$CD4^+$ T cell responses to myelin autoantigens - activation, memory and tolerance

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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.

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Abstract

Experimental autoimmune encephalomyelitis (EAE) is a $CD4^+$ T cell mediated autoimmune disease of the central nervous system and shares many characteristics with multiple sclerosis (MS). Induction of EAE is mediated by myelin reactive $CD4^+$ T helper (Th) cells, particularly Th1 and Th17 cells, which can be provoked by the immunization with myelin derived protein (or peptide) and Toll-like receptor (TLR) stimulus (eg, complete Freund¡s adjuvant, CFA). If given an injection of soluble peptide before immunization, mice do not develop EAE (they are tolerant). This approach has been widely applied, evoking tolerance in primary responses (i.e., in naive T cells). Therefore the first hypothesis of this thesis is that peptide induced protection from EAE is a result from T cell deletion or / and anergy. As MS patients have ongoing disease and over 85% of MS patients develop a relapsing-remitting course, memory T cells are key targets when considering peptide-induced tolerance as a therapeutic strategy. Thus, a model for ¡memory EAE¡ was established to test a second hypothesis that the myelin reactive memory T cells can be controlled by the administration of soluble peptide.

Here, adoptive transfer of T cells from T cell receptor transgenic mice (2D2) recognizing myelin oligodendrocyte glycoprotein 35-55 (pMOG) was used to investigate the pMOG-reactive memory responses. Soluble pMOG administration could induce a transient expansion of 2D2 T cells followed by their loss through apoptosis. A model using double immunization was established by immunizing mice first with pMOG together with unmethylated CpG oligonucleotide (CpG) as an adjuvant, and subsequently immunizing with pMOG in CFA. This produced EAE with early onset and high incidence compared to mice which received pMOG/CFA only. Cells from mice that received the double immunization protocol produced high levels of IFN-γ, suggesting that memory T cell responses have been triggered in the mice. Administration of soluble peptide before secondary immunization could ameliorate EAE, indicating that memory T cells are susceptible to tolerance induction. pMOG-reactive memory T cells were further assessed by isolating $CD4^+$ $CD25^-$ $CD44^{high}$ $CD62L^{low}$ cells from pMOG-experienced 2D2 mice. These cells showed early and high production of IFN-γ, and early but transient production of IL-2, compared with naive population. These data provide basic information relevant to translating peptide-induced T cell tolerance from mice to humans.

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List of abbreviations

Abbreviation	Full name
ACAD	activated T cell autonomous death
AIRE	autoimmune regulator
AICD	activation induced cell death
APC	antigen presenting cell
AP-1	activator protein 1
APL	altered peptide ligand
BBB	blood-brain-barrier
B cell	B lymphocyte
Bcl	B-cell lymphoma
BTLA	B and T lymphocyte attenuator
c-Cbl	casitas B-lineage lymphoma
CCR	chemokine C-C motif receptor
CFA	complete Freund¡s adjuvant
CLR	C-type lectin receptor
CNS	central nervous system
CpG	oligonucleotides containing unmethylated CpG
CSF	cerebral spinal fluid
CTLA	cytotoxic T lymphocyte antigen
CXCR	C-X-C chemokine receptor
DC	dendritic cell
EAE	experimental autoimmune encephalomyelitis
EAU	experimental autoimmune uveitis
EBNA	EBV nuclear antigen
Egr	early growth response family protein
ER	endoplasmic reticulum
FcR	Fc receptor
Foxp3	forkhead transcription factor 3
GATA	transcription factors GATA binding protein
GRAIL	gene related to anergy in lymphocyte
HBV	Hepatitis B virus
HCoV	human coronaviruses
HLA	human leukocyte antigen
ICAM-	intercellular adhesion molecule

List of abbreviations (continue)

Abbreviation	Full name
ICOS	inducible T-cell co-stimulator
IDO	indoleamine 2,3-dioxygenase
IFN	interferon
ITAM	immunoreceptor tyrosine-based activation motif
ITCH	itchy homologue E3 ubiquitin protein kinase
iTreg	inducible regulatory T cell
i.v.	intravenous
i.p.	intraperitoneal
i.n.	or intranasal
LAT	linker of activation of T cells
LFA	lymphocyte function-associated antigen
LPS	lipopolysaccharide
MBP	myelin basic protein
MC	Mast cells
MHC	major histocompatibility complex
MOG	myelin oligodendrocyte glycoprotein
MRI	magnetic resonance imaging
MS	multiple sclerosis
mTEC	medullary thymic epithelial cells
NFAT	nuclear factor of activated T cell
NFκB	nuclear factor kappa B
OVA	ovalbumin
PAMP	pathogen-associated molecular pattern
PD-1	programmed cell death 1
PD-L	programmed cell death ligand
PGN	peptidoglycan
pMHC	peptide loaded MHC
PLP	proteolipid protein
pMOG	Peptide 35 to 55 of MOG
PRR	pattern-recognition receptor
PTX	pertussis toxin
RA	rheumatoid arthritis
RANK	receptor activator of nuclear factor κB

List of abbreviations (continue)

Abbreviation	Full name
RANKL	receptor activator of NF-κB ligand
ROR	retinoid-related orphan nuclear receptor
SLE	systemic lupus erythematosus
STAT	signal transducer and activator of transcription
s.c.	subcutaneously
T-bet	T-box expressed in T cells
T cell	T lymphocyte
TCL	T cell lines
T_{CM}	central memory T cells
TCR	T cell receptor
T_{EM}	effector memory T cells
TGF	transforming growth factor
Tg mice	Transgenic mice
Th cell	T helper cell
TID	type I diabetes
TLR	Toll-like receptor
TNF	tumor-necrosis factor receptor
Treg	regulatory T cell

Chapter 1. Introduction

1.1 Immune response and adaptive immunity

Immunity is defined as resistance to disease, specifically infectious disease. The immune response involving cells, tissues and molecules, is the reaction made by the immune system to protect the host against disease by identifying and killing pathogens. The immune system consists of innate immunity, which mediates the initial protection against infection and adaptive immunity, which mediates the later defense. When microorganisms successfully invade an organism, the non-specific innate immunity responds to pathogens immediately. Providing synergy with innate immunity, jawed vertebrates (fish, birds and mammals) have developed adaptive immunity against particular antigens to provide a specific, long-lasting and recallable protection (Janeway *et al*., 2005). There are two arms to adaptive immunity, humoral immunity and cellular immunity. In humoral immunity, responding B lymphocytes (B cells) secrete antibodies that eliminate extracellular pathogens. In cellular immunity, T lymphocytes (T cells) can provide synergistic molecules for eliminating pathogens and destroying infected cells. Both B and T cells are designed to specifically recognize and respond to foreign antigens by their antigen receptors, which are extremely diverse for different antigens. In addition, adaptive immunity has different responses against distinct microorganisms, specialized by mounting distinct effector cells (i.e. effector cytokine producing T cells or antibody secreting B cells). If an individual encounters an antigen and receives the same antigen in later life, the immune system can mount greater and faster responses due to the development of ¡memory cells¡ at the first encounter with the antigen. This immunological memory makes immunity more efficient to repeated infections

(Janeway *et al.*, 2005). Based on the properties of specificity, memory and specialization of adaptive immunity, this introduction concentrates on adaptive immunity and particularly on the role of $CD4^+$ T cells in experimental autoimmune encephalomyelitis (EAE).

1.1.1 T cell activation

T cells, that develop and mature in the thymus, are an essential subset of lymphocytes in adaptive immunity. T cells can be separated into $CD4^+$ and $CD8^+$ cells and the expression of the CD4 molecule restricts $CD4^+$ T cells to recognize major histocompatibility complex (MHC) class II molecules on antigen presenting cells (APC) (Guermonprez *et al.*, 2002). APC include dendritic cells (DC), macrophages and B cells which are the initiators and modulators of the adaptive immune response. APC can respond to pathogens by up-regulating surface molecules and by secreting cytokines for T cell activation. T cells need antigens to be processed and presented to them by APC. The T cell receptor (TCR) can recognize particular peptide bound to MHC. The ligation of TCR / peptide / MHC initiates TCR downstream signalling that consequently triggers T cell activation. Despite the fact that the common portals of entry for pathogens are skin, gastrointestinal tract and respiratory tract, the only subset of APC located in the epithelium is DC. Despite that macrophages and B cells are able to activate naive $CD4^+$ T cells *in vitro*, DC are the major mediators for T cell activation *in vivo* (Villadangos and Heath, 2005; Askew *et al.*, 1995; Cassell and Schwartz, 1994). DC can capture antigens and transport them to draining lymph nodes, serving as the major MHC class II provider in the T cell area of lymph nodes, activating T cells (Steinman *et al.*, 1997; Inaba *et al.*, 1997).

1.1.2 Signal 0 for DC activation

$CD4^+$ cells are classified as naive T cells before they experience antigens. To activate naive $CD4^+$ T cells, three signals have to be provided : A) ligation of TCR / peptide / MHC, B) co-stimulatory molecules, C) cytokines for polarization. Ahead of these three signals provided, DC have to be primed to mature by pathogen-associated molecular patterns (PAMP). PAMP are microorganism derived, repeating patterns of molecular structures. For example, gram-positive bacteria have a thick layer of peptidoglycan (PGN) in the cell wall, gram-negative bacteria typically have a lipopolysaccharide (LPS) layer surrounding the outside of the cell wall (Draing *et al*., 2008), a specific sequence motif in bacterial or viral DNA consists of repeated unmethylated CpG, which is 20 times less common in mammalian than in bacterial DNA (Klinman *et al*., 2004). Receptors that recognize PAMP are pattern-recognition receptors (PRR), which are a set of evolutionary conserved proteins. Toll-like receptors (TLR) and C-type lectin receptors (CLR) are PRR which respond to PAMP and induce DC activation (van Vliet *et al*., 2007). As members of the IL-1 receptor (IL-1R) / TLR superfamily, TLR can recognize multiple PAMP, including PGN detected by TLR2, LPS detected by TLR4 and unmethylated CpG DNA detected by TLR9 (Iwasaki and Medzhitov, 2004). On the other hand, immature DC are rich in membrane- associated C-type lectin (CLR), a set of cell to cell adhesion receptors, pivotal in pathogen recognition and antigen presentation, making immature DC efficient at capturing pathogens (van Vliet *et al*., 2007).

Immature DC reside in the epithelia of skin and mucosal tissues for a period of time. They have properties of high endocytic lasting, low levels of surface MHC and co-stimulatory molecules (Banchereau and Steinman, 1998). After TLR binding to

PAMP, immature DC are activated and matured to become matured DC, together with up-regulation of MHC molecules (for signal 1) and co-stimulatory molecules (for signal 2). In addition, different types of microorganisms have diverse PAMP which have different preferences for triggering particular TLR on DC and for provoking distinct cytokine patterns for T cell polarization (Kapsenberg, 2003). Thus, PAMP / TLR signalling provides a ¡signal 0¡ in DC and specializes the DC¡s preference for T cell activation and differentiation. DC are present in an immature state in tissues when there is no stimuli and are thus unable to stimulate T cells. After they receive signals from TLR, the immature DC are activated, rapidly lose their ability for endocytosis, up-regulate MHC molecules and express high levels of adhesion and co-stimulatory molecules, including ICAM-1, LFA-3, CD80, CD86 and CD40 (Banchereau and Steinman, 1998). Moreover, activated DC are able to migrate from non-lymphoid tissue (epidermal and mucosal, the sites DC are normally located in) to lymph nodes where they provide pathogen-specific $CD4^+$ T cells with the three essential signals for activation and differentiation (Steinman *et al.*, 1997).

1.1.3 Signal 1 for T cell activation

The first signal results from the ligation of the TCR by peptide associated with MHC class II, which determines the specificity of response. Extracellular microorganisms or proteins are internalized by APC into vesicles where the foreign proteins are broken down by proteolytic enzymes, generating various peptides. APC constantly synthesize class II MHC molecules in the endoplasmic reticulum (ER). Class II MHC molecules can be transported by exocytic vesicles which can fuse with foreign peptides containing vesicles, resulting in peptide binding to MHC II molecules. Subsequently the peptide loaded MHC (pMHC) molecules are delivered to the

surface, making APC ready to prime T cells (Rocha and Neefjes, 2008). Ligation of TCR / pMHC delivers the earliest T cell activation signal called signal 1. The TCR includes α and β subunits which provide the pMHC-binding site. The TCR also includes CD3 and TCRξ subunits containing immunoreceptor tyrosine-based activation motifs (ITAM). Ligation of pMHC to TCRαβ, synergistically with the binding of the CD4 molecule to MHC, leads to phosphorylation of the ITAM, which delivers a series of signals for activating transcription factors such as nuclear factor kappa B (NFκB), nuclear factor of activated T cells (NFAT) and activator protein 1(AP-1), which are essential for T cell activation and proliferation (Janeway *et al*., 2005). TCR downstream signalling is also important for up-regulating cell adhesion molecules and co-stimulatory molecules, which provide assistance for the ligation of TCR / pMHC and co-stimulatory signals (Banchereau and Steinman, 1998). Adhesion molecules on T cells recognize their ligands on APC and stabilize the binding of TCR / pMHC, providing physical facilitation for T cell activation. The most important adhesion molecule on T cells is lymphocyte function-associated antigen-1 (LFA-1). On naive T cells, LFA-1 is in a low-affinity state against its ligand intercellular adhesion molecule-1 (ICAM-1) on APC. When TCR / pMHC ligation occurs, the affinity of LFA-1 to ICAM-1 increases, resulting in a prolonged and stronger T cell / APC contact, subsequently strengthening the signal transduction of TCR and pMHC (Lebedeva *et al*., 2005).

However, signal 1 alone is insufficient to provoke a valid T cell response. TCR signalling needs to synergize with co-stimulatory signals otherwise the inefficient signals may drive T cell tolerance rather than activation.

1.1.4 Signal 2 for T cell activation

The second signal for T cell activation is mediated by co-stimulatory molecules which are surface molecules expressed on APC and T cells. Co-stimulation can amplify signals for T cell activation, promoting T cell proliferation and survival (Kroczek *et al.*, 2004). In the absence of foreign antigens, immature DC have low expression of co-stimulatory molecules and MHC class II molecules, thus are unable to trigger co-stimulatory signalling in T cells. Co-stimulatory molecules are classified into two major groups : A) the immunoglobulin superfamily, such as CD28 and inducible T-cell co-stimulator (ICOS) and B) tumor-necrosis factor receptor (TNFR) family, such as CD154, OX-40 (CD134), and receptor activator of NF-κB ligand (RANKL) (Figure 1.1; Croft, 2003). The most pivotal co-stimulation for T cell activation is the interaction between CD28 on the T cell and CD80 / CD86 (B7-1/B7-2) on the APC. Ligation of CD28-CD80 / CD86 is essential for T cell proliferation, differentiation and survival. (Greenwald *et al*., 2005). As the TCR initially binds to pMHC, signals through CD28 facilitate TCR signalling to activate transcription factors (for example, NFκB and AP-1) and subsequently drive IL-2 production, an essential growth factor for naive T cell proliferation (Parry *et al*., 2003). CD28 signalling also up-regulates the anti-apoptotic molecule Bcl-$_{XL}$ and the expression of CD154 (CD40L) which support T cell proliferation (Sharpe and Freeman, 2002).

On the other hand, some inhibitory molecules such as cytotoxic T lymphocyte antigen 4 (CTLA4), programmed cell death 1 (PD-1) and B and T lymphocyte attenuator (BTLA), can negatively regulate TCR mediated responses. These inhibitory molecules can antagonize T cell activation by delivering signals directly or

by acting as competing ligands with agonistic co-stimulators (Kroczek *et al*., 2004; Chen, 2004). As CD28 plays an essential part in T cell activation, its inhibitory competitor, CTLA-4, should be highlighted. CTLA-4 is an inducible surface molecule expressed on activated T cells (Carreno and Collins, 2002; Teft *et al*., 2006). However, CTLA-4 binds to CD80 and CD86 with greater affinity and avidity than CD28, thereby reducing the amount of CD28 dependent co-stimulation to the cell (Tivol *et al*., 1997; Linsley *et al*., 1994). Moreover, CTLA-4 has an inhibitory effect on the recruitment of LAT into the raft (Martin *et al*., 2001). The engagement of CTLA-4/B7 can up-regulate E3 ubiquitin ligases which may inhibit TCR and CD28 signalling, thereby interrupting the cell cycle (Brunner *et al*., 1999; Li *et al*., 2004). Hence, ligation of inhibitory co-stimulatory molecules can abrogate T cell activation, driving T cells to tolerance.

1.1.5 Signal 3 for T cell differentiation

A T cell response needs to differentiate naive T cells into effector T cells (i.e. effector cytokine producing $CD4^+$ T cells or $CD8^+$ cytotoxic T cells) for eliminating foreign antigens. Apart from signal 1 and 2 mediated by membrane bound receptors and ligands on T cells and APC, soluble cytokines mainly secreted from APC and T cells, provide signals that trigger the differentiation and polarization of naive T cells (Figure 1.2). Effector $CD4^+$ T cells, also called T helper (Th) cells, play an important role in establishing and maximizing the capabilities of the immune system (Kapsenberg, 2003). DC and macrophages respond to intracellular pathogens which trigger the production of IL-12. IL-12 binds to the IL-12 receptors on naive T cells, promoting the transcription factors T-box expressed in T cells (T-bet) and signal transducer and activator of transcription 4 (STAT4) for the further differentiation of

IFN-γ producing Th1 cells (Figure 1.2) (Kalinski *et al*., 1999; Afkarian, 2002). Extracellular pathogens can trigger T cells to produce autocrine cytokine IL-4, that polarizes naive T cells to differentiate towards Th2 cells. Polarization of Th2 cells is mediated by transcription factors GATA binding protein 3 (GATA3) and STAT6 (Figure 1.2) (Yamashita *et al*., 2004). Recently, IL-25 (also known as IL-17E, produced by Th2 cells and mast cells) has been reported to be implicated in Th2 cell¡ mediated immunity and to promote GATA3 expression, suggesting that CD25 is another polarizing cytokine for Th2 differentiation (Angkasekwinai *et al*., 2007; Tato *et al*., 2006).

Recently studies have indicated that naive T cells can differentiate into IL-17 producing T cells, independently of the development Th1 and Th2 cells. This novel lineage of effector CD4 T cells is called Th17 cells (Harrington *et al*., 2005; Park *et al*., 2005). Polarization of Th17 cells is triggered by IL-6 and tumor growth factor β (TGF-β) which activate the transcription factor orphan nuclear receptors RORα, RORγt and STAT3, that promote the differentiation from Th0 towards Th17 cells (Figure 1.2) (Yang *et al*., 2008; Bettelli, 2006; Veldhoen *et al*., 2006; Mangan *et al*., 2006; Zhou *et al*., 2007). However, Th17 cells can still be generated *in vivo* in IL-6 deficient mice in the absence of regulatory T cells, suggesting an alternative pathway other than IL-6 / TGF-β driven polarization for the differentiation of Th17 cells may exist. An exogenous *in vitro* supply of IL-21 and TGF-β can also trigger STAT3 and RORγt in naive T cells, indicating that IL-21 can also trigger the polarization of Th17 cells (Wei *et al*., 2007; Korn *et al*., 2007; Nurieva *et al*., 2007).

Over and above these effector T cells, naive T cells can be differentiated to inducible

regulatory T cells. Regulatory T cells are a specialized subpopulation of T cells that act to suppress T cell activation. Regulatory T cells can be classified into naturally occurring regulatory T cells (Treg) and inducible regulatory T cells (iTreg). The former constitutively express CD25 and the forkhead transcription factor 3 (Foxp3) without any stimulation. The latter originate from $CD4^+$ $CD25^-$ naive cells that can be triggered to express high levels of CD25 and Foxp3 *in vitro* by an exogenous supply of TGF-β and IL-2 (Fontenot *et al*., 2003; Fontenot and Rudensky, 2005; Chen *et al*., 2003; Zheng *et al*., 2004; Selvaraj and Geiger, 2007). Moreover, a transient pulse of exogenous TGF-β can expand $CD4^+$ $CD25^+$ $Foxp3^+$ T cells that can suppress autoimmune disease (Peng *et al*., 2004). Thus, in naive T cells receiving signal 1, signal 2 and the polarizing cytokines TGF-β and IL-2, Foxp3 can be activated to trigger the differentiation of iTreg cells (Figure 1.2) (Zheng *et al*., 2004; Chen *et al*., 2003; Horwitz *et al*., 2008).

1.1.6 $CD4^+$ T cell subsets and their interactions

Different T cell subsets can interact with each other and therefore give balance to the immune response. Naive $CD4^+$ T cells begin a process of differentiation upon TCR stimulation with different polarizing cytokines. The Th1 / Th2 hypothesis was developed over 20 years ago (Coffman and Carty, 1986; Mosmann *et al*., 1986), showing that Th1 cells respond to intracellular pathogens and produce IFN-γ and TNF-α that drive cell-mediated immune responses and the production of IgG2a, leading to tissue inflammation. In contrast, Th2 cells respond to extracellular infections and produce IL-4, IL-5, IL-10 and IL-13 that promote allergy, IgE, mast cell and eosinophil responses (Coffman, 2006). Reciprocally, Th1 cells can negatively regulate the generation of Th2 cells through IFN-γ, whereas Th2 cells can

inhibit the development of Th1 cells via IL-4 (Figure 1.3). The third member of helper $CD4^+$ T cells, Th17, is characterized by IL-17 secretion, which is involved in inflammation. An exogenous supply of IL-4 and IFN-γ in clonal activation conditions *in vitro* reduces the generation of Th17 cells and neutralizing antibodies against either IL-4 or IFN-γ greatly increase IL-17 production, suggesting that both Th1 and Th2 cytokines can inhibit Th17 differentiation (Harrington *et al*., 2005; Park *et al*., 2005). IL-23, an IL-12 family member produced predominantly by DC, comprises a unique p19 subunit that can associate with IL-12p40 (Hunter, 2005; Langrish *et al*., 2004). Although IL-23 can slightly enhance IFN-γ responses, its importance in maintaining the proliferation and pathogenicity of Th17 cells has more recently been recognized (Langrish *et al*., 2005; McGeachy *et al*., 2007). Another member of IL-12 family, IL-27, can enhance the early phase of Th1 responses and suppress Th2 and Th17 differentiation (Yashida *et al*., 2001). However, enhancement of Th1, Th2 and Th17 responses has been seen in IL-27Rα knockout mice, indicating an immunosuppressive role of IL-27. Recently it is found that IL-27 can promote IL-10 producing cells and suppress the production of IL-6 and IL-17, suggesting an anti-inflammatory role of IL-27 (Awasthi *et al*, 2007; Batten *et al*., 2006; Stumhofer *et al*., 2007, Batten *et al*., 2008).

Regulatory T cells (Treg) can regulate effector Th1, Th2 and Th17 responses by surface inhibitory receptors and anti-inflammtory cytokines. Treg can secrete IL-10 and TGF-β, suppressing the generation of Th1 and Th2 cells. Recent data also show that Treg cells can produce IL-35 which promotes the expansion of Treg and down-regulate the differentiation of Th17 cells (Collison *et al*., 2007; Niedbala *et al*., 2007). Although TGF-β can trigger the generation of Treg cells, an exogenous supply

of TGF-β and IL-6 can also promote the differentiation of Th17 cells (Chen *et al*., 2003; Veldhoen *et al*., 2006; Bettelli *et al*., 2006). In addition, IL-6 is a pro-inflammatory cytokine produced from Th1, Th2, and Th17 cells, and is capable of inhibiting TGF-β dependent Treg cells (Bettelli *et al*., 2006). IL-21 may be another inhibitor of Treg cells. IL-21 is produced by activated $CD4^+$ T cells and NKT cells and was first characterized to promote Th2 responses. Experiments in IL-21 receptor knockout mice or using anti-IL-21 neutralizing antibodies have been reported to have an enhancement on Th1 and Th17 responses and a reduction on Treg cells, suggesting an inhibitory role of IL-21 in Th1 and Th17 differentiation (Liu *et al*., 2008; Piao *et al*., 2007). However, current data suggest that Th17 cells can also produce IL-21 which enhances the generation of Th17 cells and down-regulates Treg in the absence of IL-6, suggesting IL-21 may have an autocrine role in Th17 differentiation and an inhibitory role in Treg generation (Nurieva *et al*., 2007).

1.1.7 Immunological memory

When an antigen experienced individual contacts the same antigen in later life, the immune system responds more rapidly and aggressively than in the primary response and this is defined as immunological memory (Sprent, and Surh, 2002). Immunological memory is mediated by memory T cells and memory B cells. By skipping the stage of activation and expansion of the naive cell, memory cells can control the secondary infection more efficiently than naive cells, giving the individual an immediate protection (Sprent, 1997).

1.1.8 Differentiation of memory T cells

Mature APC trigger the activation and expansion of antigen-reactive T cells and

evoke the differentiation of naive T cells towards effector T cells which produce high levels of effector cytokines (eg. IFN-γ for Th1 cells; IL-4, IL-5 and IL-13 for Th2 cells) (Kaech *et al*., 2002). It has been suggested that the initiation of naive T cell activation takes 24 to 48 hours *in vivo* after immunization with protein in adjuvant and needs a further 5 days for full activation (Bradley *et al*., 1991). Effector T cells are able to migrate into non-lymphoid tissues where they can trigger inflammatory responses (Campbell *et al*., 2003). This is because effector T cells express high levels of adhesion molecules such as CD44 and P-selectin that facilitate their homing to non-lymphoid tissues (Swain *et al*.,1999). In addition, the expression of the lymph homing molecule, L-selectin (CD62L), and chemokine receptors involved in lymphoid tissue location, chemokine C-C motif receptor 5 (CCR5) and CCR7, are down-regulated on effector T cells, allowing them to enter into non-lymphoid tissues (Roman *et al*., 2002). After acute infection, antigen-specific T cells expand dramatically, acquire effector function and mediate clearance of the pathogen. After resolution of the infection, 90%¡95% of antigen-specific T cells die, leaving behind around 5-10% that can become long-lived memory T cells to provide protection upon re-infection (Hataye *et al*., 2006; Williams and Bevan, 2007). However, whether the memory T cells are differentiated from effector T cells is still being debated. Some studies have provided evidence that memory T cells are originated from effector T cells, while others have clues that memory T cells are a lineage of activated T cells (Hu *et al*., 2001; Geginat *et al*., 2001; Lanzavecchia, and Sallusto, 2000; Sprent and Tough, 2001). It is believed that the strength of the overall signal that is received by a T cell determines its differentiation program, although the minimal requirements of activation signal (including magnitude and longitude) for T differentiation from naive cells to memory cells are not known (Lanzavecchia *et al*., 2002). Studies in

$CD8^+$ T cells showed that memory cells need to undergo at least 7-10 divisions to develop from naive $CD8^+$ T cells, but do not require the existence of continuing antigen. Cell division may be an important parameter regulating the differentiation of memory $CD8^+$ T cells (Opferman *et al*., 1999; Kaech and Ahmed, 2001). For the development of memory $CD4^+$ T cells, in contrast to $CD8^+$ T cells, prolonged or repeated contact with antigen-bearing APC throughout the effector phase is required to induce optimal proliferation (Obst *et al*., 2005). Prolonged antigen expression, even after clearance of virus, has been reported for influenza infection and may contribute to efficient memory generation (Jelley-Gibbs *et al*., 2005). It is unknown how the degree of expansion influences the efficiency of memory generation. For example, adoptively transferred IFN-γ positive Th1 cells appear to have a lower potential to develop into memory T cells, compared with IFN-γ negative $CD4^+$ T cells (Wu *et al*., 2002). In addition, IFN-γ can limit the expansion of Th1 cells during the effector phase and makes Th1 cells more susceptible to apoptosis (Zhang *et al*., 1997). Thus, proliferation in the effector phase (which may favor AICD) must be balanced to provide for differentiation into a pool of resting memory cells.

1.1.9 Maintenance of memory T cells

Once established, memory T cells can persist for a long time in the absence of antigenic exposure. As smallpox has been eradicated since 1978, a test of smallpox vaccination observed that more than 90% of volunteers maintained humoral or cellular responses against the original antigen after 25 to 75 years post inoculation, suggesting that memory cells can survive in an antigen-free environment for a lifetime (Hammarlund *et al*., 2003). $CD8^+$ memory T cells are maintained in an individual by mechanisms of homeostatic proliferation and survival, which are

dependent on IL-15, IL-7 and anti-apoptotic molecules, but not dependent on TCR / pMHC signalling (Sprent and Surh, 2002; Becker *et al*., 2002; Judge *et al*., 2002; Surh *et al*., 2006). On the other hand, memory $CD4^+$ T cells are dependent on IL-7 for survival and homeostatic proliferation, together persistent TCR signalling, (Seddon *et al*., 2003; Kondrack *et al*, 2003). IL-15 has also been reported to have a mild but significant effect in supporting background memory $CD4^+$ T cell turnover in the presence of TCR stimulation (Lenz *et al*., 2004). Notably, memory $CD4^+$ T cells can survive in the absence of MHC class II molecules, but the presence of MHC class II is required for their production of effector cytokines, suggesting that TCR signalling is dispensable in maintenance but is required for effector functions of memory $CD4^+$ T cells (Kassiotis *et al*., 2002). For $CD4^+$ T cells, survival of naive and memory cells have different requirements for cytokines. Adoptive transfer studies in the common cytokine receptor γ-chain deficient mice suggest that memory $CD4^+$ T cells do not need common γ-chain cytokines for survival but naive $CD4^+$ T cells do in the absence of TCR stimulation (Lantz *et al*., 2000). The common γ-chain is used by receptors for IL-2, IL-4, IL-7, IL-9 and IL-15. In addition, memory $CD4^+$ T cells from wild type and γ-chain deficient mice proliferate similarly in response to antigenic stimulation *in vivo*, suggesting that γ-chain cytokines are dispensable for the proliferation of $CD4^+$ memory T cells (Lantz *et al*., 2000).

1.1.10 Subsets and features of memory T cells

Memory T cells have been classified into central memory T cells (T_{CM}) and effector memory T cells (T_{EM}). Both subsets express IL-7 receptor α for maintaining survival and express high levels of CD44 (Kaesh *et al*., 2003). T_{CM} share some characteristics with naive T cells, they express the high levels of lymph homing markers CD62L and

CCR7, which enable T_{CM} to localize to secondary lymphoid organs (Sallusto *et al*., 1999; Masopust *et al*., 2001). T_{CM} also express the chemokine receptor CXCL12, which helps retain them in peripheral lymph nodes (Sallusto *et al*., 1999; Scimone *et al*., 2004). In contrast, T_{EM} do not express CCR7 and express low levels of CD62L. This property makes T_{EM} capable of penetrating blood vessel walls and entering tissues rather than being restricted in the secondary lymphoid organs (Weninger *et al*., 2001; Sallusto, 1999; Masopust *et al*., 2001; Lanzavecchia and Sallusto, 2005). $CD4^+$ T_{EM} produce large amounts of effector cytokines (IFN-γ, IL-4 or IL-5) after receiving a polyclonal stimulation, whereas T_{CM} do not (Sallusto, 1999). Thus, T_{EM} are more efficient at responding to second encounter with antigens.

Although it is not known how activated or effector T cells undergo transition to memory T cells, many features of effector T cells are maintained in T_{EM} cells including rapid production of effector cytokines after re-stimulation, lower threshold of TCR stimulation and independence of co-stimulatory requirement (Swain *et al*., 2006; London *et al*., 2000). Secondary recall responses to antigen *in vivo* are characterized by more rapid kinetics and greater magnitude compared with primary responses. This efficient response of memory $CD4^+$ T cells may result from their properties of migration and co-stimulatory requirement (Masopust *et al*., 2001; London *et al*., 2000). T_{EM} cells have a reduced expression of lymphoid organ homing markers (CD62L and CCR7), an increased expression of adhesion markers (CD44) and tissue specific chemokine receptors (CXCR4), suggesting a better ability to seek antigen in peripheral tissues than naive T cells (Seder and Ahmed, 2003; Lanzavecchia and Sallusto, 2005). These properties make $CD4^+$ T_{EM} cells capable of circulating around lymphoid and non-lymphoid tissues, even in the absence of

inflammation. The advantage of migration also allows T_{EM} cells to detect and eliminate the foreign pathogen long before it reaches the secondary lymphoid organs (Reinhardt *et al.*, 2001).

Memory T cells have a rapid turnover rate compared to naive T cells (Tough and Sprent, 1994; Macallan *et al.*, 2004; Sojka *et al.*, 2004). Croft and colleagues have reported that memory $CD4^+$ T cells can produce IL-2 with immobilized anti-CD3 antibodies in the absence of APC, while naive T cell can not. In addition, antigen specific memory $CD4^+$ T cells can respond to inactivated B cells or macrophages in the presence of antigens, suggesting that memory $CD4^+$ T cells can be triggered by a wider range of APC types which are inefficient at providing co-stimulation (Croft *et al.*, 1994; Ott *et al.*, 2007). With the DO11.10 TCR transgenic mice, it has been reported that ovalbumin (OVA) specific memory $CD4^+$ T cells divide faster at lower antigen concentrations than naive T cells. Moreover, normal effector cytokine production was observed when OVA specific memory $CD4^+$ T cells were stimulated with antigen-loaded APC from B7 or CD40 deficient mice, compared with naive $CD4^+$ T cells. These data indicate that memory $CD4^+$ T cells have a lower TCR threshold and are less dependent on co-stimulation (London *et al.*, 2000). Recent data has indicated that NFATc1 and NFATc2 proteins are more abundant in memory $CD4^+$ T cells than in naive T cells before stimulation (Dienz *et al.*, 2007). NFAT family proteins, mediated by TCR and CD28 signalling, are essential for IL-2 production in naive $CD4^+$ T cells (Isakov and Altman, 2002). The pre-accumulation of NFAT proteins in memory T cells can facilitate the rapid production of IL-2, suggesting that memory $CD4^+$ T cells are less dependent on CD28 signalling for IL-2 production than naive $CD4^+$ T cells. This confirms the feature of reduced co-stimulatory

requirement in memory $CD4^+$ T cells (Dienz *et al*., 2007).

1.2 Immunological tolerance

Immunological tolerance is a process by which the immune system does not respond to antigens. Naturally, the immune system of an individual develops immunological tolerance to self-antigens to avoid immune responses that would damage itself. However, tolerance can also be induced artificially. The Nobel Prize in Physiology and Medicine was awarded in 1960 to Medawar and Burnet for discovering that skin allografts can be accepted in mice if they had been pre-inoculated neonatally with allogeneic lymphoid cells (Burnet, 1991). They first proposed that exposure to antigens before the development of the immune response can abrogate immune responses to that antigen in later life. Therefore, immunological tolerance is an important mechanism not only for discriminating self and non-self antigens but also for avoiding harmful immune responses and autoimmune diseases.

1.2.1 Central tolerance

Central tolerance occurs in the thymus and is required due to the random generation of receptors that occurs during T cell differentiation, whereby a proportion of the cells recognize self-antigens of the host. In the thymus, T cell progenitors whose antigen receptors bind to MHC-self-antigen complexes on medullary thymic epithelial cells (mTEC) can survive, whereas cells which do not bind to MHC-self-antigen complexes undergo programmed cell death. This procedure is termed positive selection ensuring that all of the T cells are self-MHC restricted (Anderton, 2006). T cell progenitors are also divided into $CD8^+$ T cells and $CD4^+$ T cells based on the restriction of MHC. $CD8^+$ T cells are restricted by MHC class I

and $CD4^+$ T cells are restricted by MHC class II (Janeway *et al*., 2005). In the thymus, autoimmune regulator (AIRE) has been found to be expressed by mTEC and slightly expressed by DC (Anderson *et al*., 2002; Cheng *et al*., 2007). It has been shown that AIRE controls the ectopic transcription of the peripheral tissue antigens in mTEC, therefore triggering apoptosis in self-reactive T cells (Liston *et al*., 2003; Mathis and Benoist, 2007). This procedure of apoptosis is called negative selection. T cell progenitors bearing TCR of high affinity for self-antigens die in the thymus, so potentially dangerous self-reactive T cells are eliminated (Hogquist *et al*., 2005). Only the T cells that survive from the positive and the negative selection can enter the periphery. However, central tolerance can not eliminate all of the self-reactive T cells. Although thymic selection limits the majority of self-reactive T cells, some of them entering the periphery may damage tissues (Anderton, 2006). As unwanted T cell activation occurs, peripheral tolerance provides a second line that has evolved to guard against peripheral self-reactivity.

1.2.2 Peripheral tolerance

Three pillars of peripheral tolerance, deletion, anergy and regulation supply a protective shield against self-reactive T cells evading central tolerance. They may work individually or collectively to restrain T cell from survival, proliferation or producing effector cytokines (Hochweller *et al*., 2006).

1.2.3 Peripheral tolerance : Deletion

Immune responses against foreign antigens involve the generation of effector T cells through a process of activation and expansion; subsequently the majority of the expanded T cells are programmed to die after the foreign antigens have been

removed (Strasser and Pellegrini, 2004). T cell death can occur through two mechanisms, involving extrinsic and intrinsic pathways (Krammer *et al*., 2007). Extrinsic cell death is mediated by death receptors, including Fas (CD95) and tumor necrosis factor receptor (TNFR) that drive apoptosis. Through death receptors, pre-activated and expanded T cells can undergo activation induced cell death (AICD) (Krammer *et al*., 2007; Krueger *et al*., 2003). AICD is thought to play a role in the maintenance of self-tolerance by eliminating self-reactive lymphocytes and cannot be prevented by anti-apoptotic proteins of the Bcl family (Van Parijs 1998a). For example, the Fas / FasL-dependent AICD occurs when T cells encounter high doses of antigens or repeated stimulation (Ju *et al*., 1995; Van Parijs 1998b). The ligation of death receptors transmits death signals to cells and drives a caspase 8 / 10-dependent apoptosis (Hildeman *et al*., 2002). Caspases are a family of cytosolic proteases that cleave proteins essential to sustain cell life (Krammer *et al*., 2007). Administration of superantigen to wild type mice or peptide to peptide relevant TCR transgenic mice can trigger apoptosis, whereas antigen specific T cells are resistant to apoptosis when Fas or FasL are non-fuctional (Bonfoco *et al*., 1998; Pinkoski *et al*., 2002).

The intrinsic pathway of T cell death, also called activated T cell autonomous death (ACAD), is mediated by the unbalance of anti-apoptotic and pro-apoptotic molecules. B-cell lymphoma 2 (Bcl-2) and Bcl-2 related proteins, which have Bcl-2 homology domains (Such as Bcl-$_{XL}$, Bim, Bad, Bax and Bak), participate in this pathway (Hildeman *et al*., 2002). Anti-apoptotic proteins, such as Bcl-2 and Bcl-$_{XL}$, are located on outer membrane of mitochondria, sequestering pro-apoptotic proteins and preventing cell death. During apoptotic stimulation, Bcl-2 molecules are occupied by Bim, driving the formation of pores in mitochondria by Bax and Bak, subsequently

releasing cytochrome C into the cytosol to initiate caspase 9 and caspase 3 dependent cell death (Krammer *et al*., 2007). In intrinsic cell death, the existence of IL-2 is crucial to up-regulate Bcl-2 and to prevent cell death (Arnold at al., 2006). However, production of IL-2 is strongly dependent on TCR and CD28 signalling in naive $CD4^+$ T cells. Mice challenged with soluble antigens do not receive sufficient CD28 signalling, which results in a deficit of IL-2 production and leads to intrinsic cell death (Krammer *et al.*, 2007). While both extrinsic and intrinsic cell death involve caspases to regulate apoptosis, recent reports suggest that the strong TCR stimulation may induce a release of cathepsin from lysosomes and subsequently induce apoptosis, indicating that a caspase-independent cell death may play a role in peripheral tolerance (Michallet *et al*., 2004).

1.2.4 Peripheral tolerance : Anergy

T cell anergy is a mechanism of tolerance in which T cells are intrinsically and functionally inactivated following an antigen encounter, but cells remain alive for a period in the hyporesponsive state. First identified *in vitro*, anergy has been defined as an unresponsive state in which T cells can not proliferate and produce IL-2 (Schwartz, 2003). It can be induced by TCR ligation in the absence of co-stimulation, very low dose stimulation with agonist, or by exogenous supply of suppressive cytokines (Andris *et al*., 1996; Mirshahidi *et al*., 2001; Faria and Weiner, 2005). Schwartz categorized T cell anergy into clonal anergy and adaptive tolerance. The former can be induced in T cells *in vitro* by delivering a strong TCR signal in the absence of co-stimulation or by stimulation with low-affinity agonist with co-stimulation. Once anergy is induced, T cells are resistant to further strong stimulation with anti-CD3 and anti-CD28 antibodies. Adaptive tolerance was

described using *in vivo* models in which antigen-reactive transgenic T cells are transferred into antigen expressing mice (Schwartz, 2003). The endogenous antigens can drive and then maintain those antigen-reactive T cells in a hyporesponsive state (Liblau *et al*., 1996; Raimondi *et al*., 2006). Apart from clonal anergy and adaptive tolerance, the Jenkins Lab has shown with an adoptive transfer system that an administration of soluble peptide without adjuvant results in limited cell expansion of antigen reactive T cells and the residual subset remained unresponsive to further re-stimulation *in vivo* and *in vitro* (Kearney *et al*., 1994; Pape *et al*., 1998). Clonal anergy, adaptive tolerance and peptide-induced tolerance all exhibit a reduction of proliferation and IL-2 production. However, only adaptive tolerance and peptide-induced tolerance appear to require a persistence of antigen and are able to limit the production of pro-inflammatory cytokines (Schwartz, 2003, Saibil *et al*., 2007).

Although T cell anergy requires signals from the TCR, this signalling is qualitatively distinctive from cells in an activated state (Mueller, 2004). In hyporesponsive T cells induced by a adaptive tolerance model, the linker of activation of T cells (LAT) was found to be hypophosphorylated after a subsequent stimulation by CD3 / CD28 and the recruitment of LAT to the immunological synapse was defective (Hundt *et al*., 2006). As phosphorylated LAT is evoked by TCR / pMHC ligation and is a scaffold to deliver downstream signalling for T cell activation (Lin *et al*., 1999), activation and localization of LAT contributes to the induction of T cell anergy (Hundt *et al*., 2006).

TCR / pMHC ligation without CD28 signals also induces the expression of transcription factor of early growth response family proteins (Egr, such as Egr2 and

Egr3) that promote the expression of E3 ubiquitin ligases (Safford *et al*., 2005). The E3 ubiquitin ligases, such as casitas B-lineage lymphoma (Cbl), gene related to anergy in lymphocyte (GRAIL), and itchy homologue E3 ubiquitin protein kinase (ITCH), can further trigger the degradation of proteins that are involved in T cell activation, subsequently driving T cells to anergy (Mueller, 2004). Unresponsiveness in clonal anergy is reversible by adding IL-2, whereas it is irreversible in adaptive tolerance. Moreover, adaptive tolerance was relevant to the expression CTLA-4 (Perez *et al*., 1997; Well *et al*., 2001). CTLA-4 can compete with CD28 for ligands and deliver negative signals, arrest cell cycle and block T cell proliferation (Ratts *et al*., 1999; Eagar *et al*., 2002; Fathman and Lineberry, 2007). Li and colleagues have reported that CTLA-4 deficient mice express a reduced amount of Cbl-b, which is a protein that negatively regulates TCR signalling by ubiquitinating the downstream proteins of CD28 and TCR, suggesting that CTLA-4 can affect CD28 signalling through Cbl-b (Li *et al*., 2004; Zhang *et al*., 2002; Fang *et al*., 2001). Moreover, peptide-induced tolerance can be abrogated by gene deficiency of Egr or by retroviral transduction of dominant negative Grail gene (Safford *et al*., 2005; Seroogy *et al*., 2004). Taken together, synergy of TCR and CD28 signalling delivers signals for T cell activation, whereas TCR stimulation alone delivers distinct signalling that down-regulates T cell proliferation and drives T cell anergy.

1.2.5 Peripheral tolerance : Regulation

There is a special subset of T cells which has regulatory properties, aiding in the prevention of aggressive immune responses (Itoh *et al*., 1999). These cells were named after their functions as regulatory T cells (Treg). The ¡naturally occurring¡ Treg was first recognized as a $CD4^+$ T cell population expressing the IL-2 receptor α

chain (CD25) in non-primed mice. If $CD4^+$ $CD25^+$ cells are eliminated, mice develop autoimmune diseases, whereas these diseases can be prevented by reconstituting with $CD4^+$ $CD25^+$ cells (Sakaguchi *et al*., 1995). Foxp3 is the other hallmark other than CD25 in Treg cells and is crucial in the Treg development and function (Hori, 2003; Sakaguchi, 2005). Foxp3 deficient or mutant mice fail to develop Treg cells and succumb to a lethal autoimmune syndrome, whereas naive T cells retrovirally transduced with the Foxp3 gene can develop a Treg-like phenotype and obtain the ability to suppress T cell activation and inflammation (Hori *et al*., 2003; Fontenot, 2003).

Inducible Treg (iTreg) can be induced from naive $CD4^+$ $CD25^-$ cells by a polyclonal stimulation with TGF-β and IL-2. The expression of Foxp3 in iTreg is transient *in vitro* (removal of TGF-β led to a loss of Foxp3 expression 4 days later) but can persist for 28 days *in vivo* (Chen *et al*., 2003; Zheng *et al*., 2004; Selvaraj and Geiger, 2007). Recently Foxp3 has also been proven to interact with NFAT and NF-κB (nuclear factor-kappa B) (Bettelli *et al*., 2005), which are key transcription factors for TCR and CD28 signalling. The engagement between Foxp3 and NFAT suppresses IL-2 production and up-regulates CD25 and CTLA-4 on Treg cells (Bettelli *et al*., 2005; Wu *et al*., 2006). As many reports observe that Treg cells are able to inhibit proliferation and cytokine production of effector cells in a contact-dependent manner (Thornton *et al*., 1998), the expression of CTLA-4 and surface bound TGF-β contribute to their suppressive function by interacting with responder T cells or DC **(**Read *et al*., 2000; Nakamura *et al*., 2001). On the other hand, TGF-β and IL-10 produced from Treg cells also mediate the suppression of T cell activation and inflammation, providing a contact-independent suppression (Asseman *et al*., 1999;

Green *et al*., 2003; Zuany-Amorim, 2002; von Boehmer, 2005).

Apart from Foxp3$^+$ Treg cells, some subsets of CD4$^+$ T cells also have regulatory functions. Tr1 and Th3 cells are differentiated from naive T cells in the periphery after antigen specific activation (Roncarolo *et al*., 2006; Faria and Weiner, 2005). Tr1, characterized by the properties of IL-10 secretion, can be generated *in vitro* and *in vivo* upon priming naive T cells with antigen in the presence of IL-10 (Groux *et al*.,1997). Once activated, Tr1 cells can mediate anti-inflammatory and suppressive activity against naive CD4$^+$ T cells through releasing IL-10 (together with TGF-β in some cases (Groux, 2003). Th3 cells were identified during the induction of oral tolerance (Faria and Weiner, 2005). In oral tolerance models, administration of high dose antigen triggers T cell anergy or deletion, whereas low dose antigen induces the generation of TGF-β secreting Th3 cells (Weiner, 2001). TGF-β secreting Th3 cells can suppresses both Th1 and Th2 responses during oral tolerance, while treatment with neutralizing antibodies against TGF-β reverses this suppression (Chen *et al*., 1994).

