfer more severe disease than wild type T cells into wild type recipients, suggesting that expression of endogenous MOG exhibits some level of tolerance to this brain determinant (Linares, Mana et al. 2003). However, another study using MOG-deficient mice showed that responses to MOG were the same in MOG-deficient mice as in wild type mice (Delarasse, Daubas et al. 2003). That endogenous MOG peptide is not expressed highly enough in the periphery for the development of anergy or for the deletion of MOG-specific clones is supported by the fact that if MOG peptide is injected intravenously prior to EAE induction then the development of autoimmunity is prevented (Fazilleau, Delarasse et al. 2006).

In summary, tolerance to MOG is incomplete, however under normal circumstances MOG-specific T cell clones do not become activated by their antigen in the periphery. Specific signals induced by immunisation with MOG in CFA and pertussis toxin can break tolerance to this peptide and induce encephalitogenic T cells, capable of initiating a pathological immune response in the CNS.

To investigate the implications of this immunisation we first investigated the sequence of events following MOG CFA immunisation, an immunisation which in most cases does cause disease pathology.

CFA containing heat-killed *Mycobacterium tuberculosis* is used to elicit a number of autoimmune models (Billiau and Matthys 2001) however the specific implications of injection with this adjuvant are not clearly defined. In this study, immunisation with

MOG peptide in CFA alone was sufficient to up-regulate peripheral IL-17A and IFN-γ responses detected after PdBu and ionomycin *ex vivo* stimulation. More specifically, an increase in the frequency of IL-17A-producers was detected within the CD4 and γδ T cell populations in the draining lymph nodes 7 days following immunisation (**Figures 3.3B,3.4A**), which was accompanied by an increase in the number of these cells in the blood (**Figure 3.6A**). No change in the percentage of IFN-γ-producing cells within the CD4 T cell population was observed in the lymph nodes at this time point (**Figure 3.3B**), however an increase in the number of IL-17A/IFN-γ- double producing CD4 T cells was observed in the blood (**Figure 3.6A**). Whether these observations are a result of the outgrowth or recruitment of already IL-17A or IFN-γ-expressing cells, or *de novo* generation cannot be determined from these experiments but could be investigated further using cytokine reporter mice. *In vitro*, the generation of Th17 cells can be induced by activation of CD3 in the TCR complex using an anti-CD3 antibody, stimulation of CD28 using an anti-CD28 antibody, as well as stimulation with the cytokines IL-6 and TGF-β (Veldhoen, Hocking et al. 2006), and *in vivo* these signals are also required for lineage commitment of Th17 cells given the absence Th17 cell generation in IL-6 knockout mice and in mice expressing a dominant negative form of TGF-β receptor II under the control of the CD4 promoter (Bettelli, Carrier et al. 2006; Veldhoen, Hocking et al. 2006) In addition, generation of IFN-γ-expressing Th1 cells can be induced *in vitro* by anti-CD3, anti-CD28, plus addition of IL-12. *Mycobacterium tuberculosis* consists of a number of PAMPs including cell wall lipoproteins and polysaccharides that are capable of activating PRRs on DCs and macrophages (Akira, Uematsu et al. 2006). For example, *Mycobacterium tuberculosis* stimulated the production of IL-12p40 from a human monocyte line (Brightbill, Libraty et al. 1999) and TNFα from a mouse macrophage cell line (Underhill, Ozinsky et al. 1999), which was dependent upon TLR2 signalling, and has also been shown to stimulate TLR1 and TLR4 (Akira, Uematsu et al. 2006). *Mycobacterium tuberculosis* has been shown to drive the production of IL-17 and IFN-γ-producing cells in *in vitro* systems using bone marrow-derived dendritic cells rather than triggering with anti-CD3 and anti-CD28, suggesting that these signals are also delivered by the antigen presenting cells that

are activated by *Mycobacterium tuberculosis* (Veldhoen, Hocking et al. 2006; Zenaro, Donini et al. 2009). The generation of IL-17-producing CD4 T cells in these cultures was inhibited by blocking the effects of TGFβ or IL-6 (Veldhoen, Hocking et al. 2006) and was dependent upon Dectin-1 receptor engagement (Zenaro, Donini et al. 2009). In addition, *in vivo* infection with *Mycobacterium tuberculosis* has been shown to induce IL-17- and IFNγ-producing CD4 T cells (Khader, Pearl et al. 2005; Cruz, Khader et al. 2006; Khader, Bell et al. 2007), which is in agreement with the *in vivo*, up-regulation of IL-17A and IFN-γ-expressing CD4 T cells in this study.

γδ T cells have also been described to have a number of surface PRRs including TLR2, TLR1 and Dectin-1 (Mokuno, Matsuguchi et al. 2000; Deetz, Hebbeler et al. 2006; Schwacha and Daniel 2008; Martin, Hirota et al. 2009) and are activated to produce IL-17 in response to various microbial stimuli including *Mycobacterium tuberculosis* (Martin, Hirota et al. 2009), as well as cytokines such as IL-23 and IL-1 (Sutton, Lalor et al. 2009) which supports the observation of IL-17A-expressing γδ T cells induced after immunisation with MOG CFA. The MOG CFA immunisation alone also induced marginal accumulation of granulocytes in the blood (**Figure 3.6B**), which are predominately neutrophils. This could be due to increased mobilisation of neutrophils from the bone marrow, increased survival of these cells or an accumulation of these cells in the blood due to an inhibition of the influx of these cells into peripheral tissue. Neutrophils were also observed as part of the cellular infiltrate in the spinal cord from day 7 after MOG CFA immunisation in the absence of pertussis toxin (**Figure 3.13A**), suggesting that the increase in neutrophils in the blood was at least not solely due to an inhibition of cells to access peripheral tissue. γδ T cells have been closely associated with neutrophil-dependent inflammatory responses (King, Hyde et al. 1999; Moore, Moore et al. 2000; Toth, Alexander et al. 2004) and IL-17-producing γδ T cells have been shown in other systems to induce recruitment of neutrophils after direct interaction with pathogens including *Mycobacterium tuberculosis* (Martin, Hirota et al. 2009). In addition, IL-17 has been associated with neutrophil accumulation in peripheral tissues (Forlow,

Schurr et al. 2001; Ye, Rodriguez et al. 2001) and has been shown to be involved in the mobilisation of neutrophils from the bone marrow via upregulation of G-CSF (Fossiez, Djossou et al. 1996). It is therefore conceivable that IL-17A production from activated CD4 T cells and γδ T cells in response to *Mycobacterium tuberculosis* in CFA may contribute to up-regulation of neutrophil mobilisation after MOG CFA immunisation.

The frequency of Foxp3-expressing cells within the CD4 T cell population was also increased in the lymph nodes by day 7 after immunisation with MOG CFA (**Figure 3.4B**). *De novo* generation of Foxp3 expression in naïve CD4 T cells has been shown to be dependent on the presence of TGF-β, however in the presence of additional IL-6, naïve T cells can become Th17 cells. Moreover, pro-inflammatory Th17 cells and suppressive Foxp-3 expressing T-regs can develop in a mutually exclusive fashion (Bettelli, Carrier et al. 2006). Although this *de novo* generation has been shown to occur *in vitro* it is impossible to deduce from the experiments in this study whether the increase in this population is a result of *de novo* generation from naïve CD4 T cells or the outgrowth of an already differentiated population. However, studies using adoptive transfer experiments with *Foxp3gfp* mice, in which Foxp3 expression is reported by the presence of GFP, have suggested that in EAE the expansion of Foxp3-expressing regulatory T cells, is a result of the outgrowth of already Foxp3-expressing cells, rather than *de novo* generation (Korn, Reddy et al. 2007).

However, the MOG CFA-induced up-regulation of peripheral IL-17A and IFN-γ-responses (detected after PdBu ionomycin re-stimulation) and granulocyte induction is insufficient alone to induce autoimmunity. For EAE to develop, administration of pertussis toxin is also required.

EAE is understood to be mediated by CD4 T cells because elimination of CD4 T cells prevented disease onset or reversed ongoing disease (Pettinelli and McFarlin 1981; Brostoff and Mason 1984; Waldor, Sriram et al. 1985) and also auto-antigen

specific CD4 T cell lines could induce disease following transfer (Ben-Nun and Cohen 1982; Zamvil, Nelson et al. 1985; Satoh, Sakai et al. 1987; Bourdette, Vandenbark et al. 1989; van der Veen, Trotter et al. 1990; Kuchroo, Sobel et al. 1991; Whitham, Bourdette et al. 1991; Kuchroo, Sobel et al. 1992). EAE was for a long time believed to be mediated by Th1 effector cells, which was due to an association between IFN-γ expression in the target tissue and disease pathology, the transfer of disease by IFN-γ-expressing Th1 T cell lines (Kuchroo, Martin et al. 1993; Ramirez and Mason 2000), and disease resistance in mice lacking T-bet and STAT-4, which are transcription factors involved in Th1 cell development (Bettelli, Sullivan et al. 2004). However, genetic deletion of IFN-γ (Ferber, Brocke et al. 1996), the characteristic cytokine produced by Th1 cells, as well as the IFN-γ receptor (IFN-γ R) (Willenborg, Fordham et al. 1996) resulted in enhanced disease severity. In addition, deletion of genes involved in Th1 development such as IL-12p35 (Gran, Zhang et al. 2002), IL-12Rβ2 (Zhang, Gran et al. 2003) and IL-18 (Gutcher, Urich et al. 2006) had the same phenotype. Molecules involved in the Th17 pathway, on the other hand, have been found important for disease development. For example, genetic deletion of the IL-17A cytokine (Komiyama, Nakae et al. 2006) and neutralisation of IL-17A with a monoclonal antibody (Lubberts, Koenders et al. 2004) led to reduced disease severity in EAE. Removal of IL-23p19, which is required for the stability of Th17 cells *in vivo*, rendered mice resistant to EAE (Cua, Sherlock et al. 2003). In addition, RORγt-deficient mice and mice that had defective signalling through TGFβ receptor II in CD4 T cells had undetectable Th17 cells also had attenuated disease development (Ivanov, McKenzie et al. 2006; Veldhoen, Hocking et al. 2006). Enhancing the production of Th17 cells *in vivo* by AHR ligation with FICZ enhanced EAE severity (Quintana, Basso et al. 2008; Veldhoen, Hirota et al. 2008), which again supported a role for the Th17 pathway in the development of EAE. Nevertheless, adoptive transfer studies are inconclusive and auto-reactive clones polarised to both Th1 and Th17 phenotypes have been shown to be able to induce the development of EAE, and both IFN-γ-producing Th1 cells and IL-17-producing Th17 cells have been detected in the CNS during EAE development in this study as well as others (Murphy, Lalor et al. 2010)

(Ivanov, McKenzie et al. 2006; Suryani and Sutton 2007; O'Connor, Prendergast et al. 2008).

