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Characteristics of induced regulatory T cells and bystander suppression

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Declaration

I declare that this thesis has been written by myself, describes my own work and that this work has not been submitted for any other higher degree. Richard O'Connor performed cytokine bead arrays. Experiments in allergic airways inflammation were performed in collaboration with Karen Mackenzie and Dominika Nowakowska.

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Abstract

Regulatory T cells expressing the transcription factor Foxp3 have a critical role in the maintenance of tolerance to both self and innocuous exogenous antigens. Humans and mice die from overwhelming autoimmunity in the absence of $Foxp3^+$ Treg whilst administration of regulatory T cells has shown promise therapeutically in ameliorating autoimmunity in several animal models. Regulatory T cells arise naturally in the thymus (nTreg) but may also be induced from naïve $Foxp3^-$ cells in the presence of TGF-β (iTreg), both *in vitro* and *in vivo*. This thesis focuses on *in vitro* generated mouse iTreg, testing the hypothesis that they are able to effect bystander suppression; iTreg activated by a given antigen are able to suppress other responding cells with different antigen reactivities.

Chapter 3 details an *in vitro* assay system using iTreg and responder cells recognising different antigens (TCR transgenic cells). Evidence for bystander suppression is presented and that did not require the presence of iTreg-relevant antigen but did require iTreg-relevant MHC Class II. The kinetics of iTreg suppression are discussed, with evidence presented that iTreg exert their effects early in co-culture. Chapter 4 identifies the production of three pro-inflammatory cytokines by iTreg - IFN-γ, GM-CSF, and TNF. These were not involved in the *in vitro* suppressive mechanism, but early abrogation of TGF-β signalling did inhibit suppression. Chapter 5 describes the *in vivo* function of iTreg under various experimental protocols. iTreg did not limit initial proliferation of naïve T cells in response to antigen but did limit the development of effector cells producing pro-inflammatory cytokines. Exposure to a pro-inflammatory environment *in vivo* led to iTreg producing IFN-γ and TNF, but not GM-CSF. This could be replicated *in vitro* by exposure to IL-6, IL-12 or IL-27. Finally, evidence for bystander suppression by iTreg *in vivo* is presented, with a reduction in effector cells producing pro-inflammatory cytokines shown in an allergic airways diease model.