1.2.6 Peripheral tolerance : steady state DC

DC have the potential to induce both immunity and tolerance in the periphery. When proteins or peptides are presented without danger signals (PAMP), subsets of antigen-presenting DC do not develop to mature DC but stay in a quiescent state, called steady state DC or tolerogenic DC (Banchereau and Steinman, 1998). Steady state DC remain quiescent after capturing and processing exogenous antigens, express low levels of surface MHC molecules and have a low frequency of co-stimulatory molecules, therefore they induce insufficient activation of T cells,

triggering T cell deletion and / or unresponsiveness (Morelli and Thomson, 2007). Anti-inflammatory or immunosuppressive conditions *in vitro* can trigger steady state DC to produce of IL-10, TGF-β1 and indoleamine 2,3-dioxygenase (IDO), enhancing expression of FasL, programmed cell death ligand 1 (PD-L1) in DC which can subsequently suppress inflammation (Hackstein *et al*., 2004). IDO is an immunomodulatory enzyme that regulates the degradation of tryptophan, serotonin and melatonin and initiates the production of neuroactive and immunoregulatory metabolites, including pro-apoptotic molecules. IDO expressed by DC greatly affects T cell proliferation and survival (Grohman *et al*., 2003). Moreover, Treg cells can trigger IDO production in DC by the ligation of CTLA-4 (Treg) and CD80 / 86 (DC), which results in the inhibition of T cell proliferation and T cell apoptosis (Puccetti and Grohmann, 2007).

1.2.7 Peptide-induced tolerance

DC are the most effective APC to present antigens. When attempting to prime naive $CD4^+$ T cells, a protein antigen is presented poorly by DC, whereas antigen delivered as a short peptide is presented most efficiently to $CD4^+$ T cells by DC (Constant *et al*., 1995). Thus peptide antigens are also used for tolerance induction. Administration of soluble peptide through oral, mucosal, intravenous (i.v.), intraperitoneal (i.p.), or intranasal (i.n.) routes have been shown to successfully induce tolerance and suppress T cell-mediated autoimmune diseases, giving prospects for developing antigen-specific therapies (Anderton, 2001). Peptide-induced T cell tolerance can result from many causes. First, tolerance induction can affect T cell migration. Naive T cells spend their lives re-circulating through the secondary lymphoid organs. After priming with antigen in adjuvant subcutaneously (s.c), the antigen specific T cells

receive TCR signal from pMHC on DC in the paracortical region of the lymph nodes. T cells proliferate in the paracortical region for several days before migrating into the B cell rich follicle zone to interact with B cells (Kearney *et al*., 1994; McLachlan and Jenkins, 2007). However, injection of soluble antigen alone makes antigen specific T cells accumulate in the paracortical region but they fail to enter follicles (Kearney *et al*., 1994). This may prevent B cell maturation and antibody production. Also, avoidance of T-B cells contact can prevent the pMHC and co-stimulatory signal provided by B cells and subsequently prevents the potential activation signals delivered through CD40 and CD40L (Ranheim and Kipps; 1993; van Essen *et al*., 1995; Grewal *et al*., 1995).

Second, administration of high dose peptide in soluble form can induce cell death. High dose of soluble peptide results in reduced proliferation and enhanced apoptosis of the peptide-reactive T cells after immunization with peptide in adjuvant (Critchfield *et al*., 1994; Liblau *et al*., 1996; Klugewitz *et al*., 2002; Weishaupt *et al*., 1997). In addition, it has been reported in adoptive transfer system that oral administration of OVA peptide to the mice leads to Fas / FasL mediated cell (OVA-reactive cells) death in the host liver (Watanabe *et al*., 2002).

Third, tolerance induction actively triggers peptide-reactive T cell deletion and / or anergy. According to the two signal hypothesis for T cell activation, naive $CD4^+$ T cells require both signal 1 and 2 to provide proliferative and survival signals. After the administration of soluble peptide without adjuvant, DC present pMHC complexes to T cells in a tolerogenic manner resulting from the insufficient expression of co-stimulatory molecules (Anderton, 2001). With the adoptive transfer system with

pOVA and transgenic mice specific to pOVA (OT-II), surface expression of co-stimulatory markers have been compared between OT-II cells re-isolated from tolerant (injection of soluble pOVA alone) and primed (injection of pOVA peptide and LPS) groups. DC from mice receiving peptide alone had a basal expression of CD80 / 86 and CD40 and a lack of receptor activator of nuclear factor κB (RANK) expression, whereas DC from the primed mice had a stronger expression of CD86, CD40 and RANK. In addition, OT-II cells from the primed mice showed a longer expression of OX40 compared to OT-II cells from the tolerance group (Hockweller and Anderton, 2005). As co-stimulatory markers deliver activation and survival signals into both T cells and DC, T cells which receive no co-stimulation by the steady state DC after soluble peptide treatment would undergo apoptosis or become unresponsive (Soroosh *et al*., 2006; Hochweller and Anderton, 2004; Josien *et al*., 2000; Appleman and Boussiotis, 2003). Moreover, peptide-induced T cell tolerance can be abrogated by an exogenous injection of agonistic antibodies against co-stimulatory molecules (eg, anti-CD80, anti-CD86, anti-CD40 or anti-OX-40), indicating that the absence of co-stimulatory signals are crucial in peptide-induced tolerance (Bell *et al*., 2003; Bansal-Pakala, 2001; Hochweller, 2006). As a basal expression of MHC and CD80 / 86 exist, immature DC may drive a transient T cell expansion within a short time. However, these insufficient activation and survival signals cannot sustain prolonged T cell expansion. Instead, they trigger T cells apoptosis and anergy (Huang *et al*., 2003; Kearney *et al*., 1994; Liblau *et al*., 1996; Deng *et al*., 2001). Thus administration of soluble peptide (without adjuvant) fails to trigger IL-2 production and can induce cell death or anergy (Burstein *et al*., 1992, Critchfield *et al*., 1994; Gaur *et al*., 1992).

With oral and i.n. routes, administration of peptide can provoke the production of suppressive cytokines to Th1 responses (such as IL-4, IL-10 and TGF-β) (Burkhart *et al*., 1999; Maron *et al*., 2002; Khoury *et al*., 1992). In addition, administration of low doses peptide orally can induce Th3 cells within the target organ and provide tolerance by immunosuppressive cytokines or by physical contact inhibition, suggesting that the route of peptide administration can also affect T cell tolerance (Figure 3; Miller *et al*., 1992; Chen *et al*., 1994; Miller *et al*., 2007).

1.3 Autoimmunity

The immune system needs to discriminate between antigens of self and non-self origin to avoid a specific adaptive immune response mounted against self-antigens. Autoimmunity is the failure of an organism to recognize its own antigens which results in an immune response against its own cells and tissues (Janeway, 2005). Although the majority of self-antigen-responsive T cells are deleted in the thymus, some self-reactive T cells may escape deletion and become sustained within the periphery and cause a risk of autoimmunity if they are activated to respond against their own cells or tissues (Anderton, 2006). Prominent examples of autoimmune disease include type I diabetes (TID), rheumatoid arthritis (RA), systemic lupus erythematosus (SLE) and multiple sclerosis (MS).

Autoimmunity is associated with both genetic and environmental factors **(**Martin and McFarland, 1995**)**. Twin studies have demonstrated that if one monozygotic twin develops an autoimmune disease, the other twin has higher incidence to get disease, whereas dizygotic twins have significant low concordance of the same disease. In MS, the concordance to get MS in monozygotic twin is 25% and 5% in dizygotic

twins (Willer *et al*., 2003). The risk to get type I diabetes is also higher in monozygotic twins (long term risk is estimated 44% if one monozygotic twin develops diabetes) than in dizygotic twins (risk of 19%) (Kumar *et al*., 1993). Genetics therefore contributes to disease susceptibility. Studies in human leukocyte antigen (HLA) appear that class II HLA molecules are associated with susceptibilities of several autoimmune diseases (Ebringer and Wilson, 2007). In RA, over 90% of patients possess either HLA-DR1 or some subtypes of HLA-DR4, while the frequency of HLA-DR1 in the general population is 35%. Moreover, class I HLA molecule (HLA-B27) has been reported to be involved in ankylosing spondylitis (Khan *et al*., 2007).

Environmental factors, such as infection, also affect autoimmunity. Infection may have a bystander effect on autoimmune disease. For example, the composition of the neuronal microenvironment is maintained by the blood-brain-barrier (BBB) in the CNS, making the CNS an inaccessible region for lymphocytes. Some bacterial or viral infections can break the BBB and damage the CNS, subsequently releasing CNS derived self-antigens. This release of self-antigens may further trigger CNS derived protein-reactive T cells, causing antigen specific demyelination in the CNS (Gay, 2007). Another potential mechanism for infection-induced autoimmunity is molecular mimicry. This can occur when a microorganism and its host share a similar immunological epitope (Oldstone, 2005; Fujinami *et al*., 2006). For example, coxsackievirus infections were associated with the induction of TID and mice infected with coxsackievirus developed diabetes (Tracy and Drescher, 2007). An immune response induced by a foreign pathogen having a cross-reactive determinant may therefore recognize the similar host epitope (Fujinami, 2001), providing the risk

of developing autoimmune disease.

1.3.1 Multiple Sclerosis

Multiple sclerosis (MS) is a chronic inflammatory and autoimmune disease of the central nervous system (CNS) affecting young adults. It is characterized by focal lesions of inflammatory demyelination in the white matter of the brain (Lassmann *et al*., 2007). Recently magnetic resonance imaging (MRI) studies have revealed a much more widespread and global damage of the brain and spinal cord in MS patients (Miller *et al*., 2002). Clinical signs of MS include pain, fatigue and problems with sight, mobility and coordination. Although MS has been known for over one hundred years, its causes remain unknown (Martin and McFarland, 1995). Studies in inheritance show the occurrence of MS is greater in close relatives of MS patients than in a matched control population. Similarly, the concordance rate is greater in monozygotic twins (25 %) than in dizygotic twins (5 %) (Willer *et al*., 2003). The increased familial risk indicates a role for genetic factors in the aetiology of MS. Moreover, a number of genes may influence the susceptibility to MS. An MS susceptibility locus in MHC (or human leukocyte antigen, HLA) is reported and a dataset of 98 multiplex families demonstrated a strong genetic linkage for an association to the HLA-DR2 allele (HLA-DR2 is a HLA-DR serotype that recognizes several DRB1*15 and DRB1*16 alleles) in familial MS (Haines *et al*., 1998), suggesting that sporadic and familial MS share at least one common susceptibility marker (Kalman and Lublin, 1999). In addition, allelic variants of the HLA-DRB1 and HLA-DQB1 genes are associated with susceptibility to MS (Rubio *et al*., 2002). HLA-DR15 is one of the HLA-DR serotypes that recognizes the DRB1*15 alleles and is associated with MS (Masterman *et al*., 2000). Humanized

transgenic mice that express MS-associated HLA-DR15 spontaneously developed paralysis (Ellmerich *et al*., 2006). In addition to MHC class II, HLA class I alleles that increase and decrease the genetic susceptibility to MS were identified. The HLA-A*0301 allele increases the risk of MS, HLA-A*0201 decreases the overall risk (Rubio *et al*., 2007; Fogdell-Hahn *et al*., 2000), suggesting an effect on MS susceptibility of class I HLA molecule (Burfoot *et al*., 2008).

Another possible cause of MS is molecular mimicry originated from infection. Hepatitis B virus (HBV) polymerase was found to share 6 consecutive amino acids with rabbit myelin basic protein (MBP). HBV infection may trigger the production of antibodies and mononuclear cells that cross-react with MBP (Fujinami and Oldstone, 1985). Synthesized peptides have been tested on MBP-specific T cell clones from MS patients and several viral and bacterial peptides have been found to stimulate MBP_{85-99}-reactive T cell clones, suggesting that peptides from some infectious pathogens (including Herpes simple virus, Epstein-Barr virus, Adenovirus type 12, Influenza type A virus, Human papillomavirus, Reovirus and Pseudomonas aeruginosa) may act as molecular mimics of immunodominant MBP_{85-99} peptide (Wucherpfennig and Strominger, 1995). Infectious virus may contribute to MS. For example, human coronaviruses (HCoV), which are responsible for up to one third of common colds, have been reported to be associated with MS. T cell clones derived from MS patients can be activated by either HCoV proteins (HCoV-OC43 or HCoV-229E) or myelin antigens (MBP or proteolipid protein, PLP), suggesting a cross-reactivity between myelin and HCoV proteins (Boucher *et al*., 2007).

Epstein-Barr virus (EBV) is another strong candidate in MS etiology. Based on the

epidemiological observations in MS, all MS patients are seropositive (95 % seropositive) to EBV in contrast to healthy controls (90-95 % seropositive), an increased risk of developing MS has been associated with infectious mononucleosis and higher serum levels of anti-EBV antibodies, suggesting a link among EBV, B cells and MS (Haahr and Hollsberg, 2006). EBV is a human DNA herpesvirus that establishes asymptomatic latent infection in B cells in most individuals. The genome of EBV has been detected in cerebral spinal fluid (CSF) and MS plaques, and the replicating viruses exist in peripheral blood mononuclear cells prior to MS exacerbations (Christensen *et al*., 2007; Fatheringham and Jacobson, 2005; Ascherio and Munger, 2007). Previous studies concluded that the presence of high titres of IgG antibodies against the EBV nuclear antigen 1 (EBNA-1) or EBNA complex in healthy individuals is a strong risk factor for MS (Ascherio *et al*., 2001; Sundstrom *et al*., 2004; DeLorenze *et al*., 2006). In longitudinal analyses, a 4-fold increase in anti¡ EBNA-1 or anti-EBNA complex titres was associated with a 3-fold increase in MS risk (Levin *et al*., 2005). Moreover, a study with long-term follow-up appeared that the elevations of anti-EBNA antibodies among MS patients first occurred 15-20 years before the onset of MS and remained constant thereafter, suggesting that the elevation of anti-EBNA titres may be an early event in the pathogenesis of MS (DeLorenze *et al*., 2006). Recent data using T cell lines (TCL) from MS patients indicated that some EBNA-1-specific CD4 TCL can selectively cross-react to myelin antigens and produce IFN-γ, suggesting that these EBNA-1-specific CD4 may contribute to the development of MS by cross-recognition of myelin antigens (Lunemann *et al*., 2008). Recent studies show that intra-cerebral accumulation of EBV-infected B cells and plasma cells is a regular feature of MS patients and that the B cell follicle is the main site of viral persistence. In addition, expansion and

cytotoxic activity of $CD8^+$ T cells against EBV was found at sites of accumulation of EBV-infected cells in the MS brain, indicating an ongoing anti-viral immune response in the MS brain (Serafini *et al*., 2007; Jilek *et al*., 2008).

Regardless of the precise etiology of the disease, most evidence suggests that MS is an autoimmune disease which is influenced by both environmental and genetic factors and is mediated by the adaptive immune system (Lassmann *et al*., 2007). Studies in animal models indicate that the adaptive immune response targets the CNS and that myelin protein specific T cells drive the inflammation in the CNS. Myelin protein-reactive T cells are activated and migrate into the CNS, where they trigger macrophage and microglia cells to damage the myelin sheath that surrounds axons. The damage of myelin subsequently causes apoptosis in oligodendrocytes, loss of axonal and subsequent blocks the neuron transmission ultimately leading to paralysis (McFarland and Martin, 2007; Kuhlmann *et al*., 2008).

1.3.2 MS and immunological memory

There are four subtype definitions in MS: relapsing remitting, secondary progressive, primary progressive and progressive relapsing. Around 85% to 90% of MS patients suffer with the relapsing-remitting form (Lublin and Reingold, 1996). A possible cause of MS relapse is the existence of memory cells. It has been reported that the majority of T cells in the CSF of MS patients exhibit the phenotype of memory cells ($CD45RA^+$ $CD45RO^+$) and this is the case for both $CD4^+$ and $CD8^+$ cells (Vrethem *et al*., 1998). A study with relapsing¡remitting MS patients demonstrated that the percentage of $CD25^+CD45RO^+CD4^+CD3^+$ cells was significantly increased in peripheral blood and CSF of active MS patients compared with inactive MS patients.

A longitudinal study also showed a higher percentage of $CD25^{+}CD45RO^{+}CD4^{+}CD3^{+}$ cells in peripheral blood at the phase of exacerbation than during remission in relapsing¡ remitting MS patients, suggesting that the activation of memory $CD4^{+}$ T cells is associated with the exacerbation of MS (Okuda *et al*., 2005). Recently, a population of $CD4^{+}$ $CD45RO^{+}$ $CD26^{high}$ $CD62L^{low}$ memory T cell has also reported to be correlated with MS severity (Krakauer *et al*., 2006). MS relapse therefore appears highly likely to reflect the activation of $CD4^{+}$ memory T cells.

1.3.3 Experimental Autoimmune Encephalomyelitis

Experimental autoimmune encephalomyelitis (EAE) is the most widely used inducible animal model for MS. In Louis Pasteur¡s landmark vaccination against rabies, people inoculated with the dry spinal cord from rabies virus infected rabbits were protected from disease. However, many cases developed encephalitogenic signs. Five decades later, Thomas M. Rivers injected monkeys and rabbits with rabbit brain extract and subsequently observed perivascular infiltrates and demyelination in the brain and spinal cord of the inoculated animals (Baxter, 2007). Now River¡s model and its derivatives are known as EAE. Clinically, EAE runs a monophasic or a remitting-relapse course of disease with weakness, limb paralysis, optic neuritis and cerebral signs. It shares many characteristics with MS including genetic susceptibility, lymphocyte infiltration in the CNS, production of anti-myelin antibody, demyelination and clinical presentation (inflammatory lesions in white matter, myelitis, inflammation, optic neuritis) (Steinman and Zamvil, 2005; Martin and McFarland, 1995). In practice, EAE can be induced by immunizing susceptible mice with myelin derived antigens emulsified with complete Freund¡s adjuvant (CFA), together with doses of pertussis toxin (PTX) which opens the BBB. EAE can be

induced by immunization with crude CNS tissue or myelin derived proteins such as myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG), and proteolipid protein (PLP). $CD4^+$ T cells are central to the induction of EAE. Effector cells, including microglial cells and monocytes are triggered by IFN-γ and TNFα produced from myelin-reactive $CD4^+$ T cells and participate in tissue destruction and demyelination (Martin and McFarland, 1995; Elenkov and Chrousos, 2002). As T cells recognize peptides bound to MHC molecules on the APC, synthetic peptides with immunodominant regions derived from MBP, PLP, and MOG have been identified to be able to trigger T cells and provoke EAE in susceptible mice (Zamvil *et al*., 1986; Tuohy *et al*., 1989; de Rosbo and Ben-Nun, 1998). The development of synthesized myelin peptides and their corresponding T cell line derived-TCR transgenic (Tg) mice give practical models to address the disease nature and the potential strategies for therapy.

1.3.4 TCR transgenic models of EAE

Looking into the immunodominant region of myelin proteins can provide important information for TCR recognition, MHC binding and for designing peptide vaccination. For example, MOG, a minor component of myelin protein, is expressed on the surface of oligodendrocytes and is a member of the Ig superfamily with high homology between species. Unlike other myelin proteins which are integral components and are not easily accessible to immune cells, exposed MOG has more chance to encounter antibodies (Martin and McFarland, 1995). Different synthetic peptides derived from MOG have been tested in $H\text{-}2^b$ mice. Peptide 35 to 55 ($MOG_{35\text{-}55}$ or pMOG) gives mice an encephalomyelitis with a paralytic disease, indicating that pMOG is the immunodominant peptide (Mendel *et al*., 1995). A TCR

transgenic mouse (2D2) has been generated from a pMOG-specific T cell clone bearing the TCRVα3.2 and Vβ11 from C57BL/6 mice. These 2D2 mice are on a C57BL/6 background and thus bear the I-A^b MHC class II molecules (Bettelli *et al*., 2003; Mendel *et al*., 2004). Furthermore, analysis of overlapping short peptides, single residue substitutions and T cell lines have identified the core sequence of MOG as residue 40-48 (Ben-Nun *et al*., 2006; Sweenie *et al*., 2006). Recent data suggests that positions 41, 44, 46, 47 of pMOG are TCR contact sites, whereas positions 40, 43, 45, 48 of pMOG are MHC class II interacting sites (Petersen *et al*., 2004; Sweenie *et al*., 2006).

The MBP / Tg4 / B10.PL system is based on myelin basic protein (MBP), which is an abundant component of myelin comprising 30 % of myelin protein. MBP is located in the cytoplasm of oligodendrocytes (Martin and McFarland, 1995). The acetylated N-terminal peptide 1-11 (Ac1-11) of MBP was the first myelin epitope to be identified (Zamvil *et al*., 1986). As the residues 1 to 9 of MBP are the immunodominant residues in H-2^U mice for EAE (Kumar and Sercarz, 1993), acetylated N-terminal peptide (Ac1-9) was applied in this thesis. Ac1-9 mediates encephalitogenicity in PL/J and B10.PL mice, the TCR transgenic mouse (Tg4) is on the B10.PL background and was generated from an encephalitogenic $CD4^+$ T cell clone which expresses the TCR Vα4 and Vs8.2 and is specific for Ac1-9 (Liu *et al*., 1995; Kurschus *et al*., 2006). Ac1-9 binds to the I-A^u class II molecule through residues 4 (lysine) and 5(arginine), with residue 4 contributing the major part of MHC binding. As the lysine at residue 4 interacts with the A^u peptide binding cleft poorly, altered peptide ligands (APL) with various binding affinities have been developed by substituting 4Lys with Ala, Val, and Tyr (ordered by MHC affinity

from low to high) (Zamvil *et al*., 1986; Wraith *et al*., , 1992; Pearson *et al*., 1997a).

1.3.5 Central tolerance in EAE

The idea that EAE is the result of a failure in central tolerance is outwardly logical because the CNS is an immune privileged site with the BBB that physically separates the peripheral lymphocytes from the CNS (Persidsky *et al*., 2006). Therefore, myelin antigens should be excluded during central tolerance. However, evidence indicates that myelin antigens, including PLP, MBP and MOG, are expressed in the thymus, suggesting that negative selection against myelin antigens should occur in the thymus (Fritz and Zhao, 1996; Pribyl *et al*., 1993; Bruno *et al*., 2002; Pagany *et al*., 2003). Contradictorily, an analysis of TCRαβ repertoires expressed by MOG-specific $CD4^+$ T cells showed that T cells are identical in $MOG^{-/-}$ and WT mice in the periphery, suggesting that MOG-reactive T cells are not depleted in the thymus (Fazilleau *et al*., 2006). In Tg4 TCR transgenic mice relevant to MBP, an administration of MBP APL peptide with high affinity for MHC affinity causes a strong deletion in double positive thymocytes, whereas administration of low affinity peptide causes no depletion of thymocytes, suggesting that MBP-reactive T cells with moderate avidity avoid central tolerance and enter the peripheral T cell repertoire (Liu *et al*., 1995; Anderton *et al*., 1999).

1.3.6 $CD4^+$ T cells subsets in EAE

Depletion of $CD4^+$ T cells *in vivo* or *in vitro* with anti-CD4 antibody (Ab) before EAE induction can prevent disease, proving that $CD4^+$ T cells are pivotal to EAE (Brostoff *et al*., 1984; Pettinelli *et al*., 1981). Myelin protein reactive $CD4^+$ T cells can migrate and accumulate in the CNS and trigger inflammation (Flugel *et al*., 1999;

Flugel *et al*., 2001). It was initially believed that Th1 cells were pathogenetic T cells in EAE because myelin reactive T cells produced large amounts of IFN-γ (Zamvil and Steinman, 1990). However, many exceptions appear in this idea of ¡Th1 cell driven EAE¡. For example, administration of IFN-γ or TNFα protects from EAE, blockage of or gene deficiency of IFN-γ or IFN-γR signaling worsens EAE (Billiau *et al*., 1988; Liu *et al*., 1998; Chu *et al*., 2000; Willenborg *et al*., 1996). Recent data on IL-23 and Th17 cells have adjusted the previous concept. IL-12 which consists of p40 and p35 subunits is required for the development of optimal Th1 responses and mice lacking IL-12p40 show a resistance to EAE development (Segal *et al*., 1998, Becher *et al*., 2002). IL-23 consists of a unique p19 subunit and shares a common p40 subunit with IL-12 (Oppmann *et al*., 2000). IL-12p35 or IL-12R knockout mice are susceptible to EAE and either IL-23p19 or p40 knockout mice are less susceptible to EAE, indicating that IL-23 plays an essential role in EAE (Cua *et al*., 2003; Becher *et al*., 2002; Gran *et al*., 2002; Zhang *et al*., 2003). Moreover, IL-23 was recently proposed to contribute to EAE pathology through the expansion of Th17 cells. Stimulation of PLP-reactive T cells with TGF-β plus IL-6 abrogates their pathogenic function and pro-inflammatory chemokine secretion despite up-regulation of IL-17 production, whereas stimulation with IL-23 promotes expression of IL-17 and pro-inflammatory chemokines (McGeachy *et al*., 2007; Langrish *et al*., 2005). Th17 cells are thought to be important in EAE as Ab blockage or gene deficiency of IL-23 or IL-17 reduces severity and incidence of EAE in mice (Aggarwal *et al*., 2003; Park *et al*., 2005; Chen *et al*., 2006; Komiyama *et al*., 2006). In addition, adoptive transfer of Th17 cells can induce EAE and IL-17 and IL-17^{+} perivascular lymphocytes are up-regulated in brain lesions from patients with active MS, indicating an important pathogenic role for IL-17^{+} cells in EAE and MS pathogenesis

(Langrish *et al*., 2005; Lock *et al*., 2002; Tzartos *et al*., 2008).

Notably, there was a significant population of IFN-γ producing Th1 cells (5-7 % in splenocyte culture) generated in T cell culture supplemented with IL-23 and polyclonal stimulus (Langrish *et al*., 2005). The adoptive transfer with IL-23 driven ¡Th17 cells¡ usually contains a certain proportion of Th1 cells. In addition, sick mice which had received the ¡IL-23 stimulated population¡ had 5 % and 15 % of IFN-γ producing donor cells harvested from spleen and CNS, respectively (McGeachy *et al*., 2007). ¡Cleaner¡ populations of *in vitro* polarized Th1 and Th17 were generated and transferred into recipient mice and found that Th17 cells lack the capacity of inducing EAE, whereas Th1 cells were highly pathogenic (O¡Connor *et al*., 2008 in press). In active EAE, the frequency of IFN-γ^+ cells was greater than IL-17^+ cells in the CNS (Suryani and Sutton, 2007). In passive transfer models, IFN-γ producing cells were always over-represented in the CNS after Th17 transfer, compared with their frequency at the time of transfer, suggesting that Th1 cells are better able to access non-inflamed CNS and the establishment of Th1 cells in the CNS may facilitate the entry of Th17 cells (O¡Connor *et al*., 2008 in press). *In vitro* IL-12 polarized PLP-reactive T cells have been reported to up-regulate the β-chemokine receptor and CCR5 in correlation with the CNS infiltration and encephalitogenic capacity. Also, The CCR5 ligands are expressed in the spinal cords at EAE onset (Bagaeva *et al*., 2003). Recent studies in experimental autoimmune uveitis (EAU), an autoimmune disease involved in Th1 and Th17 cells, showed that IL-17 knockout mice develop EAU (these mice showed a high enhanced frequency of Th1 cells) and either Th17 or Th1 effector cells can provoke disease (Luger *et al*., 2007). Thus, both Th1 and Th17 appear to contribute to EAE and EAU, possibly via different

mechanisms of pathology and migration.

Transfer of Treg cells can limit the severity of EAE (Kohm *et al*., 2002). In addition, a marked proportion of $CD4^+$ $CD25^+$ $Foxp3^+$ cells (about 30 % of $CD4^+$ T cells) are seen to accumulate in the inflamed CNS during the recovery phase of EAE, indicating that Treg cells may directly correlate to disease alleviation (McGeachy *et al*., 2005). Depletion or inactivation of Treg cells prior to immunization can increase the severity of disease and enhance IFN-γ, IL-6 and IL-17 production, suggesting that Treg cells may suppress EAE by inhibiting the self-reactive effector T cells (McGeachy *et al*., 2005; Reddy *et al*., 2004; Stephens *et al*., 2006; Reddy *et al*., 2005).

1.3.7 B cells and mast cells in EAE

In some models, B cells and antibodies appear to be dispensable in the induction of EAE, as EAE can be triggered in B cell deficient mice (Wolf *et al*., 1996; Hjelmstrom *et al*., 1998; Fillatreau *et al*., 2002). However, a correlation has been found between anti-MOG antibody titers from patients with primary and secondary progressive MS and disease severity (Mantegazza *et al*., 2004). Moreover, MS patients with anti-MOG and anti-MBP antibodies have an increased frequency of relapses than patients without these antibodies (Berger *et al*., 2003). Thus, the significance B cells and antibodies in the CNS is still a clinical hallmark in the diagnosis of MS (Walsh and Tourtellotte, 1986). In EAE, the administration of anti-myelin antibodies has been reported to enhance demyelination, suggesting an aggressive role of B cells and anti-myelin antibodies in EAE (Schluesener *et al*., 1987; Raine *et al*., 1999). B cells also have another role as an immune regulator. B

cell deficient mice fail to progress to the recovery phase of disease and a requirement of IL-10-producing B cells has been identified during the recovery phase of EAE (Fillatreau *et al*., 2002). In addition, IL-10-producing B cells have been identified in humans (Duddy *et al*., 2004). Preliminary evidence shows that B cells from MS patients produce decreased amounts of IL-10, suggesting a regulatory role of B cells derived IL-10 in MS (Duddy *et al*., 2007). Recent data show that TLR / MyD88 signals can regulate IL-10 production in B cells, suppress inflammatory Th1 and Th17 cells and drive the recovery phase of EAE (Lampropoulou *et al*., 2008).

Mast cells (MC) contain granules rich in histamine, proteases and various hormonal mediators which are enhancers in allergy and anaphylaxis. Antigen specific IgE or IgG1 can bind to the Fc receptor (FcR) on MC after a primary humoral response. After secondary challenge, ligation between the antigen and the MC bound antibodies can activate MC to rapidly release granules, resulting in allergy or anaphylaxis (review in Bischoff, 2007). During the progression of EAE, accumulation of MC has been observed at the inflammatory sites in the CNS (Brenner *et al*., 1994). MC associated histamine and hormonal mediators are proven to be involved in demyelination in EAE and MS (El Behi *et al*., 2005). Moreover, the blockade of MC degranulation or MC deficiency can both reduce the severity of encephalomyelitis, suggesting an aggressive role of MC in EAE (Seeldrayers *et al*., 1989; Secor *et al*., 2000).

1.3.8 Peptide based tolerance in EAE

Immunization with immunodominant myelin derived peptides mixed with CFA can provoke and overt EAE, whereas administration of soluble peptide before priming

induces T cell tolerance and prevents disease (Anderton, 2001). Extensive studies have used the administration of soluble peptides (containing immunodominant epitopes) in tolerance induction through oral, intravenous (i.v.), intraperitoneal (i.p.), or intranasal (i.n.) routes which all protect mice from disease development (Miller *et al*., 1993; Liu and Wraith, 1995; Metzler and Wraith, 1993; Burkhart *et al*., 1999). It has been reported that the development of tolerance requires an interval of 4-6 days between peptide administration and subsequent immunization, suggesting that systemic T cell tolerance takes a few days to become established (Metzler and Wraith, 1993; Liu and Wraith, 1995). As mentioned in section 1.2.7, administration of soluble peptide may affect T cell migration and trigger T cell deletion and anergy. However, the efficacy of tolerance could be correlated with the affinity of pMHC ligation. Studies using APL have highlighted the flexibility in TCR recognition of pMHC complexes (Kersh and Allen, 1996). The MBP Ac1-9 (or Ac1-11 in early reports) has a low affinity for the class II MHC, I-A^u molecule. Making a substitution of lysine at position 4 with Alanine or Tyrosine can increase the affinity of peptide-MHC ligation by 10^3 or 10^6 times, respectively (Zamvil *et al*., 1986; Fairchild *et al*., 1993; Mason *et al*., 1995; Anderton, 2001). Administration of the wild-type Ac1-9 peptide in soluble form has a mild influence on subsequent priming (mice develop a mild course of EAE after priming), whereas the tolerance effect can be greatly increased by using the soluble Ac1-9_{4A} and Ac1-9_{4Y} APL. Thus, increasing the avidity of the interaction during tolerance induction leads to a more complete tolerance (Liu and Wraith, 1995; Metzler and Wraith, 1993).

Although soluble peptide administration can induce tolerance in non-primed mice, the same strategy is problematic in EAE experienced mice. Administration of soluble

$PLP_{139–151}$ (pPLP) to pPLP experienced SJL/J mice at the recovery phase of EAE (day 21 or day 28) induced an anaphylactic reaction characterized by respiratory distress, erythema, decreased body temperature and often death. At day 21 or day 28 after immunization, enhanced anti-PLP IgG1 and total IgE were found in the mice, indicating that those signs of hyperreactivity may be resulted from anti-pPLP-antibodies (Pedotti *et al.*, 2001). As antibody-dependent anaphylaxis in mice can be mediated by the cross-linking of IgE bound to FcεRI on mast cells or by the cross-linking of IgG1 bound to FcγRIII on macrophages, mast cells, and / or other cell types, soluble pMOG challenge was tested in the pMOG experienced wild type mice with anti-IgE treatment or in the FcγR deficient mice. Data suggested that antibody-induced anaphylaxis depends on IgE / FcεRI but not IgG1/ FcRIII (Smith *et al*., 2005). However, FcγR deficient mice are less susceptible to EAE and injections of anti-pMOG antibodies can aggravate EAE in an FcR-independent but a complement-dependent fashion (Urich *et al*., 2006).

1.3.9 Translation of peptide tolerance from mouse to human

Antigen specific immune modulation could successfully be induced in animal models, leading to evaluation of synthetic peptides for immunotherapy in humans. In the studies of allergy, peptides derived from cat and bee venom allergens have been tested in human and gave encouraging results. Administration of soluble, allergen-derived peptides has been found to reduce sensitivity to allergen, to change the isotype ratio of allergen-specific antibodies, to enhance the production of anti-inflammatory cytokines, to down-regulate allergen specific proliferation and to reduce the production of Th1 and Th2 cytokines in the blood (Oldfield *et al*., 2002; Muller *et al*., 1998; Fellrath *et al*., 2003). In autoimmune disease, administration of

synthetic peptides derived from heat shock proteins (HSP, a group of self antigens involved in the inflammation in TID and RA) reduces Th1 responses, enhance Th2 responses, increases C-peptide in TID patients and increase Treg cells in RA patients (Raz *et al*., 2001; Prakken *et al*., 2004). However, peptide based therapy in MS has been slower to translate into the clinic compared to other diseases. An antagonistic APL of MBP_{83-99} (NBI 5788) was constructed by altering TCR contact sites (91 Lysine to 91 Alanine) and was tested in MS patients. In a placebo-controlled phase II trial, weekly administrations of NBI 5788 increased IL-5 and IL-13 production in the MBP-reactive T cells and enhanced antibodies against NBI 5788 (Kappos *et al*., 2000). Although treatment with 5 mg NBI 5788 reduced the inflammatory lesions in brain, exacerbations of disease were found in patients received 20 mg and 50 mg NBI 5788. Moreover, 9 % of MS patients developed allergic signs in APL treatment, (Kappos *et al*., 2000). Cytokine secretion by MBP_{83-99} APL (NBI-5788)-reactive T-cell lines from NBI-5788-treated MS patients was more frequently Th2-like compared with T-cell lines from untreated MS patients (Crowe *et al*., 2000), suggesting that peptide-induced tolerance through the mechanism of Th1 / Th2 skew has an associated risk of hypersensitivity. Another MBP_{83-99} APL (CGP77116) with substitutions at positions 1, 2, 7 and 8 was tested in MS patients. Four in 8 patients developed enhanced lesions in brains and 2 patients had enhanced frequencies of MBP-reactive T cells in peripheral blood and CSF after CGP77116 treatment (Bielekova *et al*., 2000). Recently a long-term clinical trial with MBP_{82-98} has reported that administration of high dose (500 mg) soluble MBP_{82-98} can delay disease progression in patients with progressive MS and with HLA-DR2 and / or DR4 (Warren *et al*., 2006).

Clinically, immunomodulators (such as interferon-β, glatiramer acetate, mitoxantrone and azathioprine) are major therapeutic drug for easing the symptoms and slowing the progression of MS (Stuart, 2007). However, the systemic treatment of immunomodulaor can affect different cells globally and usually cause side effects in patients. In addition, MS patients carry myelin-reactive memory T cells that may result in disease relapse. Therefore, a specific strategy for controlling myelin-reactive T cells, especial memory T cells, is of importance in MS therapy.

1.4 Hypothesis

The central hypothesis of this thesis is that peptide induced tolerance can be used to control an autoaggressive memory T cell population. However, the effect of peptide on memory T cells may differ from the effect of naive T cells.

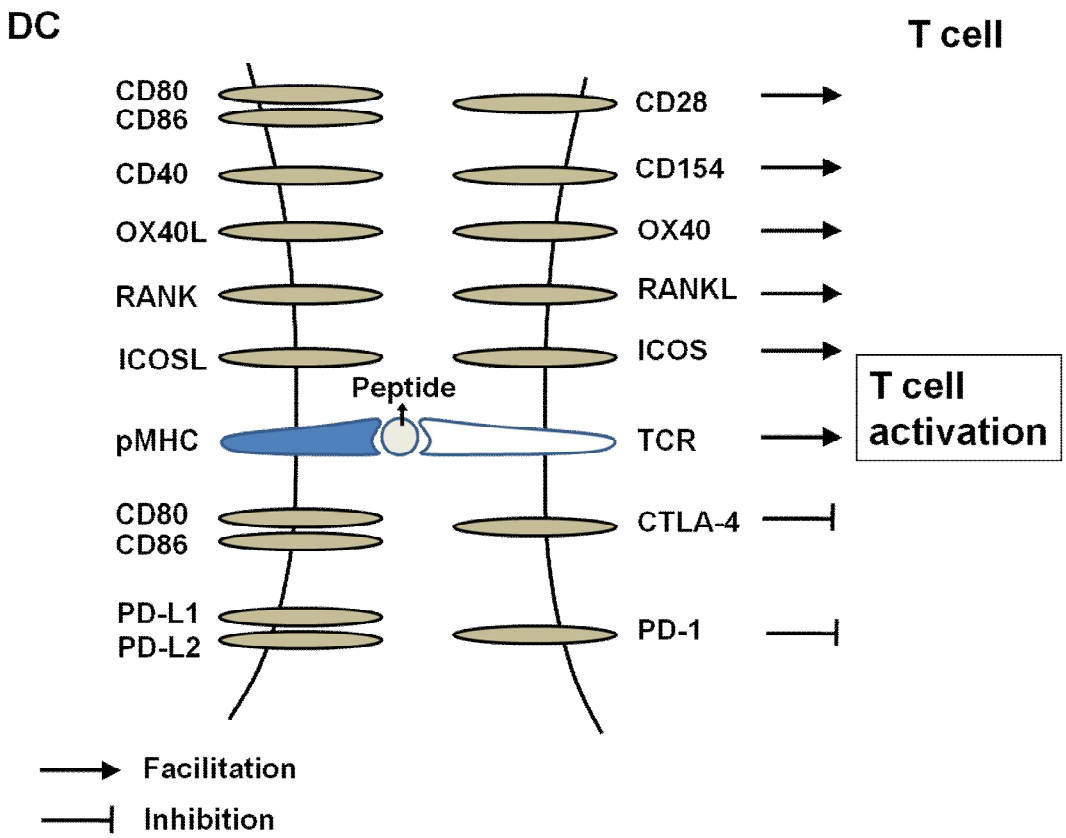


Figure 1.1 Co-stimulatory molecules on T cells and DC. Signal 1 delivered by the ligation of TCR and pMHC alone can induce T cell tolerance. With the ligation and signalling through co-stimulatory molecules, such as CD28, CD154, OX40, RANK and ICOS, signal 1 and co-stimulatory signals (signal 2) can induce T cell activation. Co-inhibitory molecules including CTLA-4 and PD-1 can deliver negative signals that attenuate the activation signals.

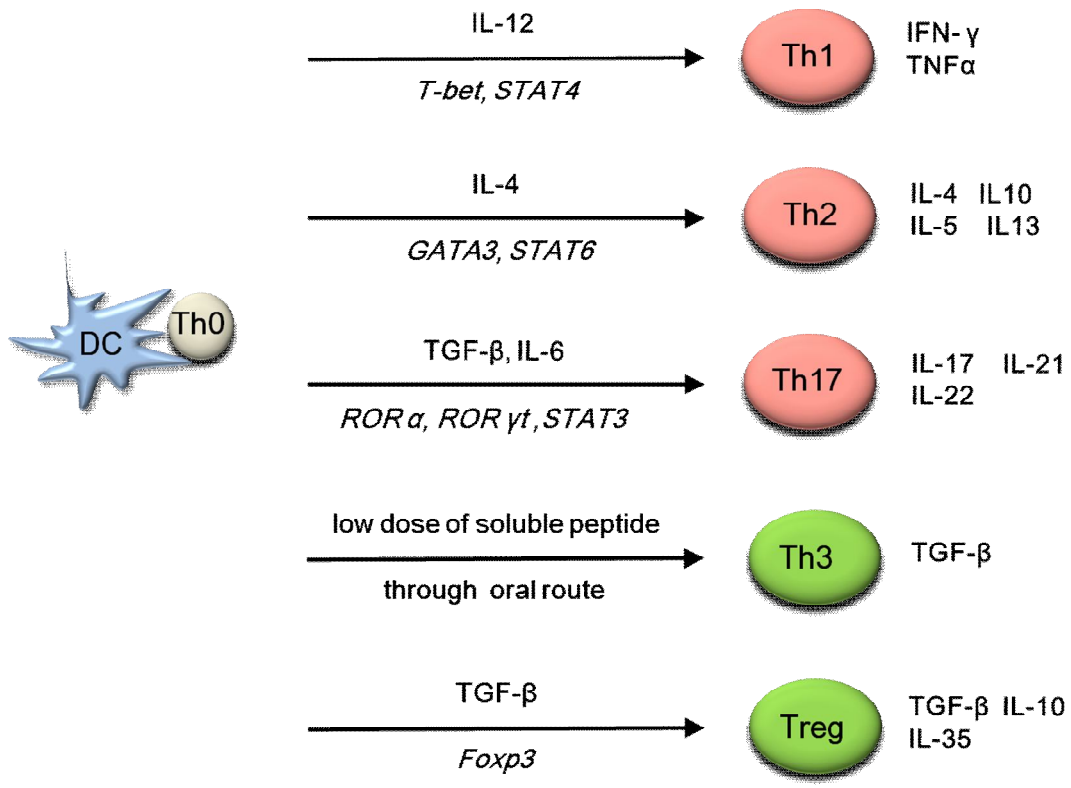


Figure 1.2 T cell polarization. Naive $CD4^+$ T cells can be polarized to different effector subsets of $CD4^+$ T cells. Th1 cells are driven by IL-12 and transcription factors T-bet and STAT4, triggering production of IFN-γ and TNFα. Th2 cells are driven by IL-4 and transcription factors GATA3 and STAT6, triggering production of IL-4, IL-5, IL-10 and IL-13. Th17 cells are driven by TGF-β, IL-6 and transcription factors RORα, RORγt and STAT3, triggering production of IL-17, IL-21 and IL-22. Th3 cells were identified during the induction of oral tolerance in which an administration of low dose peptide can induce the generation of TGF-β secreting Th3 cells. Treg cells are driven by TGF-β and transcription factor Foxp3 and triggering cytokine production of TGF-β, IL-10 and IL-35.

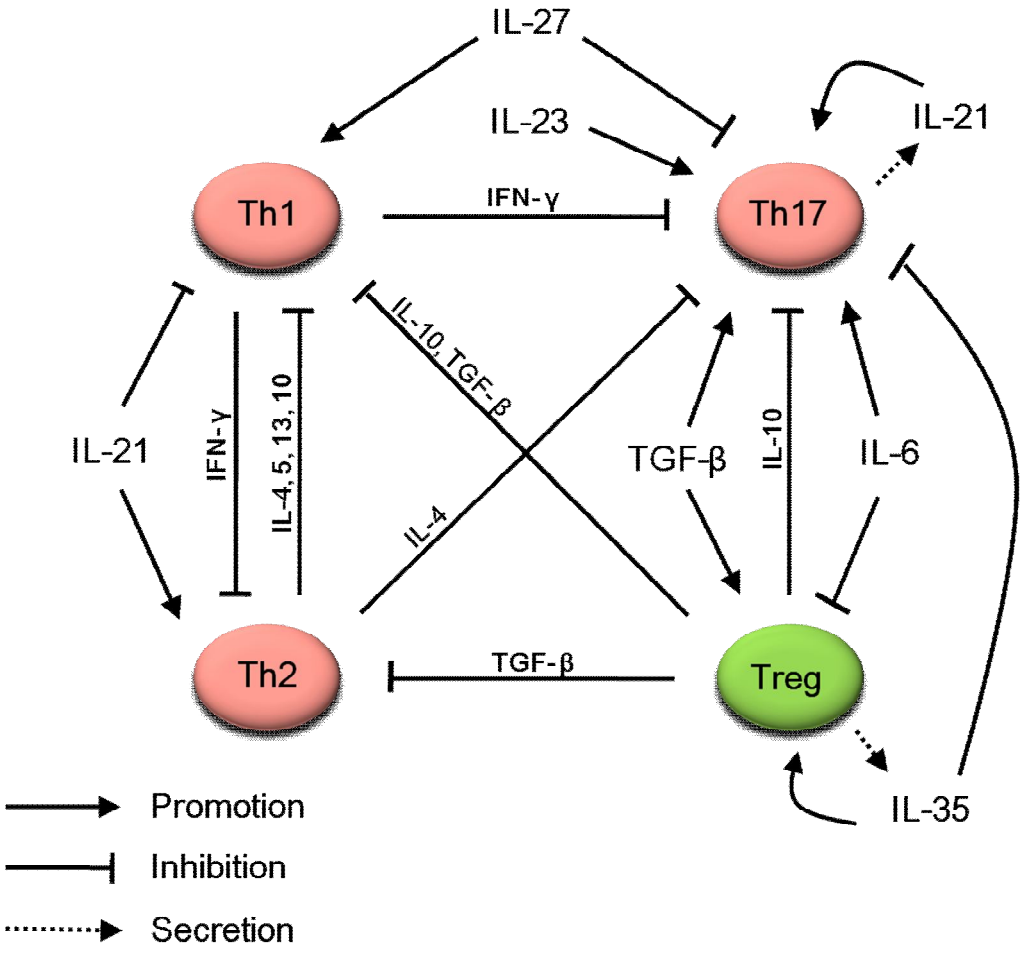


Figure 1.3 Subsets of CD4$^+$ T cells can interact with each other. IFN-γ secreted by Th1 cells can inhibit Th2 and Th17 cells. Reciprocally, IL-4, 5, 10, 13 secreted by Th2 cells can inhibit Th1 and Th17 cells. TGF-β and IL-10 secreted by Treg cells can inhibit Th1, Th2 and Th17 cells. Cytokines shown in the picture represent promoters or inhibitors for polarization of particular subsets of CD4$^+$ T cell.