For an auto-reactive T cell to be pathogenic it must have the ability to home to the target tissue, to be able to recognise its target antigen, and to secrete the appropriate pro-inflammatory molecules upon activation to initiate tissue injury. Although IL-6 and TGF-β are required *in vitro* and *in vivo* for lineage commitment of Th17 cells (Veldhoen, Hocking et al. 2006) (cytokines which can be induced from DCs by *Mycobacterium tuberculosis in vitro)*, these two cytokines are not sufficient to promote full differentiation of inflammatory pathogenic Th17 cells as IL-23p19 mice (Cua, Sherlock et al. 2003) and IL-1R1-deficient mice have reduced Th17 responses (Sutton, Brereton et al. 2006). Furthermore, one report showed that IL-23 was found necessary for full pathogenic function of myelin-specific T cells in EAE (McGeachy, Bak-Jensen et al. 2007) and another showed that the IL-17 production from γδ T cells produced in response to IL-23 and IL-1β could enhance CD4 T cell IL-17 responses, providing a mechanism by which IL-23 may amplify Th17 responses *in vivo* (Sutton, Lalor et al. 2009). This therefore suggests that development of autoimmune pathology in EAE requires more than TGF-β and IL-6-induced Th17 cells that can be induced by MOG CFA immunisation.

One of the proposed functions of pertussis toxin in EAE is that it opens up the blood brain barrier, facilitating the movement of activated auto-antigen specific CD4 T cells into the CNS. Indeed, administration of pertussis toxin is associated with an increase in vascular permeability (Kerfoot, Long et al. 2004). However, it is known that activated CD4 T cells can access the CNS in the absence of pertusss toxin in non-immunised individuals, as exemplified in humans where T cells can be detected in the CSF of healthy individuals, albeit in low frequencies (Svenningsson, Hansson et al. 1993) and pertussis toxin is infrequently injected into the recipient mice when disease is induced by transfer of auto-reactive cells. In this study, a basal level of IL-17A and IFN-γ-expressing CD4 T cells was observed in the spinal cords of mice immunised with MOG CFA alone (**Figure 3.15A**), suggesting that activated cells

can access the spinal cord to some extent in the absence of pertussis toxin. In addition, an effect of pertussis toxin on the blood brain barrier can occur within 5 hours of injection (Kerfoot, Long et al. 2004) and so one would expect that administration of pertussis toxin at later time points in EAE development could induce EAE as efficiently. However the most optimal time point at which pertussis toxin should be administered for the development of disease was day 0 and 2 suggesting that pertussis toxin influences EAE development in other ways.

Pertussis toxin is commonly used as an agent to inhibit signalling through G-protein receptors such as chemokine receptors. In these experiments, pertussis toxin administration was associated with a non-selective reduction in the number of CD4, CD8 and γδ T cells, as well as B cells in the lymph nodes after immunisation with MOG CFA (**Figure 3.8**) which could be due to the effect of pertussis toxin on G-protein associated-chemokine receptors such as CCR7 and sphingosine-1-phosphate which are responsible for cell movement into and out of the lymph nodes (Matloubian, Lo et al. 2004; Forster, Davalos-Misslitz et al. 2008). In addition, pertussis toxin reduced signalling through CCR7, measured by migration of CD4 T cells to CCL21, for at least 10 days after administration (**Figure 3.18B**). It seems paradoxical that inhibition of G-protein receptor signalling by pertussis toxin would allow development of disease given that chemokine signalling is important for leukocyte trafficking in both steady state and inflammatory environments (Rebenko-Moll, Liu et al. 2006) and is also important in the movement of activated, encephalitogenic T cells into the central nervous system at the blood brain barrier (Ransohoff, Kivisakk et al. 2003). One report showed that encephalitogenic T cells were treated with pertussis toxin *in vitro* before being injected into mice in order to inhibit G-protein receptor signalling and these mice exhibited delayed onset of disease (Alt, Laschinger et al. 2002). In addition, various chemokine receptor deficient mice are resistant to EAE, such as the CCR7-deficient mice (Kuwabara, Ishikawa et al. 2009), CCR2 deficient mice (Fife, Huffnagle et al. 2000) and in some reports CCR6-deficient mice (Liston, Kohler et al. 2009; Villares, Cadenas et al. 2009). This suggests that perhaps the dose and timing of pertussis injection given in

EAE are not enough to completely inhibit the aspects of migration that are important in EAE. Pertussis toxin is an AB exotoxin. The A subunit possesses the ADP-ribosylase activity capable of inactivating G-protein coupled receptors and the B subunit is responsible for binding to cell surface receptors and facilitating the entry of the A subunit into the cell. In order to assess whether the G protein inhibitory activity of pertussis toxin is required for EAE induction, the encephalitogenic potential of a toxin that lacked the A subunit or that had been mutated to lack the ADP-ribosylase activity could be investigated.

It was observed that pertussis toxin injected in combination with MOG CFA immunisation enhanced peripheral MOG-specific CD4 T cell IL-17A and IFN-γ cytokine responses *in vitro,* when compared to the response of CD4 T cells from mice immunised with MOG CFA alone. More specifically, stimulation of lymph node cells from pertussis toxin-treated and untreated mice with PdBu and ionomycin for 24 hours did not reveal any substantial differences in the amount of IL-17A and IFN-γ protein detected in the supernatant (**Figure 3.21B**) and stimulation for 4 hours with these stimuli did not reveal any difference in the percentage of IL-17A and IFN-γ -expressing cells within the CD4 T cell population (**Figure 3.9B**). However, stimulation of lymph node cells from pertussis toxin-treated and untreated mice with MOG peptide for 24 hours resulted in IL-17A and IFN-γ detection in the supernatants of cell cultures from pertussis toxin-treated mice only (**Figure 3.21A**), and a substantial increase in the percentage of IL-17A and IFN-γ-expressing cells within the CD4 T cell population in the pertussis toxin-treated mice when detected by flow cytometry (**Figure 3.22**). This observation could be due to a greater proportion of MOG-specific T cells producing IL-17A and IFN-γ in the lymph nodes of pertussis toxin-treated mice or it could be that the there are a similar proportion of MOG-specific cells in the two groups of mice but that MOG-specific T cells from pertussis toxin un-treated mice do not produce IL-17A and IFN-γ in response to MOG peptide. Furthermore, PdBu and ionomycin are mitogenic stimuli and can activate protein kinase C and up-regulate intra-cellular calcium respectively, however MOG peptide is processed and presented on MHC class II molecules and

can be presented to T cells for their activation in a conventional manner. It could be envisaged that stimulation by PdBu and ionomycin overrides the effects of conventional T cell activation, triggering the production of cytokines that the cell is able to make, however MOG peptide stimulation specifically captures cytokine triggered by re-stimulation with MOG peptide, a response which may be different. Detection of MOG-specific cells using a MOG tetramer would help to investigate this issue further. Nevertheless, this enhanced responsiveness to MOG is an effect which could be directly attributed to pathogenesis in EAE, as the ability of CD4 T cells to respond to MOG peptide in the CNS is a trigger for further inflammation to promote recruitment and activation of other effector cells such as macrophages and neutrophils to the target organ (Sobel and Kuchroo 1992).

In this study disease development was associated with an increase in the total number of CD4 and γδ T cells recovered from the spinal cord starting at day 10, before the onset of disease (**Figure 3.13B**) which then increased further at the peak of disease (**Figure 3.16B)**, and was not detected in groups of mice that did not develop disease at the same time points. It must be noted that the anatomical organization of the CNS is highly complex and contains numerous fluid compartments, membranes and vascular beds (Ransohoff, Kivisakk et al. 2003). The mechanisms and consequences of leukocyte entry into these different compartments will differ and have not been assessed in the context of this thesis. Furthermore, in EAE, although infiltration of mononuclear cells into the spinal cord parenchyma is a frequent observation, it has been suggested that T cells first enter the CNS via the choroid plexus in the brain (Reboldi, Coisne et al. 2009). This provides the first wave of cells into the CNS, which can subsequently be activated by local antigen presenting cells present in the subarachnoid space (Kivisakk, Healy et al. 2009).

Disease development was also associated with the persistence of neutrophils and monocytes in the spinal cord (**Figure 3.16A**). These effector cells could be detected in the spinal cord following immunisation with MOG CFA in the presence and

absence of pertussis toxin treatment before the onset of disease (**Figure 3.13A**) but only persisted in the mice that developed disease. Although a PBS perfusion was carried out prior to removal of the spinal cord in order to reduce the possibility of contaminating blood cells in the spinal cord preparation, this possibility can obviously not be completely excluded. However, other studies have also reported the presence of neutrophils or polymorphonuclear cells in the CNS infiltrate of animals with EAE using both flow cytometry and immunohistochemistry methods (Allen, Baker et al. 1993; Maatta, Sjoholm et al. 1998; Bettelli, Sullivan et al. 2004; Wu, Cao et al. 2010) which supports these findings. Monocytes encompass a heterogeneous cell population based upon differential expression of surface molecules. However, they are typically blood-derived cells that migrate into tissue and differentiate into antigen presenting macrophages. Mice that lacked CCR2 were relatively resistant to the development of EAE, even in the presence of wild type T cells and this has been attributed to a lack of monocytes in the CNS of these mice suggesting a role for CCR2 in the transport of monocytes across the BBB and in the development of disease (Fife, Huffnagle et al. 2000). In EAE, monocytic cells can migrate into the CNS and differentiate into antigen presenting cells for the reactivation of myelin-reactive CD4 T cells (Deshpande, King et al. 2007; Miller, McMahon et al. 2007). Activated macrophages can secrete numerous cytokines including IL-1 and TNF-α, nitrous oxide, free oxygen radicals and protelolytic enzymes that can contribute to tissue damage (Scheurich, Thoma et al. 1987; Segal 2005). However, monocytic and macrophage populations have also been described to have suppressive implications in EAE (King, Dickendesher et al. 2009). In the experiments in this thesis, monocytes were defined based on detection of a surface epitope by 7/4 antibody. Reduction in the number of these cells could mean that the cells had down-regulated this epitope, indeed monocytes have been reported to reduce expression of the epitope recognised by 7/4 as they differentiate into macrophages (Henderson, Hobbs et al. 2003).

