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The effect of tolerogenic peptide administration on pathogenic antigen-experienced T cells

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Declaration

I declare that this thesis has been composed by myself, describes my own work and has not been submitted in any other application for a higher degree.

The experiments described in this thesis involving the use of BrdU were done in collaboration with Dr Richard O'Connor.

Rhoanne Catherine McPherson
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Abstract

The administration of soluble antigenic peptides is known to be effective at inducing tolerance in naïve antigen-reactive $CD4^+$ T cells. This observation forms the basis of antigen-based therapy, which offers the potential to specifically target the auto-reactive $CD4^+$ T cells involved in driving autoimmune disease pathogenesis, whilst leaving the rest of the immune system intact.

The prophylactic administration of soluble autoantigen-derived peptides has proven to be effective at inhibiting disease induction in various experimental models of autoimmune disease. However, the clinical requirement is to switch off the activated antigen-experienced $CD4^+$ T cells that are present during an ongoing immune response. The effect of soluble peptide administration of antigen-experienced $CD4^+$ T cells is poorly understood, and several clinical trials using peptides in multiple sclerosis patients had to be halted due to the exacerbation of disease. This thesis characterises the effect of soluble peptide administration on pathogenic antigen-experienced $CD4^+$ T cells, using experimental autoimmune encephalomyelitis (EAE) as a model of autoimmune disease of the central nervous system.

Using traceable myelin-reactive T cells from Tg4 mice, it was determined that soluble peptide administration induces substantial expansion of antigen-experienced $CD4^+$ T cells. Despite the increase in number, these cells were no longer able to induce EAE. Production of effector cytokine was significantly decreased in peptide treated antigen-reactive $CD4^+$ T cells, and this correlated with high level expression of the co-inhibitory molecule PD-1. The induction of tolerance in both naïve and antigen-experienced $CD4^+$ T cells was found to be dependent upon PD-1 expression, whereby peptide treatment of naïve and antigen-experienced $CD4^+$ T cells that were deficient in PD-1, did not inhibit disease induction.

This thesis identifies a novel mechanism of peptide-induced tolerance in $CD4^+$ T cells, and demonstrates that soluble peptide administration can induce tolerance in antigen-experienced T cells.

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List of abbreviations

AIRE	Autoimmune regulator
APC	Antigen presenting cell
APL	Altered-peptide ligand
BBB	Blood-brain barrier
CFA	Complete Freund's adjuvant
CFSE	Carboxyfluorescein succinimidyl ester
CLTA-4	Cytotoxic T lymphocyte antigen 4
CNS	Central nervous system
CPM	Counts per minute
cTEC	Cortical thymic epithelial cell
DN	Double negative
DP	Double positive
EAE	Experimental autoimmune encephalomyelitis
EBV	Epstein-Barr virus
ECDI	ethylene carbodiimide
ELISA	Emzyme-linked immunosorbant assay
FACS	Fluorescence-activated cell sorting
FCS	Fetal calf serum
GM-CSF	Granulocyte-macrophage colony stimulating factor
GWAS	Genome-wide association studies
HEV	High endothelial venules
HHV-6	Human Herpes virus-6
HLA	Human leukocyte antigen
i.p.	Intraperitoneal
i.v.	Intravenous
ICS	Intracellular cytokine staining
IFN-γ	Interferon-γ
IL-	Interleukin-
IPEX	Immunodysregulation polyendocrinopathy enteropathy X-linked syndrome
ITAM	Immunoreceptor tyrosine-based activation motif
iTreg	Induced regulatory T cell
ITSM	immunoreceptor tyrosine-based switch motif
LN	Lymph node
MBP	Myelin basic protein
MHC	Major histocompatability
MOG	Myelin oligodendrocyte glycoprotein
MS	Multiple sclerosis
MTB	Mycobacerium tuberculosis
mTEC	Medullary thymic epithelial cell
NOD	Non-obese diabetic
nTreg	Natural regulatory T cell
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD-1	Programmed death-1 receptor
PDL-1/2	Programmed cell death ligand-1/2
PFA	Paraformaldehyde
PMA	Phorbol myristate acetate

pMHC	Peptide:MHC complex
PML	Progressive multifocal leukoencephalopathy
PPMS	Primary progressive MS
PRR	Pattern recognition receptor
PTK	Protein tyrosine kinase
Ptx	Pertussis toxin
RA	Rheumatoid arthritis
RAG-1/2	Recombinase activating gene-1/2
RRMS	Relapsing-remitting MS
s.c.	Sub-cutaneous
SP	Single positive
SPMS	Secondary-progressive MS
T1D	Type-1 diabetes
TCR	T-cell receptor
Teff	Effector T cell
TGF-β	Transforming growth factor β
Th	T helper
TNF-α	Tumour necrosis factor-α
Treg	Regulatory T cells
TSA	Tissue-specific antigen
VLA-4	Very late antigen-4
WT	Wild-type

1. Introduction

Context

The immune system has evolved to protect the body from invasion by a host of diverse pathogens, including bacteria, viruses, fungi and parasites. The immune system harnesses several mechanisms by which to protect from infectious challenge, including physical barriers and the innate and adaptive immune components. The innate and adaptive immune systems are tasked with the challenge of discriminating between foreign and self in order to mount an appropriate defensive response. Although many safety mechanisms exist to ensure inappropriate immune responses to self do not occur, in situations where these are circumvented the onset of autoimmune disease can result.

The innate immune system is one of the first lines of defence, and as such possesses a detection system to recognise harmful pathogens. Cells of the innate immune system can express pattern-recognition receptors (PRR) that enable them to detect pathogen-associated molecule patterns (PAMPs) expressed on harmful invading organisms. Upon detection of infectious agents these cells raise the alarm, and activate cells of the adaptive immune response. The adaptive immune response involves T cells, which add specificity to the response and can instruct cells of the innate immune system. T cells recognise antigenic peptides presented by specialised innate immune cells through their T cell receptor (TCR). This can recognise peptides up to 20 amino acid residues in length, and therefore there are postulated to be 10^{15} different antigenic peptides that the TCR repertoire needs to be able to recognise (Wooldridge et al., 2012). Despite this, there are only thought to be a pool of 10^{8} T cells in humans, therefore a certain degree of degeneracy needs to exist whereby one TCR can recognise more than one peptide. Although highly efficient, this characteristic increases the potential for T cells to recognise self-peptides. As such, there are several mechanisms are in place that act to prevent the activation of self-reactive T cells; these include the processes of central and peripheral tolerance. It is the breakdown of these mechanisms that is thought to be

associated with the onset of autoimmune disease. Currently, the mainstay of therapeutics for autoimmune disease involves non-specific immunosuppression, which leaves individuals immuno-compromised. As such, therapeutic approaches that specifically act to switch-off self-reactive T cells and re-instate tolerance to self-antigens, offer a more targeted method of treating autoimmune disease.

In this thesis the ability to switch-off autoimmune pathology by specifically targeting the cells driving the disease is investigated.

1.1 T cell biology

1.1.1 T cell development

There are two distinct T cell lineages; the alpha beta (αβ) and gamnmadelta (γδ) T cells. The αβ T cell lineage represent the majority of the T cell repertoire and are generated in the thymus. T cell precursors originate in the bone marrow and migrate into the thymus where they develop into mature T cells. T cell progenitors, which are $CD3^-CD4^-CD8^-$ double negative thymocytes (DN), enter the thymus at the cotico-medullary junction through high-endothelial venules and they migrate to the subcaspular region of the cortex (Lind et al., 2001). During the migration from the cortico-medullary junction to the subcapsular region, thymocytes receive signals from cortical thymic epithelial cells (cTEC's) which act to instigate the initial differentiation stages. These differentiation states are classified as DN1, DN2 and DN3 and are characterised by different surface marker expression of CD44 and CD25 (Godfrey et al., 1993). During this process there is rearrangement of the genes which encode the TCRβ chain through a process called VDJ recombination, which is controlled by the enzymes recombination activating genes 1 & 2 (RAG-1 and RAG-2) (Mombaerts et al., 1992, Shinkai et al., 1992). In order for developing thymocytes to transition from the DN3 to DN4 stage the TCRβ chain must be assembled into the pre-TCR complex which consists of the TCRβ chain, pre-TCRα (pTα) chain and CD3 components (Wolfer et al., 2002). The ability of these cells to

receive signalling through the pre-TCR, in addition to NOTCH signalling, enables their differentiation into $CD4^+CD8^+$ double positive (DP) cells (Huang et al., 2003, Ciofani et al., 2004). Cells that do not express a functional pre-TCR complex do not survive the transition from DN3 to DP cells (Fehling et al., 1995, Falk et al., 2001, Hathcock et al., 2011); this overall process is called β selection. DP thymocytes can subsequently interact with cTEC's which express high levels of major histompatability complex (MHC I and MHC II) molecules associated with self-peptides (Bousso et al., 2002). Cells with TCRs that interact with a high avidity for self-peptide MHC complexes are deleted through apoptosis (a process termed negative selection)(Takahama et al., 2010), whereas cells that do not engage with peptide:MHC (pMHC) complexes die through neglect (von Boehmer et al., 1989, Chung et al., 2002). Only cells that interact with pMHC complexes with a weak affinity receive the necessary signals for survival, this process is termed positive selection (Jameson et al., 1995). DP thymocytes that form an interaction with peptide bound to MHC I mature into $CD8^+$ single positive cells (SP), whereas cells that interact with pMHC II complexes mature into $CD4^+$ SP cells (Germain, 2002). These SP cells subsequently relocate from the cortical region into the medulla where they encounter tissue-restricted self-antigens presented by medullary TEC's (mTEC) and other antigen presenting cells (APCs) such as dendritic cells (DC) (Nitta et al., 2009, Koble and Kyewski, 2009). The interaction of DP T cells with mTEC and APCs enables deletion of remaining self-reactive thymocytes that have escaped negative selection in the cortex (Douek et al., 1996). Once SP cells have passed this process and are fully matured, they exit the thymus where they enter either the blood or the lymphatic system.

The deletion of self-reactive thymocytes in both the cortex and the medulla of the thymus forms the basis of central tolerance. Through this process it is thought that the majority of highly self-reactive T cells are removed before they enter the periphery.

1.1.2 $CD4^+$ T cell activation

Bretscher and Cohen were the first to propose a two-signal model of B lymphocyte activation (Bretscher and Cohn, 1970). Similarly, a two-signal model of T cell activation was later proposed (Lafferty and Cunningham, 1975). More recent studies have revealed the requirement for additional signals in T cell activation, and have led to the development of a four signal model.

1.1.2.1 Signal 0

Innate immune cells with the capacity to process and present antigen to T cells in the context of an MHC molecule are called antigen presenting cells (APC). These include macrophages and DC (Poulter, 1983). DC are the most efficient cell type at inducing T cell activation, and have therefore been termed 'professional' APC (Guery and Adorini, 1995). These cells reside within the peripheral tissues and upon encounter with pathogen they capture antigen and migrate to the draining lymph nodes (Guermonprez et al., 2002). Antigen capture can occur through pinocytosis, endocytosis or phagocytosis and is followed by the processing and presentation of antigen in the context of an MHC molecule (Burgdorf et al., 2007).

Although antigen can be presented by immature DC (in the steady state), this does not provide T cells with the full compliment of signals required for effective T cell activation, and rather can result in the induction of T cell tolerance to antigen (Miller et al., 2007). Signal zero refers to the pathogenic stimulus required to induce maturation of the APC, whereby the presence of danger signals or PAMPs induces the up-regulation of pMHC and co-stimulatory molecules by the APC (Maddur et al., 2010). Only mature APC have the necessary equipment to drive the activation of T cells. Therefore, naïve T cell activation first requires the activation of the innate immune system.

1.1.2.2 Signal 1

The primary signal that T cells require for activation is the recognition of antigen bound to the MHC of APC by the TCR. Naïve $CD4^+$ T cells circulate the body and transit between lymphoid organs via the lymphatic and circulatory systems (Berard and Tough, 2002). T cells enter the lymph nodes (LN) through high endothelial venules (HEV) and make contact with APC residing there. Contact between APC and T cells is facilitated by the adhesion molecules LFA-1 and ICAM-1 (Friedl and Gunzer, 2001), enabling the T cell to sample peptide presented by MHC molecules on the surface of the APC. If the TCR does not recognise the pMHC complex, contact is broken between the T cell and APC and the T cell leaves the LN through the efferent lymphatics (Catron et al., 2004). However, if the αβ subunit of the TCR does recognise the pMHC complex, cell contact is sustained, an immunological synapse is formed and signalling through the TCR ensues. The TCR complex is designed so that the αβ subunits are for antigen recognition and the associated CD3 ε, γ, δ and ζ subunits are responsible for signal transduction (Love and Hayes, 2010).

Binding of the TCR to pMHC results in the phosphorylation of immune-receptor tyrosine-based activation motifs (ITAMs) located within the cytoplasmic tails of the CD3 subunits (Weiss, 1993). Phosphorylation of these ITAMs is conducted by Src-family protein tyrosine kinases (PTK) such as Fyn and Lck, and subsequently enables the binding of Zap70 to the phosphorylated motifs (Maltzman and Koretzky, 2008). Lck associated with the TCR co-receptor (CD4) can then activate Zap70, which in turn can lead to activation of multiple signalling pathways such as the PI3K-Akt pathway. Downstream mediators of TCR signalling include the transcription factors NF-κB, NFAT, ERK and JNK, which facilitate functions such as activation, proliferation and apoptosis (Coudronniere et al., 2000) (Diehn et al., 2002, Su et al., 1994)

1.1.2.3 Signal 2

The suggestion that signalling through the TCR alone is insufficient to drive full T cell activation was first proposed by Lafferty and Cunningham. They proposed a two-signal model of T cell activation whereby in addition to TCR signalling a secondary "inductive stimulus" is required (Lafferty and Cunningham, 1975). This signal 2 acts in conjunction with the TCR signal and is also known as 'co-stimulation'.

The best characterised co-stimulatory molecule, CD28, is constitutively expressed by $CD4^+$ T cells and is engaged upon ligation with CD80 (B7.1) or CD86 (B7.2) on APC (Gross et al., 1992, Hathcock et al., 1994). Signalling through CD28 is known to enhance production of IL-2 and the anti-apoptotic molecule Bcl-xL (Powell et al., 1998, Boise et al., 1995). TCR signalling alone results in increased levels of pro-apoptotic factors such as Fas, FasL and Bim (Brunner et al., 1995, Dhein et al., 1995, Sandalova et al., 2004), and therefore CD28 signalling promotes survival of the cell. Additional co-stimulatory molecules include CD40L, OX-40 and ICOS, these bind respectively with CD40, OX40L and ICOSL on APC. CD40L is up-regulated upon T cell activation and functions to promote cytokine production and T cell activation (Daoussis et al., 2004). One of the mechanisms by which CD40:CD40L interactions are thought to facilitate this is by increasing expression of B7.1 and B7.2 on APC, and therefore enhancing signalling through CD28 (Caux et al., 1994). Likewise, CD40 signalling has been shown to up-regulate expression of OX40L on APC, thereby facilitating signalling though OX40 (Fillatreau and Gray, 2003). The co-stimulatory molecule OX40 is not constitutively found on resting T cells but is transiently expressed upon activation (Paterson et al., 1987). Ligation of OX40 with OX40L results in up-regulation of the anti-apoptotic molecules Bcl-xL and Bcl-2 (Rogers et al., 2001), and is thought to be important in the generation of memory T cells by promoting cell survival following activation (Soroosh et al., 2006). Expression of ICOS is a co-stimulatory molecule also up-regulated on T cells upon TCR ligation (Yoshinaga et al., 1999). ICOS:ICOSL interactions have been shown to be important for proliferation and cytokine production by T cells in response to antigen (Hutloff et al., 1999, Dong et al., 2001).

The full extent of how co-stimulatory molecules function and how they may interact with one another are not yet fully understood, however, loss of any of these molecules results in impaired T cell function (Green et al., 1994, Stout et al., 1996, Kopf et al., 1999, Totsuka et al., 2009). Despite the expression of co-stimulatory molecules on T cells upon TCR signalling, for T cells to receive the necessary co-stimulatory signals for full activation, the APC need to express the signalling counterparts for these molecules. This highlights the importance of Signal 0 in T cell activation.

An additional feature involved in T cell activation is the expression of co-inhibitory molecules such as cytotoxic T-lymphocyte antigen 4 (CTLA-4) and programmed death-1 (PD-1) receptor (discussed in further detail in 1.5.2). These molecules are also up-regulated upon engagement of the TCR and have been shown to negatively regulate T cell activation (Walunas et al., 1996, Parry et al., 2005). Therefore, it is the balance of co-stimulatory and co-inhibitory signals that the cell receives which dictates whether the cell achieves full activation.

In summary, activation of naive T cells will occur only when an APC displays an antigen in the context of MHC and concurrently displays the partners of co-stimulatory molecules. The requirement of Signal 0 for this to occur ensures that T cells respond only to antigens associated with infection and limits inappropriate responses to innocuous antigens such as self-peptides.

1.1.2.4 Signal 3

Although Signal 3 is not a requirement for T cell activation, this acts to determine the phenotype of the T cell response. Signal 3 is the APC-derived cytokine milieu dictated by the type of PAMPs encountered by the APC, and acts to instruct the differentiation of $CD4^+$ T cells into different helper T cell subsets. This occurs due to the expression of a range of PRR such as Toll-like receptors (TLR) by APC, which can detect different types of pathogenic stimulus. In mammals, there are currently 13 known toll-like receptors TLR1-TLR13, which are thought to

recognise distinct PAMPs (Akira et al., 2006, Tabeta et al., 2004). Signalling through TLRs is triggered by the ligation of PAMPs, and requires signalling through the adaptor proteins Myd88 and TRIF, resulting in the transcription of genes necessary for cytokine production (Yamamoto et al., 2003, Takeda and Akira, 2004, Kissner et al., 2010). The activation of specific TLRs is thought to instruct the APC to secrete a defined cytokine profile which influences the differentiation of the T cell upon activation. However, the effects of each TLR on the cytokine milleu produced by APC are not clearly defined at present (Re and Strominger, 2004). The influence of Signal 3 on T cell differentiation is discussed in further detail in section 1.1.3.

1.1.3 $CD4^+$ T cell subsets

The division of $CD4^+$ helper T cells into distinct functional subsets was first described by Mossman and Coffman in 1986, who defined the T helper type-1 (Th1) and T helper type-2 (Th2) subsets based upon the cytokine profiles secreted by $CD4^+$ mouse T cell clones. Since then, several other $CD4^+$ T cell subsets have been identified including the pro-inflammatory Th17 cells and the suppressive regulatory T cells (Treg). Each of these subsets is described in greater detail below and is illustrated in Figure 1.1.

1.1.3.1 Th1 cells

Th1 cells were originally defined as $CD4^+$ T cells that secreted IFN-γ in response to antigenic stimulation (Mosmann et al., 1986). The current understanding of Th1 cells is that they mainly secrete IFN-γ and IL-2, but can also produce other cytokines such as TNF-α and GM-CSF (Romagnani, 1999, Wan, 2010, Ponomarev et al., 2007). Differentiation of Th1 cells is driven by the secretion of IL-12 by APC during signal 3 and is enhanced by IL-18 (Yoshimoto et al., 1998). IL-12 binds to the IL-12 receptor on $CD4^+$ T cells and induces signalling through STAT4, a transcription factor that is required for differentiation of Th1 cells (Bacon et al., 1995, Kaplan et al., 1996). IL-12 signalling can lead to the up-regulation of T-bet,

the Th1-associated transcription factor which drives production of IFN-γ and expression of the chemokine receptor CXCR3 (Ylikoski et al., 2005, Szabo et al., 2000, Lord et al., 2005). IFN-γ produced by Th1 cells can act in an autocrine manner to activate STAT1 signalling and further promote expression of T-bet (Afkarian et al., 2002). IFN-γ has also been shown to inhibit the proliferation of Th2 cells (Oriss et al., 1997).

Th1 cells are thought to orchestrate cell-mediated immune response and are involved in the clearance of intracellular pathogens through the activation of macrophages (Zhu and Paul, 2008). This is mediated by the IFN-γ secreted by the Th1 cells, which activates macrophages and enhances their microbicidal activity (Kagaya et al., 1989). Th1 cells have also been implicated in harmful immune responses and are thought to be involved in the induction of autoimmune pathology.

1.1.3.2 Th2 cells

Th2 cells were initially characterised as $CD4^+$ T cells that secreted IL-3 and IL-4 upon stimulation (Mosmann et al., 1986). It is now understood that the main Th2-associated cytokines are in fact IL-4, IL-5, IL-13 and IL-10 (Hartenstein et al., 2002, Johnson and Graham, 1999). Differentiation of Th2 cells occurs in the presence of IL-4, which mediates signalling through STAT6 (Wurster et al., 2000). IL-4 and STAT6 signalling regulate the expression of GATA-3, the master transcription factor that drives production of Th2-asscoiated cytokines (Kurata et al., 1999, Zhu et al., 2004). Similar to IFN-γ signalling in Th1 cells, IL-4 can also act in an autocrine manner to provide a positive feedback loop for Th2 cells.

Th2 cells direct humoral immune responses and play a role in the clearance of certain parasites such as helminths. The Th2 cytokines IL-4, IL-13 and IL-5 mediate these responses through the activation of eosinophils and by promoting IgE production by B cells (Gleich and Loegering, 1984, Deo et al., 2010). IgE can bind to Fc receptors located on mast cells and also facilitate the clearance of extracellular

pathogens (Gurish et al., 2004). Th2 responses to innocuous antigens are also associated with allergic immune responses (Maggi, 1998).

The Th2-associated cytokine IL-4 has been shown to directly inhibit the induction of Th1 cells (Vercelli et al., 1990, Tanaka et al., 1993). It has also been reported that IL-4 and IL-10 can antagonise Th1 responses by inhibiting the activation of macrophages (Appelberg et al., 1992, O'Farrell et al., 1998). Due to the reciprocal inhibitory effects of IFN-γ on Th2 responses and IL-4 on Th1 responses it was proposed that these two subsets mutually regulated each other. This Th1/Th2 paradigm was thought to provide a mechanism of immune regulation, whereby a shift in the helper T cell response would inhibit harmful excessive responses such as Th1-associated autoimmune disease or Th2-associated allergic disease (Romagnani, 1997). However, the dogma was challenged when a new subset of $CD4^+$ T cells were discovered; the IL-17 secreting Th17 cells.

1.1.3.3 Th17 cells

Before the discovery of Th17 cells there appeared to be certain contradictions to the Th1/Th2 hypothesis. These included the observation that in "Th1-classified" models of autoimmune disease, inhibition of IFN-γ could exacerbate disease, and reciprocally, treatment with IFN-γ could ameliorate disease (Billiau et al., 1988, Voorthuis et al., 1990, Nakajima et al., 1991). These models were thought to be Th1 mediated diseases as blockade or deficiency in the p40 subunit of IL-12 protects from disease induction (Leonard et al., 1995, Segal et al., 1998, McIntyre et al., 1996). However, in 2000, a novel member of the IL-12 family was identified which shared the p40 subunit with IL-12, named IL-23 (Oppmann et al., 2000). IL-12 is a heterodimer comprised of a p40 and a p35 subunit, whereas the IL-23 heterodimer is composed of p40 and p19 subunits. Subsequent studies revealed that mice deficient in the p19 subunit were protected from disease induction in certain models of supposed Th1-classified disease, but mice deficient in p35 were not (Cua et al., 2003, Murphy et al., 2003). These finding implicated a novel helper T cell response, which was verified by the discovery of a distinct IL-17 secreting $CD4^+$ T

cell subset induced by IL-23, called Th17 cells (Langrish et al., 2005, Weaver et al., 2006).

Although Th17 cells were originally thought to require IL-23 for their differentiation (Aggarwal et al., 2003), it has recently been suggested that IL-23 only functions to stabilise the phenotype of these cells (Stritesky et al., 2008). It is now believed that IL-6 and TGF-β are the cytokines required the differentiation of Th17 cells, through the activation of the Smad2 and STAT3 signalling pathways (Bettelli et al., 2006, Tanaka et al., 2008, Malhotra et al., 2010). These cells have been shown to secrete IL-17A, IL-17F, and IL-22, and are regulated by the transcription factor RORγt (Ivanov et al., 2006, Korn et al., 2009). To further complicate the original Th1/Th2 paradigm, both IFN-γ and IL-4 have been shown to inhibit the differentiation of Th17 cells (Harrington et al., 2006). However, it is not currently understood whether Th17 responses can inhibit Th1 and Th2 cells. Th17 responses are thought to play a role in the clearance of extracellular bacteria and fungi (Stockinger and Veldhoen, 2007, Milner et al., 2008), and since their discovery, have been implicated in several autoimmune diseases previously thought to be driven by Th1 cells.

The requirement of TGF-β for Th17 differentiation means that these cells are closely linked with regulatory T cells which can also be induced by TGF-β. However, regulatory T cells play a role in limiting effector T cell responses and therefore these subsets have opposing actions.

1.1.3.4 Regulatory T cells

In addition to the effector T helper subsets (T_{eff}) there are $CD4^+$ T cells that possess immunosuppressive characteristics called regulatory T cells or Treg. A cardinal feature of a regulatory cell population is the ability to dampen immune responses to antigen upon transfer to mice. This ability was first proposed in the 1970's, and these cells were termed 'suppressor T cells' (Gershon and Kondo, 1970, Gershon et al., 1972). However, the concept of suppressor T cells was largely abandoned in the

1980's due to difficulties in characterising these cells (Moller, 1988, Sakaguchi et al., 2007). The existence of suppressive T cells, now called Treg, was resurrected in the mid 1990's as a result of experiments that demonstrated the existence of $CD4^+$ T cells that could suppress effector $CD4^+$ T cells responses (Chen et al., 1994, Groux et al., 1997). Regulatory T cells have since been broadly segregated into two subpopulations; the natural thymically-derived Treg (nTreg) and the adaptive or induced Treg which are generated in the periphery.

1.1.3.4.1 Natural regulatory T cells

$CD4^+CD25^+$ natural regulatory T cells (nTreg) were first identified by Sakaguchi et al, who demonstrated that adoptive transfer of $CD4^+$ cells into naïve recipient mice that had been depleted of $CD25^+$ cells resulted in the onset of autoimmune pathology (Sakaguchi et al., 1995). These $CD4^+CD25^+$ cells were later shown to be important in the maintenance of self-tolerance and were found to express the transcription factor Foxp3 (Asano et al., 1996, Suri-Payer et al., 1998, Hori et al., 2003). Indeed, people with mutations in the Foxp3 gene have been shown to develop Immune dysregulation Polyendocrinopathy Enteropathy X-linked (IPEX) syndrome, where autoimmune and allergic responses are observed (Le Bras and Geha, 2006). nTreg constitute ~5-10% of the circulating $CD4^+$ population and are generated as a distinct $CD4^+$ T cell lineage in the thymus during development (Itoh et al., 1999). This is thought to occur during the later stages of development as CD25 is only first expressed upon transition of DP $CD4^+CD8^+$ cells to SP $CD4^+CD8^-$, and $Foxp3^+$ cells are only observed within the thymic medulla (Papiernik et al., 1998, Fontenot et al., 2005). Foxp3 expression is induced in SP thymocytes by a process thought to involve IL-2 and TGF-β signalling, as absence of these cytokines results in reduced numbers of circulating $CD4^+CD25^+Foxp3^+$ cells (Liu et al., 2008). Similar to $CD4^+Foxp3^-$ cells, once nTreg cells have been generated, they can leave the thymus and enter the circulation.

Although the exact mechanisms by which nTreg suppress T_{eff} responses are not well defined, multiple potential mechanisms have been identified. These are discussed in further detail in section 1.1.3.4.3.

1.1.3.4.2 Adaptive regulatory T cells

Adaptive and induced $CD4^+Foxp3^+$ regulatory T cells (iTreg) are generated from $CD4^+Foxp3^-$ cells within the periphery. These include the generation of $CD4^+Foxp3^+$ iTreg and the Tr1 and Th3 $CD4^+Foxp3^-$ regulatory T cell subsets.

It has been demonstrated that $CD4^+CD25^+Foxp3^+$ cells can be induced from conventional $CD4^+Foxp3^-$ cells both *in vivo* and *in vitro* (Curotto de Lafaille et al., 2004, Chen et al., 2003b). The generation of these iTreg cells is dependent upon the presence of TGF-β, and they have been shown to have immunosuppressive activities in several experimental models (Zheng et al., 2006, DiPaolo et al., 2007). It has also been reported that the generation of iTreg *in vivo* can be induced by the administration of antigen in the absence of DC activation (Apostolou and von Boehmer, 2004, Mahnke et al., 2003). The ability to generate large numbers of iTreg from $CD4^+Foxp3^-$ cells offers many advantages over the use of nTreg as a cellular therapy for the treatment of autoimmune conditions. However, the observation that Foxp3 expression is unstable in iTreg has prompted concerns over the potential of these cells to convert to a pathogenic phenotype. Reassuringly, recent studies have demonstrated that loss of Foxp3 expression does not abrogate the ability of iTreg to suppress T_{eff} responses (O'Connor et al., 2010).

In addition to regulatory T cells there are $CD4^+Foxp3^-$ cells that have suppressive functions; these include the Tr1 and Th3 subsets. Tr1 cells can be induced from naïve precursors by the presence of IL-10, and have been shown to down-regulate immune responses mediated by T_{eff} cells (Groux et al., 1997). Activation of naïve $CD4^+$ T cells by immature DC has also been implicated in the induction of Tr1 cells (Levings et al., 2005). These regulatory cells function through the production of large amounts of the immunosuppressive cytokine IL-10 (Roncarolo et al., 2006).

In contrast, the Th3 cells predominantly produce TGF-β, and were defined in models of oral tolerance (Chen et al., 1994, Weiner, 2001). Due to their secretion of TGF-β, Th3 cells might also prompt the induction of $Foxp3^+$ iTreg from "bystander" naïve $CD4^+$ T cells (Carrier et al., 2007).

Despite the characterisation of the above regulatory populations, the precise mechanisms of immunosuppression by these cells are not fully understood. However, several possible mechanisms have been identified.

1.1.3.4.3 Mechanisms of Immune-suppression

The potential mechanisms of suppression by Treg, Tr1 and Th3 cells include the secretion of inhibitory cytokines, metabolic disruption, alteration of APC function, and cell-cell contact mediated cell death.

Tr1 and Th3 cells have been shown to secrete IL-10 and TGF-β respectively, as have nTreg cells under certain conditions (Liu et al., 2003, Joetham et al., 2007). IL-10 inhibits both proliferation and cytokine secretion by T cells (Fiorentino et al., 1991, Taga and Tosato, 1992), and can inhibit the activation of T cells as well as suppress memory and effector $CD4^+$ T cells (Perrin et al., 1999, Brooks et al., 2010). Furthermore, IL-10 can inhibit maturation of DC and therefore hinder their ability to activate T cells (Cavani et al., 2000). TGF-β has also been shown to inhibit proliferation of T cells (Bright et al., 1997), and is thought to act both as a soluble and membrane bound mediator of T cell suppression (Nakamura et al., 2001, Shevach, 2002). An additional immunosuppressive cytokine produced by Treg is IL-35, which has been shown to inhibit effector T cell responses and may also play a role in expanding nTreg populations (Collison et al., 2007, Castellani et al., 2010).

Despite the ability of nTreg to secrete immunosuppressive cytokines, these cells are also thought to mediate suppression through a contact-dependent, cytokine-independent mechanism. This is due to the observation that neutralisation of IL-10

and TGF-β *in vitro* does not inhibit suppression by nTreg, and that nTreg do not suppress responder cells when they are separated by a permeable membrane (Thornton and Shevach, 1998, Takahashi et al., 1998). A potential contact-dependent mechanism of suppression is through cytolysis. nTreg have been shown to produce Granzyme A (Granzyme B in mice) and can therefore kill target cells (Gondek et al., 2005, Cao et al., 2007). It has recently also been suggested that nTreg can suppress through metabolic disruption. nTreg are generally thought to be anergic as they fail to produce their own IL-2 upon stimulation, and therefore rely on IL-2 from other cells for their survival and growth (Housley et al., 2011). Therefore, a proposed mechanism of suppression by nTreg is through deprivation of IL-2 which can induce apoptosis of T_{eff} cells (Pandiyan et al., 2007). A further mechanism by which nTreg can suppress through metabolic disruption is through the expression of cell-surface CD39 and CD73. These molecules act to degrade extracellular ATP which can have pro-inflammatory effects and aid T cell activation (Deaglio et al., 2007, Romio et al., 2011).

Treg also harness the potential to modulate APC function. In the case of nTreg, these cells have been shown to express high levels of the inhibitory molecules CTLA-4 and LAG-3. These molecules can interact with ligands on APC (CD80/CD86 and MHC II) and inhibit their ability to activate naïve T cells (Liang et al., 2008, Onishi et al., 2008).

These examples demonstrate that there are many potential mechanisms of suppression by regulatory cells, and it is likely that a selection of these are utilised depending upon the conditions.

1.1.3.5 Novel $CD4^+$ T cells subsets

Several novel helper T cell subsets have recently been described. These include the IL-9 secreting Th9 cells (Veldhoen et al., 2008, Dardalhon et al., 2008) and IL-22 secreting Th22 cells (Trifari et al., 2009). However, the role of these cells in immune pathology and their existence as discrete $CD4^+$ T cell subsets is not yet fully understood.

Figure 1.1 Key $CD4^+$ T cells subsets

1.1.3.6 Plasticity of T cells

There is an emerging field in T cell biology which challenges the paradigm that $CD4^+$ T cell differentiation is an irreversible process. This so-called 'plasticity' of $CD4^+$ T cells was first described in nTreg, whereby culture in the presence of IL-6 was shown to promote loss of Foxp3 expression and gain of IL-17 production (Xu et al., 2007). iTreg cells appear to be resistant to this conversion (Zheng et al., 2008). However, recent studies have demonstrated that they can adopt a Th1-like phenotype when cultured in the presence of IL-12 (O'Connor et al., 2010). Similarly, it has been demonstrated that Th2 cells can be re-programmed to produce IFN-γ (Hegazy et al., 2010) and more recently, both Th1 and Th17 cells have been shown to acquire a Th2-like phenotype and produce IL-4 (Panzer et al., 2011). Th1 cells have also been shown to produce the immunsuppressive cytokine IL-10 under certain conditions (Jankovic et al., 2007). In addition, the presence of IFN-γ and IL-17 double positive $CD4^+$ have been observed during inflammation (Annunziato et al., 2007, Nistala et al., 2008).

These observations demonstrate that T cell differentiation is not absolute and that segregation of $CD4^+$ T cells into discrete subsets does not accurately reflect the characteristics or phenotype of these cells. In addition, the ability of so-called lineage-specific cytokines to inhibit the differentiation of different T cell subsets adds further complexity to understanding the role of these cells in the induction and resolution of immune pathology.

1.2 Peripheral tolerance

As mentioned in section 1.1.1 during the development of T cells within the thymus, many self-reactive T cell clones are thought to be eliminated by negative selection. However, not all self-antigens are expressed in the thymus and therefore T cells that recognise these self-peptides escape this negative selection process. Also, due to the requirement for T cells to recognise and protect from a diverse array of

pathogens and the degeneracy of the TCR, there are T cells in the periphery that are cross-reactive and are capable of recognising both foreign and self-antigens. Therefore, mechanisms need to exist in order to prevent the activation of these autoreactive T cells. These peripheral tolerance mechanisms include cell death, anergy and regulation.

1.2.1 Death

In addition to the deletion of self-reactive T cells in the thymus, these cells can also be programmed to undergo apopotosis in the periphery (Jones et al., 1990, Wells et al., 1999). This is thought to occur due to encounter with antigen in the absence of co-stimulation. As discussed in section 1.1.2.3, co-stimulation provides necessary survival signals required for full T cell activation. Therefore if the TCR of self-reactive T cells are engaged by self pMHC complexes under steady state conditions, the up-regulation of pro-apoptotic molecules such as Fas, FasL and Bim leads to deletion (Walker and Abbas, 2002, Mueller, 2010). The importance of cell death in maintaining tolerance to self antigens has been demonstrated by the occurrence of autoimmune disease in mice with mutations in Fas, FasL or Bim (Nagata and Suda, 1995, Bouillet et al., 1999, Weant et al., 2008).