Table 1.1 Adoptive transfer systems of EAE

	B6/2D2/pMOG	**B10.PL/Tg4/MBP**
Immunodominant peptide	MOG35-55 (pMOG)	MBP Ac1-9 (Ac1-9)
Transgenic mice	2D2	Tg4
MHC molecule	I-A^b	I-A^u
Tracking marker	Thy1.1	Ly5.1
Recipient mice	C57BL/6	B10.PL
TCR usage	TCRVα3.2 TCRVβ11	TCRVα4 TCRVβ8.2

Chapter 2. Materials and Methods

2.1 Mice

TCR transgenic Thy1.1^{+} or Ly5.1^{+} 2D2 mice on the C57BL/6 (H-2^{b}) background, Ly5.1^{+} Tg4 mice on the B10.PL (H-2^{u}) background, as well as C57BL/6 and B10.PL mice were bred in the Institute of Immunology and Infection Research, University of Edinburgh under specific pathogen-free conditions. All mice were sex-matched within experiments and used at 6-10 weeks of age. (PIL No 60/10170 approved by the Home Office)

2.2 Reagents

2.2.1 Antigens and adjuvants

MOG_{35-35} (pMOG, MEVGWYRSPFSRVVHLYRNGK), wild type Ac1-9 (Ac-ASQKRPSQR) and the APL Ac1-9_{4Y} and Ac1-9_{4V} were synthesized by the Advanced Biotechnology Centre, Imperial College (London, UK). Complete Freund¡s adjuvant (CFA) containing heat-killed *Mycobacterium tuberculosis* H37Ra (1mg/ml), incomplete Freund¡s Adjuvant (IFA), *Staphylococcus aureus* peptidoglycan (PGN) and *Escherichia coli* lipopolysaccharide (LPS) were purchased from Sigma-Aldrich Company Ltd (Poole, UK). CpG oligodeoxynucleotides (ODN) 5¡-TCCATGACGTTCCTGACGTT-3¡ was synthesized with a phosphorothioated backbone by MWG-biotech AG (Germany). Pertussis toxin (PTX, Speywood Pharmaceuticals, Maidenhead, UK) for induction of EAE was stored in glycerol before diluting in PBS (PBS : glycerol = 1:1) for further administration.

2.2.2 Antibodies and dyes

Different cell types were identified using biotin- or fluorochrome- (including Fluorescein isothiocyanate, FITC; Phycoerythrin, PE; PerCP-Cy5.5, PerCP; Allophycocyanin, APC; Alexa Fluor 700, AF700) conjugated antibodies, specific for Thy1.1 (clone H1S5), Ly5.1 (clone A20), CD4 (clone RM4-5 or GK1.5), CD25 (clone PC61), CD44 (clone IM7), CD62L (clone MEL-14), CD69 (clone H1.2F3), Fas (clone 15A7), FasL (clone MFL3) and Foxp3 (FJK-16S), TCR Vα3.2 (clone RR3-16), TCR Vβ11 (clone CTVB11), IL-2 (clone JES6-5H4), IFN-γ (clone XMG1.2), IL-17 (clone eBio17B7) and secondary reagents for biotin detection (FITC-conjugated streptavidin, SA-FITC; PE-conjugated streptavidin, SA-PE; PerCP-conjugated streptavidin, SA-PerCP; APC-conjugated streptavidin, SA-APC) which were purchased from eBioscience (SD, US) and Becton Dickinson (NJ, US) for surface staining (see section 2.5.5 and 2.5.6). For ELISA, coating and detecting antibodies were from Becton Dickinson. Carboxy fluoroscein succinimidyl ester (CFSE, Molecular Probes, US) was use for cell division assays (see section 2.5.7). 7-amino-actinomycin D (7-AAD, BD) and annexin V (Becton Dickinson) were used for apoptotic staining (see section 2.5.8). See table 2.1 for the working concentration of antibodies.

2.2.3 Tissue culture media

Cells were cultured in X-VIVO15 serum free medium (BioWhittaker, Maidenhead, UK) supplemented with 2mM L-glutamine and 50 μM 2-mercaptoethanol (2-ME) unless indicated. RPMI 1640 medium, supplemented with 5 % of fetal calf serum (FCS, Sigma), 2mM L-glutamine and 50 μM 2-ME, 100U / ml penicillin and 100 £g / ml streptomycin (all from Gibco Life Technologies, Paisley, UK) was used in indicated experiments.

2.2.4 General buffers

MACS buffer consisted of Hank¡s buffer (Gibco) supplemented with 5 x 10^{-5} M 2-ME, 100U / ml penicillin and 100 £g / ml streptomycin and 2% FCS. FACS buffer for staining and flow cytometry consisted of phosphate buffered saline (PBS) supplemented with 2 % FCS. PBS used for *in vivo* injection was calcium- and magnesium-free PBS (Gibco). ELISA wash buffer (PBST) consisted of PBS and 0.1 % Tween-20 (Sigma)

2.3 Cell purification

2.3.1 Isolation of naive $CD4^+$ T cells

Peripheral lymph nodes (LN) (in some experiments the draining LN, including inguinal nodes, lumbar nodes and sacral nodes were used) and spleens were collected, disaggregated by grinding tissues on sterile gauzes and re-suspended in MACS buffer. Red blood cells (RBC) were lysed by incubating cells in RBC lysis buffer (Sigma) for 2 minutes at room temperature, followed by washing in MACS buffer 3 times. Cells were re-suspended in MACS buffer, counted and incubated with anti-CD4 antibody-conjugated beads (90 £l MACS buffer and 10 £l beads were added per 10^7 cells) (Miltenyi Biotec, Germany) for 15 minutes at 4^0C. Cells were washed, re-suspended in MACS buffer and run through a MACS LS column (positive selection). The purified cells consistently contained 90-95 % $CD4^+$ T cells.

2.3.2 Sorting of memory and naive $CD4^+$ T cells

LN and spleens were collected from immunized mice, disaggregated and incubated in digest buffer containing 300 £l of 10 mg/ml collagenase (Lorne Ltd, Reading, UK),

100 £l of 10 mg/ml deoxribonuclease (Sigma) and 600ul of RPMI medium (final concentration of collagenase and deoxribonuclease were 3 mg/ml and 1 mg/ml, respectively) and incubated at 37^0C for 30 min. After depleting RBC, cells were washed and re-suspended in MACS buffer, counted and incubated with rat IgG cocktail (20 £l antibody cocktail to 10^7 cells) which includes antibodies against CD8 (clone 53.6.72), B220 (clone RAB832), MAC-1 (M1/70) and class II MHC (M5/114.15.2) (Invitrogen, CA, USA) for 20 minutes at 4^0C. Cell were washed and re-suspended in MACS buffer with anti-rat IgG conjugated Dynabeads (200 ul Dynalbeads to 10^7 cells in total 1 ml buffer) (Invitrogen, CA, USA) for 20 minutes at 4^0C, subsequently $CD4^+$ T cells were collected by negative selection using a Dynal magnetic stand. The purity of $CD4^+$ T cells was normally 90-95 %. The isolated cells were stained with anti-CD4-AF700 (0.125 £g to 10^7 cells), anti-CD25-APC (0.2 £g to 10^7 cells), anti-CD44-PE (0.5 £g to 10^7 cells) and anti-CD62L-FITC (1.25 £g to 10^7 cells) (with 1.25 £g anti-TCR Vα3.2-FITC and 0.25 £g anti-Vβ11-biotin in indicated experiments). Cells were sorted by flow cytometry (BD FACS Aria) into $CD4^+$ $CD25^-$ $CD44^{high}$ $CD62L^{low}$ memory T cells and $CD4^+$ $CD25^-$ $CD44^{high}$ $CD62L^{low}$ naive T cells.

2.3.3 Preparation of bone marrow derived DC (BMDC)

Femurs and tibias were collected from mice, cut and cells were flushed out by injecting PBS from one end. RBC were depleted and cells were seeded into bacteriological petri dishes at 2 x 10^5 / ml in 10 ml RPMI medium supplemented with 10% FCS and 20 £g / ml GM-CSF (Peprotech, London, UK). At day 3, a further 10 ml of medium containing 20 £g / ml GM-CSF was added. At days 6 and 8, 10 ml culture supernatant was removed and replaced with 10 ml fresh culture medium

containing 20 £g / ml GM-CSF. Cells were collected at day 7 for further experiments (*MacDonald et al., 2001*).

2.4 *In vivo* manipulations

2.4.1 Cell transfer

For all experiments in which cells were transferred into mice, the prepared cells were re-suspended in PBS and i.v. injected in a total volume of 200 µl into the tail vein of mice.

2.4.2 EAE induction in the naive setting

For induction of primary disease, C57BL/6 or B10.PL mice were immunized in both hind legs s.c. with a total volume of 100 £g pMOG or Ac1-9 emulsified with CFA. Each leg was injected with 50 £l peptide / CFA. Mice additionally received 200 ng of PTX i.p. on the same day and two days later.

To induce EAE with CpG or PGN, 60 µg of CpG or 250 µg of PGN were emulsified with 100 µg pMOG and IFA (the ratio of volume was CpG or PGN : pMOG : IFA = 1 : 1 : 2), followed by s.c. injection in the hind limbs and PTX injection i.p. on the same day and two days later.

Clinical signs of EAE were assessed daily with a 0-6 scoring range (Grade 0, healthy; Grade 1, flaccid tail; Grade 2, impaired righting reflex and /or abnormal gait; Grade 3, partial hind leg paralysis; Grade 4, total hind leg paralysis; Grade 5, hind leg paralysis with partial front leg paralysis; Grade 6, moribund or dead). Differences in disease burden were analyzed using Mann-Whitney U test.

2.4.3 EAE induction in memory setting

For inducing a memory response, mice were primarily given 50 μg of peptide in 60 μg of CpG or 250 μg of PGN and IFA in the left hind leg. Secondary immunization was given 4 weeks later with 100 μg of peptide / CFA in the right hind leg. Mice received 200 ng of PTX i.p on day 0 and day two of the secondary immunization.

In some experiments, memory responses in EAE were tested by immunizing with 100 μg peptide / CFA and followed by 100 μg peptide in IFA / CpG or IFA / PGN (60 μg of CpG or 250 μg of PGN, the ratio of volume was CpG or PGN : pMOG : IFA = 1 : 1 : 2) 4 weeks later .

In some experiments, memory responses in EAE were tested by administering antigen-loaded DC. BMDC were purified and re-suspended at 2.5 x 10^6 cells / ml in RPMI medium and pulsed with 50 μM pMOG in the presence of 0.1 μg / ml LPS for 2 hours. Cells were washed and re-suspended in PBS and 5 x 10^5 antigen-loaded DC were injected i.v. into recipient mice. PTX was injected i.p. on the same day and two days later.

2.4.4 Tolerance induction in memory experiments

Mice received peptide in PBS (soluble peptide) 7 days before EAE induction through the i.v. (200 μg) or i.p. (500 μg) route. For tolerance induction with i.v. injection, mice received soluble peptide 7 days before secondary immunization. For tolerance induction with i.p. injection, multiple doses of soluble peptide were applied at day -10, -8, -6 before secondary immunization.

2.4.5 Administration of LPS

For inducing T cell immunity by the i.v. route, LPS was used as an adjuvant. Mice received 200 μg pMOG and 30 μg LPS in 200 μl of total volume through i.v. route at day 0.

2.5 *Ex vivo* and *in vitro* assessment

2.5.1 *Ex vivo* recall proliferation assays

Spleen and/or lymph nodes were collected, disaggregated, washed and subsequent lysis the RBS to make single cells suspensions. Cells suspensions were re-suspended in X-VIVO15 medium containing various doses (serial 4-fold dilutions from a top concentration of 50 £M) of peptide and seeded in 96-well plates with concentrations of 8 x 10^5 cells / well spleen cells and 6 x 10^5 cells / well LN cells. After 54 hours of culture, 0.5 μCi / well ^{3}H-thymidine (Amersham Biotech, Buckinghamshire, UK) solution was added. Cells were harvested and ^{3}H-thymidine incorporation was measured at 72 hours by a liquid scintillation β counter (PerkinElmer, MA, US). Results are presented as mean counts per minute (CPM) of triplicate cultures.

2.5.2 Primary i*n vitro* stimulation of TCR transgenic T cells

Cells from the lymph node and spleen of TCR transgenic mice were collected and cultured in RPMI medium containing 10 £M of relevant peptide for 24 hours. Cells were washed with medium twice and seeded in fresh RPMI medium, without peptide, supplemented with 20U / ml IL-2. Activated blastocytes were harvested 48 hours later by ficoll (NycoPrep, AXIS-SHIELD, Oslo, Norway) gradient separation. Cells were suspended in 30 ml RPMI medium and layered over 8 ml of ficoll. Cells were centrifuged at 350 x *g* without brake for 15 minutes and the cells located at the

interface were collected and washed 2 times with a large amount of medium. Cells were re-suspended in PBS prior to adoptive transfer.

2.5.3 *Ex vivo* cytokine production assay

For cytokine production, single cell suspensions were prepared and cultured in X-VIVO15 medium as in the proliferation assay (section 2.5.1). Cells were cultured for the time indicated and 100 µl aliquots of supernatant were removed for cytokine ELISA assay. Microtitre plates (NUNC, Roskilde, Denmark) were coated overnight at 4^0C with 50 µl per well of 2 £g cytokine specific capture antibodies (anti-IL-2, clone JES-1A12; anti-IL4, clone 11B11; anti-IL-10, clone JES5-2A5; anti-IFN-γ, clone R4-6A2; anti-IL-17, clone TC11-18H10, all from BD Pharmingen) prepared in bicarbonate coating buffer (0.05 M, pH 9.6). After washing twice in PBST buffer, 200 µl PBS / BSA (1%) was added to each well and plates were incubated at 37^0C for an hour. After 6 washes with PBST, cytokine standards (BD Pharmingen) diluted in PBS / BSA were added at 100 µl per well in duplicate and two-fold dilutions (starting at 5 ng / ml for IL-2, 5 ng / ml for IL-4, 100 ng / ml for IL-10, 100 ng / ml for IFN-γ and 10 ng / ml for IL-17) performed to give a standard curve for each plate. Samples from each culture were added at 100 µl per well in duplicate and plates were incubated at room temperature in a humidified atmosphere for 2 hours. Plates were washed 6 times with PBST and 100 µl diluted (0.5 µg / ml) biotinylated anti-cytokine detecting antibody (anti-IL-2, clone E56-5H4; anti-IL4, clone BVD6-24G2; anti-IL-10, clone SXC-1; anti-IFN-γ, clone XMG1.2; anti-IL-17, clone TC11-8H4.1, all from BD Pharmingen) was added and incubated for 1 hour at room temperature. Plates were washed 6 times with PBST and 100 µl per well of 2µg / ml extravidin peroxidase (Sigma) in PBS / BSA were added to each well and incubated

for 30 minutes at room temperature. Plates were washed 6 times with PBST and 100 µl per well of tetramethylbenzidine (TMB, Sigma) substrate buffer (100 µl of 10 mg / ml TMB in DMSO to 9.9 ml of phosphate citrate buffer pH5 and 3µl of hydrogen peroxidase) was added followed by 100 µl of 2M H_2SO_4 to stop the reaction. Optical absorbance values were read at 450 nm with a Multiskan plate reader (Labsystems, Basingstoke, UK).

2.5.4 ELISA for serum antibody measurement

Microtitre plates were coated with 50 µl / well of pMOG (4 µg / ml) in 0.1M bicarbonate buffer and incubated at 4^0C overnight. Plates were washed and blocked with 200 µl / well of PBS / BSA (1%) for an hour at 37^0C. After washing, 50µl / well of serially diluted serum samples (starting at 1 in 5 and then 2-fold dilutions) were added in duplicate and the plates were incubated for one hour at 37^0C. Plates were washed with PBST and 50 µl / well of alkaline phosphatase-conjugated anti-mouse Ig secondary antibody (2 µg / ml) were added. Plates were incubated at room temperature for one hour. Plates were washed 5 times with PBST and bound secondary antibody was detected with substrate buffer (para-nitrophenyl phosphate, 5 mg pNPP in 5 ml of substrate buffer containing 50 mM $MgCl_2$ and 9.7 % of diethanolamine solution). Plates were read at 405 nm with a Multiskan plate reader.

2.5.5 Cell surface markers analysis by flow cytometer

Single cell suspensions were obtained as described in section 2.3.1. Cells were re-suspended in FACS buffer containing anti-Fc receptor antibodies (clone 2.4G2) for 15 minutes on ice. 10^6 cells were washed and stained with 50 µl of antibodies diluted in FACS buffer and incubated at 4^0C for 15 minutes. After washing, cells

were re-suspended in cold paraformaldehyde (1 % paraformaldehyde in PBS) and incubated for at least 20 minutes at 4^0C. All samples were collected on BD FACS Calibur or BD LSR II flow cytometer and analysed with FlowJo (Treestar, US).

In some experiments, biotinylated antibodies were applied. 10^6 cells were stained with 50 µl of biotinylated antibodies and incubated at 4^0C for 15 minutes. Cells were washed and stain with fluorescently conjugated streptavidin. After incubation on ice for 15 min, cells were washed with FACS buffer and fixed with cold paraformaldehyde solution as described above for flow cytometer analysis.

2.5.6 Intracellular staining

Cells were prepared as described in section 2.3.1 and incubated in 24 well plates at 2.5 x 10^6 cells / well with 1 ml RPMI medium with 10 £M pMOG for 12 hours. One µl of Brefeldin A (Becton Dickinson) was added into each well and cells were washed and harvested 4 hours later. Cells were stained with surface markers as described in 2.5.5, washed once with PBS and twice with permeabilizing buffer (0. 1 % saponin and 1% BSA in PBS) and stained with anti-cytokine antibodies (0.2 µg to 2.5 x 10^6 cells in 100 µl permeabilizing buffer) for 30 minutes at 4^0C. Cells were washed once with permeabilizing buffer and once with FACS buffer and re-suspended in FACS buffer for analysis and collection by flow cytometry.

For Foxp3 staining, cells were prepared and stained with antibodies against surface markers as described in section 2.5.5. 2 x 10^6 cells were re-suspended in 500 µl Fix / Perm solution (eBioscience) and incubated at 4^0C overnight. Cells were washed once with PBS and once with permeabilization buffer (eBioscience). Cells were

re-suspended in 80 ul of eBioscience permeabilization buffer supplemented with 2 % normal rat serum and left at room temperature for 15 minutes. Cells were split into two 2 tubes (50 ul each) and 50 µl of eBioscience permeabilization buffer containing 0.2 £g anti-Foxp3 antibodies were added to one tube and 0.2 £g isotype (IgG2a) antibodies were added to the other tube. Cells were incubated at 4^0C for 20 minutes followed by washing and re-suspension in FACS buffer for analysis and collection by flow cytometry.

2.5.7 CFSE labelling

Single cell suspensions were collected and prepared as described in section 2.3.1. Cells were washed twice in large volumes of serum free RPMI medium and re-suspended in serum free RPMI medium at a concentration of 10^7 cells / ml. 1 £M CFSE was added and incubated at 37℃ in a water bath for 8 min. Excess CFSE was quenched with equal volume of FCS to stop the reaction. Cells were washed twice in RPMI medium and re-suspended in PBS prior to adoptive transfer.

2.5.8 Apoptotic staining

A single cell suspension was prepared and stained with antibodies to surface markers as described in section 2.5.5. Cells were washed with cold PBS and re-suspended in annexin-binding buffer (10 mM HEPES, 140 mM NaCl, and 2.5 mM $CaCl_2$, pH 7.4) with annexin V (1:20 in annexin-binding buffer). Cells were incubated in the dark at room temperature for 15 minutes and washed twice with annexin-binding buffer. Immediately before collection, 300 £l annexin-binding buffer and 0.25 £g of propidium iodide (PI) or 4 £l of 7-AAD were added and re-suspended in FACS

buffer for flow cytometry.

Table 2.1 Antibodies and reagents used for staining

Target	**Clone**	**Fluorescent / biotin conjugation**	**Concentration used**
Thy1.1	H1S5	FITC	2.5 £g /ml
Ly5.1	A20	FITC	2.5 £g /ml
CD62L	MEL-14	FITC	2.5 £g /ml
IFN-γ	XMG1.2	FITC	5 £g /ml
Biotin		SA-FITC	2.5 £g /ml
CD44	IM7	PE	1 £g /ml
IL-2	JES6-5H4	PE	2 £g /ml
IL-17	eBio17B7	PE	2 £g /ml
Biotin		SA-PE	1 £g /ml
CD4	RM4-5	PerCP	1 £g /ml
Biotin		SA-PerCP	1 £g /ml
CD4	GK1.5	APC	1 £g /ml
CD25	PC61	APC	1 £g /ml
Foxp3	FJK-16S	APC	1 £g /ml
Biotin		SA-APC	1 £g /ml
CD4	RM4-5	AF700	0.3 £g /ml
CD69	H1.2F3	Biotin	2.5 £g /ml
Fas	15A7	Biotin	2.5 £g /ml
FasL	MFL3	Biotin	2.5 £g /ml
TCR Vα3.2	RR3-16	Biotin	2.5 £g /ml
TCR Vβ11	CTVB11	Biotin	2.5 £g /ml

Abbreviation : Fluorescein isothiocyanate, FITC; Phycoerythrin, PE; PerCP-Cy5.5, PerCP; Allophycocyanin, APC; Alexa Fluor 700, AF700; streptavidin, SA.

Chapter 3. Characteristics of T cell tolerance induced in MBP-reactive versus pMOG-reactive naive T cells

3.1 Introduction

Peptide antigens have been used as an effective means of inducing T cell tolerance. Mice injected with the soluble peptide in PBS prior to antigen / CFA priming can be protected from EAE induced by the subsequent immunization of peptide / CFA. (Anderton, 2001). The basic protocol of tolerance is to inject mice with soluble peptide before immunization with antigen in CFA 7 days later. Liu and colleagues have suggested that peptide induced T cell tolerance needs at least 4 days to establish (Liu, 1995). It is established that activated $CD4^+$ T cells are highly sensitive to deletion by AICD and high dose of antigen can lead to cell death of antigen-reactive T cells *in vivo* (Liblau *et al*., 1996; Critchfield *et al*., 1994). In addition, APL with increased MHC-binding properties are highly effective at inducing T cell tolerance after systemic administration in soluble form, thereby preventing EAE, whereas peptides with low MHC avidity are less capable at inducing T cell tolerance (Anderton, 2001; McCue *et al*., 2004). In the MBP Ac1-9 / Tg4 / B10.PL system, administration of the superagonist Ac1-9_{4Y} in CFA failed to trigger EAE and 70% of Ac1-9-reactive T cells showed annexin V positive when injected the Tg4 cells transferred mice with soluble Ac1-9_{4Y} (compared with 8 % of annexin V positive cells in soluble Ac1-9 injected mice (Anderton *et al*., 2001). Therefore, the first hypothesis of this chapter is that the administration of the superagonist Ac1-9_{4Y} in soluble form may induce T tolerance by cell deletion due to its ability to deliver a strong TCR signaling. To this hypothesis, mice were transferred with Tg4 T cells and next injected with either soluble Ac1-9_{4Y} or PBS, subsequently immunized with

Ac1-9 / CFA. T cell tolerance was assessed by cell proliferation and Tg4 cell number.

In pMOG / 2D2 / C57BL/6 system, administration of soluble pMOG prior to EAE induction can reduce T cell proliferation and EAE (Fazilleau *et al*., 2006; Hochweller *et al*., 2006). Many studies of peptide induced tolerance on EAE focus on the effect at least 10 days after peptide / CFA priming (Gaur *et al*., 1992; Meyer *et al*., 1996; Smith and Miller, 2006; Galazka *et al*., 2007). However, less data are available on the effect made by soluble myelin before priming or after a short time of priming (i.e. within 10 days). Administration with antigen in lipopolysaccharide (LPS) can markedly increase antigen loading onto MHC class II and surface display of pMHC (Turnbull *et al*., 2005; Netea *et al*., 2002; Reis e Sausa, 2004). In addition, injection with peptide and LPS is a systemic and immediate stimulus that can induce strong but transient responses (Khoruts *et al*., 1998; Maxwell *et al*., 2002). Unlike LPS, CFA contains heat killed *Mycobaterium tuberculosis* and mineral oil, the mixture of peptide / CFA is a slow-releasing, water-in-oil emulsion of immunogens that can trigger strong and long-lasting responses. Since pMOG is not a superagonist for pMOG-reactive T cells, the second hypothesis of this chapter is that administration of soluble pMOG may induce tolerance by triggering T cell anergy, rather than T cell deletion. To approach this, mice were injected with 2D2 T cells, soluble pMOG and pMOG in LPS in turn to investigate the effect of soluble pMOG upon 2D2 T cells before and after priming (particularly at early time points after priming). The transferred 2D2 cells were harvested before and after pMOG / LPS priming for further analysis.

3.2 Results

3.2.1 Administration of soluble Ac1-9_{4Y} prior to EAE induction can induce T cell unresponsiveness and ameliorate EAE

To test the ability of Ac1-9_{4Y} to induce tolerance, B10.PL mice were injected with 200 £g of soluble Ac1-9_{4Y} or PBS i.v. at day -7, immunized with 100 £g of Ac1-9 / CFA and PTX for developing EAE at day 0 and day 2 and subsequently sacrificed for assays at day 27 (Figure 3.1A). Ac1-9_{4Y} treated mice had a delayed onset (day 10) and a reduced disease incidence, compared to control mice which developed EAE from day 7 post immunization (Figure 3.1B). Two in 6 mice developed EAE in Ac1-9_{4Y} treated mice, whereas the incidence was 4 in 6 in control mice. In addition, Ac1-9_{4Y} treated mice had a significantly ($p < 0.001$) reduced severity and an early recovery course, compared with the control mice. Spleen cells harvested at day 27 from Ac1-9_{4Y} treated mice were unresponsive in the *ex-vivo* proliferation assay, whereas those from control mice gave a dose-dependent response to Ac1-9 (Figure 3.1C). Cytokine ELISA showed that cells produced low amounts of IFN-γ in both groups (Figure 3.1D), whereas there was a trend of reduced IL-17 production in cells after Ac1-9_{4Y} treatment, compared to the cells from the control mice (Figure 3.1E).

To assess peptide induced T cell tolerance in the Ac1-9 / Tg4 / B10.PL system, B10.PL mice received 3 x 10^5 $CD4^+$ $Ly5.1^+$ (Tg4) T cells at day -8 and were injected with 200 £g of soluble Ac1-9_{4Y} or PBS i.v. at day -7 and immunized as above. If we estimate that there are 10^8 CD4 cells in secondary lymphoid tissues in a mouse, 3 x 10^5 of Tg4 cells is still a large number (3 ?) compared to the endogenous Ac1-9-reactive $CD4^+$ T cells (1 / 10^5). The populations of $Ly5.1^+$ $CD4^+$ Tg4 cells harvested from different groups could easily be assessed after a transfer of 3 x 10^5

Tg4 cells. Draining lymph nodes (LN) and spleens were harvested at day 10 post immunization (Figure 3.2A). The frequency of $Ly5.1^+$ $CD4^+$ Tg4 T cells was assessed and there was no significant difference between Ac1-9_{4Y} treated and control mice, in both LN and spleen (Figure 3.2B). In the *ex-vivo* recall response, spleen cells from the Ac1-9_{4Y} treated mice had a significantly reduced proliferative response compared to the control mice (Figure 3.3A, $p < 0.05$). The same pattern was observed in the LN cells, indicating that the administration of soluble Ac1-9_{4Y} induces T cell tolerance (Figure 3.3B). The outcome of reduced proliferation could be caused by A) the reduced number of transferred Tg4 cells or B) the reduced ability for *in vitro* expansion of Tg4 cells. Data of Tg4 cell frequency showed that the reduced proliferation of Ac1-9_{4Y} treated mice did not result from reduced cell number of Ac1-9 reactive T cells (Figure 3.2B). Tg4 cells harvested from Ac1-9_{4Y} treated mice were relatively less capable of responding to Ac1-9, compared with cells from control mice. Thus, administration of soluble Ac1-9_{4Y} does not induce cell deletion but causes unresponsiveness in Tg4 T cells.

3.2.2 Administration of soluble pMOG prior to EAE induction can prevent disease

To estimate peptide induced tolerance in the MOG / 2D2 / C57BL/6 system, C57BL/6 mice were injected with 200 £g of pMOG or PBS i.v. at day -7 and immunized with 100 £g of pMOG / CFA and PTX at day 0 and day 2 (Figure 3.4A). In the experiment shown in figure 3.4B, pMOG-treated mice had a reduced incidence of 20% (1 in 5), whereas control mice had an EAE incidence of 71% (5 in 7). The control mice developed clinical signs from day 11 and reached the peak score at day 15 before entering a recovery phase by day 20. One mouse in the pMOG-treated

group developed disease on day 22 and maintained score 1 to 3 thereafter. After day 22, the mean score of both pMOG-treated and control mice remained below 1. These results confirm previous reports that administration of soluble pMOG can prevent EAE (Fazillean *et al*., 2006; Hochweller *et al*., 2006).

3.2.3 Administration of soluble pMOG prior to immunization triggers T cell deletion

To assess how the administration of soluble pMOG achieves tolerance, 2 x 10^6 of Thy1.1$^+$ CD4$^+$ 2D2 T cells were transferred into C57BL/6 mice 1 day prior to pMOG injection. Mice were injected with 200 £g of soluble pMOG or PBS at day -7 and immunized as above (Figure 3.5A). Ten days after immunization, there were 0.68% of 2D2 cells among CD4$^+$ T cells in the LN of pMOG-treated mice, whereas control mice had 1.7% (Figure 3.5B, $p < 0.05$). The same pattern was observed in the spleen cells. pMOG treated mice showed a two-fold reduction in 2D2 cell survival compared to control mice, indicating that the administration of soluble pMOG results in a reduced number of pMOG-reactive T cells in the secondary lymphoid organs by day 10 post immunization. Cells harvested at day 10 from pMOG treated mice showed reduced proliferation over the dose range in spleen and LN (Figure 3.6A and B), compared with PBS treated group, suggesting that soluble pMOG treatment prior to priming induced T cell deletion. The reduced proliferation of cells from pMOG-treated mice may reflect to the reduced frequency of 2D2 cells in the secondary lymphoid organs, rather than reflecting an inability to proliferate in response to pMOG. This demonstrates that administration of soluble pMOG induces T cell tolerance by depleting the pMOG-reactive T cells.

3.2.4 Administration of pMOG induces transient activation in pMOG-reactive T cells

So far, T cell tolerance has been demonstrated in mice which were immunized following peptide treatment. To assess how soluble pMOG acts on pMOG-reactive T cells at earlier time points before priming, 2 x 10^6 CFSE labeled 2D2 $CD4^+$ $Thy1.1^+$ T cells were transferred into C57BL/6 mice. One day later, mice were injected with 200 £g of pMOG and spleens were subsequently collected for analysis (Figure 3.7A). Spleen cells were harvested, stained and analyzed by flow cytometry. $CD4^+$ $Thy1.1^+$ 2D2 cells were gated and plotted by CFSE dilution for cell division or by levels of 7-AAD / Annexin V for apoptosis (Figure 3.7B). The frequency of transferred 2D2 cells was assessed by staining at the day indicated (Figure 3.8A). The frequency of 2D2 cells from pMOG-treated mice was enhanced at day 2, peaked at day 3 and subsequently contracted by day 4 (Figure 3.8A), whereas the frequency of 2D2 cells in control mice continuously decreased from day 1 and was sustained at a lower frequency than cells in pMOG-treated mice. To assess whether the administration of pMOG triggered mitosis *in vivo*, loss of CFSE fluorescence was assessed in the transferred 2D2 T cells. Cells in the PBS treated mice remained undivided throughout the time course (Figure 3.8B). In the pMOG-treated mice, very few 2D2 cells had divided at day 1 and this was followed by a burst of division from day 2. In the pMOG-treated mice, 72%, 89%, 70% and 42% of 2D2 cells had divided in the spleens harvested at day 2, 3, 4 and 7, respectively (Figure 3.8B). 2D2 T cells expanded within 3 days and disappeared from the spleen afterwards, indicating that T cell division upon soluble pMOG stimulation is not maintained, suggesting that cell expansion under soluble pMOG stimulation occurred transiently within 3 days. 2D2 cells from pMOG-treated mice also showed an enhanced ability to proliferate *in vitro*

in recall assays to pMOG at day 1, 2 and 3 after pMOG injection, whereas cells from control mice only proliferated at high pMOG concentrations *in vitro* (Figure 3.9). The enhanced frequency of 2D2 cells enhanced $CFSE^{+}$ cells and enhanced proliferation seen at day 3 indicated the initial activation of cells in pMOG treated mice, whereas the later decline in frequency suggested that those activated 2D2 cells may have undergone cell death, or migrated out of the spleen. Further experiments for assessing apoptosis and migration are needed.

CD69 is the earliest inducible surface marker on T cells and is a functional co-stimulatory molecule acquired during T cell activation (Sancho *et al.*, 2005). As Figure 3.10 shows there was a high expression of CD69 in 2D2 cells from pMOG-treated mice at day 1. This expression of CD69 in pMOG-treated mice dropped to basal level comparable to that found in 2D2 cells from PBS-treated mice by day 3. Another surface activation marker, CD44, a glycoprotein involved in cell adhesion and migration was analyzed (Ponta *et al.*, 2003). The expression of CD44 was slightly up-regulated at day 1 and this up-regulation was further increased at day 2 and day 3 and dropped to basal level thereafter. There were also changes in the expression of CD62L, an adhesion and homing marker which plays an important role in cell interaction between leukocytes and endothelial cells (Bevilacqua *et al.*, 1991). Naive T cells tend to express high-levels of CD62L, whereas activated and effector memory T cells express low-levels of CD62L. CD62L expression fluctuated over the time course studied from $CD62L^{high}$ to $CD62L^{low}$ expression. On day 1, a heterogeneous population was measured in cells expressing $CD62L^{high}$ or $CD62L^{low}$. The $CD62L^{low}$ population in pMOG treated group was increased at day 1, 3 and 4, compared with the PBS treated group. At day 7, the majority of cells in both groups

expressed CD62L^{high} (Figure 3.10). These data suggested that 2D2 cells from soluble pMOG injected mice acted like activated T cells up to day 4 in terms of the expression of surface markers. However, this pattern of CD69^{high} CD44^{high} CD62L^{low} can not be sustained and cells can revert to a CD69^{low} CD44^{low} CD62L^{high} phenotype at later time point, suggesting that the cells cannot develop to memory T cells following soluble pMOG administration.

To measure apoptosis of 2D2 cells, spleen cells were stained with annexin V and 7-amino-actinomycin D (7-AAD) and analyzed by flow cytometry. CD4^{+} Thy1.1^{+} Annexin V^{+} 7-AAD^{-} cells were gated as apoptotic cells (Figure 3.12A). Results in figure 3.11B show a reduction of apoptotic 2D2 cells in pMOG-treated mice on day 2, a trend that was also seen on day 3 compared to the PBS-treated mice, whereas no apparent difference was observed between two groups at other indicated time points (Figure 3.11B). On the other hand, the frequency of apoptotic host cells was constantly between 2% to 5% and there was no difference in the frequency of CD4^{+} Thy1.1^{-} (host) apoptotic cells in both mice (Figure 3.11C). As the frequency of apoptotic cells was low (ranging from 1 % to 7.5 %) in both pMOG treated and PBS treated mice, administration of pMOG may not change the survival rate of 2D2 T cells in the spleen. Collectively, these data suggested that most of the 2D2 cells sampled from pMOG treated mice at day 7 are non-activated cells (based on the patterns of CD69^{low} CD44^{low} CD62L^{high} in figure 3.10) and those activated 2D2 cells might have left the spleen.

3.2.5 Administration of pMOG induces T cell unresponsiveness to further stimulation

In mice primed with peptide / CFA, only local T cells in the draining lymph nodes can be activated immediately. To assess the early effects of soluble peptide after priming, it requires an adjuvant that can facilitate a systemic and immediate T cell activation. Thus, LPS was substituted for CFA as an adjuvant in this experiment. C57BL/6 mice received 2 x 10^6 $CD4^+$ $Thy1.1^+$ (2D2) T cells on day -8 were injected with 200 £g of soluble pMOG or PBS i.v. on day -7 and subsequently were primed with 100 £g of pMOG and 30 £g of LPS on day 0 (Figure 3.12A). Spleen cells were harvested, stained and analyzed by flow cytometry at various time points from day 1 to day 10. $CD4^+$ $Thy1.1^+$ cells were gated as transferred 2D2 cells and plotted by levels of 7-AAD / Annexin V for apoptosis (Figure 3.12B). Notably, the frequency of 2D2 cells in the mice which received soluble pMOG was lower than the frequency of 2D2 cells in the PBS-treated mice on day 0 (Figure 3.12C). It remains possible that a certain proportion of 2D2 cells in pMOG treated mice might have migrated out of the spleen.

In the mice first treated with PBS, the frequency of transferred 2D2 cells increased dramatically at day 3, a phenomenon which was not seen in mice first given soluble pMOG. Mice which received pMOG prior to priming were unresponsive at all time points. Mice which received PBS prior to priming were able to respond and proliferate in response to *in vitro* restimulation with pMOG. This capacity of proliferation peaked at day 2 and subsequently decreased over time (Figure 3.13A). At day 10, cells from both groups were unresponsive to pMOG in *ex-vivo* culture. It suggested that the administration of pMOG induces T cell unresponsiveness against further priming. Consistent with this, ELISA data showed that very few IL-2 was produced from the cells of pMOG-treated mice after priming at every time-point,

whereas cells from PBS-treated mice produced high levels of IL-2 at day 2 and day 3 (Figure 3.14A). However, there was no IFN-γ produced by cells from either group except for low-level IFN-γ detected at day 2 in PBS treated mice (Figure 3.14B). 2D2 cells from spleen cells were stained for 7-AAD / Annexin V to represent apoptotic cells (Figure 3-15A). As low frequencies of 2D2 cells appeared after pMOG / LPS priming (Figure 3.12C), levels of apoptotic cells seemed relatively high. Increased frequencies of apoptotic 2D2 cells appeared at day 1 to day 3 in both groups, compared with day 0 and no significant difference was observed between pMOG-treated and the control mice (Figure 3.15B). Host cells had a low frequency (5-15 %) of apoptotic cells that showed no difference between groups (Figure 3.15C).

There were no apparent differences in the expression of CD69 between both groups after pMOG / LPS priming, whereas pMOG-treated mice had more $CD62L^{low}$ 2D2 cells at day 0 and day 1, suggesting that some of the 2D2 T cells in soluble pMOG treated mice may be activated with a faster kinetics than the control group (Figure 3.16. Interestingly, staining with antibodies against Fas and FasL suggested that 2D2 cells from pMOG-treated mice had a trend of enhanced expression of Fas and FasL at day 1 after pMOG / LPS administration, compared with the control mice. By day 2, 2D2 T cells in both mice expressed Fas. The expression level of Fas / FasL in 2D2 cells from pMOG-treated mice seemed to decline at day 2, compared with day 1 (Figure 3.16) Collectively, these results suggest that the administration of soluble pMOG before priming can inhibit cell proliferation and IL-2 production and might also induce an early expression of Fas / FasL.

3.3 Discussion

The degree of antigen aggregation, the presence of adjuvant and the route of antigen administration determine whether T cells become fully activated or tolerant (Kearney *et al*., 1994). In this chapter, administration of peptide in soluble form through the i.v. route can reduce T cell proliferation induced by subsequent immunization and prevent EAE in mice. In the Ac1-9 / Tg4 system, the occurrence of AICD has been reported when Tg4 T cells were cultured with APC plus Ac1-9_{4Y} (Ryan *et al*., 2005). Data in this chapter showed that administration of soluble Ac1-9_{4Y} did not trigger a cell loss of Tg4 T cells in Ac1-9_{4Y} treated mice but led to T cell unresponsiveness after subsequent Ac1-9 / CFA immunization (Figure 3.3C and D). It is possible that Tg4 T cells in Ac1-9_{4Y} treated mice had undergone activation, expansion and apoptosis and subsequently returned to an equivalent cell number to the control group. As the high affinity analogue Ac1-9_{4Y} can persist longer on MHC and provide a high density of pMHC complexes compared to wild type Ac1-9 (Konkel and Anderton, unpublished data; Anderton *et al*., 1998, McCue *et al*., 2004), administration of soluble Ac1-9_{4Y} may provide a persistent TCR signal that maintains the state of unresponsiveness.

The existence of persisting antigen (without adjuvant) has been reported to be essential in keeping the hyporesponsive state of antigen- reactive T cell *in vivo* (Tanchot *et al*., 2001; Singh and Schwartz, 2003; Raimondi *et al*., 2006). The Schwartz lab have developed an *in vivo* anergy model in which $CD4^+$ TCR transgenic cells specific for pigeon cytochrome *c* (PCC) and I-E^k are transferred to a T cell deficient transgenic mice that constitutively expresses PCC. The transferred cells underwent transient activation within 4 days but remained hyporesponsive from

day 7 to day 56 when cultured with PCC *in vitro* (Tanchot *et al*., 2001). A recent report found a reduction in quality and quantity of TCR signaling (Zap70, calcium / NFAT, NFκB) in this model (Chiodetti *et al*., 2006; Knoechel *et al*., 2006). On the other hand, T cell unresponsiveness in the adoptive transfer model with PCC transgenic mice (the tolerance induction protocol is similar to this chapter using soluble PCC peptide to induce T cell tolerance in mice given with PCC transgenic T cell) has been reported to be mediated by the ineffective recruitment of linker for activation of T cells (LAT) which affects the activity of Zap70, phospholipase C-γ1 (PLC-γ1), PI3K and Ras and restrains T cell proliferation (Hundt *et al*., 2006). This data suggests that peptide induced T cell tolerance has different signalling characters from the adaptive tolerance model.

In this chapter, Tg4 cells were harvested 27 days after EAE induction for proliferation and cytokine analysis (Figure 3.1). Presumably the time that effector cell numbers reached their peak would be close to (or before) the time of peak EAE score (around day 10-14), so that the effector cells sampled at day 27 may have died in both groups or migrated from secondary lymphoid organs to non-lymphoid tissues (Reinhardt and Jenkins, 2003). Thus, a broader range of tissues (especially the spinal cord and the brain) and different timing for cell harvest (for example, day 10, day 14) should be assessed in the future experiments.

A more in depth investigation of peptide-induced tolerance was undertaken in the pMOG / 2D2 / C57BL6 adoptive transfer system in this chapter. The lower level of proliferation reflected to the lower frequency of 2D2 cells in pMOG treated mice (Figure 3.5 and 3.6). Unlike tolerance in the Ac1-9 / Tg4 system, the gross reduction

of proliferation in the soluble peptide treated group resulted from the loss of 2D2 T cells, therefore preventing EAE (Figure 3.4B and 3.5B). T cell deletion in response to antigen has been found in models in which superantigens or high dose soluble peptides are injected into normal mice (Kawabe and Ochi, 1991; Critchfield *et al.*, 1994), or the relevant peptides are injected into TCR transgenic mice (Liblau *et al.*, 1996). In peptide-induced tolerance, the Jenkins lab has attempted to address the mechanisms of tolerance induction by using an adoptive transfer model with OVA peptide and OVA specific TCR transgenic mice (DO11.10). It was demonstrated that soluble OVA peptide induced a transient accumulation and a subsequent loss of transferred DO11.10 $CD4^+$ T cells that was accompanied by unresponsiveness in the surviving DO11.10 T cells (Kearney *et al.*, 1994). This was confirmed in the pMOG / 2D2 system in this chapter, with 2D2 T cells undergoing a state of transient activation and expansion for 3 days after the administration of tolerogenic pMOG (Figure 3.8A and 3.9). Normally the duration of the transient activation state can persist for 2-5 days depending on the model used (Kearney *et al.*, 1994; Huang *et al.*, 2003; Pape *et al.*, 1998). So there were more 2D2 cells in the pMOG treated mice on day 2 after pMOG treatment (Figure 3.11B). After pMOG / LPS priming, 2D2 T cells from pMOG treated mice expanded, produced no IL-2 and remained hyporesponsive, whereas cells in PBS treated mice produced IL-2 and proliferated vigorously (Figure 3.12C, 3.13 and 3.14). In the OVA / DO11.10 adoptive transfer system, this unresponsive state could be reversed after 2 weeks of soluble peptide administration, but weekly booster injections of soluble peptide could extend the unresponsive state (Pape *et al.*, 1998). This matches the adaptive tolerance model in which persisting antigens maintain the unresponsiveness (Knoechel *et al.*, 2006), presumably in those cells that survive soluble pMOG-induced tolerance is also a transient and reversible.

However, there were conflicting results in 2D2 T cells from soluble pMOG treated mice between the priming with pMOG / LPS (unresponsiveness, figure 3.13) and with pMOG / CFA (responsiveness, figure 3.6). There were no differences between pMOG-treated and PBS-treated groups in the frequency of 2D2 T cells and in proliferation 10 days after pMOG / LPS priming, whereas a reduced frequency of 2D2 T cells was found in pMOG treated mice 10 days after pMOG / CFA immunization (Figure 3.5B and 3.12C). Priming with pMOG / CFA provides a persisting, chronic release of antigen, whereas priming with pMOG / LPS is a systemic but not persisting source of antigen **(**Maxwell *et al*., 2002**).** Thus, a persisting stimulation of antigen and TLR stimuli (pMOG / CFA) may reverse the unresponsive cells into antigen responsive cells.

Interestingly, 2D2 T cells from pMOG treated mice seemed to have an early expression of Fas / FasL one day after pMOG / LPS immunization, compared with the control group (Figure 3.16). It has been reported that OVA induced cell deletion in DO11.10 or OT-II cells does not happen in normal mice transferred with T cells from DO11.10 x *lpr* or OT-II x *lpr* mice (or in *gld* mice transferred with T cells from OVA transgenic mice), suggesting that the Fas / FasL pathway is possibly involved in peptide induced T cell deletion (Pinkoski *et al*., 2002; Herndon *et al*., 2005). In Pinkoski¡s report, soluble OVA administration alone in wild type mice given OVA specific T cells could up-regulate the expression of FasL in OVA specific T cells, whereas mice given with CFA immunization without soluble OVA injection had a reduced FasL expression compared to soluble OVA injected mice (Pinkoski *et al*., 2002). However, the time point of these peptide induced Fas / FasL mediated apoptosis experiments were determined before priming. There is little evidence on

whether soluble peptide administration can facilitate Fas / FasL expression on CD4 T cells after subsequent priming. The rapid up-regulation of Fas / FasL in 2D2 T cells in pMOG treated mice after pMOG / LPS priming in this chapter suggests that peptide tolerized cells might be more sensitive to death receptor mediated apoptosis following stimulation. However, because activated cells may migrate into non-lymphoid tissues, assessment of apoptosis and proliferation in spleen cells might not represent the *in vivo* condition of most of the 2D2 cells. Therefore, other non-lymphoid tissues should be tested in the future experiments.

Taken together, administration of soluble peptide induces transient T cell activation, T cell deletion and T cell unresponsiveness in the pMOG / 2D2 / C57BL6 system. The major effect of reduced severity of EAE in pMOG induced tolerance may result from the deletion of pMOG-reactive T cells. In contrast, administration of soluble Ac1-9_{4Y} in the Tg4 / B10.PL system maintains T cells in an unresponsive state.