Pertussis toxin has been described to have mitogenic effects on T cells *in vitro* (Kong and Morse 1977) so it could be conceived that treatment with pertussis toxin

promotes the outgrowth of auto-reactive T cells. However, it was observed that immunisation with MOG CFA in the absence of pertussis toxin led to the induction of activated IL-17A and IFN-γ-expressing cells that were induced in the lymph nodes and were detectable in the blood circulation in similar numbers as in the presence of pertussis toxin (**Figure 3.11A**) suggesting that pertussis toxin was not merely enhancing activated cell proliferation. In addition, it has been shown in other studies that a Vβ8-specific superantigen (SEB), which specifically targets the outgrowth of T cells expressing the TCR Vβ8 chain which is expressed in some encephalitogenic CNS T cell clones, and another mitogenic stimulus, Con A cannot substitute pertussis toxin in the induction of EAE (Kamradt, Soloway et al. 1991)

As previously mentioned, pertussis is not usually required to be administered in adoptive transfer models of EAE suggesting that the effect of pertussis toxin, whether indirect or direct is on the T cell itself. Furthermore, the effect of pertussis toxin is overridden by stimulation of cells *in vitro* with peptide and an antibody cocktail before adoptive transfer, suggesting that the effects of pertussis toxin *in vivo* could be due to induction of an environment which can alter the encephalitogenic potential of the T cell. One study showed that IL-1 -deficient mice are resistant to EAE and these mice also have reduced MOG-specific IL-17A and IFN-γ responses in the peripheral lymph nodes after immunisation with MOG CFA (Matsuki, Nakae et al. 2006). In this same study, IL-1R antagonist-deficient mice, which lack an endogenous antagonist for IL-1 signalling, develop MOG-induced EAE in the absence of pertussis toxin, unlike wild type mice. This suggests that pertussis toxin may act through a mechanism dependent upon IL-1 signalling to induce enhanced MOG-specific IL-17A and IFN-γ responses in EAE. IL-1 is produced by a number of cell types including APCs and can induce the expression of CD40 ligand and OX40 expression on T cells (Nakae, Asano et al. 2001), molecules which strengthen the interaction between T cells and APCs which can lead to more efficient T cell priming. Pertussis toxin has been shown to enhance antigen-specific responses previously (Sewell, Munoz et al. 1983; Sewell, de Moerloose et al. 1986; Mu and Sewell 1993; Shive, Hofstetter et al. 2000) and the toxin has been shown to

upregulate cytokine production by a method dependent upon its effects on G-proteins (He, Gurunathan et al. 2000). Pertussis toxin has been reported to act on macrophages and B cells, by upregulating B7.1 and B7.2 molecules, and on T cells by upregulating CD28, a process which could be envisaged to enhance T cell activation (Ryan, McCarthy et al. 1998). In addition, pertussis toxin has been shown to trigger the TCR directly, activating a number of signalling molecules downstream of the receptor (Schneider, Weiss et al. 2007; Schneider, Weiss et al. 2009), a mechanism which again may lead to enhanced T cell activation.

Pertussis toxin is a microbial agent capable of breaking immunological tolerance to MOG peptide. A number of studies have clearly shown that administration of pertussis toxin can affect vascular permeability at the blood brain barrier, facilitating the entry of radioactive isotopes and serum proteins into the CNS that would normally be excluded by tight junctions, and this could also be attributed to EAE pathogenesis by facilitating entry of auto-reactive T cells into the CNS. The results in this study, however show that the effects of pertussis toxin are most potent for EAE development if given at the same time as the MOG CFA immunisation, in the priming stage of disease, suggesting that its effects may extend beyond enhancing vascular permeability, which can occur within a few hours of pertussis toxin injection (Kerfoot, Long et al. 2004). Enhancing antigen-specific cytokine production may be one of the ways it does this. The priming of MOG peptide-specific CD4 T cells in the periphery is partially dependent upon the specific interaction between MOG peptide presented by MHC class II and the MOG-specific TCR, as well as other factors such as costimulatory molecules and cytokines. This interaction may vary between strains of animal, dependent partly upon the repertoire of MOG-specific CD4 T cells selected in the thymus, which might explain differences in the requirement of pertussis toxin for disease development in different strains of animal. In one study it was suggested that that the effect of pertussis toxin on permeability at the blood brain barrier could be a consequence of the interaction between T cells and endothelial cells of the blood brain barrier, as blocking this interaction, blocked the increase in permeability of the blood brain barrier to FITC-

dextran, observed with pertussis toxin administration (Kerfoot, Long et al. 2004). Thus, it could be that an effect of pertussis toxin on the encephalitogenic T cell in the priming stage of disease can enhance interactions between the T cell and the endothelial cells at the blood brain barrier, which consequently promotes vascular permeability. That T cells can induce permeability at the blood brain barrier has also been suggested by a study showing that T cells enter the CNS in two waves in EAE: the first wave through the choroid plexus in the brain which triggers the subsequent recruitment of another wave of effector T cells across the blood brain barrier (Reboldi, Coisne et al. 2009).

Further investigations are required to understand the mechanisms by which pertussis toxin enhances responsiveness to antigen and can break immunological tolerance. In order to specifically address how pertussis toxin is influencing the expression of effector cytokines, mice which report cytokine expression could be utilised. Specific effects of pertussis toxin on T cells and APCs in the context of EAE could be assessed and the receptors through which pertussis toxin is exerting its effects should be considered.

Chapter 4: Generation and characterization of an IL-17A reporter mouse

4.1 Introduction

Effector T cell subsets are typically identified by the profile of cytokine genes they express. Th1 cells are identified by the expression of IFN-γ, Th2 cells by the expression of IL-4 and Th17 cells by the expression of IL-17A and IL-17F. The phenotype of individual cells can be determined by the expression of extra-cellular surface proteins and intra-cellular cytokine protein using flow cytometry methods. Indeed, this is currently the predominant method for identification of effector T cell subsets. The process of intra-cellular cytokine staining uses a fixation and permeablisation step, which results in death of the cells and therefore does not permit their use in further experiments. In addition, an *in vitro* stimulation which is usually given by a mitogenic stimulus such as PdBu and ionomycin is usually also required in order to detect intra-cellular cytokine protein, a process which bypasses physiological signaling mechanisms through which the cell would be responding in its natural environment and is therefore not necessarily reflective of the *in vivo* situation. Expression of cytokines can also be detected in cell populations by quantitative PCR, which measures mRNA levels, or by stimulating cell populations and assessing secreted protein in the supernatant. This however, does not give information about gene expression in individual cells.

Reporter mice have been used to circumvent these problems for IL-4, IFN-γ and IL-10 genes (Mohrs, Shinkai et al. 2001; Stetson, Mohrs et al. 2003; Kamanaka, Kim et al. 2006), all of which inserted a fluorescent reporter gene immediately before the polyadenylation site of the target cytokine gene and made use of an IRES (internal ribosomal entry site) element to allow translation of the reporter gene. The aim of this chapter was to generate an IL-17A reporter mouse to track the induction and the fate of cells that activate the IL-17A promoter in the periphery. As we wanted to

determine the fate of these cells, we chose to employ the Cre recombinase/Loxp system, rather than directly inserting the reporter gene as in the reporter mice described above, in order to irreversibly report transcriptional activation of IL-17A. Expression of the reporter gene would allow a pure, live population of these cells to be sorted for use in other experiments.

We aimed to generate this reporter mouse by directly targeting the IL-17A gene using homologous recombination in Embryonic Stem (ES) cells (Thomas and Capecchi 1987). Cre recombinase from the bacteriophage P1 is a 38kDa recombinase (Sternberg and Hamilton 1981) which promotes intra and inter-molecular recombination at specific 34bp sites, called LoxP sites. Recombination of two LoxP sites in the same orientation leads to excision of the intervening DNA, leaving a single LoxP site behind (Sauer and Henderson 1988). Insertion of the Cre recombinase gene immediately downstream of the IL-17A promoter would lead to expression of Cre recombinase in cells that had activated IL-17A transcription. Crossing the IL-17A_Cre heterozygous mice with a ROSA26_EYPF strain, in which expression of Enhanced Yellow Fluorescence Protein (EYFP) in the constitutively expressed ROSA26 locus is usually prevented by the presence of a LoxP-flanked-transcriptional stop sequence, would lead to Cre recombinase-mediated excision of the transcriptional stop sequence and expression of EYFP.

We chose to insert the Cre recombinase gene so that as little of the IL-17A gene was disrupted as possible. Although there would be no expression of IL-17A from the targeted allele, we did not want to remove any potential regulatory elements that may disrupt expression of IL-17A from the other allele or of any other nearby genes such as IL-17F which is 45kb downstream of IL-17A and has a similar expression pattern. The generation of the IL-17A reporter would also allow us to generate an IL-17A knockout strain.

4.2 Generation of an IL-17A_Cre construct

In order to generate the IL-17A_Cre mice, where Cre Recombinase is expressed under the control of the IL-17A promoter, a targeting vector was first constructed. This vector would briefly consist of a modified Cre Recombinase (iCre) gene inserted immediately downstream of the transcriptional start (the first ATG codon) of the IL-17A gene and would be followed by a LoxP-flanked neomycin resistance gene (*neo*) that would enable ES cells that had taken up the targeting construct to be positively selected.

For the generation of this targeting vector, an iCrefloxneo knock-in cassette was first assembled by ligating a LoxP-flanked neomycin resistance (*neo*) gene downstream of a modified iCre exon with a Simian virus 40 (SV40) intron and SV40 polyA tail to enhance mRNA stability **(Figure 4.1A).** This generated the iCrefloxneo cassette, which was verified by sequencing and also in three analytical digests as shown in **Figure 4.1B**. The *neo* gene would be constitutively expressed by the presence of the upstream PKG (phosphoglycerate kinase) promoter. The PKG promoter is a promoter of the yeast gene encoding phosphoglycerate kinase and is widely used when a high level of constituitive gene expression is required. *Neo* encodes for neomycin phosphotransferase, which inhibits the effects of the aminoglycoside antibiotic, G418 and would therefore allow positive selection of ES cell clones containing the construct. Cre Recombinase is a bacteriophage protein, normally expressed in bacteria. The iCre gene used for this cloning had been previously modified for mammalian codon usage, so that it would be efficiently translated in mouse cells (Shimshek, Kim et al. 2002).