1.2.2 Adaptation

As an alternative fate to apoptosis, stimulation of naïve T cells through the TCR in the absence of co-stimulation can also result in a hypo-responsive phenotype termed anergy (Choi and Schwartz, 2007). Clonal anergy is classically an *in vitro* phenomenon that has been shown to occur when T cells are stimulated in the absence of co-stimulation (Jenkins and Schwartz, 1987, Harding et al., 1992). Anergic T cells lack the ability to produce IL-2 and therefore cannot undergo clonal expansion upon recall stimulation with antigen (Mueller et al., 1989). Production of effector cytokines such as IFN-γ is not impaired in T cells that have been anergised *in vitro*. However, the ability to induce this precise form of clonal anergy *in vivo* and it's relevance in peripheral tolerance to self-antigens remains a mater of debate.

Another form of hypo-responsive state is adaptation, also known as adaptive tolerance or *in vivo* anergy. This can occur *in vivo* due to the persistent presence of antigen (Rammensee et al., 1989, Oxenius et al., 1998, Rocha et al., 1993). The induction of adaptive tolerance is thought to be due to TCR signalling in an environment deficient in co-stimulation and or high in co-inhibition (Schwartz, 2003). Upon encounter with antigen, the T cells undergo an initial phase of proliferation, which is followed by cell death and a lack of proliferation and cytokine production in the cells that remain (McCormack et al., 1993, Pape et al., 1998). Unlike clonal anergy, adaptive tolerance cannot be reversed through the addition of exogenous IL-2, but is reversed by the removal of antigen (Tanchot et al., 2001, Choi and Schwartz, 2007). Investigation into the role of co-inhibitory signals in the maintenance of adaptive tolerance has demonstrated that blockade of CTLA-4 can prevent the induction of adaptive tolerance (Perez et al., 1997). A further co-inhibitory molecule thought to play a role in maintaining peripheral tolerance is PD-1 (Fife and Pauken, 2011) (described in further detail in section 1.5.2.2.2)

In situations where antigen is sequestered in an immune privileged site adaptive tolerance cannot be induced. However, the inability of self-reactive T cells to encounter their antigen in this situation prevents their activation due to clonal ignorance (described in further detail in 1.3.2.2).

1.2.3 Regulation

The third pillar of peripheral tolerance is regulation, which involves the aforementioned regulatory T cells (as described in 1.1.3.4). The obligate role of nTreg in peripheral tolerance has been demonstrated by the spontaneous development of multi-organ autoimmune conditions in mice and humans that either lack or have mutations within the Foxp3 gene (Fontenot et al., 2003, Le Bras and Geha, 2006). One theory is that nTreg cells stem from thymic T cell precursors that have a high affinity to self antigens, and therefore can compete with conventional T cells that have a lower affinity for self-antigens (Jordan et al., 2001, Hsieh et al.,

2004, Stritesky et al., 2011). Th3 and Tr1 cells have also been implicated in maintaining peripheral tolerance, whereby Th3 cells can encourage the generation of iTreg (Carrier et al., 2007), and Tr1 cells can induce tolerance by the secretion of IL-10 (Bacchetta et al., 1994, VanBuskirk et al., 2000).

1.3 Autoimmune disease: The breakdown of tolerance

In 1901 Paul Ehrlich famously postulated that a living organism would not endanger itself by the production of toxic auto-antibodies, a phenomenon termed *horror autotoxicus* (Silverstein, 2001). However, work by Julius Donath and Karl Landsteiner in 1904 demonstrated that auto-antibodies were a causative agent in Paroxysmal cold haemoglobinuria (Schwartz, 2005) (a haemolytic disease which occurs upon exposure to cold temperatures), and thus the concept of autoimmune disease was born.

Autoimmune disease is now understood to be driven by auto-reactive T cells that have escaped mechanisms of tolerance, and mounted an inappropriate immune response against target self-antigens. Autoimmune disease must reflect deficiencies in both central and peripheral tolerance.

1.3.1 Breakdown of central tolerance

Central tolerance was initially thought to be an incomplete mechanism of deleting auto-reactive T cell progenitors due to a lack of tissue-restricted or tissue-specific antigen (TSA) expression within the thymus (Sprent and Surh, 2003). Therefore, T cells that could recognise these TSAs could escape central tolerance and potentially induce organ-specific autoimmune disease. However, it later became evident that this theory needed refinement when several studies demonstrated that the thymus can in fact express TSAs, and that these antigens were largely expressed by mTEC (Pribyl et al., 1996, Smith et al., 1997, Derbinski et al., 2005). The discovery of the

autoimmune regulator (AIRE), a transcription factor required for the expression of TSAs within the thymus, supported these findings (Anderson et al., 2002). Deficiency in AIRE expression has been shown to result in the inhibition of negative selection and the onset of autoimmune pathology in both humans and mice (Nagamine et al., 1997, Ramsey et al., 2002).

Even when AIRE is expressed, there are clear examples where autoantigenic epitopes are inefficiently presented in the thymus, allowing T cells to avoide negative selection (Anderton and Wraith, 2002). This has been demonstrated with the encephalitogenic Ac1-9 peptide of myelin basic protein (MBP); whereby the presence of Ac1-9 in the thymus does not induce deletion of T cells that recognise this peptide (Liu et al., 1995). This is thought to be due to the poor MHC-binding affinity and therefore low functional avidity of Ac1-9, as systemic administration of Ac1-9 peptide analogs with higher MHC-binding affinities resulted in deletion of the Ac1-9-reactive T cells. Despite the mechanisms by which auto-reactive T cells can escape central tolerance, these T cell clones also need to circumvent mechanisms of peripheral tolerance in order to induce autoimmune pathology.

1.3.2 Breakdown of peripheral tolerance

1.3.2.1 Defects in peripheral tolerance mechanisms

There are various defects in peripheral tolerance that have been associated with autoimmune disease. As mentioned in section 1.2.1, mutations in the genes encoding the pro-apoptotic molecules Fas, FasL and Bim limits the potential for self-reactive T cells to be deleted within the periphery and can result in the onset of autoimmune pathology. In humans, mutations in these genes are associated with autoimmune lymphoproliferative syndrome (Straus et al., 1999). Defects in the expression and function of co-inhibitory molecules thought to play a role in peripheral tolerance, such as CTLA-4 and PD-1, have also been implicated in autoimmune pathology. Genetic polymorphisms at the CTLA-4 and PD-1 loci have been associated with several autoimmune diseases including type 1 diabetes (T1D),

Graves' disease and multiple sclerosis (MS) (Yanagawa et al., 1995, Nistico et al., 1996, Awata et al., 1998, Nielsen et al., 2003, Kroner et al., 2005). A further contributing factor to the breakdown in peripheral tolerance can be attributed to deficiencies in regulation by Treg. Multiple proposed defects in regulation by Treg have been associated with autoimmunity; including a reduction in numbers of Treg and functional inadequacies (Buckner, 2010). As mentioned in section 1.1.3.4.1, mutations affecting Foxp3 expression are associated with the autoimmune condition IPEX syndrome (Le Bras and Geha, 2006). Studies into the function of $CD4^+CD25^+$ cells from patients with T1D or MS have also shown that they have a reduced *in vitro* suppressive capacity compared to those from healthy controls (Lindley et al., 2005, Viglietta et al., 2004).

1.3.2.2 Antigen release from immune privileged sites

Clonal ignorance is a further mechanism that prevents activation of autoreactive T cells and relies upon the permanent separation of antigen and the self-reactive T cells. This can occur due to the location of the target self-antigen within an immune privileged site, such as the eye and central nervous system (CNS). Tissue damage associated with infection or physical injury is thought to compromise the immune privileged site and release these hidden antigens, which can result in the onset of autoimmune pathology (Caspi, 2006). This has been demonstrated in sympathetic ophthalmia, whereby physical damage to one eye can induce an inflammatory response in the uninjured contralateral eye. This is thought to occur due to the release of self-antigens by the initial insult (Rao et al., 1983) which instigates autoimmune uveitis that can target both eyes (Hellmund et al., 1998).

1.3.2.3 Bystander activation of T cells

Although the exact mechanisms that enable the bystander activation of T cells are not fully understood, it is thought to occur non-specifically as a result of an immune response triggered by infection (Burt et al., 2002). The presence of TLR ligands and inflammatory cytokines present during infection are thought activate APC that

can subsequently stimulate auto-reactive T cells. This has been demonstrated using TCR transgenic mice, whereby the $CD4^+$ cells from these mice can be activated during infection with a virus that the T cells do not recognise (Gangappa et al., 1999, Di Genova et al., 2010). However, the exact role this phenomenon plays in the onset of autoimmune disease is not currently known.

1.3.2.4 Molecular mimicry

Many autoimmune diseases have also been linked with particular pathogenic infections.. One attractive idea is that the infection might provide an antigenic trigger, sowing the seeds (activated/memory T cells) for autoimmune disease to develop. This is thought to occur due to the degeneracy required of TCR, where the requirement to recognise a broad range of foreign antigens means there is the potential that a specific TCR can be cross-reactive with both foreign and self-antigens. Molecular mimicry is the hypothesis that T cells activated by foreign antigen during infection can cross-react with self-antigen and lead to the onset of autoimmune disease (Oldstone, 1987). Evidence of this has been provided by the observation that potential target auto-antigens bear resemblance to viral peptides (Honeyman et al., 1998, Atkinson et al., 1994), and that viral infection can trigger autoimmune pathology in several experimental models (Fujinami and Oldstone, 1985, Yuki et al., 2004, Ohashi et al., 1991, Olson et al., 2001). In addition to this, it has also been observed that viral peptides can activate auto-reactive T cells (Ufret-Vincenty et al., 1998), most notably MBP-reactive T cell clones isolated from MS patients (Wucherpfennig and Strominger, 1995). Despite these observations, direct evidence of this in humans is difficult to obtain. This might be because the infection is cleared before the onset of clinical symptoms of autoimmune disease.

Due to the multitude of mechanisms in place to prevent the activation of autoreactive T cells, it is thought that a combination of factors needs to coincide in order to permit the escape from tolerance. Depending upon the contributing factors leading to the activation of self-reactive T cells, a variety of different autoimmune

diseases can result. This thesis focuses on the organ-specific autoimmune disease of the CNS, MS.

1.4 Multiple Sclerosis & EAE

1.4.1 Multiple Sclerosis

MS is thought to be an autoimmune disease of the CNS, whereby $CD4^+$ cells orchestrate immune-mediated damage to the myelin sheath surrounding axons (Korn, 2008, Prat and Antel, 2005). MS is epidemiologically associated with extreme geographical latitudes, with the highest incidence occurring in northern Europe, North America and south Australasia (Kurtzke, 1991, Simpson et al., 2011), and more frequently occurs in females than males (Sellner et al., 2011). The exact eitiology of MS is currently unknown, but is thought to include both environmental and genetic risk factors (Marrie, 2004).

1.4.1.1 Clinical courses of MS

There are three principal clinical courses recognised in MS; relapsing-remitting MS (RRMS), secondary progressive MS (SPMS) and primary progressive MS (PPMS) (Figure 1.2). The majority of cases present with an initial relapsing-remitting course which often later advances to a secondary progressive phase, where cumulative neurodegeneration and an absence of remission are hallmark features (Venken et al., 2010, Spain et al., 2009). In RRMS, it is thought that relapse is associated with active inflammation, and remission reflects the resolution of inflammation and repair of the myelin sheath (Bruck, 2005). Multiple relapses lead to an accumulation in neurological damage and axonal loss; once this reaches a critical level there is thought to be a change in the disease phenotype from inflammation to neurodegeneration, and the patient goes on to develop SPMS (Trapp *et al.,* 1999, Spain *et al.,* 2009). In contrast, PPMS is characterised by progressive axonal degeneration in the absence of any overt inflammation, and therefore is primarily thought of as a neurodegenerative disease (Matthews, 2004, Trapp and Nave, 2008).

Figure 1.2 Clinical courses of MS

1.4.1.2 Symptoms & Immunopathology of MS

MS is a highly heterogeneous disease and therefore symptoms are many and varied, and largely depend upon the location of the demyelinating lesions within the CNS (Kincses et al., 2010). Symptoms can arise from both sensory and motor defects and include; loss of co-ordination, numbness and tingling, vision loss, neuropathic pain, fatigue, spasticity and imbalance (Thompson, 2001, Ben-Zacharia, 2011). There are no symptoms that are specifically unique to MS, and therefore diagnosis is usually aided by magnetic resonance imaging (MRI) to detect the presence of lesions within the CNS.

Although the exact mechanisms involved in the pathogenesis of MS are not fully defined, most of what is known has been aided by studies in animal models of CNS autoimmune disease (described in more detail in 1.4.2). In MS, myelin-reactive $CD4^+$ T cells are thought to be activated within the periphery, which enables them to cross the blood brain barrier (BBB) and enter the CNS (Goverman, 2009). Once there, these cells are thought to orchestrate immune-mediate damage directed at the myelin sheath (Constantinescu et al., 2011). Other immune cells implicated in the pathogenesis of MS include $CD8^+$ T cells, B cells and innate immune cells such as macrophages and CNS-resident microglia (Hemmer et al., 2006). Evidence that MS is a $CD4^+$ T cell mediated disease stems from the observation that experimental autoimmune encephalomyelitis (EAE), an animal model of CNS autoimmune disease, can be induced by the passive transfer of encephalitogenic $CD4^+$ T cells into naïve mice (Zamvil et al., 1985, Ben-Nun et al., 1981) (described in more detail in 1.4.2). $CD4^+$ T cells can also be observed within MS lesions (Raine, 1994, Hauser et al., 1986), and myelin-reactive T cells have been shown to exist in a heightened state of activation in MS patients compared to healthy controls (Zhang et al., 1994). Furthermore, the monoclonal antibodies Natalizumab (Tysabri®) and Alemtuzumab (Campath®), which block entry of lymphocytes to the CNS or induce deletion of lymphocytes respectively, have shown clinical efficacy in the treatment of relapsing-remitting MS (Pucci et al., 2011, Coles et al., 2008). More in detail mechanisms of MS pathology can be gleaned from animal models and are discussed in further detail in 1.4.2.

1.4.1.3 Suspected causes of MS

As the exact mechanism of disease induction in MS is not understood, no direct cause has been identified. However, several genetic and environmental risk factors have been proposed to play a role in the induction of disease. In addition to potential genetic mutations associated with the function of central and peripheral tolerance (section 1.2), there is an associated risk between MHC II allele expression and MS. Individuals with the human leukocyte antigen (HLA)-DRB1*15 genotype have a genetic susceptibility to MS, and comprise over 60% of cases (Haines et al., 1998, Barcellos et al., 2003, Schmidt et al., 2007). Genome-wide association studies (GWAS) have also identified other potential genetic associations, the majority of which include genes associated with immune function (Table 1.1) (Kemppinen et al., 2011). In addition to providing further evidence that MS is an immune-mediated disease, the results of GWAS and linkage studies may give an insight into the pathology of the disease. For example, several of the MS risk loci identified are associated with activation, proliferation and differentiation of T cells, namely the IL-12 responsive Th1 cells and the STAT3-regulated Th17 cells. Despite the implications of these GWAS findings, the concordance rate between monozygotic twins is only thought to be ~25-30% (Sadovnick et al., 1993), which implicates an important contribution of environmental factors in disease onset and or progression. Potential environmental triggers thought to be associated with MS include viral infection and vitamin D deficiency.

As mentioned in section 1.3.2.4 infection with certain viruses are thought to enable self-reactive T cells to escape tolerance and become activated. Several viral infections have been linked to MS including Epstein-Barr virus (EBV) and human herpes virus 6 (HHV-6) (Ascherio et al., 2001, Soldan et al., 1997). Epidemiological studies have identified these as risk factors in MS, and several potential molecular mimics have been found in both viruses for MBP (Ascherio and Munch, 2000, Challoner et al., 1995, Holmoy et al., 2004, Tejada-Simon et al., 2003). These associations provide a potential mechanism by which MBP-reactive T cells can become activated in the periphery.

Nearest gene to risk allele	*p*-value	Odds ratio	Function of encoded protein
KIF1B	2.50E-10	1.34	Motor protein belonging to the Kinesin family involved in axonal transport (Aulchenko et al., 2008, Nangaku et al., 1994)
TMEM39A	3.09E-08	1.24	Transmembrane protein, function unknown (IMSGC, 2007, IMSG 2010)
KIF21B	6.56E-10	1.22	Kinesin family member involved in neuronal transport unknown (IMSGC, 2007, IMSG 2010, Marszalek et al., 1999)
CLEC16A*	1.6E-16	1.20	c-type lectin highly expressed in B cells, NK cells and DC (Hoppenbrouwers et al., 2009, Hakonarson et al., 2007)
IRF8*	3.73E-09	1.25	Transcription factor involved in lineage commitment of myeloid cells and B cells (De Jager et al., 2009, Wang & Morse 2009)
CD6*	3.79E-09	1.18	Expressed by T cells and B cells, can function as an accessory molecule in T cell activation (De Jager et al., 2009, Gimferrer et al., 2004))
MPHOSPH9	3.96E-08	1.10	M-phase phosphoprotein-9; plays a role in mitosis (De Jager et al., 2009, Matsumoto-Taniura et al., 1996)
TNFRSF1A*	1.59E-11	1.20	Major receptor for TNF-α (De Jager et al., 2009, Tartaglia et al., 1993)
IL2RA*	2.38E-23	1.25	The IL-2 receptor alpha chain, expressed on immune cells such as T cells and B cells, and promotes cell proliferation via IL-2 signalling (IMSGC 2007, Minami et al., 1993)
CD58*	4.0E-09	1.23	Expressed by APC and facilitates interactions with T cells through ligation with CD2 (IMSGC 2007, Moingeon et al., 1989)
RGS1	3.55E-09	1.15	Attenuates signalling activity of G-proteins (De Jager et al., 2009, Druey et al., 1996)
TYK2*	5.08E-09	1.30	Facilitates interferon-mediated signalling through STAT3 (WTCCC & TASC 2007, Karaghiosoff et al., 2000)
IL12A*	3.08E-08	1.11	The alpha subunit of IL-12, a cytokine that plays a role in Th1 cell differentiation (De Jager et al., 2009, Athie-Marales et al., 2004)
IL7R*	1.21E-17	1.20	IL-7 receptor
METTL1	5.4E-11	1.23	Methyl-transferase responsible for post-transcriptional modification of tRNA's (ANZgene 2009, Alexandrov et al., 2002)
STAT3*	2.75E-10	1.15	Signalling transducer of activation that plays a role in T cell activation (De Jager et al., 2009, Takeda et al., 1998)
CBLB*	1.60E-10	1.40	E3 ubiquitin ligase which acts as a negative regulator of adaptive immune responses (Sanna et al., 2010, Bachmaier et al., 2000)

Table 1.1: MS risk loci as identified by GWAS

Table illustrates the nearest genes to 16 identified non-HLA loci associated with multiple sclerosis. Criteria used to identify associations were based on p-values ≤5x10E-8. * Represents genes with known immunological function. Table adapted from Kempinnen et al., 2011.

In addition, a potential link between vitamin D deficiency and levels of sunlight exposure in MS has also been proposed due to the prevalence of MS cases in geographically extreme latitudes (Cantorna, 2008). It has been observed that individuals with MS have lower circulating levels of vitamin D compared to healthy controls (Nieves et al., 1994), and there is an increased prevalence of MS in people born in the northern hemisphere during spring months, which also implicates an association with vitamin D exposure during pregnancy (Willer et al., 2005). Furthermore, the frequency of MS cases is found to be lower in populations residing at high latitudes with high dietary intake of vitamin D compared to those with low dietary intake of vitamin D (Kakalacheva and Lunemann, 2011). The observation that vitamin D possesses immunomodulatory properties may explain the link between MS and vitamin D levels. The active form of vitamin D (1,25-dihydroxyvitamin D_3) has been shown to inhibit the activation and maturation of DC, and enhance the function and generation of Treg (Ben-Zvi et al., 2010, Jeffery et al., 2009, Smolders et al., 2009) Various studies have also demonstrated that administration of vitamin D can inhibit the pathogenesis of EAE (Cantorna et al., 1996, Spach and Hayes, 2005, Mayne et al., 2011). Although the exact mechanism by which vitamin D acts to modulate immune responses is not understood, it appears to act by beneficial immunoregulatory mechanisms.

Despite the association of several risk factors with MS, it is unlikely that there is a single responsible cause, but rather a combination of risk factors acting in concert to provoke autoimmune pathology, and the induction of MS.

1.4.1.4 Treatment of MS

Current treatments for MS largely rely upon non-specific immunomodulation of the immune system. These include the use of mitoxantrone, glatiramer acetate, interferon-β and natalizumab (Lopez-Diego and Weiner, 2008, Berger, 2009) .

Interferon-β therapeutics (Avonex®, Ribef®, Betaferon®, Extavia®) include both interferon-β-1a and interferon-β-1b, and have been shown to reduce the frequency

of relapses in RRMS (Paty and Li, 1993, Jacobs et al., 1996, Kappos et al., 2007). The mode of action is not fully understood, but potential reported mechanisms include the inhibition of antigen presentation, T cell proliferation, and effector cytokine production (Markowitz, 2007, Noronha et al., 1993, Barna et al., 1989).
Glatiramer acetate (Copaxone®) is a synthetic amino acid polymer designed to mimic MBP which has been shown to reduce the relapse rate and delay the onset of disability in RRMS (Teitelbaum et al., 1971) (Johnson et al., 1995, Simpson et al., 2002). Although glatiramer acetate has been shown to switch off an immune response targeted at the MBP 82-100 epitope (Aharoni et al., 1999), the main mode of action is thought to be non-specific and functions through effects on DC rather than T cells (Weber et al., 2004, Vieira et al., 2003).

Mitoxantrone (Novantrone®) is a cytotoxic drug typically used to treat aggressive forms of MS that are refractory to treatment with interferon-β and glatiramer acetate (Correale et al., 2005). Treatment with mitoxantrone results in long-lasting immunosuppression and is thought to decrease the number of demyelinating lesions and inhibit the progression of disability (Edan et al., 1997, Hartung et al., 2002), but also renders the patient immunocompromised.

Natalizumab (Tysabri®) is a monoclonal antibody that blocks the adhesion molecule α-4 integrin (a component of VLA-4), inhibiting the entry of lymphocytes into the CNS (Hutchinson, 2007). In clinical trials natalizumab has been shown to reduce the relapse rate and the development of gadolinium-enhancing lesions in RRMS (Polman et al., 2006, Rudick et al., 2006). However, this drug was briefly withdrawn from the market due to associations with the deaths of several patients following treatment (Kleinschmidt-DeMasters and Tyler, 2005, Langer-Gould et al., 2005). These patients had developed progressive multifocal leukoencephalopathy (PML), an often fatal condition caused by the reactivation of JC virus (as a result of the inhibition in immune surveillance), and leads to demyelination within the CNS (Wenning et al., 2009, Stuve et al., 2006). Although natalizumab was allowed back onto the market, JC virus is a relatively common infection (Taguchi et al., 1982), and therefore the development of PML remains a risk.

Additional new-line therapies include Fingolimod (FTY7220, Gilenya®), alemtuzumab (Campath®) and rituximab (Rituxan®). Figolimod is an oral therapy that blocks the sphingosine 1-phosphate receptor, $S1P_1$, and inhibits the egress of lymphocytes from lymph nodes (Brinkmann et al., 2010). Despite demonstrating clinical efficacy (Kappos et al., 2010), fingolimod is currently under review by the FDA due to some fatalities (Lindsey et al., 2012). Alemtuzumab is a monoclonal antibody that targets CD52 and depletes T and B cells (Minagar et al., 2010). This drug is currently in phase III clinical trials for MS, however, an associated risk is the development of secondary autoimmune conditions (Jones et al., 2009, Cossburn et al., 2011). Rituximab, a monoclonal antibody that targets CD20 and selectively depletes B cells is also in clinical trials for MS (Hauser et al., 2008). However, the exact role of B cells in MS is not currently understood (Ireland and Monson, 2011).

All of these therapeutic regimes, including the new-line therapeutics act non-specifically and therefore fail to treat underlying mechanisms of the disease. Current therapeutics are also frequently associated with adverse side effects and can render patients susceptible to secondary infections and potentially neoplasia. (Barker et al., 2007). As such, there is a pressing need to develop methods of specifically targeting the relevant pathogenic factors that instigate and maintain disease, whilst leaving normal immune function intact. Antigen-specific therapy offers the possibility of selectively switching-off only the T cells involved in driving disease. Such approaches have been developed in EAE using peptide antigens (discussed in further detail in section 1.5) and are currently in clinical trials for MS (clinical trials identifier: NCT01097668).

1.4.2 EAE

Development of the EAE model began after Louis Pasteur's vaccine against rabies was found to induce encephalomyelitis (Balaguer 1888). The vaccine involved the injection of spinal cord matter from rabbits infected with rabies into humans. Later studies investigating this phenomenon demonstrated that injection of emulsified brain or spinal cord material into rabbits and rhesus monkeys also induced

encephalomyelitis, and was characterised by perivascular inflammatory infiltrates and demyelination within the CNS (Baxter, 2007). Through the use of complete Freund's adjuvant (CFA, a mineral oil containing heat-inactivated *mycobacterium*) emulsified with CNS homogenate, the reliability of encephalomyelitis induction was improved, and could be induced by a single injection (Kabat et al., 1946, Freund et al., 1947). These experiments form the basis of the EAE model used today. EAE is a useful model of organ-specific autoimmune disease and due to its demyelinating nature, is often referred to an animal model of multiple sclerosis.

1.4.2.1 Immunopatholgy of EAE

Through the identification of several CNS auto-antigens it has been demonstrated that EAE can be induced in susceptible strains of mice through immunisation with whole protein or encephalitogenic peptides from myelin basic protein (MBP) (Laatsch et al., 1962, Zamvil et al., 1986), Proteolipid protein (PLP) (Tuohy et al., 1988, Tuohy et al., 1989), and myelin oligodendrocyte glycoprotein (MOG) (Amor et al., 1994). Disease can also be induced by the adoptive transfer of activated $CD4^+$ T cells isolated from immunised WT mice or *in vitro* activated TCR transgenic cells, into naïve recipient mice (Stromnes and Goverman, 2006).

EAE is a $CD4^+$ T cell mediated disease, as demonstrated by the inhibition of disease induction by treatment with α-CD4 depleting antibodies (Waldor et al., 1985), and the induction of disease in naïve mice by the transfer on encephalitogenic $CD4^+$ T cells (Ben-Nun et al., 1981, Zamvil et al., 1985). Although $CD8^+$ T cells have been implicated in the pathology of MS, it has been demonstrated that MOG-reactive $CD8^+$ T cells do not induce EAE, whereas MOG-reactive $CD4^+$ T cells do (Leech et al., 2011). A role for B cells has also been suggested in MS, however, it has been observed that EAE can be induced in B cell-deficient mice (Wolf et al., 1996, Hjelmstrom et al., 1998, Fillatreau et al., 2002). These studies implicate that both $CD8^+$ T cells and B cells may not play a role in the induction of MS.

During EAE, immunisation with CNS antigens in CFA activates antigen-reactive $CD4^+$ T cells within the draining lymph nodes, and once activated these cells can then migrate into the CNS (Furtado et al., 2008, Goverman, 2009). In the passive transfer models of EAE, pre-activated myelin-reactive T cells are administered (usually intravenously, or intraperitoneally) and these subsequently migrate into the CNS (Baron et al., 1993). Once there, they are re-activated and produce pro-inflammatory cytokines that activate CNS-resident microglia and recruit macrophages from the peripheral circulation and tissues (Ponomarev et al., 2005, Ajami et al., 2011). Activated microglia up-regulate antigen presentation and secrete pro-inflammatory cytokines such as IL-1, IL-6 and TNF-α, which acts to further disrupt the BBB and enhance the recruitment of immune cells into the CNS (Conrad and Dittel, 2011, Prendergast and Anderton, 2009). Inflammatory macrophages induced by the pro-inflammatory cytokines secreted by $CD4^+$ T cells are thought to be directly responsible for the destruction of the myelin sheath and damage to oligodendrocytes (Epstein et al., 1983, Benveniste, 1997).

The mechanisms of T cell entry into the CNS are not understood but several chemokine receptors and adhesion molecules have been implicated. These include the P-selectin glycoprotein ligand-1 (PSGL-1), CXCR5, and CXCR3. PSGL-1 binds to P-selectin on endothelial surfaces and is thought to mediate the initial stages of cell extravasation through the endothelial wall into sites of inflammation (Kerfoot and Kubes, 2002). Although T cells are thought to up-regulate PSGL-1 upon activation, PSGL-1 requires glycosylation before it can bind to P-selectin (McEver and Cummings, 1997). Despite this requirement, studies in MS patients have shown elevated levels of PSGL-1 expression on circulating $CD4^+$ cells, and enhanced migration of these cells through a brain-derived endothelial cell layer, compared to healthy controls (Bahbouhi et al., 2009). Using a passive transfer model of EAE it has also been demonstrated that pre-incubation of activated myelin-reactive T cells with α-PSGL-1 blocking antibody antagonises or delays the induction of EAE (Deshpande et al., 2006)(Prendergast, 2011). The frequency of $CCR5^+$ and $CXCR3^+$ $CD4^+$ cells has also been found to be higher in the CSF of MS patients compared to healthy controls (Balashov et al., 1999). It has been

demonstrated that blockade of CXCR3 can inhibit the induction of passive but not active EAE (Sporici and Issekutz, 2010). Whereas, in the case of CCR5, it has been shown that CCR5-deficient mice are fully susceptible to EAE (Tran et al., 2000). Regardless of these findings, the ligands for CXCR3 and CCR5 are not constitutively expressed but are up-regulated in the inflammatory milieu (Lacotte et al., 2009, Fujita et al., 2004). Therefore, expression of these chemokine receptors on pathogenic $CD4^+$ T cells may not be required for the initial entry of the pathogenic T cells into the CNS, but could act to help later waves of these cells migrating to the CNS. Another chemokine receptor that is over-represented in MS patients is the Th17-associated CCR6. In EAE, it has been demonstrated that CCR6 may enable the migration of Th17 and Treg cells into the CNS (Yamazaki et al., 2008). However, investigation into the requirement of CCR6 for the induction of EAE has yielded conflicting results. Several studies have reported a reduction in the severity of EAE in CCR6 deficient mice (Reboldi et al., 2009, Yamazaki et al., 2008, Liston et al., 2009), whereas studies by Elhofy et al., and Villares et al., have observed that CCR6 deficient mice developed a more severe disease course than WT mice (Elhofy et al., 2009, Villares et al., 2009). The disparity in these observations may reflect the contentious issue regarding the role Th17 cells play in EAE.

EAE was long considered as a Th1-mediated autoimmune disease (Ando et al., 1989, Bright et al., 1998). However, despite the observation that blockade of the IL-12p40 subunit protected from the induction of EAE (Bright et al., 1998), mice deficient in IFN-γ were found to be susceptible to disease induction (Ferber et al., 1996). These conflicting results cast doubt over the requirement for Th1 cells in the induction of EAE. The subsequent discovery of IL-23, a cytokine which shares the p40 subunit of IL-12 appeared to solve this paradox. As mentioned in section 1.1.3.3, studies by Cua et al., demonstrated that IL-12 deficient mice were fully susceptible to EAE induction, whereas IL-23 deficient mice were resistant (Cua et al., 2003). This observation, coupled with the discovery of a novel T cell subset thought to be induced by IL-23 (Th17 cells), challenged the standing dogma that EAE was a Th1-mediated disease (Langrish et al., 2005). However, the idea that EAE was a Th17-mediated disease was also later challenged when it was

determined that the induction of EAE was not abrogated in the absence of IL-17 (Komiyama et al., 2006). In addition, studies from the Anderton laboratory demonstrated that myelin-reactive T cells polarised to a Th1 phenotype could effectively induce EAE whereas cells polarised to a Th17 phenotype could not (O'Connor et al., 2008). Further experiments suggested that Th1 cells are in fact the pioneer cells required for the induction of disease, and that Th17 cells can migrate into the CNS at a later stage (Prendergast, 2011). This paradox was further complicated by the observation that both T-bet and IL-23 are required for the induction of EAE (Bettelli et al., 2004, Cua et al., 2003, Lovett-Racke et al., 2004, Thakker et al., 2007). Taken together, all of this evidence suggests that EAE is not explicitly a Th1 or Th17 driven disease. This paradox has been some-what explained by recent studies that have demonstrated the absolute requirement of GM-CSF production by T cells in the induction of EAE (Ponomarev et al., 2007, Kroenke et al., 2010). In addition, GM-CSF is found to be produced by both Th1 and Th17 cells (El-Behi et al., 2011). Therefore, rather than an absolute requirement of Th1 or Th17 cells for the induction of EAE, it is the ability of these cells to produce GM-CSF that dictates their potential to induce disease. It is thought that the production of GM-CSF may play a role in instigating disease through the activation of macrophages and other APC within the CNS (Ponomarev et al., 2007).

Although pathogenic T cells mediate disease induction it is thought that regulatory T cells may be required for the resolution of disease. Evidence of this stems from the observation that $CD25^{+}Foxp3^{+}$ cells accumulate in the CNS during EAE, and this coincides with recovery from disease (McGeachy et al., 2005). In addition, depletion of $CD25^{+}$ cells during the peak of disease was shown to inhibit recovery from EAE. Treg accumulation in the CNS has been shown to occur through the activation and proliferation of these cells within the inflamed CNS rather than recruitment of these cells from the periphery (O'Connor et al., 2007). Therefore, these data suggest that inflammation drives accumulation of Treg cells in the target organ, and that these cells can then induce resolution of inflammation and enable disease recovery. The use of Treg therapeutically has also been demonstrated

whereby transfer of MBP-reactive Treg from TCR-transgenic Tg4 mice can inhibit disease induced by immunisation of MBP peptide, and can also be used to accelerate recovery of disease when administered during a chronic EAE disease course.

In the context of MS, although no differences have been observed in the number of Treg cells in the peripheral blood of MS patients compared to WT controls, the Treg in MS patients have been shown to be functionally impaired (Viglietta et al., 2004). It was observed that $CD4^+CD25^+$ cells isolated from the peripheral blood of MS patients were unable to suppress $CD4^+CD25^-$ responder cells, whereas those from healthy controls could. It has also been demonstrated that Treg cells from MS patients have a lower level of Foxp3 expression that those from healthy controls (Venken et al., 2008). These observations may indicate a lack in the ability to control inflammation, and may explain why multiple relapses can occur in MS patients.

1.4.2.2 TCR transgenic models of EAE

The use of traceable $CD4^+$ T cells from TCR transgenic mice such as the Tg4 ($H\text{-}2^u$) and 2D2 ($H\text{-}2^b$) strains specifically enables the study of the antigen-reactive T cells involved in disease pathogenesis. Tg4 TCR transgenic mice have $CD4^+$ T cells that recognise the Ac1-9 peptide of MBP (Liu et al., 1995)(section 1.5.5), whereas $CD4^+$ T cells from 2D2 mice recognise the 35-55 peptide of MOG (pMOG) (Bettelli et al., 2003); both MOG and MBP are CNS antigens that have been identified as potential target self-antigens in MS (Ben-Nun et al., 1996). Active EAE can be induced by the transfer of naive $CD4^+$ cells from Tg4 or 2D2 mice into B10.PL (Tg4), C57BL/6xB10.PL (Tg4) or C57BL/6 (2D2) hosts, before subsequent immunisation with antigen in CFA (O'Connor et al., 2010)(Chung, 2008). The antigen reactive T cells become activated in the peripheral lymph nodes and are subsequently able to migrate into the CNS, where they instigate disease pathogenesis. Although active EAE can be induced in C57BL/6 and B10.PL mice without the transfer of antigen-reactive T cells, in C57BL/6xB10.PL mice the

induction of EAE requires the presence of antigen-reactive Tg4 cells. Therefore, use of the C57BL/6xB10.PL model explicitly enables investigation into the population of cells responsible for driving disease pathogenesis. T cells from TCR transgenic mice can also be used to induce a passive transfer model of EAE, whereby $CD4^+$ T cells from Tg4 or 2D2 mice can be activated *in vitro* in the presence of antigen and Th1-polarising cytokines, before transfer into recipient mice (O'Connor et al., 2008, Williams et al., 2011). Following transfer, these activated cells care able to migrate into the CNS and induce disease.