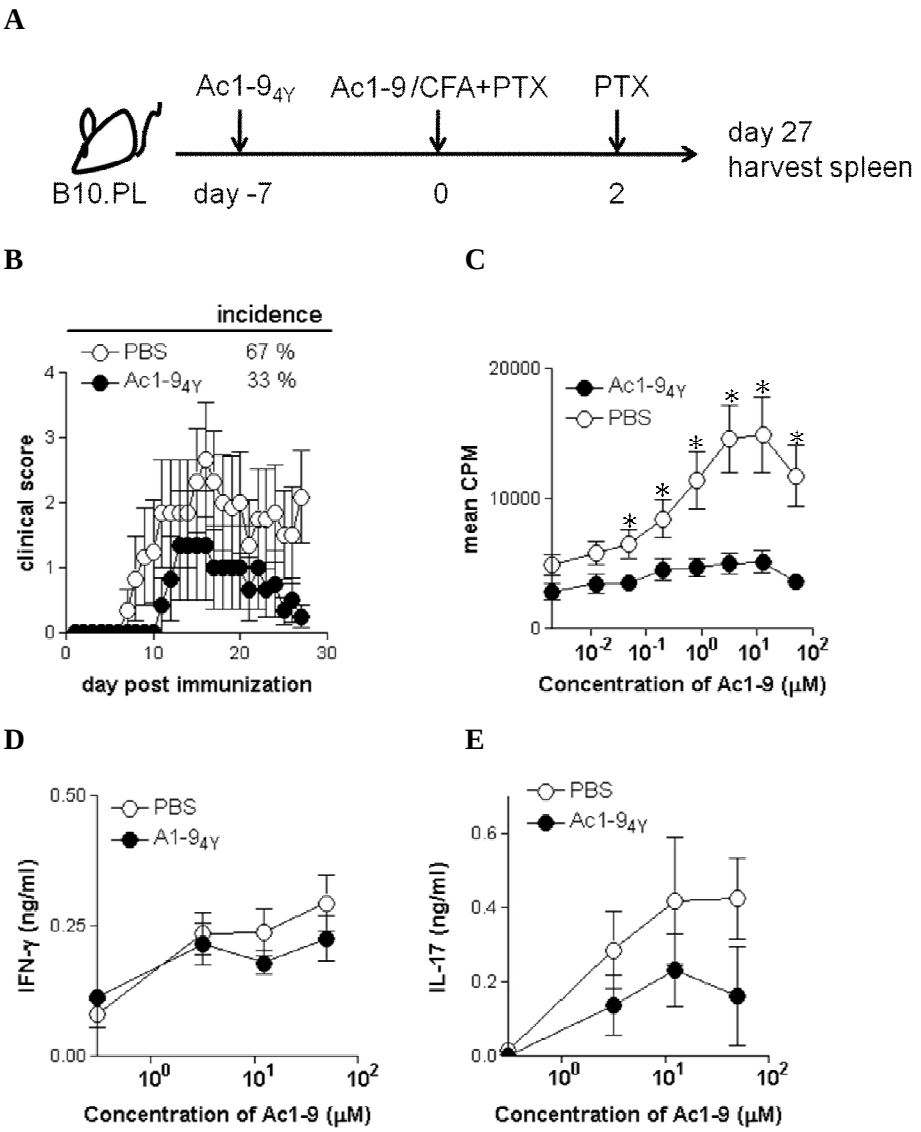
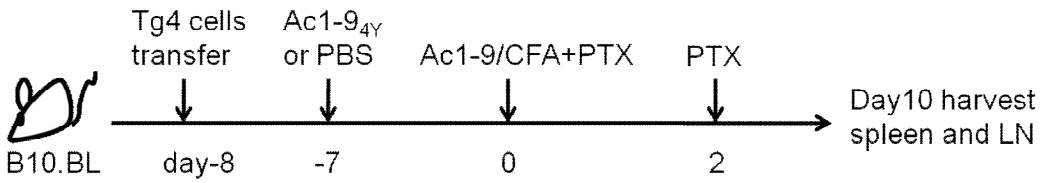


Figure 3.1 Administration of peptide Ac1-9$_{4Y}$ ameliorates EAE. *A*,B10.PL mice were injected with 200 £g of soluble Ac1-9$_{4Y}$ or PBS i.v. on day -7, immunized with 100 £g of Ac1-9 in CFA on day 0 (with administration of PTX on day 0 and day 2). *B*, disease severity of the mice was scored daily post immunization ($p < 0.001$ by Mann-Whitney U test). *C*, on day 27 post immunization, cells were harvested from the spleen and cultured for 72 hours with a pulsing of thymidine for the last 18 hours. Asterisks represent significant difference between peptide treated and PBS mice ($p < 0.05$ by unpaired T-test). *D* and *E*, Culture supernatants was harvested for cytokine ELISA assay. Plots show the amount of IFN-γ (D) and IL-17 (E) against various doses of Ac1-9. Data are from one experiment and represent the mean ¡ SEM (6 mice in each group, SEMs reflect samples from different mice).

A



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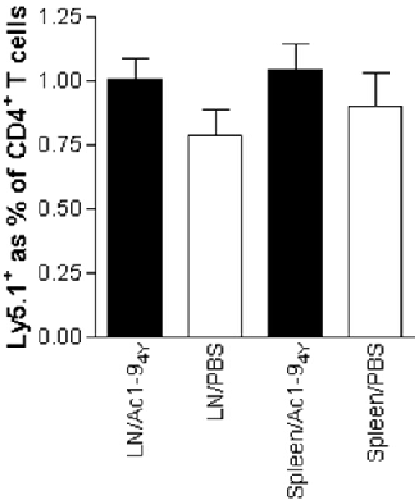


Figure 3.2 Administration of tolerogenic Ac1-9$_{4Y}$ maintains Tg4 T cells. *A*, B10.PL mice were given 3 x 10^5 CD4$^+$Ly5.1$^+$ Tg4 T cells on day -8, injected with 200 £g of Ac1-9$_{4Y}$ or PBS i.v. on day -7 and immunized with 100 £g of Ac1-9/CFA on day 0 (with administration of PTX on day 0 and day 2). *B*, Ten days after immunization, cells were harvested from draining lymph node (LN) and spleen and stained for CD4 and Ly5.1. Plot shows the frequency of Ly5.1$^+$ Tg4 cells as a percentage of CD4$^+$ T cells. Data are from one experiment and represent the mean ¡ SEM (3 mice in each group, SEMs reflect samples from different mice).

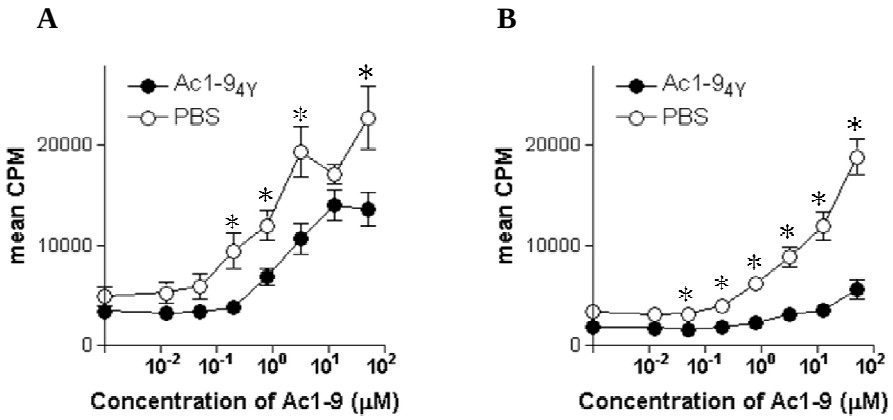
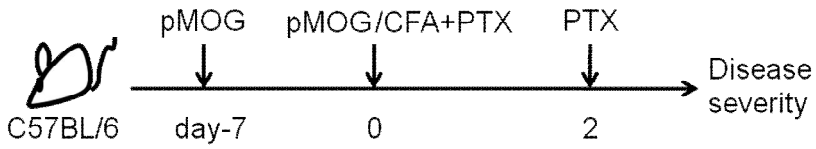


Figure 3.3 Administration of soluble Ac1-9$_{4Y}$ causes T cell unresponsiveness. Mice were immunized with the protocol as shown in figure 3.2A. Cells were harvested from spleen (A) and LN (B) on day 10 post immunization and cultured for 72 hours with a pulsing of thymidine for the last 18 hours. Asterisks represent significant difference between peptide treated and PBS mice ($p < 0.05$ by Unpaired T-test). Data are from one experiment and represent the mean ¡ SEM (3 mice in each group , SEMs reflect samples from different mice).

A



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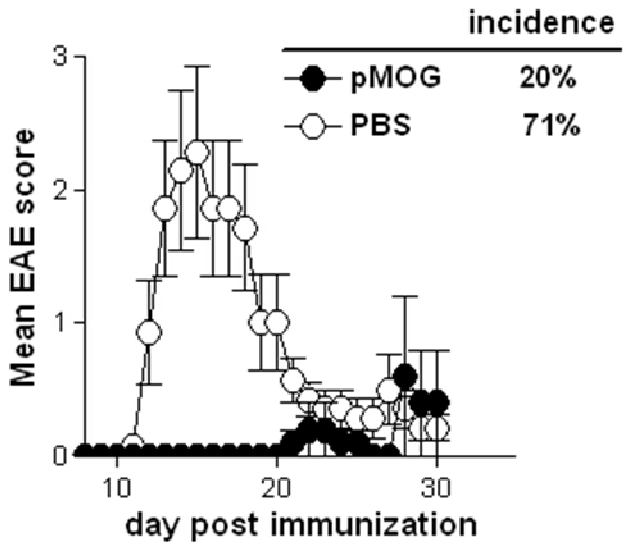

Figure 3.4 Administration of soluble pMOG protects mice from EAE. *A,* C56BL/6 mice were injected with 200 £g of soluble pMOG or PBS i.v. on day -7 and immunized with pMOG/CFA on day 0 (with administration of PTX on day 0 and day 2). *B,* disease severity was scored daily post immunization. Data represent the mean ¡ SEM gathered from individual mice ($p < 0.0001$ by Mann-Whitney U test, 5-7 mice in each group, data are from one of three repeated experiments, SEMs reflect samples from different mice).

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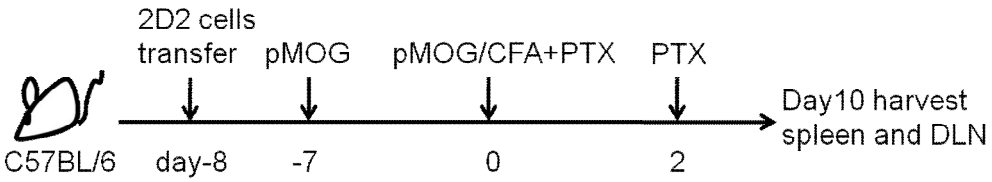

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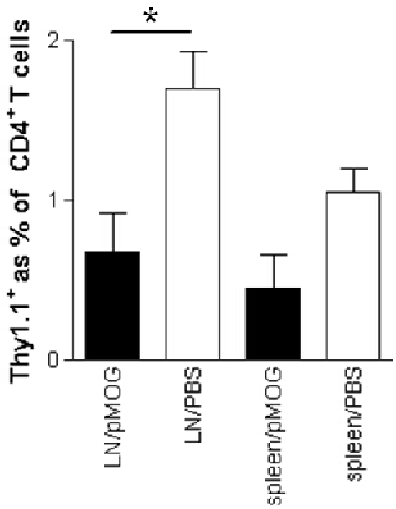

Figure 3.5 Administration of soluble pMOG reduces 2D2 T cells. *A*, C57BL/6 mice were given 2 x 10^6 $CD4^+Thy1.1^+$ T cells from 2D2 mice on day -8, injected with 200 £g of pMOG or PBS i.v. on day -7 and EAE was induced with pMOG/CFA (with administration of PTX on day 0 and day 2). *B*, 10 days post immunization, cells were harvested from spleen and LN and stained for CD4 and Thy1.1, with the frequency of $Thy1.1^+$ 2D2 cells shown as a percentage of total $CD4^+$ T cells. Asterisks represent a significant difference between pMOG treated and PBS mice ($p < 0.05$ by Unpaired T-test). Data are from on e experiment and represent the mean ¡ SEM gathered from individual mice (3 mice in each group, SEMs reflect samples from different mice).

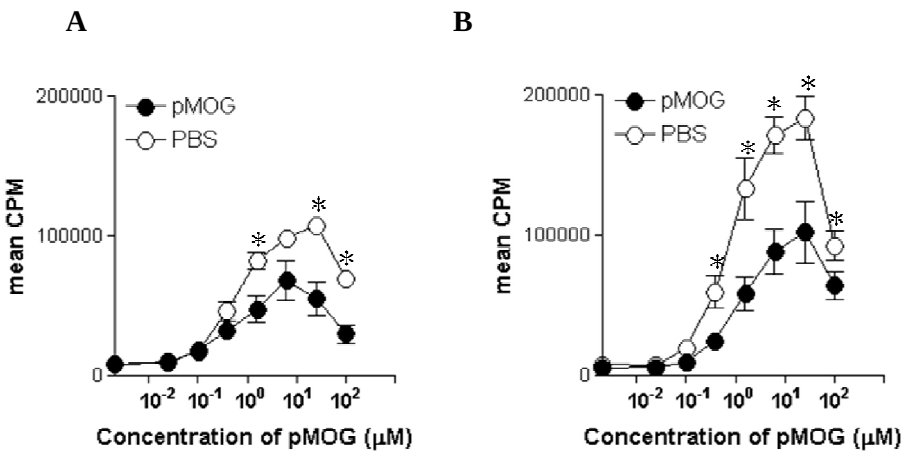

Figure 3.6 Administration of tolerogenic pMOG reduces T cell proliferation. Mice were immunized with the protocol shown in figure 3.5A. Cells were harvested from spleen and LN on day 10 post immunization. Cells were harvested from spleen (A) and LN (B) and cultured for 72 hours with a pulsing of thymidine for the last 18 hours. Asterisks represent a significant difference between pMOG treated and PBS treated mice ($p < 0.05$ by Unpaired T-test). Data are from one experiment and show the mean ¡ SEM (3 mice in each group, SEMs reflect samples from different mice).

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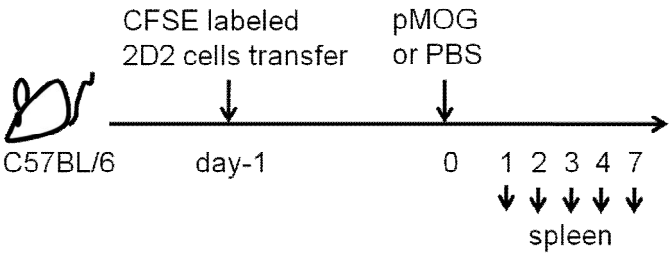

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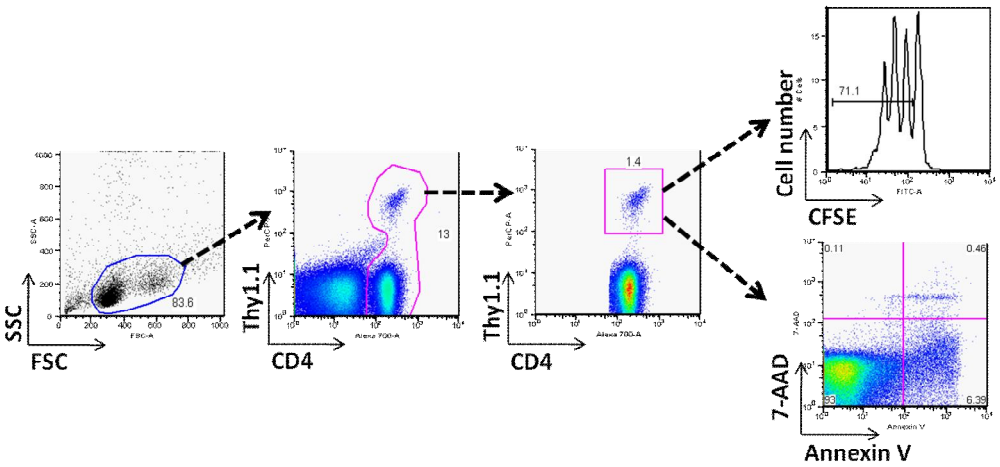
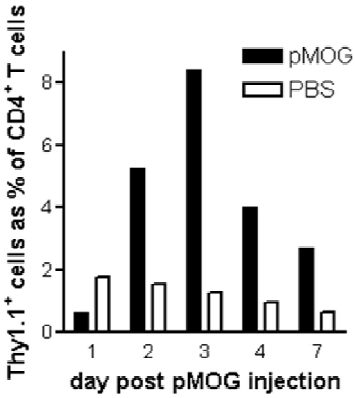


Figure 3.7 Administration of soluble pMOG to mice receiving 2D2 cells. *A*, C57BL/6 mice were injected with 2 x 10^6 CFSE labelled, $CD4^+$ $Thy1.1^+$ 2D2 T cells and injected with 200 £g of pMOG or PBS i.v. one day later. *B*, at the day indicated, spleen cells were harvested and stained for CD4 and Thy1.1. 7-AAD and Annexin V staining represent dead cells and apoptotic cells. Cells were gated on CD4+Thy1.1+, next plotted by CFSE level or by 7-AAD/Annexin V. Data are from one of three repeated experiments, 2 mice in each group. Data from this experiment continue in Fig 3.8-3.10.

A



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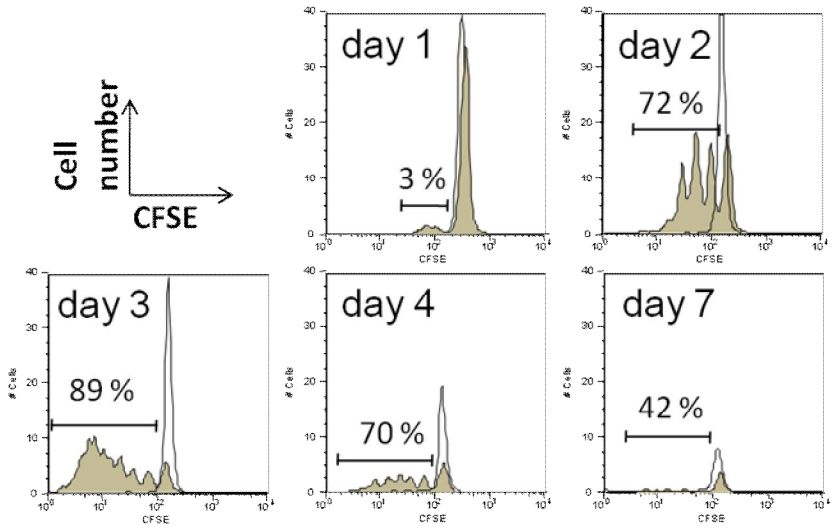

Figure 3.8 Administration of soluble pMOG induces cell division in 2D2 T cells. See figure 3.7A for experimental protocol. Cells were gated on $CD4^+$ $Thy1.1^+$ (see Fig 3.7 B). *A*, The frequency of $Thy1.1^+$ 2D2 cells is as a percentage of total $CD4^+$ T cells at the day indicated. *B*, Histograms show the dilution of CFSE versus the number of 2D2 cells at the day indicated. Filled histograms represent the pMOG treated mice and open histograms represent the control mice. The frequency in each plot represents the percentage of dividing 2D2 T cells in pMOG treated mice. Data are from one of three repeated experiments, 2 mice in each group.

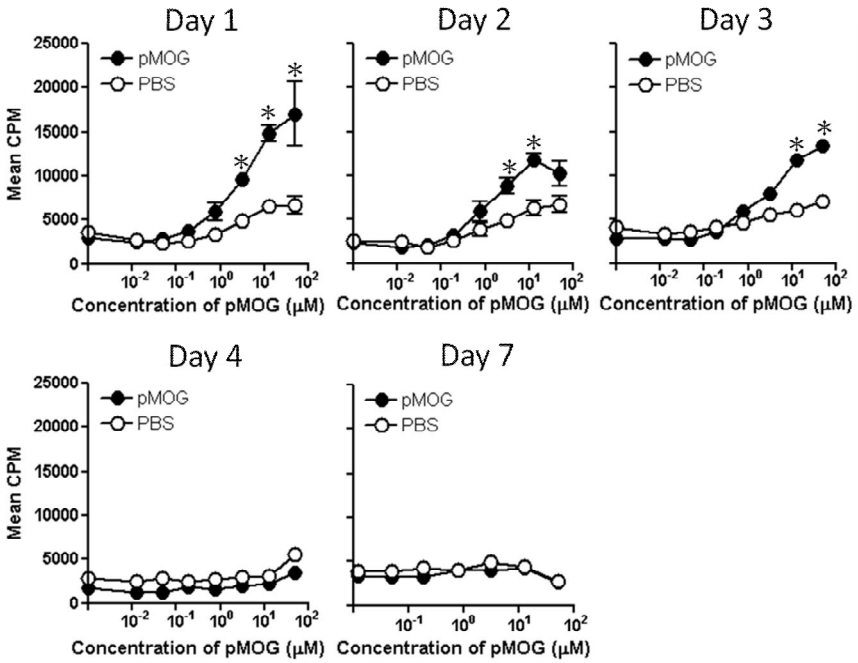

Figure 3.9 Administration of soluble pMOG induces transient proliferation. See figure 3.7A for experimental protocol. Spleen cells were harvested at the day indicated and cultured for 72 hours with a pulsing of thymidine for the last 18 hours. Asterisks represent significant difference between peptide treated and PBS treated mice ($p < 0.05$ by Unpaired T-test). Data are from one of three repeated experiments, 2 mice in each group (triplicate in each dose, each point represents mean CPM ¡ SEM).

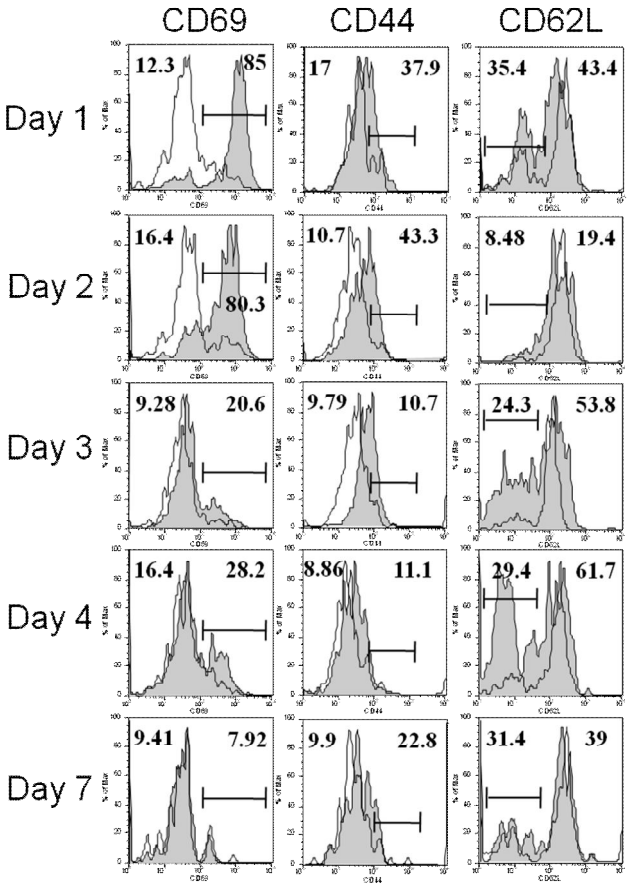

Figure 3.10 Administration of soluble pMOG enhances the expression of T cell activation markers. See figure 3.7A for experimental protocol. Spleen cells were stained for CD4, Thy1.1, CD69, CD44 and CD62L at the day indicated. Cells were gated on $CD4^+$ $Thy1.1^+$cells (2D2 cells). Filled histograms represent cells from pMOG treated mice and open histograms represent cells from the control mice. Numbers represent the percentage of gated cells (pMOG group at the right and PBS group at the left). Data are from one of two repeated experiments, 2 mice in each group

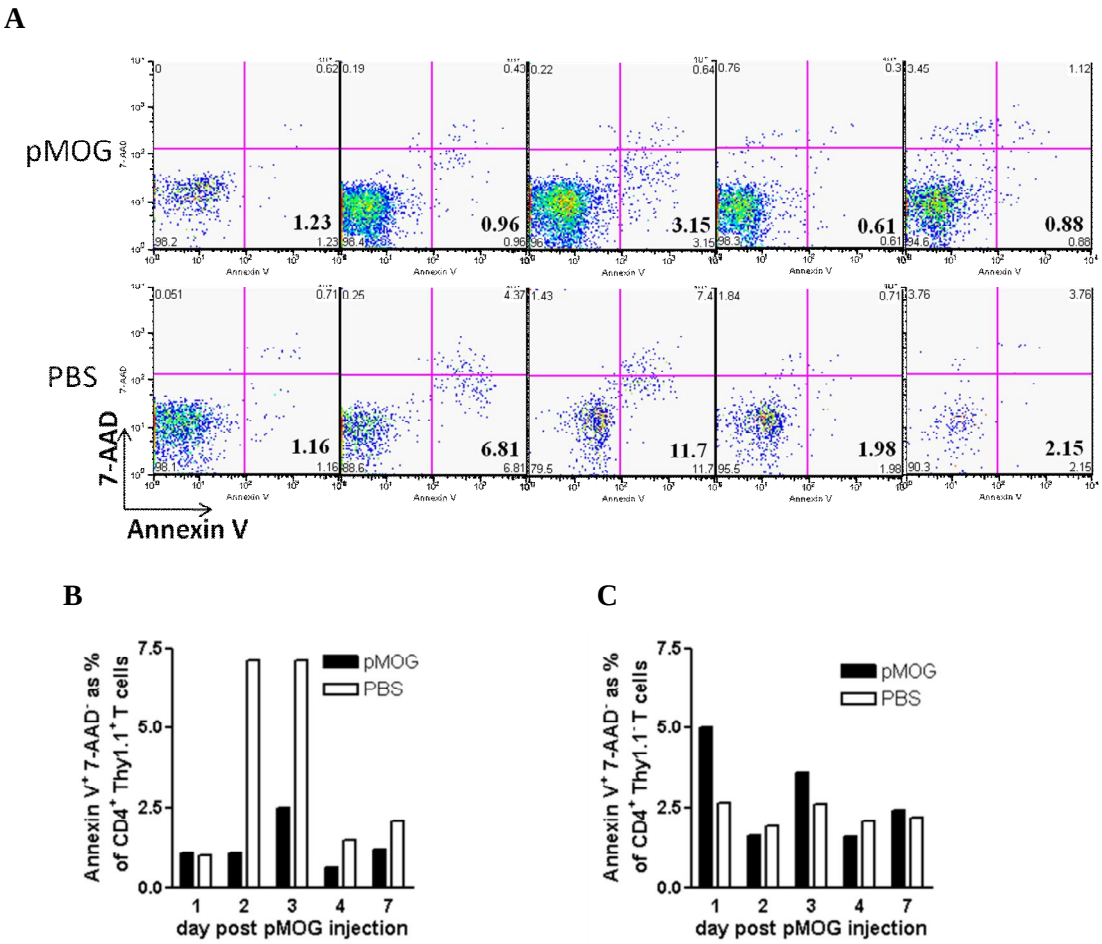

Figure 3.11 pMOG-treated mice have fewer apoptotic cells. See figure 3.7A for experimental protocol. *A*, Spleen cells were gated on $CD4^+$ $Thy1.1^+$ (2D2) cells. *B*, Annexin V^+ 7-AAD^- T cells as a percentage of $CD4^+$ $Thy1.1^+$ cells at the day indicated. *C*, Plot shows the Annexin V^+ 7-AAD^- T cells as a percentage of $CD4^+$ $Thy1.1^-$ (host) cells at the day indicated. Data are from one of two repeated experiments, 2 mice in each group.

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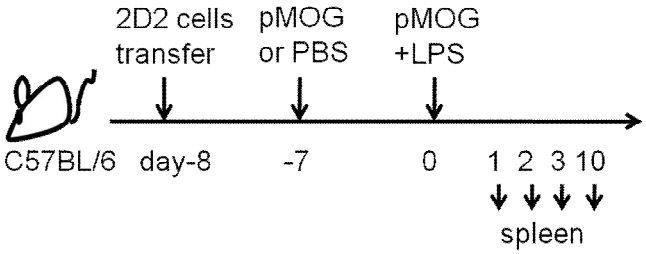

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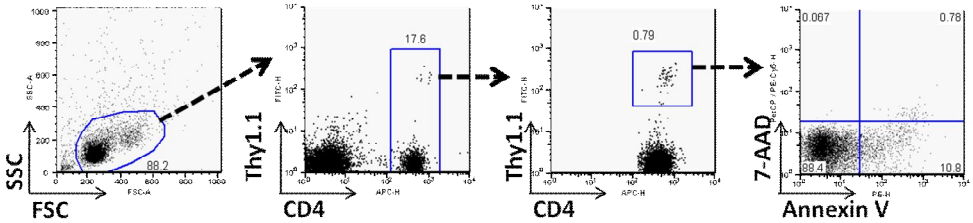

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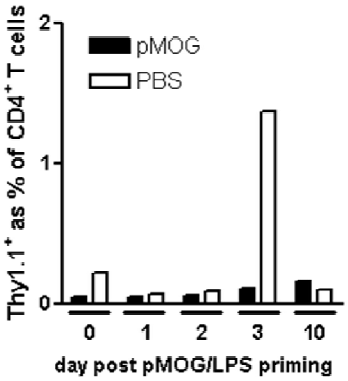


Figure 3.12 Administration of soluble pMOG prevents the expansion of 2D2 cells by pMOG/LPS priming. *A*,C57BL/6 mice were injected with 2 x 10^6 $CD4^+$ $Thy1.1^+$ 2D2 T cells on day -1, injected with 200 £g of pMOG or PBS i.v. on day 0 and subsequently primed i.v. with 100 £g of pMOG in 30£g of LPS. *B*, Spleen cells were gated on $CD4^+$ $Thy1.1^+$ (2D2) cells. Annexin V^+ 7-AAD^- cells are representative of apoptotic cells. *C*, The frequency of $Thy1.1^+$ 2D2 cells as a percentage of $CD4^+$ T cells at the day indicated. Data are from one of two repeated experiments, 2 mice in each group.

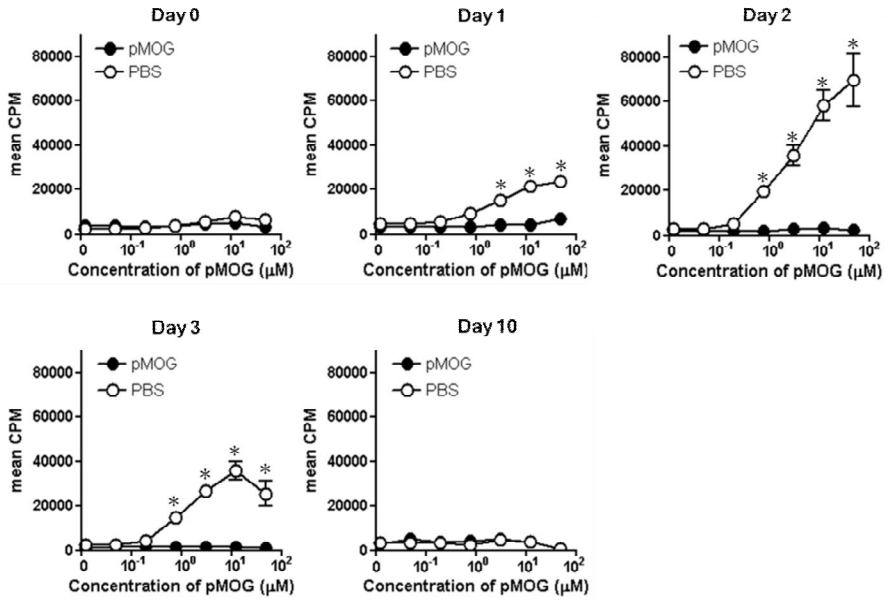
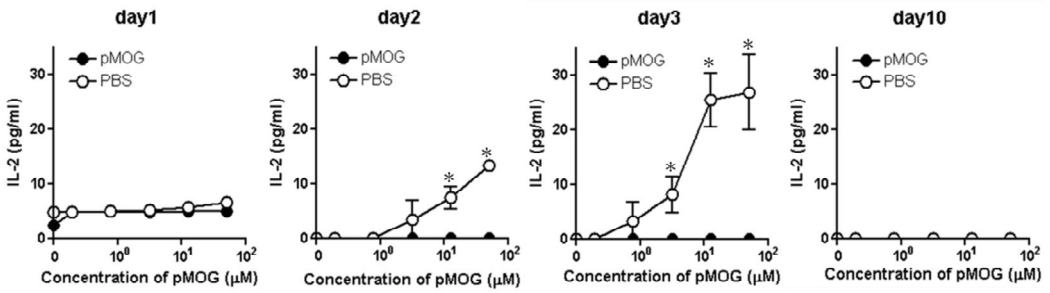


Figure 3.13 Administration of soluble pMOG prior to priming inhibits pMOG/LPS triggered T cell expansion. *A,* The injection protocol is shown in figure 3.13A. Spleen cells were harvested at the day indicated and cultured with pMOG for 72 hours with a pulsing of ^{3}H-thymidine for the last 18 hours. Asterisks represent significant difference between pMOG treated and PBS treated mice ($p < 0.05$ by Unpaired T-test). Data are from one of two repeated experiments, 2 mice in each group (Triplicate in each dose. Data represent the mean ¡ SEM).

A



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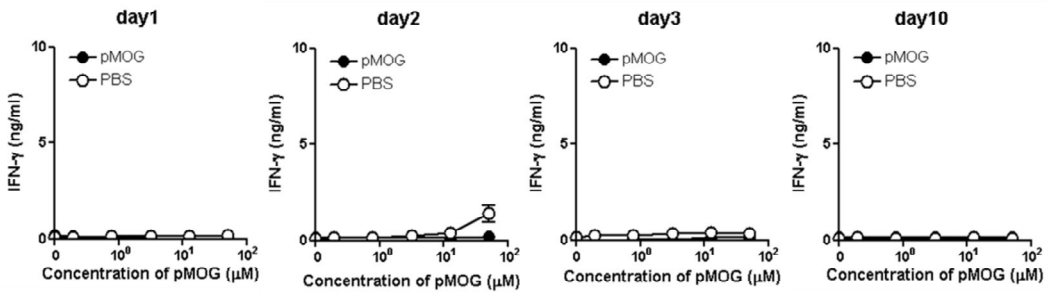


Figure 3.14 Administration of pMOG prior to pMOG/LPS priming inhibits IL-2 production. The experimental protocol is shown in figure 3.13A, spleen cells were harvested at various time points and cultured for 48 hours (IL-2 assay) or for 72 hours (IFN-γ) with pMOG. Culture supernatant was collected for cytokine assay by ELISA. Plots show the amount of IL-2 (A) and IFN-γ (B) secreted against various doses of pMOG. Asterisks represent significant difference between pMOG treated and PBS treated mice ($p < 0.05$ by Unpaired T-test). Data are from one of two repeated experiments, 2 mice in each group (Triplicate in each dose. Data represent the mean ¡ SEM).

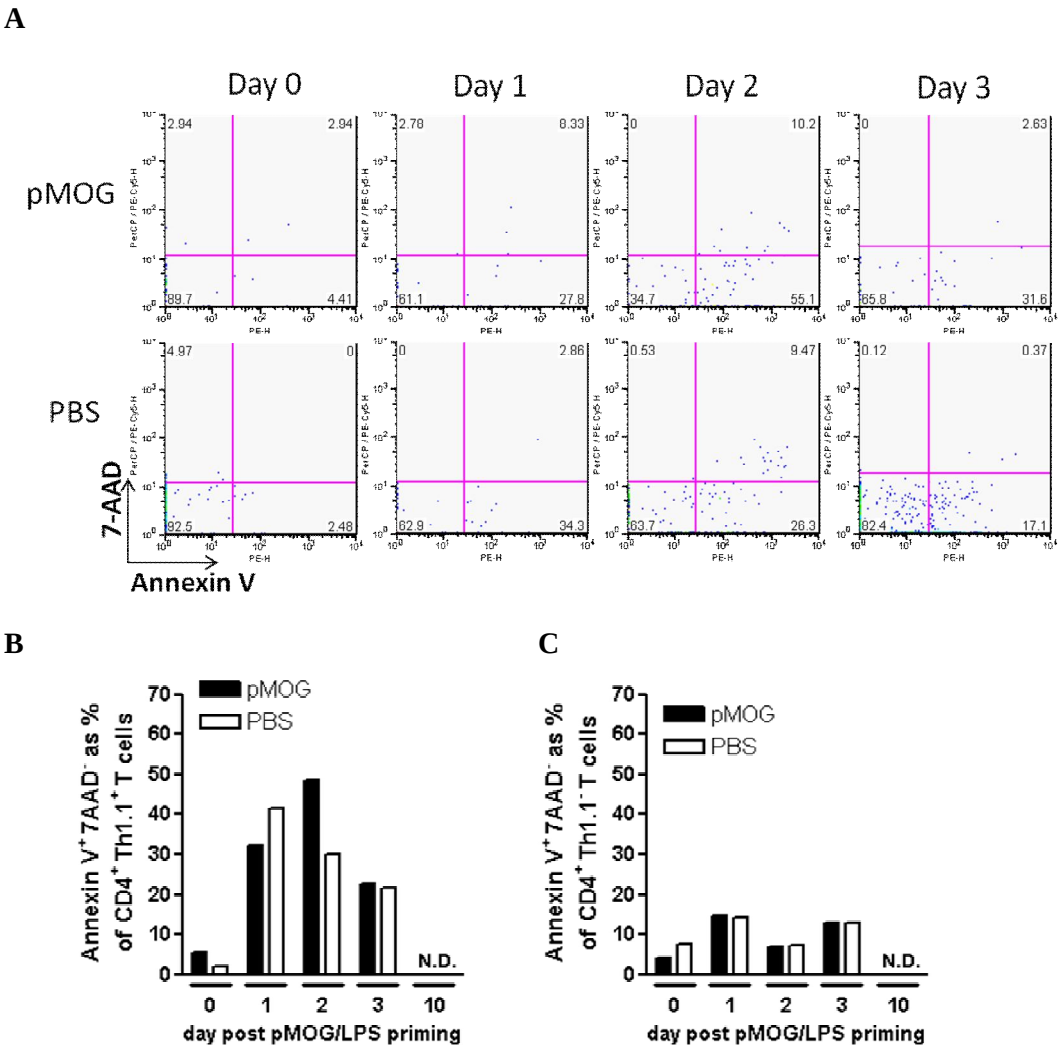


Figure 3.15 T cell apoptosis after soluble pMOG administration and pMOG / LPS priming. The experimental protocol is shown in figure 3.13A. Spleen cells were harvested and stained for CD4 and Thy1.1. *A*, cells were gated on $CD4^{+}Thy1.1^{+}$ as transferred 2D2 cells. Annexin V and 7-AAD staining represent dead cells and apoptotic cells. *B*, Annexin V^{+} 7-AAD^{-} T cells as a percentage of $CD4^{+}$ $Thy1.1^{+}$ 2D2 cells at the day indicated. *C*, Plot shows the Annexin V^{+} PI^{-} T cells as a percentage of $CD4^{+}$ $Thy1.1^{-}$ cells (host) at the day indicated. Data are from one of two repeated experiments, 2 mice in each group (N.D. represents not detected).

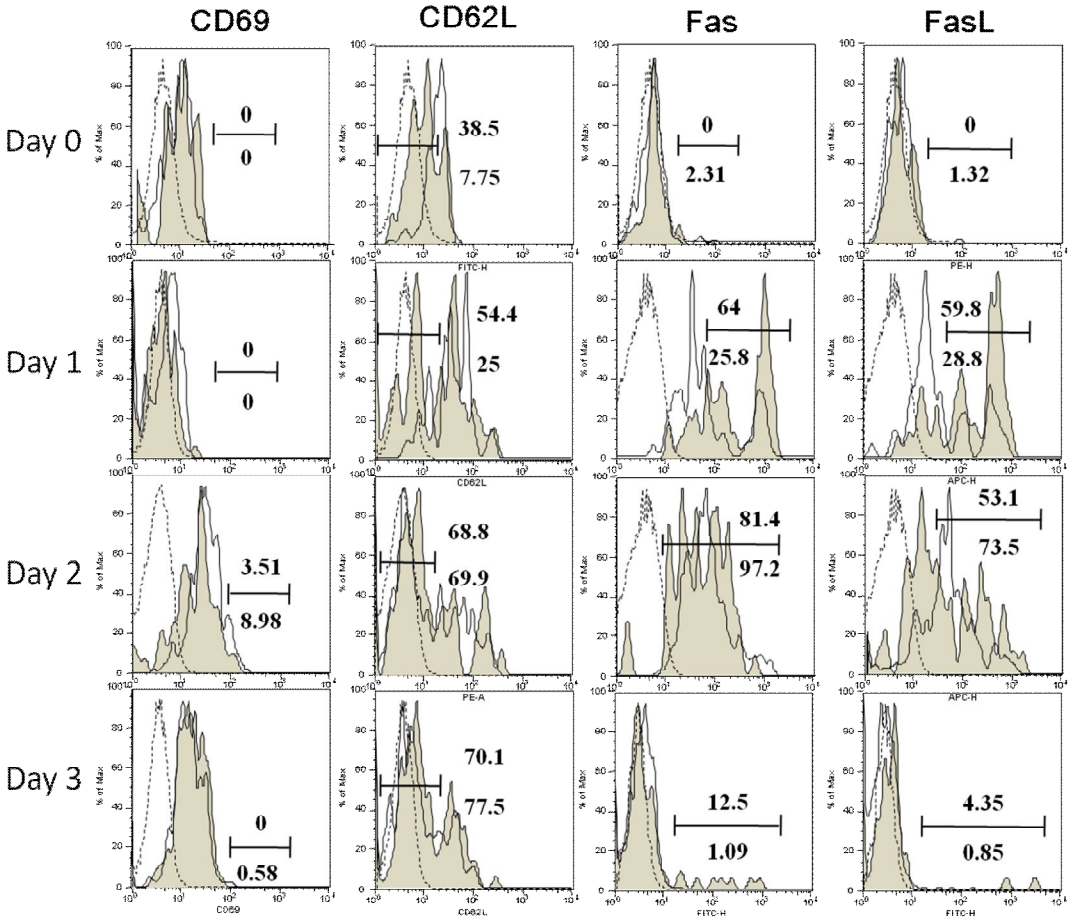


Figure 3.16 Administration of soluble pMOG enhances Fas/FasL expression after pMOG/LPS priming. The experimental protocol is shown in figure 3.13A. Spleen cells were harvested at the day indicated and stained for CD69, CD62L, Fas and FasL. Plots show the expression of surface markers versus the percentage of $CD4^{+}Thy1.1^{+}$ cells. Filled histograms represent cells from pMOG treated mice and open histograms represent cells from the control mice. Dash histograms represent un-stained control. Numbers represent the percentage of gated cells (pMOG group on the top and PBS group on the bottom). Data are from one of two repeated experiments, 2 mice in each group

Chapter 4. Development of a memory model for EAE

4.1 Introduction

Clinical translation of antigen-based tolerance will require the ability to control autoreactive memory T cells. To mimic the pathological situation of MS patients, we need models to study the development and control of memory T cells. Models of tolerance in ¡ongoing EAE¡ have been reported by injecting soluble peptide, recombinant peptide-MHC complexes, or fixed peptide-pulsed APC to diseased mice (Margot *et al.*, 2005; Huan *et al.*, 2004; Smith *et al.*, 2005). However, some of the mice in these models developed fatal anaphylaxis immediately after the peptide administration (Pedotti *et al.*, 2001). Presumably, anti-myelin peptide antibodies were generated after primary immunization, causing anaphylactic shock upon systematic administration of the same peptide. This chapter focuses on the feasibility of applying a ¡double immunization system¡ to investigate memory response of EAE. In the EAE model where mice are given primary immunization, the frequency of myelin-reactive T cells declines to a low level after 4 weeks and presumably the number of effector T cells contracts at this time point. To provoke the memory response, a secondary immunization was applied to the same mice, 28 day after the primary immunization. As we are allowed to use CFA in the same animal only once, mice were therefore immunized with peptide in alternative adjuvants to generate memory T cells prior to secondary immunization. CpG can activate DC through TLR9, whereas PGN can activate DC through TLR2 and nucleotide-binding oligomerization domain 2 (NOD2) signalling (Iwasaki and Medzhitov, 2004; Strober *et al.*, 2006). EAE has been reported to be induced by administering myelin protein with CpG /IFA or PGN / IFA (Segal *et al.*, 2000 Ichikawa *et al.*, 2002; Visser *et al.*,

2005). These two adjuvants were tested in this chapter. To establish a memory response, several protocols were tested in mice with: A) Peptide pulsed DC injection first and peptide / CFA immunization secondarily. B) Primary immunization with peptide CFA and second immunization with peptide IFA. C) Primary immunization with peptide / CFA and second immunization with peptide / CpG / IFA or peptide / PGN / IFA. D) Primary immunization with peptide / CpG / IFA or peptide / PGN / IFA and second immunization with peptide / CFA.

4.2 Results

4.2.1 Maintenance of transferred cells after antigen priming

The adoptive transfer of TCR transgenic T cells was applied in several protocols to study the persistence of these cells under various conditions. 2 x 10^6 $Ly5.1^+CD4^+$ cells from Tg4 mice were transferred into B10.PL mice prior to immunization with 100 £g of wild type Ac1-9 in CFA (Figure 4.1A). Based on previous observations in Chapter 3, Tg4 T cells would initially expand after priming and subsequently contract over time. As figure 4.1B shows in spleen cells harvested 10 days after Ac1-9 immunization, frequency of Tg4 cells was between 0.34 % and 0.92 %. However, this proportion decreased rapidly and dropped below 0.1% and 0.02% at day 20 and day 30, respectively (Figure 4.1B). Meanwhile the percentage of transferred cells stayed constantly low in the control group (0.01 %, 0.065 % and 0.006 % at days 10, 20 and 30, respectively). There was no significant accumulation of Tg4 cells at the time point of day 30 post priming. High expression of CD44 and down-regulation of CD62L are both markers of activated, effector and memory T cells. To assess whether the cells that persisted were memory T cells, cells were analyzed for surface expression of CD44 and CD62L (memory T cells are $CD44^{high}$

and CD62L^{low}). Non-immunized mice had a proportion of 10-15 % of CD44^{high} Tg4 cells and 15-30 % of CD62L^{low} Tg4 cells (Figure 4.2A and B). Tg4 cells in the Ac1-9 primed mice had enhanced frequencies (24 %, 39 % and 22 % on days 10, 20 and 30, respectively) of CD44^{high} T cells and enhanced frequencies (78.1 %, 77.3 % and 67 % on days 10, 20 and 30, respectively) of CD62L^{low} T cells (Figure 4.2C and D). These data suggest that although the cell number of Tg4 cells in Ac1-9-primed mice expanded and contracted back to a basal level by 4 weeks after immunization, antigen priming resulted in a persistent memory-like phenotype (CD44^{high} CD62L^{low}) in the surviving Tg4 cells.

4.2.2 Priming with peptide-pulsed DC is inefficient to trigger EAE in pMOG experienced mice

To develop a model of ¡memory EAE¡, the protocol was adapted to give the mice a primary immunization for the generation of memory T cells without disease followed by a secondary immunization for the induction of clinical EAE. Mice were transferred with 3 x 10^5 CD4$^+$ Ly5.1$^+$ Tg4 cells and immunized with 100 £g of Ac1-9 in CFA. Induction of EAE was attempted by injection of Ac1-9$_{4Y}$-pulsed DC and PTX four weeks later (Figure 4.3A). This treatment did not trigger EAE (Figure 4.3B). Ten days after DC injection, spleen cells were analyzed in *ex-vivo* recall response assays. Although there was a high background of CPM in the DC group, no group showed a dose response to Ac1-9 *in vitro* (Figure 4.3C). Unlike the primary response measured at day 10 with the immunization of Ac1-9 / CFA (Figure 3.3), secondary challenge with Ac1-9$_{4Y}$-pulsed DC did not expand Tg4 T cells (Figure 4.3D).

A similar pattern of low incidence (20 % in pMOG-loaded DC injected mice) and mild severity was observed in pMOG / 2D2 / C57BL/6 system (Figure 4.4), indicating that double immunization with peptide / CFA primarily and peptide-pulsed DC secondarily was insufficient to reliably re-activate pathogenic cells.

4.2.3 Peptide administration in the absence of adjuvant does not re-induce EAE in Ag-experienced mice.

B10.PL mice were transferred with 3 x 10^5 $CD4^+$ $Ly5.1^+$ Tg4 T cells at day -1, induced to develop EAE with 100 £g of Ac1-9 / CFA one day later, then injected with soluble Ac1-9_{4Y} or PBS at day 27 (Figure 4.5A). As figure 4.5B shows, mice developed disease after primary immunization and were in the recovery phase with mean clinical scores lower than 1 at day 27. After injection with soluble Ac1-9_{4Y}, none of these Ac1-9 experienced mice developed disease. There was a trend of increased frequency of Tg4 T cells in soluble Ac1-9_{4Y} treated mice compared to the control group, at both day 30 and day 47 (day 3 and day 20 after peptide injection, respectively) (Figure 4.6). This suggested that soluble Ac1-9_{4Y} might sustain the survival of the Tg4 T cells. *Ex-vivo* proliferation assays showed that cells from Ac1-9_{4Y} and PBS injected mice remained equally responsive to Ac1-9 at day 30 and day 47 post immunization (Figure 4.7A and B), suggesting that Ac1-9_{4Y} does not re-activate T cells in Ac1-9 experienced mice.