The targeting vector was generated using Bacterial Artificial Chromosome (BAC) DNA from the 129SvEV/AB2.2 mouse strain made at the Wellcome Trust Sanger institute, as a source of genomic DNA. The 5' long arm of homology was generated by PCR amplification of a 4.5kb fragment from the IL-17A BAC clone. Restriction sites were incorporated into the primers used for the PCR reaction, and these were

subsequently used for cloning the PCR fragment into the iCrefloxNeo cassette. The ligation of the 5' long arm of homology in this way resulted in generation of a five amino acid-linker peptide between the first ATG codon of IL-17A and the first ATG codon of iCre (**Figure 4.2A**). An N-terminal peptide addition to iCre has been used successfully in other systems such as in a RAG1_Cre mouse (McCormack, Forster et al. 2003) and was not expected to have any effects on the functionality of the enzyme. The 5' amino acid linker sequence also encompassed a kozak consensus sequence (Kozak 1991) so that translation of Cre recombinase would be enhanced.

For the generation of the 3' short arm of homology, a 5.2kb BamHI fragment of DNA was first isolated from the BAC clone, which encompassed the 3 exons of the IL-17A gene and was cloned into a pBluescript vector (not shown in figure). From this sub-cloned fragment a 2.8kb 3' short arm of homology, downstream of exon 1 and encompassing exon 2 and part of exon 3 was ligated into a pBluescript vector. A double point mutation was introduced into exon 2 by PCR (AT→CA) to remove a potential start codon that may have lead to expression of a truncated form of IL-17A. The 3' short arm of homology was cloned into the 5' long arm of homology_iCrefloxNeo vector in order to complete the targeting vector (**Figure 4.2**). This vector was confirmed by sequencing and by six analytical digests as shown in **Figure 4.2B**.

It was observed that in some plasmid preparations of the iCrefloxNeo cassette or containing the iCrefloxNeo cassette described above (**Figure 4.1**), an unexpected digestion pattern appeared. An example of this was in a HindIII digest of the final construct. This digest was predicted to give a 7.6kb band encompassing the plasmid backbone and 5' long arm of homology, a 3.9kb band encompassing the iCrefloxNeo cassette and a 1.8kb band encompassing part of the 3' short arm of homology. In some large scale plasmid DNA preps the 3.9kb band disappeared or became fainter and there was appearance of a 1.7kb band. These digestion patterns suggested removal of the *neo* gene (**Figure 4.3**), although sequencing both the iCrefloxneo (**Figure 4.1**) and the final construct (**Figure 4.2**) plasmid preps

indicated that the *neo* gene was still present. However, this could be due to the presence of plasmids that had not lost the *neo* fragment in the DNA used for sequencing. We hypothesised that the LoxP sites either side of the *neo* gene could be mediating recombination of the gene and therefore used competent cells lacking the recombination enzymes RecB and RecJ (SURE cells) and competent cells lacking RecA (INV alpha f' cells) for transformation of the plasmid. This reduced, but did not prevent the extent of the recombination seen in the analytical digests. Other possibilities for the loss of the *neo* gene included the expression of Cre recombinase from the plasmid or another endogenous prokaryote recombinase which may have been able to facilitate excision of the *neo* gene via the flanking LoxP sites. Another possibility was the presence and close proximity of the Cre Recombinase and *neo* gene polyA tails, which may have lead to removal of the *neo* gene. Nevertheless, enough targeting vector containing the *neo* gene was obtained by repeated, small scale cultures and this was used for targeting the IL-17A locus in ES cells.

4.3 Targeting the IL-17A locus in ES cells and generation of IL-17A_Cre^{EYFP} reporter mice

The final targeting vector was linearised and electroporated into 129Sv Embryonic Stem (ES) cells. *Neo*-containing clones were positively selected by growth in G418 and correct incorporation of the targeting construct into the IL-17A locus by homologous recombination was verified by Southern blot.

For southern blot analysis, 5' and 3' probes were generated by PCR amplification of the IL-17A BAC DNA. Primers for these PCR reactions were designed with incorporated restriction sites to allow cloning of the probes into commercial vectors. A 537bp external 5' probe hybridised to a 5.8kb band on an EcoRV digest of 129/Sv genomic DNA (**Figure 4.4A**). After incorporation of the targeting construct into the IL-17A locus by homologous recombination this probe was expected to hybridise to a 9kb band (**Figure 4.4A**). This would give a clear distinction of the wild type and

targeted bands on a Southern blot. Three 3' external probes were also tested for hybridisation to an XbaI digest of 129Sv and C57BL/6 genomic DNA, however none of these probes clearly hybridised to a band of the correct size. A 3' probe was predicted to bind to a 10.3kb band on an XbaI digest of wild type DNA and a 6.8kb band on targeted DNA (**Figure 4.4B**).

The IL-17A_Cre construct as generated above did not result in the generation of any ES cells clones which had incorporated the targeting construct by homologous recombination. This was verified by Southern blot analysis using the 5' external probe to analyse a total of 1200 clones in two separate targeting experiments. In order not to compromise my chances to finish the PhD in a timely manner I stopped working on this project at this stage and the design of the targeting vector was re-assessed by someone else. As the targeting vector was highly prone to recombination as described in the previous section it was subsequently modified by insertion of a *neo* gene, flanked by frt sites rather that LoxP sites.

Targeting of this modified construct into ES cells resulted in the generation of two positive clones which were subsequently injected into 3.5 day old blastocysts from C57BL/6 mice. These blastocysts were injected into the uteri of foster mothers and were allowed to develop. This process gave rise to chimeric animals that would have been derived from both the donor stem cells and the host blastocyst. In mice where the targeted ES cells were in the germline, breeding with C57BL/6 wild type animals would give mice that were heterozygous for the IL-17A_Cre allele. These mice were bred with ROSA26 _EYFP mice to derive F1 generation reporter mice.

4.4 Assessment of reporter functionality

EYFP and IL-17A expression were analysed in the F1 generation IL-17A_Cre^{EYFP} reporter mice. In an ideal situation all EYFP expressing cells would be positive for IL-17A expression detected by intra-cellular staining.

EYFP expression and intra-cellular IL-17A expression were first analysed in a non-immunised mouse. The cells were stimulated with PdBu and ionomycin for 4 hours and analysed for IL-17A and EYFP expression. EYFP expression was also analysed in non-stimulated cells to check that *ex vivo* stimulation of the cells had not altered EYFP expression (data not shown). Cells were gated on counted cells, singlets and lymphocytes as shown previously in chapter 3.

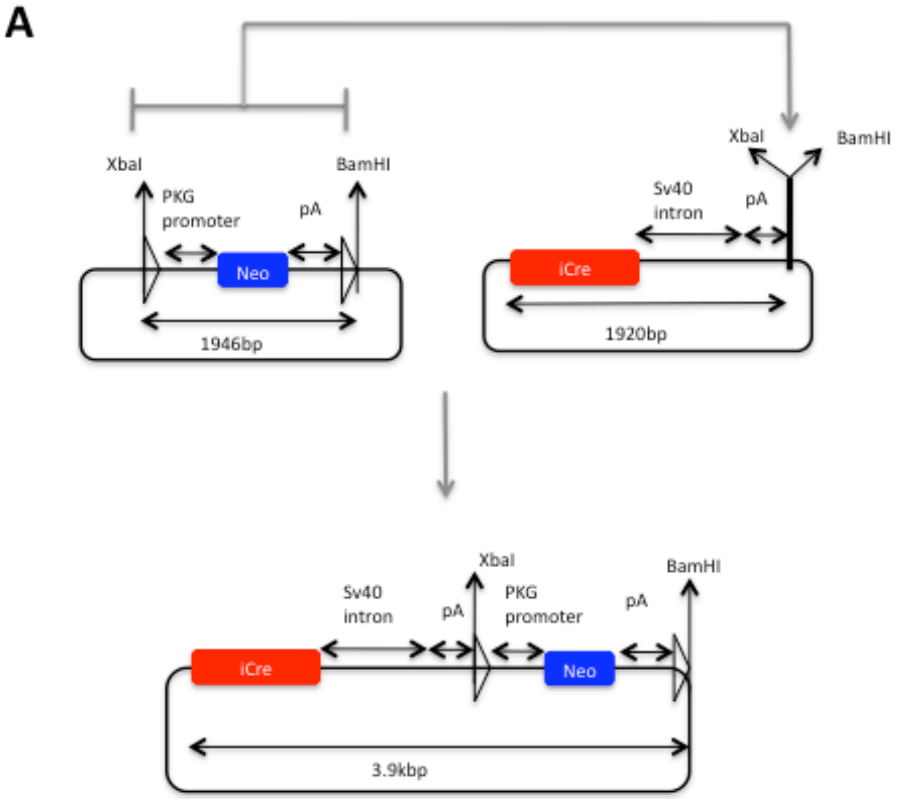
Figure 4.5A shows expression of IL-17A measured by intra-cellular staining versus EYFP expression in CD4 T cells and γδ T cells in the lymph nodes of a non-immunised mouse. Littermate controls have the ROSA26_EYFP locus but no targeted IL-17A locus. The vast majority of EYFP expressing cells expressed IL-17A detected by intra-cellular staining, indicating that EYFP faithfully reported IL-17A expression in non-immunised mice. However, of the IL-17A-expressing CD4 T cells within the lymph nodes measured by intra-cellular cytokine staining, approximately 80% of them were $EYFP^-$ and of the γδ T cells, approximately 50% were $EYFP^-$.

Given the fact that in chapter 3, using a 4 hour stimulation with PdBu and ionomycin and intra-cellular cytokine staining, there was no difference observed in IL-17A expression between mice immunised with MOG CFA alone and those immunised with MOG CFA and pertussis toxin and yet after 24 hour stimulation in the presence of MOG peptide, the pertussis toxin-treated mice had a higher percentage of IL-17A-producers, we wanted to know how EYFP expression correlated with these results. In order to assess this, F1 generation IL-17A_Cre^{EYFP} mice and littermate controls were immunised with MOG CFA into the base of the tail in the presence and absence of pertussis toxin. Mice were sacrificed 7 days later and the lymph nodes were analysed for IL-17A expression by intra-cellular cytokine staining as well as EYFP expression.