The many similarities in the immunopathology and demyelination between EAE and MS, makes this animal model a useful tool in the development of therapeutics for use in treating MS. The use of transgenic models of EAE enables the effect of potential therapeutic regimens on the pathogenic cells driving disease to be investigated.

1.5 Antigen-specific therapy

As mentioned in section 1.4.1.4 the mainstay of current therapeutics for MS, and indeed the majority of autoimmune diseases, relies upon non-specific immunosuppression. In contrast, antigen-specific therapy offers the opportunity to specifically target the T cells driving autoimmune pathogenesis.

Antigen-specific therapy was first utilised in the treatment of allergy over 100 years ago by Noon & Freeman, whereby subcutaneous injection of pollen was used to prevent hayfever in humans (Cohen et al., 2003). The use of whole protein antigen to treat allergy is still in use today, however, due to the repeated exposure to antigen an associated risk is the development of anaphylactic reactions. Anaphylaxis occurs due to the generation of antigen-specific IgE and IgG_1 antibodies upon initial sensitisation; additional contact with antigen leads to the cross-linking of these antibodies on the surface of mast cells and the release of inflammatory factors such

as histamine (Moote and Kim, 2011, Peavy and Metcalfe, 2008). The use of immunodominant peptides in place of whole antigen offers a safer alternative as the short peptides are less likely to bind IgE and/or IgG1 (Francis and Larche, 2005).

The basis of antigen-specific or peptide therapy involves the induction of tolerance in the antigen-reactive $CD4^+$ T cells that are driving pathogenesis. This is achieved through the administration of antigen in a tolerogenic form i.e. soluble peptides in the absence of adjuvant (Figure 1.4). Peptide therapy offers great potential for the treatment of autoimmune disease, and the administration of soluble peptides has proven to be effective when administered prophylactically in animal models of disease (Gaur et al., 1992, Hoyne et al., 1993). There are various modes and routes of tolerogenic peptide administration, and the specific mechanisms of tolerance induction are thought to vary depending on these factors. Administration of peptide via the oral, intranasal, subcutaneous, intraperitonal and intravenous routes have all been shown to effectively lead to the induction of tolerance (Aichele et al., 1995, Daniel and Wegmann, 1996, Sayegh et al., 1996, Kearney et al., 1994). In addition to other protocols, peptides can induce tolerance when delivered within the context of a DNA vaccine, bound to ethylene carbodiimide (ECDI) fixed-APC or simply in a soluble form (Turley and Miller, 2007, Hochweller and Anderton, 2004, Ferrera et al., 2007). Much akin to mechanisms of peripheral tolerance, the administration of tolerogenic peptides has been shown to induce death, anergy and regulation of antigen-reactive T cells, depending on the precise model under investigation (Miller et al., 2007).

1.5.1 Models to define T cell behaviour during peptide-induced tolerance

Initial studies into the effects of antigen-specific therapy were conducted using non-transgenic wild-type (WT) mice (Swanborg, 1973, Kennedy et al., 1988, Gaur et al., 1992). Although these models demonstrated the effective protection from autoimmune disease induction by prophylactic administration of tolerogenic

peptides, the effect of soluble peptide administration on the antigen-reactive T cells could not be demonstrated.

The advent of TCR transgenic mice enabled this to be investigated, as all of the T cells within these mice recognise a particular peptide epitope. However, administering antigenic peptides to intact TCR transgenic mice makes it impossible to accurately track the effects of the peptide, given that essentially all T cells are responsive and will likely be stimulated to differing degrees depending on their location and state of differentiation. This problem was overcome by the Jenkins group who first described the transfer of a cohort of naïve TCR transgenic T cells into MHC-syngeneic wild type recipients (Kearney et al., 1994). That study used OVA-reactive DO11.10 TCR transgenic T cells which could be traced in recipient BALB/c by flow cytometry using a clonotypic antibody recognizing the DO11.10 TCR. Since then, the use of such "Jenkins chimera" experiments has become a standard *in vivo* tool for the assessment of antigen-reactive T cells in many experimental scenarios, including antigen-induced tolerance. Very few TCR transgenic models benefit from the availability of clonotypic antibodies, and so rely on antigenic disparities between the donor TCR transgenic cells and the recipient immune system in either CD90 (expressed by T cells) or CD45 (expressed by all leukocytes).

The use of these models has enabled investigation into the effect of peptide-induced tolerance on both $CD4^+$ and $CD8^+$ antigen-reactive T cells. In the context of $CD4^+$ T cells, many TCR transgenic strains have been utilised, examples of which include DO11.10, OT-II, 2D2 and Tg4 mice. T cells from both DO11.10 ($H\text{-}2^d$) and OT-11 ($H\text{-}2^b$) mice recognise the 323-339 peptide of ovalbumin (pOVA); a protein found in hen egg whites (Robertson et al., 2000). In contrast, T cells from 2D2 ($H\text{-}2^b$) and Tg4 ($H\text{-}2^u$) mice recognise peptides from self-antigens, namely pMOG and the Ac1-9 of MBP respectively (section1.4.2.1) (Liu and Wraith, 1995, Bettelli et al., 2003). As both 2D2 and Tg4 $CD4^+$ T cells can be used to induce EAE (section 1.4.2.2), the use of these cells enables the effect of peptide-induced tolerance to be investigated in antigen-reactive T cells within the context of autoimmune disease.

Furthermore, due to the explicit requirement of Tg4 cells in the induction of EAE in C57BL/6 x B10.PL mice (section 1.4.2.2); this model permits investigation into the effects of peptide therapy on the cells responsible for driving disease.

Although the use of TCR transgenic models permits the investigation of T cells that recognise a specified target antigen, human autoimmune disease is a more complex situation where polyclonal T cell populations are present. Moreover, the diverse TCR repertoire between individuals and the ability of antigenic peptides to be presented by a variety of different HLA molecules means that in a given human population any one peptide can be displayed and recognised in a vast number of different configurations. These differences in the fine specificity of T cells could mean that a peptide with an antagonistic effect in one individual could prove to have an aganostic effect in another. Indeed, murine models have already demonstrated that a TCR antagonist altered peptide ligand (APL) (as defined by in vitro responses of T cell lines derived from TCR transgenic mice) could act agonistically when given in immunogenic form to non-transgenic mice (Anderton et al., 1998). Therefore, it is important to note that although studies utilising TCR transgenic models can identify important underlying mechanisms of peptide-induced tolerance, they should not form the sole basis of the argument for the clinical translation of peptide therapy.

1.5.2 Mechanisms of peptide-induced tolerance

1.5.2.1 Tolerance as a result of insufficient co-stimulation

As mentioned in section 1.1.2.3, signalling through the TCR induces the up-regulation of Fas, FasL and Bim expression, whereas co-stimulation through CD28 and OX40 counteracts the effect of these molecules by up-regulating Bcl-2 and Bcl-xL (Boise et al., 1995, Rogers et al., 2001). Peptide-induced tolerance relies upon the presentation of peptide in the absence of inflammation. This lack of activating stimuli enables the peptide to be presented by immature APC, which can engage the TCR of peptide-reactive T cells but do not provide necessary

costimulatory signals. Therefore, soluble peptide administration can result in the death of peptide-reactive T cells due to a failure to up-regulate anti-apoptotic molecules following TCR stimulation.

There are two mechanisms by which T cells can be instructed to undergo apoptosis; via cell intrinsic and extrinsic pathways. The extrinsic pathway is induced by signalling through death receptors on the cell surface, such as the binding of Fas with FasL (Elmore, 2007). In contrast, the intrinsic pathway is regulated by the balance of intracellular pro- and anti-apoptotic Bcl-2 family proteins. Pro-apoptotic molecules such as Bim induce permeabilisation of the mitochondrial outer membrane, which leads to release of cytochrome c and activation of the caspase cascade (Bouillet and O'Reilly, 2009). The anti-apoptotic Bcl-2 family members such as Bcl-2 and Bcl-xL antagonise the actions of the pro-apoptotic molecules and act to prevent the induction of apoptosis (Chipuk et al., 2008). In addition to regulating the intrinsic apoptotic pathway, Bcl-2 and Bcl-xL have also been reported to regulate the Fas-mediated extrinsic pathway (Srinivasan et al., 1998, Jaattela et al., 1995, Sun et al., 2002). Ultimately, both the intrinsic and extrinsic pathways drive apoptosis by triggering caspase cascades; the intrinsic pathway leads to activation of capsase 9 (Pop et al., 2006), whereas the extrinsic pathway activates caspase 8 (Walczak and Krammer, 2000). Both caspase 8 and capase 9 activate the executioner caspases, such as caspase 3, which mediate cell death (Fulda and Debatin, 2006). The role of each pathway in the control of T cell populations is a controversial area. The intrinsic pathway is thought to be involved in passive cell death which can be triggered due to cytokine and growth factor deprivation (Wiegers et al., 2011, Park et al., 2002). In contrast, Fas-mediated apoptosis is thought to be involved in activation-induced cell death (AICD), which can occur through repetitive TCR stimulation in the presence of IL-2 (Arnold et al., 2006). Both the intrinsic and extrinsic pathways have been implicated in the induction of apoptosis during resolution of a primary immune response (Hildeman et al., 2002, Tischner et al., 2010).

The induction of cell death by administration of soluble peptides has been shown in various experimental models; these include the deletion of $CD8^+$ LCMV-reactive T cells by administration of LCMV glycoprotein peptide (Kyburz et al., 1993), and of $CD4^+$ OT-II and 2D2 cells by treatment with soluble pOVA or pMOG respectively (Pape et al., 1998, Hochweller and Anderton, 2005, Konkel et al., 2010). Tolerance induction has been shown to occur through an active process, whereby peptide-reactive T cells undergo and initial phase of clonal expansion before deletion occurs, a process that takes three days to occur (Liu and Wraith, 1995, Hochweller et al., 2006b). In addition, it has been observed that the induction of tolerance can be abrogated by ligation of OX-40 and CD40 (both of which promote OX-40 signalling) (Hochweller and Anderton, 2005, Hochweller et al., 2006a, Bansal-Pakala et al., 2001). The ability of OX-40 to up-regulate Bcl-2 has led to the consensus view that the deletion of antigen-reactive T cells in peptide-induced tolerance is mediated by the intrinsic pathway (Hochweller et al., 2006b).

Although deletion of antigen-reactive T cells by soluble peptide administration has been shown to play a major role in the induction of tolerance in many experimental models, the induction of anergy has also been observed (see section 1.5.2.2) (Kearney et al., 1994). Furthermore, the induction of tolerance in the absence of profound cell death has also been described in some models of tolerogenic peptide administration (Getts et al., 2007)(Konkel, 2009). Thus the idea that peptide-induced tolerance revolves solely around the induction of apoptosis is over-simplistic.

1.5.2.2 Tolerance due to dominant co-inhibition

Several studies have demonstrated the survival of a small population of antigen-reactive cells that have been rendered hyporesponsive to further stimulation following soluble peptide administration (Kearney et al., 1994, Pape et al., 1998, Dubois et al., 1998). These cells are long lasting and are found to have reduced capacity for proliferation, and production of IL-2 and effector cytokines when re-challenged with antigen *in vitro* (Pape et al., 1998, Hochweller and Anderton,

2005). The induction of this "anergic" population (note that this phenotype differs from classical anergy in that effector cytokine production is inhibited) may depend on the dose of antigen, as high dose antigen has been associated with deletion and the induction of anergy in cells that survive, whereas low dose antigen is thought to induce suppression (Friedman and Weiner, 1994, Gregerson et al., 1993). Furthermore, Choi & Schwartz have suggested that different biochemical states of anergy may be induced depending on the mode and route of tolerogenic antigen administration (Choi and Schwartz, 2007). Factors responsible for the induction and maintenance of peptide-induced anergy are not fully understood, but the role of co-inhibitory molecules such as CTLA-4 and PD-1 has been implicated.

1.5.2.2.1 The role of CTLA-4 in peptide-induced tolerance

CTLA-4 is an inhibitory cell-surface molecule up-regulated on conventional $CD4^+$ and $CD8^+$ T cells upon activation (Brunet et al., 1987, Linsley et al., 1992). CTLA-4 is a member of the immunoglobulin superfamily and is thought to inhibit T cell responses through negative signalling and competition with CD28 for binding to CD80 and CD86 (Carreno et al., 2000). A cell intrinsic negative signalling mechanism has been demonstrated by engagement of CTLA-4 using an antibody, which results in the inhibition of IL-2 production and T cell proliferation (Krummel and Allison, 1995, Krummel and Allison, 1996). CTLA-4 has also been shown to have a much higher binding affinity for CD80 than CD28 and therefore is effective at inhibiting co-stimulatory signals through CD28 (van der Merwe et al., 1997, Engelhardt et al., 2006). The function of CTLA-4 is important in controlling primary immune responses and tolerance to self. This is highlighted by the observation that mice lacking CTLA-4 develop fatal autoimmunity and die within three-to-four weeks of birth (Waterhouse et al., 1995, Tivol et al., 1995).

In addition to immunogenic antigen exposure, CTLA-4 is also found to be up-regulated on T cells following tolerogenic peptide administration (Metzler et al., 1999). Blockade of CTLA-4 can prevent the induction of tolerance in antigen-reactive T cells following soluble administration of antigen (Perez et al., 1997,

Samoilova et al., 1998). In contrast, studies by Eagar et al., using ECDI fixed antigen-coupled splenocytes have implicated a role for CTLA-4 in the maintenance but not the induction of tolerance, whereby blockade of CTLA-4 did not inhibit the induction of an unresponsive state in antigen-reactive T cells but could reverse unresponsiveness upon recall stimulation (Eagar et al., 2002, Eagar et al., 2004). Despite these observations, other models of antigen-specific tolerance have been shown to occur through CTLA-4-independent mechanisms (Ratts et al., 1999, Sotomayor et al., 1999, Tsitoura et al., 1999, Frauwirth et al., 2001). Therefore, the exact role that CTLA-4 plays in peptide-induced tolerance is not clear-cut, and may likely depend upon the mode of treatment application.

1.5.2.2.2 The role of PD-1 in peptide-induced tolerance

A further co-inhibitory molecule implicated in the induction of tolerance is the PD-1. Like CTLA-4, PD-1 is up-regulated on $CD4^+$ and $CD8^+$ T cells upon activation and also plays a role in controlling primary immune responses and maintaining peripheral tolerance (Agata et al., 1996, Keir et al., 2008). The ligands for PD1, PDL-1 and PDL-2 are expressed on a variety of cell types; PDL-1 is widely expressed and is found on epithelial cells, parenchymal cells, lymphocytes and myeloid cells including APC (Freeman et al., 2000, Keir et al., 2006), whereas PDL-2 expression is more restricted and is found mainly on activated APC (Tseng et al., 2001). It has recently been reported that PDL-1, which is up-regulated on T cells upon activation, can also bind to CD80 and therefore may play a similar role in competitive inhibition as CTLA-4 (Butte et al., 2007). However, the main mechanism by which the PD-1 pathway is thought to function is through delivery of negative signals to the T cell (Figure 1.3). Studies by Parry et al., have demonstrated that although PD-1 and CTLA-4 both inhibit the PI3K/AKT signalling pathway, they act by distinct mechanisms, whereby PD-1 inhibits CD28-mediated PI3K phosphorylation and CTLA-4 acts downstream to inhibit AKT phosphorylation (Parry et al., 2005). PD-1 contains an immunoreceptor tyrosine-switch motif (ITSM) that is required for suppression of T cell activation, and is thought to function by recruiting SHP-2, a phosphatase that can inhibit the PI3K

pathway (Zhang et al., 2002, Chemnitz et al., 2004). Signalling through PD-1 upon TCR stimulation has been shown to inhibit proliferation, and the production of IL-2 and effector cytokines (Freeman et al., 2000, Sandner et al., 2005, Keir et al., 2006). A further mechanism by which PD-1 signalling can potentially inhibit immune responses is by promoting reverse signalling through PDL-1 and PDL-2, which is thought to inhibit APC maturation (Kuipers et al., 2006, Keir et al., 2008). The expression of both PD-1 and PDL-1 on activated T cells also suggests that populations of activated T cells could potentially self-regulate and that negative signalling could be received through either PD-1 or PDL-1. In addition to controlling primary immune responses, the role of PD-1 signalling in maintaining peripheral tolerance has also been demonstrated by the observation that PD-1 deficient mice develop autoimmune pathology, albeit with a delayed onset and less severe phenotype than CTLA-4 deficient mice (Nishimura et al., 1999).

Evidence that implicates PD-1 signalling in peptide-induced tolerance stems from the observation that PD-1 is up-regulated on antigen-reactive T cells following soluble peptide administration (Hochweller and Anderton, 2005, Konkel et al., 2010). In addition, blockade of, or deficiency in, PD-1 has been shown to inhibit the induction of tolerance, or reverse established unresponsiveness, in antigen-reactive cells $CD8^+$ T cells after soluble peptide administration (Tsushima et al., 2007, Chikuma et al., 2009). In particular, the study by Chikuma et al., demonstrated that PD-1 may function to induce tolerance via inhibition of IL-2 production, as addition of IL-2 prevented the induction of anergy in the presence of PD-1 signalling (Chikuma et al., 2009). It has also been demonstrated that lack of PD-1 signalling can convert a tolerogenic stimulus into an immunogenic one (Probst et al., 2005), which in certain models can lead to the onset of autoimmune pathology (Reynoso et al., 2009). The role of PD-1 signalling in the maintenance of tolerance has been observed in the nonobese diabetic (NOD) mouse model of diabetes induced by the transfer of transgenic $CD4^+$ T cells specific for an islet antigen. It was observed that blockade of PD-1 signalling was able to reverse the tolerogenic effects of ECDI-fixed peptide-coupled splenocytes, and resulted in the development of disease (Fife et al., 2006).

Figure 1.3 Potential mechanisms of PD-1 mediated inhibition of T cell activation

The majority of studies that demonstrate a requirement for PD-1 signalling in the induction of tolerance have been in the context of $CD8^+$ T cells. Although the study by Fife et al., observed roles for both PD-1 and CTLA-4 signalling in the induction of tolerance in $CD4^+$ T cells using ECDI-fixed APC (Fife et al., 2006), another study using a deletional model of tolerance induction specifically identified the requirement of PD-1 signalling for the induction of tolerance in $CD8^+$ T cells but not $CD4^+$ T cells (Haspot et al., 2008). This is consistent with a recent study showing that PD-1 signalling could restrict the clonal expansion of $CD4^+$ T cells in response to an immunogenic administration of peptide, but was not required for peptide-induced tolerance (Konkel et al., 2010). These conflicting results may reflect differences in the mode or route of tolerogenic antigen administration and highlight the need for further clarification of the role of PD-1 signalling in the induction and maintenance of tolerance in $CD4^+$ T cells.

1.5.2.2.3 Immune deviation

In addition to anergy several models of peptide-induced tolerance have described a change in the phenotype of response. This deviation in immune response is associated with a reduction in pro-inflammatory Th1-associated cytokine production such as IFN-γ and TNF-α and increased production of Th2 cytokines such as IL-4, IL-5 and IL-10 upon tolerogenic antigen administration (Degermann et al., 1996, Prakken et al., 2004, Rocken et al., 1996). However, the role by which this occurs is not fully understood and in light of the recent findings surrounding the plasticity of T cell subsets (section 1.1.3.6), may not provide a desirable outcome for antigen-specific therapy if deviation can be reversed.

1.5.2.3 Peptide-induced tolerance: regulation

The induction of T cells with a regulatory phenotype has also been described in several models of antigen-specific therapy. Due to the immunosuppressive environment of the gut, oral administration of protein antigen has been shown to induce cells with a Th3 phenotype, and inhibit the induction of autoimmune disease

upon subsequent challenge (Chen et al., 1994, Hafler et al., 1997, Miller et al., 1992). Oral tolerance has also been shown to activate nTreg (Zhang et al., 2001) and under certain circumstances can promote the generation of iTreg (Mucida et al., 2005). In contrast, intranasal administration of antigen is associated with the induction of IL-10 producing Tr1 cells, and has been shown to be effective at inhibiting various models of autoimmune and allergic disease (Burkhart et al., 1999, Akbari et al., 2002, Chen et al., 2003a, O'Neill et al., 2006). Furthermore, studies by Gabrysova et al., have demonstrated that by repeated administration of intranasal peptide, IL-10 secreting cells can be induced from IFN-γ producing, T-bet^{+} Th1 cells (Gabrysova et al., 2009). The induction of T cells with regulatory function versus deletion or anergy of antigen-reactive T cells upon oral or nasal administration of antigen is likely a factor of the dose of antigen treatment, with high doses of antigen inducing deletion and anergy and low doses resulting in regulation (Weiner, 2001). Induction of cells that secrete immunosuppressive cytokines such as Tr1 and Th3 cells may also enable bystander suppression, where tolerogenic antigen administration of one peptide epitope can induce tolerance to different epitopes (Anderton and Wraith, 1998). This characteristic would be favourable in the context of autoimmune disease where the number of target autoantigens may have expanded due to the phenomenon of epitope spreading, provoked by chronic inflammatory tissue damage (Vanderlugt and Miller, 2002). The role of T cells with regulatory function in tolerance induced by systemic peptide administration is less well understood. However, studies by Chappert et al., have demonstrated expansion of CD4^{+}Foxp3^{+} cell populations following intravenous administration of peptide (Chappert et al., 2008).

As is clear from the mechanisms of peptide-induced tolerance described, the predominant phenotype of the response seems to be dependent upon the route of tolerogenic peptide administration and the method in which the antigen is targeted and presented to the antigen-reactive T cell.

1.5.3 Peptide therapy and ongoing disease

Despite the characterisation of peptide-induced tolerance in naïve antigen-reactive T cells, and the effective prophylactic use of peptide therapy to inhibit animal models of autoimmune disease, the clinical requirement is to switch-off ongoing disease where antigen-experienced activated or memory T cells may be present. The exacerbation of clinical disease observed in several clinical trials of peptide therapy highlights the necessity to understand the effect of peptide therapy on the pathogenic antigen-experienced T cells driving disease.

The administration of soluble peptides has been shown to be effective at switching-off naïve autoreactive T cells and can inhibit the induction of autoimmune disease when administered prophylactically (Critchfield et al., 1994). However, the effect of tolerogenic peptide administration on traceable antigen-experienced TCR transgenic T cells has been less widely characterised. As shown in Figure 1.4, the basis of peptide-induced tolerance in naïve T cells relies upon the presentation of antigen in the absence of co-stimulatory signals. However, activated and memory T cells are less dependent upon co-stimulation, and can become re-activated through TCR signalling alone (Croft et al., 1994). This implies that soluble peptide administration given during ongoing disease may act to enhance the activation of antigen-experienced effector T cells, with the potential to exacerbate rather than ameliorate disease.

Several studies have reassuringly demonstrated that soluble peptide administration given after the onset of EAE can reduce the clinical signs of disease (Samson and Smilek, 1995, Devaux et al., 1997). However, investigation into the effect of soluble peptide administration on antigen-experienced T cells has been hindered due the occurrence of severe anaphylactic reactions upon repeat encounter with the peptide (Pedotti et al., 2001, Marshall, 2001). These observations demonstrate that despite the use of peptides instead of whole antigen, anaphylaxis can still occur. In addition, studies by Getts et al., have demonstrated that administration of antigen-coupled splenocytes targeting activated or memory $CD8^+$ T cells during ongoing pathology in a model of Theiler's murine encephalomyelitis virus-induced

Figure 1.4 Signals required for $CD4^+$ T cell activation vs. peptide-induced tolerance

demyelinating disease, can result in a fatal systemic reaction thought to be mediated by a "cytokine storm" (Getts et al., 2007). Furthermore, a study by Weishaupt et al., in experimental autoimmune neuritis observed increased production of the pro-inflammatory cytokines IFN-γ and TNF-α following tolerogenic peptide administration to treat disease (Weishaupt et al., 1997).

Despite these observations, studies conducted in the Anderton laboratory developed an APL based on the MOG(35-55) sequence that was unable to bind antibody but still ligated the TCR (thereby circumventing anaphylaxis) to show that peptide-induced tolerance can effectively treat EAE. Administration of the APL at the peak of disease led to a dramatic reduction in clinical signs within 24 hours, which coincided with a significant decrease in the total number of $CD4^+$ T cells present in the CNS (Leech et al., 2007). Activated and memory $CD4^+$ T cells are more susceptible to the induction of apoptosis compared to naïve T cells due to up-regulated expression of pro-apoptotic molecules (Brunner et al., 2000, Green et al., 2003). Leech et al., therefore postulated that administration of the pMOG APL in soluble form may have induced deletion of antigen-reactive T cells through AICD, thereby accelerating resolution of disease. In addition, due to the observation that accumulation of $CD4^+Foxp3^+$ Treg within the CNS is required for the resolution of EAE (O'Connor and Anderton, 2008), it can therefore be hypothesised that antigen-reactive Treg might be resistant to AICD following peptide-induced tolerance.

1.5.4 Clinical trials of antigen-specific therapy

Although antigen-specific therapy holds great promise for the treatment of autoimmune disease, the results of clinical trials have been mixed. Several clinical trials of antigen-specific therapy have demonstrated no significant benefit on disease outcome (Weiner, 1997, Weiner, 2004, Skyler et al., 2005, Chaillous et al., 2000). In particular, a phase III clinical trial using an MBP-derived peptide in MS showed no difference in disease outcome compared to placebo (Freedman et al., 2011). In contrast, several other clinical trials of antigen-specific therapy have reported clinical efficacy. A phase I clinical trial into the use of a proinsulin peptide

in the treatment of T1D has reported increased IL-10 responses in peptide-specific T cells (Thrower et al., 2009). Similarly, a clinical trial using nasal administration of insulin in recent onset T1D reported decreased IFN-γ responses to proinsulin (Fourlanos et al., 2011). Peptide immunotherapy in the context of allergic asthma has also demonstrated the induction of IL-10 responses, whereby administration of peptides derived from the cat allergen Fel d 1, decreased production of cytokines such as IL-4 and IFN-γ, and increased production of IL-10 by allergen-specific T cells (Oldfield et al., 2001, Oldfield et al., 2002). As a result, treatment with Fel d 1-derived peptides has been shown to result in clinical improvement of asthma (Alexander et al., 2005). A phase II clinical trial using peptide therapy in rheumatoid arthritis (RA) has also demonstrated a reduction in effector cytokine production by T cells, and found that this correlated with enhanced expression of PD-1 (Koffeman et al., 2009). Furthermore, a phase I/II clinical trial using a DNA vaccine encoding full-length MBP in RRMS and SPMS reported a decrease in the IFN-γ response of $CD4^+$ T cells to myelin antigens, reduced anti-myelin antibody titres in the cerebrospinal fluid, and a reduction in gadolinium-enhancing lesions within the CNS (Bar-Or et al., 2007).

Despite these encouraging observations, some clinical trials have reported adverse effects such as exacerbation of disease following antigen-specific therapy. In a phase I/II clinical trial using oral administration of multiple retinal antigens in uveitis, disease was worse in the peptide-treated individuals compared to placebo (Nussenblatt et al., 1997). In addition, two separate phase II clinical trials using an altered peptide ligand (APL) based on an immunodominant region of MBP (MBP_{83-99}) in MS had to be halted due to either the development of hypersensitivity reactions (Kappos et al., 2000), or apparent clinical exacerbation (Bielekova et al., 2000).

These conflicting reports on the therapeutic use of peptides to treat ongoing disease, and the observation that antigen-specific therapy has the potential to exacerbate disease, highlights the importance of investigating the effect of soluble peptide

administration on antigen-experienced T cells in circumstances where disease is already established.

1.5.5 The Tg4 TCR transgenic model for studying peptide-induced tolerance in EAE

The observation that encephalitogenic T cell clones from B10.PL (H-2^u) mice recognise the Ac1-9 peptide of MBP (Zamvil et al., 1986) prompted development of the Ac1-9-specific Tg4 TCR transgenic mouse. Tg4 mice were created by the generation of TCR constructs from Ac1-9 specific T cell hybridoma derived from an encephalitogenic T cell clone (Liu et al., 1995). As described in section 1.4.2.2, Tg4 mice are a useful tool in the study of autoimmune disease. A particular advantage to using T cells from Tg4 mice is the availability of APL of Ac1-9 that have a range of effects on Tg4 cells due to their differing binding affinities for MHC II (Liu and Wraith, 1995). The native sequence for Ac1-9 is Ac-ASQKRPSQR. Residues 3Q and 6P six are contact residues for the Tg4 TCR, whereas residues 4K and 5R bind to I-A^u (Wraith et al., 1989, Wraith et al., 1992, Anderton et al., 2001) (Figure 1.5). The WT Ac1-9 peptide (4Lys) has a very weak binding affinity due to the unfavourable interaction of the Lys residue at position four with a hydrophobic pocket within the I-A^u peptide binding grove (Lee et al., 1998, Pearson et al., 1999). APL in which residue four is substituted with Ala (4Ala), or particularly the hydrophobic resides Val (4Val) or Tyr (4Tyr), show graded increases in the binding affinity for I-A^u. This is most extreme for 4Tyr, which shows >100,000-fold increase affinity compared with the wild type 4Lys peptide (Anderton et al., 2001). These alterations in MHC-binding affinities translate directly into improved antigenicity when the APL are used to stimulate Tg4 cells (or other Ac1-9-reactive T cells) in vitro (McCue et al., 2004).

In vivo, although the WT 4Lys Ac1-9 peptide can induce EAE when used to immunize H-2^u mice, it is a relatively poor tolerogen (Metzler and Wraith, 1993). In contrast, the APL with increased I-A^u binding affinities are potent tolerogens. This has been demonstrated by the observation that prophylactic treatment with

4Lys offers little protection from the subsequent induction of EAE, whereas 4Tyr offers the greatest degree of protection (Liu and Wraith, 1995). Both intranasal and intraperitoneal administration of the 4Tyr peptide can protect WT mice from the induction of EAE when given prophylactically (Metzler and Wraith, 1993, Liu and Wraith, 1995). Although the exact mechanisms behind this protection are not fully understood, treatment of intact Tg4 mice with intranasal administration of 4Tyr can result in the induction of IL-10 producing cells (Burkhart et al., 1999). However, using this mode of application in intact Tg4 mice, multiple doses of peptide are required to offer full protection from disease induction (Gabrysova et al., 2009), and it is impossible to accurately assess a cohort of T cells with defined starting characteristics (functional and differentiation state). In other models of peptide-induced tolerance using cells from TCR transgenic mice, it has been shown that a single intravenous high dose of peptide is sufficient to induce tolerance (Hochweller and Anderton, 2005, Konkel et al., 2010).

Despite the observation that 4Tyr administration can also be protective when administered on the first day after disease onset (Liu and Wraith, 1995), the effect of 4Tyr administration on activated and antigen-experienced T cells is not known. For the effective clinical translation of peptides, the effect of soluble peptide administration on T cell behaviour during ongoing disease needs to be characterised.

Figure 1.5 TCR and MHC II I-Au binding residues of Ac1-9

1.6 Hypothesis & Aims

Hypothesis

Antigen-experienced autoimmune effector T cells will undergo apoptosis upon soluble peptide administration, whereas protective antigen-reactive Treg will be resistant to this effect and will persist, giving long-term protection.

The aims of this thesis were:

1) To determine the effect of tolerogenic peptide administration on antigen-experienced pathogenic $CD4^+$ T cells from Tg4 mice, in comparison to naïve Tg4 $CD4^+$ T cells.
2) To investigate the response of Tg4 Treg to tolerogenic peptide administration.
3) To elucidate the mechanisms of peptide-induced tolerance during ongoing EAE.

2. Materials & Methods

2.1 Mice

C57BL/6 (H-2^b), B10.PL (H-2^u), C57BL/6xB10.PL, PD-$1^{-/-}$ (H-2^b) (Nishimura et al., 1998), Tg4 (Liu et al., 1995) (H-2^u, CD45.1 or CD90.1), Tg4×Foxp3.LuciDTR-4 (CD45.1) (O'Connor et al., 2010) and Tg4xPD-$1^{-/-}$ (CD90.1) mice were bred under specific pathogen-free conditions at the University of Edinburgh. Characterisitcs of mouse strains used can be seen in Table 2.1. Tg4 mice are transgenic for a TCR specific for the Ac1-9 peptide of MBP. Tg4xPD-$1^{-/-}$ mice were generated at the University of Edinburgh by crossing Tg4 mice with PD-$1^{-/-}$ mice. Tg4xPD-$1^{-/-}$ mice were genotyped using PCR to detect the presence of the neo-cassette (Tables 2.2 & 2.3), and blood samples were used to detect the presence of the V$\beta8^+$ Tg4 TCR and CD90.1 congenic marker using flow cytometry (Figure 2.1). Cells obtained from Tg4xPD-$1^{-/-}$ and Tg4xFoxp3.LuciDTR-4 mice before 10 generations of back-crossing had been completed were transferred into C57BL/6xB10.PL hosts. Mice were sex matched within experiments and used at 6-10 weeks of age. All experiments had University of Edinburgh ethical approval and were performed in accordance with UK legislation

2.2 Peptides and adjuvant

Acetylated myelin basic protein peptides (Ac1-9) 4Lys (Ac-ASQKRPSQR), 4Ala (Ac-ASQARPSQR), 4Val (Ac-ASQVRPSQR) and 4Tyr (Ac-ASQYRPSQR) were synthesised by the Advanced Biotechnology Centre (Imperial College, London, UK). Complete Freund's adjuvant (CFA) containing 1mg/ml heat-killed *Mycobacterium tuberculosis* H37Ra (MTB) was purchased from Sigma-Aldrich (Poole, UK).

2.3 Buffers and Media

2.3.1 Wash buffer

RPMI 1640 medium containing 25mM Hepes (Gibco, Life Technologies, Paisley, UK)

2.3.2 RPMI-5% & RPMI-10%

RPMI 1640 medium, supplemented with 2mM L-Glutamine (PAA Laboratories Ltd, Somerset, UK), 100U/ml Penicillin (PAA), 100µg/ml Streptomycin (PAA), 50µM 2-mercaptoethanol (Gibco) and 5% or 10% heat-inactivated fetal calf serum (FCS, Sigma).

2.3.3. X-VIVO serum-free medium

X-VIVO 15 serum-free medium (BioWhittaker, USA) supplemented with 2mM L-Glutamine and 50µM 2-mercaptoethanol.

2.3.4 MACS buffer

HANKS balanced salt solution (PAA) supplemented with 2% heat-inactivated FCS.

2.3.5 FACS buffer

Phosphate-buffered saline solution (PBS) supplemented with 2% heat-inactivated FCS and 0.1% sodium azide (Sigma).

2.3.6 Solutions for ELISA

10x Bicarbonate Buffer: 0.1M Na_2CO_3 (Sigma) and 0.2M Na_2HCO_3 (Sigma) in ddH_2O pH 9.6.

Phosphate citrate-buffer: 0.05M Na_2HPO_4 (Sigma) and 0.02M citrate (Fisher Scientific, UK), pH5.

2.4 Antibodies

The RMP1-14 anti-PD-1 Ig (rat IgG2a) was a gift from Dr Hideo Yagata (Juntendo University, Japan). The isotype control antibody was purified rat IgG (Sigma).