To attempt to induce a secondary episode of disease, mice were immunized with Ac1-9 in IFA (plus PTX) on day 33 after primary immunization. Soluble Ac1-9_{4Y} or PBS 6 was injected 6 days before secondary immunization to induce T cell tolerance (Figure 4.8A). No matter whether these mice received soluble Ac1-9_{4Y} before

secondary immunization or not, they did not develop any signs of EAE (Figure 4.8B). The Ac1-9_{4Y} treated mice had a trend of increasing the frequency of Tg4 cells 14 days after secondary immunization, although this was not significant (Figure 4.9A). However, spleen cells from Ac1-9_{4Y} treated mice proliferated significantly less than the PBS treated mice (Figure 4.9B). Normalization of CPM with relative cell number showed a state of unresponsiveness in cells from the Ac1-9_{4Y} treated mice (Figure 4.9C). Although clinical signs of EAE were not observed after secondary immunization, the reduced proliferation in cells from the Ac1-9_{4Y} treated mice suggests that the i.v. administration of soluble Ac1-9_{4Y} may have induced T cell tolerance in the antigen-experienced mice. Thus the memory Tg4 T cells may have been driven to unresponsiveness by the administration of soluble Ac1-9_{4Y}.

4.2.4 Administration of soluble myelin peptide after primary immunization can induce anaphylactic shock

Anaphylactic shock is clinically defined by vasodilation of arterioles, constriction of the airways and bronchioles in the lungs. When administering soluble peptide to peptide-experienced mice as the protocol shown in figure 4.5A, it was observed that some of the mice developed breathing difficulties. These mice subsequently died within 20 minutes of peptide injection. This was seen in 15% of Ac1-9-experienced mice (Table 4.1). When a similar protocol was applied in the pMOG / C57BL/6 system (with primary immunization of pMOG/CFA at day 0, and various doses of soluble pMOG through i.v. or i.p. route at day 28), no anaphylaxis occurred in mice receiving 10 £g or 50 £g of soluble pMOG, while 75 % and 50 % of the mice developed anaphylaxis after receiving pMOG injection of 200 £g and 500 £g respectively. No pMOG-specific IgE was detected in sera collected 4 weeks after

pMOG / CFA immunization. However, high titers of anti-pMOG IgG1 were observed (Figure 4.10), suggesting that pMOG specific IgG1 is possibly one of the causes of anaphylactic shock induced by re-challenge of soluble peptide.

4.2.5 Development of memory EAE with pMOG / CpG / IFA or pMOG / PGN / IFA

Segal and Laman¡s groups have reported that the immunization of encephalitogenic peptide in IFA, together with bacteria based TLR stimulus, unmethylated CpG (CpG) or peptidoglycan (PGN), can induce EAE (Segal *et al*., 2000; Laman *et al*., 2005). A double immunization protocol was tested as Figure 4.11. Mice received 2 x 10^6 of 2D2 T cells at day -29 were injected with 100 £g of pMOG in CFA one day later and were subsequently induced to develop EAE with pMOG / CpG / IFA or pMOG / PGN / IFA. Unexpectedly, only 1 in 3 mice in pMOG / CpG / IFA immunized group developed disease, whereas mice had no clinical signs after receiving the immunization of pMOG / PGN / IFA (Figure 4.11B).

The protocol was then tested in reverse. C57BL/6 mice received 2 x 10^6 $CD4^+$ $Thy1.1^+$ 2D2 T cells at day -29, immunized with either pMOG / CpG / IFA , pMOG / PGN / IFA or pMOG / IFA on day later and subsequently induced to develop EAE with 100 £g of pMOG in CFA at day 0 (Figure 4.12A). As Figure 4.12B shows, pMOG / CpG / IFA and pMOG / PGN / IFA immunized mice started to develop EAE from day 8 and day 10, which were 5 days and 3 days earlier than primary immunization control (day 13), respectively. Even the group that received a primary immunization with pMOG/IFA had an earlier onset of disease compared to the control mice. All mice that initially received pMOG / CpG / IFA and pMOG / PGN /

IFA immunized mice developed EAE, contrasting with 2 in 4 mice in pMOG / CFA control group. These data suggested that the double immunization with pMOG / CpG / IFA or pMOG / PGN / IFA primarily and pMOG / CFA secondarily triggers a more severe and faster ¡memory¡ form of EAE. However, t ransferred 2D2 T cells could not be detected in either spleen, LN or CNS harvested at the time of peak EAE score after the secondary immunization, suggesting that pMOG-reactive T cells from 2D2 mice may not persist in LN, spleen or CNS (data not shown). When the same protocol was applied to C57BL/6 mice without a transfer of 2D2 T cells (Figure 4.13A), mice still had an early onset of EAE if initially immunized with pMOG / CpG / IFA. Without transfer of 2D2 T cells, mice immunized with pMOG / PGN / IFA did not show earlier onset and had a faster recovery, compared with the mice immunized only with pMOG / CFA (Figure 4.13B).

Table 4.2 shows a summary of the data from different experiments using double immunization protocols. Mice with primary immunization of CpG or PGN as adjuvants had a similar incidence of disease as the pMOG / CFA immunized control mice (74% to 79%). The mean day of disease onset for mice receiving pMOG / CFA immunization only was day 11, whereas CpG, PGN or IFA based double immunization started disease in treated mice at day 9.9, 11.1 and 13.3, respectively. Although the mice with double immunization with pMOG / CFA first and pMOG / CpG / IFA secondarily started disease 2.7 days ahead of the control mice, this protocol was not chosen for future experiments due to its low incidence (50 %). For the mice with pMOG / CpG / IFA first and pMOG / CFA secondarily, disease onset was 1-2 days ahead of the control mice, giving an indication that a memory response of pMOG had developed in these mice.

4.2.6 Mice primed with pMOG/CpG/IFA or pMOG/PGN/IFA primarily develop a mild immune response

Having obtained data suggesting that immunization with pMOG / CpG / IFA or pMOG / PGN / IFA could generate pMOG-reactive, memory T cells, it was of interest to analyze the primary response made in response to CpG or PGN based immunization. C57BL/6 mice were immunized with pMOG / CpG / IFA or pMOG / PGN / IFA plus PTX, with control mice receiving pMOG / CFA and PTX. Unexpectedly, mice did not develop EAE except in the control group receiving pMOG / CFA (Figure 4.14A). Spleen cells taken at day 28 were assayed for pMOG recall responses. In terms of proliferation, cells from pMOG / CpG / IFA immunized mice were as responsive as the cells from pMOG / CFA immunized mice (Figure 4.14B). Cells from pMOG / PGN / IFA immunized mice were less responsive than pMOG / CpG / IFA and pMOG / CFA immunized mice, suggesting that PGN based immunization induces a milder T cell activation in mice. On day 28 after secondary immunization, there was a trend of less IL-2 production in cells of the pMOG / CpG / IFA group compared to pMOG / CFA group, whereas cells in pMOG / CpG / IFA group produced more IL-2 than cells in the pMOG / PGN / IFA group (Figure 4.15A). Likewise, cells from pMOG / CpG / IFA immunized mice produced greatly reduced amounts of IFN-γ and IL-17 compared to cells from pMOG / CFA immunized mice (Figure 4.15B and C), whereas cells in pMOG / PGN / IFA and pMOG / IFA groups did not produce IFN-γ and IL-17. These data suggest that compared with CFA, CpG / IFA is a milder adjuvant for activating T cells and for triggering Th1 and Th17 responses. Also, PGN / IFA is a mild stimulus for T cell activation but it is insufficient to induce Th1 and Th17 responses in this system.

Because disease initiation in mice primarily immunized with pMOG / CpG / IFA was consistently ahead of the control (pMOG / CFA only) mice by 1 to 2 days in every individual experiment and was able to induce Th1 and Th17 responses, primary immunization with peptide / CpG / IFA was utilized in subsequent ¡¡memory¡¡ experiments.

4.2.7 Comparison of the mice receiving pMOG/CpG/IFA and pMOG/CFA

To further assess the immune response made after CpG based immunization, mice were immunized with pMOG / CpG / IFA with PTX injection, 4 weeks later spleen cells were analyzed for cell proliferation and intracellular cytokine production in response to pMOG. Two control groups were added in these experiments, pMOG / CFA / PTX with a supply of PTX and pMOG / CFA without a supply of PTX. All of the mice given pMOG / CFA / PTX developed EAE, whereas 4 in 7 mice in the pMOG / CFA group had mild disease (NOTE: this is highly unusual). There were no clinical signs of EAE observed in pMOG / CpG / IFA mice (Figure 4.16B). Cells from pMOG / CFA / PTX injected mice proliferated slightly greater than cells from pMOG / CpG / IFA and pMOG / CFA mice (Figure 4.16C), confirming the previous observation in figure 4.14 B. The percentage of $CD4^+$ T cells producing cytokines in response to pMOG was assessed by intracellular staining. Production of IFN-γ and IL-17 in cells from pMOG / CFA / PTX immunized mice were significantly greater than in cells from pMOG / CFA and pMOG / CpG / IFA immunized mice (Figure 4.16D and E). In addition, the pMOG / CpG group generated less $IL\text{-}17^+$ T cells than pMOG / CFA group. There was a trend of reduced $IFN\text{-}\gamma^+$ producing cells in pMOG / CpG / IFA immunized mice compared to other groups. This suggested that CFA

based immunization has a stronger ability to produce IFN-γ and IL-17 producing effector T cells and the supply of PTX can augment this. To understand whether CpG based immunization affects regulatory T cells (Treg), cells harvested at day 28 post immunization were stained with antibodies against CD4, CD25 and Foxp3. Immunization with pMOG / CFA / PTX resulted in an enhanced frequency of $CD4^+$ $CD25^+$ $Foxp3^+$ Treg cells in the spleen, compared with other groups (Figure 4.17). This suggested that at the time of 4 weeks post immunization (the time we applied the secondary immunization in the double immunization protocol), pMOG / CpG / IFA immunization does not affect the number of Treg cells.

Sera were collected from individual mice for detection of anti-pMOG Abs. Although anti-pMOG IgE was not detectable, sera from mice treated with pMOG / CpG / IFA or pMOG / CFA had enhanced titres of anti-pMOG IgG1, compared with the pre-immune serum collected from the same mice (Figure 4.18A). Sera from pMOG / CFA / PTX immunized mice had higher titres than sera in pMOG / CFA / no PTX group, suggesting that the injection of PTX can trigger a stronger humoral immune response. However, not all of the mice produced anti-pMOG IgG1. In the pMOG / CFA / PTX group, 3 in 7 mice had comparative high titres of anti-pMOG IgG1 (Figure 4.18B), but this did not correspond with the highest scores of EAE. In fact, the mouse with the highest disease score accumulated low titres of anti-pMOG IgG1, indicating that disease severity is independent of anti-pMOG IgG1.

4.2.8 Comparison of primary and memory response

So far the protocol was fixed with immunization of pMOG / CpG / IFA first and pMOG / CFA plus PTX secondarily (double immunization) for investigating

¡memory EAE¡. To confirm the presence of a memory response to the secondary immunization, mice were immunized with pMOG / CpG / IFA, and a group of non-immunized mice were kept as a control. Four weeks after the primary immunization, all of the mice were immunized for EAE with pMOG / CFA plus PTX (Figure 4.19A). Similar to the observations in figure 4.12 (with a transfer of 2D2 cells) and 4.13 (without adoptive transfer), mice with double immunization developed a disease with earlier onset and resolved more slowly compared with the primary immunized mice ($p < 0.01$, Figure 4.19B). Spleen cells were collected from the mice at day 28 post EAE induction. The recall response assays showed that cells from primary and double immunization proliferated equally against pMOG *ex-vivo* (Figure 4.20A), but cells from double immunized mice produced more IFN-γ and less IL-17, compared with the primary immunized mice (Figure 4.20B and C). Previous data showed that cells from mice immunized only with pMOG / CpG / IFA produced very little IFN-γ and IL-17 in response to pMOG (figure 4.15B). The enhanced production of IFN-γ in double immunized mice suggested that double immunization generated a strong IFN-γ-producing memory response.

4.3 Discussion

This chapter aimed to establish a memory model of EAE by developing a double immunization system. Mice immunized with pMOG / CpG / IFA can develop a weak Th1 and Th17 response and generate anti-pMOG IgG1 but a subsequent injection of soluble pMOG did not induce anaphylaxis. Mice receiving a primary immunization of pMOG / CpG / IFA and a secondary immunization of pMOG / CFA supplemented with PTX can develop EAE with an early onset and delayed recovery.

In adoptive transfer systems, immunization can activate and expand the transferred TCR transgenic cells whose number subsequently contracts to a low level. Tg4 T cell transferred mice receiving Ac1-9 / CFA had an equal frequency of Tg4 T cells compared to non-primed mice 4 weeks after immunization (Figure 4.1). Of course, it is possible that a certain frequency of memory T cells may have entered the non-lymphoid tissues. In the spleen, the surviving Tg4 T cells in primed mice expressed more CD44 and less CD62L compared to PBS treated mice (Figure 4.2), suggesting that the primary immunization had provided an Ac1-9 reactive memory T cell pool.

Different protocols of double immunizations were tested for inducing a memory response of EAE in mice adoptively transferred with $CD4^+$ T cells from TCR transgenic mice, but most protocols failed to induce disease (Figure 4.3, 4.4 and 4.8). Notably, transferred Tg4 cells could be triggered to proliferate by primary immunization with Ac1-9 / CFA and an injection of soluble Ac1-9_{4Y} after Ac1-9 / CFA immunization abrogated T cell proliferation, no matter whether the Ac1-9 / CFA immunized mice were given secondary immunization or not (Figure 4.7 and 4.9). This suggests that memory T cells can be tolerized with the administration of soluble peptide. However, soluble peptide injection through the i.v. route to antigen experienced mice can also induce a fatal anaphylaxis (Table 4.1). This side effect is risky in peptide-based therapy.

The Steinman Lab has reported that mouse death occurs within 30 minutes in 43 % of $PLP_{139-151}$ / CFA primed mice challenged with soluble $PLP_{139-151}$ through the i.p. route 28 days after primary immunization (Pedotti *et al*., 2001). In this chapter, 15 %

of Ac1-9 / CFA immunized B10.PL mice and 50-75 % of pMOG / CFA immunized C57BL/6 mice developed anaphylaxis after soluble peptide ($Ac1\text{-}9_{4Y}$ and pMOG, respectively) injection i.v. In another study, i.v. injection of soluble myelin derived peptides was tested in different strains of mice and 90 % of $PLP_{139\text{-}151}$ / CFA immunized mice and showed that 80 % of pMOG / CFA immunized mice developed anaphylactic responses after relevant soluble peptide challenge post immunization (Smith *et al*., 2005). In the antigen-experienced mice, antigen-specific antibodies are generated and sustained in the serum. These antibodies can bind to mast cells by ligations of high-affinity IgE receptors (FcεRI) or IgG receptors (FcγRs) (Miyajima, 1997). Upon challenge with the same soluble antigen, ligation of mast cell-bound IgE or IgG1 can activate mast cells, triggering the release of several immune mediators including histamine, prostaglandin and leukotriene, causing anaphylaxis (Malbec and Daeron, 2007). It has been shown that an administration of anti-pMOG IgG1 can aggravate EAE in a complement-dependent but not FcR-independent fashion (Urich *et al*., 2006). It has been reported that antibody-induced anaphylaxis depends on IgE / FcεRI, but not IgG1/ FcRIII (Smith *et al*., 2005). However, in that study, 25 % of anti-IgE treated mice still suffered anaphylaxis after secondary challenge of peptide in soluble form, suggesting that factors other than IgE could induce anaphylaxis. Data in this chapter suggest that high titres of anti-pMOG IgG1 in pMOG / CFA immunized mice were a possible factor for inducing anaphylaxis in subsequent peptide challenge (Figure 4.18B). As antigen-specific IgE is often pre-bound to mast cells, it is possible that pMOG-specific IgE had been generated, but that this could not be assessed by ELISA of serum (Chen *et al*., 1995). To clarify the existence of anti-pMOG IgE, a protocol for labeling mast cell-bound, pMOG specific IgE would be needed in the future.

In the later experiments using pMOG / CpG / IFA as a primary immunization mice did not develop anaphylactic signs even after an i.v. injection of high doses of soluble pMOG (Table 4.1), although anti-pMOG IgG1 were still generated in primed mice (Figure 4.18A). It is not clear why mice primed with pMOG / CFA developed anaphylaxis after soluble pMOG challenge but mice primed with pMOG / CpG / IFA did not. In mice, IgG1 isotype switching is known to be partially dependent upon IL-4 (Coffman *et al*., 1988). IgG1 has been reported to comprise two functionally distinct phenotypes. One of these IgG1 phenotypes is able to induce passive cutaneous anaphylaxis (PCA) which is IL-4 dependent and the other type of IgG1 lacks PCA ability which is IL-4 independent (Faquim-Mauro *et al*., 1999). The difference between two types of IgG1 has been found to be caused by the binding ability to the MC that is closely related to N-glycosylation of IgG1 molecules (Faquim-Mauro *et al*., 2003). In addition, IL-10, IL-12 and IFN-γ can inhibit the generation of IL-4-dependent IgG1, suggesting that anaphylaxis active IgG1 molecules tend to be generated in a ¡Th2 environment¡ (Faquim-Mauro *et al*., 1999; Faquim-Mauro and Macedo, 2000; Silva *et al*., 2006). Different TLR stimulation is thought to be able to affect antibody class switching, therefore different types of antibodies may be generated in response to the different adjuvants (Faquim-Mauro *et al*., 2000). Therefore mice immunized with pMOG / CFA may have generated ¡dangerous IgG1¡ whilst mice immunized with pMOG / CpG / IFA did not (without anaphylaxis, table 4.1). Recently, we used a novel strategy of APL administration to test a therapeutic potential in antigen-experienced mice, by avoiding anaphylaxis. Single residue substitutions were made in $MOG_{35¡50}$ and it was found that an APL with a substitution from Valine to Alanine on position 37 ($MOG_{35¡50}$ 37Ala) can

retain TCR binding but not antibody binding, suggesting that position 37 is a possible BCR binding site. Therefore administration of $MOG_{35¡50}$ 37Ala did not induce anaphylaxis in antigen experienced mice, suggesting that the TCR contact and antibody contact residues were sufficiently diverse (Leech *et al*., 2007).

Injection of PGN derived from different bacterial strains can induce chronic arthritis and colitis in susceptible rodents (Sartor *et al*., 1996; Onta *et al*., 1993). However, in this chapter, mice immunized with pMOG / PGN / IFA induced a weak T cell proliferation, failed to induce production of IL-2, IFN-γ and IL-17 and failed to induce EAE (Figure 4.14 and 4.15). PGN has been shown to facilitate development of autoimmune disease. The Laman group has compared soluble PGN (sPGN, prepared from *S. aureus* by gel-permeation chromatography) and in insoluble form of PGN (iPGN, as used in this chapter) and found that immunization with low dose (25 £g) of pMOG / sPGN / IFA can induce EAE in C57BL/6 mice. In comparison, administration of high dose of iPGN (250 £g, as used in this chapter) induces a delayed-onset and weak disease (*Visser et al., 2005*). PGN triggers TLR2 and induces IFN-γ producing Th1 response, however, iPGN is less capable of activating DC *in vitro* (Salgame, 2005; *Visser et al., 2005*), suggesting that mice need a stronger stimulation for Th1 and Th17 responses when using iPGN as an adjuvant *in vivo*. In the result shown in this chapter, double immunization with pMOG / PGN / IFA first and pMOG / CFA secondarily resulted in development of EAE with early onset in 2D2 T cell transferred mice. In the absence of 2D2 T cells, pMOG / PGN / IFA induced early initiation of EAE compared to pMOG / CFA, suggesting a weak memory response is induced in mice compared to pMOG / CpG / IFA (Figure 4.12 and 4.13). On the other hand, mice immunized with pMOG / CpG / IFA had a

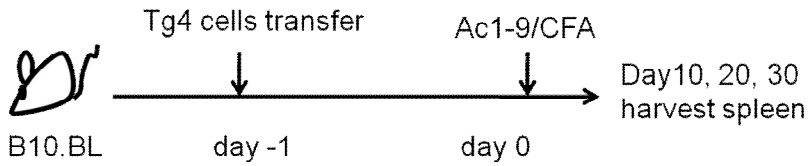
comparable T cell proliferation and IL-2 production and a mild Th1 and Th17 response, compared to mice immunized with pMOG / CFA (Figure 4.14 and 4.15). Segal has reported that SJL mice can develop EAE with an immunization with MBP_{87-106} / CpG /IFA (Segal *et al*., 2000), immunization with pMOG / CpG / IFA did not induce EAE in C57BL/6 mice in this project. Nevertheless, the weak Th1 and Th17 responses triggered by primary immunization with pMOG / CpG / IFA were not themselves pathogenic but did allow a memory response which could be triggered by subsequent exposure to pMOG / CFA (Figure 4.14, 4.15). This provoked an EAE that developed one day ahead of the pMOG / CFA immunized control mice and showed a delayed-recovery phase (Figure 4.19 and Table 4.2). As the high levels of IFN-γ production is the hallmark of Th1 memory response (Foulds *et al*., 2006), the enhanced production of IFN-γ in cells from CpG-CFA double immunized mice indicates that such a memory response is induced.

Notably, mice receiving a primary immunization with pMOG / CpG or pMOG / CFA without injections of PTX had a reduced frequency of IFN-γ and IL-17 producing cells, suggesting PTX can enhance Th1 and Th17 responses (Figure 4.16D and E). PTX can induce the maturation of DC, which results in the expansion of effector T cells and the differentiation of both Th1 and Th2 cells (Hofstetter *et al*., 2002; Wakatsuki *et al*., 2003; Hou *et al*., 2003). Recent data found that *in vivo* treatment with PTX induced IL-17-secreting T cells in wild type mice, suggesting that PTX can augment Th17 responses (Chen *et al*., 2007). Interestingly, data in this chapter show that primary immunization with PTX also increased the $CD4^+$ $CD25^+$ $Foxp3^+$ Treg cells in mice, compared to non-immunized, pMOG / CpG / IFA and pMOG / CFA immunized groups (without PTX, Figure 4.17). This seems to conflict with the

previous reports that PTX treatment can suppress Treg cell numbers and functions (Chen *et al*., 2006; Cassan *et al*., 2006). This difference observed may relate to the timing of investigation. Other groups measured Treg cells within 2 weeks of PTX treatment, whereas in this project, data were collected at day 28 to fit with the timing of the secondary immunization. Cassan¡s paper also showed a full recovery and even a modest increase in Treg frequency 4 weeks after PTX treatment, suggesting that PTX can reduce Treg cells in a short time but the increased inflammation triggered by PTX can further induce Treg (probably iTreg cells) generation (Chen *et al*., 2006).

Overall, immunization of pMOG / CpG / IFA triggers a weak Th1 and Th17 response which is not strong enough to induce EAE by itself. Although mice receiving pMOG / CpG / IFA generate anti-pMOG IgG1, it does not trigger anaphylaxis after subsequent challenge of soluble pMOG, indicating a safe system in which to test for tolerance induction. Double immunization with pMOG / CpG / IFA and then pMOG CFA provides a system to study pMOG-reactive memory responses. Thus, the work described in this chapter allowed the subsequent studies on whether peptide induced tolerance could silence an autoaggressive memory response.

A



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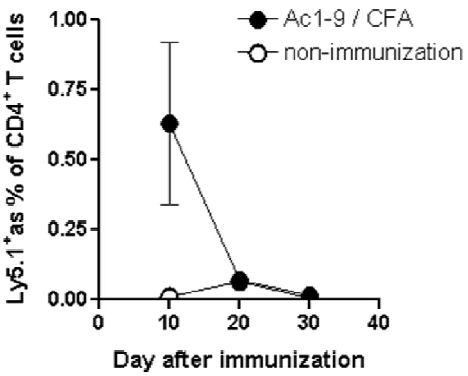

Figure 4.1 Maintenance of Tg4 cells in host mice. *A*, B10.PL mice received 2 x 10^6 $CD4^+Ly5.1^+$ Tg4 T cells and primed with 100 £g of Ac1-9 / CFA through s.c. route one day later. On days 10, 20 and 30 after immunization, spleens were harvested for staining. *B*, data show $CD4^+Ly5.1^+$ T cells as a percentage of $CD4^+$ T cells. Data are from one experiment, 3 mice in each group. Symbols represent mean ¡ SEM and SEMs reflect samples from different mice.

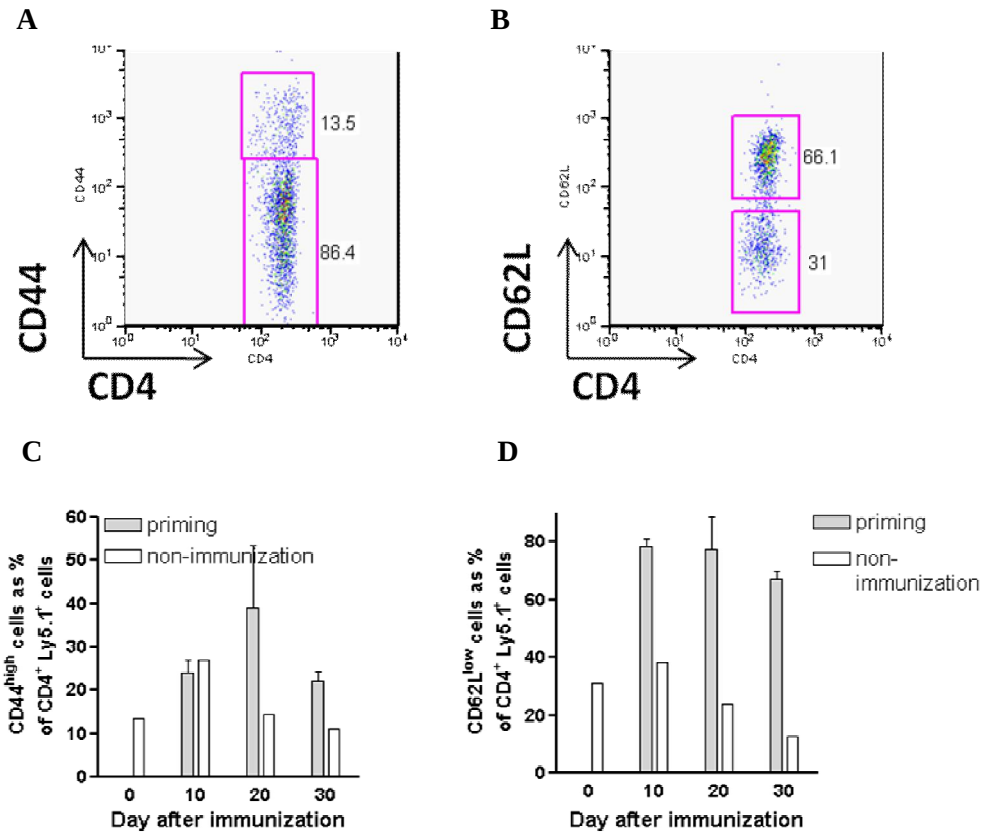

Figure 4.2 Tg4 T cells express memory markers after priming. The immunization protocol is shown in figure 4.1A. $CD4^+$ $Ly5.1^+$ (Tg4) cells were gated into two populations by the expression of CD44 (A) or CD62L (B) (A and B represent the cells from PBS treated mouse). *C* and *D* show the frequency of $CD44^{high}$ cells (C) and $CD62L^{low}$ cells (D) as a percentage of Tg4 cells. Data are from one experiment, 3 mice in each group. Bars represent mean ¡ SEM and SEMs reflect samples from different mice.

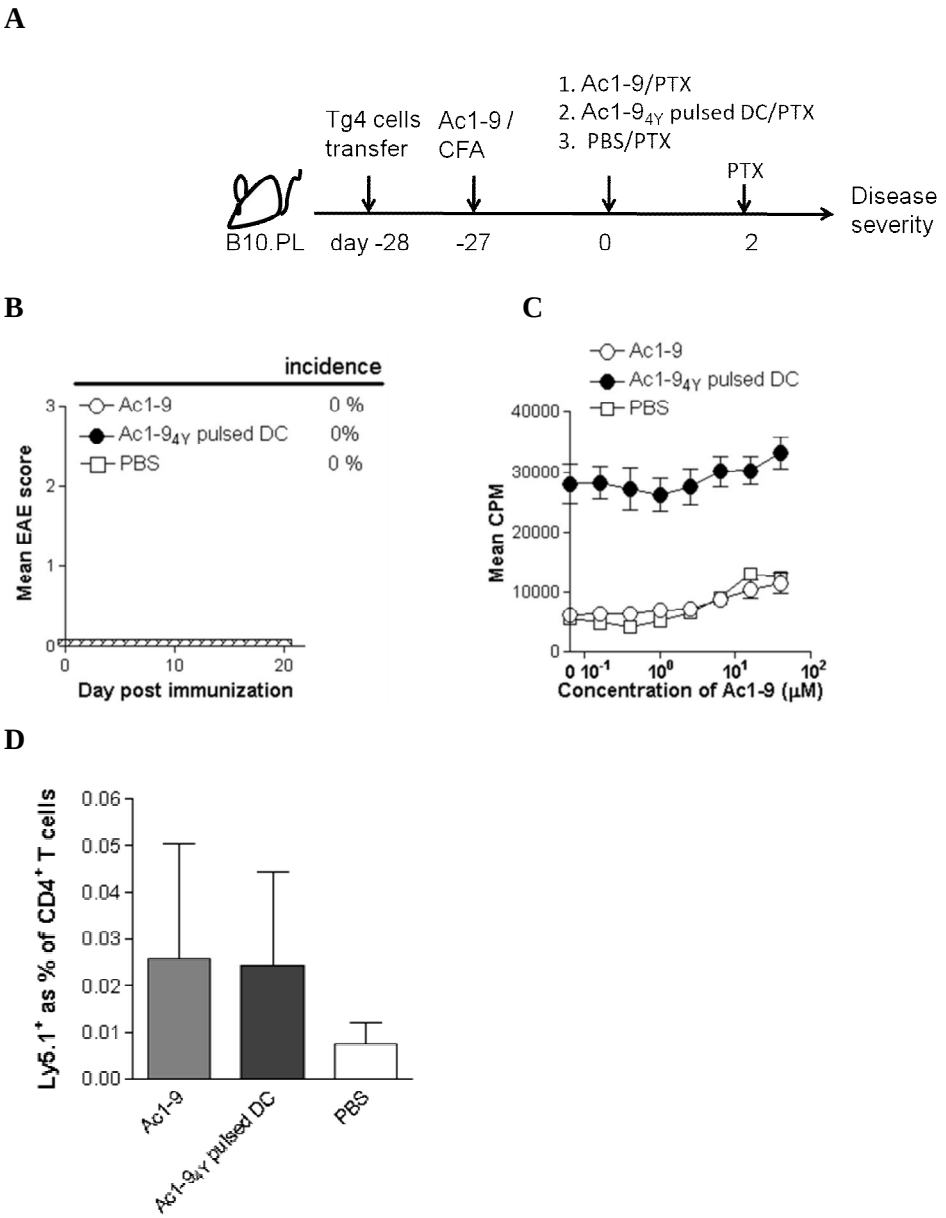

Figure 4.3 Ac1-9$_{4Y}$ pulsed DC are insufficient to induce EAE in antigen experienced mice. *A*, B10.PL mice were given 3 x 10^5 CD4$^+$Ly5.1$^+$ Tg4 cells on day -28 and immunized with 100 £g of Ac1-9/CFA s.c. one day later. On day 0, mice were injected with either 200 £g of Ac1-9 in PBS, 5 x 10^5 of Ac1-9$_{4Y}$-pulsed DCs or PBS i.v. 200 ng of PTX was given on day 0 and day 2. *B*, Disease severity post secondary immunization. *C*, 10 days post secondary immunization, spleen cells were harvested and cultured for 72 hours with a pulsing of thymidine for the last 18 hours. *D*, plot shows the frequency of CD4$^+$ Ly5.1$^+$ (Tg4) cells as a percentage of total CD4$^+$ cells. Data are from one experiment and represent the mean ¡ SEM. SEMs reflect samples from different mice (4-5 mice in each group).

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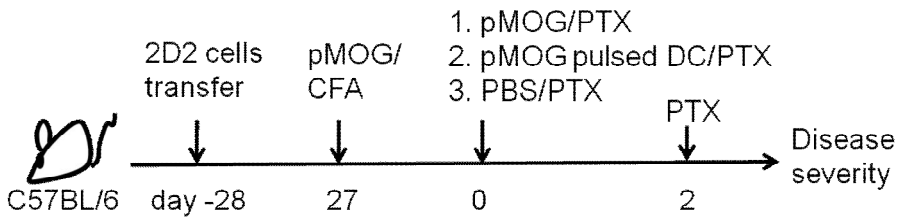

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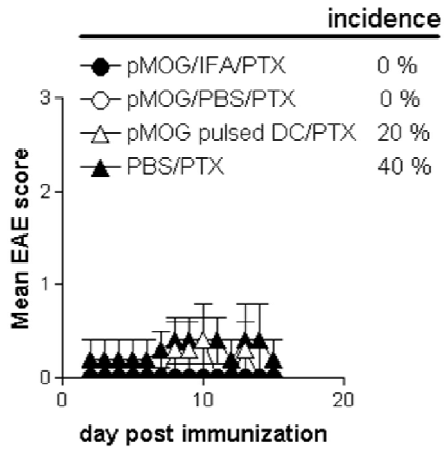
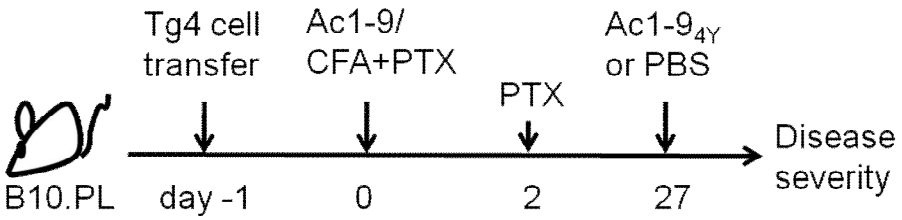


Figure 4.4 pMOG pulsed DC are insufficient to induce EAE in antigen experienced mice. *A*, C57BL/6 mice were given 2 x 10^6 $CD4^+$ $Thy1.1^+$ 2D2 cells on day -28 and immunized with 100 £g of pMOG/CFA s.c. one day later. On day 0, mice were injected with either 200 £g of pMOG in PBS, 5 x 10^5 of pMOG-pulsed DC or PBS i.v. 200 ng of PTX was given on day 0 and day 2. *B*, data show disease severity post immunization. Data are from one experiment and represent the mean score ¡ SEM, 5 mice in each group.

A



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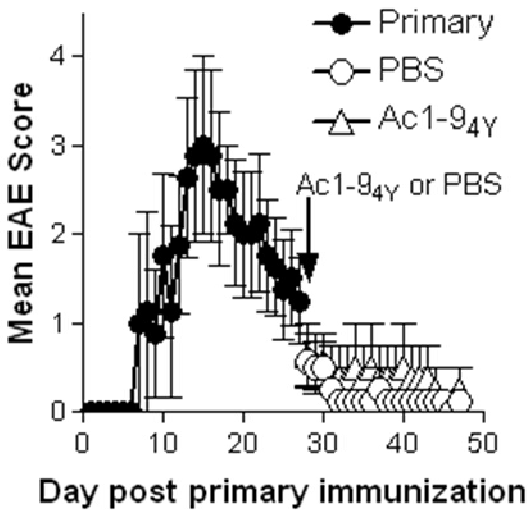


Figure 4.5 Peptide administration in the absence of adjuvant does not re-induce EAE in Ag-experienced mice. *A*, B10.PL mice were given 3 x 10^5 $CD4^+Ly5.1^+$ Tg4 cells and were immunized with 100 £g of Ac1-9/CFA one day later (with PTX on day 0 and day 2). Mice were i.v. injected with 200 £g of Ac1-9_{4Y} or PBS on day 27. *B*, disease severity was scored daily post primary immunization. Data are from one experiment and show mean ¡ SEM (3-4 mice in each group).

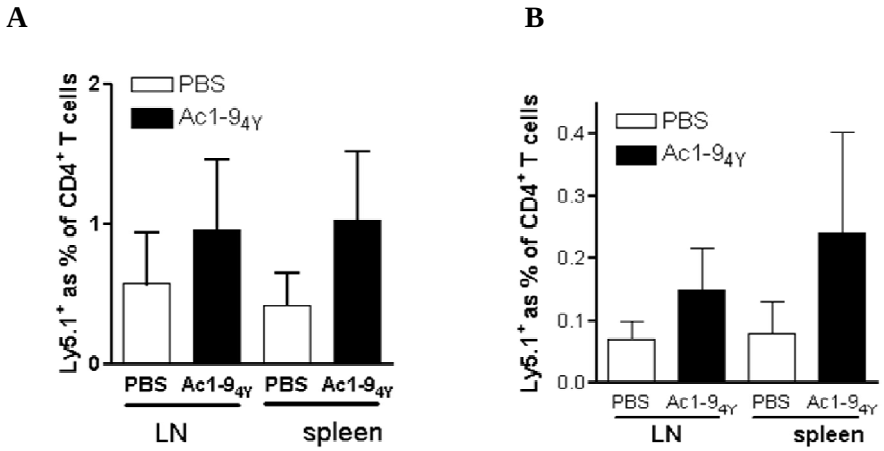


Figure 4.6 Number of Tg4 cells in Ac1-9-experienced mice given soluble Ac1-9$_{4Y}$. The immunization protocol is shown in figure 4.5A. Spleen and draining lymph node (LN) cells were harvested on day 30 (A) an day 47 (B) post immunization. Plots show the proportion of CD4$^+$ Ly5.1$^+$ Tg4 cells as a percentage of total CD4$^+$ T cells. Data are from one experiment and show the mean ¡ SEM (3 mice in each group).

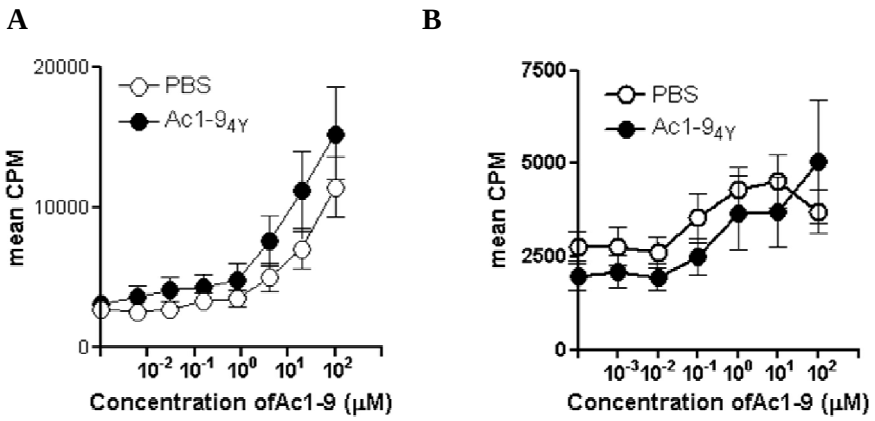
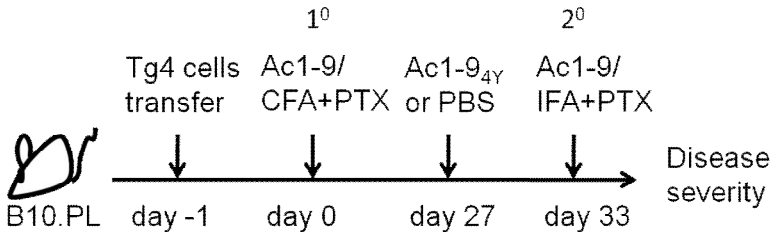


Figure 4.7 Soluble Ac1-9$_{4Y}$ administration does not re-activate T cells in Ag-experienced mice. The immunization protocol is shown in figure 4.5A. Spleen cells were harvested on day 30 (A) and day 47 (B) post immunization and cultured for 72 hours with a pulsing of thymidine for the last 18 hours. Data are from one experiment and represent the mean CPM ¡ SEM (3 -4 mice in each group).

A



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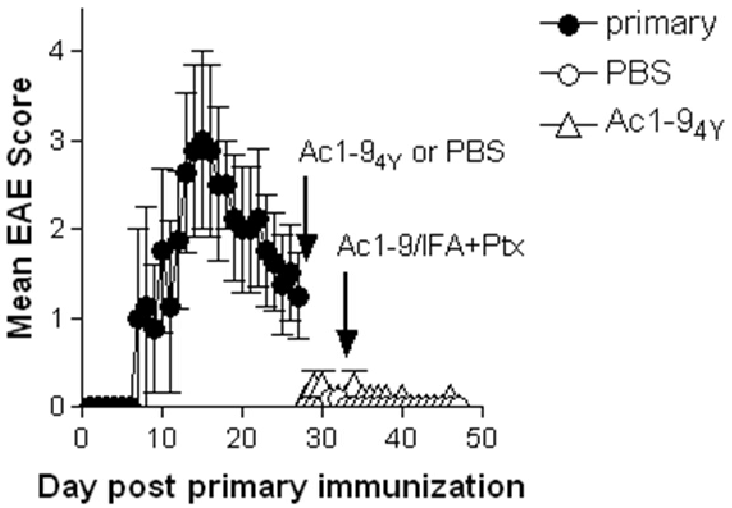

Figure 4.8 Immunization with Ac1-9 in IFA does not re-induce EAE in Ac1-9 experienced mice. *A*, B10.PL mice were given 3 x 10^5 of $CD4^+Ly5.1^+$ Tg4 cells and immunized with 100 £g of Ac1-9/CFA plus PTX one day later. Mice were injected with 200 £g Ac1-9_{4Y} or PBS through i.v. route on day 27 and subsequently given secondary immunization with Ac1-9/IFA (with PTX on day 33 and day 35). *B*, disease severity was scored daily after primary immunization. Data are from one experiment and show mean ¡ SEM (4 -5 mice in each group).

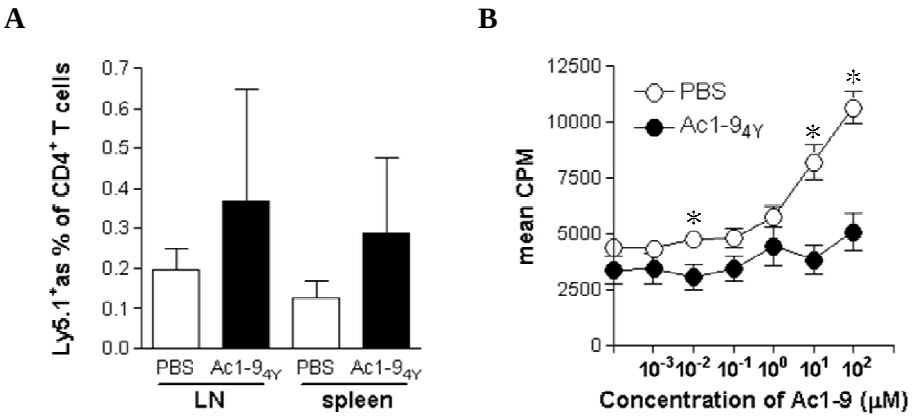

Figure 4.9 Administration of soluble Ac1-9$_{4Y}$ induces T cell unresponsiveness. The immunization protocol is shown in figure 4.8A. Spleen and draining lymph node (LN) cells were harvested on day 47 (day 14 after secondary immunization). *A*, plot shows the frequency of $CD4^+$ $Ly5.1^+$ cells as a percentage of $CD4^+$ T cells. *B*, spleen cells were cultured for 72 hours with a pulsing of thymidine for the last 18 hours. Data are from one experiment and show mean ¡ SEM (4 -5 mice in each group).

Table 4.1 Incidence of anaphylaxis in the antigen-experienced mice after i.v. or i.p. injection of soluble peptide 4 weeks after immunization

Primary immunization	Injection of soluble peptide	Dose and route	Frequency of mice with anaphylaxis
Ac1-9 / CFA	$Ac1\text{-}9_{4Y}$	200 μg i.v.	15 % (2 / 13)
pMOG / CFA	pMOG	10 μg i.p.	0 % (0 / 3)
pMOG / CFA	pMOG	50 μg i.p.	0 % (0 / 1)
pMOG / CFA	pMOG	100 μg i.p.	0 % (0 / 1)
pMOG / CFA	pMOG	200 μg i.p.	75 % (3 / 4)
pMOG / CFA	pMOG	300 μg i.v.	50 % (1 / 2)
pMOG/CpG/IFA	pMOG	10 μg i.v.	0% (0 / 6)
pMOG/CpG/IFA	pMOG	50 μg i.v.	0% (0 / 16)
pMOG/CpG/IFA	pMOG	200 μg i.v.	0% (0 / 16)
pMOG/CpG/IFA	pMOG	500 μg i.v.	0% (0 / 6)

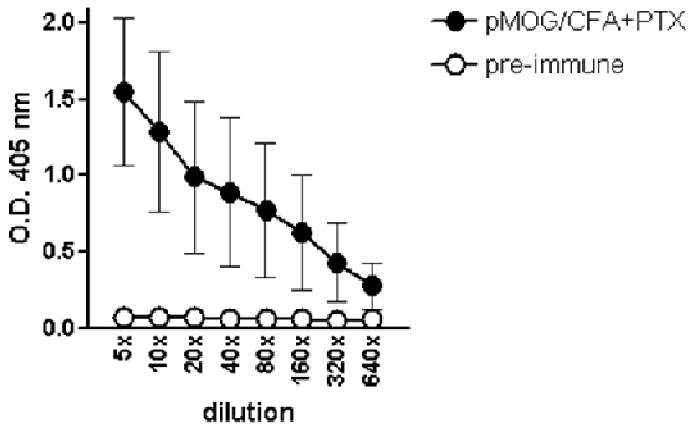

Figure 4.10 Mice produce anti-pMOG antibodies after pMOG/CFA immunization. Pre-immune sera were from C57BL/6 mice collected 7 days before immunization. The mice were immunized with 100 £g of pMOG/CFA plus PTX and bled on day 28 after immunization. The titre of anti-pMOG IgG1 was assessed by ELISA. Asterisks represent significant difference between samples from immunized and non-immunized mice at indicated dilution ($p < 0.05$ by Unpaired T-test). Data are from one experiment and represent the mean ¡ SEM (7 mice in each group).

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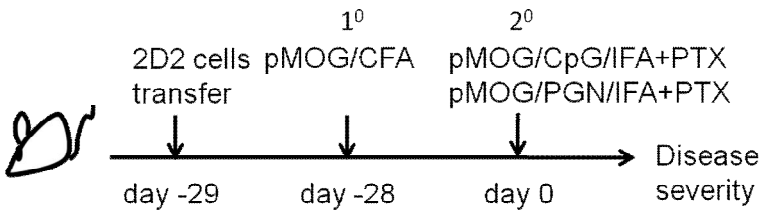

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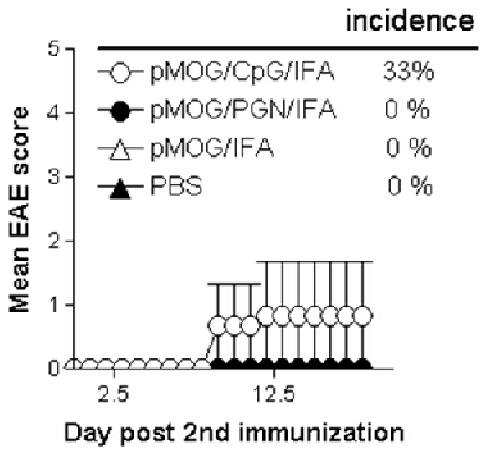

Figure 4.11 Primary immunization with pMOG/CFA, followed by pMOG plus alternative adjuvant, is insufficient to induce EAE. *A,* C57BL/6 mice received 2 x 10^6 $CD4^+$ $Thy1.1^+$ (2D2) T cells on day -29 and were immunized with either 100 £g of pMOG/CFA on day -28. Secondary immunization with pMOG/CpG/IFA (pMOG : CpG = 50 £g : 60 £g), pMOG / PGN / IFA (pMOG : PGN= 50 £g : 250 £g), 50 £g of pMOG/IFA or PBS was on day 0 (with administration of PTX on day 0 and day 2). *B,* disease severity was scored daily post secondary immunization. Data are from one of two repeated experiments and represent the mean ¡ SEM (3 mice in each group).