The percentage and numbers of CD4 and γδ T cells expressing IL-17A and EYFP are shown in **Figure 4.5B**. In the initial experiments we saw an increase in the percentage of CD4 and γδ T cells expressing IL-17A on day 7 following immunisation in the draining lymph nodes. Interestingly, although the number of CD4 T cells expressing IL-17A as well as the percentage of IL-17A-expressing cells within the CD4 T cell population expanded at day 7 as observed in the original kinetics experiments, this population was predominantly $EYFP^-$. In contrast, like in the original kinetics experiments the number of γδ T cells expressing IL-17A as well as the percentage of IL-17A-expressing cells within the γδ T cell population expanded on day 7 after immunisation, however this population of IL-17A-expressing cells was predominantly $EYFP^+$. There were no obvious differences in the EYFP expression in the lymph nodes of mice treated with and without pertussis toxin, indicating pertussis toxin does not modulate transcriptional activation of IL-17A.

Figure 4.1 Cloning of an iCrefloxneo knock-in cassette.

LoxP-flanked neomycin resistance gene (*neo*) containing a PKG promoter and polyA tail (pA) was cloned immediately down-stream of a modified Cre Recombinase (iCre) exon with a Simian virus 40 (SV40) intron and SV40 polyA tail in a commercial pBluescript vector using *XbaI* and *BamHI* restriction sites. LoxP sites are indicated by open triangles (A). The product was verified by a three digests shown in (B). Digest 1 was a *HindIII* and *BamHI* digest, predicted to give bands at 4.9kb and 1.9kb. Digest 2 was a *PstI* digest, predicted to give bands at 6.8kb and 0.2kb. Digest 3 was a *PvuII* digest, predicted to give bands at 2.5kb, 2kb, 1.4kb and 0.8kb. M=marker. In ascending order the bands on the marker = 1kb, 1.5kb, 2kb, 3kb, 4kb, 5kb, 6kb, 8kb, 10kb.


A
XbaI
BamHI
PKG
promoter
pA
Neo
1946bp
XbaI
BamHI
Sv40
intron
pA
iCre
1920bp
XbaI
Sv40
intron
pA
PKG
promoter
pA
BamHI
iCre
Neo
3.9kbp

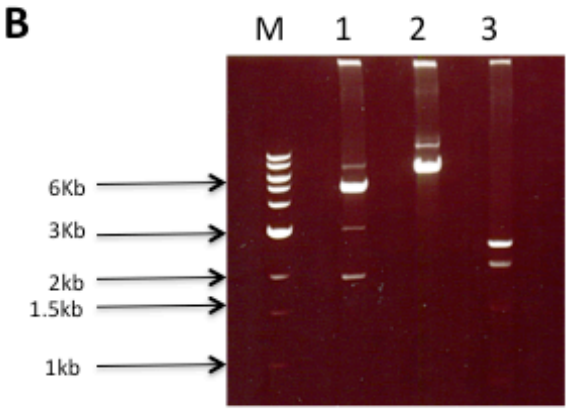
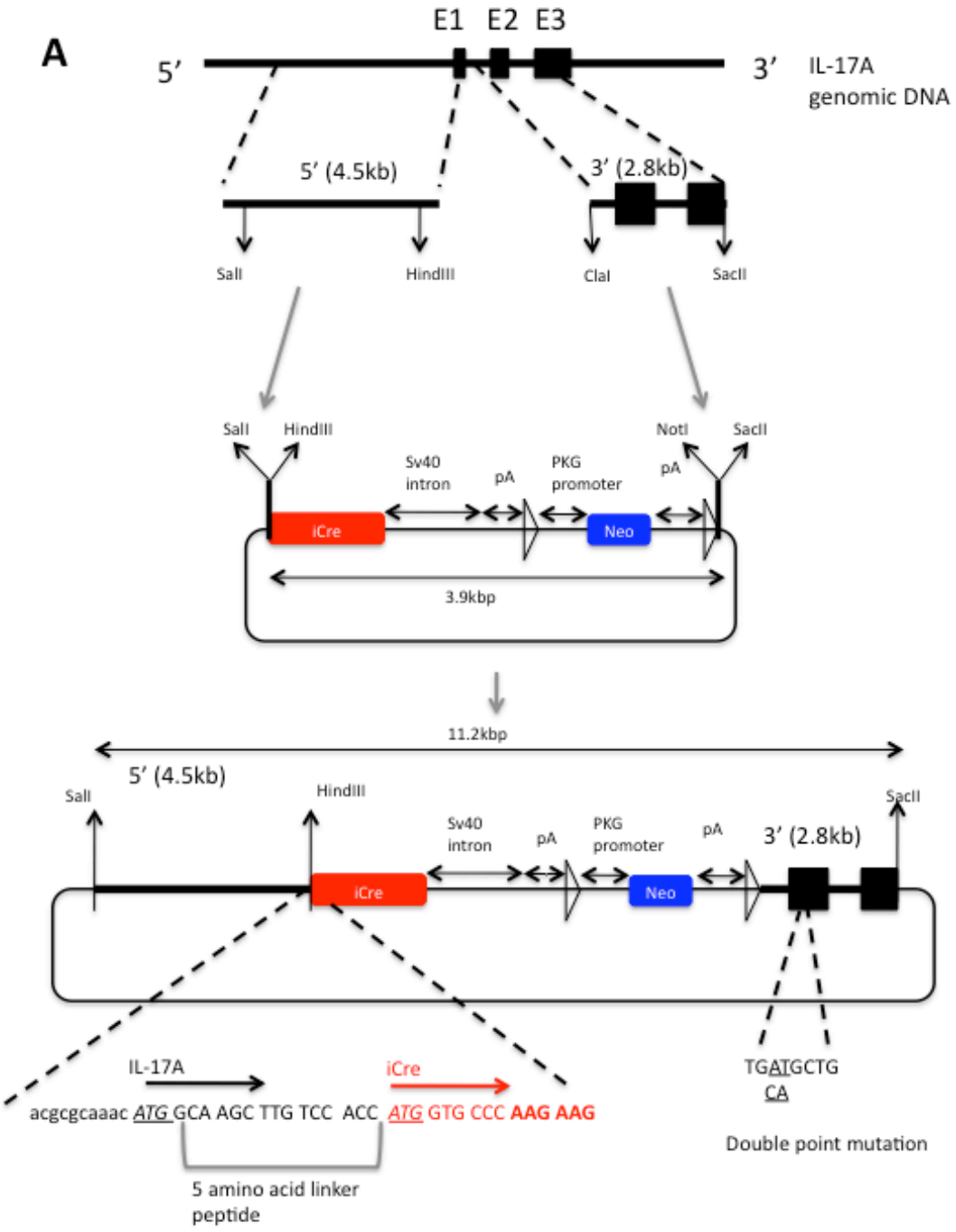

B
M
1
2
3
6Kb
3Kb
2kb
1.5kb
1kb

Figure 4.2 Generation of an IL-17A Cre targeting construct.

The 5' long arm of homology was generated by PCR amplification of a 4.5kb fragment from an IL-17A BAC clone. *SalI* and *HindIII* restriction sites were incorporated into the primers used for the PCR reaction, and these were subsequently used for cloning the PCR fragment into the iCrefloxNeo cassette. The ligation of the 5' long arm of homology in this way resulted in the generation of a five amino acid-linker peptide between the first ATG codon of IL-17A and the first ATG codon of iCre. A 2.8kb 3' short arm of homology, downstream of exon 1 and encompassing exon 2 and part of exon 3 was ligated downstream of *neo* poly A into the 5' long arm of homology_iCrefloxNeo vector using *SacII* and blunted *ClaI* and *NotI* restriction sites. A double point mutation (AT was mutated to CA) was introduced into exon 2 by PCR to remove a potential start codon that may have lead to expression of a truncated form of IL-17A. LoxP sites are indicated by open triangles (A). The final targeting vector was confirmed by six analytical digests as shown in (B). Digest 1= *SalI* digest, predicted to give a 13.9kb band. Digest 2= *SacII*, predicted to give a 13.9kb band. Digest 3= *BamHI*, predicted to give a 10kb band and 3.4kb band. Digest 4 = *HindIII*, predicted to give a 7.6kb band, 3.9kb band, 1.8kb band, 0.3kb band and a 0.09kb band. Digest 5 = *EcoRV*, predicted to give a 11kb band, a 2.2kb band and a 0.6kb band. Digest 6 = *EcoRI*, predicted to give a 9.3kb band and 4.7kb band. UD= undigested. M= molecular weight marker. In ascending order the bands on the marker = 0.5kb, 1kb, 1.5kb, 2kb, 3kb, 4kb, 5kb, 6kb, 8kb, 10kb.


A
E1 E2 E3
5′
3′
IL-17A
genomic DNA
5′ (4.5kb)
3′ (2.8kb)
SalI
HindIII
ClaI
SacII
SalI
HindIII
NotI
SacII
Sv40
intron
pA
PKG
promoter
pA
iCre
Neo
3.9kbp
11.2kbp
5′ (4.5kb)
SalI
HindIII
SacII
Sv40
intron
pA
PKG
promoter
pA
3′ (2.8kb)
iCre
Neo
IL-17A
iCre
acgcgcaaac ATG GCA AGC TTG TCC ACC ATG GTG CCC AAG AAG
5 amino acid linker
peptide
TGATGCTG
CA
Double point mutation

B

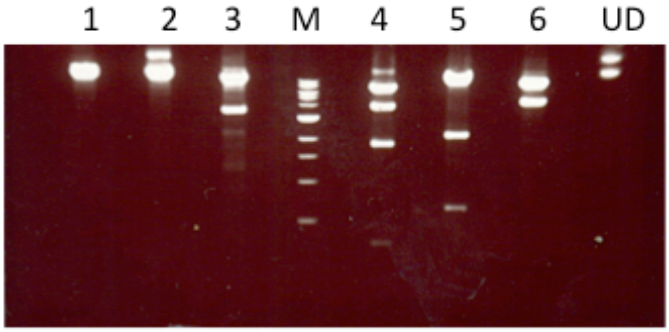


Figure 4.3 The iCrefloxNeo targeting construct was prone to recombination

A *HindIII* digest of the targeting construct was expected to yield a 7.6kb fragment, a 3.9kb fragment and a 1.8kb fragment as illustrated in digest 1. In some large-scale preparations the 3.9kb band was reduced and an additional 1.7kb band appeared which corresponded to the sizes of fragments expected after removal of the LoxP-flanked *neo* gene as illustrated in digest 2. Red rectangle indicates *iCre* exon, blue rectangle indicates *neo* exon and open triangles indicate LoxP sites. M = molecular weight marker.