Table of Contents

List of Figures

List of Tables

List of abbreviations used

6-MP – 6-mercaptopurine

α3(IV)NC1 – non-collagenous domain of α3-chain of type IV collagen

AAI – allergic airways inflammation

ACAD – activated cell autonomous death

AHR – aryl hydrocarbon receptor

AICD – activation induced cell death

AIF – apoptosis inducing factor

AIG – autoimmune gastritis

Aire – Autoimmune regulator

ALDH1A1 – retinaldehyde dehydrogenase 1

ALK5 – Activin receptor-like kinase receptor 5

ALPS – autoimmune lymphoproliferative syndrome

AP-1 – activator protein-1

Apaf-1 – apoptotic protease-activating factor 1

APC – antigen presenting cell

APECED - autoimmune polyendocrinopathy, candidiasis, ectodermal dystrophy

APL – altered peptide ligand

APS1 – autoimmune polyglandular syndrome type 1

ATP – adenosine triphosphate

BAL – bronchoalveolar lavage

BLIMP-1 – B-lymphocyte-induced maturation protein 1

Bcl – B-cell lymphoma

BSA – bovine serum albumin

cAMP – cyclic adenosine monophosphate

CCR – chemokine receptor

CD – cluster of differentiation

CDR – complementarity determining region

CFA – complete Freund's adjuvant

CFSE – carboxyfluorescein succinimidyl ester

CIITA – MHC Class II transactivator

CLIP – Class II associated Ii peptide

CO_2 – carbon dioxide

CREB – cAMP-response element-binding protein

CTLA-4 – cytotoxic T-lymphocyte antigen 4

CNS – central nervous system

dATP – deoxyadenosine-5-triphosphate

DC – dendritic cell

DIABLO – direct IAP binding protein with low pI

DISC – death inducing signaling complex

DMSO – dimethyl sulfoxide

DTR – diphtheria toxin receptor

DTX – diphtheria toxin

EAE – experimental autoimmune encephalomyelitis

ELISA – enzyme-linked immunosorbent assay

Eomes - eomesodermin

ER – endoplasmic reticulum

ERK – extracellular signal-regulated kinase

FACS – fluorescence-activated cell sorting

FADD – Fas-associated protein with death domain

Fas – Fs7 associated cell surface antigen

Fc – constant fragment

FCS – fetal calf serum

FITC – fluorescein isothiocyanate

FLICE – FADD-like interleukin-1 beta-converting enzyme

FLIP – FLICE/caspase 8 inhibitory protein

Foxp3 – Forkhead box P3

GAD-65 – glutamic acid decarboxylase 65

GARP – glycoproteins A repetitions predominant

GITR – glucocorticoid-induced T cell receptor

GM-CSF – granulocyte monocyte colony stimulating factor

Grb2 – Growth factor receptor-bound protein 2

GVHD – graft versus host disease

HA –haemagglutinin

HDR – hyperparathyroidism, sensorineural deafness and renal dysplasia

HLA – human leukocyte antigen

IBD – inflammatory bowel disease

ICAM -1 – Intercellular adhesion molecule-1

ICCS – intracellular cytokine staining

ICER – inducible cAMP early repressor

IDO – indoleamine 2,3-dioxygenase

IFN-γ – Interferon-γ

IgG – Immunoglobulin G

Ii – invariant chain of MHC Class II

IL – interleukin

iLC - innate lymphoid cell

i.p. - intraperitoneal

IPEX – immune dysregulation, polyendocrinopathy, enterocolitis, X-linked

IRF-1 – immune regulatory factor-1

IRF-8 – interferon regulatory factor-8

ITAM – immunoreceptor tyrosine-based activation motif

iTreg – induced regulatory T cell

i.v. - intravenous

Jak – Janus tyrosine kinase

JCV – John Cunningham virus

JNK – c-Jun N-terminal kinase

KO - knockout

LAG – lymphocyte activation gene

LAP – latency associated peptide

LAT – linker for activation of T cells

Lck – lymphocyte-specific protein tyrosine kinase

LCMV – lymophocytic choriomeningitis virus

LIGHT – lymphotoxin-like, exhibits inducible expression

LN – lymph nodes

LPAM-1 – lymphocyte Peyer's patch adhesion molecule-1

LPS – lipopolysaccharide

LT-α – lymphotoxin-α

LTBP – latent TGF-β binding protein

Lys - lysine

MACS – magnetic activated cell sorting

MAPK – mitogen-activated protein kinase

MARCH1 – membrane-associated ring finger (C3CH4) -1

MBP – myelin basic protein

MIIC – MHC Class II compartment

MHC – major histocompatibility complex

MOG – myelin oligodendrocyte glycoprotein

MPT – mitochondrial permeability transition

mRNA – messenger ribonucleic acid

MS – multiple sclerosis

mTEC –medullary thymic epithelial cell

mTOR – mammalian target of rapamycin

MyD88 – myeloid differentiation primary response gene 88

nTreg – natural T regulatory cell

NF-AT – nuclear factor for activated T cells

NF-κB – nuclear factor-κB

NK – natural killer

OVA – ovalbumin

P38MAPK – p38 mitogen-activated protein kinase

PAMP – pathogen associated molecular pattern

PBMC – peripheral blood mononuclear cell