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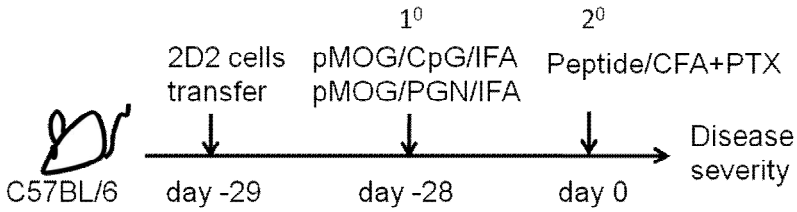

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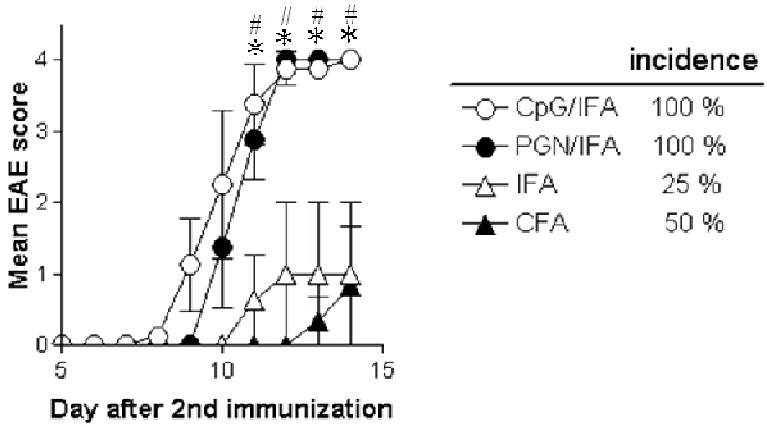
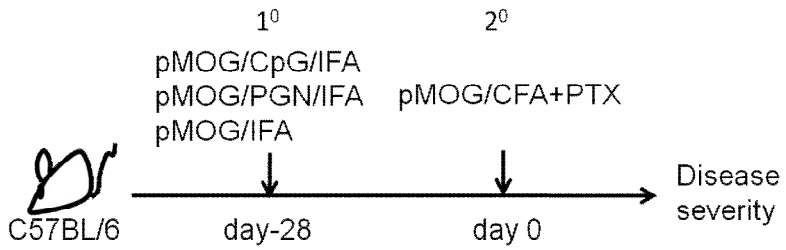


Figure 4.12 Mice receiving a double immunization develop a memory response. *A*, C57BL/6 mice received 2 x 10^6 $CD4^+$ $Thy1.1^+$ 2D2 cells on day -29 and were immunized with pMOG/IFA, pMOG/CpG/IFA (pMOG : CpG = 50 £g : 60 £g) or pMOG/PGN/IFA (pMOG : PGN= 50 £g : 250 £g) on day -28. Secondary immunization was with pMOG/CFA on day 0 (with PTX on day 0 and day 2). Control mice were given an immunization of pMOG/CFA plus PTX on day 0. *B*, disease severity was scored daily post secondary immunization. Data represent the mean ¡ SEM disease. Asterisks represent significant difference between CpG and CFA groups. Hash symbols represent significant difference between PGN and CFA groups ($p < 0.05$T test). Data are from one of two experiments and represent mean ¡ SEM (4 mice in each group).

A



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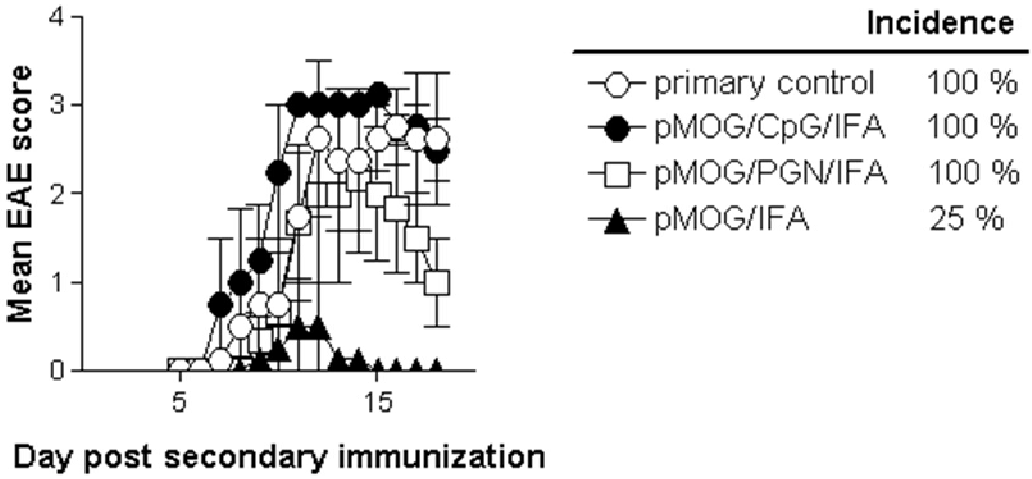

Figure 4.13 Mice receiving double immunization develop EAE with earlier onset without adoptive transfer of autoreactive T cells. *A*, C57BL/6 mice were immunized with 50 £g of pMOG/IFA, pMOG/CpG/IFA (pMOG : CpG = 50 £g : 60 £g) or pMOG/PGN/IFA (pMOG : PGN= 50 £g : 250 £g) on day -28. Secondary immunization was with 100 £g of pMOG/CFA on day 0 (with administration of PTX on day 0 and day 2). Control mice were given an immunization of pMOG/CFA plus PTX on day 0. *B*, disease severity was scored daily post secondary immunization. Data are from one experiment and represent mean ¡ SEM (3-4 mice in each group). There was no significant difference between control and CpG groups and between control and PGN groups. ($p < 0.05$ between CpG and PGN groups, analyzed by Mann-Whitney U test).

Table 4.2 Incidence of EAE by immunization with pMOG in different adjuvants

Primary immunization	Secondary immunization	Incidence of EAE	Mean of disease onset (day)
pMOG/CFA	pMOG/CpG	4/8 (50%)	8.3
pMOG/CFA	pMOG/PGN	0/8 (0 %)	—
pMOG/CFA	pMOG/IFA	0/3 (0 %)	—
pMOG/CpG	pMOG/CFA	29/39 (74%)	9.9
pMOG/PGN	pMOG/CFA	12/16 (75%)	11.1
pMOG/IFA	pMOG/CFA	4/16 (25%)	13.3
—	pMOG/CFA	34/43 (79%)	11

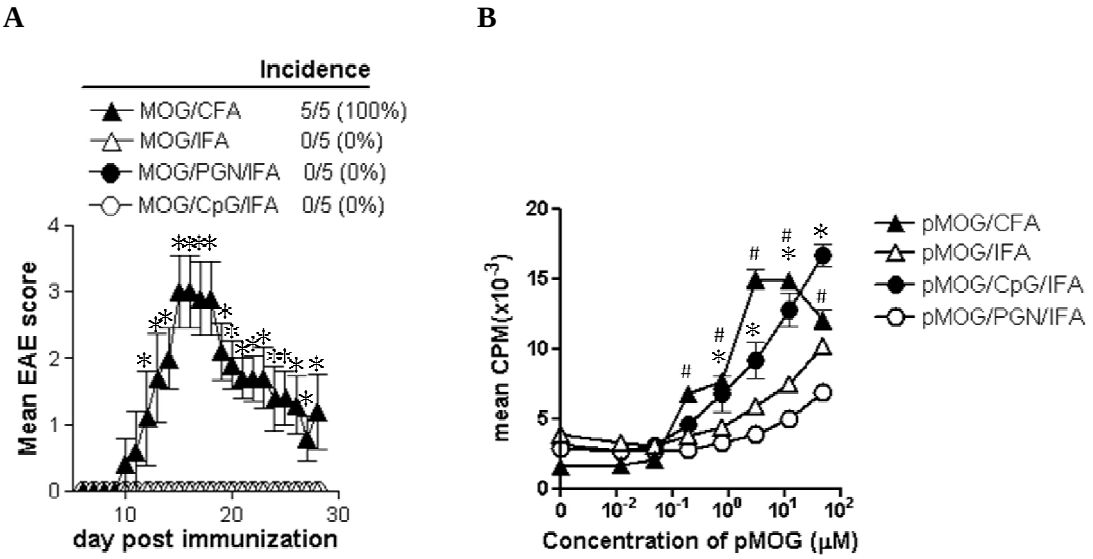

Figure 4.14 Mice given a single immunization with pMOG/CpG/IFA or pMOG/PGN IFA do not develop EAE. C57BL/6 mice were immunized with 100£g of pMOG/CFA, 100£g of pMOG/IFA, pMOG/CpG/IFA (pMOG : CpG = 100 £g : 120 £g) or pMOG/PGN (pMOG : PGN= 100 £g : 250 £g) (with administration of PTX on day 0 and day 2). *A*, disease severity was scored daily post immunization. Asterisks represent significant difference between CFA and other groups ($p < 0.01$ by Unpaired T-test). *B*, spleen cells were harvested on day 28 post immunization and cultured for 72 hours with a pulsing of thymidine for the last 18 hours. Data are from one experiment and represent the mean CPM ¡ SEM. Asterisks represent significant difference between CpG and PGN groups. Hash symbols represent significant difference between CFA and PGN groups ($p < 0.05$ by Unpaired T-test, 5 mice in each group).

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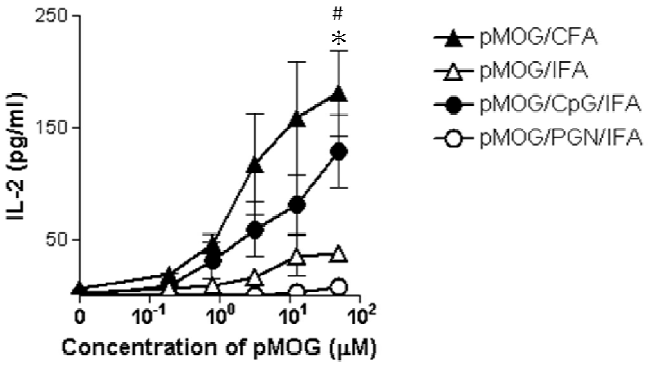

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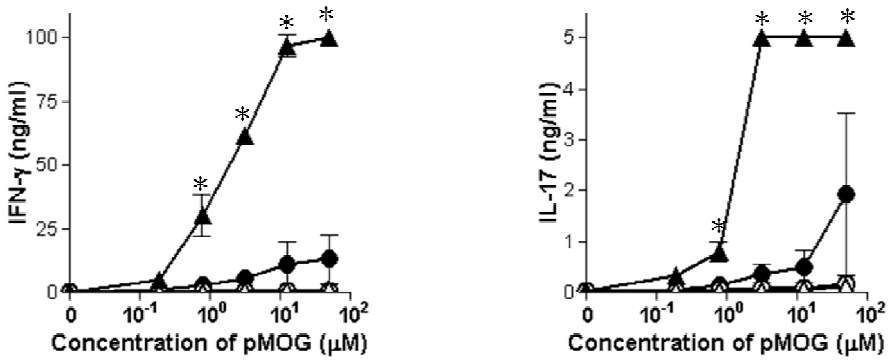


Figure 4.15 Cytokine productions in mice receiving pMOG/CpG/IFA or pMOG/PGN/IFA. The immunization protocol is shown in figure 4.13. On day 28, spleen cells were harvested and cultured with pMOG for cytokine ELISA. Data represent the mean ¡ SEM. *A*, production of IL-2 (48hours). Asterisk represents significant difference between CFA and PGN groups ($p < 0.05$). Hash symbol represents significant difference between CpG and PGN groups ($p < 0.05$). *B* and *C*, production of IFN-γ and IL-17 (72 hours). Asterisks represent significant difference between CFA and other groups ($p < 0.05$). Data are from one of three experiments (3 mice in each group).

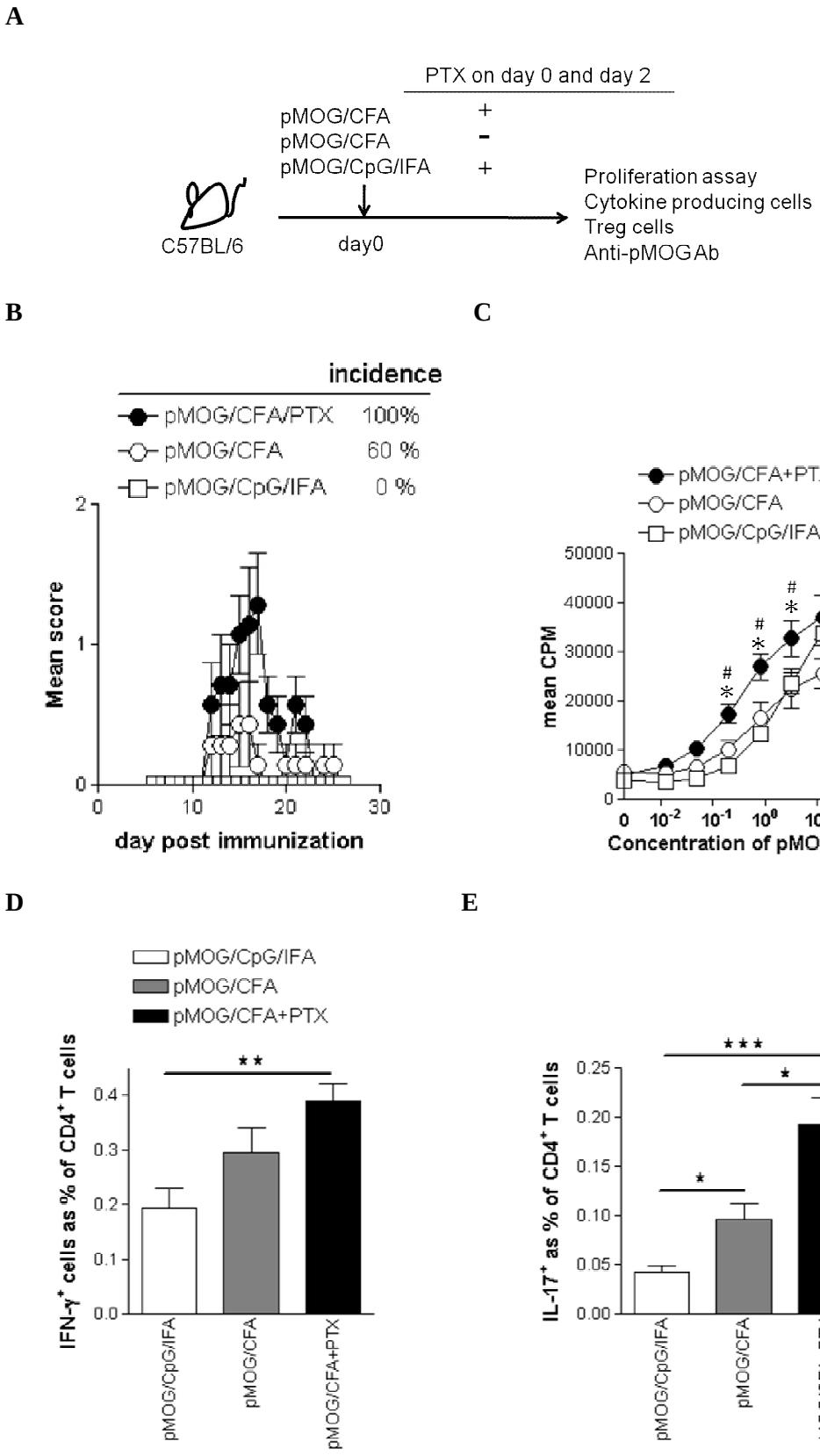


Figure 4.16 Fewer Th1 and Th17 cells develop in mice immunized with

pMOG/CpG/IFA. *A*, mice were immunized with either pMOG/CFA (together with administration of PTX on day 0 and day 2), 100 £g of pMOG/CFA (without PTX) or 100 £g of pMOG/CpC/IFA (pMOG : CpG = 100 £g : 120 £g, with PTX). Spleens were harvested 28 days after immunization for assays. *B*, disease severity was scored daily post immunization. *C*, spleen cells were cultured for 72 hours with a pulsing of thymidine for the last 18 hours. *D* and *E*, cells were cultured in medium with 10 £g of pMOG overnight and stained for intracellular cytokines. Plots show IFN-γ^+ cells (C) and IL-17^+ cells (D) as a percentage of $CD4^+$ T cells. Data represent the mean ¡ SEM. Asterisks represent significant difference (★ represents $p < 0.05$; ★★ represents $p < 0.01$; ★★★ represents $p < 0.001$ by Unpaired T-test). Data are from one experiment (6-7 mice in each group).

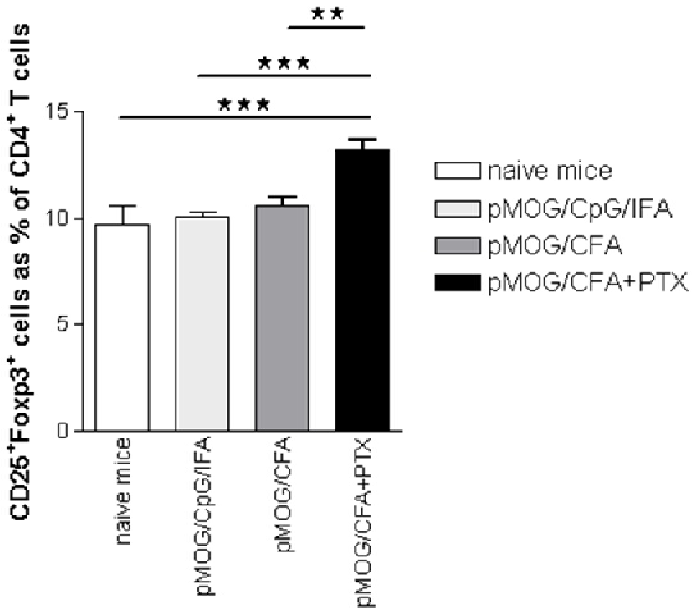
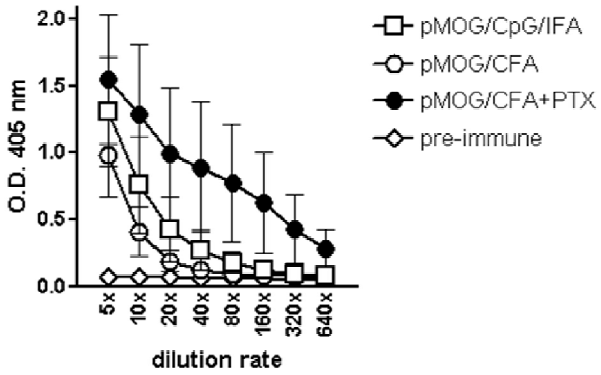


Figure 4.17 Immunization with pMOG / CFA / PTX results in an increased frequency of Treg cells within the spleen. The immunization protocol is shown in figure 4.16A. Spleen cells were stained for CD4, CD25 and Foxp3 on day 28 post immunization. Plot shows the $CD4^+$ $CD25^+$ $Foxp3^+$ cells as a percentage of $CD4^+$ T cells. Data represent the mean ¡ SEM. Asterisks represent significant difference (★ represents $p < 0.05$; ★★ represents $p < 0.01$; ★★★ represents $p < 0.001$ by Unpaired T-test). Data are from one experiment (6-7 mice in each group).

A



B

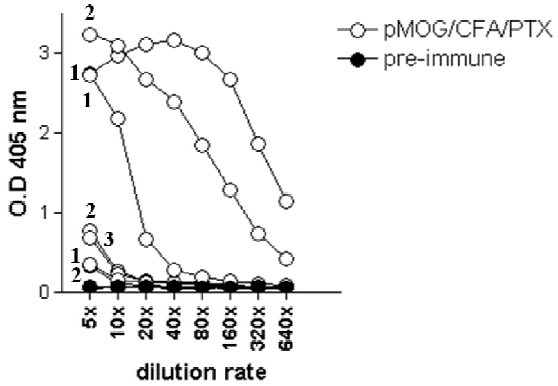
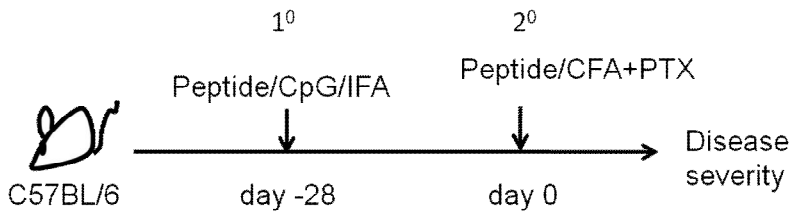


Figure 4.18 Mice immunized with pMOG/CFA plus PTX develop high titres of anti-pMOG IgG1. The immunization protocol is shown in figure 4.16A. *A*, sera were collected on day 28 post immunization and assessed for anti-pMOG IgG1 by ELISA. Data show mean optical density (O.D.) ¡ SEM at 405 nm (6 mice in the group). *B*, Titres of anti-pMOG IgG1 were assessed from the serum of individual mice in the pMOG/CFA/PTX immunized group. Numbers represent the highest EAE score of each mouse. Data are from one experiment (6-7 mice in each group).

A



B

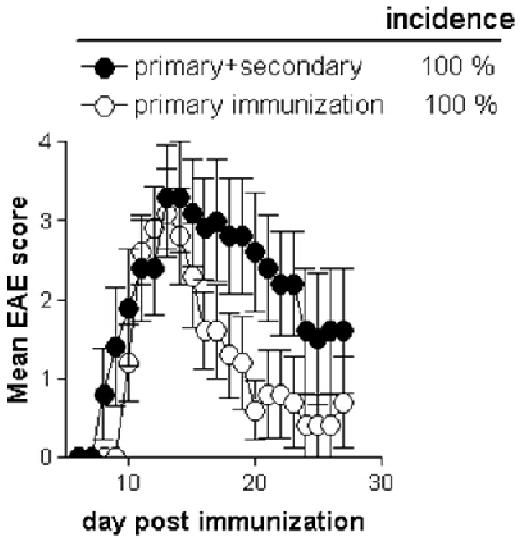


Figure 4.19 Mice receiving pMOG/CpG/IFA as a primary immunization develop EAE with an earlier onset and slower recovery after a secondary injection of pMOG/CFA and PTX. *A,* mice were s.c. immunized with pMOG/CpC/IFA (pMOG : CpG = 50 £g : 60 £g) on day -28 and induced to develop EAE with 100 £g of pMOG/CFA on day 0 (with PTX on day 0 and day 2). Control group was given 100 £g of pMOG/CFA on day 0 with PTX on day 0 and day 2. *B,* disease severity was scored daily post secondary immunization ($p < 0.01$ by Mann-Whitney U test. Data are from 3 experiments and show mean ¡ SEM (5 mice in each group).

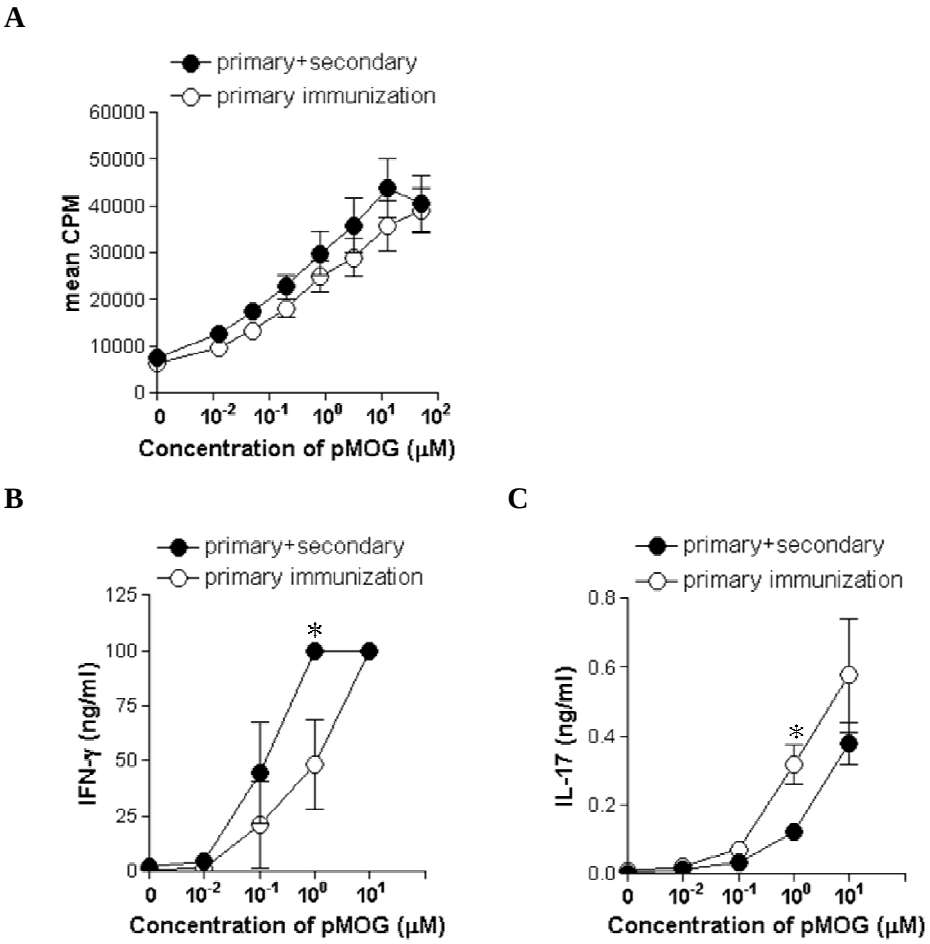


Figure 4.20 Comparison of proliferation and cytokines production in the cells from primary and secondary immunized mice. The immunization protocol is shown in figure 4.19A. *A*, spleen cells were harvested on day 27 and cultured for 72 hours with a pulsing of thymidine for the last 18 hours. *B* spleen cells were culture for 72 hours and medium was collected for cytokine assay by ELISA. Plots show the amount of IFN-γ (B) and IL-17 (C) in the medium with various doses of pMOG. Asterisks represent significant difference ($P < 0.05$). Data are from one of three and experiments show mean ¡ SEM (3 mice in each group).

Chapter 5. Peptide induced tolerance in ¡memory EAE¡

5.1 Introduction

In previous chapters, two models for investigating peptide induced T cell tolerance were developed; the first a ¡naive EAE model¡ and the second a ¡memory EAE model¡. The latter was designed to assess memory T cells in myelin immunized mice. Memory T cells possess several properties crucial for their function, including rapid reactivation upon antigen stimulation, wide tissue distribution, and their ability to survive and self-renew for long periods in the absence of cognate antigen (Sprent and Surh, 2002; Sojka *et al.*, 2004; Williams and Bevan, 2007). Also, memory T cells have been shown to have a lower TCR threshold for activation and to enter cell cycle faster than naive T cells, suggesting that memory T cells are easier to trigger than naive T cells (Rogers *et al.*, 2000). In human studies of MS, the frequency of memory CD4 T cells ($CD4^+$ $CD3^+$ $CD25^+$ $CD45RO^+$) was significantly increased in the peripheral blood and cerebrospinal fluid (CSF) of active MS patients and patients undergoing relapse, compared with inactive MS patients and patients in remission (Okuda *et al.*, 2005). In addition, increases of $CD8^+$ $CCR7^+$ $CD45RA^-$ central memory and $CD8^+$ $CCR7^-$ $CD45RA^-$ effector memory T cells were found in peripheral blood of patients with relapsing-remitting MS compared to healthy controls (Haegele *et al.*, 2007; Liu *et al.*, 2007). These data indicate that memory T cells are key targets when applying peptide induced tolerance in antigen-experienced individuals. We hypothesized that pMOG reactive memory T cells could be controlled by soluble peptide in the same way as naive T cells. To approach these questions, soluble peptide was applied to antigen experienced mice. To further characterize features of autoreactive memory T cells, effector memory T cells from

pMOG experienced 2D2 mice were sorted for *in vitro* analysis.

5.2 Results

5.2.1 Peptide induced tolerance in memory EAE using the pMOG/C57BL/6 system

The initial aim of this chapter was to determine if peptide induced tolerance could be established in the ¡memory EAE¡ model. C57BL/6 mice were immunized with pMOG / CpG / IFA (pMOG : CpG = 50 £g : 60 £g), injected with 200 £g or 50 £g of soluble pMOG i.v. 4 weeks later and subsequently challenged with 100 £g of pMOG / CFA plus PTX for EAE induction (Figure 5.1A). Injection of soluble pMOG through the i.v. or i.p. route and multiple doses were tested in order to find the best protocol for tolerance. As Figure 5.1B shows 50 £g and 200 £g of soluble pMOG i.v. treated mice had incidences of 40 % and 20 %, respectively, whereas the control group was 57 %. Treatment with 50 £g of pMOG slightly reduced severity in mice compared to control group, whereas mice which received 200 £g of pMOG had almost no clinical signs (Figure 5.1B). Pooled data from two individual experiments show a similar pattern with the 200 £g pMOG injected group showing better protection than the 50 £g pMOG injected group ($p < 0.05$, Figure 5.1C). Both the 200 £g and the 50 £g pMOG injected groups had a reduced incidence of 45 %, compared to 73 % in the PBS group (Figure 5.1C). *Ex-vivo* assays on spleen cells at day 30 post secondary immunization showed that cells from 200 £g and 50 £g pMOG-treated mice proliferated identically to cells from the control mice (Figure 5.1D). Assays of cytokine production showed no difference between groups. However, there was a trend that cells from 50 £g pMOG-treated mice produced less IL-4 and IL-17, more IL-10 and similar IFN-γ, whereas cells from 200 µg

pMOG-treated mice had a trend of reduced production of IL-4, IL-10, IL-17 and IFN-γ, compared with the cells from the control mice on day 30 (Figure 5.2A-D). These data suggest that cells in the peptide injected group had a trend for reduced production of pro-inflammatory cytokines and that 200 £g of pMOG i.v. could provide effective protection from EAE in mice already exposed to pMOG.

To assess the feasibility of multiple doses of soluble peptide in tolerance induction, mice were immunized with pMOG / CpG / IFA (pMOG : CpG = 50 £g : 60 £g) at day -35, injected with soluble pMOG i.p on day -8, -6 and -4 and subsequently challenged with 100 £g of pMOG / CFA (with PTX on day 0 and day 2) (Figure 5.3A). Two doses of soluble pMOG, 200 £g or 50 £g, were also applied in this experiment. Compared with the disease course of the control mice, mice receiving 50 £g of pMOG developed EAE with delayed onset, reduced severity and reduced incidence (40 %). In contrast, mice receiving 200 £g of pMOG developed EAE with delayed onset, normal severity but enhanced incidence (80 %) (Figure 5.3B). Pooled data from two individual experiments show that the 200 £g and 50 £g pMOG-treated groups had similar disease course and severity (Figure 5.3C). The incidence was 54 % in the 200 £g pMOG-treated group and 45 % in the 50 £g pMOG treated group, compared to 73 % in PBS injected group (Figure 5.3C). On day 30 post secondary immunization, spleen cells from 50 £g and 200 £g pMOG-treated mice proliferated in response to pMOG similarly to cells in control group (Figure 5.3D). Assays of cytokine production showed no difference between groups, but there was a trend which showed that cells from 50 £g pMOG-treated mice produced less IL-10, IFN-γ and IL-17, compared with cells from the control mice (Figure 5.4). Meanwhile, there was a trend which showed that cells from 200 £g pMOG-treated mice had a reduced

production of IL-4 at the low doses of pMOG, a reduced production of IL-17 and similar levels of IL-10 and IFN-γ. These observations suggest that the administration of multiple injections with low dose of pMOG i.p. is sufficient to alleviate EAE, although no pronounced *in vitro* phenotype of T cell tolerance was observed at the time point been sampled.

Sera from individual mice were collected at day 30 post secondary immunization for measurement of anti-pMOG antibody. As Figure 5.5 shows, double immunized mice did not produce more anti-pMOG IgG, IgG1 and IgG2a, compared with the primary immunized control. The mice receiving soluble pMOG, in spite of showing reduced EAE, did not reduce their ability to generate anti-pMOG antibodies. Mice receiving 50 £g of pMOG i.v. showed a tendency towards of higher titers of pMOG specific IgG, however, one mouse failed to produce any antibody, thus, resulting in no significant difference to control group (Figure 5.5A). These results suggest that peptide-induced tolerance in antigen-experienced mice does not correlate with a reduction of anti-pMOG IgG, IgG1 and IgG2a.

5.2.2 Peptide induced tolerance in memory EAE in the Ac1-9/B10.PL system

A similar protocol was tested in the Ac1-9 / B10.PL system. B10.PL mice were immunized with Ac1-9 / CpG / IFA (Ac1-9 : CpG = 50 £g : 60 £g), dosed with soluble Ac1-9_{4Y} or PBS 4 weeks later and subsequently challenged with 100 £g of Ac1-9 / CFA plus PTX for EAE induction 7 days after soluble peptide injection (Figure 5.6A). Unexpectedly, soluble Ac1-9_{4Y} had no protective effect on EAE development. The disease incidence in Ac1-9_{4Y} treated mice was 100 %, the disease

course proceeded rapidly and reached the peak score of 3.5, whereas control group had a peak score of 2.5 (Figure 5.6B). In contrast, spleen cells collected from Ac1-9_{4Y} treated mice at day 27 showed a reduced proliferative response, compared with cells from the control mice (Figure 5.6C). Also, cytokine production assays showed a trend of reduced production of IFN-γ and IL-17 in cells from Ac1-9_{4Y} treated mice (Figure 5.7A and B). These conflicting data showed that T cells were tolerized by the soluble peptide injection after secondary immunization but mice were not protected.

5.2.3 pMOG induced tolerance in pMOG experienced 2D2 T cells

What does soluble peptide do to the antigen experienced T cells? In an attempt to answer this question, Ly5.1^+ 2D2 spleen cells were cultured in medium containing 10 £M of pMOG. One day later, cells were transferred to fresh medium contained 10U/ml of IL-2 for 2 days. The activated cells were subsequently separated from the dead cells by a ficoll gradient as described in *Materials and Methods*. The live activated cells were harvested and transferred into C57BL/6 mice. Mice were i.v. injected with 200 £g pMOG or PBS the next day. Spleen cells were collected at various time points for quantitative and phenotypic analysis of the transferred 2D2 cells (Figure 5.8A). The frequency of transferred 2D2 cells contracted continuously in pMOG-treated and control mice (Figure 5.8B). However, the frequency was consistently 2-3 folds lower in pMOG-treated mice than in control mice, indicating that the administration of soluble pMOG either rapidly reduces the number 2D2 T cells or prevents an expansion continuing from the *in vitro* activation.

Completely in reverse to the frequency of live 2D2 cells, the frequency of apoptotic

2D2 cells ($CD4^+Ly5.1^+$Annexin V^+7-AAD^-) continuously increased over time from 0.51% (day 1) to 23.8% (day 7) in pMOG-treated mice (Figure 5.9A). Apoptotic 2D2 cells in the control group increased in frequency from 0.41% (day 1) to 3.86% (day 3), and remained constant at 5% Annexin V^+ 7-AAD^- cells thereafter. From day 2 to day 7, the frequency of apoptotic 2D2 cells in pMOG-treated mice was 2-3 folds higher than the frequency in control mice (Figure 5.9B). This enhanced frequency of apoptotic 2D2 cells in pMOG-treated mice suggests that soluble pMOG triggers cell death in pMOG experienced T cells.

5.2.4 Administration of MBP Ac1-9 APL to antigen experienced Tg4 T cells

To estimate the relationship between peptide-MHC binding affinity and peptide induced tolerance in Ag-experienced T cells, a similar protocol as used in 5.2.3 was tested in the Ac1-9 / Tg4 / B10.PL system using altered peptide ligands (APL). *In vitro* activated $CD4^+$ $Ly5.1^+$ Tg4 cells were transferred to B10.PL mice. Different Ac1-9 derived APL were given i.v. one day later. Spleen and LN were analyzed at day 2, 3 and 4 (Figure 5.10A). The peptides used were 4Tyr (Ac1-9_{4Y}), 4Val (Ac1-9_{4V}), wild type Ac1-9 (Ac1-9) (ordered by MHC affinity from high to low). In the spleens collected from peptide treated mice, the proportion of transferred Tg4 cells in Ac1-9_{4Y} injected mice was greater than in Ac1-9_{4V} or Ac1-9 injected mice at day 2 and day 3, whereas the proportion in Ac1-9_{4V} injected mice was greater than the proportion in Ac1-9 injected mice (Figure 5.10B). Comparison of cells exposed to the same peptides shows that frequency of Tg4 cells started to decline from day 4 in Ac1-9_{4Y} injected mice, while frequencies of Tg4 cells in Ac1-9_{4V} and Ac1-9 injected mice continuously decreased over time, suggesting that soluble Ac1-9_{4Y}

induces a transient T cell expansion from day 1 to day 3 (Figure 5.10B). Transferred Tg4 cells from Ac1-9 injected mice showed an enhanced proportion of Annexin V^+ 7-AAD^- apoptotic cells (30 % and 19 % at day 3 and day 4, respectively), whereas Tg4 cell apoptosis in Ac1-9_{4Y} and Ac1-9_{4V} injected mice remained a comparable proportion of 7 to 10 %, comparable with the control mice (Figure 5.10C).

The transferred Tg4 cells from Ac1-9_{4Y} injected mice proliferated vigorously to Ac1-9 *in vitro* when sampled at day 2, whereas T cells in Ac1-9_{4V}, Ac1-9 and PBS injected mice remained only weakly responsive (Figure 5.11A). However, the higher responsive state of T cells from Ac1-9_{4Y} injected mice declined over time resulting in similar proliferative responses between all groups. This suggests that the increased proliferation Ac1-9_{4Y} group is a result of increased cell number. Collectively, these observations suggest that administration of APL with high MHC affinity in soluble form induces a transient T cell activation in antigen experienced T cells, whereas administration of soluble peptide with low MHC affinity triggers the apoptosis of antigen experienced T cells.

To further assess the effect made by soluble Ac1-9_{4Y} in this system, mice were given *in vitro* activated Tg4 cells and either 200 £g of Ac1-9_{4Y} , 10 £g of Ac1-9_{4Y},or PBS i.v. The frequency of Tg4 T cells was assessed in the spleen at days 3, 6 and 9 (Figure 5.12A). Mice receiving 200 £g or 10 £g of Ac1-9_{4Y} had a tendency for a reducing frequency of Tg4 T cells over time, whereas the frequency of Tg4 T cells in mice receiving 200 £g Ac1-9 or PBS remained on 3% to 6 % at all indicated time points (Figure 5.12B). Mice receiving 200 £g of Ac1-9_{4Y} also had an enhanced frequency of Tg4 cells at day 6, compared with the Ac1-9 and PBS injected mice.

The total transferred cell number was calculated by combining Tg4 cell numbers from the spleen and LN and the result showed a similar trend as Figure 5.10B in that the Tg4 cell number at day 3 and day 6 in the mice receiving Ac1-9_{4Y} was greater than the original number of cells transferred (Figure 5.12C). The number of transferred cells in Ac1-9-treated and control mice was constantly lower than the original number transferred (Figure 5.12C). Over time, the number of Tg4 cells in mice receiving 200 £g or 10 £g Ac1-9_{4Y} was greatly reduced (Figure 5.12B and C). At day 9, the number of Tg4 cells in both Ac1-9_{4Y} groups reached a lower level than the Ac1-9 injected mice, indicating a fast contraction in Ac1-9_{4Y} groups. When comparing the frequency of Tg4 cells between treatments of high or low dose of Ac1-9_{4Y}, injection of 200 £g Ac1-9_{4Y} resulted in both a faster expansion and a faster contraction of Tg4 cells. Thus, the Tg4 cell number was lower in 200 £g Ac1-9_{4Y} treated mice than in 10 £g Ac1-9_{4Y}-treated mice at day 9.

Consistent with Tg4 cell number, the cells from Ac1-9_{4Y}-treated mice showed an enhanced proliferation at day 3 and a reduced proliferation at day 9, compared to the control group (Figure 5.13A). These observations suggest that the administration of the high MHC affinity Ac1-9_{4Y} peptide can trigger a transient expansion in Ac1-9 experienced Tg4 cells, but the expanded cells cannot remain active for long and the rapid contraction of Tg4 cells number in the spleen may reflect to either cell migration, or cell deletion.

5.2.5 The $CD4^+$ $CD44^{high}$ T cell population contains $CD25^+$ $Foxp3^+$ Treg cells

To further estimate the pathogenicity of effector memory T cells *in vivo*, adoptive

transfer of memory T cells was performed. Tg4 mice were immunized with 50 £g of Ac1-9 / CFA and $CD4^+$ $CD44^{high}$ (memory T cell) or $CD4^+$ $CD44^{low}$ (naive T cell) cells were sorted from these mice 6 weeks later. $CD4^+$ $CD44^{high}$ or $CD4^+$ $CD44^{low}$ Tg4 cells were transferred into B10.PL mice one day prior to EAE induction with Ac1-9 / CFA plus PTX (Figure 5.14A). The disease course showed that mice receiving naive T cells developed EAE with a incidence of 75 %, whereas those receiving memory T cells were totally protected from EAE (Figure 5.14B). B10.PL mice normally develop disease in response to Ac1-9 immunization without a cell transfer, thus adoptive transfer of $CD44^{high}$ Tg4 cells must have protected against disease development. Analysis of the Tg4 populations used showed that over 50 % of the $CD44^{high}$ population expressed Foxp3 and 89% of the $Foxp3^+$ cells were $CD25^{high}$ (Figure 5.15A and B), indicating that a mixed population with $CD25^+$ $Foxp3^+$ Treg and $CD4^+$ $CD44^{high}$ T cells were co-transferred initially. To avoid the contamination with Treg, $CD4^+$ $CD25^+$ cells were excluded for further experiments.

5.2.6 Features of pMOG-reactive memory T cells

Results shown in 5.2.3 indicated that *in vitro* activated pMOG-reactive T cells undergo rapid apoptosis in response to soluble pMOG. However, it is not clear how closely these cells resemble memory T cells generated *in vivo*. In order to generate a population of memory T cells *in vivo*, 2D2 mice were immunized with pMOG / CpG / IFA. Six weeks later, LN and spleen were collected and stained for memory markers prior to cell sorting (Figure 5.16A). None of the pMOG immunized 2D2 mice developed EAE. $CD4^+$ $CD25^-$ cells were separated into two populations, $CD44^{high}$ $CD62L^{low}$ and $CD44^{low}$ $CD62L^{high}$ (Figure 5.16B). The population of $CD44^{high}$ $CD62L^{low}$ represented to effector memory T cells, whereas the population

of $CD44^{low}$ $CD62L^{high}$ represented to naive T cells. The sorted cells were cultured with irradiated APC and pMOG for 72 hours. T_{EM} cells showed a reduced proliferation, a trend of reduced IL-2 production and enhanced IFN-γ production, compared to the naive T cells (Figure 5.17). As high IFN-γ production is a hallmark of memory T cells, these data indicated that the $CD4^{+}$ $CD25^{-}$ $CD44^{high}$ $CD62L^{low}$ cells were functional effector memory T cells. In a time course study of cytokine production (Figure 5.18A), IFN-γ production by memory cells was evident at 12 hr and peaked at 48 hr (100 ng/ml), whereas in naive cells IFN-γ production was only evident after 48 hr and reached only 20 ng/ml by 72 hr. On the other hand, IL-2 production in T_{EM} cells was evident at 6 hr, peaked at 24 hr and reduced thereafter (Figure 5.18B). At 72 hr, there was no detectable IL-2 in the medium from the T_{EM} culture. Naive T cells started to produce IL-2 at 24 hr, peaked at 48 hr and continued producing IL-2 until 72 hr (Figure 5.18B). These observations show that memory T cells can respond to pMOG and this response is faster than naive T cells, in terms of the production of IL-2 and IFN-γ. However, detection of IL-2 production by memory T cells is time dependent, as IL-2 is secreted at early time points and is rapidly utilized by the population responding.

Memory T cells produced cytokines more rapidly than naive T cells (Figure 5.18), but why did memory T cells proliferate less well than naive T cells (Figure 5.17A)? To answer this, the expression of TCR molecules in memory and naive T cells was assessed. Not all of the T_{EM} cells expressed high levels of TCR Vβ11, with a population having a reduced expression of TCR Vβ11 (Figure 5.19A). In addition, two populations appeared in the T_{EM} cells based on expression of TCR Vα3.2 with only 16.2 % of T_{EM} cells showing high expression. In contrast, the naive T cells

appeared homogeneous as a single $V\beta11^{high}$ $V\alpha3.2^{high}$ population. Overlapping plots from naïve and memory T cells showed a reduced expression of TCR Vα3.2 and Vβ11 molecules on the T_{EM} cells (Figure 5.19B and C).

Comparing $CD4^+$ $CD25^-$ $V\alpha3.2^+$ and $CD4^+$ $CD25^-$ $V\alpha3.2^-$ T cells showed that $CD4^+$ $CD25^-$ $V\alpha3.2^-$ were less responsive to antigen *in vitro* (Figure 5.20A), indicating that the expression of Vα3.2 is essential for responding to pMOG. The sorting protocol was adjusted to collect $CD4^+$ $CD25^-$ $V\alpha3.2^+$ T cells for further experiments. Unexpectedly, $V\alpha3.2^+$ memory T cells proliferated less than $V\alpha3.2^+$ naive T cells in response to pMOG (Figure 5.20B). This is probably a result of the timing. Because the $V\alpha3.2^+$ T_{EM} cells still showed enhanced production of IFN-γ and rapid production of IL-2, compared to the naive T cells (Figure 5.20C and D). Presumably the *in vitro* system cannot supply sufficient IL-2 (mostly produced by T_{EM} themselves) for the proliferation of $V\alpha3.2^+$ T_{EM} cells. Consistent with data from cells separated as $CD44^{high}$ $CD62L^{low}$ $V\alpha3.2^{+/-}$ (Figure 5.18), these data suggest that $V\alpha3.2^+$ memory 2D2 cells produce both IFN-γ and IL-2 faster than naive 2D2 T cells.

5.2.7 Adoptive transfer of effector memory 2D2 T cells

$CD4^+CD25^-V\alpha3.2^+$ cells from pMOG-immunized 2D2 mice were sorted into $CD44^{high}$ $CD62L^{low}$ (memory) and $CD44^{low}CD62L^{high}$ (naive) populations for adoptive transfer into C57BL/6 mice. One day later the recipients were injected with soluble pMOG (Figure 5.21A). The frequency of 2D2 cells from both memory and naive T cell transferred mice expanded by day 3 and contracted by day 7. However, no significant difference was found on days 1-3, whereas a reduced frequency of 2D2 cells was observed in mice transferred with T_{EM} cells (Figure 5.21B). Proliferation

assays showed that cells from both groups responded to pMOG at day 1, whereas cells from the mice transferred with T_{EM} cells were less responsive to pMOG than cells from the naive T cell group (Figure 5.22). However, cells from memory T cell transferred mice were unresponsive to pMOG *in vitro* at day 2 and thereafter, while cells from naive T cells transferred mice proliferated greater at day 2 but remained unresponsive thereafter. These results suggest that antigen experienced $V\alpha3.2^{+}$ T_{EM} cells can be activated transiently by soluble peptide administration *in vivo*. However, naive T cells were better at responding to pMOG than T_{EM} cells.