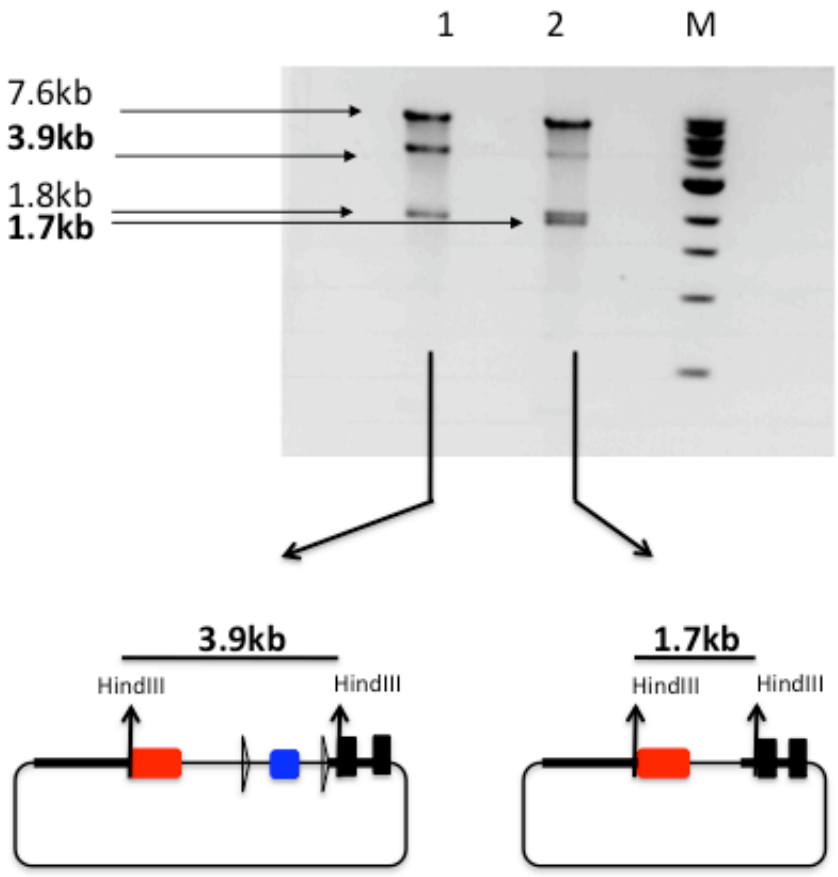

1
2
M
7.6kb
3.9kb
1.8kb
1.7kb
3.9kb
HindIII
HindIII
1.7kb
HindIII
HindIII

Figure 4.4 Southern Blot targeting strategy to detect integration of the targeting construct into the IL-17A locus in ES cells.

A 5' probe was generated by PCR amplification, which hybridised to a 5.8kb *EcoRV* fragment of wild type DNA in a Southern blot, outside the region of the targeting construct. Upon integration of the targeting construct into the IL-17A locus an additional *EcoRV* site would be introduced into the IL-17A locus and the size of the fragment recognised by the 5' probe would be increased to 9kb (A). 3' probes were generated that were predicted to hybridise to a 10.3kb *XbaI* fragment outside the region of the targeting construct. Upon integration of the targeting construct into the IL-17A locus an additional *XbaI* site would be introduced into the locus and the size of the fragment recognised by the 3' probe would be reduced to 6.8kb (B). None of the 3' probes tested, hybridised to genomic wild type DNA in a Southern blot.

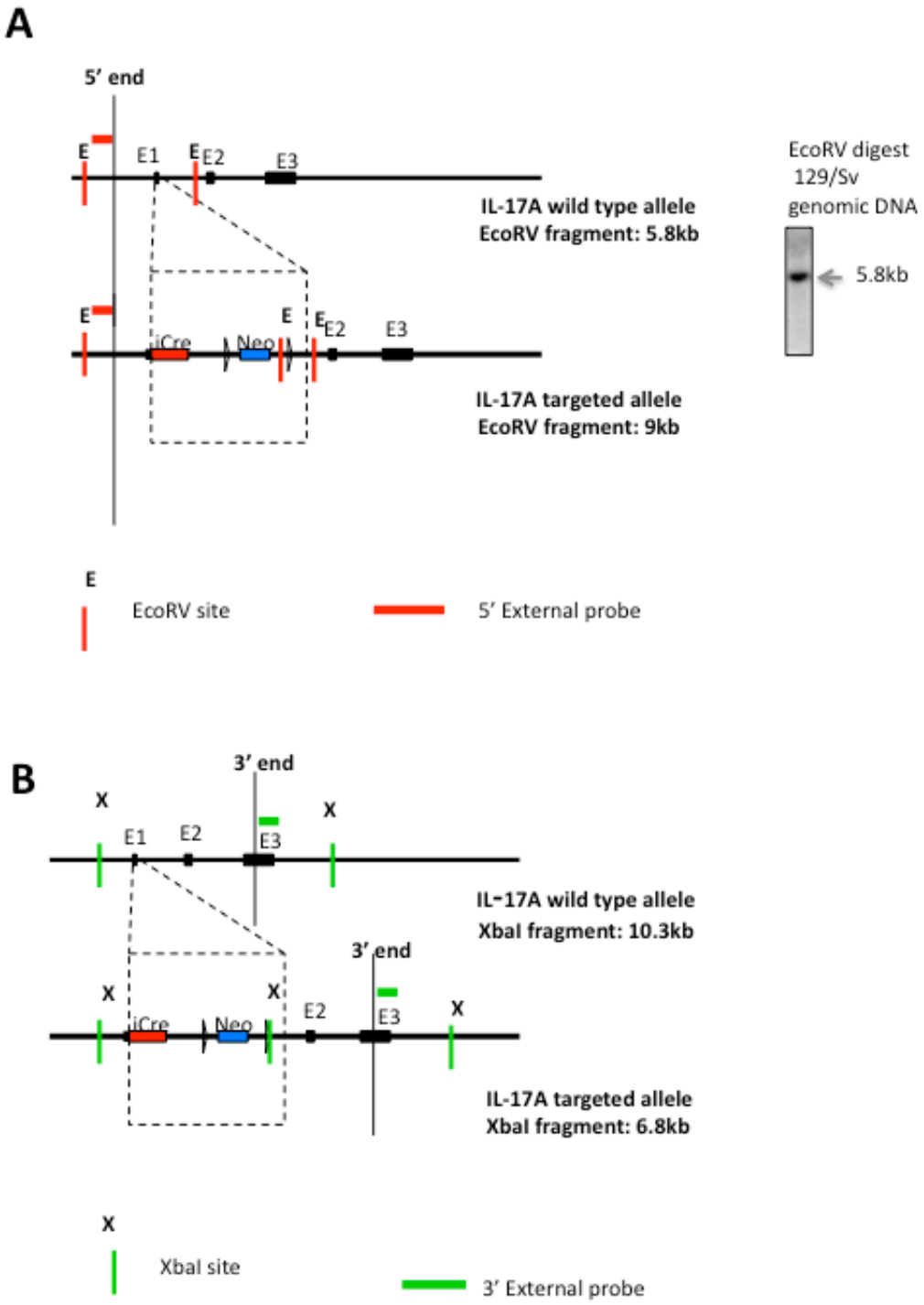
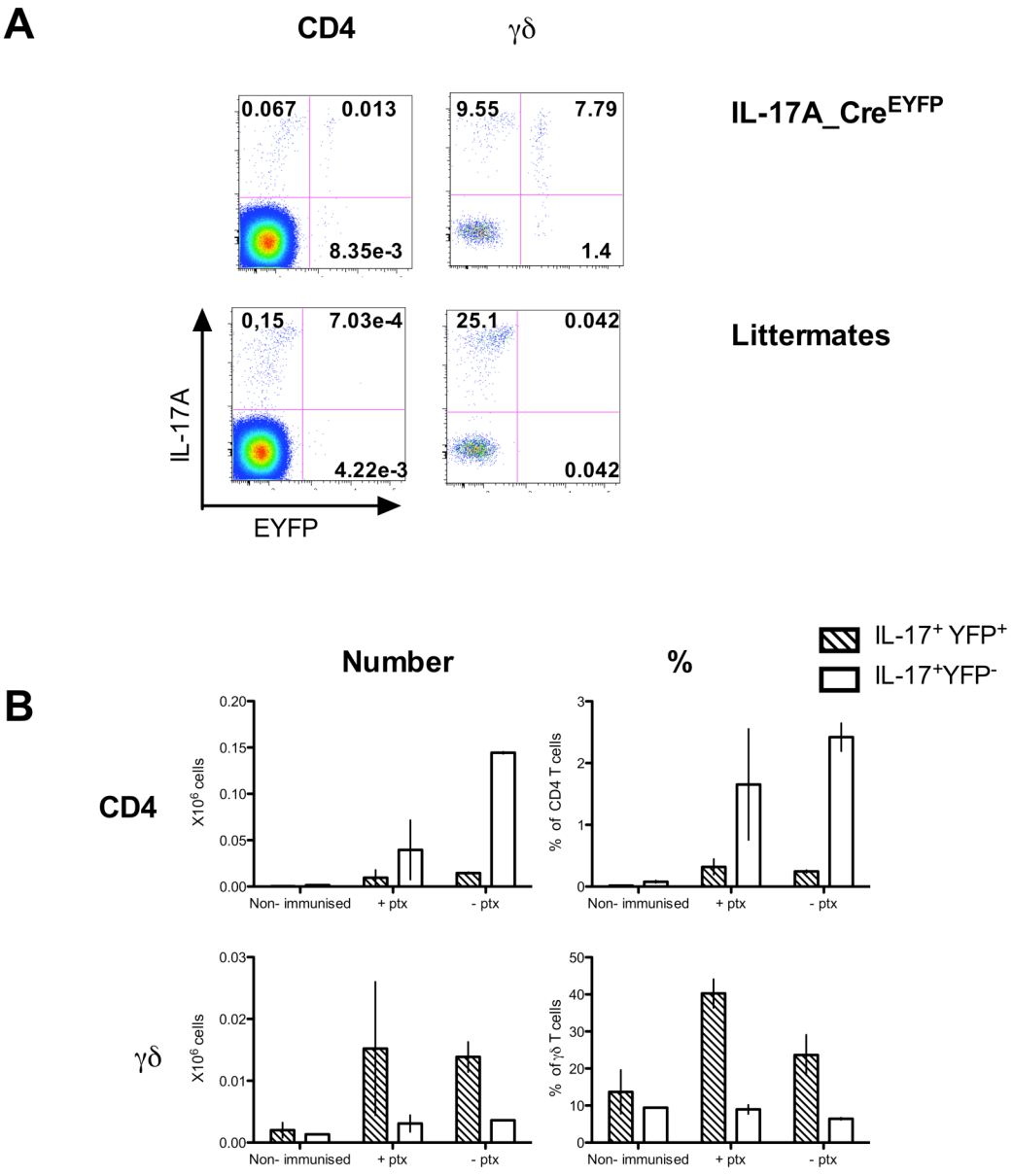