2.5 Cell purification & preparations

2.5.1 Preparation of mononuclear cells from spleen and lymph nodes

Splenocytes and peripheral lymph nodes (LN) cells were obtained by mechanical disaggregation of the tissue (between layers of sterile gauze) and re-suspended as a single-cell suspension in wash buffer. For all wash steps, cells were centrifuged at 350g for five minutes. Red blood cells (RBC) were lysed at room temperature for two minutes using RBC lysis buffer (Sigma), and washed twice in wash buffer. Cells were re-suspended in wash buffer and counted using trypan blue (Sigma) exclusion.

2.5.2 Purification of $CD4^+$ T cells

Cells were isolated as in 2.5.1 and incubated with 45µl MACS buffer and 5µl anti-CD4 conjugated magnetic beads (Miltenyi Biotec, Germany) per 10^7 cells, for 15 minutes at 4^oC. Cells were washed in MACS buffer and the pellet re-suspended at 10^8 cells/ml MACS buffer. $CD4^+$ cells were positively selected using an autoMACS Pro Seperator or LS column and MidiMACS separator as per manufacturer's instructions (Miltenyi Biotec). Purity of $CD4^+$ T cells was routinely 95% ± 5%.

2.5.3 CFSE-labelling of $CD4^+$ T cells

$CD4^+$ T cells were obtained (as in 2.5.2) and re-suspended at $5x10^7$/ml in wash buffer and incubated with 5µM carboxyfluorescein succinimidyl ester (CFSE) (Sigma) for 5 minutes at 37^oC. Unbound CFSE was quenched with the addition of serum-containing medium (RPMI-10%). Cells were washed twice in RPMI-10%.

CFSE-labelled cells were cultured *in vitro* (as described in section 2.8.5) and dilution of CFSE was measured using flow cytometry.

2.5.4 Isolation of naïve CD4$^+$ T cell populations by FACS

CD4$^+$ cells were prepared as in 2.5.2 from Tg4×Foxp3.LuciDTR-4 mice and naïve CD4$^+$ T cells were isolated by fluorescence-activated cell sorting (FACS) according to their GFP and/or cell surface marker expression. Cells were stained with anti-CD4-AlexaFluor700 / eFluor450, anti-CD62L-PE and anti-CD25-APC in MACS buffer at 4^{o}C for 20 minutes. Cells were washed and re-suspended at ~1x10^7/ml in MACS buffer. CD4$^+$CD62L^{hi}CD25$^-$GFP$^-$ cells were obtained using a FACSAria II FACS sorter (Becton Dickinson, USA). Purity of naïve CD4$^+$CD62L^{hi}CD25$^-$GFP$^-$ T cells was consistently > 90%.

2.5.5 Isolation of nTreg cells by FACS

CD4$^+$ T cells from Tg4×Foxp3.LuciDTR-4 mice were stained as in 2.5.4. CD4$^+$CD62L^{hi}CD25$^+$GFP$^+$ cells were FACS sorted. Purity of nTreg cells was consistently >95%.

2.5.6 Re-isolation of donor cells from mixed host-donor cell populations

CD4$^+$ cells were prepared as in 2.5.2 from B10.PL host mice and donor CD4$^+$ T cells were isolated by FACS according to their cell surface expression of CD45.1. Cells were stained with anti-CD4-AlexaFluor700 / eFluor450 and anti-CD45.1-PE and obtained as in 2.5.2 by FACS sorting. Purity of donor CD4$^+$ T cells was consistently > 90%.

2.5.7 Isolation of mononuclear cells from the CNS

Mice were sacrificed by CO_2 asphyxiation and subjected to transcardial perfusion with 10mls PBS. Spinal cords were removed by intrathecal hydrostatic pressure and brains were removed by dissection (McGeachy et al., 2005). Brain and spinal cord were cut into small pieces and disrupted using a 1ml syringe before digestion in 2.5mg/ml collagenase (Lorne laboratories, UK) and 1mg/ml deoxyribonuclease

(Sigma) for 40 minutes at 37°C. Mononuclear cells were isolated from the interface of a 30:70% discontinuous Percoll gradient (GE healthcare, Sweden) by centrifugation at 850xg for 20 minutes and washed twice in wash buffer.

2.6 *In vivo* manipulations

2.6.1 T cell transfer

Purified $CD4^+$ T cells, Th1 polarised (2.8.1) and iTreg cells (2.8.2) were washed and re-suspended in sterile PBS at stated numbers. Cells were injected intravenously (i.v.) via the tail vein in a total volume of 200µl.

2.6.2 Administration of soluble peptide

Mice received a single 200µg dose of 4Lys, 4Ala, 4Val or 4Tyr in a total volume of 200µl PBS i.v. Peptide was administered one day after cell transfer or at the peak of disease.

2.6.3 Administration of BrdU

Mice received intraperitoneal (i.p.) injection of 2mg BrdU in a total volume of 200µl per mouse, on day 1 and day 3 following peptide administration.

2.6.4 Immunisations

Mice received 100µg 4Lys or 10µg 4Tyr emulsified in CFA containing 50µg heat-killed *Mycobacterium tuberculosis* H37Ra (Sigma). A total volume of 100µl was injected subcutaneously (s.c.); 50µl into each hind leg.

2.7 Induction and assessment of EAE

2.7.1 Induction of active EAE

C57BL/6xB10.PL mice received 0.5–2x10^6 Tg4 cells i.v. On the day following or 8 days after cell transfer, mice were immunised (as in 2.6.4) and given 200ng of pertussis toxin (PTX) (Health Protection Agency, Dorset, UK) in 500µl PBS, i.p. on the day of immunisation and 2 days later. Clinical signs of EAE were assessed daily from day 6 post-immunisation, and scored as follows:

0 – No disease
1 – Flaccid tail
2 – Impaired righting reflex and/or abnormal gait
3 – Partial hind limb paralysis
4 – Total hind limb paralysis
5 – Partial forelimb paralysis
6 – Moribund or dead

2.7.2 Induction of passive EAE

Tg4 effector cells activated *in vitro* as in 2.8.1, were harvested and washed in PBS. Cells were re-suspended in PBS and 2x10^6 blasts were transferred per host in a total volume of 200µl i.v. On the same day as cell transfer mice also received 200ng PTX in 500µl PBS i.p. Clinical signs of EAE were assessed daily (as in 2.7.1) from day four post-cell transfer.

2.8 *In vitro* cell culture and T cell polarisations

2.8.1 Polarisation of Tg4 effector (Th1) cells

Cells were obtained from the spleen and peripheral LN of Tg4 mice as in 2.5.1 and re-suspended in RPMI-10% at 4x10^6/ml. Cells were stimulated with 10µg/ml 4Lys in the presence of 10U/ml rIL-2 (purified from the x63-IL-2 hybridoma, a gift from

David Gray, University of Edinburgh), 25ng/ml rIL-12 and 25ng/ml rIL-18 (R&D systems) in 6-well plates (Corning, Costar, UK) at 37°C in a humidified atmosphere at 5% CO_2 (O'Connor et al., 2008). After 48 hours the cells were split and the concentration of rIL-2 was increased to 20U/ml for the final 24 hours.

2.8.2 Generation of iTreg cells

Naïve $CD4^+$ T cells were FACS sorted as in 2.5.4 and re-suspended in RPMI-10% at a concentration of $2.5x10^5$cells/ml. Each well of a 24-well plate (Corning, Costar, UK) was coated with anti-CD3 and anti-CD28 antibodies (eBioscience, Hatfield, UK), both at a concentration of 2µg/ml in PBS for 2 hours at 37°C. The plates were washed three times in wash buffer and cells were seeded at $2.5x10^5$/well in the presence of 100U/ml rIL-2 and 5ng/ml rTGF-β, and cultured for 120 hours at 37°C in a humidified atmosphere at 5% CO_2 (O'Connor et al., 2010). After culture, cells were harvested, washed and re-suspended in RPMI-10% at $1x10^7$/ml. GFP^+ cells were purified using FACS as in 2.5.4.

2.8.3 Expansion of nTreg cells

nTreg cells were obtained as in 2.5.5 and re-suspended at $1x10^6$/ml in RPMI-10%. $1x10^5$ cells were added per well of a 96-well round bottomed micro-titre plate (Corning, Costar). Cells were expanded in the presence of mouse α-CD3/α-CD28 T cell expander Dynabeads (Dynal Biotech, Invitrogen, UK) at a 4:1 bead to cell ratio and 1000U/ml rIL-2. Cells were maintained at a concentration of $0.5\text{-}1x10^6$/well and incubated at 37°C in a humidified atmosphere at 5% CO_2 for 21 days.

2.8.4 Stimulation assays

$CD4^+$ T cells were obtained from the spleens of Tg4-PD-$1^{+/+}$ and Tg4-PD-$1^{-/-}$ mice as described in section 2.5.2 and re-suspended in RPMI-5%. These were cultured at $1x10^4$ or $2x10^4$ per well (as stated) with $2x10^5$/well gamma-irradiated splenocytes (40Gy) from C57BL/6xB10.PL mice. Cells were cultured in 96-well flat-bottomed plates (Corning, Costar) in the presence of increasing antigen concentrations (0-100µM) 4Lys at 37°C for 48 or 72 hours

2.8.5 CFSE-proliferation assays

$CD4^+$ T cells were from Tg4-PD-$1^{+/+}$ and Tg4-PD-$1^{-/-}$ mice were CFSE-labelled as described in section 2.5.3 and re-suspended in RPMI-5%. These were cultured at $1x10^5$/well or $2x10^5$/well (as stated) with $2x10^6$/well gamma-irradiated splenocytes (40Gy) from C57BL/6xB10.PL mice. Cells were cultured in 48-well plates (Corning,Costar) in the absence or presence of 1μM or 10 μM 4Lys at 37°C for 72 hours. The dilution of CFSE within the $CD4^+CD90.1^+$ Tg4-PD-$1^{+/+}$ and Tg4-PD-$1^{-/-}$ cells was measured using flow cytometry.

2.8.6 Suppression assays

GFP^+ and GFP^- $CD4^+CD45.1^+$ iTreg cells were isolated from the spleens of C57BL/6xB10.PL recipient mice four days after soluble peptide (4Tyr) administration using FACS. GFP^+ and GFP^- cells were re-suspended in RPMI-5% at $3.2x10^5$/ml and 100μl was added in duplicate to the top wells of a 96-well round bottomed plate. 50μl of GFP^+ or GFP^- cells were taken from the top wells and diluted two-fold in 50μl RPMI-5% down the plate. Irradiated splenocytes from C57BL/6xB10.PL mice were suspended in RPMI-5% at $4x10^6$/ml and 50μl were added to each well. $CD4^+$ Tg4 responder cells were isolated (as in 2.5.2) and were re-suspended at $3.2x10^5$/ml in RPMI-5%, 50μl of these cells were added to each well. In addition, 50μl of 40μM 4Lys in RPMI-5% was added to each well. Cells were cultured at 37°C for 72 hours before 0.5μCi of [^{3}H]-thymidine (Perkin-Elmer, UK) was added to each well. After 18 hours of further culture incorporation of [^{3}H]-thymidine was measured using a liquid scintillation β-counter (Wallac) and expressed as mean counts per minute (cpm).

2.8.7 *Ex vivo* recall proliferation assays

Cells were isolated from the spleen and peripheral LN of experimental mice and re-suspended at $8x10^6$/ml and $6x10^6$/ml respectively in X-Vivo medium. Cells were cultured in duplicate at $8x10^5$/well or $6x10^6$/well respectively in 96-well flat bottomed micro-titre plates (Corning, Costar) in the presence of increasing antigen concentrations (0-100μM) 4Lys at 37°C for 72 hours. 0.5μCi of [^{3}H]-thymidine (Perkin-Elmer, UK) was added to each well for the final 18 hours of culture.

Incorporation of [^{3}H]-thymidine was measured using a liquid scintillation β-counter (Wallac) and expressed as mean counts per minute (cpm).

2.8.8 Cytokine assays

Recall assays were set up as in 2.8.7 and supernatants were used to measure cytokine production of IL-2 at 48 hours and IFN-γ and IL-17 at 72 hours using an enzyme-linked immunosorbant assay (ELISA).

Cytokine capture antibodies (Table 2.4) were diluted in bicarbonate buffer and used to coat 96-well plates (maxisorp, Nunc) overnight at 4^{o}C. Plates were washed twice in PBS containing 0.1% Tween (PBS-Tween; Sigma) and subsequently blocked with 200µl/well PBS containing 1% bovine serum albumin (BSA) (Sigma) for one hour at 37^{o}C. Plates were washed twice with PBS-Tween and supernatants from recall assays were loaded onto the plates in duplicate 100µl/well and incubated for two hours at room temperature. Known cytokine standards were generated by doubling dilution of recombinant mouse cytokine in 1% BSA-PBS, top concentration for each cytokine was as follows; IL-2: 2ng/ml, IFN-γ and IL-10: 100ng/ml and IL-17: 10ng/ml (all BD Pharmingen, UK). Plates were washed four times in PBS-Tween before biotinylated detection antibody (Table 2.4), diluted in 1% BSA-PBS, was added 100µl/well and plates were incubated for one hour at room temperature. Plates were washed six times with PBS-Tween and were incubated with 100µl/well extravidin peroxidase (Sigma) diluted 1:1000 in 1% BSA-PBS, for 30 minutes at room temperature. Plates were washed eight times in PBS-Tween. The ELISA was developed by adding 100µl/well tetramethyl-benzidine (TMB) (Sigma) solution (prepared by adding 100µl of 10mg/ml TMB in DMSO to 9.9ml phosphate-citrate buffer and 3µl of hydrogen peroxide (sigma). The reaction was stopped by adding 100µl/well 2M sulphuric acid. Plates were read for optical absorbance at 450nm using a Multiskan plate reader (Labsystems, UK).

2.8.9 Recall stimulation for analysis of intracellular cytokines

Splenocytes or cells from LN were re-suspended at $8x10^6$/ml in X-VIVO medium and cultured at $8x10^6$/well in 48-well plates overnight in the presence or absence of 20µg/ml 4Lys. Brefeldin A (eBioscience) was added to cultures at a 1:1000 dilution for the final four-to-five hours of culture, prior to intracellular cytokine staining.

2.9 Flow cytometric analysis

2.9.1 Antibodies for flow cytometric analysis

Antibodies used for flow cytometric analysis were from eBioscience unless otherwise stated (Table 2.5).

2.9.2 Surface staining

Cells were washed in FACS buffer and stained with the indicated antibodies (see Table 2.5 for a list of all antibodies, clones and concentrations used) in 50µl FACS buffer at 4°C for 20 minutes. Cells were washed in FACS buffer and re-suspended in FACS buffer for immediate analysis or 1% paraformaldehyde solution (PFA) and stored at 4°C for analysis the next day.

2.9.3 Intracellular cytokine staining

Cells were incubated with brefeldin A for the final four-to-five hours of culture. Where stated, cells were also incubated with 50ng/ml phorbol myristate acetate (PMA) and 1µg/ml ionomycin (both Sigma) for the final four-to-five hours. Cells were stained for cell surface markers as in 2.9.2 and re-suspended in 200µl BD Cytofix/Cytoperm (BD Pharmingen) solution and incubated at 4°C for 20 minutes. Cells were washed twice in 1x BD Perm/Wash buffer (BD Pharmingen) and stained for intracellular cytokines using indicated antibodies (Table 2.5) diluted in 50µl Perm/Wash buffer at room temperature for 30 minutes. Cells were washed twice in Perm/Wash buffer and re-suspended in FACS buffer for immediate or next day analysis. Alternatively, after incubation with brefeldin A cells were washed in

FACS buffer and stained for intracellular cytokines using indicated antibodies diluted in 50µl FACS buffer containing 0.1% saponin (Sigma) and incubated at room temperature for 30 minutes. Cells were washed in FACS buffer and stained for cell surface markers as in 2.9.2 and re-suspended in FACS buffer for immediate analysis or 1% PFA and stored overnight at 4^{o}C for next day analysis.

2.9.4 Intracellular staining for transcription factors

Cells were stained for cell surface markers as in 2.9.2 and re-suspended in 400µl Foxp3 Fixation/Permeabilisation buffer (ebioscience) overnight at 4^{o}C. Cells were washed in FACS buffer and stained for transcription factors using indicated antibodies (Table 2.5) diluted in 50µl permeabilisation buffer (ebioscience) at room temperature for 30-40 minutes. Cells were washed twice in FACS buffer and re-suspended in PBS for immediate or next day analysis.

2.9.5 Intracellular BrdU staining

Cells were stained for cell surface markers as in 2.9.2 and re-suspended in 400µl Foxp3 Fixation/Permeabilisation buffer (ebioscience) overnight at 4^{o}C. Cells were washed in 1x BD Perm/Wash buffer and re-suspended in 200µl of 10mg/ml DNase diluted 1:10 in 1x Perm/Wash buffer and incubated at 37^{o}C for one hour. Cells were washed in 1xPerm/Wash buffer and re-suspended in 50µl Perm/Wash containing diluted α-BrdU and other intracellular antibodies where indicated, and incubated at room temperature for 20 minutes. Cells were washed and re-suspended in FACS buffer.

2.9.6 Flow cytometric data analysis

Flow cytometric data were acquired using a BD LSRFortessa cell analyzer (BD biosciences) and data analysed using FlowJo software (Treestar version 3.2.1, USA). FACS was performed using a BD FACSAria II. Relevant gating strategies are shown in results and all intracellular staining analysis gates were determined using appropriate isotype controls.

2.10 Statistics

Statistics were performed using GraphPad Prism software (USA). In order to verify that data were normally distributed D'Agostino & Pearson normality tests were conducted. In experiments where n numbers were not sufficient to test for normality, data were assumed to follow a Gaussian distribution and significance was tested using an unpaired Student's t-test when comparing two experimental groups, and a one-way ANOVA with Tukey's Post-Hoc test when comparing three or more experimental groups. Bonferroni's Post-Hoc test was used for analysis of ELISA data. Data were considered significantly different with p values of <0.05. Due to the ranked nature of EAE disease scores the non-parametric Mann Whitney U-test was used to determine differences in the mean maximal score and a Fisher's exact test was used to determine differences in disease severity (i.e. number of mice with score 2 and below, versus number of mice with grade 3 or above) and incidence.

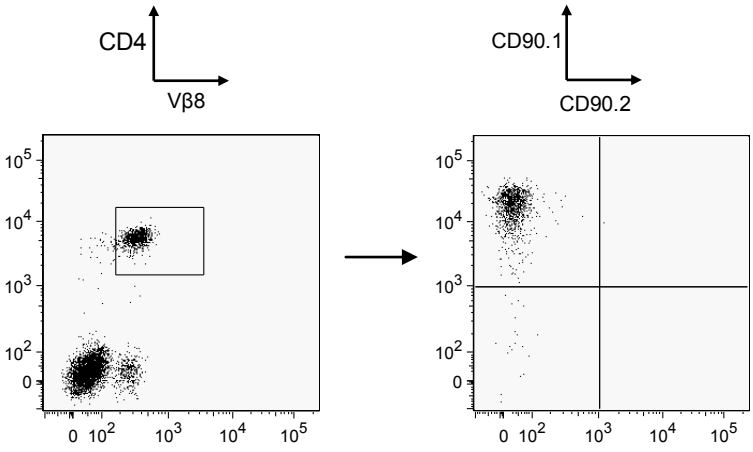

Figure 2.1: Representative flow cytometry plots of Tg4-PD-1$^{+/+}$ and Tg4-PD-1$^{-/-}$ screening.
Red blood cells were lysed from blood samples using RBC lysis buffer. Cell surface markers were stained as described in section 2.9.2 for CD4, Vβ8, CD90.1 and CD90.2

Strain	Genetic background	Description
C57BL/6	H-2^b	Wild-type strain
B10.PL	H-2^u	Wild-type strain
C57BL/6xB10.PL	H-2^b/H-2^u	Wild-type strain
PD-$1^{-/-}$	H-2^b	Knock-out for PD-1 (Nishimura et al., 1998)
Tg4	H-2^u (CD45.1 or CD90.1)	TCR trangenic; T cells specific for the Ac1-9 peptide of MBP (Liu et al., 1995)
Tg4xFoxp3.LuciDTR-4	H-2^b/H-2^u (CD45.1)	TCR transgenic (as above) crossed with BAC trangenics co-expressing eGFP, luciferase and the diphtheria toxin receptor under the Foxp3 promoter (O'Connor et al., 2010)
Tg4xPD-$1^{-/-}$	H-2^b/H-2^u (CD90.1)	TCR transgenic (as above) crossed with PD$1^{-/-}$ (as above)

Table 2.1 : Genetic background and properties of mouse strains utilised

Reagent	Concentration	Volume per reaction
dH_20		16.3
PCR buffer	10x	2.5
dNTP	2.5mM	2.0
Primer 1 (Reverse WT) (CTCGGCCATGGGACGTAGGG)	10pmol/µl	1.0
Primer 2 (Forward) (GGGTCTGCAGCATGCTAATGGCTG)	10pmol/µl	1.0
Primer 3 (Reverse KO) (TTGTGTAGCGCCAAGTGCCCAGCG)	10pmol/µl	1.0
DNA		1.0
Taq		0.2

Table 2.2 : PCR screening reagents for murine PD-1$^{-/-}$ gentyoping.
Primers were from VWR International (USA), PCR buffer, dNTP and Taq polymerase were from Qiagen (Holland)

Step	Temperature (^{o}C)	Time (sec)
1	94	180
2	94	30
3	60	30
4	72	60
5	72	300
	Repeat steps 2-4 for 30 cycles	

Table 2.3: PCR reaction conditions for PD1$^{-/-}$ genotyping.
PCR's were conducted using a G-Storm thermal cycler (UK). Products were subsequently run on a 1.5% agarose (Invitrogen, UK) gel. WT band: 125bp, KO band: 207bp.

Capture antibody	Clone	Final concentration	Detection antibody (biotinylated)	Clone	Final concentration
α-IL-2	JES6-1A12	2μg/ml	α-IL-2	JES6-5H4	0.5μg/ml
α-IFN-γ	R4-6A2	2μg/ml	α-IFN-γ	XMG1.2	0.5μg/ml
α-IL-17	TC11-18H10	0.5μg/ml	α-IL-17	TC11-8H4.1	0.5μg/ml
α-IL-10	JES5-2A5	2μg/ml	α-IL-10	SXC-1	0.5μg/ml

Table 2.4: Capture and detection antibodies used for ELISA
All BD Pharmingen.

Antibody	Conjugate	Clone	Final Concentration
CD3	PerCP-Cy5.5*	145-2C11	1µg/ml
CD4	AlexaFluor700[&] eFluor450*	RM4-5	2µg/ml
CD8	APC*	53-6.7	1µg/ml
CD45.1	FITC*, PE^	A20	2µg/ml
CD90.1	APC*	HIS-51	1µg/ml
CD90.2	PE^	53-2.1	1µg/ml
Vβ8.1/Vβ8.2	FITC*	KJ16	2µg/ml
CD44	APC-Cy7[+]	IM7	1µg/ml
CD62L	PE*, Biotinylated*	MEL-14	2.5µg/ml
CD25	APC*	PC61.5	1µg/ml
CD19	APC-Cy7[+]	6D5	1µg/ml
CD11b	APC*, eFluor450*	M1/70	1µg/ml
CD11c	APC*	N418	1µg/ml
Ly6G	AlexaFluor700^	1A8	1µg/ml
MHC II (H-2I-Ak/s)	FITC[$]	OX6	0.5µg/ml
PD-1	PE*	J43	1µg/ml
PD-L1	APC[+]	10F.992	1µg/ml
PD-L2	PE*	122	1µg/ml
PSGL-1	PE^	2PH1	1µg/ml
CXCR3	PE*	CXCR3-173	1µg/ml
CCR5	PE*	7A4	1µg/ml
IFNγ	APC*, FITC*, PE^	XMG1.2	2µg/ml
IL-17A	PerCP-Cy5.5*, APC*	eBIO17B7	2µg/ml
TNFα	eFluor450*	MP6-XT2	2µg/ml
GM-CSF	PE^	MP1-22E9	4µg/ml
IL-10	APC^	JES5-16E3	2µg/ml
Foxp3	eFluor450*	FJK-16a	2µg/ml
T-Bet	PerCP-Cy5.5*	4B10	2µg/ml
Streptavidin	PerCP-Cy5.5^		2µg/ml
IgG1	PerCP-Cy5.5*	P3.6.2.8.2.1	2µg/ml
IgG1	FITC*, PE*, APC*	eBRG1	2µg/ml
IgG2a	APC*, eFluo450*, PE*,	eBR2a	2µg/ml
IgG2a	PerCPCy5.5[+]	MOPC-173	2µg/ml
Armenian Hamster IgG	PE*, APC[+]	eBIO299Arm, HTK888	2µg/ml

Table 2.5: Antibodies used for flow cytometry.
* eBioscience, [+] BioLegend, ^BD Pharmingen, [$] AbD Serotech, [&] Invitrogen

3. The effect of soluble peptide administration on naïve antigen-reactive T cells

3.1 Introduction

As mentioned in chapter 1 (section 1.5), the prophylactic administration of peptide has been shown to be effective at inducing tolerance in naive antigen-reactive T cells and inhibiting disease induction in various experimental models of autoimmune and allergic disease (Gaur et al., 1992, Hoyne et al., 1993, Aichele et al., 1994). As such, the effect of soluble peptide administration on naïve $CD4^+$ T cells has been extensively investigated and several mechanisms of peptide-induced tolerance have been identified. These include the induction of anergy in antigen-reactive T cells (Miller et al., 2007), the switch in phenotype of response from pathogenic to regulatory (Sundstedt et al., 1997, Burkhart et al., 1999) and deletion of the antigen-reactive T cells (Konkel et al., 2010). Despite this, the definitive underlying mechanisms involved in peptide-induced tolerance have not yet been identified, and may vary depending on the mode of peptide application.

Using $CD4^+$ T cells from the transgenic Tg4 strain (which have TCR specific for the Ac1-9 peptide of MBP) it is possible to investigate the effects of soluble peptide administration using peptides with different tolerogenic properties. The WT Ac1-9 peptide, 4Lys, has an extremely weak binding affinity for MHC II I-A^u and is relatively poor at inducing tolerance in antigen-reactive T cells (Metzler and Wraith, 1993). In contrast, the 4Tyr APL of Ac1-9 binds with a 100000-fold greater binding affinity for I-A^u, and is extremely effective at inducing tolerance in antigen-reactive T cells (Liu and Wraith, 1995). The use of Ac1-9 peptides that have a range of binding affinities for I-A^u enables associations to be drawn between observed effects and the tolerogenic properties of the peptide. As such, these associations will facilitate the identification of mechanisms involved in tolerance induction. Previous studies within the laboratory have focused on characterising the

effect of 4Tyr administration on antigen-reactive Tg4 cells, and have identified several candidate mechanisms to be investigated.

3.1.1 Aims

1) To demonstrate that prophylactic administration of peptide can effectively inhibit induction of EAE
2) To characterise the effect of soluble peptide administration on naïve antigen reactive $CD4^+$ T cells
3) To verify the mechanism of tolerance induction in naïve $CD4^+$ T cells.

3.1.2 Experimental approach

Due to the low frequency of $CD4^+$ cells that recognise the Ac1-9 peptide in B10.PL mice, induction of EAE requires immunisation with a high dose of the 4Lys Ac1-9 peptide. In C57BL/6xB10.PL mice the frequency of these antigen-reactive cells is further reduced, and as a result immunisation with Ac1-9 peptide is not sufficient to induce EAE. However, in both of these strains a robust disease course can be induced through the pre-transfer of naive $CD4^+$ Ac1-9-reactive Tg4 cells prior to immunisation with 100μg 4Lys or 10μg 4Tyr in CFA. The use of $CD4^+$ Tg4 cells that express the congenic markers CD45.1 or CD90.1 enables the fate of these cells to be traced, and effects of therapeutic interventions on the actual cells driving disease to be investigated. Previous studies within the Anderton laboratory have established the use of these models to investigate the effect of soluble peptide administration on naïve antigen-reactive $CD4^+$ T cells. In this chapter the same models are utilised in order to further define the mechanisms of peptide-induced tolerance.

3.2 Results

3.2.1 Prophylactic administration of the Ac1-9 (4Tyr) APL reduces EAE severity

Previous studies have demonstrated that prophylactic administration of soluble 4Lys has little effect on the subsequent EAE disease course compared to treatment with PBS (Metzler and Wraith, 1993, Liu and Wraith, 1995). This result has been attributed to the poor tolerogenic properties of 4Lys, which has a low functional avidity due to its weak binding affinity for MHC II I-A^u. In contrast, prophylactic administration of the 4Tyr APL, which has a much stronger binding affinity for MHC II I-A^U, has been shown to be effective at inhibiting induction of EAE (Liu and Wraith, 1995). In order to confirm this observation also held true in the T cell transfer model used in this project, $CD4^+CD45.1^+$ Tg4 T cells were transferred into B10.PL ($CD45.1^-$) hosts one day prior to intravenous administration of soluble 4Tyr; EAE was induced seven days later by immunisation (Figure 3.1A).

Induction of EAE in the PBS-treated control group led to a robust monophasic disease course which began six days after immunisation and had resolved by day 21 (Figure 3.1B). The mean maximal disease score in the PBS-treated group was 3.8 and incidence was 100%. In accordance with previous studies, treatment with soluble 4Tyr before induction of EAE inhibited the subsequent disease course. This was evident by the significant reduction in the mean maximal score of disease (1.6 in the 4Tyr-treated group, $p<0.05$) and the overall severity of disease as judged using a Fisher's exact test ($p<0.001$). No significant differences were observed in the incidence of disease (80% in the 4Tyr-treated group). Although there was no difference in the onset of disease between groups the duration of disease was also shorter in the 4Tyr-treated group, where complete resolution had occurred by day 13, as opposed to day 21 in the PBS group. These results demonstrate that soluble administration of the Ac1-9 peptide, 4Tyr, is effective at inhibiting the subsequent antigen-driven immune response instigated by challenge with the same peptide in an immunogenic form.

This effect has also previously been demonstrated in a separate model of EAE, whereby soluble administration of the CNS-derived peptide pMOG inhibits subsequent induction of disease by immunisation with pMOG (Leech et al., 2007). Through the use of pMOG-reactive 2D2 $CD4^+$ T cells, it was observed that soluble pMOG administration induced deletion of the 2D2 cells within three days (Konkel et al., 2010). Therefore, the removal of the antigen-reactive T cells by soluble pMOG administration was responsible for inhibiting disease induction. The next step was to determine whether the inhibition of disease induction seen here in the Tg4 model following 4Tyr administration was due to deletion of the $CD4^+$ Tg4 cells.

3.2.2 Administration of 4Tyr does not induce deletion of naive antigen-reactive T cells

To test this, the presence of Tg4 cells was investigated fours days after peptide administration. Naïve $CD4^+$ Tg4 cells were transferred into B10.PL hosts one day prior to intravenous administration of soluble peptide or PBS, spleens were taken four days after peptide administration and the presence of Tg4 donor cells was determined (Figure 3.2). In addition to 4Tyr, the administration of Ac1-9 peptides with varying affinities for $I\text{-}A^u$ was investigated, by comparing the effects of 4Lys, 4Ala, 4Val and 4Tyr treatment.

No difference was found in the total cellularity of the spleens between any of the treatment groups (data not shown). There was also no difference in the total numbers and proportion of $CD4^+$ T cells within the spleen between any of the groups (Figure 3.3A). However, the total numbers and proportion of Tg4 $CD4^+$ cells appeared to increase in line with the affinity of the peptide for $I\text{-}A^u$; with 4Tyr administration resulting in a significant increase in the total numbers and proportion of Tg4 cells compared to PBS-treated and all other groups (Figure 3.3B). Administration of 4Lys, 4Ala, and 4Val did not result in a significant difference in the number or proportion of donor cells when compared to PBS.

These results demonstrate that administration of Ac1-9 peptide does not induce deletion of Tg4 cells within 4 days of peptide administration. Instead, the increased presence of Tg4 cells in the 4Tyr-treated group implies that an inhibitory mechanism is employed, which acts to limit the pathogenicity of these cells. This observation correlates with previous studies within the laboratory that suggested 4Tyr administration did not cause deletion of $CD4^+$ Tg4 cells (Konkel, 2009)

3.2.3 Administration of 4Tyr does not selectively expand donor $CD4^+Foxp3^+$ cells

A potential role of regulatory T cells in limiting the immune response was investigated by assessing the presence of $Foxp3^+$ cells within the host and Tg4 donor $CD4^+$ T cell populations. Spleens were taken four days after peptide administration and intracellular staining was used to identify $Foxp3^+$ cells by flow cytometry (Figure 3.4A). There was no difference in the number or proportion of host $CD4^+Foxp3^+$ cells between any of the groups (Figure 3.4B). However, within the donor compartment a greater number of $CD4^+Foxp3^+$ cells were observed in the 4Tyr-treated group compared to all other treatment groups (Figure 3.4C). When translated into proportion of $Foxp3^+$ cells within the donor $CD4^+$ population there was no difference between groups (Figure 3.4C). The proportion of $CD4^+Foxp3^+$ Tg4 cells was no different to the proportion at the time of transfer (data not shown).

3.2.4 Naïve antigen-reactive T cells up-regulate expression of PD-1 upon treatment with Ac1-9 peptides

A further candidate inhibitory mechanism is the expression of co-inhibitory molecules. Previous studies within the laboratory have identified that treatment with 4Tyr leads to up-regulated expression of PD-1 on Tg4 $CD4^+$ T cells (Konkel, 2009). Therefore, the effect of treatment with the panel of Ac1-9 peptides, that have a range of affinities for $I\text{-}A^u$, was investigated.

Spleens were taken on day four after peptide treatment and PD-1 expression was determined by flow cytometry. All Ac1-9 peptides led to up-regulation of PD-1 expression on Tg4 $CD4^+$ cells (Figure 3.5A). The degree of up-regulation appeared to be dependent upon the affinity of the peptide for $I\text{-}A^u$ with 4Lys causing the least degree of up-regulation and 4Tyr the most. When comparing the geometric mean fluorescence intensity (MFI) of PD-1 expression, 4Tyr and 4Val were the only peptides that induced a significant increase in PD-1 (Figure 3.5B). Host $CD4^+$ did not show elevated PD-1 expression in any of the treatment groups (Figure 3.5B)

3.2.5 Tg4 T cells persist for at least 7 days after 4Tyr treatment and are capable of producing IL-2

PD-1 was first identified as a ligand expressed on T cells before they undergo apoptosis (Ishida et al., 1992). Therefore, up-regulation of PD-1 on peptide treated Tg4 cells four days after peptide may imply that these cells are indeed deleted, but that the process occurs after four days post-treatment. To test whether 4Tyr-treated Tg4 cells were still present at the point of disease induction, spleens were taken seven days after treatment with 4Tyr (the time at which mice would be immunised for EAE, as in Figure 3.1) and donor populations were analysed.

There was no difference in the total number of $CD4^+$ T cells present within the spleen. However, there was a significantly higher proportion of $CD4^+$ cells in the 4Tyr-treated group compared to PBS (Figure 3.6A). As seen at day four after peptide administration, there was still significantly higher numbers and proportion of Tg4 $CD4^+$ cells in the 4Tyr-treated group compared to PBS (Figure 3.6B). These data further confirmed that antigen-reactive Tg4 T cells were not deleted by administration of the 4Tyr peptide, despite their expression of PD-1.

PD-1 has also been implicated in the reduced ability of T cells to produce effector cytokines in response to stimulation with antigen (Freeman et al., 2000). In order to investigate the ability of Tg4 cells to produce effector cytokines following 4Tyr

administration, *ex vivo* recall assays were performed. Spleens were taken seven days after treatment with 4Tyr or PBS, and cells were stimulated *in vitro* with a dose range of 4Lys; production of IL-2 and IFN-γ was determined by ELISA. Levels of IFN-γ production were not significantly higher than background in either the PBS- or 4Tyr-treated group. In contrast, IL-2 production was observed in both groups in a dose-dependent response to antigenic stimulation (Figure 3.6C). The low levels of cytokine production observed in the PBS group is likely due to the low frequency and total numbers of Tg4 cells within samples. These results demonstrate that 4Tyr-treated cells can produce IL-2 upon recall stimulation but not the effector cytokine IFN-γ.