Further memory or naive 2D2 T cells were transferred one day before injection of pMOG / LPS (pMOG : LPS = 100 £g : 30 £g) (Figure 5.23A). From day 3 to day 7 after priming, the mice receiving memory T cells showed a trend of reduced frequency of transferred 2D2 cells, compared to those receiving naive T cells (Figure 5.23B), suggesting that effector memory T cells are less capable of expanding than naive T cells after antigen priming *in vivo*. As memory T cells are more capable of entering non-lymphoid tissues upon re-stimulation, it is possible that the majority of T_{EM} cells might not remain in the spleen. Thus, the frequency of transferred 2D2 cells in spleen might not truly reflect the real frequency in the mouse. This point needs to be further assessed by checking the existence of 2D2 cells in non-lymphoid tissues.

5.3 Discussion

This chapter aimed to induce tolerance in antigen-experienced mice and to characterize myelin reactive memory T cells. With a double immunization system, administration of pMOG in mice can induce tolerance in pMOG-experienced mice. A

single dose with a high concentration of soluble pMOG i.v. gave the best protective effect, whereas multiple doses of a low concentration of pMOG i.p. also alleviated EAE (Figure 5.1 and 5.3). In addition, cells exposed *in vivo* to a high concentration of pMOG i.v. or multiple doses of a low concentration of pMOG i.p. had a trend of reduced production of IFN-γ and IL-17, suggesting that effector Th1 / Th17 cells in the spleen had been tolerized. Is the mechanism of tolerance in antigen-experienced mice or in a memory setting the same as a naive setting? Administration of pMOG to mice hosting activated 2D2 T cells showed an enhanced frequency of apoptosis of the 2D2 cells (Figure 5.8 and 5.9), consistent with antigen-induced apoptosis seen in other adoptive transfer models with effector or activated T cells (Klugewitz *et al.*, 2002; Tischner *et al.*, 2007). This suggests that if there were pMOG reactive-effector 2D2 T cells remaining in pMOG-experienced mice 28 days after primary immunization, cell deletion of those effector T cells would occur immediately after soluble pMOG administration.

In the adoptive transfer model, few transgenic cells can survive 4 weeks after primary immunization (Figure 4.1), indicating that most of the effector T cells have contracted and it is the memory T cells that remain in the antigen-experienced mice. The Abbas lab has reported that memory $CD4^+$ T cells are resistant to tolerance induction. In the Abbas model, *in vitro* activated DO11.10 T cells were transferred into recipient mice and rested for at least 6 weeks, generating a population of resting memory T cells. Administration of soluble OVA to mice containing resting memory DO11.10 T cells did not inhibit their ability to respond to a subsequent immunization (London *et al.*, 2000). However, soluble pMOG administration to the memory EAE model presented within this thesis clearly protects pMOG-experienced mice from

EAE. Despite the belief that memory T cells have lower co-stimulatory requirements and can respond to antigen faster than naive T cells (Rogers *et al*., 2000; London *et al*., 2000; Merica *et al*., 2000; Dienz *et al*., 2007), data in this thesis show that memory T cells may be susceptible to peptide induced-tolerance, if we study a longer period after immunization (the London report only analyzed 3 days after immunization and did not use an disease model). Presumably, the hyperresponsiveness of memory T cells using a resting-memory T cell transfer model in London¡s report is a transitional state and is a prelude to further cell deletion or unresponsiveness. In the long run, memory T cells could possible be induced to deletion or unresponsiveness.

In the Ac1-9 / Tg4 / B10.PL system, less susceptibility to EAE induction has been evident in mice pre-treated with the soluble, high affinity analogues Ac1-9_{4A} and Ac1-9_{4Y} before immunization, whereas mice given the low affinity peptide (wild type peptide Ac1-9) are only moderately protected (Liu and Wraith, 1995; Anderton *et al*., 2001). This difference in tolerance induction may be related to the ability of the high affinity analogue Ac1-9_{4Y} to persist for up to 12 days *in vivo* (Konkel and Anderton, unpublished data) and therefore to provide a high density of pMHC complexes compared with Ac1-9. The different tolerogenic effect among the different APL analogues suggests that TCR avidity against pMHC is important for tolerance induction (Anderton *et al*., 1998, McCue *et al*., 2004). In the model of memory EAE with double immunization, administration of soluble Ac1-9_{4Y} to Ac1-9 / CpG / IFA primed mice did not protect the Ac1-9-experienced mice from EAE and resulted in a severe disease course upon subsequent immunization (Figure 5.6). This could be a result of re-activation of Tg4 cells. It indicated that an administration of a strong

agonist (like Ac1-9_{4Y}) may re-activate memory T cells, and these re-activated cells may be harmful (inducing EAE). Reciprocally, the ability of proliferate (assessed by spleen cells collected on day 27) was reduced, compared with the PBS treated group. This point suggested that the peptide induced re-activation is transient (at least in the spleen cells). It has to be emphasized that the cells that remained in the spleen are not the cells that cause disease, so that it is would be necessary to check the cells in the CNS in the future experiments.

Unlike the pMOG system, transferred pre-activated Tg4 cells showed transient expansion within 6 days after Ac1-9_{4Y} treatment, with a subsequent contraction. In contrast, treatment with wild type Ac1-9 increased the frequency of apoptotic cells immediately (Figure 5.10-5.13). This suggests that soluble peptide with high binding affinity for MHC can induce transient T cell activation, whereas soluble peptide with low MHC affinity can trigger apoptosis in antigen-experienced T cells directly. Ac1-9_{4Y} treatment to mice receiving activated Tg4 cells did not protect the mice from EAE (Figure 5.6). It is possible that a significant number of effector Ac1-9 reactive-cells still remain in mice at day 7 after Ac1-9_{4Y} injection, the time of secondary immunization, so that the administration of soluble Ac1-9_{4Y} fails to induce tolerance. However, mice receiving activated Tg4 cells were hyporesponsive after Ac1-9_{4Y} treatment when spleen cells were harvested on day 27, after secondary immunization (Figure 5.7), suggesting that the T cell activation may be a prelude of unresponsiveness in this system. Notably, the frequency of Tg4 T cells was significantly lower in Ac1-9_{4Y} treated mice than in Ac1-9 treated mice on day 9 after soluble peptide injection, suggesting a better deletion effect of Ac1-9_{4Y} compared to Ac1-9. Of course it is also possible that Tg4 cells in the Ac1-9_{4Y} treated mice are

better able to migrate out from the spleen than Tg4 cells in the Ac1-9 treated mice. Future experiments should address these questions by collecting as many tissues (including lymphoid and non-lymphoid tissues) as possible and delaying the secondary immunization to a suitable time-point after soluble Ac1-9_{4Y} administration.

Naive ($CD4^+$ $CD44^{low}$ $CD62L^{high}$) and T_{EM} ($CD4^+$ $CD44^{high}$ $CD62L^{low}$) populations were isolated from 2D2 mice that had been given pMOG / CpG / IFA 6 weeks before. As the $CD4^+$ $CD44^{high}$ $CD62L^{low}$ population contained $CD25^+$ $Foxp3^+$ Treg cells that may inhibit T cell responses (Figure 5.14 and 5.15), $CD25^+$ cells were excluded during cell sorting. At first, it seemed that T_{EM} cells had a reduced proliferation, enhanced IFN-γ and transient IL-2 production compared to naive cells (Figure 5.17 and 5.18). However, the T_{EM} cells showed two heterogenous populations, TCR $V\alpha3.2^+$ $V\beta11^{high}$ and $V\alpha3.2^-$ $V\beta11^{low}$ (Figure 5.19). As the 2D2 mice are not on a RAG-deficient background, it is possible that $CD4^+$ T cells carrying other TCRα subunits contaminate the T cell repertoire (Rogers *et al*., 2000). Heterogeneous T cells have been reported in other adoptive transfer models in which only 65 % of DO11.10 T cells that encounter OVA *in vivo* divide, whereas adoptive transfer with DO11.10 RAG-$2^{-/-}$ T cells exhibit a responder frequency of 95-98 %, suggesting that the proliferative potential may depend on complete allelic exclusion at the TCRα locus (Gudmundsdottir *et al*., 1999). Moreover, TCR Vβ allelic exclusion has been reported to be incomplete, since a significant proportion of peripheral T cells express dual Vβ in both TCR transgenic and normal mice (Balomenos *et al*., 1995). Allelic exclusion at the TCRα locus is relatively inefficient compared with allelic exclusion at the TCRβ locus (Borgulya *et al*., 1992). Thus, a proportion of mature transgenic T

cells can express an endogenously rearranged TCRα chain (different from the transgenic TCRα chain) (Padovan *et al*., 1993).

To improve the analysis of MOG-reactive cells, $CD4^+$ $CD25^-$ $V\alpha3.2^+$ cells from immunized 2D2 mice were isolated and separated into T_{EM} and naive T cells. Only $V\alpha3.2^+$ T_{EM} responded to pMOG, $V\alpha3.2^-$ T_{EM} cells did not respond (Figure 5.10). Comparing $V\alpha3.2^+$ T_{EM} and naive T cells, T_{EM} cells started to produce IFN-γ and IL-2 earlier than naive T cells (Figure 5.20). Notably, IL-2 production in T_{EM} cells was transient and ceased after 72 hours, matching the observations on memory T cells in the PCC / AND system (Rogers *et al*., 2000). Sojka and colleagues have shown a rapid secretion of IL-2 *in vivo* after stimulation of naive T cells for 1-2 hours and as early as 30 min in memory T cells. Maximal secretion was achieved within 1-2 h for memory cells or 6-8 h for naive T cells (Sojka *et al*., 2004), confirming the idea of rapidly responding memory T cells (Macallan *et al*., 2004).

Unlike the previous reports, T_{EM} appeared to proliferate less than naive T cells *ex vivo* in this chapter. Reduced proliferation and reduced expansion of T_{EM} cells were also found in T_{EM} within host mice receiving soluble pMOG or pMOG / LPS, compared with naive T cell transferred groups (Figure 5.21-5.23). One possibility is that the early response of memory T cells does not persist. The Jenkins Lab has reported a DO11.10 adoptive transfer model with OVA / CFA as primary immunization and OVA / IFA as secondary immunization. They found the cell expansion and IL-2 production was earlier in DO11.10 T cells from OVA-experienced mice (memory cells) and the expansion was later but greater in cells from primary immunized mice (naive cells) (Merica *et al*., 2000). A recent study

suggested that CD4 memory T cells do not divide as efficiently as primary responding cells at later time points (after day 5) and fewer of the re-activated memory cells made IL-2 than primary responding cells (but re-activated memory CD4 T cells can still make IFN-γ) (Macleod *et al.*, 2008). In other words, the timing of sampling is important. Memory T cells respond to stimulation faster but naive T cells are able to maintain their activated state for longer. The other possibility is that Vα3.2$^+$ T_{EM} cells might be more sensitive to AICD in our system, therefore Vα3.2$^+$ T_{EM} cells seem less responsive to antigen than naïve Vα3.2$^+$ T cells. Fas deficiency prompts the accumulation of CD8 memory phenotype T cells in mice, suggesting Fas may play a more prominent role in apoptosis of memory T cells (Giese and Davidson; 1992; Aranami *et al.*, 2004). Recently it was reported that human CD4$^+$ memory T cells have more efficient assembly and activation of proximal Fas signaling components (including Caspase 8 and FADD), suggesting that memory T cells are intrinsically more sensitive to Fas-induced apoptosis **(Cruz et al., 2008).** Therefore, memory T cells may be more sensitive to signals that trigger cytokine production and probably also apoptosis, compared with naive T cells.

Taken together, this chapter shows that EAE can be ameliorated in pMOG experienced mice by a single high dose of tolerogenic pMOG injection through the i.v. route and by multiple low doses of tolerogenic pMOG injection through the i.p. route. Administration of soluble pMOG to activated 2D2 T cells in host mice induces apoptosis in the 2D2 T cells, whereas soluble Ac1-9_{4Y} injection induces a transient expansion of activated Tg4 cells *in vivo* and a later cell death. T_{EM} cells from pMOG immunized 2D2 mice expressed less transgenic TCRαβ. TCR Vα3.2$^+$ T_{EM} cells have faster kinetics of responses to pMOG than TCR Vα3.2$^+$ naive T cells, suggesting that

transgenic memory T cells may respond faster, but also die faster than naive T cells after antigen stimulation.

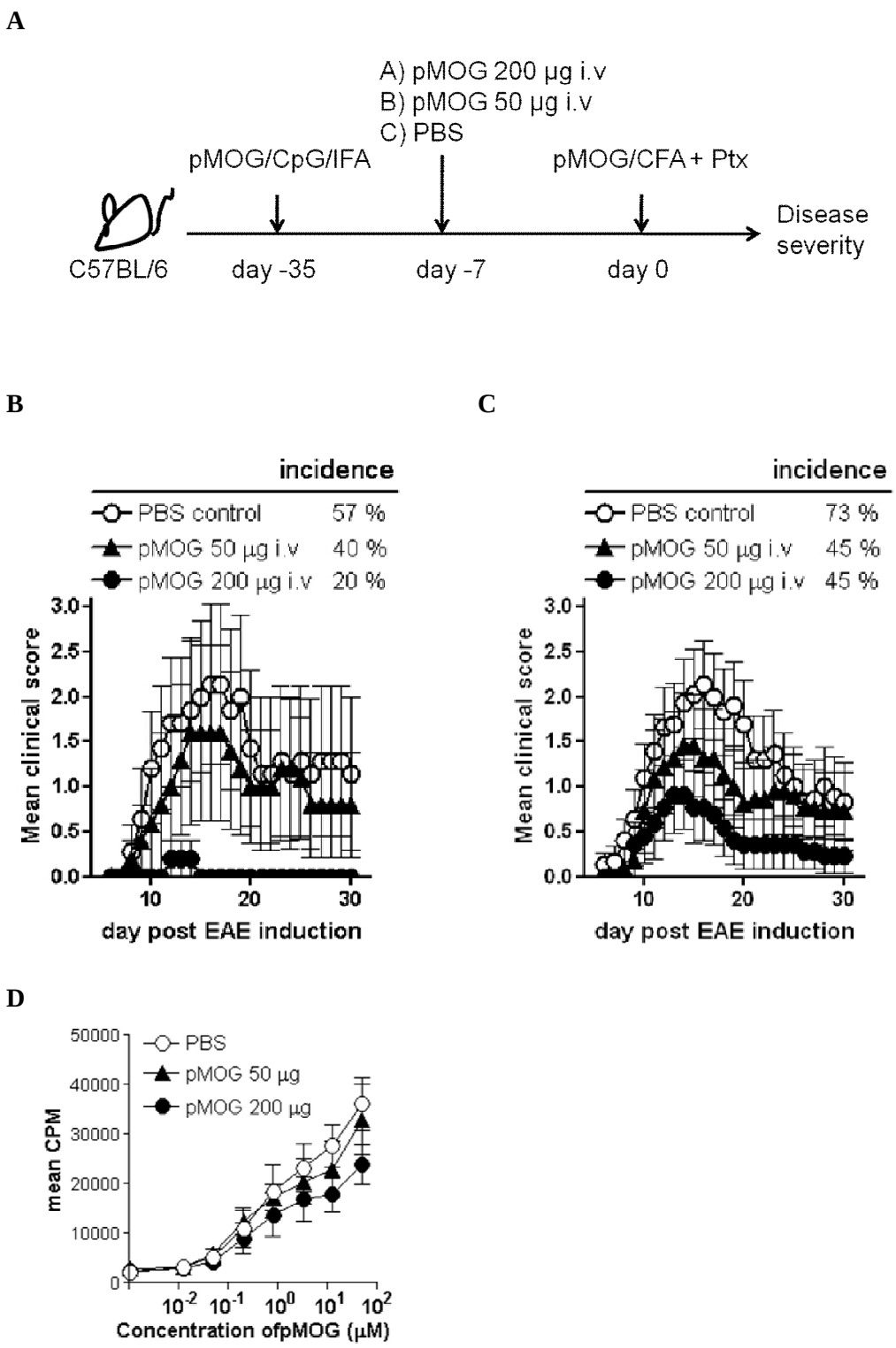


Figure 5.1 Peptide induced tolerance in memory EAE through the i.v. injection of a single dose of soluble pMOG. *A*, mice were immunized with pMOG/CpG/IFA (pMOG : CpG = 50 £g : 60 £g) on day -35, injected with soluble pMOG on day -7 and subsequently immunized with 100 £g of pMOG/CFA on day 0 (with PTX on day 0 and

day 2). Mice received either 200 £g of pMOG, 50 £g of pMOG or PBS on day -7. *B*, disease severity was scored daily post secondary immunization (PBS versus 50 £g pMOG, $p < 0.01$; PBS versus 200 £g pMOG, $p < 0.0001$ by Mann-Whitney U test). Data are from one of two repeated experiments and show mean ¡ SEM (5 -7 mice in each group, SEMs reflect samples from different mice). *C*, data are pooled by two individual experiments (PBS versus 50 £g pMOG, $p < 0.05$; PBS versus 200 £g pMOG, $p < 0.05$ by Mann-Whitney U test. 11-12 mice in each group). *D*, spleen cells were harvested on day 30 post secondary immunization and cultured for 72 hours with a pulsing of thymidine for the last 18 hours. Data are from one of two repeated experiments and show mean ¡ SEM (5-7 mice in each group)

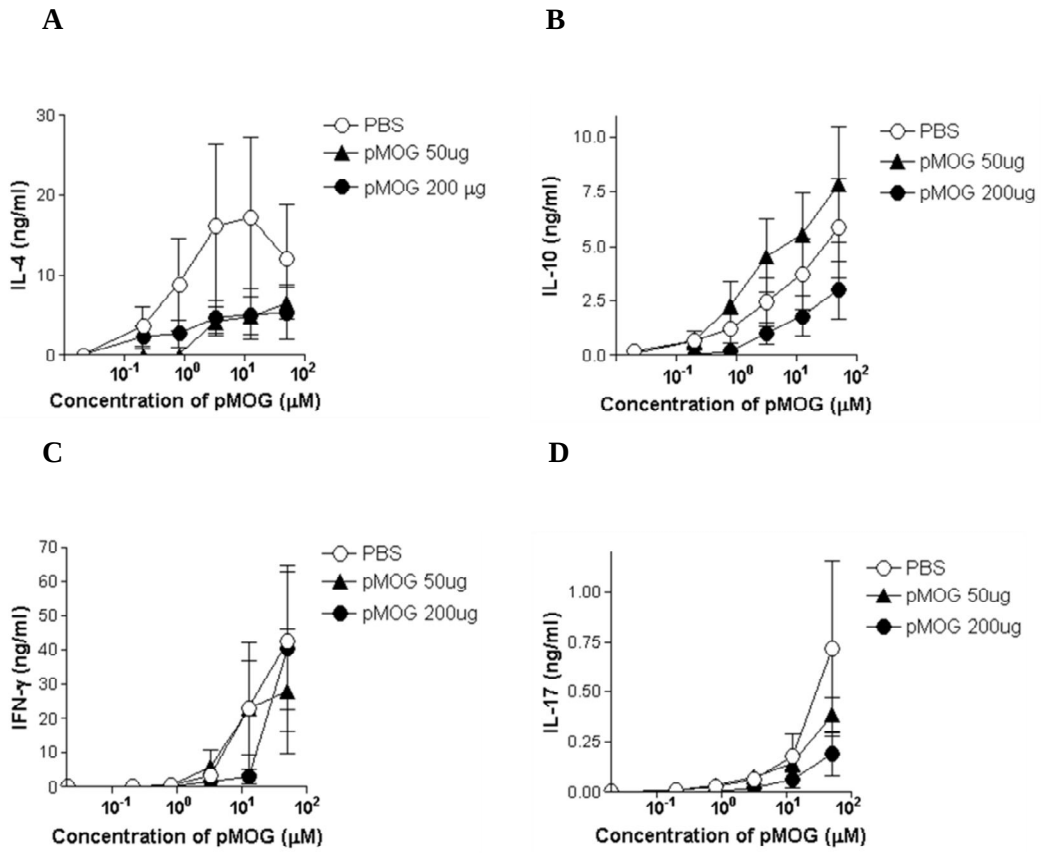


Figure 5.2 Cytokine productions after peptide induced tolerance through the i.v. injection of soluble pMOG. Mice were immunized as shown in figure 5.1A. Spleen cells were harvested on day 30 post secondary immunization and cultured for 72 hours for cytokine assay by ELISA. Plots show the amount of IL-4 (A), IL-10 (B), IFN-γ (C) and IL-17 (D) against various doses of pMOG. Data are from one experiment and represent mean ¡ SEM (5 -7 mice in each group).

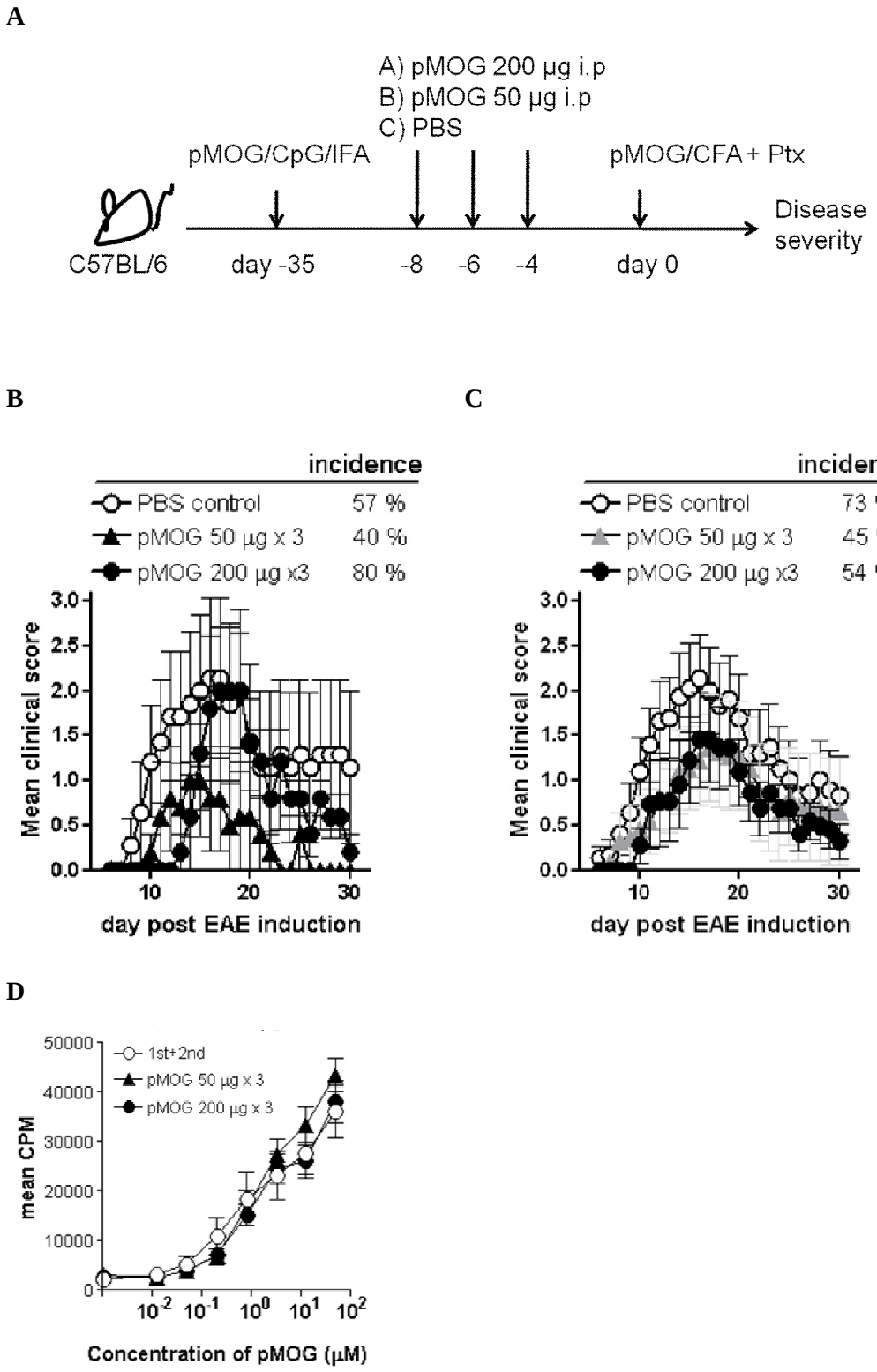


Figure 5.3 Peptide induced tolerance in memory EAE through the i.p. injection of multiple doses of soluble pMOG. *A*, mice were immunized with pMOG/CpG/IFA (pMOG : CpG = 50 £g : 60 £g) on day -35, injected with 3 shots of soluble pMOG on

days -8, -6 and -4 and subsequently challenged with 100 £g of pMOG/CFA on day 0 (with PTX on day 0 and day 2). Mice received either 200 £g of pMOG, 50 £g doses of pMOG or PBS on days -8, -6 and -4. *B*, disease severity was scored daily post secondary immunization (PBS versus 50 £g pMOG, $p < 0.001$; PBS versus 200 £g pMOG, $p < 0.01$ by Mann-Whitney U test). Data are from one of two repeated experiments and show mean ¡ SEM (5 -7 mice in each group). *C*, data are pooled by two individual experiments (PBS versus 50 £g pMOG, $p < 0.05$; PBS versus 200 £g pMOG, $p < 0.05$ by Mann-Whitney U test. 11-12 mice in each group). *D*, spleen cells were harvested on day 30 post secondary immunization and cultured for 72 hours with a pulsing of thymidine for the last 18 hours. Data are from one of two repeated experiments and show mean ¡ SEM (5-7 mice in each group)

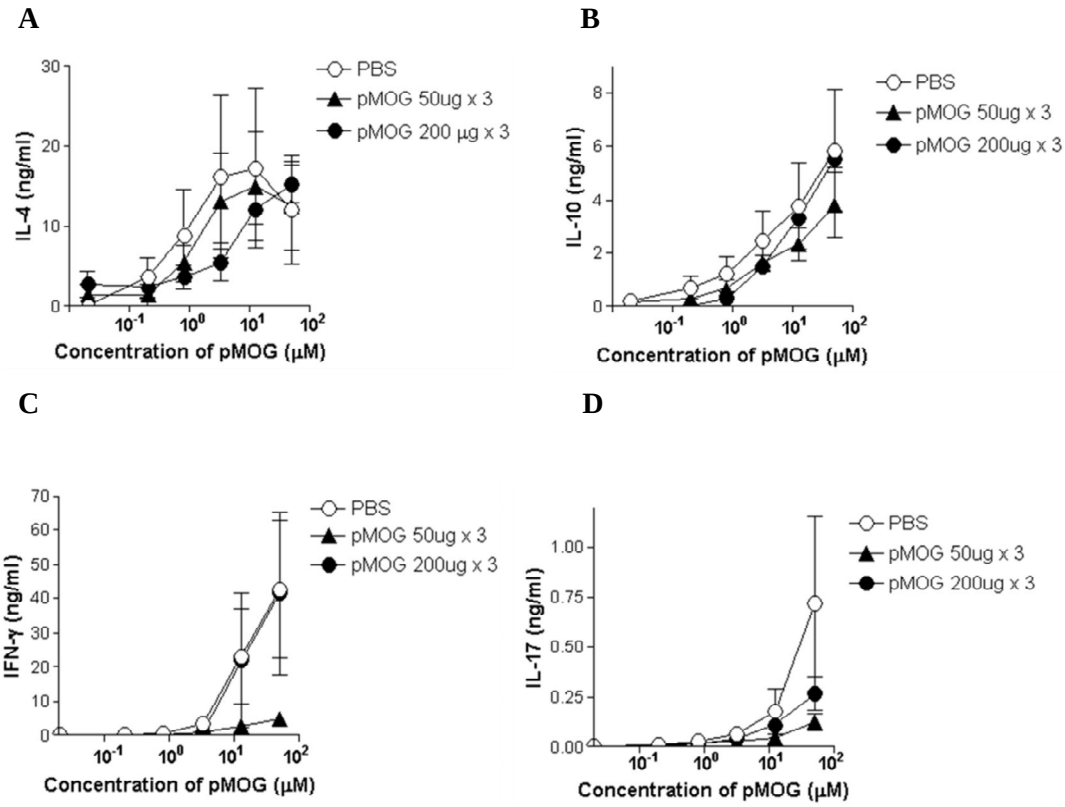


Figure 5.4 Cytokine productions after peptide induced tolerance through the i.p. injection of soluble pMOG. Mice were immunized as shown in figure 5.3A. Spleen cells were harvested on day 30 post secondary immunization and cultured for 72 hours for cytokine assay by ELISA. Plots show the amount of IL-4 (A), IL-10 (B), IFN-γ (C) and IL-17 (D) against various doses of pMOG. Data are from one experiment and represent mean ¡ SEM (5 -7 mice in each group).

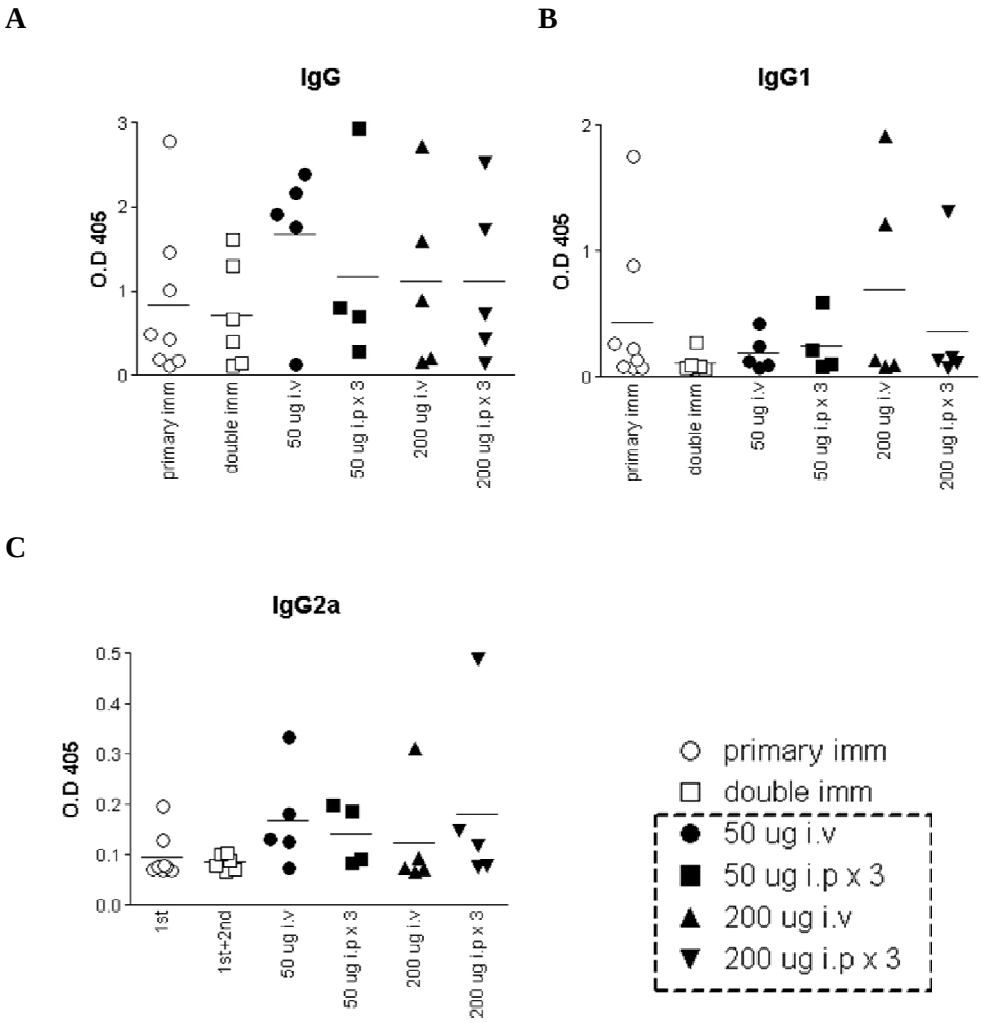


Figure 5.5 Peptide induced tolerance is independent of the production of anti-pMOG antibodies. Mice were immunized as shown in figures 5.1A and 5.3A. Sera were collected on day 30 and diluted by 100X post secondary immunization for detection of anti-pMOG IgG (A), anti-pMOG IgG1 (B), anti-pMOG IgG2a (C). Plots represent optical density obtained from ELISA. Mean values of O.D are shown in each group (5-7 mice in each group) Data are from one experiment.

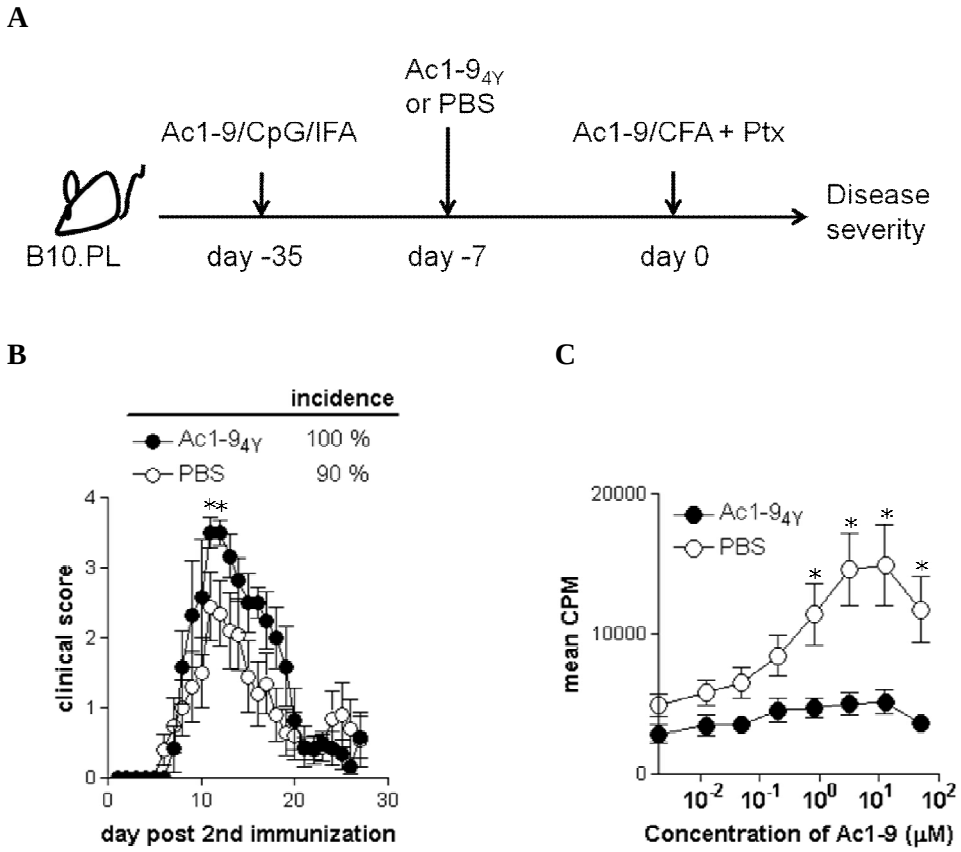


Figure 5.6 Administration of soluble Ac1-9$_{4Y}$ does not induce tolerance in memory EAE. *A*, mice were immunized with 50 £g Ac1-9/CpG/IFA (Ac1-9 : CpG = 50 £g : 60 £g) on day -35, i.v. injected with 200 £g of soluble Ac1-9$_{4Y}$ or PBS on day -7 and subsequently immunized with 100 £g of Ac1-9/CFA on day 0 (with PTX on day 0 and day 2). *B*, disease severity was scored daily post secondary immunization. Asterisks represent significant difference between Ac1-9$_{4Y}$ treated and PBS groups ($p < 0.05$ by Mann-Whitney U test). *C*, spleen cells were harvested on day 27 post secondary immunization and cultured for 72 hours with a pulsing of thymidine for the last 18 hours. Asterisks represent significant difference between Ac1-9$_{4Y}$ treated and PBS groups ($p < 0.05$ by Unpaired T-test) Data are from one experiment and show mean ¡ SEM (6 -10 mice in each group, SEMs reflect samples from different mice).

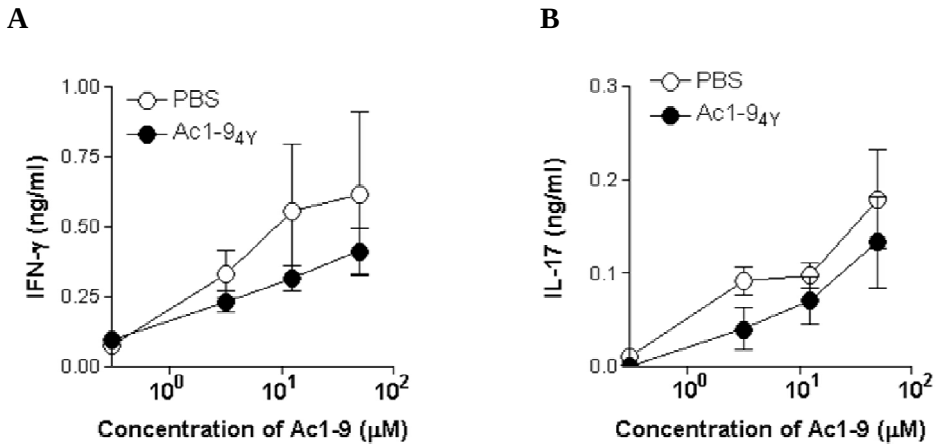


Figure 5.7 Cytokine productions after the administration of soluble Ac1-9$_{4Y}$ in memory EAE. The immunization protocol is shown in figure 5.6A. Cells were harvested from the spleen on day 27 post secondary immunization and cultured for 72 hours for cytokine assay by ELISA. Plots show the amount of IFN-γ (A) and IL-17 (B) against various doses of Ac1-9 peptide. Data represent the mean ¡ SEM (Data are from one experiment, 6-10 mice in each group).

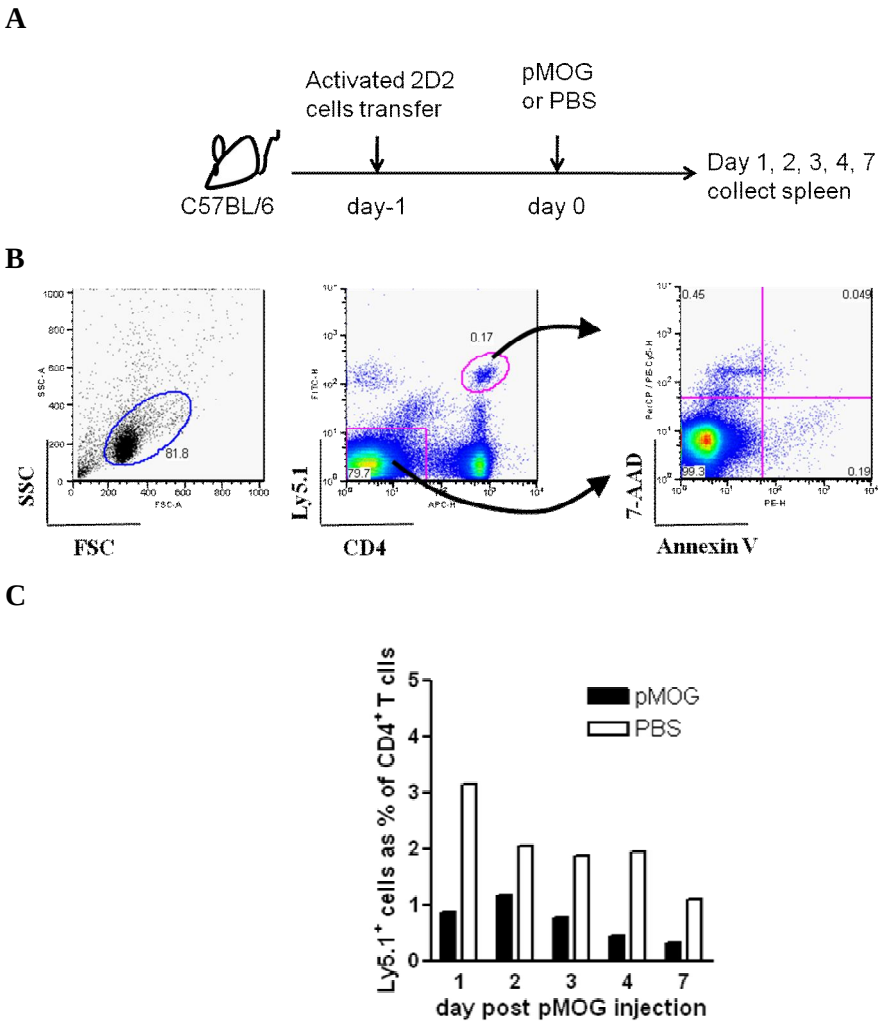


Figure 5.8 Administration of pMOG in activated 2D2 T cells. Cells from Ly5.1^{+} 2D2 mice were cultured in medium with 10 £g/ml of pMOG for 24 h and rested in the pMOG-free medium with 10U/ml of IL-2 for 2 days. Live activated cells were harvested by Ficoll separation as described in *Materials and Methods*. *A*, C57BL/6 mice received 2 x 10^{6} activated cells on day -1 and injected with 200 £g of pMOG or PBS through i.v. route on day 0. *B*, at the day indicated, spleen cells were harvested and stained for CD4, Ly5.1, 7-AAD and Annexin V. Cells were gated on CD4^{+}Ly5.1^{+} and plotted by 7-AAD / Annexin V. Annexin V single positive cells were showed as apoptotic cells (the frequency of apoptotic cells were showed in figure 5.9) *C*, The frequency of CD4^{+}Ly5.1^{+} (2D2) T cells as a percentage of spleen CD4^{+} T cells. Data are from one of three repeated experiments (2 mice in each group).

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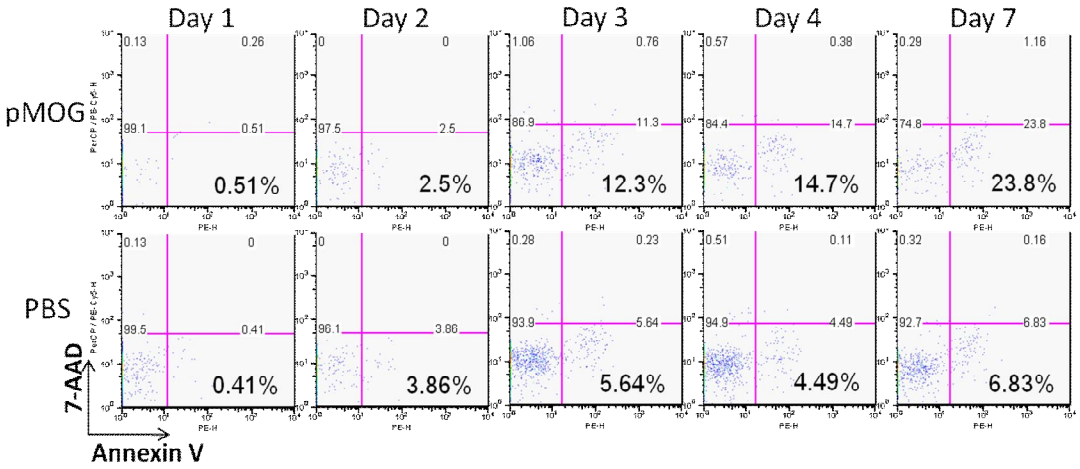

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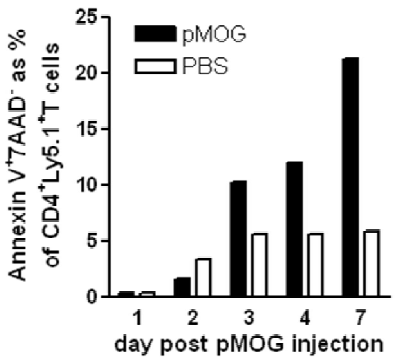

Figure 5.9 Administration of soluble pMOG induces apoptosis in activated 2D2 T cells. Mice were treated and spleen cells were gated as shown in figure 5.8. *A*, Cells were stained for Annexin V and 7-AAD to represent dead or apoptotic 2D2 cells. *B*, The percentage of Annexin $V^+7\text{-}AAD^-$ cells as a percentage of $CD4^+Ly5.1^+$ T cells on the day indicated. Data are from one of three repeated experiments (2 mice in each group).

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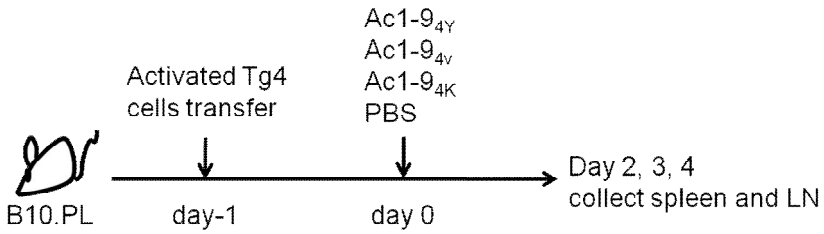

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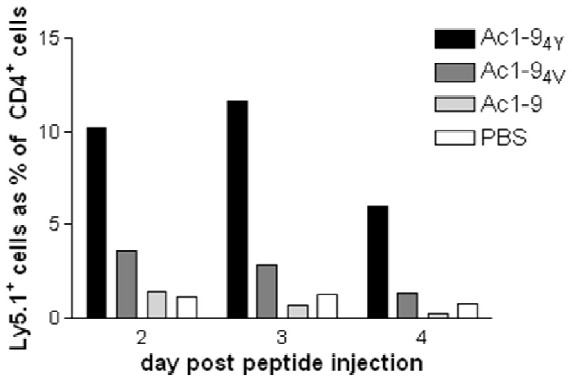

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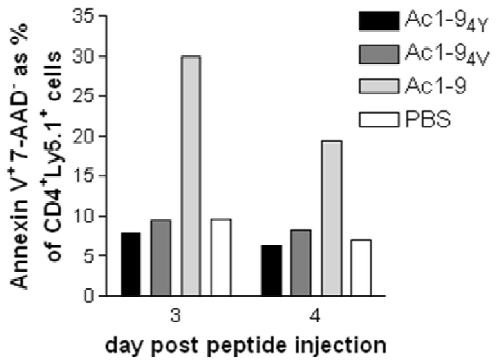

Figure 5.10 Administration of Ac1-9$_{4Y}$ expands activated Tg4 T cells transiently. Cells from Tg4 mice were cultured in medium with 10 £g/ml of Ac1-9 and rested in the Ac1-9 free medium with 10U/ml of IL-2 for 2 days. Live activated cells were harvested by Ficoll separation as described in *Materials and Methods*. *A*, B10/PL mice received 2 x 10^6 activated cells on day -1 and were injected with 200 £g of Ac1-9$_{4Y}$, Ac1-9$_{4V}$, Ac1-9 or PBS on day 0. *B*, plot shows CD4$^+$ Ly5.1$^+$ Tg4 cells as a percentage of CD4$^+$ T cells on the day the indicated. *C*, Annexin V$^+$ 7-AAD$^-$ cells as a percentage of CD4$^+$Ly5.1$^+$ Tg4 T cells on the day indicated. Data are from one experiment (2 mice in each group).