A
5' end
E
E1
E
E2
E3
IL-17A wild type allele
EcoRV fragment: 5.8kb
E
iCre
Neo
E
E
E2
E3
IL-17A targeted allele
EcoRV fragment: 9kb
EcoRV digest
129/Sv
genomic DNA
5.8kb
E
EcoRV site
5' External probe
B
3' end
X
X
E1
E2
E3
IL-17A wild type allele
XbaI fragment: 10.3kb
3' end
X
X
iCre
Neo
E2
E3
X
IL-17A targeted allele
XbaI fragment: 6.8kb
X
XbaI site
3' External probe

Figure 4.5 Assessing the functionality of the reporter system

Lymph nodes were harvested from non-immunised IL-17A_Cre^{EFYP} F1 generation reporter mice and littermate controls containing the ROSA26_EYFP locus but no targeted IL-17A locus and were assessed for EYFP and IL-17A expression following a 4 hour stimulation with PdBu, ionomycin and brefeldin A. Representative flow cytometry plots are shown for staining in CD4 and γδ T cells (A). A mixture of male and female IL-17A_Cre^{EFYP} F1 generation reporter mice and littermate controls mice were immunised with MOG CFA into the base of the tail and injected with and without pertussis toxin at the same time and also two days later. Lymph nodes were harvested 7 days following MOG CFA immunisation and analysed for IL-17A and EYFP expression following a 4 hour stimulation with PdBu, ionomycin and brefeldin A. The number of CD4 and γδ T cells expressing IL-17A and EYFP as well as the percentage of IL-17A and EYFP-expressing cells within the CD4 and γδ T cell population are shown (B) The value shown on the bar chart is the mean and standard deviation of the results from two individual mice from each group


Draining lymph nodes
A
CD4
γδ
0.067
0.013
8.35e-3
9.55
7.79
1.4
IL-17A_Cre^EYFP
0,15
7.03e-4
4.22e-3
25.1
0.042
0.042
Littermates
IL-17A
EYFP
B
Number
%
IL-17+ YFP+
IL-17+YFP-
CD4
γδ
X10^6 cells
% of CD4 T cells
% of γδ T cells
Non- immunised
+ ptx
- ptx

4.5 Discussion

The Cre_LoxP system was employed in order to allow the identification of cells which had activated the IL-17A promoter without the need for intra-cellular cytokine staining. Targeting of ES cells with an IL-17A_CrefloxNeo construct did not result in the generation of any ES clones which had incorporated the gene mutation by homologous recombination. In addition, this targeting vector displayed some instability, possibly due to the LoxP sites surrounding the *neo* gene. Subsequent modification of this original vector by incorporation of frt sites flanking the *neo* gene lead to the successful generation of ES clones which had incorporated the targeting construct into the correct location in the genome and this was confirmed using 3' and 5' external probes in a Southern blot. These clones were injected into developing blastocysts which were implanted into foster mothers to generate chimeric mice. Chimeras were then bred with C57BL/6 wild type mice to determine germ line transmission of the targeted ES cells. Mice that were heterozygous for the targeted IL-17A gene were bred with ROSA26_EYFP mice to generate IL-17A_Cre^{EYFP} mice. In these mice expression of IL-17A transcription should drive the expression of Cre recombinase which should excise a LoxP-flanked transcriptional stop sequence upstream of EYFP in the ubiquitously expressed ROSA26 locus, leading to irreversible expression of EYFP in all cells that had activated IL-17A.

A litter of IL-17A_Cre^{EYFP} F1 generation mice was used to investigate the functionality of the reporter system in steady state conditions and also after immunisation. These mice were a mixture of C57BL/6 and 129 strains and contained the whole targeting construct including the frt-flanked *neo* gene. For future experiments these mice will eventually be backcrossed onto the C57BL/6 background, which is the standard strain used for many experiments. It will also be important to remove the frt-flanked *neo* gene as there have been reports that the presence of positive selection markers can lead to a number of unanticipated effects. For example, the presence of the *neo* gene, often with its own promoter, can alter the

expression of neighbouring loci (Olson, Arnold et al. 1996; Pham, MacIvor et al. 1996). This could be achieved by breeding the mice with a FLP recombinase-expressing mouse strain to initiate FLP recombinase-mediated recombination of the *neo* gene, with subsequent backcrossing onto a wild type background.

Analysis of the F1 reporter mice showed that EYFP expression reliably reported IL-17A expression detected by intra-cellular cytokine staining after a 4 hour stimulation with PdBu, ionomycin and brefeldin A, however not all IL-17A-expressing cells were EYFP positive. Expression of EYFP and IL-17A, measured by intra-cellular cytokine staining were more comparable in γδ T cells where approximately 50% of the IL-17A-expressing cells were EYFP positive, versus only about 20% in CD4 T cells. Following immunisation with MOG CFA in the presence or absence of pertussis toxin, the number of IL-17A-expressing CD4 and γδ T cells increased as well as the percentage of IL-17A-expressing cells within each of these populations, as determined in chapter 3. However in the γδ T cell population, the IL-17A-expressing cells that increased following immunisation were predominantly $EYFP^+$ whereas in the CD4 T cell population the IL-17A-expressing cells were mostly $EYFP^-$. This confirmed the fact that EYFP more reliably reports expression of IL-17A in γδ T cells.

There are a number of reasons why EYFP expression and IL-17A expression may not reflect each other. It is possible that EYFP may leak out of cells upon permeabilisation, leading to absence of EYFP in IL-17A-expressing cells, however it is clear that this does not occur to the same extent in CD4 T cells and γδ T cells suggesting that either EYFP leaks more readily out of CD4 T cells or perhaps that there is another reason. It is also possible that differential expression between EYFP and IL-17A may be due to the ability of the IL-17A promoter to drive the expression of Cre recombinase. It may be that the induction of IL-17A and therefore Cre recombinase transcription by the IL-17A promoter in CD4 T cells and γδ T cells is different and that in γδ T cells the IL-17A promoter more readily drives Cre expression. Furthermore, it could be that there is a certain degree of monoallelic

expression, and that in CD4 T cells in particular, the IL-17A promoter induces IL-17A transcription from the intact allele but not expression of Cre recombinase on the targeted allele. This however, might be expected to also work in the opposite way, so that in some cases the IL-17A promoter induces Cre recombinase transcription from the targeted allele without the expression of IL-17A from the intact allele, however the expression of such $EYFP^{+}IL\text{-}17A^{-}$ cells were not detected. Differential expression of IL-17A and EYFP could also be due to a delay in expression of EYFP relative to expression of IL-17A, which is perhaps more delayed in CD4 T cells than γδ T cells. One group generated an IL-17F transgenic reporter using a similar Cre_LoxP system (Croxford, Kurschus et al. 2009) and also found a proportion of IL-17F-expressing cells that did not express EYFP, which they attributed to a delay in the expression of EYFP relative to IL-17F, although they did not confirm this. This could partly be assessed in the IL-17A_Cre^{EYFP} mice using *in vitro* analysis of EYFP expression after stimulation of CD4 T cells with Th17-polarising cytokines in order to investigate whether CD4 T cells do eventually express the reporter gene.

The experiments in chapter 3 established that IL-17A-expressing CD4 and γδ T cells increased in number and the percentage of IL-17A-expessing cells within these populations also increased upon immunisation with MOG CFA. However from those results it was not possible to say whether the increase in these populations was due to an increase in recruitment, proliferation, survival or *de novo* generation. One way to address whether the increase in these populations was the result of *de novo* generation would be to transfer $EYFP^{-}$ cells from the reporter mice into wild type hosts and investigate whether these cells up-regulate EYFP upon immunisation. Up-regulation of EYFP in this experiment could be attributed to *de novo* generation of IL-17A-expressing cells *in vivo* in response to MOG CFA immunisation. The experiments in chapter 3 also established that pertussis toxin was enhancing MOG-specific IL-17A (and IFN-γ) responses in the periphery. This could be the result of an enhanced number of MOG-specific CD4 T cells in the pertussis toxin-treated mice or an enhanced ability of MOG-specific cells to produce IL-17A. In order to investigate this, MOG tetramer staining could be used in combination with

intracellular cytokine staining after both MOG and PdBu and ionomycin stimulation and also using the reporter mice. It would be interesting to investigate whether the MOG-specific CD4 cells respond differently to PdBu and ionomycin staining and MOG stimulation. It could be that PdBu and ionomycin stimulation is reflective of transcriptional expression of IL-17A staining, something which should correlate with the expression of the EYFP in the reporter mice, but that MOG stimulation could highlight post-transcriptional expression of the cytokine, something that may be different in mice treated with and without pertussis toxin.

The experiments in chapter 3 also showed that immunisation with MOG CFA induced double producing IL-17A and IFN-γ-expressing CD4 T cells that were detected in the blood and spinal cord. This indicates that there is a certain degree of plasticity between the Th1 and Th17 cell lineages. Experiments *in vitro* using an IFN-γ reporter mouse, have shown that Th17 cells can be induced without opening the IFN-γ locus (Veldhoen, Hocking et al. 2006), however it will be of interest to check using the IL-17A_Cre^{EYFP} mice, whether IFNγ-expressing cells in both *in vitro* and *in vivo* settings, activate IL-17A expression.