3.2.6 Numbers of Tg4 cells in the spleen and LN of 4Lys-, 4Tyr- and PBS-treated mice after immunisation

In order to determine the response of peptide-treated Tg4 cells upon subsequent immunisation, such as that used to induce EAE, mice were immunised with 4Lys in CFA seven days after tolerogenic administration of 4Lys or 4Tyr (Figure 3.7). The spleen and draining LN (Para aortic and inguinal) were taken ten days after immunisation and host and donor $CD4^+$ cell populations investigated.

There was no difference in the total numbers and proportion of $CD4^+$ T cells, or the number and proportion of donor $CD4^+$ cells within the spleen ten days after immunisation (Figure3.8A & 3.8B). In the LN there was also no difference in the total number or proportion of $CD4^+$ T cells (Figure 3.9A). However, there was a significantly lower number and proportion of Tg4 cells in the 4Lys- and 4Tyr-treated groups compared to PBS (Figure 3.9B).

Due to the presence of Tg4 cells in the spleen and LN in all groups after immunisation, it was possible to further characterise the phenotype of the response in these cells.

3.2.7 Treatment with 4Lys or 4Tyr increases the proportion of Tg4 $CD4^+Foxp3^+$ cells after immunisation

The presence of $CD4^+Foxp3^+$ cells within the host and donor populations ten days after immunisation was investigated. In the spleen there was no difference in the numbers or proportion of $Foxp3^+$ cells within the host $CD4^+$ T cell population (Figure 3.10B). However, within the Tg4 population there was a significant increase in the proportion of $Foxp3^+$ cells in the 4Tyr group compared to PBS (Figure 3.10C). No differences were observed in the proportion of Tg4 $Foxp3^+$ cells in the 4Lys group compared to PBS, and no significant differences were observed in the numbers of Tg4 $Foxp3^+$ cells between any of the groups.

In the LN there was also no difference in the numbers or proportion of host $CD4^+Foxp3^+$ cells (Figure 3.11B). A significantly higher proportion of Tg4 $Foxp3^+$ cells were observed in both the 4Lys- and 4Tyr-treated groups compared to PBS (Figure 3.11C), and similarly to the spleen there were no significant differences in the numbers of Tg4 $Foxp3^+$ cells between groups.

3.2.8 Peptide treated Tg4 cells produce IL-2 upon recall stimulation but have a limited capacity to produce IFN-γ and IL-17

Due to the differences observed in cytokine production between PBS- and 4Tyr-treated groups seven days after soluble peptide administration, the ability of 4Tyr-treated Tg4 cells to produce effector cytokines after immunisation was investigated. Recall stimulation assays were set up using cells obtained from the spleen and LN ten days after immunisation; cells were stimulated with a range of 4Lys concentrations and cytokine production was assessed by ELISA.

There were no significant differences in the production of IFN-γ between treatment groups in the spleen or LN (Figures 3.12A & 3.12B). However, a significant reduction in the production of IL-17 was observed in the spleen and LN of the

4Tyr-treated group compared to PBS (Figure 3.12A & 3.12B). IL-17 production in the 4Tyr-treated group was also lower than the 4Lys group in the spleen but not LN. Despite the reduced capacity of 4Tyr-treated cells to produce IL-17, there were similar levels of IL-2 production in response to recall stimulation in all treatment groups (Figure 3.12A).

In order to verify that it was the Tg4 cells that were responsible for producing effector cytokines in response to recall stimulation, intracellular cytokine staining was used. Cells were taken from the spleen and LN ten days after immunisation and incubated overnight in the presence or absence of 4Lys. No intracellular cytokine staining was seen within the host $CD4^+$ population when stimulated with peptide (representative data shown in Figure 3.13A). Although no significant differences were observed in the donor cohort, there was a trend towards a lower proportion of Tg4 cells that could produce IFN-γ and IL-17 in the 4Tyr-treated group compared to 4Lys and PBS (Figure 3.13B). Similar results were obtained in repeat experiments.

Figure 3.1 Prophylactic administration of 4Tyr reduces EAE severity

Figure 3.2 Experimental approach to determine the effect of soluble peptide administration on naïve antigen-reactive Tg4 T cells

Figure 3.3 There are higher numbers of Tg4 cells in the 4Tyr-treated group compared to PBS

Figure 3.4 Administration of 4Tyr increases the number of Tg4 Foxp3^{+} cells

Figure 3.5 PD-1 is up-regulated on peptide-treated Tg4 cells

Figure 3.6 4Tyr-treated Tg4 donor cells persist at greater levels than PBS-treated donor cells for at least seven days after peptide treatment and are capable of producing IL-2 in response to *ex-vivo* stimulation with Ag

Figure 3.7 Experimental approach to assess the fate of 4Tyr- and 4Lys-treated Tg4 cells after subsequent immunisation with 4Lys in CFA

Figure 3.8 4Tyr- and 4Lys-treated Tg4 cells are present at the same number and frequency as PBS-treated cells in the spleen after immunisation

Figure 3.9 4Lys and 4Tyr treatment reduces the number of Tg4 cells compared to PBS treatment within the draining lymph nodes after immunisation

Figure 3.10 Administration of 4Tyr led to an increase in the proportion of Foxp3$^+$ cells within the donor CD4$^+$ population in the spleen after immunisation

Figure 3.11 Treatment with 4Lys and 4Tyr led to an increase in the proportion of Foxp3$^+$ cells within the donor CD4$^+$ population in the draining lymph nodes after immunisation

Figure 3.12 4Tyr-treated cells produce IL-2 in response to recall stimulation with Ag, but do not produce IL-17

Figure 3.13 No significant differences in effector cytokine production between 4Ty-, 4Lys- and PBS-treated groups, as determined by ICS ten days after immunisation

3.3 Discussion

3.3.1 Tolerance induction in Tg4 cells by administration of 4Tyr is not due to deletion

It is well established that prophylactic administration of soluble peptides is extremely effective at inhibiting subsequent disease induction using the same peptide in an immunogenic form (Metzler and Wraith, 1993, Dick et al., 1994, Staines et al., 1996). The induction of tolerance through the administration of soluble peptides is largely attributed to the deletion of the majority of antigen-reactive cells (Liblau et al., 1996, Hawiger et al., 2001, Bonifaz et al., 2002, Miller et al., 2007). It has been shown that antigen-reactive cells undergo an initial proliferative phase upon tolerogenic peptide administration, but subsequently undergo apoptosis and numbers are greatly reduced within 3 days after peptide (Huang 2003).

In agreement with previous studies (Metzler and Wraith, 1993, Anderton et al., 1998), the results described within this chapter demonstrate that soluble administration of 4Tyr effectively inhibits EAE disease course (Figure 3.1). This observation cannot be attributed to the deletion of the antigen-reactive Tg4 cells upon administration of 4Tyr. Contrary to the reduction in antigen-reactive cell numbers seen following peptide administration in other models, 4Tyr-treated Tg4 cells were in fact present in greater numbers and constituted a greater proportion of $CD4^+$ cells within the spleen at both day four and day seven compared to PBS-treated mice (Figures 3.3B & 3.6B). This was also confirmed by the observation that 4Tyr-treated cells persist following subsequent challenge with 4Lys in CFA (Figure 3.8B). Although fewer Tg4 cells were observed in the LN of 4Tyr-treated mice compared to PBS controls upon immunisation, lower Tg4 numbers were also observed in the LN of 4Lys-treated mice (Figure 3.9B). As prophylactic 4Lys administration does not inhibit the induction of EAE compared to PBS (Konkel, 2008), the differences in numbers of Tg4 cells in the LN does not explain the inhibition of disease induction by 4Tyr administration. The lower number of Tg4

cells in the LN of 4Tyr- and 4Lys-treated mice is unlikely to be due to deletion of the cells upon challenge, as a distinct Tg4 population remained in all groups. It is more likely that the decrease in numbers reflects a sub-optimal expansion of Tg4 cells upon re-stimulation compared to those in the PBS-treated group, or from migratory differences between groups.

Investigating the effects of administration of Ac1-9 peptides with differing affinities for I-A^u revealed that in fact none of the Ac1-9 peptides induce deletion of the Tg4 cells. Treatment with 4Lys had a minimal effect on the number of Tg4 cells present in the spleen four days after treatment and as the affinity of the peptide for I-A^u increased the number of Tg4 cells present escalated (Figure 3.3). The elevated number of Tg4 cells present following treatment with 4Tyr and their inability to induce disease implies that these cells have undergone a significant degree of clonal expansion, but in the space of four days have been rendered hypo-responsive.

3.3.2 4Tyr administration induces non-classical anergy in Tg4 cells

T cell hypo-responsiveness can occur through the processes of clonal anergy or adaptive tolerance. In addition to signalling through the TCR, T cells require further signals from the APC to enable full activation of the T cell. Engagement of the TCR in the absence of these necessary co-stimulatory signals results in abortive T cell activation and the induction of clonal anergy (Choi and Schwartz, 2007). Upon re-stimulation, cells that are clonally anergic do not proliferate or produce IL-2 (Mueller et al., 1989), and therefore do not undergo clonal expansion. However, this growth arrest state can be reversed by the addition of exogenous IL-2 (Beverly et al., 1992, Schwartz, 2003). Importantly, although clonal expansion is impaired in anergic T cells, they retain the capacity to produce effector cytokines (Beverly et al., 1992). In contrast, adaptive tolerance occurs through chronic antigenic stimulus and results in shutdown of both IL-2 and effector cytokine production (Tanchot et al., 2001, Choi and Schwartz, 2007, Chiodetti et al., 2006). Upon antigenic stimulation, T cells initially undergo a proliferative and productive

response. However, under circumstances where the antigen persists, these cells are eventually rendered hypo-responsive to further TCR stimulation and switch off both IL-2 and effector cytokine production (Tanchot et al., 2001). This state is known as adaptive tolerance. The addition of exogenous IL-2 does not reverse this hypo-responsive state as it is the continued presence of antigen that is maintaining adaptive tolerance (Schwartz, 2003). As a result, adaptive tolerance can be reversed over time following the removal of antigen (Tanchot et al., 2001, Fathman and Lineberry, 2007). It is worth noting that in both clonal anergy and adaptive tolerance production of IL-2 is switched off.

In models of peptide-induced tolerance where the majority of antigen-reactive $CD4^+$ T cells are deleted, it has also been shown that a small population of antigen-reactive cells persist. These persisting cells were found to be hypo-responsive, and did not produce IL-2 or proliferate upon recall stimulation (Jenkins and Schwartz, 1987, Kearney et al., 1994, Konkel et al., 2010). In order to investigate the hypo-responsiveness of 4Tyr-treated Tg4 cells, cytokine production by these cells was measured upon ex vivo recall stimulation. In contrast to both the clonally anergic and the adaptively tolerised phenotypes described above, 4Tyr-treated Tg4 cells could produce IL-2 in response to recall stimulation with 4Lys. However, despite the production of IL-2 by these cells, production of IFN-γ was not observed (Figure 3.6C). Similar results were also observed following challenge with 4Lys in CFA, whereby cells from the spleen of PBS-treated mice produced IL-17 and IL-2 in response to recall stimulation with 4Lys; whereas 4Tyr-treated cells produced IL-2 but not the effector cytokine IL-17 (Figure 3.12). Production of IL-17 was also seen in cells from the LN of PBS-treated mice but not 4Tyr-treated. Furthermore, data from intracellular cytokine staining demonstrated limited production of IFN-γ and IL-17 in the 4Tyr-treated Tg4 cells (Figure 3.13).

Despite variations in the mode and phenotype of anergy induction that have been characterised in the literature, all models of anergy demonstrate the shut down of IL-2 production (Choi and Schwartz, 2007). The lack of effector cytokine production observed in 4Tyr-treated Tg4 cells would indicate that clonal anergy has

not been induced. Also, the ability of these cells to produce IL-2 would suggest that adaptive tolerance has not occurred either. However, adaptive tolerance requires an initial phase of IL-2 production and clonal expansion before production of cytokines is eventually inhibited. Therefore, it is possible that the 4Tyr-treated Tg4 cells may represent cells that are in the process of undergoing adaptive tolerance, whereby effector cytokine production has been inhibited but IL-2 production has not yet been switched off. Regardless, the results shown in this chapter demonstrate a previously unreported state of anergy whereby 4Tyr-treated Tg4 cells switch off effector cytokine production but have retained the capacity to produce IL-2.

3.3.3 Relevance of PD-1 expression in peptide induced tolerance?

Many studies have identified the up-regulation of co-inhibitory molecules on antigen-reactive cells following tolerogenic peptide administration, which may act to limit the responsiveness of cells upon re-stimulation (Perez et al., 1997, Konkel et al., 2010). Previous studies within the Anderton laboratory have specifically identified the up-regulation of the co-inhibitory molecule PD-1 following peptide-induced tolerance (Hochweller and Anderton, 2005, Konkel et al., 2010).

Signalling through PD-1 has been reported to limit the expansion, proliferation and secretion of cytokines such as IL-2 and IFN-γ upon TCR ligation (Keir et al., 2006, Sandner et al., 2005, Freeman et al., 2000, Parry et al., 2005). Several studies have also demonstrated that PD-1 signalling plays a critical role in tolerance of $CD8^+$ cells (Reynoso et al., 2009, Probst et al., 2005). Despite a study demonstrating the role of PD-1 in the maintenance of tolerance in $CD4^+$ cells (Fife et al., 2006), it has been observed that PD-1 may not be required for the induction of tolerance in $CD4^+$ T cells (Haspot et al., 2008, Konkel et al., 2010) However, Konkel et al., used models of peptide-induced tolerance in which the main mechanism of tolerance induction was deletion of the antigen-reactive cells, not the induction of hypo-responsiveness. Although the persisting antigen-reactive cells were found to express PD-1, the frequency of these cells was extremely low. Therefore, the

assays used in those experiments would not be sensitive enough to detect cytokine production or proliferation from such few cells, even if PD-1 blockade released those few cells from a hypo-responsive state. As a consequence, the conclusion that PD-1 signalling is not important in peptide-induced tolerance of $CD4^+$ cells may not be correct. The results in this chapter demonstrate that, in the Tg4 model, the mechanism of tolerance induction by 4Tyr administration is not due to deletion of the antigen-reactive cells but the induction hypo-responsiveness. Therefore, an inhibitory mechanism that prevents the 4Tyr-treated Tg4 cells from responding to antigenic stimulus must be at work. A possible explanation lies in the marked upregulation of PD-1 expression by Tg4 T cells in response to 4Tyr.

Although PD-1 was first identified as a molecule expressed on cells about to undergo apoptosis (Ishida et al., 1992), further experiments failed to confirm the role of PD-1 in the programmed cell death of these cells (Agata et al., 1996). The experiments described within this chapter provide further evidence to support these findings, as PD-1 was found to be highly expressed on 4Tyr-treated Tg4 cells four days after peptide administration (Figure 3.5). However, these cells were not deleted and persisted for at least seven days after treatment (Figure 3.6).

Comparison of PD-1 expression after administration of PBS, 4Lys, 4Ala, 4Val and 4Tyr revealed that up-regulation of PD-1 expression correlated with the affinity of the Ac1-9 peptide for $I\text{-}A^u$, with 4Lys inducing the least up-regulation of PD-1 and 4Tyr the highest levels of PD-1 (Figure 3.5). The increase in PD-1 expression as the tolerogenic ability of the peptide increases implicates a role for PD-1 in the induction or maintenance of 4Tyr-induced tolerance, and may provide an indication as to the degree of tolerance induced in Tg4 cells.

PD-1 is up-regulated on T cells upon stimulation through the TCR and remains high in the presence of persisting antigen (Francisco et al., 2010). As such, PD-1 has been shown to be highly expressed on exhausted T cells during chronic viral infection (Freeman et al., 2006, Barber et al., 2006). Signalling through PD-1 on T cells has typically been shown to inhibit the production of IL-2 and effector

cytokines such as IFN-γ (Keir et al., 2006, Sandner et al., 2005, Freeman et al., 2000). It has also been demonstrated that addition of exogenous IL-2 can override the effects of PD-1 inhibition (Carter et al., 2002). Therefore, the ability of 4Tyr treated Tg4 cells to produce IL-2 upon recall stimulation with antigen creates an interesting paradox.

Although high levels of PD-1 expression on peptide-treated Tg4 cells appears to correlate with a reduced ability of the cells to produce effector cytokines upon re-stimulation, PD-1 is known to be up-regulated as a consequence of TCR ligation. Therefore, administration of Ac1-9 peptides may only induce expression of PD-1 on Tg4 cells as a response to TCR signalling rather than as a mechanism responsible for tolerance induction in these cells. Taken together, these data provide impetus for resolving the role of PD-1 in the induction of tolerance.

3.3.4 A role for regulatory T cells in the induction of tolerance?

Regulatory T cells are extremely potent at inhibiting effector $CD4^+$ T cell responses and it has been reported that administration of soluble peptides can mediate induction of T cells with a regulatory phenotype. Oral administration of whole MBP protein or MBP-derived peptides has been found to induce TGF-β production by T cells (Miller et al., 1993, Santos et al., 1994). Studies by Wraith et al., have also demonstrated that repeated intranasal administration of the 4Tyr peptide can induce IL-10 production in Tg4 $CD4^+$ T cells (Gabrysova et al., 2009, Burkhart et al., 1999, Sundstedt et al., 2003). In addition, it has been shown that oral administration of peptides from target antigens can increase the number of $Foxp3^+$ $CD4^+$ T cells at the site of inflammation (van Puijvelde et al., 2007).

The results described in this chapter demonstrate an increase in the absolute number of Tg4 $CD4^+Foxp3^+$ cells following administration of 4Tyr compared to PBS (Figure 3.4C). However, the proportion of $Foxp3^+$ cells within the Tg4 $CD4^+$ population did not differ between any of the groups. This suggests that 4Tyr does

not selectively expand or maintain Tg4 $CD4^+Foxp3^+$ cells, but rather they are expanded or maintained to the same extent as the Tg4 $CD4^+Foxp3^-$ cells. Therefore, the increase in Tg4 $Foxp3^+$ cells is probably only a secondary effect of 4Tyr administration.

Upon subsequent challenge with 4Lys in CFA the proportion of $Foxp3^+$ cells within the Tg4 $CD4^+$ population was significantly greater in the 4Tyr-treated group in both the spleen and LN compared to the PBS treated group. These results could possibly be due to an increase in the proliferation of $CD4^+Foxp3^-$ cells in the PBS group driving down the proportion of donor $CD4^+Foxp3^+$ cells. Alternatively, upon challenge with 4Lys in CFA, the 4Tyr-treated Tg4 $CD4^+Foxp3^+$ population expands to a greater extent than the $CD4^+Foxp3^-$ Tg4 population. Comparison of $CD4^+Foxp3^+$ Tg4 numbers in the spleen of the 4Tyr-treated group four days after treatment with the numbers ten days after challenge with 4Lys in CFA reveals a ten-fold increase, whereas the total number of $CD4^+$ Tg4 cells only increased by approximately 2-fold (Figures 3.4 & 3.10). This perhaps represents an inhibition in the propensity of 4Tyr treated $CD4^+Foxp3^-$ cells to proliferate upon challenge with 4Lys in CFA, whereas $CD4^+Foxp3^+$ cell proliferation is not inhibited.

In the CNS of mice with EAE it has been observed that Tg4 $CD4^+Foxp3^+$ cells play no role in the resolution of disease, despite the necessity of Tg4 cells to induce disease. Instead, it is believed that the host $CD4^+Foxp3^+$ cells are responsible for driving the resolution, as demonstrated by the substantial increase in the proportion of host $CD4^+Foxp3^+$ cells within the CNS upon recovery (Leech & Anderton, unpublished observations). These observations would suggest that in order for Tg4 $CD4^+Foxp3^+$ cells to effectively inhibit the proliferation and cytokine secretion of $CD4^+Foxp3^-$ Tg4 cells and therefore subsequent induction of EAE, a large number and proportion of these cells would be required. The relatively small proportion of $CD4^+Foxp3^+$ Tg4 cells in the 4Tyr-treated group after antigenic challenge indicates that these cells are probably not present in sufficient numbers to inhibit the $CD4^+Foxp3^-$ cells, and therefore it is likely they play no role in the inhibition of EAE.

In order to confirm that donor $CD4^+Foxp3^+$ cells are not required for inhibition of EAE induction by 4Tyr administration, it is possible to make use of Tg4xFoxp3.LuciDTR-4 mice (O'Connor et al., 2010), which express GFP under the Foxp3 promoter. This offers the opportunity to sort for $CD4^+Foxp3^-$ cell populations before transfer and to determine whether 4Tyr administration can inhibit induction of EAE in the absence of any Tg4 $CD4^+Foxp3^+$ cells, or whether 4Tyr administration does indeed induce $Foxp3^+$ regulatory T cells.

In summary, Ac1-9 peptides that have a greater affinity for MHC II $I\text{-}A^U$, and therefore persist as peptide:MHC complexes for longer, are better at inducing tolerance. The prolonged TCR signalling induced by 4Tyr administration appears to expand or maintain Tg4 cells to a greater extent than all other Ac1-9 peptides, induces high-level expression of PD-1 on Tg4 cells and increases the number of Tg4 $CD4^+Foxp3^+$ cells. The persistence of Tg4 cells following 4Tyr administration, and the reduced ability of these cells to produce the effector cytokines IFN-γ and IL-17 compared to PBS- and 4Lys-treated cells, would suggest the induction of adaptive tolerance. Although, adaptively tolerised cells do not typically produce IL-2 upon recall stimulation with antigen and the observation that 4Tyr-treated cells do, suggests the combination of IL-2 production and persistent TCR signalling may push the cells into an exhausted state. Both the high level expression of PD-1 on Tg4 cells and the increase in antigen-reactive $CD4^+Foxp3^+$ cells provide potential mechanisms by which administration of 4Tyr induces tolerance and provide avenues for further investigation.

3.4 Concluding remarks

- Prophylactic administration of 4Tyr inhibits induction of EAE, where it has been previously shown that 4Lys does not.
- Antigen-reactive Tg4 T cells treated with WT peptide or with APL of Ac1-9 are not deleted either upon administration of peptide, or upon subsequent immunogenic challenge with antigen.
- 4Tyr administration increases the number of donor $CD4^{+}Foxp3^{+}$ cells, whereas other Ac1-9 peptides do not.
- Administration of Ac1-9 peptides induces the up-regulation of PD-1 expression on donor Tg4 cells. The extent of this correlates with the affinity of the peptide for $I\text{-}A^{u}$; with 4Tyr inducing the highest level of expression.
- 4Tyr administration inhibits the ability of Tg4 cells to produce IL-17, whereas 4Lys does not.
- 4Tyr administration induces non-classical anergy in Tg4 T cells; i.e. they lose effector cytokine production but retain their ability to produce IL-2

Despite the extensive characterisation of the effects of soluble peptide administration on naïve antigen-reactive T cells the clinical requirement of peptide therapeutics is to switch-off activated T cells that are involved in ongoing disease. The effect of soluble peptide administration on pathogenic antigen-reactive T cells is investigated in Chapter 4. The potential role of PD-1 in tolerance induction and the effect of soluble peptide administration on regulatory T cells will also be further investigated in Chapters 5 & 6.

4. The effect of soluble peptide administration on pathogenic antigen-reactive T cells

4.1 Introduction

Although the prophylactic administration of peptide has been proven to be effective in inhibiting disease induction, the clinical requirement is to switch-off activated T cells during ongoing inflammation. Various studies have demonstrated that soluble peptide administration can be used to treat ongoing EAE and reduce the clinical signs of disease (Samson and Smilek, 1995, Devaux et al., 1997). However, later studies identified that using soluble peptides to treat EAE could result in severe anaphylactic reactions upon repeated encounter with peptide (Pedotti et al., 2001, Marshall, 2001). Therefore, studies into the effect of soluble peptide administration during ongoing disease have been hindered.

Previous work within the Anderton laboratory circumvented the problem of anaphylaxis through the design of an APL that retained the capacity to bind to the TCR, but no longer bound anti-peptide antibodies (Leech et al., 2007). These studies used the pMOG-induced model of EAE and demonstrated that administration of the APL in PBS at the peak of disease caused a striking reduction in the clinical signs of disease within 24 hours of treatment. This curative effect was associated with a reduction in the total number of $CD4^+$ cells within the CNS, and therefore it was thought that soluble peptide administration at the peak of disease induced deletion of the antigen-reactive cells. The findings promisingly demonstrate the use of peptide therapy to effectively treat ongoing disease, and prompt investigation into the effect of soluble peptide administration specifically on the pathogenic antigen-reactive T cells present during disease.

The use of traceable Tg4 cells allows the phenotype of activated antigen-reactive T cells to be investigated following soluble peptide administration. However, unlike the pMOG model of tolerance where naïve antigen-reactive T cells are deleted by

pMOG administration (Konkel et al., 2010), the data in Chapter 3 show that administration of soluble Ac1-9 peptide does not induce deletion of naïve Tg4 cells. Therefore, it was of interest to characterise the effect of soluble peptide administration on pathogenic Tg4 effector cells in an alternative form of tolerance.

Although activated $CD4^+$ T cells are less dependent on co-stimulatory signals than naïve T cells, the administration of pMOG appears to work in a similar mechanism on both naïve and activated T cells; by inducing their deletion. However, due to the observation that naïve Tg4 T cells are not deleted by 4Tyr administration, and in fact are present in greater numbers than after PBS administration, it was hypothesised that 4Tyr may induce expansion of pathogenic Tg4 T cell populations and therefore exacerbate disease. An alternative hypothesis was that Tg4 T_{eff} cells may be driven to activation-induced cell death upon 4Tyr administration due to persistent TCR stimulation in the absence of co-stimulatory signals (Green et al., 2003). This is because activated T cells are more prone to apoptosis than naïve $CD4^+$ T cells due to their high expression of the pro-apoptotic molecule FasL (Brunner et al., 2000).

4.1.1 Aims

1) To investigate the effect of soluble peptide administration on EAE driven by Tg4 T_{eff} cells.
2) To determine the fate of Tg4 T_{eff} cells following soluble peptide administration.
3) To identify a potential mechanism of action of soluble peptide administration on EAE driven by Tg4 T_{eff} cells.

4.1.2 Experimental approach

In this chapter a passive model of EAE was used, in which Tg4 cells polarised to a Th1 effector phenotype in the presence of Ac1-9 (4Lys) were transferred into B10.PL mice. This approach results in clinical signs of disease within ~5 days. Previous work has determined that the Tg4 T_{eff} cells can first be identified in the CNS ~4 days after transfer in this model (Prendergast, 2011).

To test the effect of soluble peptide administration on pathogenic T cells, the generation of these effector cells was required. Although both IFN-γ and IL-17 producing T cells can be seen in the CNS at the peak of active EAE (Rothhammer et al., 2011), previous work within the laboratory has demonstrated that Th1 and not Th17 effector cells, are absolutely required for the induction of EAE (O'Connor et al., 2008). These studies established the protocol for the generation of pathogenic Tg4 Th1 cells. In accordance with this protocol, splenocytes from Tg4 mice were isolated and cultured *in vitro* in the presence of WT Ac1-9 (4Lys), IL-2, IL-12 and IL-18. After three days of culture, the phenotype of these cells was assessed (Figure 4.1A). Effector cytokine production was measured in a small cohort of cells by ICS and flow cytometry; cells were stimulated with PMA and ionomycin in the presence of Brefeldin A for four hours prior to cytokine staining. Production of the Th1-associated cytokine IFN-γ was detected in a large proportion of $CD4^+$ Tg4 cells, and was present at high levels in the supernatant of cultures (as measured by ELISA) (Figure 4.1B & 4.1D). TNF-α and GM-CSF production by the polarised $CD4^+$ Tg4 population was also detected; and no IL-17 production was observed (Figure 4.1B). Expression of the Th1-associated transcription factor T-bet, which has been shown to be required for the induction of EAE (Bettelli et al., 2004, Gocke et al., 2007), was also observed in the $CD4^+$ population (Figure 4.1C). These data confirm the generation of Tg4 Th1 effector cells.

In order to investigate the effect of soluble peptide administration on pathogenic T cells and disease course, two approaches were used. The first approach investigated the effect of 4Tyr administration during ongoing disease, and was given at the peak

of EAE (Figure 4.2A). The second investigated the effect of 4Tyr administration on the day following cell transfer into B10.PL hosts, before the pathogenic Tg4 cells had migrated to the CNS (Figure 4.3A).

In addition to observing the effect of 4Tyr administration on disease course, the second approach was also utilised to investigate the fate of Tg4 T_{eff} cells; whereby 4Tyr was administered on the day following cell transfer and spleens were taken for analysis four days later (Figure 4.7A). In this set of experiments Ptx was not administered in order to maintain the effector cells within the periphery. The fate of the Tg4 cells following 4Tyr administration was assessed by examining the number and phenotype of these cells. Characteristics thought to be required for the pathogenicity of Tg4 cells were investigated, including the ability to produce effector cytokines, transcription factor expression and the presence of cell surface molecules associated with entry into the CNS. To follow the effects of soluble peptide administration on Tg4 T_{eff} cells over time, a separate experiment was conducted whereby 4Tyr was administered on the day after cell transfer, and from separate cohorts of mice spleens were taken for analysis on days two, five, seven and sixteen after treatment (Figure 4.9A).

In order to identify potential mechanisms of action of soluble peptide administration on EAE driven by effector T cells, the effect of treatment with the panel of Ac1-9 analog peptides on Tg4 T_{eff} cells was investigated. In these experiments Tg4 T_{eff} cells were transferred into B10.PL hosts one day prior to treatment with 4Lys, 4Ala, 4Val, 4Tyr or PBS, and the spleens were taken for analysis four days later (Figure 4.10A). Associations between the phenoptype of the Tg4 cells and the MHC-binding affinity of the peptide were interrogated.

In this chapter the effect of Ac1-9 peptide administration of pathogenic Tg4 T_{eff} cells was investigated.

4.2 Results

4.2.1 Administration of 4Tyr at the peak of passive EAE does not alter disease course.

For the effective clinical translation of peptide therapy, it is vital to understand the consequences of soluble peptide administration during ongoing disease. Previous work from the lab has demonstrated that soluble peptide administration at the peak of EAE has the ability to induce a dramatic reduction in the clinical signs of disease (Leech et al., 2007). However, due to the differences in models, it was important to determine the effects of 4Tyr administration on EAE induced by Tg4 cells. To investigate this, EAE was induced using passive transfer of Tg4 T_{eff} cells and at the peak of disease host mice were treated with 4Tyr or PBS (Figure 4.2A).

As can be seen in Figure 4.2B, 4Tyr administration had no significant effects on disease course compared to PBS. In both groups, entry into the resolution stage of disease appeared to occur without hindrance. To investigate if 4Tyr administration had induced expansion of the Tg4 cell population within the CNS, the total cellularity of the CNS and spleen were analysed three days after treatment. However, no significant differences were observed in the total cellularity of the spleen or CNS (Figure 4.2C). From these data it can be concluded that administration of 4Tyr neither exacerbates ongoing passive EAE, nor accelerates the natural recovery phase of disease.

4.2.2 Administration of 4Tyr prevents induction of EAE by pathogenic Tg4 T_{eff} cells

Clinical signs of EAE develop ~ 5 days after Tg4 T_{eff} cell transfer. This allows the opportunity to deliver tolergenic peptide between cell transfer and development of disease. Tg4 T_{eff} cells were transferred into B10.PL hosts one day prior to administration of 4Tyr, 4Lys or PBS (Figure 4.3A). Mice also received Ptx on the

same day as peptide treatment. PBS-treated mice showed a robust disease course (mean maximal disease score 3.3, incidence 100%, Figure 4.3B). Administration of 4Lys did not alter the course of EAE. However, administration of 4Tyr completely abrogated disease induction.

These results demonstrated that in addition to inducing tolerance in naïve Tg4 cells, 4Tyr administration is capable of inducing tolerance in activated T_{eff} cells. In an attempt to investigate if the lack of disease induction was due to deletion of the Tg4 T_{eff} cells upon 4Tyr administration, spleens were taken on day 28 post-treatment and the presence of donor cells was determined (Figure 4.4). No differences in the total numbers or proportion of $CD4^+$ cells or $CD4^+$ Tg4 cells were observed between any of the groups (Figure 4.4B & 4.4C). However, distinct populations of Tg4 cells were present in all treatment groups. These results implied that 4Tyr administration did not induce deletion of Tg4 T_{eff} cells.

4.2.3 4Tyr administration inhibits the ability of Tg4 T_{eff} cells to migrate into the CNS

In subsequent experiments, the spleens and CNS of 4Tyr- and PBS-treated mice were harvested directly after the peak of disease, and the presence of $CD4^+$ Tg4 cells was determined to identify any changes in location of the Tg4 T_{eff} cells (Figure 4.5A). Within the CNS, lower total cellularity and lower total numbers of $CD4^+$ cells were observed in the 4Tyr-treated group compared to PBS (Figure 4.5B). There were also significantly lower numbers of $CD4^+$ Tg4 cells in the CNS of the 4Tyr-treated group compared to PBS (Figure 4.5B). These data show that 4Tyr-treated T_{eff} cells had not migrated to the CNS as readily as PBS-treated cells.

Tg4 numbers were also analysed in the spleen (Figure 4.6A). The total cellularity and numbers of $CD4^+$ cells within the spleen were significantly higher in the 4Tyr-treated group compared to PBS (Figure 4.6B). Despite no significant differences in the numbers or proportion of $CD4^+$ Tg4 cells between groups, the numbers of donor

cells within the spleens of the PBS-treated group were $<1x10^5$, whereas in the 4Tyr-treated group three out of the six mice had $>1x10^5$ donor cells within the spleen (Figure 4.6B).

These results provided evidence that administration of 4Tyr alters the migratory potential of Tg4 T_{eff} cells, and that these cells may be inhibited from trafficking to the CNS.

4.2.4 PSGL-1 expression is down-regulated on Tg4 T_{eff} cells upon 4Tyr administration

In this passive transfer model of EAE, the earliest CNS infiltration by Tg4 cells is evident at 3-4 days after transfer (Prendergast, 2011). To identify changes in molecules associated with CNS entry, the experimental protocol was adapted to analyse splenocytes four days after peptide administration (no Ptx was given) (Figure 4.7A). PSGL-1, CXCR3 and CCR5 have each been reported to be important in the migration of $CD4^+$ T cells into the CNS during EAE (Bahbouhi et al., 2009, Balashov et al., 1999, Sorensen et al., 1999). Previous work within the laboratory has also demonstrated a potential role for PSGL-1 expression in enabling pathogenic $CD4^+$Tg4 cells to enter the CNS and induce disease (Prendergast, 2011).

Four days after administration of 4Tyr or PBS, PSGL-1 was found to be expressed on host and donor $CD4^+$ populations in both groups (Figure 4.7B). However, PSGL-1 expression was significantly higher on $CD4^+$ Tg4 cells from PBS-treated mice compared to those from 4Tyr-treated. Levels of PSGL-1 expression on Tg4 cells from 4Tyr-treated mice were similar to those expressed on the host $CD4^+$ cells of both groups. The high level of expression on PBS treated $CD4^+$ Tg4 cells compared to both 4Tyr- treated and host $CD4^+$ populations adds further evidence to suggest that pathogenic T cells require PSGL-1 expression to enter the CNS and induce disease.

CXCR3 expression has been shown to be induced by the transcription factor T-bet and therefore its expression is associated with Th1-driven pathology. Tg4 T_{eff} cells from PBS-treated mice expressed high levels of CXCR3, and 4Tyr administration caused no loss of this molecule on $CD4^+$Tg4 cells (Figure 4.7C). The majority of host $CD4^+$ cells were not found to express CXCR3 in either group. Expression of the Th1-associated chemokine receptor CCR5, was not detectable on host or donor cells of either group (Figure 4.7D).