PBS – phosphate buffered saline

PBS-T – PBS with 0.1% Tween

PD – programmed death

PE - phycoerythrin

PFA - paraformaldehyde

PI3K – phosphatidylinositol-3-kinase

PLP – proteolipid protein

PLCγ1 – phospholipase C-γ1

PMA – phorbol 12-myristate 13-acetate

PML – progressive multifocal leukoencephalopathy

PTEN – phosphatase and tensin homologue detected on chromosome ten

RA – retinoic acid

RAG – recombination activation gene

RANKL – receptor activator of NF-κB ligand

RBC – red blood cell

RORγt – retinoic acid orphan receptor γt

RTL – recombinant TCR ligand

Runx – Runt related transcription factor

s.c. - subcutaneous

SLE – systemic lupus erythematosus

SLP-76 – SH2 domain-containing leukocyte phosphoprotein of 76kDa

Smac – second mitochondria-derived activator of caspases

SMAD – single mothers against decapentoplegic

SP1 – specificity protein 1

STAT – signal transducer and activator of transcription

Tbet – T-box expressed in T cells

TCR – T cell receptor

TGF-β – transforming growth factor – β

Th – helper T cell

TLR- toll-like receptor

TNF – tumour necrosis factor

Tr1 – type 1 regulatory T cell

TRADD – TNFR-associated death domain

TRAF – TNFR-associated factor

TRAIL – TNF-related apoptosis-inducing ligand

TRAPS – TNFR associated periodic syndrome

Treg – regulatory T cell

TSA – tissue specific antigen

TSDR – Treg specific demethylated region

Tyr – tyrosine

UCB – umbilical cord blood

ZAP-70 - Zeta-associated protein of 70kDa

1 Introduction

1.1 General Introduction

In order to counteract the multitude of potential pathogens, an intricate immune system has evolved with both innate and adaptive arms. Innate immunity includes physical barriers such as the skin, the enzymatic or anti-bacterial contents of bodily fluids, such as tears, sweat, and urine, the highly acidic pH encountered early within the gastro-intestinal tract, and indeed our own commensal micro-organisms competing for essential nutrients and 'living space' on all surfaces of the body that may be exposed to external influence.

If these early non-specific defences are breached then cellular immunity comes to the fore. Various cells are found at sites of potential intrusion acting in a non-antigen specific manner to prevent further colonization. The monocyte-macrophage lineage is of particular note in phagocytosing foreign matter that may be detrimental. Neutrophils, eosinophils, or basophils may be summoned according to the nature of the micro-organisms. Natural killer (NK) cells, γδ-T cells, and the more recently recognized innate lymphoid cells (iLC) are all relevant in an innate response. Processing of foreign proteins within antigen presenting cells leads to presentation of antigen in conjunction with the major histocompatibility complex (MHC) that may then engage with T cells. This may trigger activation of $CD8^+$ cells and cell-mediated toxicity, or of $CD4^+$ T cells with subsequent expansion and B-cell recruitment ending in clonal expansion of B cells, antibody production, activation of the complement cascade, and the development of persisting memory cells, the hallmark of adaptive immunity.

Not all foreign material is intrinsically harmful. Life occurs within a miasma, in which deliberate ingestion of multiple antigens is necessary for nourishment and a bacteria and fomite-rich atmosphere is inhaled during breathing. If immune and cellular responses were to be triggered for every antigenic encounter that occurs without discrimination, these systems would be almost immediately overwhelmed. Provocation of an immune response must also be curtailed once the offending cause has been removed. The various noxious molecules released during innate immunity are equally damaging to host tissues and a defensive system causing more damage than the initial offence is not conducive to survival. As such, various checks and balances within the immune system exist to ensure that immune responses are only triggered when the stimulus is potentially harmful, the inflammatory reactions are localized to the area of stimulation, and removal or resolution leads to a rapid termination of the ongoing immune activation. Foremost are the concepts of regulation and

tolerance, either preventing an unwanted immune response or promoting a benign response to otherwise 'foreign' antigen. Clearly, there is an absolute requirement for equilibrium between immunity against harmful 'invaders' and tolerance of benign 'passers-by' (Anderton, 2006).