It should be considered when analysing the IL-17A_Cre^{EYFP} mice that these mice are heterozygous for the Cre insertion and only have IL-17A expression from one allele. Northern blot analysis of the IL-17A knockout mice generated by Nakae et al (Nakae, Komiyama et al. 2002) showed reduced IL-17A mRNA in the mice heterozygous for the knockout indicating that the deletion of one allele does result in reduced transcription of IL-17A. However, these mice showed a phenotype comparable with wild type mice and so it is expected that the heterozygous IL-17A_Cre^{EYFP} mice will be not phenotypically different from wild type mice. It will also be of interest to investigate the IL-17F expression in these mice compared to the littermate controls with an intact IL-17 locus. Nakae et al did not show any data on the expression of IL-17F from their knockout, however the close proximity of the IL-17A and IL-17F genes on chromosome 1 and the presence of non-coding conserved sequences within their locus suggest that they share regulatory sequences

and that interference with the IL-17A gene may affect the expression of the IL-17F gene in some way.

The use of cytokine reporter mice is a recent approach whereby expression of a cytokine can be tracked *in vivo* by the expression of a fluorescent reporter gene. This method allows detection of cytokine expression on a single cell basis without the need for intracellular cytokine staining which kills the cells via fixation and involves *in vitro* stimulation with a non-physiological stimulus. Cytokine-expressing cells can also be visualised on mouse sections using fluorescent microscopy, giving information about the *in vivo* localisation and interactions of these cells (Stetson, Mohrs et al. 2003). The design of cytokine reporter mice must be taken into consideration when interpreting observations. For example, many of the fluorescence genes used such as Enhanced Green Fluorescent Protein (EGFP) or Enhanced Yellow Fluorescent Protein (EYFP) have optimized kozak sequences (Kozak 1991) surrounding their start codons to ensure that after generation of their transcript, the translation of these mRNAs is efficient. However, the process of translation itself is one that may be critically regulated in the expression of the cytokine gene and so although expression of the fluorescent gene may be representative of transcription it does not reflect secretion of the protein. In the IL-17A_Cre^{EYFP}mice, Cre recombinase has a kozak sequence surrounding the start codon which will enhance translation of the enzyme. The translation of Cre recombinase and IL-17A should therefore be disassociated when analysing the mouse. Another important factor to consider is incorporation of the knock-in gene in a way that is least likely to affect any potentially regulating elements within the target locus. Incorporation of the reporter gene directly into the coding region of the target gene, under the control of its transcriptional promoter is one way of generating a reporter system which was used in the generation of the IL-17A_Cre^{EYFP} mice, however this effectively knocks out the target gene on one allele and the effects of any deleted cytokine must be taken into consideration. One way of avoiding deletion of one of the alleles in such a knock-in system is by generation of a bicistronic mRNA via use of an IRES (internal ribosomal entry site) element. IRES-

mediated translation involves initiation factors distinct from those required for cap-mediated translation and allows initiation of translation in the middle of a mRNA sequence. The IL-4, IFNγ and IL-10 reporter mice (Mohrs, Shinkai et al. 2001; Stetson, Mohrs et al. 2003; Kamanaka, Kim et al. 2006) all exploited this technology by incorporation of an IRES element within the bicistronic mRNA allowing translation of the reporter gene even after the translational stop codon of the target gene. The desired result is that transcription of the target gene leads to transcription and translation of the reporter gene and there is no deletion of cytokine expression. However, it has to be considered that this method involves insertion of a non-mammalian DNA sequence upstream of the polyA tail of a gene which is still expressed, therefore potentially disrupting the regulation of the gene.

The IL-17A reporter mouse will be an invaluable tool, allowing the purification of a live population of cells which have activated IL-17A transcription, via FACS sorting the EYFP-expressing cells. These cells can be stained for extracellular markers to determine the phenotypes of cells expressing IL-17A and used for quantitative PCR analysis to determine expression of other genes such as transcription factors. It will be of interest to investigate the location in which IL-17A expression is induced as well as the migration and lifespan of such cells in these immune responses. Despite the concentrated study of IL-17A expression in CD4 and γδ T cells, the reporter can also be used to assess IL-17A expression in other cells such as CD8 and NK cells and the role of these cells in infection and autoimmune settings can be investigated.

Chapter 5: Final discussion

Autoimmunity occurs as a result of a specific adaptive immune response against self-peptide. In order to further understand the mechanisms that can result in autoimmunity, the aims of this thesis were to investigate the sequence of immunological events that lead to the development of an autoimmune response and to generate and characterise a reporter mouse for IL-17A.

In order to generate an IL-17A transcriptional reporter mouse, an IL-17A_Cre recombinase strain was first generated where Cre recombinase expression is driven by the IL-17A promoter. This mouse was then crossed with a ROSA-26_EYFP strain, in which expression of EYFP is usually inhibited by the presence of an upstream transcriptional stop sequence. Expression of Cre recombinase in cells that have activated IL-17A leads to deletion of this transcriptional stop sequence, which is flanked by two LoxP sites, leading to irreversible expression of EYFP. Analysis of the first generation of reporter mice indicated that expression of EYFP reliably reports IL-17A protein expression, measured after PdBu and ionomycin stimulation, however there are a proportion of cells that express intra-cellular IL-17A protein without expressing EYFP, and this proportion is higher in CD4 T cells than γδ T cells. There could be numerous reasons for these observations which need to be investigated further, but one of the reasons could be that the IL-17A promoter is less able to drive Cre recombinase expression in CD4 T cells than in γδ T cells, indicating differences in the way IL-17A may be expressed in different cell types.

The sequence of immunological events leading to development of clinical EAE was assessed. It was shown that following EAE induction, IL-17A-expressing cells were increased in frequency within the CD4 and γδ T cell populations in the draining lymph nodes, with a simultaneous increase in the number of these cell populations in the blood. Single IFN-γ-expressing cells within the CD4 T cell population did not increase in frequency within the time-frame assessed in the draining lymph nodes,

however there was an increase in the number of CD4 T cells expressing both IL-17A and IFN-γ in the lymph nodes and blood. Disease was associated with the appearance of IL-17A and IFN-γ-expressing CD4 T cells, IL-17A-expressing γδ T cells, as well as monocytes and neutrophils in the spinal cord. Although originally believed to be mediated by Th1 effector cells, IL-17-producing Th17 cells have been implicated as being important in the development of EAE after studies in which molecules involved in the generation of the two lineages are genetically knocked out, or depleted with depleting antibody. However, there are contrasting reports about the capacity of both Th1 and Th17 polarised cells to generate disease in adoptive transfer experiments. The IL-17A reporter mice could be used to further investigate the generation and plasticity of the IL-17A-expressing cells in EAE. For example, to determine the origin of the double-producing IL-17A/IFN-γ-expressing CD4 T cells.

In EAE, signals induced by MOG peptide, CFA and pertussis toxin facilitate the breakdown of peripheral immune tolerance to MOG peptide and allow the generation of encephalitogenic MOG-specific effector T cells capable of inducing a pathogenic immune response in the CNS. Effector T cells exert their effects in part by the cytokines they express. Furthermore, in this study it was shown that signals mediated by pertussis toxin in combination with MOG CFA immunisation could enhance expression of MOG-specific effector cytokines (IL-17A and IFN-γ), when compared to immunisation with MOG CFA alone. This complements other findings, which have also observed that pertussis toxin can enhance auto-antigen specific cytokine production (Caspi, Silver et al. 1996; Jee and Matsumoto 2001; Hofstetter, Shive et al. 2002; Hofstetter, Grau et al. 2007). Interestingly, in this study, this effect was masked when cells were stimulated with PdBu and ionomycin instead of MOG peptide. Enhancement of MOG-specific cytokine expression in CD4 T cells from mice treated with pertussis toxin could be due to an enhancement in the number of MOG-specific CD4 T cells in the pertussis toxin-treated mice. As MOG-specific cells are only a small percentage of the total repertoire of T cells, any difference may not be detected by stimulation with PdBu and ionomycin, which

stimulates every cell regardless of specificity. Alternatively, it could be that MOG-specific CD4 T cells express cytokine in response to MOG stimulation after immunisation with MOG CFA and pertussis toxin, but do not if immunised with MOG CFA only. If the latter is the case, this could be due to an effect of pertussis toxin on post-transcriptional expression of the cytokines, for example in stabilising cytokine mRNA or enhancing storage or secretion of the proteins. The IL-17A reporter mouse was used to analyse the effect of pertussis toxin on EYFP expression. Expression of EYFP in CD4 T cells was low compared to the total IL-17A measured by intra-cellular cytokine staining, however there was no observed difference in EYFP expression in CD4 T cells from the periphery of mice immunised in the presence and absence of pertussis toxin, suggesting that pertusss toxin was not having any effect on transcriptional activation of IL-17A in CD4 T cells.

In summary, it was established that following EAE induction, IL-17A-expressing cells were increased in frequency within the CD4 and γδ T cell populations in the draining lymph nodes, with a simultaneous increase in the number of these populations in the blood. Disease development was associated with administration of pertussis toxin and the appearance of IL-17A and IFN-γ-expressing CD4 T cells, as well as IL-17A-expressing γδ T cells in the spinal cord. It was found that pertusssis toxin enhanced antigen-specific IL-17A and IFN-γ production in the periphery. The IL-17A reporter mouse will be a useful tool to investigate IL-17A expression in EAE further.

Activated, effector CD4 T cells are central to the adaptive immune response and are important players in many models of autoimmune disease. The specific requirements for what makes a CD4 T cell pathogenic or not, are still unknown. Expression of effector cytokines at a transcriptional level, which in EAE, may be induced through signals from *Mycobacterium tuberculosis* may not always reflect translation and secretion of these cytokines *in vivo* in response to specific antigen. Moreover, immunisation with CFA may be sufficient to upregulate transcriptional expression of Th1 and Th17 cytokines in the context of EAE, but may not be

sufficient to allow cells to actually translate and secrete protein in response to MOG peptide, something which may be enhanced by the effects of pertussis toxin. Further understanding of the characteristics of pathogenic CD4 T cells in EAE, as well as the signals required to generate them is important in our understanding of how autoimmune responses arise.

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