4.2.5 4Tyr administration induces substantial expansion of the Tg4 T_{eff} population

In the above experiments there was no shortage of Tg4 T_{eff} cells in the spleens of 4Tyr-treated mice. Therefore, the protection from EAE mediated by 4Tyr administration was not due to deletion of the effector cells. In fact, four days after 4Tyr administration the overall cellularity of the spleen was found to be significantly higher than that of the PBS-treated group (Figure 4.8A). Although, no significant differences were observed in the total numbers or proportion of $CD4^+$ cells within the spleen, there were significantly higher numbers and a greater proportion of $CD4^+$ Tg4 donor cells in the 4Tyr-treated group, compared to PBS-treated (Figure 4.8B). The total numbers of $CD4^+$ Tg4 cells within the spleens were around ten-fold greater than the numbers transferred, accounting for ~50% of all $CD4^+$ cells within the spleen following 4Tyr administration. These data demonstrate that within four days, administration of 4Tyr induces considerable expansion of the Tg4 T_{eff} population.

4.2.6 Tg4 T_{eff} cell numbers decline after five days in 4Tyr-treated mice

Due to the large numbers of $CD4^+$ Tg4 cells observed in the spleen four days after 4Tyr-treatment, a time-course experiment was conducted to investigate if these cells

persisted at these numbers. Spleens were taken at day two, five, seven and 16 after peptide treatment (Figure 4.9A).

Although the total cellularity of the spleens appeared higher in the 4Tyr-treated group at all time points, this was only found to be significantly different at day two and day 16 (Figure 4.9B). The total numbers of $CD4^+$ cells were significantly higher within two days of 4Tyr administration compared to PBS-treated mice and remained so until day five (Figure 4.9C). The total proportion of $CD4^+$ cells was decreased at day two in the 4Tyr-treated group; however, by day five the proportion was significantly higher than the PBS treated controls. Thereafter, there were no significant differences in the total proportion of $CD4^+$ cells.

$CD4^+$ donor cells were present in significantly higher numbers at both day two and day five after 4Tyr administration (Figure 4.9D). By day seven, however, the number of $CD4^+$ donor cells in the 4Tyr- treated group had declined, and by day 16 there was no longer any significant difference in donor numbers between groups. These data suggest that the expansion of the Tg4 T_{eff} population following 4Tyr administration is maximal around day five.

4.2.7 Only the 4Tyr Ac1-9 peptide induces a significant increase in the number of Tg4 T_{eff} cells

As shown in Figure 4.3, 4Tyr administration inhibits induction of EAE by Tg4 T_{eff} cells, whereas administration of 4Lys has no effect on disease course. In order to try and elucidate the mechanism responsible for inhibition of disease induction, Tg4 T_{eff} cells were treated with the panel of Ac1-9 analog peptides (4Lys, 4Ala, 4Val, 4Tyr) or PBS, with the hypothesis that the mechanism of inhibition would correlate with the MHC-binding affinity of the peptide (Figure 4.10A). Tg4 T_{eff} cells were transferred into B10.PL hosts one day prior to administration of peptide and spleens were taken for analysis on day four post-treatment.

There was a significant increase in the total cellularity of the spleen and the total numbers of $CD4^+$ cells in the 4Tyr-treated group compared to PBS, but not in any other peptide-treated group (Figures 4.10B & 4.10C). Despite the lack of significant differences, the total cellularity and number of $CD4^+$ cells appeared to increase with the MHC-binding affinity of the peptide. The total proportion of $CD4^+$ cells was significantly higher in the 4Lys-treated group compared to PBS-treated, but was significantly lower in the 4Tyr-treated group compared to the PBS-treated group.

None of the Ac1-9 peptides induced deletion of the Tg4 T_{eff} cells (Figure 4.10D). The total numbers of $CD4^+$ donor cells appeared to follow a similar pattern as the total cellularity and total numbers of $CD4^+$ cells, with the numbers of donor cells increasing with the MHC-binding affinity of the peptide (Figure 4.10D). However, the only peptide that induced a significant increase in the number of $CD4^+$ Tg4 cells compared to PBS was 4Tyr. As expected this result was also mirrored in the proportion of donor cells within the $CD4^+$ population, with the greatest proportion of donor cells present in the 4Tyr-treated group (Figure 4.10D).

4.2.8 Tg4 T_{eff} cells are inhibited in their ability to produce IL-2 and effector cytokines after 4Tyr administration

Thus far, the data demonstrate a striking expansion of the Tg4 T_{eff} population within four days of 4Tyr administration, but profound inhibition of EAE, correlating with lower expression of PSGL-1 on the transferred Tg4 cells. To test additional reasons for the impaired pathogenic activity of the Tg4 T_{eff} cells, functional readouts (cytokine production and T-bet expression) were examined.

Due to the substantial difference in $CD4^+$ Tg4 numbers between the 4Tyr- and PBS-treated groups, the numbers of these cells had to be normalised in order to measure cytokine production from recall assays. This was achieved by FACS sorting the $CD4^+$ Tg4 cells from the spleen four days after treatment and subsequent culture

with irradiated B10.PL splenic APC. These cells were subjected to a recall assay with increasing doses of 4Lys and cytokine production was measured by ELISA. Tg4 cells from the 4Tyr-treated group produced significantly lower amounts of both IL-2 and IFN-γ upon recall stimulation (Figure 4.11A&B). No evidence of a change in phenotype from Th1 to a Th2 was observed, as assessed by the lack of production of IL-13, IL-4 and IL-10 (data not shown).

To achieve a measure of cytokine production on a per-cell basis, unsorted splenocytes were cultured overnight in the presence or absence of 4Lys, and effector cytokine production assessed by flow cytometry. Intracellular cytokine staining revealed that a large proportion of $CD4^+$ Tg4 cells were able to produce IFN-γ in the PBS-treated group, however, this was significantly lower in the 4Tyr-treated group (Figure 4.12B) Levels of TNF-α production were also significantly lower in the 4Tyr- treated group. Despite very low production of GM-CSF by the Th1 effector cells at the time of transfer (Figure 4.1B), a greater proportion of PBS-treated $CD4^+$ Tg4 cells were now $GM\text{-}CSF^+$ (Figure 4.12B). No GM-CSF production was observed in the donor cells of the 4Tyr- treated group, and no production of any of these effector cytokines was observed in the host $CD4^+$ cells of either group.

A possible explanation for the loss of IFN-γ production by Tg4 cells from 4Tyr-treated mice would be inhibition of the master regulator of Th1 function, T-bet. Importantly, T-bet expression is also reported to be essential for T cell pathogenicity in EAE (Bettelli et al., 2004). As shown in Figure 4.1C, Tg4 T_{eff} cells express high levels of T-bet as the time of transfer. This was maintained four days after treatment with PBS (Figure 4.12C) and somewhat surprisingly, exposure to 4Tyr *in vivo* did not inhibit T-bet expression by Tg4 T_{eff} cells. This demonstrates that the inhibition of IFN-γ production occurs downstream of T-bet expression. As expected, T-bet was not found to be expressed in the host $CD4^+$ cells of either group.

4.2.9 Production of effector cytokines by Tg4 T_{eff} cells remains low for at least 16 days after 4Tyr treatment

It was important to ascertain whether 4Tyr-treated Tg4 cells could regain effector cytokine production with time or if this function was permanently lost. Intracellular cytokine staining of IFN-γ and TNF-α after overnight stimulation with 4Lys suggested that it was the latter case (Figure 4.13B). PBS-treated Tg4 cells maintained their ability to produce IFN-γ and TNF-α until day seven, but these cytokines remained inhibited in the 4Tyr- treated group. As expected, these data show that the ability to produce effector cytokine coincides with the pathogenicity of the cell.

PBS-treated Tg4 cells were found to maintain their expression of T-bet as far as day 16 (Figure 4.13C). However, Tg4 cells exposed to 4Tyr *in vivo* maintained their expression of T-bet only until day five; at subsequent time points expression of T-bet was found to be significantly lower than that seen in PBS-treated Tg4 cells.

4.2.10 Down-regulation in effector cytokine production correlates with the MHC-binding affinity of the Ac1-9 peptide

When splenocytes were sampled four days after *in* vivo exposure to the panel of Ac1-9 analog peptides the inhibition in effector cytokine production correlated with the MHC-binding affinity (Figure 4.14B). There was no significant difference in the proportion of cells that were IFN-γ^+ following overnight stimulation with the Ac1-9 peptide between the PBS-, 4Ala- or 4Lys-treated groups. The 4Val-treated group showed some inhibition of IFN-γ production, but this inhibition was greatest in the 4Tyr-treated group. Due to the link between pathogenicity and effector cytokine production, these data may indicate why 4Lys administration has no effect on the ability of Tg4 T_{eff} cells to induce disease, whereas 4Tyr administration completely inhibits disease induction. Expression of T-bet was found to be

maintained in all treatment groups, with no significant differences found (Figure 4.14C).

4.2.11 $CD4^+$ Tg4 cells in the 4Tyr-treated group do not express CTLA-4, but do express high levels of PD-1

It has been demonstrated that Tg4 T_{eff} cells exposed to 4Tyr *in vivo* have profound inhibition of IL-2 and effector cytokine production. How might this be mediated? The expression of two co-inhibitory molecules CTLA-4 and PD-1 was studied. PD-1 was identified in Chapter 3 as being selectively up-regulated on naïve Tg4 cells following 4Tyr administration. Previous reports have also suggested that signalling through CTLA-4 may play an important role in the induction of tolerance (Eagar et al., 2004).

At the time of transfer, Tg4 T_{eff} cells were not found to express CTLA-4, but did express high levels of PD-1 (data not shown). Following *in vivo* transfer and administration of PBS, Tg4 T_{eff} cells had lost expression of PD-1 (Figure 4.15A). However, $CD4^+$ Tg4 cells in the 4Tyr-treated group had maintained PD-1 expression. Expression of CTLA-4 on $CD4^+$ Tg4 cells was not detected in either treatment group (Figure 4.15B). PD-1 and CTLA-4 were also not found to be expressed on host $CD4^+$ cells in either group. The PD-1 ligand, PDL-1, was found to be expressed on both donor and host $CD4^+$ cells, but no significant differences were observed between groups (data not shown). In addition, expression of PDL-2 was not detected on host or donor $CD4^+$ cells in either group (data not shown).

Observation of PD-1 expression over time revealed that PD-1 expression was lost on PBS treated cells by day two after PBS injection, and remained low throughout the 16-day time-course (Figure 4.16A). However, 4Tyr-treated Tg4 cells maintained their expression of PD-1 at all time points observed.

4.2.12 4Tyr and 4Val administration maintain PD-1 expression on Tg4 T_{eff} cells

Comparison of the effects of Ac1-9 analog peptides on PD-1 expression (four days after exposure) showed the influence of MHC-binding affinity (Figure 4.16B). PD-1 was expressed to a significantly higher level on Tg4 T_{eff} cells in both 4Val- and 4Tyr-treated mice compared to PBS-treated. Importantly, the levels of PD-1 expression on 4Lys-exposed cells were not different to PBS controls, and may further explain why these cells remain pathogenic. These findings are consistent with the PD-1 expression on naïve cells following peptide administration shown in Chapter 3.

4.2.13 4Tyr administration increases the total number of donor $CD4^+Foxp3^+$ cells and the proportion of host $CD4^+Foxp3^+$ cells

In Chapter 3, differences in donor and host $CD4^+Foxp3^+$ populations following administration of 4Tyr identified a potential role for these cells in the inhibition of disease induction. Therefore, in the context of pathogenic Tg4 cell transfer, $CD4^+Foxp3^+$ donor and host populations were investigated four days after 4Tyr administration.

There were no significant differences in the total numbers of host $CD4^+Foxp3^+$ cells. However, there was a trend toward higher numbers in the 4Tyr-treated group (Figure 4.17B). This coincided with an increased proportion of $Foxp3^+$ cells within the host $CD4^+$ population of the 4Tyr-treated group compared to PBS-treated. This finding suggests that following Tg4 T_{eff} cell transfer, 4Tyr administration may either directly or indirectly cause expansion of the host $CD4^+Foxp3^+$ Treg population. Despite the increase in the total numbers and proportion of donor $CD4^+Foxp3^+$ cells in the 4Tyr-treated group compared to PBS-treated, the frequency of $Foxp3^+$ cells within the donor $CD4^+$ cohort was no higher than at the time of transfer (1-3%, data not shown) (Figure 4.17B).

4.2.14 Host and donor CD4$^+$Foxp3$^+$ cell numbers begin to decline five days after 4Tyr administration

Within two days of 4Tyr administration the total numbers of host CD4$^+$Foxp3$^+$ cells were significantly greater than in the PBS-treated group (Figure 4.18B). These cells remained present in higher numbers until day five, after which time the total number of these cells declined. Unlike the data from day four after peptide administration, no differences were observed in the proportion of Foxp3$^+$ cells within the host CD4$^+$ population at any of the time points. Donor CD4$^+$Foxp3$^+$ cell numbers were only found to be significantly elevated at day five following 4Tyr administration, this effect was diminished by day seven (Figure 4.18C). No differences were observed in the proportion of Foxp3$^+$ cells within the donor CD4$^+$ population at any time point. Thus the increased Foxp3$^+$ Tg4 cell numbers appeared to reflect the general expansion of the Tg4 population caused by 4Tyr administration. This indicated that Foxp3$^+$ donor cells had neither enhanced nor reduced ability to proliferate in response to soluble peptide.

When comparing the effects of different Ac1-9 analog peptides (at day four after peptide administration) only 4Tyr was found to cause a) a significantly higher frequency of host Foxp3$^+$ cells and b) significantly higher numbers, but not frequency, of Tg4 Foxp3$^+$ cells (Figure 4.19A & B). This again suggests that in this experiment the increase in CD4$^+$Foxp3$^+$ numbers is due to the overall increase in donor cell numbers

4.2.15 4Tyr administration induces proliferation of host CD4$^+$Foxp3$^+$ cells in the presence of Tg4 effectors

To further investigate the expansion of the host CD4$^+$Foxp3$^+$ population following 4Tyr administration, mice were given BrdU on days one and three after 4Tyr- or PBS-treatment. Spleens were harvested on day four after peptide and the proportion of BrdU$^+$ cells within host CD4$^+$Foxp3$^+$ was determined (Figure 4.20A).

There were insufficient numbers of donor $CD4^+Foxp3^+$ cells to adequately analyse the proportion of $BrdU^+$ cells within this population. The proportion of $BrdU^+$ cells within the host $CD4^+Foxp3^-$ population was very low and no significant differences were observed between treatment groups (Figure 4.20B). However, in the host $CD4^+Foxp3^+$ population, the proportion of $BrdU^+$ cells ranged from 5-20% and was found to be significantly higher in the 4Tyr- treated group compared to PBS. These data demonstrate that 4Tyr administration induces proliferation of host $CD4^+Foxp3^+$ cells.

Figure 4.1 Polarisation of Tg4 cells to an effector (Th1) phenotype

Figure 4.2 4Tyr administration at the peak of EAE has no effect on disease course or the cellularity of the spleen or CNS

Figure 4.3 4Tyr administration abrogates disease induced by Tg4 effector cells

Figure 4.4 4Tyr administration does not result in the deletion of pathogenic Tg4 cells

Figure 4.5 Tg4 effector cells do not enter the CNS after 4Tyr administration

Figure 4.6 Tg4 effector cells are present in the spleen after 4Tyr administration

Figure 4.7 Tg4 effector cells have lower expression of PSGL-1 and higher expression of CXCR3 after 4Tyr administration compared to PBS

Figure 4.8 4Tyr administration causes expansion of the Tg4 T_{eff} population

Figure 4.9 Expansion of Tg4 effector cells peaks at D5 after 4Tyr administration, but Tg4 cells are still present in the spleen at D16

Figure 4.10 Only the 4Tyr Ac1-9 APL induces expansion of Tg4 effector population

Figure 4.11 Tg4 effector cells produce lower amounts of IL-2 and IFN-γ following 4Tyr administration compared to PBS

Figure 4.12 4Tyr treatment decreases the proportion of Tg4 effector cells that produce IFN-γ, TNF-α and GM-CSF upon recall stimulation, but maintains T-bet expression

Figure 4.13 4Tyr treatment decreases the proportion of Tg4 effector cells that produce IFN-γ and TNF-α within two days

Figure 4.14 Only 4Tyr and 4Val administration significantly reduces the proportion of Tg4 cells that are IFN-γ^+

Figure 4.15 Tg4 effector cells express high levels of PD-1 in the 4Tyr-treated group but not in the PBS-treated group. CTLA-4 is not detected on host or donor $CD4^+$ populations in either group

Figure 4.16 Tg4 effector cells maintain PD-1 expression up to D16 after 4Tyr administration. Expression of PD-1 correlates with the MHC-binding affinity of the peptide

Figure 4.17 4Tyr administration increases the proportion of host & donor Foxp3$^+$ cells, and the number of donor Foxp3$^+$ cells

Figure 4.18 Numbers of host $CD4^+Foxp3^+$ cells peak at D2 after 4Tyr administration whereas numbers of donor $CD4^+Foxp3^+$ cells peak at D5

Figure 4.19 4Tyr is the only Ac1-9 peptide that results in an increased proportion of host CD4+Foxp3+ cells and number of donor $CD4^{+}Foxp3^{+}$ cells

Figure 4.20 4Tyr-treatment induces proliferation of host $CD4^+Foxp3^+$ cells

4.3 Discussion

4.3.1 The effect of soluble peptide administration during ongoing disease

Antigen-experienced $CD4^+$ T cells have been shown to have a lower activation threshold and are less dependent on co-stimulation for activation than naïve T cells (Croft et al., 1994, Kimachi et al., 2003). The current consensus is that peptide-induced tolerance in naïve $CD4^+$ T cells relies upon the absence of co-stimulatory signals (Miller et al., 2007). Therefore, it is imperative to determine whether soluble peptide administration can actually induce tolerance in pre-activated $CD4^+$ T cells that have no strong requirement for co-stimulation. Due to this ability to become re-activated following only TCR signalling, it is possible that peptide administration may induce further activation of these antigen-experienced cells and therefore cause exacerbation of disease. Indeed, a phase II clinical trial involving an APL of MBP had to be halted due to a suspected exacerbation of multiple sclerosis (Bielekova et al., 2000). Alternatively, activated $CD4^+$ T cells are known to be more susceptible to apoptosis due to the up-regulation of FasL (Brunner et al., 2000) and therefore, persistent signalling through the TCR may drive the cells into activation-induced cell death (Green et al., 2003).

Investigation into the effect of 4Tyr administration during ongoing disease demonstrated no effect on disease course (Figure 4.2). This observation was supported by the lack of differences in the total cellularity of the spleen and CNS three days after treatment. As mentioned in Chapter 3, it is thought that the host and not the donor $CD4^+Foxp3^+$ cells are responsible for driving the resolution of EAE. As is evident from the disease course, resolution of disease began in both 4Tyr- and PBS-treated groups the day after treatment (Figure 4.2). This may suggest that host $CD4^+Foxp3^+$ cells had begun to suppress the pathology caused by $CD4^+$ Tg4 cells, and that the number of $CD4^+$ Tg4 cells had already started to decline. Therefore 4Tyr administration at this time point might not be expected to influence the disease course. Due to the short duration of the disease, it would be

more pertinent to administer 4Tyr administration immediately after clinical signs are established.

4.3.2 Peptide-induced tolerance in Tg4 T_{eff} cells is not due to deletion

Despite the absence of any effect on disease course by soluble peptide administration at the peak of disease, the results shown in this chapter demonstrate that soluble peptide administration can switch-off activated antigen-experienced $CD4^+$ T cells and prevent them from inducing disease (Figure 4.3). However, this could only be achieved through the administration of the 4Tyr APL, with high MHC-binding affinity, not with the WT 4Lys peptide. Neither peptide was found to induce exacerbation of disease.

Surprisingly, this effect was not due to the deletion of the antigen-reactive cells upon peptide administration. Elevated numbers of $CD4^+$ Tg4 cells were found within the spleen of the 4Tyr group at day four (a five-fold increase) compared to PBS and 4Lys treatment (Figures 4.8 & 4.10). The expansion of the Tg4 T_{eff} population appeared to peak at day five after 4Tyr administration, but they could still be identified readily at day 16 (Figure 4.9). The reduction in $CD4^+$ Tg4 numbers after day five could be due to cell death induced by cytokine-withdrawal, as $CD4^+$ Tg4 cells were no longer found to produce IL-2 or IFN-γ at four days after 4Tyr injection (Figure 4.11).

Treatment with the panel of Ac1-9 analog peptides revealed a similar pattern to that seen in the treatment of naïve $CD4^+$ Tg4 cells, whereby a correlation exists between the number of Tg4 T_{eff} cells and the MHC-binding affinity of peptide (Figure 4.10). This observation, coupled with the finding that the ability of the cells to produce effector cytokines decreased as the MHC-binding affinity of the peptide increased (Figure 4.14), suggests that the induction of tolerance in Tg4 T_{eff} cells may be a function of the sustained TCR engagement. This correlates with previous studies

within the laboratory that demonstrated that 4Tyr can persist for up to 14 days after administration (Konkel, 2008).

4.3.3 The abrogation of effector cytokine production by Tg4 T_{eff} after *in vivo* exposure to 4Tyr

A major contributing factor to the pathogenicity of a T cells is their ability to produce effector cytokines. Whilst neither IFN-γ nor TNF-α production is an exquisite requirement for the induction of EAE (Ferber et al., 1996, Krakowski and Owens, 1996, Frei et al., 1997), the standing paradigm is that the transcription factor responsible for driving IFN-γ production, T-bet, is required (Bettelli et al., 2004, Gocke et al., 2007, Yang et al., 2009).

Although a significantly lower proportion of 4Tyr- treated $CD4^+$Tg4 cells were capable of producing IFN-γ and TNF-α from as early as two days after treatment (Figure 4.13), these cells were found to maintain expression of T-bet at all time points investigated. If T-bet expression was necessary for the induction of EAE this does not explain why the 4Tyr- treated Tg4 T_{eff} cells are no longer pathogenic. However, current studies within the laboratory have suggested that T-bet may not be an absolute requirement for the induction of EAE (O'Connor & Anderton, unpublished observations).

Several recent studies have demonstrated that the production of GM-CSF by $CD4^+$ T cells is necessary for the induction of EAE (Ponomarev et al., 2007, Kroenke et al., 2010, Codarri et al., 2011, El-Behi et al., 2011). It is reported that the Th17-associated transcription factor, RORγt, and not the Th1-associated T-bet, is responsible for driving GM-CSF production in $CD4^+$ T cells (Codarri et al., 2011). The results within this chapter demonstrate that despite the very low frequency of Th1 polarised Tg4 cells that produced GM-CSF at the time of transfer (Figure 4.1), a significant proportion of $CD4^+$ Tg4 cells in the PBS treated group had gained the ability to produce GM-CSF four days after treatment (Figure 4.12). The

observation that the proportion $CD4^+$ Tg4 cells capable of producing GM-CSF was significantly lower in the 4Tyr- treated group may at least partly explain why these cells are no longer pathogenic.

The inability of Tg4 T_{eff} cells to produce effector cytokines appears to be related to the degree of expansion of the cells, which in turn is determined by the MHC-binding affinity of the peptide used as tolerogen (Figure 4.14). This could possibly be due to the proliferation of cells in the absence of Th1 polarising cytokines, and therefore the progeny of the cells are not programmed to become Th1 cells. However, expression of T-bet was maintained to a similar extent in all peptide-treated groups, which would suggest that this is not the case (Figure 4.14C). Studies by Gabrysova et al., have shown that multiple doses of 4Tyr can induce a switch in phentotype from IFN-γ producing cells to IL-10 producing Th1 cells, and that these cells maintain expression of T-bet (Gabrysova et al., 2009). In the experiments detailed in this chapter, IL-10 production by Tg4 T_{eff} cells from 4Tyr-treated mice was not detected (data not shown).

In addition to reduced effector cytokine production, four days after 4Tyr administration, $CD4^+$ Tg4 cells also produced less IL-2 than PBS treated cells upon recall stimulation (Figure 4.11). This is in contrast to naïve $CD4^+$ Tg4 cells, which retained the capacity to produce IL-2 after treatment with 4Tyr (Figure 3.6). These results suggest that due to the substantial expansion of $CD4^+$ Tg4 cells following 4Tyr administration, the cells have become exhausted by day four and therefore cytokine production has been switched-off. This implication is supported by the observation that $CD4^+$ Tg4 cells from 4Tyr-treated mice express high levels of PD-1 (Figure 4.15B), and may explain why the $CD4^+$ Tg4 population decreases after five days post-treatment (Figure 4.9D).

4.3.4 Altered migratory potential of Tg4 T_{eff} cells after *in vivo* exposure to 4Tyr

Despite the expansion of $CD4^+$ Tg4 population following 4Tyr administration, fewer of these cells reached the CNS (Figure 4.5B). This might suggest that 4Tyr administration inhibits the ability of Tg4 T_{eff} cells to migrate into the CNS in order to induce disease.

Adhesion molecules and chemokine receptors that are associated with Th1 cells and are thought to potentially play a role in the recruitment of cells into the CNS include CCR5, CXCR3 and PSGL-1. The chemokine receptors CCR5 and CXCR3 have been shown to be expressed on T cells found within the MS lesions and CSF from MS patients (Sorensen et al., 1999, Balashov et al., 1999). In addition, both of these receptors are found to be preferentially up-regulated on Th1 cells (Loetscher et al., 1998, Sallusto et al., 1998). Up-regulation of PSGL-1 on $CD4^+$ T cells can be induced by IL-12 (Deshpande et al., 2006) and has been found to be up-regulated on the circulating $CD4^+$ T cells of MS patients (Bahbouhi et al., 2009).

CCR5 was not detected on either host or donor $CD4^+$ cells (Figure 4.7D). Previous studies within the laboratory have demonstrated that administration of a CCR5 inhibitor, Tak779, had no effect on the subsequent disease induction by Tg4 T_{eff} cells (Prendergast, 2011), implying that CCR5 may not be required for Tg4 T_{eff} cells to enter the CNS.

The expression of CXCR3 on $CD4^+$ T cells is thought to be induced by T-bet (Lord et al., 2005, Beima et al., 2006). Consistent with the expression of T-bet on $CD4^+$ Tg4 cells in both the 4Tyr- and PBS-treated groups, CXCR3 was found to be expressed on these populations (Figure 4.7C). The significant increase in the level of CXCR3 expression on $CD4^+$ Tg4 cells in the 4Tyr-treated group compared to PBS contorls can not be explained by differences in the expression of T-bet between these groups. Nevertheless, these results indicate that alteration in CXCR3 expression is not responsible for the lack of $CD4^+$ Tg4 cell migration into the CNS

in the 4Tyr- treated group. This is supported by the data using Tak779 (as described above), as this inhibitor also blocks the function of CXCR3 (Gao et al., 2003).

Although CCR5 and CXCR3 do not appear to play a role in the migration of Tg4 T_{eff} cells into the CNS, PSGL-1 expression was found to be lower on 4Tyr-treated $CD4^+$ Tg4 cells compared to PBS (Figure 4.7B). Although constitutively expressed on $CD4^+$ T cells, PSGL-1 levels were elevated on $CD4^+$ Tg4 cells in the PBS-treated group compared to both 4Tyr-treated and host $CD4^+$ populations. The role of PSGL-1 in enabling pathogenic cells to traffic into the CNS to induce EAE is a contentious issue. Several studies have demonstrated that the induction of EAE in PSGL-1-deficient mice is no different to that of WT mice (Osmers et al., 2005, Engelhardt et al., 2005, Bill et al., 2011). In contrast, others have shown that incubation of pathogenic myelin-reactive $CD4^+$ cells with a PSGL-1 blocking antibody before passive transfer, results in a milder form of disease (Deshpande et al., 2006). Indeed, previous studies within the laboratory have demonstrated that incubation of Tg4 T_{eff} cells with anti-PSGL-1 before transfer results in the delayed onset of disease (Prendergast, 2011).

The down-regulation of PSGL-1 may explain why the migration of $CD4^+$ Tg4 cells into the CNS is inhibited in the 4Tyr-treated group, but this does not explain the functional impairment of these cells and their lack of effector cytokine production within two days of peptide-treatment. It would be interesting to further investigate the kinetics of PSGL-1 expression, in order to determine whether down-regulation of this molecule occurs after tolerance has already been induced in these cells.

4.3.5 A role for regulatory T cells in suppressing Tg4 T_{eff} cells upon 4Tyr administration?

The increase in numbers of donor $CD4^+Foxp3^+$ cells following administration of 4Tyr is likely due to the overall increase in the number of $CD4^+$ Tg4 cells

(Figure 4.17B). Although a significant difference was observed in the proportion of $Foxp3^+$ cells within the donor $CD4^+$ cohort at day four in several experiments, this finding was not consistently observed, and the proportion was no greater than that at the time of transfer. During the time course experiment and upon investigation of all Ac1-9 peptides, no differences were seen in the proportion of donor $CD4^+Foxp3^+$ cells (Figure 4.18C & 4.19C). Due to the small numbers of cells in this population compared to the number of donor $CD4^+Foxp3^-$ cells, it is unlikely that these cells are responsible for tolerance induction in Tg4 T_{eff} cells. In order to test this, Tg4 T_{eff} cells could be generated from Tg4xFoxp3.LuciDTR-4 cells (O'Connor et al., 2010) and sorted for $CD4^+Foxp3^-$ cells before transfer.

As mentioned in Chapter 3, during EAE there is an enrichment of host $CD4^+Foxp3^+$ cells within the CNS and it is thought that these cells are responsible for driving resolution of Tg4-mediated disease. Donor $CD4^+Foxp3^+$ cells are not thought to be required for this process. Therefore, the increase in the proportion of host $CD4^+Foxp3^+$ cells after 4Tyr administration could be implicated in the inhibition of disease induction in this group (Figures 4.17B). In support of this, the host $CD4^+Foxp3^+$ population of cells were found to have proliferated to a greater extent in the 4Tyr-treated group compared to PBS controls (Figure 4.20B).

$CD4^+Foxp3^+$ nTreg cells are generally classified as being anergic due to their lack of IL-2 production upon *in vitro* stimulation (Fehervari and Sakaguchi, 2004). Despite their own lack of IL-2 production they express high levels of the IL-2 receptor α-chain, CD25 (Sakaguchi et al., 1995). Several studies have suggested that a potential mechanism by which Treg can suppress T_{eff} cells is through competition for IL-2, and therefore the induction apoptosis in T_{eff} cells by IL-2 deprivation (de la Rosa et al., 2004, Pandiyan et al., 2007). If a large amount of IL-2 is produced due to the expansion of $CD4^+$ Tg4 population upon 4Tyr administration, this may explain the subsequent increased proliferation and number of host $CD4^+Foxp3^+$ cells after treatment with 4Tyr, compared to PBS (Figures 4.18B & 4.20B). In addition, this suggests that the consumption of IL-2 by the host $CD4^+Foxp3^+$ cells may be responsible for the decrease in $CD4^+$ Tg4 numbers five

days after peptide, due to IL-2 deprivation and the induction of apoptosis in the Tg4 cells. However, this mechanism of suppression by nTreg cells is currently under debate as other studies have demonstrated that suppression can occur by Treg deficient in the IL-2 receptor α-chain (Fontenot et al., 2005), and that Treg are capable of suppressing T cells that are resistant to apoptosis (Szymczak-Workman et al., 2011). There is also the possibility that the expansion of the $CD4^+$ Tg4 population is self-limiting and that they stop producing IL-2 and other cytokines on their own accord due to exhaustion rather than regulation.

Comparison of treatment with the panel of Ac1-9 analog peptides demonstrated that only the 4Tyr APL, which caused the greatest increase in numbers of $CD4^+$ Tg4 cells, was able to induce significant changes in the host $CD4^+Foxp3^+$ population (Figure 4.19B). This observation further implicates a role for IL-2 production in the proliferation of host $CD4^+Foxp3^+$ cells. In order to investigate whether the increased numbers in host $CD4^+Foxp3^+$ cells was due to IL-2 production by $CD4^+$ Tg4 cells upon 4Tyr treatment, an IL-2 blocking antibody could be administered to observe whether this abrogates the effect. Clearly, further investigation is warranted.

Despite these observations, administration of 4Val was also found to reduce the proportion of $CD4^+$ Tg4 cells that produced effector cytokines (Figure 4.14B), even though no changes were observed in host regulatory T cells. However, both 4Tyr and 4Val treated $CD4^+$ $Tg4^+$ cells were found to have significantly higher expression of PD-1 compared to PBS (Figure 4.16B). When $CD4^+$ Tg4 cells were isolated for recall assays, production of IL-2 and IFN-γ remained low despite the removal of any influence by host $CD4^+Foxp3^+$ cells (Figure 4.11). Also, effector cytokine production remained low in 4Tyr- treated cells even after the number of host $CD4^+Foxp3^+$ cells had decreased by day seven (Figures 4.13B & 4.18B). These results indicate that host $CD4^+Foxp3^+$ cells may play some role in controlling the expansion of $CD4^+$Tg4 cells, but not necessarily in regulating the pathogenicity of these cells.

4.3.6 A role for PD-1 in the induction of tolerance in Tg4 T_{eff} cells upon *in vivo* exposure to 4Tyr?

The results in this chapter demonstrate that PBS-treated $CD4^+$ Tg4 cells lose their PD-1 expression within two days of treatment, whereas 4Tyr-treated cells maintain elevated levels of PD-1 expression until at least day 16 after treatment (Figure 4.16A). These data provide a clear cut mechanism by which 4Tyr administration may inhibit the pathogenicity of Tg4 T_{eff} cells.

It has been demonstrated that PD-1 expression is elevated on exhausted virus-specific $CD8^+$ T cells found in chronic viral infections where antigen persists, such as LCMV, HCV and HIV (Barber et al., 2006, Urbani et al., 2006, Day et al., 2006). Exhaustion of virus-specific $CD8^+$ cells is associated with inhibited proliferative capacity and ability to produce cytokines such as IL-2, IFN-γ and TNF-α (Fuller and Zajac, 2003, Wherry et al., 2003). Blockade of PD-1 signalling on $CD8^+$ T cells has been shown to restore effector function and enable viral clearance (Barber et al., 2006, Lukens et al., 2008). Therefore, it may be possible to restore effector function in 4Tyr- treated Tg4 T_{eff} cells by blockade of PD-1 signalling, and re-establish pathogenicity in this population.

It is thought that expression of both PD-1 and its ligand PDL-1 on activated T cells supports T cell-to-T cell interactions, and therefore is important in the inhibition of the PD-1 expressing cells (Latchman et al., 2004). The observation that both host and donor $CD4^+$ cells were found to express PDL-1 (data not shown), suggests that these cells may act to support inhibition of PD-1-expressing T cells.

PD-1 has been shown to be up-regulated during T cell activation (Keir et al., 2008). As a result it is also possible that PD-1 expression on 4Tyr- treated Tg4 T_{eff} cells may simply be a result of continued TCR signalling through the presence of 4Tyr. However, PD-1 expression remained elevated until at least day 16 after 4Tyr treatment, when most of the 4Tyr peptide will have been eliminated.

The observation that levels of PD-1 expression appeared to correlate with both the MHC-binding affinity of the peptide and with the reduction in T_{eff} cytokine production, supports a role for PD-1 signalling in the inhibited pathogenicity of 4Tyr- treated Tg4 T_{eff} cells (Figures 4.16B & 4.14B). Similar results were observed in Chapter 3, whereby PD-1 signalling was implicated in the induction of tolerance in naïve $CD4^+$ Tg4 cells. Taken together, the results shown in both Chapters 3 and 4 provide evidence for PD-1 signalling in the induction and maintenance of tolerance in both naïve and antigen-experienced effector $CD4^+$ cells.