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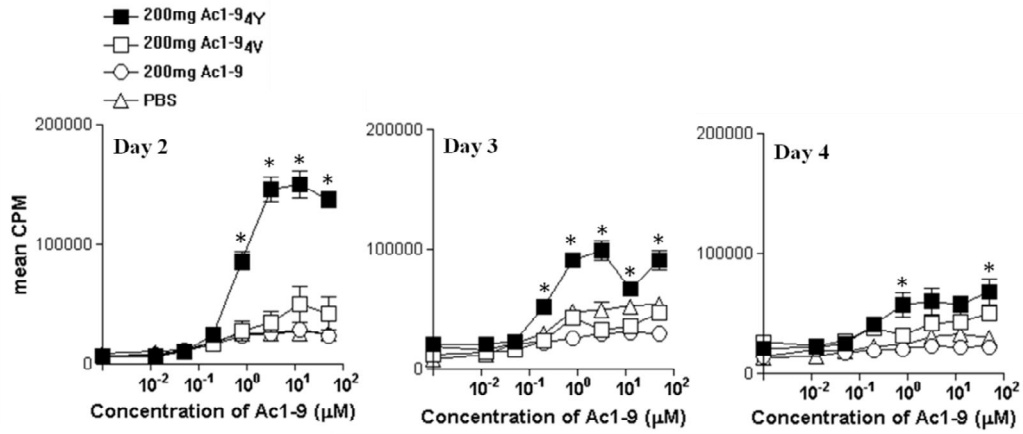

Figure 5.11 Administration of soluble, high affinity peptide induces T cell proliferation. Mice were treated as shown in figure 5.10A. *A*, spleen cells were harvested on day 2, 3, 4 and cultured for 72 hours with a pulsing of thymidine for the last 18 hours. Asterisks represent significant difference between Ac1-9_{4Y} treated group and other groups ($p < 0.05$ by Unpaired T-test). Asterisks represent significant difference between 2 groups ($p < 0.05$ by Unpaired T-test). Data are from one experiments and show mean ¡ SEM (2 mice in each group).

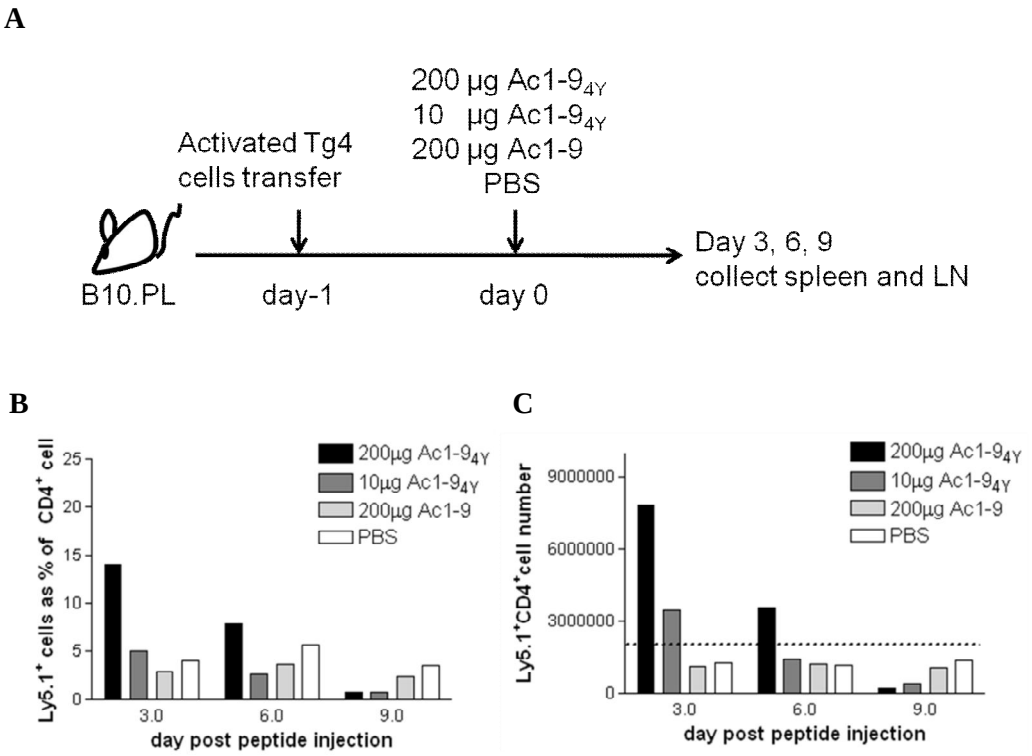

Figure 5.12 Soluble Ac1-9$_{4Y}$ treatment induces transient expansion of activated Tg4 T cells. Cells from Tg4 mice were activated as described in figure 5.10. *A*, B10.PL mice received 2 x 10^6 activated Tg4 cells on day -1 and were injected with either 200 £g of Ac1-9$_{4Y}$, 10 £g of Ac1-9$_{4Y}$,200 £g of Ac1-9, or PBS on day 0. Spleen cells were harvested and stained for CD4 and Ly5.1. *B*, CD4$^+$ Ly5.1$^+$ (Tg4) cells as a percentage of total CD4$^+$ T cells on the day indicated. *C*, plot represents the counting number of Tg4 cells isolated from spleen and LN. Dashed line represents 2 x 10^6 of Tg4 cells, the cell number for adoptive transfer. Data are from one of two repeated experiments (2 mice in each group).

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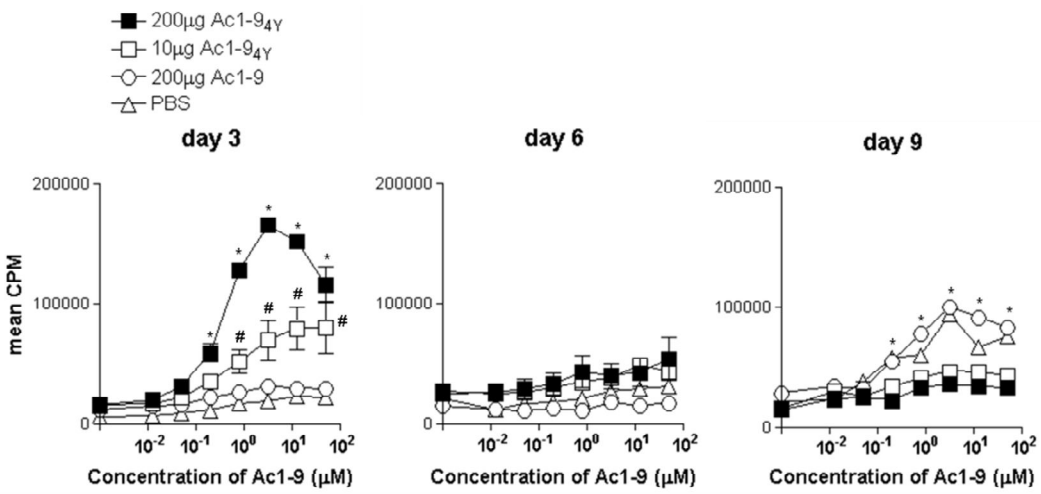

Figure 5.13 Soluble Ac1-9$_{4Y}$ treatment can induce a transient reactivation of antigen-experienced Tg4 T cells. Mice were treated as shown in figure 5.12A. *A*, spleen cells were harvested on day 3, 6 and 9 and cultured with Ac1-9 for 72 hours with a pulsing of thymidine for the last 18 hours. Asterisks represent a significant difference between 200 £g Ac1-9$_{4Y}$ treated group and other groups ($p < 0.05$ by Unpaired T-test). Hashes represent a significant difference between 10 £g Ac1-9$_{4Y}$ treated group and 200 £g Ac1-9 group ($p < 0.05$ by Unpaired T-test). Data are from one of two repeated experiments and show the mean ¡ SEM, 2 mice in each group).

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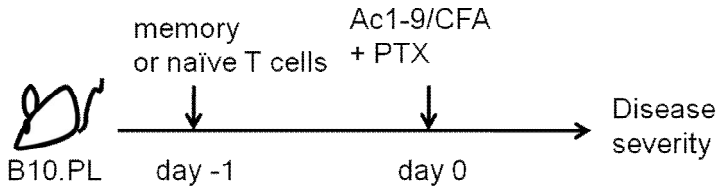

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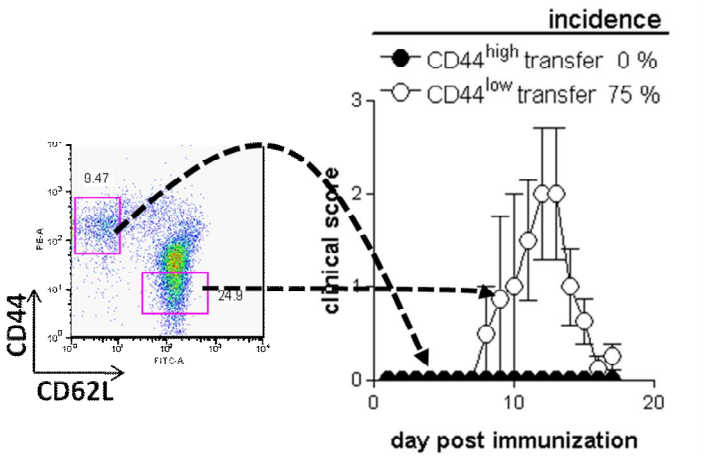

Figure 5.14 Transfer of memory and naive Tg4 cells before EAE induction. Tg4 mice were immunized with 50 £g of Ac1-9 in CpG/IFA. Six weeks later, cells were collected from spleen and LN and stained for CD4, Ly5.1, CD44 and CD62L. *A*, $CD4^+$ $CD44^{high}$ $CD62L^{low}$ and $CD4^+CD44^{low}CD62L^{high}$ cells were sorted by flow cytometry and 3 x 10^5 cells were transferred into B10.PL mice on day -1. Mice were induced to develop EAE with 100 £g of Ac1-9/CFA on day 0 (with PTX on day 0 and day 2). B, daily EAE score after disease induction ($p < 0.0001$ by Mann-Whitney U test). Data show mean ¡ SEM (Data are from one of two repeated experiments, 4 mice in each group).

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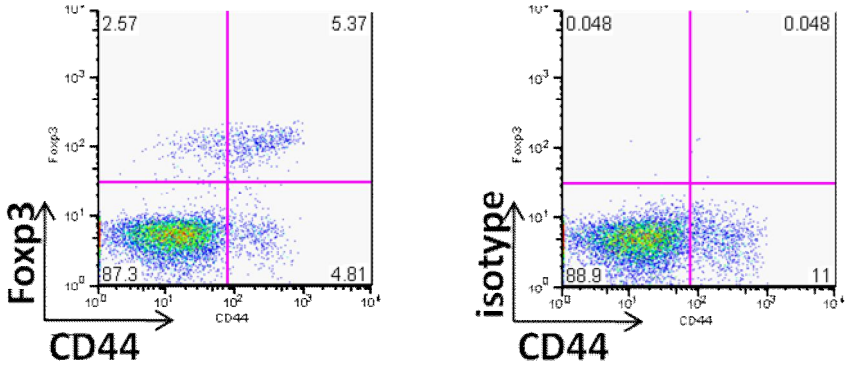

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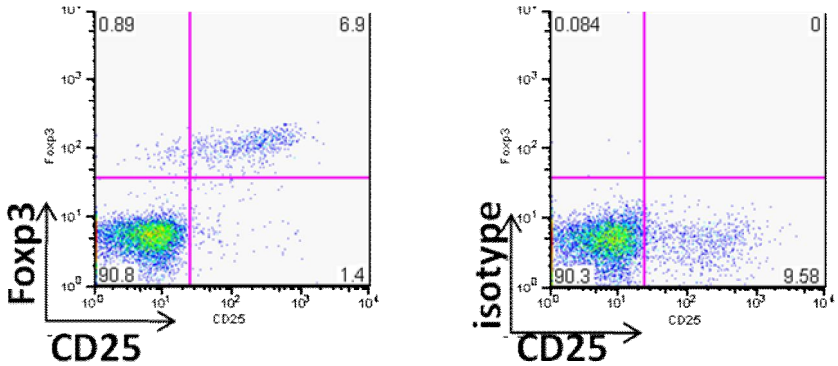

Figure 5.15 $CD4^{+}CD44^{high}$ cells express high levels of Foxp3. Tg4 mice were immunized with 50 £g of Ac1-9 in CpG/IFA. T cells from immunized Tg4 mice were harvested from spleen and LN 6 weeks post immunization and stained for CD4, CD25 and Foxp3. Plots show the expression of Foxp3 in $CD4^{+}CD44^{high}$ and $CD44^{low}$ cells (A) and $CD4^{+}CD25^{+}$ and $CD25^{-}$ cells (B).

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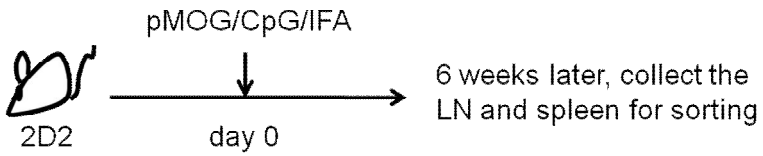

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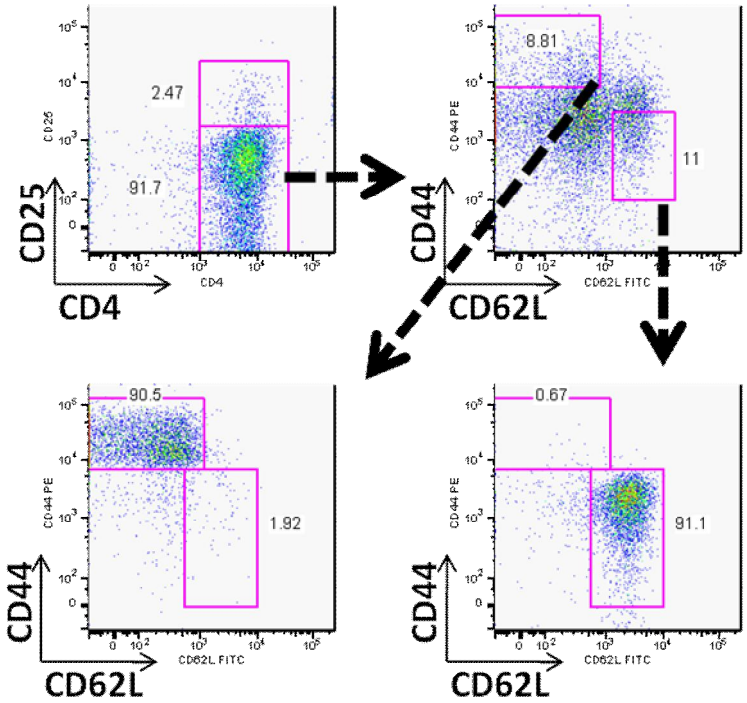

Figure 5.16 Memory and naive T cells from pMOG/CpG/IFA immunized 2D2 mice. *A*, mice were immunized with 50 £g of pMOG in CpG/IFA. Cells were harvested from draining lymph node and spleen 6 weeks after immunization. *B*, cells from immunized mice were stained for CD4, CD25, CD44 and CD62L for cell sorting. Two populations, $CD44^{high}CD62L^{low}$ (memory T cell) and $CD44^{low}$ $CD62L^{high}$ (naive T cell) were isolated.

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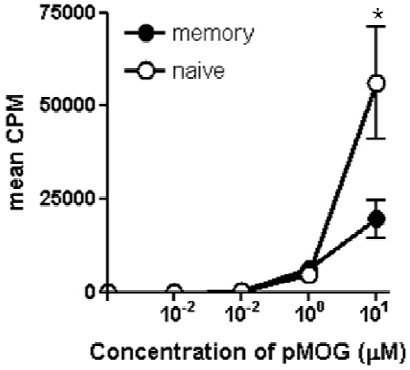

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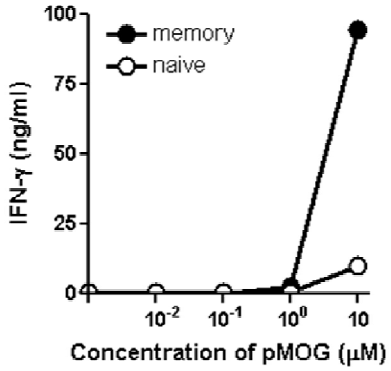

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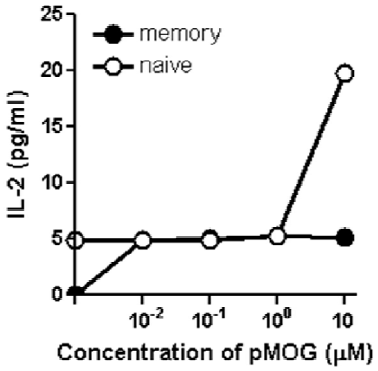

Figure 5.17 Proliferation and cytokine productions of memory and naive T cells. Mice were immunized as shown in figure 5.16. Cells were isolated based on their CD44 and CD62L expression and cultured with various doses of pMOG. 5 x 10^4 sorted cells were cultured with 5 x 10^5 of irradiated APC (72 hours with a pulsing of thymidine for the last 18 hours). *A*, Proliferation assay. Asterisks represent significant difference between 2 groups ($p < 0.05$ by Unpaired T-test). *B* and *C*, Cytokine assay by ELISA. Plots show the amount of IFN-γ (B) and IL-2 (C) at 72 hours against various doses of pMOG. The population of $CD44^{high}CD62L^{low}$ represents memory T cells and the population of $CD44^{low}$ $CD62L^{high}$ cells represents naive T cells. Data are from one of two repeated experiments.

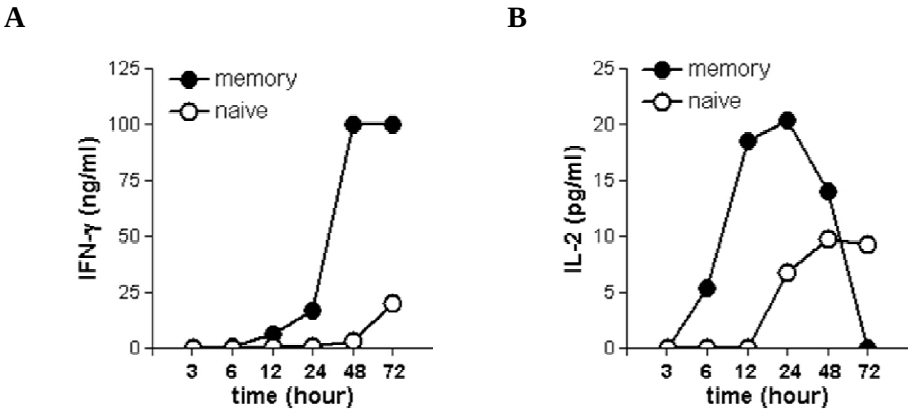

Figure 5.18 Time course of cytokine production in memory and naive T cells. Memory and naive T cells were separated as shown in figure 5.16. Cells were cultured in medium with 10 £g/ml of pMOG (5×10^4 of sorted cells with 5×10^5 of irradiated APC) for ELISA. Plots show the amount of IFN-γ (A) and IL-2 (B) at the time indicated. The population of $CD44^{high}CD62L^{low}$ represents memory T cells and the population of $CD44^{low}$ $CD62L^{high}$ cells represents naive T cells. Data are from one experiment.

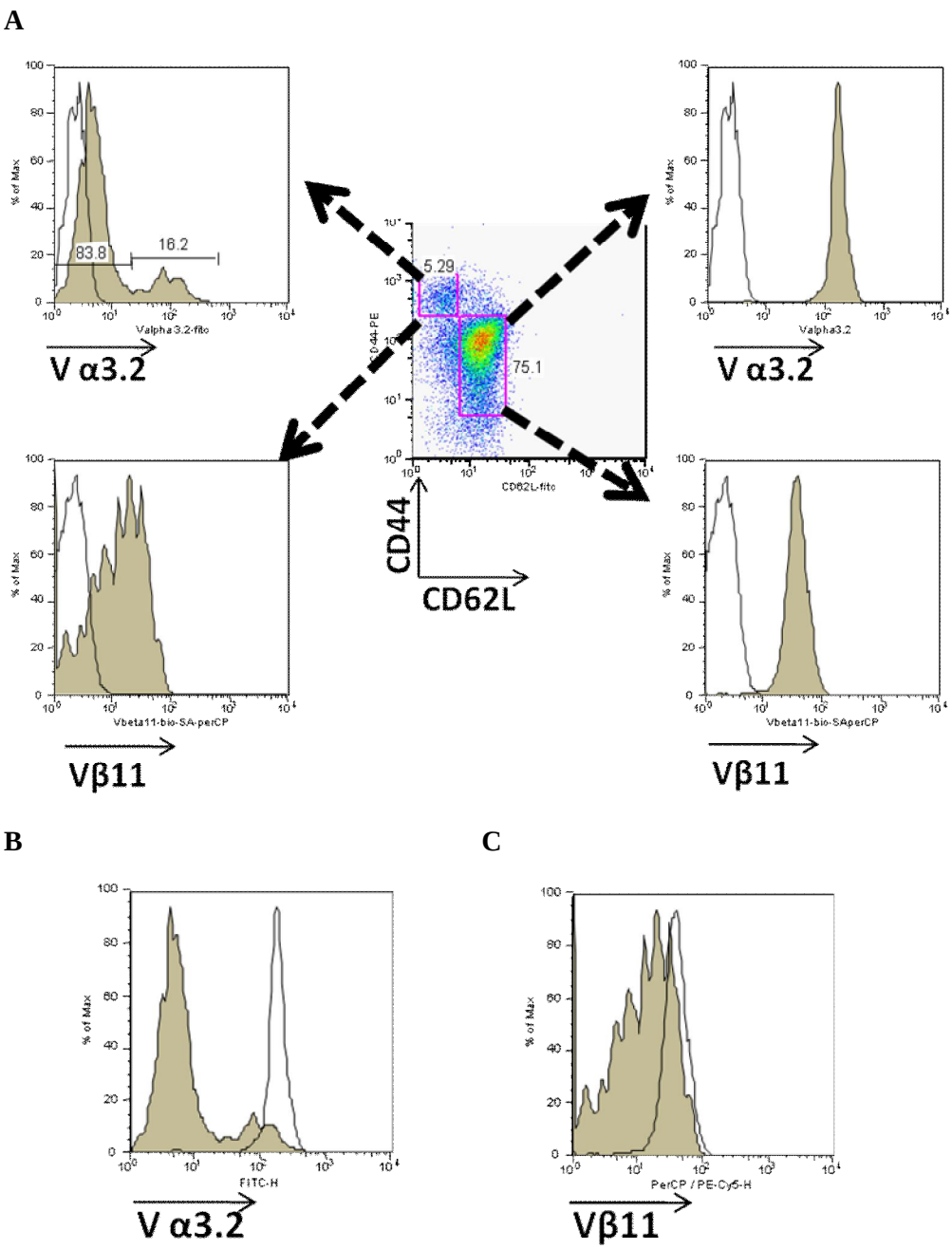

Figure 5.19 Expression of TCR Vα3.1 and Vβ11 in the memory and naïve 2D2 T cells. Cells were obtained from the immunized 2D2 mice as shown in figure 5.16 and stained for CD4, CD25, CD44, CD62L, TCR Vα3.2 and TCR Vβ11. *A*, $CD4^{+}CD25^{-}$ cells were separated into 2 populations as figure 5.16B ($CD44^{high}CD62L^{low}$ and $CD44^{low}$ $CD62L^{high}$). Expression of TCR Vα3.2 and TCR Vβ11 is shown for these populations. Open histograms show unstained baseline, shadowed histograms show stained samples. *B* and *C*, Plots represent the expression of TCR Vα3.2 (B) and TCR Vβ11 (C) with overlapping graphs of $CD44^{high}CD62L^{low}$ (shadowed histograms) and $CD44^{low}$ $CD62L^{high}$ (open histograms).

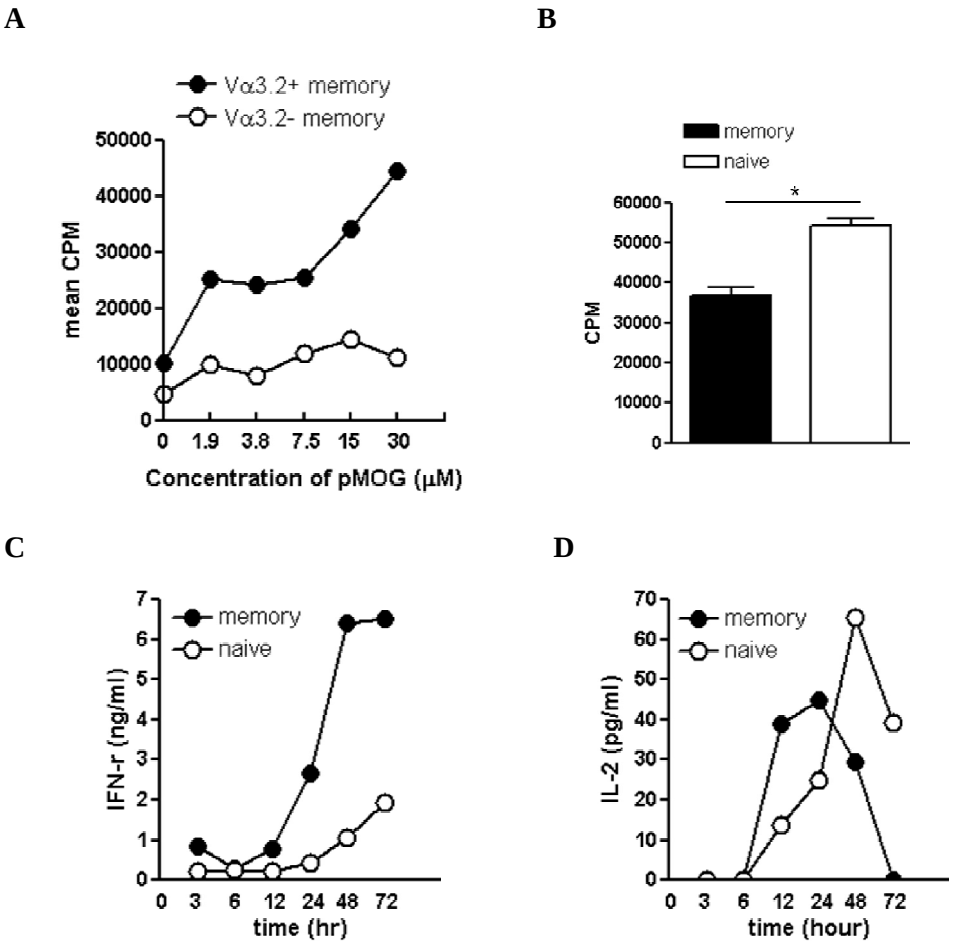

Figure 5.20 Comparison of effector memory and naive T cells. 2D2 mice were immunized as in figure 5.16A. $CD4^{+}CD25^{-}CD44^{high}$ $CD62L^{low}$cells were collected and sorted into $TCRV\alpha3.2^{+}$ and $TCRV\alpha3.2^{-}$ populations. 5 x 10^4 sorted cells were cultured with 5 x 10^5 irradiated APC for 72 hours with a pulsing of thymidine for the last 18 hours (A). *B-D*, memory and naïve T cells with $V\alpha3.2^{+}$ were cultured in medium with 10 £g/ml of pMOG for 72 hours (5 x 10^4 sorted cells with 5 x 10^5 irradiated APC with a pulsing of thymidine for the last 18 hours) (B). *C* and *D*, after culture for the time indicated, supernatants were harvested and analyzed for cytokines by ELISA. Plots show the amount of IFN-γ (C) and IL-2 (C) at the time indicated. The population of $CD44^{high}CD62L^{low}$ represents memory T cells and the population of $CD44^{low}$ $CD62L^{high}$ cells represents naive T cells. Asterisks represent significant difference between 2 groups ($p < 0.05$ by Unpaired T-test). Data are from one of two repeated experiments.

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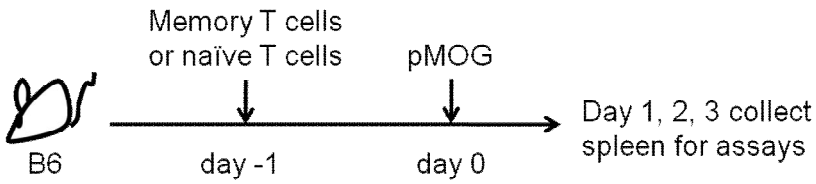

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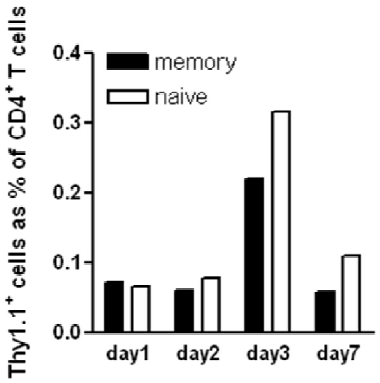


Figure 5.21 Administration of soluble pMOG triggers a transient expansion in both memory and naive T cells transferred groups. Memory ($CD4^+CD25^-V\alpha3.2^+$ $CD44^{high}CD62L^{low}$) and naive ($CD4^+CD25^-V\alpha3.2^+$ $CD44^{low}CD62L^{high}$) T cells were sorted from immunized 2D2 mice as shown in figure 5.16. *A*, Mice received either memory or naive T cells on day -1 and were injected with 200 £g of pMOG i.v. on day 0. *B*, spleen cells were harvested and stained for CD4 and Thy1.1. $CD4^+Thy1.1^+$ (2D2) cells are shown as a percentage of $CD4^+$ T cells at the day indicated. Data are from one experiment (2 mice in each group).

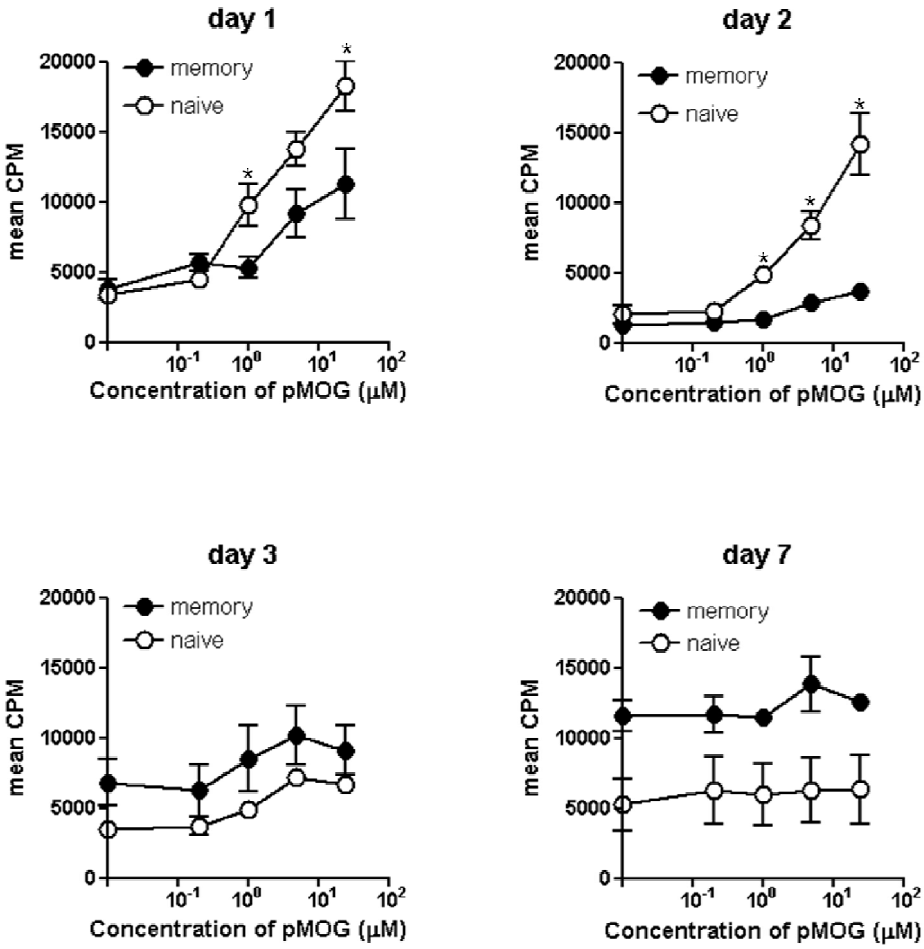
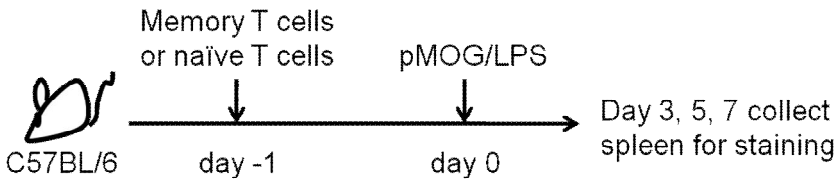


Figure 5.22 Administration of soluble pMOG can expand both memory and naive T cells transiently. The transfer and immunization protocol is shown in figure 5.21A. Spleen cells were harvested from indicated host mice and cultured for 72 hours with a pulsing of thymidine for the last 18 hours. Asterisks represent significant difference between 2 groups ($p < 0.05$ by Unpaired T-test). Data show mean ¡ SEM (Data are from one experiment, 2 mice in each group).

A



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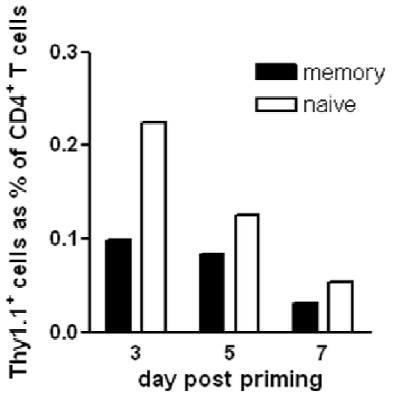

Figure 5.23 Naive 2D2 cells are more able to expand than memory 2D2 cells after pMOG /LPS priming *in vivo*. Memory ($CD4^+CD25^-V\alpha3.2^+$ $CD44^{high}CD62L^{low}$) and naive ($CD4^+CD25^-V\alpha3.2^+$ $CD44^{low}CD62L^{high}$) T cells were sorted from immunized 2D2 mice as in figure 5.16. *A*, Mice received either memory or naive T cells on day -1 and were injected with 200 £g of pMOG and 30 £g of LPS i.v. on day 0. *B*, cells were harvested from spleen and stained for CD4 and Thy1.1. Plot shows $CD4^+Thy1.1^+$ (2D2) cells as a percentage of $CD4^+$ T cells on the day indicated. Data are from one experiment (2 mice in each group).

Chapter 6. Discussion

The population of T cells specific to given antigenic peptide is undetectable in unimmunized individual (less than 1 / 10^5) and small in an immunized individual (around 1/ 10^4) (Tse *et al*., 1980). The system with adoptive transfer of transgenic cells provides a traceable *in vivo* model in which the pMHC-specific population is large enough to be detected by flow cytometry (Pape *et al*., 1997). However, there are some important issues when attempting to obtain memory T cells by immunizing 2D2 mice with pMOG / CFA and subsequently collecting these memory T cells for use in an adoptive transfer model. First, the frequency of memory T cells obtained from 2D2 mice primed with pMOG is still low (5-10 %), probably as a consequence of AICD, or migration of memory T cell (to the non-lymphoid tissues). Second, the non-transgenic population with endogenous TCR Vα (non-TCR Vα) is constantly present, recombinant activating gene (RAG) is functional in these mice. It seemed that the heterogeous TCR were only found in memory 2D2 T cells (most of naive T cells were transgenic cells), whereas only 16.2 % of T_{EM} are $V\alpha3.2^+$ $V\beta11^+$ transgenic cells in pMOG immunized 2D2 mice (Figure 6.19). In the OVA / DO11.10 system, only 6 % of memory T cells carried transgenic TCR (TCR $V\alpha2^+$ $V\beta8^+$) in non-immunized DO11.10 mice. In addition, non-immunized DO11.10 x $RAG^{-/-}$ mice lost 50 % of $CD44^{high}$ memory T cells and had a 2-fold enhancement of naive T cells compared to DO11.10 $RAG^{+/+}$ mice (Lee *et al*., 1996). As memory T cells were absent in mice raised in a germ-free environment, possibly those memory T cells bearing non-transgenic TCR Vα were derived from the stimulation of environmental antigen (Pape *et al*., 1997; Turner *et al*., 2004). Third, it has been reported that about 30% of polyclonal memory $CD4^+$ T cells express CD62L (Kassiotis and Stockinger,

2004). When collecting the memory $CD4^+$ T cells, excluding the $CD62L^{high}$ population may exclude some of memory cells. Therefore, the criteria for sorting the memory population should be adjusted. In future experiments, IL-7Rα, CCR7 and CCR5 would be considered as substitutes for CD62L for identifying the memory T cells.

The immune response against infection depends on antigen-specific memory T cells that survive for many years following initial exposure to antigen (Jameson, 2002). In adoptive transfer models, the number of transgenic cells contracts after antigen triggered expansion and it is hard to sustain a significant frequency for long (i.e. over one month) in the host. A Jenkins report showed that transgenic $CD4^+$ T cells quickly declined at high transferred frequency but persisted at low transferred frequency after adoptive transfer, indicating that the inter-clonal competition among diverse T cells can help T cell survival (Hataye *et al*., 2006). Recently CD8 and NK cells have been found to contribute to the loss of transgenic $CD4^+$ T cells after adoptive transfer, as cell deletion of either CD8 or NK cells can rescue transgenic DO11.10 cells in the wild type host (Duffy *et al*., 2008). Since the 2D2 cells are difficult to detect one month after primary immunization, the memory EAE model in this thesis was eventually applied in non-transfer systems. Recently some labs have constructed expression cassettes encoding the extracellular domains of murine $I\text{-}A^b$ α- and β-chains and pMOG peptide. The MHC II-peptide molecules were linked to streptavidin to make the MHC II tetramer, helping to track MOG-reactive T cells in non-transferred system (Korn *et al*., 2007; Sabatin *et al*., 2008). However, neither of these tetramers binds to 2D2 cells. As T cells derived from pMOG immunized B6 mice have a preference of TCR Vβ8.2 (about 50% of T cell hybridomas are TCR

Vβ8.2^{+}) (A. Carillo-Vico and S. Anderton, personal communication), the original 2D2 clone might represent a minor population in the pool of pMOG reactive T cells.

In this project, administration of tolerogenic peptide can inhibit EAE under naive and memory settings. However, cells harvested at the time of 4 weeks after EAE induction sometimes showed no significant difference in proliferation, IFN-γ or IL-17 production (figure 3.1, 5.2 and 5.4). Although primary immunization can generate Th1 and Th17 cells in mice (figure 4.16), it seems to have a weak link between IFN-γ / IL-17 and EAE score. Presumably, the timing of sampling (around day 28 in some experiments) and the organ sampled (mostly spleen) prevent a complete picture of the physical disease course. Thus, earlier harvesting (day 10-14 after EAE induction), non-lymphoid tissues, the CNS and assays for cytokine producing cells should be incorporated into future experiments. In addition, more evidence should be provided from different techniques. For example, apoptosis can be tested by TUNEL staining, staining for caspase and for Bcl-family proteins.

Both Th1 and Th17 cells can contribute to EAE (O¡Connor et al., 2008; McGeachy *et al*., 2007). A recent study showed that IL-12 polarized MBP-reactive T cells can up-regulate P-selectin glycoprotein ligand-1 (PSGL-1, CD162) which is co-localized with P-selectin (CD62P) on CNS blood vessels. Anti-PSGL-1 treatment significantly reduced passive EAE in mice, suggesting that IL-12-driven PSGL-1 expression can facilitate the infiltration of myelin-reactive T cells (Deshpande *et al*., 2006). In fact, the Segal lab has found that adoptive transfer of IL-12-modulated MBP-reactive T cells triggered a macrophage-rich infiltration in the CNS and predominantly up-regulated chemokine (C-X-C motif) ligand 9 (CXCL9), CXCL10, CXCL11 and

nitric oxide synthase 2 (NOS2, a product of activated macrophages and microglia) in the CNS and induced EAE. In contrast, adoptive transfer of IL-23-modulated MBP-reactive T cells triggered a dominant infiltration of neutrophils and up-regulated CXCL1, CXCL2 and granulocyte-colony- stimulating factor (G-CSF, activation and growth factor for neutrophils) in the CNS, indicating that IL-12- and IL-23 modulated cells transfer a passive encephalomyelitis by using distinct pro-inflammatory pathways involving distinct chemokine profiles in the CNS (Kroenke *et al.*, 2008). We have seen T cells deletion and unresponsive after the induction peptide-induced tolerance, thus it is of interest that whether treatment of tolerogenic peptide can affect chemokines / receptors and CNS infiltration.

Memory CD4 T cells promote more robust immunity than do naive cells because they respond to antigen more rapidly and promote a more vigorous effector response as compared with naive cells. As memory T cells can survive in an individual for long periods (Reinhardt *et al.*, 2001), finding that the systemic administration of peptide in soluble form can induce tolerance in memory T cell is of importance, and relevant to translational application. Recently we have used MOG_{35-50} APL ($p30\text{-}50_{37A}$) to induce tolerance in pMOG-experienced mice. The injection of MOG_{35-50} APL either before or after secondary immunization can inhibit EAE (Leech *et al.*, 2007). In this project, wild type pMOG was also proved to have protective effects in antigen-experienced mice, suggesting a feasible therapeutic strategy for MS.

When considering the translation of peptide therapy from EAE to MS, three lessons about memory T cell tolerance have arisen from this thesis. The first is that peptide

avidity matters. In the Ac1-9 system, low affinity peptide seemed to induce immediate loss of activated T cells (probably by apoptosis), whereas the high affinity peptide could induce a transient activation, before their subsequent loss (Figure 5.6). The Sadegh-Nasseri lab reported that memory, but not activated or naive, $CD4^+$ T cells were anergized upon the presentation of low densities of specific peptide, whereas peptide with a high avidity stimulation induced T cell activation (Mirshahidi *et al*., 2001; Mirshahidi *et al*., 2004), suggesting that a proper avidity should be applied to induce memory T cell tolerance. Second, the transient activated cells generated by the administration of high affinity peptide (like Ac1-9_{4Y}) to antigen experienced mice have potential to enhance pathology. It has been reported that after soluble peptide treatment *in vivo*, $CD4^+$ T cells can transiently produce IL-2 and IFN-γ and a transfer of these cells to recipient mice can damage tissues (Huang *et al*., 2003). Recently a study using an adoptive transfer system showed that memory T cells produce effector cytokines rapidly but show identical delays (3 days) in onset of cell division after *in vivo* stimulation, compared with naive T cells (Whitmire *et al*., 2008), suggesting that soluble peptide treatment may facilitate inflammation if the time point of tolerogenic injection is too closed to disease induction in the EAE model. Third, the double immunization system can provide a memory response in a mouse. However, memory responses and primary responses can overlap in time. It is possible that the secondary immunization triggers a ¡memory response¡ that is the net effect of a true memory response together with a primary response. An adoptive transfer system with memory cells or a non-adoptive system with a proper probe for memory T cells should be considered in future experiments. Therefore, a proper avidity of peptide, timing of tolerogenic injection, injection route and dose, and proper tool to identify memory T cells should be taken into consideration in

peptide-induced tolerance in memory T cells.

Figure 6.1 shows the model developed in this thesis in which soluble peptide administration provides signal 1 that can induce a transient activation to naive CD4 T cells, with subsequent cell deletion. Those cells that survive are in an anergic state. Tolerance in naive CD4 T cells protects the mice from ¡primary induced EAE¡. When cells initially encounter peptide / CFA (or peptide /CpG / IFA), signal 1 and signal 2 can fully activate naive T cells. A further administration of low affinity peptide can induce cell death immediately to those activated T cells, whereas a high affinity peptide can re-activate and expand those activated T cells transiently before they enter apoptosis. Cell death occurs in 90% to 95% of activated cells, the survivors differentiate into memory T cells. A subsequent administration of soluble peptide may induce memory T cell tolerance and control the memory responses.

To further approach the issue of memory T cell tolerance, some new directions are worth exploring. First, RAG-deficient 2D2 mice should be applied in the adoptive transfer system to assure that pMOG-specific T cells are targeted. To transfer memory T cells from 2D2 x $RAG^{-/-}$ mice can assure the mechanisms of peptide induced tolerance. Because 2D2 cells are not currently detectable by pMOG-A^b tetramers, a non-transfer system (with B6 mice in which pMOG-A^b tetramers can be used to assess the pMOG-reactive T cells) could be used. By matching the cytokine production profile, the early IFN-γ producing, tetramer positive cells can be identified as the re-activated memory CD4 T cells (although the later effector T cells are difficult to distinguish from those derived from naive cells). If a soluble peptide injection can down-regulate those IFN-γ producing, tetramer positive cells, this could

indicate that memory T cells are being controlled. Second, memory T cells can efficiently enter non-lymphoid tissues to survey for antigen, facilitating the early detection of and rapid response to infection. Whether memory T cells are substantially lost or just alter their ability of migration is unclear. A wider range of tissues can be sampled for assessing the frequency of memory T cells. Also, information about the expression of homing markers by memory T cells can be obtained by an adoptive transfer system (either with or without peptide administration). These approached should provide basic information relevant to translating peptide-induced T cell tolerance from mice to humans.

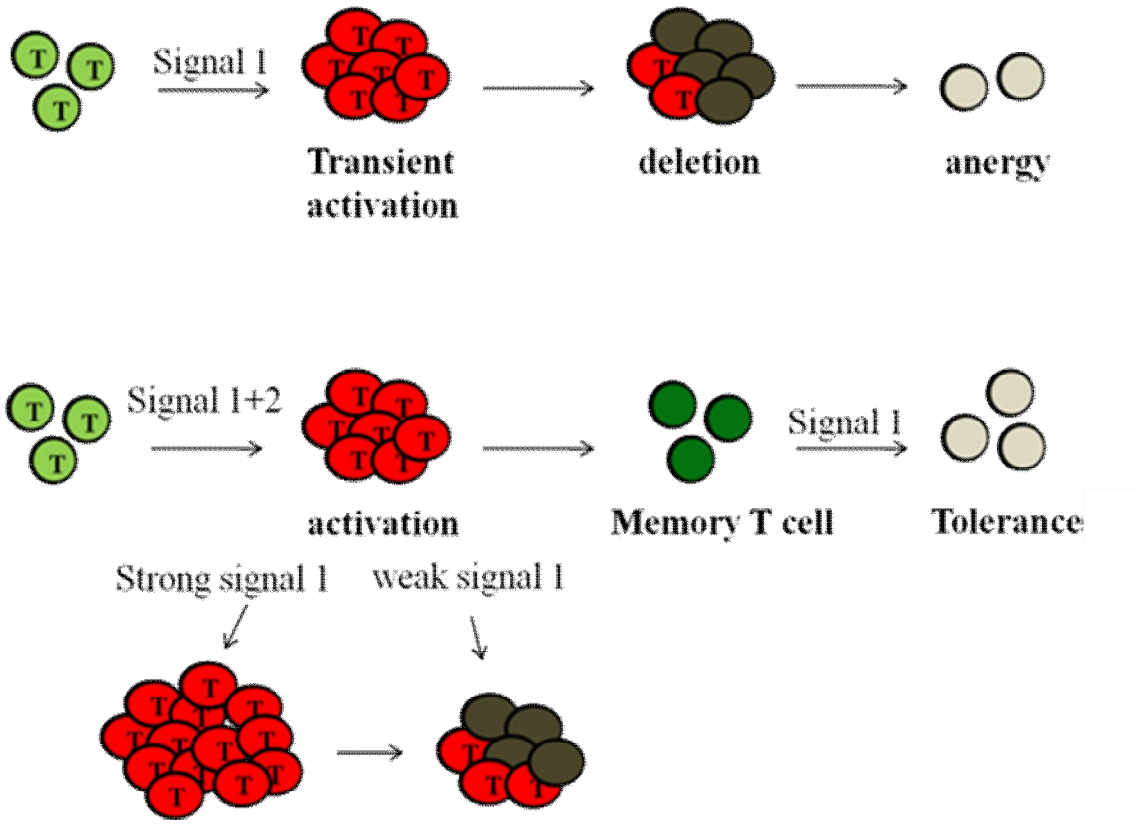

Figure 6.1 Models for peptide induced tolerance in naive, activated and memory T cells. Naive T cells received signal 1 only can be activated transiently and subsequent be deleted, leaving survival cells in anergic state. When both signal 1 and signal 2 exist, naive T cells can be fully activated. The further administration of weak signal 1 can induce cell death immediately to those activated cells, whereas a strong signal 1 can re-activate and expand the activated cells transiently before they fate to apoptosis. Most of the activated / effector T cells die after the antigen are cleared, while a few survivals become memory T cell. A further signal 1 provided by soluble peptide administration may induce memory T cell tolerance.

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Appendix. Published paper