4.4 Concluding remarks

- Administration of 4Tyr abrogates induction of EAE by Tg4 T_{eff} cells, whereas 4Lys does not.
- Tg4 T_{eff} cells from 4Tyr-treated mice have lower expression of PSGL-1 compared to PBS-treated, and are inhibited in their ability to migrate into the CNS.
- 4Tyr administration induces substantial expansion of the Tg4 T_{eff} cell population compared to PBS and 4Lys.
- The expansion of the Tg4 T_{eff} cell population correlates with the MHC-binding affinity of the peptide
- The increase in the number of Tg4 cells upon 4Tyr administration is associated with a decrease in the proportion of Tg4 cells that can produce IFN-γ, TNF-α and GM-CSF. Effector cytokine production in this population remains low until at least day seven after peptide treatment.
- 4Tyr-treatment is not associated with a change in phenotype from Th1 to Th2, as is evident by lack Th2-associated cytokine production
- Tg4 cells maintain elevated levels of PD-1 expression in 4Tyr-treated mice; whereas Tg4 cells from PBS-treated mice lose their PD-1 expression by day two after treatment.

- Levels of PD-1 expression correlate with the MHC-binding affinity of the peptide.
- 4Tyr administration also induces proliferation of host $CD4^+Foxp3^+$ cells; however, their expansion is not maintained past day five after treatment.

The results in this chapter demonstrate that although administration of 4Tyr induces a substantial increase in the numbers of Tg4 T_{eff} cells, these cells are no longer pathogenic. The inability of these cells to induce disease may be associated with down-regulation of PSGL-1, a reduction in the ability to produce effector cytokines such as GM-CSF, or due to regulation by host $CD4^+Foxp3^+$ cells. However, the evidence shown more likely indicates that 4Tyr administration drives Tg4 T_{eff} cells into an exhausted state that is regulated by PD-1 expression. In Chapter 5, the direct effect of 4Tyr administration on Tg4 Treg cells is investigated, and in Chapter 6 the role of PD-1 in the induction of tolerance by 4Tyr is explored further.

5. The effect of soluble peptide administration on antigen-reactive regulatory T cells

5.1 Introduction

One of the mechanisms by which the administration of soluble peptides has been demonstrated to induce tolerance is through the generation of T cells with a regulatory phenotype (Miller et al., 1992, Burkhart et al., 1999, Thorstenson and Khoruts, 2001). It is thought that oral administration of antigen leads to the induction of TGF-β producing Th3 cells (Chen et al., 1994, Hafler et al., 1997), whereas intranasal administration of peptide has been shown to induce IL-10 producting cells from Th1 cells, which have switched from IFN-γ to IL-10 production (Gabrysova et al., 2009). In addition, delivery of antigen via intravenous routes has previously been shown to induce expansion of antigen-reactive $CD4^+CD25^+$ Treg cells (Chappert et al., 2008).

In Chapters 3 and 4 it was demonstrated that treatment of naïve and T_{eff} Tg4 cells with 4Tyr resulted in an increase in the numbers of Tg4 $CD4^+Foxp3^+$ cells that were present in the transferred population. This population was found to expand at the same rate as the $CD4^+Foxp3^-$ population as no differences were found in the proportion of $Foxp3^+$ cells within the donor $CD4^+$ cohort. Although soluble peptide administration did not appear to induce $Foxp3^+$ regulatory T cells, the potential ability of peptide therapy to expand or maintain antigen-reactive Treg cells could be exploited in the use of Treg in cellular therapy.

The use of cells from Tg4xFoxp3.LuciDTR-4 reporter mice (O'Connor et al., 2010) enables the identification and purification of Ac1-9-responsive $Foxp3^+$ cells, that can be traced due to their expression of CD45.1. Therefore, the effect of peptide administration on the number, stability and function of antigen-reactive Treg can be investigated.

5.1.1 Aims

1) To determine whether *in vivo* administration of 4Tyr can induce the expansion of an antigen-reactive Treg population.
2) To investigate the effect of 4Tyr administration on the expression of Foxp3 by Treg.
3) To verify if 4Tyr treated Treg maintain their ability to suppress responder T cells.

5.1.2 Experimental approach

Although nTreg cells are typically characterised as being $CD4^+CD25^+Foxp3^+$, many previous studies on nTreg have used only CD4 and CD25 expression as a method for distinguishing and isolating these cells. The use of Tg4xFoxp3.LuciDTR-4 mice (O'Connor et al., 2010), which express GFP under the Foxp3 promoter, enables the isolation of nTreg based also upon their expression of $Foxp3^+$, and therefore is a more stringent method of obtaining nTreg. Although Tg4xFoxp3.LuciDTR-4 mice also express luciferase and the diphtheria toxin receptor under the Foxp3 promoter, enabling imaging and selective depletion of $Foxp3^+$ cells respectively, neither of these additional features were utilised in the experiments described in this chapter. Previous work within the laboratory has also determined that $CD62L^{hi}$ nTreg cells retain their expression of Foxp3 to a greater extent than $CD62L^{lo}$ cells (Stephens et al., 2009), and therefore nTreg cells were isolated by sorting for $CD4^+CD62L^{hi}CD25^+GFP^+$ cells.

Due to the low frequency of nTreg found in Tg4 transgenic mice (~1-3% of $CD4^+$ cells, data not shown) it is challenging to obtain sufficient numbers of these cells to transfer directly into recipient mice. Therefore, in order to obtain adequate numbers of nTreg to be able to determine the effect of 4Tyr administration on these cells, an *in vitro* expansion protocol was utilised whereby nTreg were cultured in the presence of α-CD3/α-CD28 coated beads and high dose IL-2 (1000U/ml).

An alternative method of obtaining large numbers of antigen-reactive Treg is through the *in vitro* generation of iTreg cells. This can be achieved by the purification and stimulation of naïve $CD4^{+}CD62L^{hi}CD25^{-}GFP^{-}$ cells in the presence of TGF-β and IL-2. Furthermore, the use of cells from Tg4xFoxp3.LuciDTR-4 reporter mice enables the purification of Foxp3 expressing iTreg cells at the end of culture based on GFP expression.

In this chapter the effect of soluble peptide administration antigen-reactive nTreg and iTreg is investigated, and the stability of their phenotype following peptide administration is determined.

5.2 Results

5.2.1 nTreg maintain expression of Foxp3 to a higher extent after 4Tyr administration compared to PBS

nTreg were isolated from Tg4xFoxp3.LuciDTR-4 mice and expanded *in vitro* as described in 5.1.2. After 21 days of culture, GFP^+ nTreg were purified by FACS and 0.75-1x10^6 cells were transferred into C57BL/6xB10.PL hosts one day prior to administration of 4Tyr or PBS (Figure 5.1A). The purity of the GFP^+ transferred population was consistently >98% (Figure 5.1B). Four days after 4Tyr/PBS administration, the numbers and proportion of host and donor $CD4^+$ cells were similar in both treatment groups (Figure 5.2B & 5.2C). Assessment of Foxp3 expression revealed that the majority of Tg4 nTreg had lost expression of Foxp3 in the PBS treated group (Figure 5.3B). However, a higher proportion of nTreg cells were found to maintain Foxp3 expression in the 4Tyr-treated group. This was reflected in the higher total numbers of $CD4^+Foxp3^+$ cells within the donor cohort after 4Tyr administration.

5.2.2 nTreg cells that maintain Foxp3 expression after 4Tyr treatment express PD-1

Within the donor population of the 4Tyr-treated group, bi-modal expression of PD-1 was observed (Figure 5.4A). In contrast, very few donor cells from the PBS-treated group were found to express PD-1. The majority of donor Tg4 cells in the PBS group were $Foxp3^-PD\text{-}1^-$, and the few that had maintained Foxp3 expression were largely $PD\text{-}1^-$ (Figure 5.4B). In the 4Tyr-treated group, the majority of Tg4 cells that had maintained Foxp3 expression were also found to express PD-1 (Figure 5.4C).

5.2.3 Antigen-reactive iTreg lose Foxp3 expression upon treatment with 4Tyr

Due to the limitations of using nTreg cells to determine the effect of peptide administration of regulatory T cells, iTreg were also utilised as these could be generated in large numbers. Tg4 iTreg cells were generated as stated in 5.1.2. After five days of *in vitro* culture, GFP^+ iTreg cells were FACS sorted and transferred into C57BL/6xB10.PL hosts one day prior to administration of 4Tyr or PBS. Spleens were taken for analysis four days later (Figure 5.5A). Induction of GFP expression was consistently >90% in iTreg, and the purity of the GFP^+ population transferred was consistently >98% (Figure 5.5B).

No differences were observed in the total cellularity of the spleen or the total numbers and proportion of $CD4^+$ cells between groups (Figure 5.6B). However, there were higher numbers and proportion of donor cells in the 4Tyr-treated group compared to PBS (Figure 5.6C). This suggests that 4Tyr administration maintains the iTreg population to a greater extent than PBS. Investigation into the expression of Foxp3 by Tg4 donor cells, as determined by GFP expression, revealed that PBS-treated iTreg largely maintained expression of Foxp3, whereas the majority of 4Tyr-treated iTreg had lost Foxp3 expression (Figure 5.7B). Despite the decrease in the proportion of $Foxp3^+$ donor cells, there were greater numbers of these cells in the 4Tyr-treated group compared to the PBS group. The effect of 4Tyr administration on PD-1 expression by iTreg was not investigated.

5.2.4 iTreg cells that lose Foxp3 expression upon 4Tyr administration retain their suppressive function

In order to determine if the 4Tyr-treated iTreg cells that had lost Foxp3 expression had retained their suppressive capacity, GFP^+ and GFP^- $CD4^+CD45.1^+$ cells were isolated from the spleen four days after peptide treatment using FACS. The GFP^+ and GFP^- populations were subjected to a suppression assay using Tg4 responder

cells and stimulation with 4Lys. The post-sort purity of the GFP^+ population was 99.5%, and was 100% in the GFP^- population (Figure 5.8A). Both the 4Tyr-treated GFP^+ and GFP^- Tg4 iTreg presented an anergic phenotype, whereby proliferation was not detected in either population when stimulated with 4Lys (Figure 5.8B). In contrast, T responder cells cultured with 4Lys in the absence of 4Tyr-treated iTreg were found to proliferate. Both GFP^+ and GFP^- 4Tyr treated iTreg were able to suppress the proliferation of the Tg4 responder cells in response to stimulation with 4Lys (Figure 5.8) However, there was an overall trend for increased suppression by the GFP^+ donor cells, and was significantly different at two dilution points. This data implies that although iTreg can lose Foxp3 expression upon administration of 4Tyr, they can still suppress proliferation of T_{eff} cells albeit to a potentially lower extent than the iTreg that retain Foxp3 expression.

Figure 5.1 Experimental approach to determine the effect of 4Tyr administration of antigen-reactive nTreg cells

Figure 5.2 4Tyr administration has no effect on the number or frequency of antigen-reactive nTreg

Figure 5.3 nTreg cells maintain expression of Foxp3 to a greater extent after 4Tyr administration compared to PBS

Figure 5.4 nTreg cells that maintain Foxp3 expression in the 4Tyr-treated group are PD-1^{+}

Figure 5.5 Experimental approach to determine the effect of 4Tyr administration on antigen-reactive iTreg

Figure 5.6 4Tyr maintains the peptide-reactive iTreg population to a greater degree than PBS

Figure 5.7 iTreg cells lose expression of Foxp3 upon 4Tyr administration

Figure 5.8 iTreg cells retain their suppressive function following 4Tyr administration

5.3 Discussion

5.3.1 The effect of 4Tyr administration on Treg numbers

Despite evidence in the literature to suggest that soluble peptide administration can expand nTreg cell populations (Chappert et al., 2008), no evidence of this was seen in the Tg4 nTreg population four days after administration of 4Tyr (Figure 5.2B). In the studies conducted by Chappert et al., nTreg cells were isolated by purification of $CD4^+CD25^+$ cells, and the clonal expansion of 'Treg' cells upon administration of peptide was measured based upon total donor numbers. The expression of $Foxp3^+$ in the transferred population or after peptide administration was not stated. As effector $CD4^+$ T cells are also known to express CD25 (Waldmann, 1986), there is the potential for contamination of the Treg with T_{eff} cells, and therefore it may be the T_{eff} cells which expand upon peptide administration rather than the Treg. In the experiments shown in this chapter, nTreg were isolated on the basis of Foxp3 expression using the Tg4xFoxp3.LuciDTR-4 reporter strain of mice. The results shown in this chapter demonstrate that administration of soluble peptide may not result in the clonal expansion of pure nTreg populations, and therefore the increased numbers of these populations seen in Chapter 3 & 4 are likely due to a secondary effect of peptide administration on naïve or T_{eff} cells, such as the provision of IL-2.

Alternatively, the lack of nTreg expansion observed following peptide may be due to the prolonged *in vitro* expansion of these cells before transfer. The experiments conducted by Chappert et al., had the advantage of using direct transfer of freshly isolated $CD4^+CD25^+$ cells. However, the nTreg used for experiments in this chapter were subjected to persistent TCR stimulation for 21 days and had undergone ~100-fold expansion. Therefore, once transferred *in vivo* it is possible that these cells were in an exhausted state and were therefore refractory to further expansion by the administration of peptide. Another factor to consider is the requirement for IL-2 in the expansion of nTreg populations (D'Cruz et al., 2005, Fontenot et al., 2005). During the *in vitro* expansion of nTreg they were cultured in the presence of a high concentration of IL-2, and therefore it is possible that upon

transfer *in vivo* and during the subsequent administration of peptide, there was insufficient IL-2 production to support the further expansion of these cells

Unlike nTreg cells, iTreg cells were present in greater numbers and constituted a higher proportion of the $CD4^+$ T cells within the spleen four days following 4Tyr administration (Figure 5.6C). During the generation of iTreg cells, naive $CD4^+$ cells were subjected to stimulation through their TCR for only five days before transfer, as opposed to the 21 days received by nTreg cells. Also, iTreg cells require less IL-2 for their expansion as they are generated from naïve $CD4^+$ T cells, which can produce sufficient IL-2 on their own and are therefore less dependent upon exogenous addition of IL-2. As a result, it is likely that iTreg cells were not in an exhausted state upon transfer, and were capable of expanding in response to stimulation by peptide.

The use of human nTreg in the cellular therapy of autoimmune disease would likely require *in vitro* expansion in order to obtain sufficient numbers of these cells, as the frequency of circulating nTreg in humans is very low (Trzonkowski et al., 2009). The experiments shown in this chapter demonstrate that prolonged *in vitro* expansion of nTreg may render them unresponsive to further stimulation. It would be interesting to compare the suppressive function of *in vitro* expanded nTreg cells with freshly isolated cells, in order to determine if they are functionally different.

Due to the limitations in obtaining sufficient quantities of nTreg cells from humans, and the difficulty in identifying true nTreg cell populations there is increasing interest in the thereapeutic use of *in vitro* generated iTreg cells. iTreg cells can be generated in large numbers *in* vitro and have been shown to be effective in the prevention of autoimmune disease (DiPaolo et al., 2007, Haribhai et al., 2009). In particular, the use of antigen-reactive iTreg have been shown to be more potent in treating models of autoimmune disease than polyclonal iTreg (Stephens et al., 2009). Therefore, the expansion of antigen-reactive iTreg upon administration of soluble peptide may provide a mechanism by which to enhance the numbers of iTreg cells and therefore increase suppression. However, due to the observation

that nTreg cells can acquire a Th17 phenotype in the absence of TGF-β (Xu et al., 2007), concerns about the potential ability of iTreg to convert to a pathogenic phenotype and the stability of Foxp3 expression in these cells have recently been raised (Koenecke et al., 2009, Beres et al., 2011).

5.3.2 Maintenance of Foxp3 expression in nTreg cells

Previous work within the laboratory has determined that $CD62L^{hi}$ nTreg maintain expression of Foxp3 after *in vivo* challenge with peptide in CFA, and also after *in vitro* expansion for seven days. Whereas the majority of $CD62L^{lo}$ nTreg cells lose their Foxp3 expression (Stephens et al., 2009). Similar findings have been shown in human nTreg whereby cells with a memory phenotype ($CD45RO^{+}$ or $CD45RA^{-}$) were found to lose Foxp3 expression upon activation, but those with a naïve phenotype maintained expression of Foxp3 even after three weeks of *in vitro* expansion (Hoffmann et al., 2009, d'Hennezel et al., 2011). The experiments described in this results chapter used $CD62L^{hi}$ nTreg, and the proportion of $Foxp3^{+}$ cells at the end of culture was consistently >70% (Figure 5.1B). However, despite the purification of $Foxp3^{+}$ nTreg cells before transfer, the vast majority of PBS treated nTreg were found to have lost their expression of Foxp3 (Figure 5.3B).

Stephens et al., suggested that the $CD62L^{hi}$ cells represented naïve nTreg and $CD62L^{lo}$ cells were activated nTreg, and their studies determined that upon TCR stimulation $CD62L^{hi}$ nTreg cells down-regulated expression of CD62L. This may suggest that after 21 days of *in vitro* stimulation, the $CD62L^{hi}$ cells had down-regulated expression of CD62L and therefore were more prone to loss of Foxp3 expression upon transfer *in vivo*. However, the loss of Foxp3 expression by nTreg cells has typically been shown to occur upon continued TCR stimulation, and the 4Tyr treated nTreg were actually found to maintain Foxp3 expression to a greater degree than the PBS treated cells (Figure 5.3B). This implies that after three weeks of *in vitro* expansion, nTreg cells actually require continued TCR stimulation to maintain Foxp3 expression.

Epigenetic analysis has revealed that the Foxp3 promoter is largely un-methylated in nTreg, but is methylated in $CD4^{+}Foxp3^{-}$ cells (Floess et al., 2007). The studies conducted by Hoffman et al., into the loss of Foxp3 expression in human nTreg cells determined that upon *in vitro* expansion, CpG methylation within the Foxp3 promoter increased and this correlated with a decrease in Foxp3 expression. Therefore, it may be pertinent to monitor the methylation status of the Foxp3 promoter during *in vitro* expansion of nTreg as an indication of their potential to lose Foxp3 expression.

The observation that the majority of nTreg that had maintained $Foxp3^{+}$ expression in the 4Tyr treated group also expressed PD-1, adds further evidence to suggest that continued activation of the nTreg cell is required for the maintenance of Foxp3 expression (Figure5.4C). The absence of PD-1 expression appeared to correlate with the loss of Foxp3 expression in both the PBS- and the 4Tyr-treated group (Figure 5.4B) (Figure 5.9).

The results shown in this chapter demonstrate that in order to fully determine the effect of peptide administration on nTreg it would be more pertinent to transfer freshly isolated nTreg cells. However, this would be impractical due to the extremely large number of donor mice this would require. In addition, the problems identified with the stability of nTreg cells after *in vitro* expansion, highlights the potential obstacles in using nTreg as a cellular therapy for autoimmune disease.

5.3.3 Loss of Foxp3 expression in 4Tyr-treated iTreg cells

In contrast to nTreg cells, the majority of PBS-treated iTreg cells were found to maintain their expression of Foxp3 at four days after treatment (Figure 5.7B). However, upon treatment with 4Tyr, iTreg cells lost Foxp3 expression. It has previously been demonstrated that re-stimulation of iTreg *in vivo* can result in the loss of Foxp3 expression (Chen et al., 2011)(O'Connor & Anderton, unpublished observations). These studies involved the treatment of antigen-reactive iTreg with

their cognate peptide in adjuvant. The results shown in this chapter using soluble peptide administration imply that it is only the signalling through the TCR that is required for the loss of Foxp3 expression, rather than the immunogenic challenge.

Understandably, doubts have been raised about the use of iTreg cells therapeutically due to this instability in Foxp3 expression. Although it has been demonstrated that iTreg cells are less prone to conversion to a pathogenic phenotype under pro-inflammatory conditions than nTreg cells (Zheng et al., 2008, O'Connor et al., 2010), it is not known whether the loss of Foxp3 expression is associated with a loss in suppressive capacity. The data shown in this chapter demonstrate that both iTreg cells that have maintained expression of Foxp3, and those that have lost expression upon treatment with 4Tyr, are capable of suppressing Tg4 $CD4^+$ responder cells (Figure 5.8B). Suppression by GFP^- cells was not due to contamination of GFP^+ cells, as the purity of this population was 100% (Figure 5.8A). Therefore, this establishes that iTreg that lose expression of Foxp3 can retain suppressive capabilities; however, cells that retain Foxp3 expression may be more potent at suppressing responder T cells.

5.4 Concluding remarks

- Administration of 4Tyr does not alter the number or proportion of Tg4 nTreg cells compared to PBS.
- Antigen-reactive nTreg maintain expression of Foxp3 to a higher extent upon administration of 4Tyr compared to PBS.
- Expression of PD-1 on 4Tyr-treated nTreg cells is associated with maintenance of Foxp3 expression.
- Administration of 4Tyr results in increased numbers and proportion of iTreg cells compared to PBS.
- 4Tyr treatment results in a loss of Foxp3 expression in iTreg cells, whereas the majority of PBS-treated iTreg maintain Foxp3 expression.

- 4Tyr-treated iTreg cells that lose their expression of GFP retain their capacity to suppress Tg4 $CD4^+$ responder cells.

The results shown within this chapter demonstrate that administration of 4Tyr does not directly induce expansion of antigen-reactive nTreg populations. This implies that the increase in numbers of antigen-reactive nTreg observed in Chapters 3 and 4 is likely due to a secondary effect of peptide administration on naïve and effector Tg4 cells. The expansion of naïve and T_{eff} Tg4 cells upon 4Tyr administration would suggest that IL-2 is produced, and that this is responsible for the expansion of nTreg.

The increased numbers of iTreg following 4Tyr administration and the observation that both $Foxp3^+$ and $Foxp3^-$ cells can suppress the proliferation of naïve antigen-reactive cells may provide an opportunity to use soluble peptide administration in order to enhance the function of iTreg-based cellular therapy.

Figure 5.9 Effect of 4Tyr / PBS administration on nTreg vs iTreg.

6. The role of PD-1 signalling in the establishment and maintenance of peptide-induced tolerance

6.1 Introduction

PD-1 is thought to limit the clonal expansion during a primary T cell response, thereby enabling resolution of the effector response. Blockade or lack of PD-1 results in earlier onset of diabetes in NOD mice (Ansari et al., 2003) and earlier onset and increased severity of EAE (Salama et al., 2003). The inhibition of PD-1 signalling has also been demonstrated to break tolerance in $CD4^+$ T cells in an experimental model of diabetes (Fife et al., 2006), and re-instate cytokine production and proliferation in exhausted $CD8^+$ T cells during chronic viral infection (Barber et al., 2006, Trautmann et al., 2006). Although the role of PD-1 signalling in T cell tolerance has been demonstrated, the role of PD-1 expression in the induction of tolerance in naïve $CD4^+$ T cells has yet to be fully defined. Further, the role of PD-1 signalling in the induction of tolerance in T_{eff} cells has not been characterised. In the context of peptide-induced tolerance, prophylactic administration of peptide in deletional models of tolerance showed no role for PD-1 signalling in the induction of tolerance in $CD4^+$ T cells (Haspot et al., 2008, Konkel et al., 2010). However, as demonstrated in this thesis, peptide-induced tolerance in naïve $CD4^+$ Tg4 cells occurs through a mechanism that does not involve deletion of the peptide-reactive cells. Similarly, the data in Chapter 4 demonstrate that peptide-induced tolerance can also be established in Tg4 T_{eff} cells, and this occurs through a non-deletional mechanism. In fact, the cells persist in sizeable numbers, but express high levels of PD-1.

This chapter describes the results of preliminary experiments designed to test the importance of PD-1 expression, in the tolerant phenotype seen in Tg4 naïve and effector cells after *in vivo* exposure to the 4Tyr peptide.

6.1.1 Aims

1) To verify the role of PD-1 signalling in controlling the primary stimulation of $CD4^+$ T cells.
2) To investigate the role of PD-1 signalling in the establishment and maintenance of tolerance in naïve $CD4^+$ Tg4 cells.
3) To explore the role of PD-1 signalling in the establishment and maintenance of tolerance in Tg4 T_{eff} cells.

6.1.2 Experimental approach

A PD-1 blocking antibody was used in *in vitro* recall assays for initial studies into the role of PD-1 signalling in the maintenance of tolerance in naïve Tg4 cells. The availability of $PD1^{-/-}$ ($H\text{-}2^b$) mice (kindly provided by Professor Honjo, Kyoto University, Japan) allowed the generation of Tg4-$PD1^{-/-}$ mice, thereby providing a source of MBP-reactive T cells that could not receive signalling through PD-1, and which could be transferred and monitored in PD-1 sufficient hosts. Tg4-$PD1^{-/-}$ cells expressed the congenic marker CD90.1 and therefore could be traced in $CD90.1^-$ hosts. This enabled the specific effects of PD-1 signalling on the antigen-reactive T cells to be investigated upon peptide administration. Tg4-$PD1^{-/-}$ mice had not been fully back-crossed onto the B10.PL genetic background and therefore cells from these mice were transferred into C57BL/6xB10.PL recipients. Although the development of spontaneous autoimmune disease has been reported in $PD\text{-}1^{-/-}$ mice on the C57BL/6 background, this was found to occur with a late onset appearing in mice aged 6-14 months (Nishimura et al., 1999). In order to avoid complications arising from the onset of spontaneous autoimmune disease all mice used in the experiments described in this chapter were used at under ten weeks of age.

6.2 Results

6.2.1 Absence of PD-1 signalling increases production of IFN-γ and IL-2 by $CD4^+$ T cells

In order to verify the role of PD-1 signalling in limiting a primary T cell response, *in vitro* primary stimulation assays were performed using naive $CD4^+$ Tg4 cells. These cells were cultured with C57BL/6xB10.PL splenocytes at a 1:10 ratio ($2x10^4$ Tg4 cells and $2x10^5$ splenocytes per well), with increasing concentrations of 4Lys and in the presence of a PD-1 blocking antibody or isotype control (Figure 6.1A). In the absence of α-PD-1, IL-2 was produced in response to antigenic stimulation, whereas no IFN-γ production was observed. Thus suggesting the Tg4 cells had been driven towards a Th0 phenotype under these conditions. Inhibition of PD-1 signalling by α-PD-1 increased production of IL-2 and enabled the cells to produce IFN-γ (Figure 6.1B).

Similar primary cultures were performed comparing $CD4^+$ Tg4-$PD1^{-/-}$ and Tg4-$PD1^{+/+}$ cells. These were cultured with C57BL/6xB10.PL splenocytes at 1:20 ratio ($1x10^4$ Tg4 cells and $2x10^5$ splenocytes per well) and stimulated with increasing doses of 4Lys (Figure 6.2A). Tg4-$PD1^{+/+}$ cells produced both IL-2 and IFN-γ upon stimulation; however, levels of these cytokines were low. In contrast Tg4-$PD\text{-}1^{-/-}$ cells produced significantly higher amounts of IL-2 and IFN-γ (Figure 6.2B). Together, these two lines of investigation demonstrated that PD-1 signalling acts to limit the gain of effector function (IFN-γ production) by $CD4^+$ T cells upon primary stimulation.

6.2.2 PD-1 signalling may inhibit the proliferation of $CD4^+$ T cells upon primary stimulation

To determine if PD-1 signalling acts to limit the clonal expansion of $CD4^+$ T cells upon stimulation, proliferation assays were perfomed using CFSE labelled Tg4

$CD4^+$ cells cultured with C57BL/6xB10.PL splenocytes at a 1:10 ratio. Cells were stimulated with two doses of 4Lys in the presence of α-PD-1 antibody or isotype control and the extent of cell division was determined by the dilution of CFSE using flow cytometry (Figure 6.3A). Very few $CD4^+$ Tg4 cells were found to proliferate in the absence of antigen. The proportion of dividing cells increased with the addition of 4Lys, with a larger proportion dividing at the higher concentration of 4Lys (Figure 6.3B). There appeared to be no difference in the proportion of dividing cells in the presence or absence of α-PD-1 at either of the 4Lys concentrations used.

The effect of PD-1 on clonal expansion was also assessed by comparing CFSE labelled $CD4^+$ cells isolated from Tg4-PD-$1^{-/-}$ and Tg4-PD-$1^{+/+}$ mice (cultured with C57BL/6xB10.PL splenocytes at a 1:20 ratio) (Figure 6.4A). Similarly, in the absence of antigen very few $CD4^+$ Tg4 cells were found to proliferate, and upon addition of 4Lys the proportion of both Tg4-$PD1^{-/-}$ and Tg4-PD-$1^{+/+}$ cells that had divided increased. However, the proportion of divided cells appeared markedly higher in the Tg4-PD-$1^{-/-}$ group compared to Tg4-$PD1^{+/+}$.

6.2.3 $CD4^+$ Tg4 cells can induce EAE under sub-optimal conditions in the absence of PD-1 signalling

The above observations suggested that PD-1 deficient naïve Tg4 T cells had undergone clonal expansion and had gained effector function *in vitro*. Such an enhancement would be predicted to lead to greater pathogenic activity following immunisation *in vivo*. It was also predicted that this would best be observed using conditions known to be sub-optimal when PD-1 is present. The presence of naïve $CD4^+$ Tg4 cells is required for the induction of EAE in C57BL/6xB10.PL mice. Decreasing the number of $CD4^+$ Tg4 cells transferred should therefore act to limit the severity of disease. Thus, $5x10^5$ Tg4-PD-$1^{+/+}$ or Tg4-PD-$1^{-/-}$ $CD4^+$ T cells were transferred into C57BL/6xB10.PL hosts, one day prior to the induction of EAE by immunisation with 4Tyr in CFA (Figure 6.5A).

EAE did not develop in the hosts that received Tg4-PD-$1^{+/+}$ cells; incidence was 0/8 (Figure 6.5B). However, a robust monophasic disease course was seen in the Tg4-PD-$1^{-/-}$ group, with a mean maximal score of 3.5 and 100% incidence. Although no differences were observed in the total number or proportion of $CD4^+$ cells within the spleen upon resolution of disease, in five out of the seven hosts that received Tg4-PD-$1^{-/-}$ cells the donor cells were still detectable, whereas in the Tg4-PD-$1^{+/+}$ group no donor cells were seen in any of the spleens analysed (Figure 6.6A & 6.6B). This might reflect a greater expansion of Tg4-PD-$1^{-/-}$ population upon immunisation.

These data demonstrate that Tg4-PD-$1^{-/-}$ cells can induce disease under sub-optimal conditions and therefore provides further evidence to suggest that PD-1 acts to limit a primary T cells response.

6.2.4 Inhibition of PD-1 signalling enables effector cytokine production by tolerised Tg4 cells

In Chapter 3 it was shown that naïve $CD4^+$ Tg4 cells that have been rendered tolerant by administration of soluble 4Tyr express high levels of PD-1 (Figure 3.5). These 4Tyr-treated cells were found to produce IL-2 upon recall stimulation, but their ability to produce the effector cytokines IFN-γ and IL-17 was inhibited (Figure 3.6 & 3.12). To determine if PD-1 signalling plays a role in the inhibition of effector cytokine production by $CD4^+$ Tg4 cells from 4Tyr-treated mice, the effect of PD-1 blockade was investigated. Naïve $CD4^+$ Tg4 cells were transferred into B10.PL hosts one day prior to administration of 4Tyr or PBS. Seven days later, splenocytes were isolated and subjected to a recall stimulation assay in the presence of α-PD-1 or isotype control antibody (Figure 6.7A).

In agreement with previous experiments, the total number and proportion of $CD4^+$ Tg4 cells was increased in the 4Tyr-treated group compared to PBS (Note: Figure 6.7B is a reproduction of Figure 3.6B). Production of IL-2, IFN-γ or IL-17 was not

observed in splenocytes from the PBS-treated group in either presence or absence of α-PD-1 (Figure 6.7C); this is likely due to the low number of $CD4^+$ Tg4 cells remaining in this group. The ability of cells from 4Tyr-treated mice to produce IL-2 was also not altered by the addition of α-PD-1. Production of IFN-γ and IL-17 in the absence of α-PD-1 was not detected in the 4Tyr-treated group. However, inhibition of PD-1 signalling enabled 4Tyr treated cells to produce both IFN-γ and IL-17 in response to stimulation with 4Lys. These data provide evidence to suggest that PD-1 plays a role in the maintenance of a tolerant phenotype by preventing effector cytokine production.

6.2.5 Prophylactic administration of 4Tyr does not inhibit EAE induction by $CD4^+$ Tg4 cells that lack PD-1

In order to investigate the requirement for PD-1 signalling *in vivo* during the induction of tolerance, the effect of prophylactic 4Tyr administration on the subsequent induction of EAE was determined using Tg4-$PD1^{+/+}$ versus Tg4-$PD1^{-/-}$ $CD4^+$ T cells. Cells were transferred into C57BL/6xB10.PL hosts one day prior to administration of 4Tyr, and EAE was induced seven days later by immunisation with 4Tyr (Figure 6.8A).

Despite no significant differences in the mean maximal score or severity of disease, there was a significant difference in the incidence of disease. The mean maximal score in the Tg4-PD-$1^{-/-}$ group was 3 and incidence was 80%, compared to a mean maximal score of 0.8 and incidence of 20% in the Tg4-PD-$1^{+/+}$ group. These data show that prophylactic administration of 4Tyr is not as effective at inhibiting disease induction in the absence of PD-1 signalling.

6.2.6 Greater numbers of Tg4-PD-1$^{-/-}$ cells are present after immunisation compared to Tg4-PD-1$^{+/+}$ regardless of treatment with 4Tyr or PBS

To further clarify if the induction of tolerance by 4Tyr administration was inhibited in naïve CD4^{+} Tg4-PD-1$^{-/-}$ cells, the numbers and phenotype of these cells were investigated after subsequent immunisation. Naïve CD4^{+} Tg4-PD-1$^{+/+}$ or Tg4-PD-1$^{-/-}$ cells were transferred into C57BL/6xB10.PL hosts one day prior to administration of 4Tyr or PBS. Seven days after peptide/PBS mice were immunised with 4Lys in CFA, and the spleens and LN were taken ten days later (Figure 6.9A).

There was no difference in the total numbers and proportion of CD4^{+} cells within the spleen (Figure 6.10A) or LN (Figure 6.11A) ten days after immunisation. In the spleen there were significantly higher numbers and proportion of CD4^{+} Tg4-PD-1$^{-/-}$ cells compared to Tg4-PD-1$^{+/+}$ cells in the 4Tyr-treated groups (Figure 6.10B). A higher proportion of Tg4-PD1$^{-/-}$ cells were also observed in the PBS-treated groups. Similarly, in the LN there were significantly higher numbers of Tg4-PD-1$^{-/-}$ cells than Tg4-PD-1$^{+/+}$ in the PBS-treated groups (Figure 6.11B). However, no significant differences were observed between the 4Tyr-treated groups in the LN. No change was observed in either the Tg4-PD-1$^{+/+}$ or Tg4-PD-1$^{-/-}$ 4Tyr versus PBS groups. This correlates with data shown using PD-1 sufficient Tg4 cells in Chapter 3.

6.2.7 Comparison of cytokine production by Tg4-PD-1$^{+/+}$ and Tg4-PD-1$^{-/-}$ CD4^{+} cells after *in vivo* exposure to 4Tyr

Due to the differences observed in the numbers of Tg4-PD-1$^{+/+}$ and Tg4-PD-1$^{-/-}$ cells after 4Tyr administration, it was important to determine if there were any differences in the ability of these cells to produce effector cytokines. To test this,

spleens and LN were taken ten days after immunisation and cells were cultured overnight in the presence or absence of 4Lys; cytokine production was measured by ICS.

No differences were observed in the proportion of donor cells that were able to produce IFN-γ or TNF-α between any of the groups (Figure 6.12A&B). However, a lower proportion of both Tg4-PD-$1^{-/-}$ and Tg4-PD-$1^{+/+}$ cells were able to produce IL-17 in the 4Tyr-treated groups compared to PBS-treated (Figure 6.12C). No differences were seen in the proportion of IL-17^{+} donor cells within the PBS- or the 4Tyr-treated groups.

These data demonstrate that despite the differences in the total numbers and proportion of donor cells, effector cytokine production appears to be inhibited to a similar level in both Tg4-PD-$1^{+/+}$ and Tg4-PD-$1^{-/-}$ cells after prophylactic treatment with 4Tyr.

6.2.8 In the absence of PD-1 signalling 4Tyr no longer inhibits the induction of EAE by Tg4 T_{eff} cells

In Chapter 4 it was demonstrated that administration of 4Tyr abrogated the induction of EAE by Tg4 effector cells. Therefore, using Tg4-PD-$1^{+/+}$ and Tg4-PD-$1^{-/-}$ effector T cells the role of PD-1 signalling in disease inhibition by 4Tyr was investigated (Figure 6.13A).

In agreement with previous experiments, administration of 4Tyr abrogated the induction of EAE by Tg4-PD-$1^{+/+}$ effector cells (Figure 6.13B). In contrast, administration of 4Tyr did not prevent the induction of disease by Tg4-PD-$1^{-/-}$ T_{eff} cells. Clinical signs of EAE were evident from day 15 post-cell transfer and a monophasic disease course ensued with a mean maximal score of 1.3 and incidence of 50%. This experiment suggests that 4Tyr administration does not induce tolerance in Tg4 T_{eff} cells in the absence of PD-1 signalling.

6.2.9 Exposure of Tg4 T_{eff} cells to 4Tyr enhances clonal expansion and induces splenomegaly in the absence of PD-1 signalling

To disect the effect of 4Tyr administration on Tg4-PD-$1^{-/-}$ cells, Tg4-PD-$1^{+/+}$ and Tg4-PD-$1^{-/-}$ effector T cells were transferred into C57BL/6xB10.PL hosts one day prior to administration of 4Tyr or PBS and spleens were analysed four days later (Figure 6.14A).

4Tyr-treated mice that had received Tg4-PD-$1^{-/-}$ effector cells developed splenomegaly, as demonstrated by significant increases in the weight and total cellularity of the spleen in the 4Tyr-treated Tg4-PD-$1^{-/-}$ group compared to all other groups (Figure 6.14B).

A significant increase in the total numbers and proportion of $CD4^+$ cells was observed in the Tg4-PD-$1^{-/-}$ group after 4Tyr administration, compared to all other groups (Figure 6.15A). The total numbers of $CD4^+$ donor cells was also found to be significantly higher in the 4Tyr-treated Tg4-PD-$1^{-/-}$ group compared to all other groups (Figure 6.15B). No further significant differences were observed in the total number of $CD4^+$ donor cells in any of the groups. In agreement with previous experiments the proportion of donor cells was significantly higher in the 4Tyr-treated Tg4-PD-$1^{+/+}$ compared to PBS (Figure 6.15B). Strikingly, Tg4-PD-$1^{-/-}$ cells were found to constitute nearly 80% of total $CD4^+$ T cells within the spleen after 4Tyr administration, and this was a significantly greater proportion than that seen in all other groups. Despite the vast differences observed in the number of donor $CD4^+$ cells between groups, no differences were observed in the number of host $CD4^+$ cells (data not shown).

These data demonstrate that 4Tyr administration induces considerable expansion of the Tg4 T_{eff} population in the absence of PD-1 signalling, and this surpasses the increase of Tg4-PD-$1^{+/+}$ numbers upon 4Tyr treatment. Therefore, PD-1 signalling in Tg4 effector cells is implicated in controlling the expansion of this population upon 4Tyr administration.

6.2.10 Tg4 effector cells from 4Tyr-treated mice maintain expression of T-bet in the absence of PD-1 signalling

As shown in Chapter 4, both 4Tyr- and PBS-treated Tg4 effector T cells are found to maintain expression of T-bet four days after treatment (Figure 4.12C). However, in contrast to previous experiments, Tg4-PD-$1^{+/+}$ and Tg4-PD-$1^{-/-}$ cells did not maintain T-bet expression in the PBS treated group to the same extent as the 4Tyr-treated on this occasion (Figure 6.16A). No differences were observed in T-bet expression between Tg4-PD-$1^{+/+}$ and Tg4-PD-$1^{-/-}$ cells in either the 4Tyr- and PBS-treated group.

6.2.11 4Tyr administration reduces the ability of Tg4 effector cells to produce effector cytokines independently of PD-1 signalling

In Chapters 3 & 4, it was demonstrated that the administration of 4Tyr reduces effector cytokine production by naïve and effector $CD4^+$ Tg4 cells. PD-1 signalling in T cells is thought to inhibit production of effector cytokines such as IFN-γ (Freeman et al., 2000, Latchman et al., 2001). Therefore, the ability of Tg4-PD-$1^{-/-}$ effector cells to produce effector cytokines after 4Tyr administration was investigated as a possible reason for their maintained pathogenic activity. Splenocytes were isolated four days after administration of 4Tyr or PBS and were cultured overnight in the presence or absence of 4Lys.

No significant differences were observed in the proportion of IFN-γ^+ cells within the donor $CD4^+$ population between any of the groups (Figure 6.16B). No differences were observed in the proportion of Tg4-PD-$1^{+/+}$ or Tg4-PD-$1^{-/-}$ cells that could produce IFN-γ or TNF-α in the PBS-treated group. This demonstrates that cytokine production was not exaggerated in the absence of PD-1 signalling. Upon exposure to 4Tyr the proportion of both Tg4-PD-$1^{+/+}$ and Tg4-PD-$1^{-/-}$ cells that could produce TNF-α was reduced.

These data imply that PD-1 signalling may not be directly involved in the inhibition of effector cytokine production by Tg4 effector cells upon 4Tyr administration.

Figure 6.1 PD-1 signalling limits cytokine production upon stimulation

Figure 6.2 Tg4-PD-1$^{-/-}$ CD4^{+} cells produce more IFN-γ and IL-2 upon stimulation with 4Lys

Figure 6.3 Blocking PD-1 signalling does not enhance proliferation upon stimulation

Figure 6.4 Tg4-PD-1$^{-/-}$ CD4^{+} cells proliferate to greater extent than Tg4-PD-1$^{+/+}$ cells upon stimulation

Figure 6.5 Tg4-PD-1$^{-/-}$ CD4^{+} cells can induce EAE under sub-optimal conditions

Figure 6.6 There are no significant differences in the numbers of Tg4-PD-1$^{-/-}$ CD4^{+} cells in the spleen after resolution of EAE

Figure 6.7 Blocking PD-1 signalling can induce effector cytokine production by 4Tyr treated Tg4 cells

Figure 6.8 There is an increased incidence of EAE in mice that received Tg4-PD1$^{-/-}$ cells following prophylactic administration of 4Tyr

Figure 6.9 Experimental approach to assess the fate of 4Tyr-treated Tg4-PD-1$^{+/+}$ and Tg4-PD-1$^{-/-}$ cells after immunisation with 4Tyr in CFA

Figure 6.10 There is a larger number of Tg4-PD1$^{-/-}$ cells in the spleen of the 4Tyr treated group compared to Tg4-PD-1$^{+/+}$ after immunisation

Figure 6.11 There is a larger number of Tg4-PD1$^{-/-}$ cells in the LN of the PBS treated group compared to Tg4-PD-1$^{+/+}$ after immunisation

Figure 6.12 4Tyr administration decreases the proportion of both Tg4-PD-1$^{+/+}$ and Tg4-PD-1$^{-/-}$ cells that can produce IL-17 upon recall stimulation

Figure 6.13 4Tyr treated Tg4-PD-1$^{-/-}$ T_{eff} cells can induce EAE

Figure 6.14 4Tyr administration results in splenomegaly in the group that received Tg4-PD1$^{-/-}$ T_{eff} cells

Figure 6.15 Tg4-PD-1$^{-/-}$ T_{eff} cells expand to a greater extent than Tg4-PD-1$^{+/+}$ upon 4Tyr administration

Figure 6.16 4Tyr treated Tg4-PD-1$^{-/-}$ T_{eff} cells maintain T-bet expression but have a reduced capacity to produce effector cytokines

6.3 Discussion

6.3.1 Regulation of primary T cells responses by PD-1

It is well established that PD-1 is up-regulated on T cells upon activation, and that signalling through this co-inhibitory molecule can act to limit their expansion and effector cytokine production (Freeman et al., 2000, Keir et al., 2008, Carter et al., 2002). PD-1 signalling is thought to inhibit the downstream activity of Akt, through inhibition of PI3K (Parry et al., 2005). The exact mechanism of PI3K inhibition is unknown. However, it is thought that the recruitment of SHP-2, known to be a negative regulator of PI3K in the EGFR pathway (Zhang et al., 2002), to the ITSM domain of PD-1 may play a role (Latchman et al., 2001, Chemnitz et al., 2004). The activation of Akt in T cells is required for effective proliferation and effector production (Sun et al., 2010). Therefore, inhibition of this pathway through PD-1 signalling can limit T cells responses.

The inhibition of PD-1 signalling has been shown to enhance proliferation and production of IFN-γ and IL-2 of T cells upon stimulation (Freeman et al., 2000, Konkel et al., 2010). In accordance with this, the results shown in this chapter demonstrate that either the inhibition of PD-1 signalling by α-PD-1 or the absence of PD-1 expression increases the production of IFN-γ and IL-2 by naïve Tg4 T cells upon stimulation with antigen (Figures 6.1B& 6.2B). The investigation into the effect of PD-1 signalling inhibition on the proliferation of T cells has yielded conflicting results. Although no differences were seen in the proliferation of Tg4 cells in the presence of PD-1 blocking antibody, the observation that permanent lack of PD-1 signalling resulted in increased proliferation upon stimulation suggests that PD-1 can act to limit clonal expansion of cells (Figure 6.3B & 6.4B). Similarly it has also been reported in other studies that PD-1 blockade can enhance proliferation of T cells (Iliopoulos et al., 2011). The lack of differences observed in the proliferation of cells in presence of α-PD-1 are possibly due to the turnover of antibody in culture, and sub-optimal inhibition of PD-1 signalling compared to the constant absence of PD-1 signalling in Tg4-PD-$1^{-/-}$ cells. The increased IL-2

production in the presence of α-PD-1 would support the idea that inhibition of PD-1 signalling would enhance clonal expansion.

Despite the differences in proliferation, these results illustrate that upon stimulation $CD4^{+}PD1^{-/-}$ cells display a similar phenotype to $CD4^{+}PD1^{+/+}$ cells stimulated under conditions of PD-1 inhibition.

In experimental models of autoimmune disease, inhibition or lack of PD-1 can bring about alterations in the disease course. It has been reported that blockade or deficiency of PD-1 in NOD mice results in disease with an earlier onset and more severe insulitis (Ansari et al., 2003, Wang et al., 2005). Similar results have also been reported in models of EAE whereby blockade of PD-1, or PD-1 deficiency, can induce an accelerated and more severe disease course (Salama et al., 2003, Carter et al., 2007). The findings in this chapter correlate with these reports, whereby Tg4-$PD1^{-/-}$ cells can induce a robust disease course under suboptimal conditions, whereas Tg4-$PD1^{+/+}$ cells do not (Figure 6.5B). The continued presence of Tg4-$PD1^{-/-}$ cells in the spleen at the point of resolution of disease (Figure 6.6B), coincides with the observation that severity of disease in PD-$1^{-/-}$ myelin mutant mice correlated with increased clonal expansion of T cells (Kroner et al., 2009).

Taken together these data demonstrate that inhibition or lack of PD-1 signalling upon T cell activation increases the production of IL-2 and IFN-γ, and results in increased severity and incidence of EAE.

6.3.2 The role of PD-1 signalling in the maintenance of peptide induced tolerance in naïve $CD4^{+}$ T cells

In Chapter 3 it was demonstrated that tolerised naïve $CD4^{+}$ Tg4 cells were able to produce IL-2 in response to recall stimulation with peptide, however, these cells did not have the capacity to produce the effector cytokines IFN-γ and IL-17. Shut-down of effector cytokine production was most profound in the 4Tyr-treated group,

which displayed the highest expression of PD-1. PD-1 has been shown to be up-regulated as a consequence of TCR stimulation (Parry et al., 2005). Previous studies within the laboratory have demonstrated that the 4Tyr peptide can persist in an immunologically relevant form, and induce proliferation of naïve Tg4 cells for up to 14 days after administration (Konkel, 2009). Therefore, the prolonged exposure of naive $CD4^+$ Tg4 cells to 4Tyr may be responsible for the high expression of PD-1, and the shut down in effector cytokine production. Indeed the ability of tolerised Tg4 cells to produce IFN-γ and IL-17 upon inhibition of PD-1 signalling (Figure 6.7C) provides evidence to suggest that PD-1 has a role in the maintenance of tolerance induced by 4Tyr administration. The lack of differences observed in IL-2 production upon PD-1 inhibition is most likely due to the ability of 4Tyr-treated cells to produce this cytokine even in the presence of PD-1 signalling. These data, suggest that expression of PD-1 on 4Tyr-treated $CD4^+$ Tg4 cells is responsible for the inhibition of effector cytokine production and for maintaining these cells in a tolerant state.

6.3.3 The role of PD-1 signalling in the induction of peptide induced tolerance in naïve $CD4^+$ T cells

Expression of PD-1 is thought to play a role in both peripheral and central T cell tolerance. Thymocytes are shown to express PD-1 and PDL-1, and signalling through these molecules is thought to play a role in both positive and negative selection (Nishimura et al., 2000, Blank et al., 2003). Also, the observation that PD-1 deficient mice develop spontaneous autoimmune pathology implicates a role for PD-1 signalling in the maintenance of peripheral tolerance (Nishimura et al., 1998).

Several studies have demonstrated that PD-1 signalling is required for the induction of tolerance in experimental models of autoimmune disease. Inducible expression of antigen by resting DC in the absence of PD-1 signalling has been shown to convert a tolerogenic stimulus into immunogenic response (Probst et al., 2005).

Another study has demonstrated that blockade of PD-1 signalling upon transfer of OT-I $CD8^+$ cells into RIP-mOVA mice results in the onset of diabetes (Martin-Orozco et al., 2006). It has also been observed that tolerance can not be induced in PD-$1^{-/-}$ OT-I $CD8^+$ cells upon transfer into RIP-OVA^{high} mice, whereas tolerance is readily induced in PD-$1^{+/+}$ OT-I cells (Keir et al., 2007). The requirement of PD-1 signalling in the induction of tolerance in $CD4^+$ cells is poorly understood. Previous studies within the laboratory have demonstrated that PD-1 signalling is not required for the induction of tolerance in naïve $CD4^+$ T cells (Konkel et al., 2010). However, those studies were conducted using a model of deletional tolerance, whereas here it has been shown that the induction of tolerance in naïve $CD4^+$ Tg4 cells does not rely upon deletion of the cells.

The observation that prophylactic treatment of Tg4-PD-$1^{-/-}$ cells with 4Tyr is not as effective at inhibiting subsequent disease induction as it is in Tg4-PD-$1^{+/+}$ cells (Figure 6.8B), suggests that PD-1 signalling does play a role in the induction of tolerance in this model.

The ability of 4Tyr to inhibit effector cytokine production in both Tg4-PD-$1^{+/+}$ and Tg4-PD-$1^{-/-}$ T cells suggests that this may not be the mechanism responsible for inhibiting pathogenicity (Figure 6.12). The lack of differences observed in IFN-γ and TNF-α production between groups is most likely due to the small group sizes and lack of statistical power. However, due to the significantly elevated numbers of Tg4-PD-$1^{-/-}$ cells compared to Tg4-$PD1^{+/+}$ in the 4Tyr treated group this may in fact indicate a higher number of effector cytokine producing cells in the Tg4-PD-$1^{-/-}$ group. Therefore, this may explain their increased propensity to induce disease.

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6.3.4 The role of PD-1 signalling in the induction of peptide tolerance in T_{eff} cells

As demonstrated in Chapter 4, the induction of tolerance in antigen-experienced Tg4 T_{eff} cells by 4Tyr administration was also found to coincide with expression of

PD-1. Data in this chapter indicate that administration of 4Tyr does not protect from disease if the Tg4 T_{eff} cells lack PD-1 (Figure 6.14B). Thus, PD-1 appears to be required for the induction of tolerance in effector $CD4^+$ T cells in this model.

The splenomegaly observed four days after 4Tyr administration in host mice that had received Tg4-PD-$1^{-/-}$ T_{eff} cells was striking. Although PD-1 deficient mice have been reported to develop splenomegaly under steady state (Nishimura et al., 1998), this was not observed in the PBS-treated Tg4-PD-$1^{-/-}$ group. The reason for this enlargement of the spleen was found to be almost entirely due to the substantial expansion of $CD4^+$ Tg4-PD-$1^{-/-}$ cells upon 4Tyr administration.

Despite the considerable numbers of $CD4^+$ Tg4-PD-$1^{-/-}$ cells in the spleen four days after 4Tyr administration, the disease course induced by Tg4-PD-$1^{-/-}$ effector cells in 4Tyr-treated mice was relatively mild (mean maximal score of 1.3), and clinical signs were not observed until 14 days after treatment (Figure 6.13B). Phenotypic analysis of the Tg4 T_{eff} cells four days after peptide treatment, revealed that T-bet expression was maintained in both Tg4-PD-$1^{-/-}$ and Tg4-PD-$1^{+/+}$ groups. However, even with the lack of PD-1 signalling, the proportion of cells that could produce effector cytokines was reduced in both the 4Tyr-treated Tg4-PD-$1^{-/-}$ and Tg4-PD-$1^{+/+}$ groups (Figure 6.16B). These data imply that PD-1 signalling may not be directly responsible for the inhibition of effector cytokine production by 4Tyr treated Tg4 T_{eff} cells.

The expansion of both Tg4-PD-$1^{+/+}$ and Tg4-PD-$1^{-/-}$ effector populations upon administration of 4Tyr may be a contributing factor in decreasing the proportion of cells that produce effector cytokines upon recall stimulation.

In Chapter 3 it was indicated that 4Tyr administration may alter the migratory potential of Tg4 T_{eff} cells, as fewer of these cells were present in the CNS of the 4Tyr treated group compared to the PBS treated group. This observation could be due to direct changes in molecules associated with migration into the CNS on the Tg4 cells, or perhaps it is the prolonged presence of 4Tyr in the periphery that

removes any requirement for the Tg4 T_{eff} cells to migrate into the CNS in search of antigen. The demonstration that 4Tyr persists for up to fourteen days, may explain why 4Tyr treated Tg4-PD-$1^{-/-}$ effector cells do not induce EAE until this time point. It would be interesting to monitor the migration of these cells in order to determine if they do not migrate into the CNS before this time point. In Chapter 4 it was also demonstrated that after five days post-4Tyr administration, the number of Tg4 T_{eff} cells steadily declined. Therefore, there may be insufficient numbers of Tg4-PD-$1^{+/+}$ effector cells to induce disease once the peptide is no longer present, whereas Tg4-PD-$1^{-/-}$ effector cells, which expand to a greater extent upon 4Tyr administration, may be present in greater numbers at this later time point. An alternative but related possibility is that Tg4-PD-$1^{-/-}$ effector cells regain pathogenicity due to the absence of PD-1 signalling once the peptide is no longer present, whereas Tg4-PD-$1^{+/+}$ cells which maintain PD-1 expression can not.

In order to answer these questions it would be pertinent to conduct time-course experiments to monitor the migration and effector cytokine production by the 4Tyr treated Tg4 T_{eff} cells in the presence or absence of PD-1. Experiments should also be conducted to ascertain whether the presence of 4Tyr within the periphery does prevent migration of 4Tyr treated Tg4 cells into the CNS. This question can be answered through the isolation of 4Tyr treated Tg4 T_{eff} cells and the transfer of these cells into hosts that have not been treated with 4Tyr. In addition, one of the caveats of using Tg4-PD-1 deficient cells is the potential differences in the initial generation of the pathogenic Th1 cells compared to Tg4-PD$1^{+/+}$ cells. Therefore, it would be beneficial to conduct experiments using the α-PD-1 blocking antibody in order to provide substantiating evidence.

6.4 Concluding remarks

- Inhibition or lack of PD-1 signalling enhances production of IFN-γ and IL-2 by naïve Tg4 T cells upon *in vitro* stimulation with peptide.
- PD-1 signalling may limit proliferation of naive Tg4 cells upon *in vitro* stimulation with 4Lys.
- Tg4 cells that lack PD-1 can induce EAE under sub-optimal conditions.
- Blockade of PD-1 signalling can break tolerance of naïve Tg4 cells and allow IFN-γ and IL-17 production upon recall stimulation.
- PD-1 is required for the induction of tolerance in naïve $CD4^+$ Tg4 cells upon 4Tyr administration.
- 4Tyr administration does not protect from induction of EAE by Tg4-PD-$1^{-/-}$ T_{eff} cells.
- 4Tyr treated Tg4-PD-$1^{-/-}$ effector cells induce EAE but not until 14 days after peptide treatment
- 4Tyr treated Tg4-PD-$1^{-/-}$ T_{eff} cells expand to a greater extent than Tg4-PD-$1^{+/+}$ T_{eff} cells upon 4Tyr administration.
- Administration of 4Tyr reduces the proportion of both Tg4-PD-$1^{+/+}$ and Tg4-PD-$1^{-/-}$ cells that produce effector cytokines upon recall stimulation.

In summary, the results shown in this chapter implicate a role for PD-1 signalling in the induction of tolerance in naïve and Tg4 T_{eff} cells. These initial observations warrant further detailed analysis of the functions of PD-1 in the development of a therapeutic intervention in this EAE model.

7. General discussion

This thesis tested the hypothesis that antigen-experienced autoimmune effector T cells will undergo apoptosis upon soluble peptide administration, whereas protective antigen-reactive Treg will be resistant to this effect and will persist, giving long-term protection.

The results can be summarised as follows:

- 4Tyr administration induces tolerance in naïve antigen-reactive $CD4^+$ Tg4 cells, as prophylactic administration of 4Tyr can inhibit the induction of EAE.
- 4Tyr administration does not delete naive $CD4^+$ Tg4 cells; in fact there are higher numbers of these cells evident following 4Tyr administration.
- These tolerant T cells express high levels of PD-1, and are capable of producing IL-2 but not effector cytokines upon recall stimulation.
- PD-1 is required for maintaining tolerance in naïve Tg4 T cells (blockade of PD-1 signalling during recall stimulation enables effector cytokine production).
- PD-1 signalling is also required for the induction of tolerance in naïve Tg4 T cells (prophylactic administration of 4Tyr does not inhibit EAE induction in the absence of PD-1).
- 4Tyr also induces tolerance in Tg4 T_{eff} cells and abrogates induction of EAE.
- A striking expansion is seen in the number of Tg4 T_{eff} cells within four days of 4Tyr treatment.
- Tg4 T_{eff} cells maintain high levels of PD-1 expression for at least 16 days after 4Tyr administration.
- 4Tyr does not inhibit the induction of EAE by Tg4 T_{eff} cells that cannot express PD-1.
- 4Tyr administration does not induce deletion of antigen-reactive iTreg or nTreg cells.
- nTreg cells maintain expression of Foxp3, whereas iTreg lose Foxp3 expression after 4Tyr administration. Loss of Foxp3 expression does not correlate with a loss in suppressive activity

Therefore, tolerance can be induced in both naïve and effector $CD4^+$ Tg4 cells by the administration of 4Tyr. The mechanism behind tolerance induction in both types of cells relies upon signalling through PD-1. In addition, soluble peptide administration may function to maintain antigen-reactive Treg populations.

7.1 The role of PD-1 signalling in peptide-induced tolerance

In contrast to many described models of peptide-induced tolerance, the mechanism by which 4Tyr administration induced tolerance in naïve $CD4^+$ Tg4 cells did not involve deletion of the cells. Instead, 4Tyr administration induced a non-classical form of anergy, as the tolerised Tg4 T cells retained the capacity to produce IL-2 (Figure 7.1). The observation that PD-1 signalling was required to induce and maintain tolerance in this model, and that PD-1 expression correlated with the MHC-binding affinity of the peptide, provides an explanation as to why 4Lys is very poor at inducing tolerance in naïve $CD4^+$ T cells, whereas 4Tyr is a very effective tolerogen (Metzler and Wraith, 1993, Liu and Wraith, 1995).

Determining the mechanism of tolerance induction in naïve antigen-reactive T cells provides useful insights into the use of peptide therapy to switch-off autoreactive T cells. However, the clinical requirement of peptide therapy is to switch-off antigen-experienced activated and memory T cells that may be present during ongoing disease. The model described in this thesis provided the opportunity to characterise the effect of soluble peptide administration on pathogenic effector T cells, and in addition provided a clinical readout of therapeutic intervention. Furthermore, the use of a passive transfer model of EAE also enabled investigation into the effect of peptide administration during ongoing disease without the risk of anaphylaxis. Therefore, these models may provide significant advantages for the clinical translation of peptide therapy.

Figure 7.1 The effects of 4Tyr administration on naïve, effector & regulatory T cells

The discovery that 4Tyr administration could induce tolerance in Tg4 T_{eff} cells holds promise for the clinical use of peptides to treat autoimmune disease. However, the significant expansion in the number of T_{eff} cells upon 4Tyr administration highlights the need to determine the mechanisms by which tolerance is induced, and how easily these cells could potentially revert to a pathogenic phenotype.

The identification of PD-1 as a requirement for tolerance induction in Tg4 T_{eff} cells means further investigation into this co-inhibitory molecule in the context of peptide therapy is warranted. Despite similarities in the effect of peptide-administration on naïve and effector Tg4 T cells, the inhibition of IL-2 production and the significant expansion in the number of Tg4 T_{eff} cells following 4Tyr administration suggests a different mechanism of tolerance induction (Figure 7.1).

The induction of tolerance by 4Tyr may be a function of its ability to persist in an immunologically relevant form for a long period of time. Previous studies within the laboratory have demonstrated that 4Tyr can form stable functional complexes with MHC which persist for at least 14 days after administration (Konkel, 2009). This ability of 4Tyr to provide consistent TCR stimulation would favour the induction of adaptive tolerance in naïve Tg4 T cells, a process that is thought to require persistent antigenic signalling in the absence of co-stimulation (Schwartz, 2003). Adaptive tolerance typically takes longer than seven days to become established, and requires an initial phase of proliferation (Tanchot et al., 2001, Choi and Schwartz, 2007). Therefore, the phenotype of tolerised naïve Tg4 T cells may represent the initial stages of adaptive tolerance, before IL-2 production is switched-off. Regardless of the exact process involved in tolerance induction, PD-1 was required for both the establishment and maintenance of tolerance in naïve Tg4 cells.

The phenotype of Tg4 T_{eff} cells after 4Tyr administration may be more akin to clonal exhaustion rather than adaptive tolerance. Similarly to naïve T cells, the induction of tolerance coincided with the level of PD-1 expression maintained by the Tg4 T_{eff} cells. Expression of PD-1 has also been associated with exhaustion of

$CD8^+$ T cells (Day et al., 2006, Urbani et al., 2006). Persistent antigenic stimulus, for example during chronic LCMV or HIV infection, is thought to be responsible for driving and maintaining the expression of PD-1 on virus-reactive $CD8^+$ cells (Hokey et al., 2008). The inhibition of PD-1 signalling on exhausted $CD8^+$ T cells, enabled the production of effector cytokines and increased cytolytic capacity, thereby enhancing viral clearance (Barber et al., 2006, Trautmann et al., 2006, Lukens et al., 2008). Those studies, together with the data described in this thesis, demonstrate that PD-1 plays a functional role in maintaining T cell hypo-responsiveness. It has also been shown that expression of PD-1 can be influenced by epigenetic modification of the *Pdcd1* gene as a result of antigenic stimulation.

Studies by Youngblood et al, into the expression of PD-1 in $CD8^+$ cells during viral infection, identified a CpG island in the promoter region of the PD-1 encoding *Pdcd1* gene that plays a role in the transcriptional regulation of PD-1 expression. It was demonstrated that upon initial T cell activation, the *Pdcd1* promoter is demethylated which correlates with the expression of PD-1. Once the antigenic stimulus is removed the *Pdcd1* promoter can be remethylated, which is associated with the down-regulation of PD-1 expression on memory $CD8^+$ cells. However, under conditions of persistent antigenic stimulation, such as during chronic viral infection, the *Pdcd1* promoter remains un-methylated and the ability to re-methylate the promoter is eventually lost, therefore maintaining PD-1 expression. In the case of the 4Tyr-treated Tg4 T_{eff} cells, PD-1 expression was maintained for at least 16 days after peptide treatment, when the majority of the administered 4Tyr peptide will no longer be present. Therefore, it is possible that similar epigenetic modifications occur in the Tg4 T cells upon 4Tyr administration, whereby PD-1 expression is maintained even once 4Tyr has cleared from the system.

This may explain the delayed EAE disease course induced by Tg4-PD-$1^{-/-}$ T_{eff} cells cells after 4Tyr administration. One hypothesis would be that the presence of 4Tyr:I-A^u complex within the periphery prevents the Tg4-PD-$1^{+/+}$ T_{eff} cells from migrating into the CNS in search of antigen. Once the peptide has been cleared, any remaining Tg4 cells can traffic to the CNS but due to their maintained

expression of PD-1 are unable to mount an immune response. Tg4-PD-$1^{-/-}$ cells would also migrate into the CNS once 4Tyr has been cleared, but their lack of PD-1 would allow them to mount a pathogenic response.

In light of this potential mechanism, it would be pertinent to investigate the epigenetic modification of the *pdcd1* promoter in Tg4 cells upon 4Tyr administration, and to determine whether removal of the peptide can enable the loss of PD-1 expression and regain of effector function.

7.3 Therapeutic implications

The crucial role of PD-1 signalling in the induction and maintenance of tolerance, as demonstrated in this thesis, provides a mechanism that can be exploited therapeutically. Although the induction of clonal exhaustion in pathogenic antigen-reactive T cells by soluble peptide administration would be clinically benenficial, this therapeutic regime poses a danger in the continued presence of autoreactive T cells that could potentially revert to a pathogenic phenotype. In order to minimise this risk and to achieve optimal tolerance induction during peptide therapy, signalling through PD-1 on T cells should be instigated from the time of peptide administration. This could be achieved using an agonistic PD-1 antibody, but as all T cells express PD-1 during a normal primary immune response, this could inhibit T cells involved in beneficial inflammatory responses and therefore would remove the specificity of peptide therapy. An alternative mechanism of ensuring PD-1 signalling is through the delivery of peptide coupled to APC that constitutively express high levels of PD-1 ligand. This would ensure delivery of a negative signal to antigen-experienced T cells upon encounter with peptide. This method has already been used effectively in a model of EAE, whereby administration of dendritic cells transfected with PDL-1 and a peptide of MOG inhibited the induction of disease (Hirata et al., 2005). However, the observation that antigen-reactive T cells can express PDL-1 following 4Tyr administration, suggests that

both counterparts required for PD-1 signalling can be induced by peptide administration alone.

Additionally, the up-regulated expression of PD-1 seen on tolerised antigen-reactive T cells offers the potential use of PD-1 expression as a biomarker in peptide therapy. Peptide-reactive T cells could be identified through the use of tetramers and PD-1 expression on these cells could be monitored during treatment. This could inform whether peptide-induced tolerance has been achieved, and conversely whether loss of tolerance is occurring. The potential use of PD-1 as a biomarker is supported by a phase II clinical trial of peptide therapy in RA, where PD-1 expression on T cells was found to be enhanced in patients that responded to treatment (Koffeman et al., 2009). Such approaches would be useful in developing optimal dosing regimes and would enable the success of treatment to be determined without having to wait for clinical read-outs. Likewise, the use of tetramers to identify peptide-reactive T cells enables the phenotype of these cells to be assessed during treatment, and could provide a quicker, safer mechanism of determining if the peptide therapy is likely to induce exacerbation of disease.

In contrast to the induction of tolerance, PD-1 signalling has also been implicated in the inhibition of protective immune responses against tumours, parasites and chronic viral infections (Mumprecht et al., 2009, Zhang et al., 2009, Butler et al., 2012, Taylor et al., 2007). Therefore, in situations where antigen persists, and where immune responses are beneficial, PD-1 signalling has a detrimental effect. As a result, therapeutic vaccines targeting PD-1 signalling are currently in development for use in cancer. This includes the use of an anti-PD-1 humanised antibody currently in clinical trials (Rosenblatt et al., 2011, Berger et al., 2008). In contrast to the induction of tolerance, the desired outcome in cancer therapeutics is the inhibition of PD-1 signalling in order to re-invigorate tumour-reactive $CD8^+$ T cells and enable tumour clearance. Therefore, the observation that PD-1 signalling is beneficial in the induction of tolerance demonstrates that the role PD-1 plays in immune responses is a double-edged sword. As a consequence, therapeutic regimes

that block PD-1 signalling may have implications in the context of autoimmunity and vice versa.

An additional note of caution with respect to therapeutic manipulation of PD-1 signalling is the observation that a polymorphism in the PD-1 gene is associated with autoimmunity. Autoimmune diseases found to be associated with this polymorphism include; systemic lupus erythematosus (Prokunina et al., 2002), T1D (Nielsen et al., 2003), and RA (Prokunina et al., 2004). Furthermore, in MS patients this polymorphism has been associated with disease progression and an inhibited capacity to switch-off effector cytokine production by T-cells (Kroner et al., 2005). Therefore, if PD-1 signalling is crucial for the induction of tolerance, peptide-induced tolerance may not be as effective in these individuals.

The potentially detrimental outcome associated with defects in PD-1 signalling highlights the fundamental risk of using a therapeutic regime that can increase the number of antigen-reactive T cells. Despite the tolerant status of these cells there remains the very real possibility that under favourable conditions they are poised to revert to a pathogenic phenotype which could prove to have catastrophic repercussions for the patient. In light of this fact, it would perhaps be beneficial to combine peptide therapy with a therapeutic regime that involved the induction or transfer of Treg, thereby utilising peptides to target and switch-off the antigen-reactive T cells in the first instance and Treg to prevent the subsequent activation/re-activation of pathogenic T cells.

7.4 Future work

The primary question that needs to be answered now is, how easily can antigen-reactive T effector cells revert to a pathogenic phenotype following 4Tyr administration? As PD-1 expression is required for the maintenance of tolerance in naïve Tg4 T cells, the role of PD-1 signalling in maintaining tolerance in Tg4 T_{eff}

cells needs to be investigated. This can be determined using α-PD-1 antibodies during recall stimulation of 4Tyr-treated Tg4 T_{eff} cells.

In order to investigate whether the continued presence of antigen is required for the maintenance of tolerance (perhaps through PD-1 expression), tolerised Tg4 T_{eff} cells can be isolated from recipient mice and transferred into secondary hosts that have not been exposed to 4Tyr. The ability of these cells to regain pathogenicity and potentially induce disease can be monitored. This protocol can be repeated, reisolating Tg4 effector cells at various time points after 4Tyr administration to the primary host. In conjunction with epigenetic analysis of the *Pdcd1* promoter, this will determine a) if irreversible tolerance can be induced in antigen-experienced T cells and b) if this coincides with prolonged expression of PD-1 due to epigenetic modifications of the *pdcd1* promoter. In addition, epigenetic analysis of the *Pdcd1* promoter will help determine the requirements for stable PD-1 expression and whether this can be achieved by only a single peptide administration or whether continued treatment is required.

It would also be pertinent to determine the effect of soluble peptide administration during peak of EAE, i.e. at a time point where T_{eff} cells are actively prosecuting their inflammatory activity. The ability of 4Tyr administration to expand the number of antigen-experienced Tg4 cells may have a detrimental effect on disease course if this occurs once the cells are established in the CNS.

7.5 Concluding remarks

This thesis demonstrates a crucial role for PD-1 signalling in the induction of tolerance in naïve and effector Tg4 cells by 4Tyr administration, and therapeutic implications include the use of PD-1 as a biomarker of peptide-induced tolerance.

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