A major mediator of this balance is a cell population dedicated to suppression, rather than promotion, of the inflammatory response, regulatory T cells (Treg). Various subsets exist (discussed in section 1.7.3) but they universally down-regulate effector functions, and deficiency in numbers or function can be associated with the development of abnormal responses to harmless antigen (Haas et al., 2005, Kukreja et al., 2002). This can clinically manifest as allergy to respiratory antigens, food intolerances or colitis to ingested antigens, or autoimmunity in the case of self antigen reactivity. Following their relatively recent identification, much effort has gone into exploring the fundamental biology and mechanisms behind these regulatory populations. As ever, an understanding in health leads to an understanding in disease.

A great deal of Treg biology remains to be unraveled, including the exact mechanisms by which they suppress (Vignali et al., 2008). The existence of multiple subsets makes it highly probable that varying mechanisms are used by different cells, promoting redundancy within the immune system, and offering alternative means if one mechanism of suppression is ineffective. That autoimmunity exists dictates that this is inadequate, but offers the prospect of restoration of the aberrant function leading to resolution of autoimmune disease in a much more 'physiological' sense than the current use of broad immunosuppressants (Riley et al., 2009).

One crucial phenomenon seen in Treg biology is the ability to effect bystander suppression. Once a Treg is activated, suppression affects all cells in the neighbouring response, not just the cells sharing antigen specificity with the Treg (Thornton et al., 2000). This is analogous to a schoolteacher's raised voice in a crowded classroom with one child misbehaving – the naughty child stops what they're doing, but so do all the other 'bystanding' children. Bystander suppression offers both positive and negative aspects. It extends beyond antigen specificity and so may offer use of Treg as a therapy in diseases where the exact auto-antigen is not yet identified, or multiple antigens are implicated without any clear immunodominance in the human setting (e.g. multiple sclerosis (MS)) (Fisson et al., 2006, Miller et al., 1993). Conversely, the purpose of targeted cellular therapy would be to achieve specific immunosuppression of the pathogenic cells without impacting on the overall ability of the immune system to respond to pathogens (Soulillou et al., 2001).

How this bystander suppressive effect is mediated, and indeed whether it truly occurs, has been under investigation since it was first described. This thesis will attempt to unravel some of the mechanisms behind this bystander suppression effect, to hopefully take another small step forward in its eventual application in the clinical setting.

1.2 T cell biology

A defining feature of the T-lymphocyte is the ability to recognise peptide bound to major histocompatibility complex (MHC) molecules found on the surface of antigen presenting cells (APC) (Marrack et al., 1986). Binding to the T cell receptor (TCR), the peptide-MHC drives the downstream activation of the T cell that causes up-regulation of transcription factors (Weiss et al., 1994). The 'classical' response of T cells to TCR stimulation is clonal expansion, with differentiation to effector phenotypes and cytokine release (notably IL-2) (Cantrell et al., 1984). The exact nature of the phenotype differentiation and cytokine release depends on the intercellular milieu and nature of the signals received. These events will now be considered in detail.

1.2.1 The T cell receptor

The T cell receptor is a heterodimeric receptor consisting of two polypeptide chains, α andβ, with a disulphide bond between the two. Each chain has a variable amino-terminal region and a constant region, the latter of which has a short transmembrane domain (Acuto et al., 1983). The variable region of the TCR provides heterogeneity in T cell recognition of antigen, but each T cell expresses multiple copies of identical receptors. The variable region is genetically determined through random rearrangements of the germline during T cell development within the thymus (Tonegawa, 1983). The germline has multiple loci within the variable (V), diversity (D) and joining (J) segments (reviewed in Alt et al., 1992). Through random recombination, a single V locus is joined to a single J locus forming a new exon. This then undergoes transcription together with the C_α locus to form a complete α chain region mRNA that is subsequently translated to the α-chain protein. The β chain is similarly formed but consists of V, D and J loci, all of which recombine together prior to transcription and splicing to a C_β chain. The human germline contains over 70 Vα domains, 61 Jα domains, 52 Vβ domains, and 13 Jβ domains (Rowen et al., 1996). The structure and antigen specificity of the TCR is predominantly determined by the complementarity

determining region 3 (CDR3) (Gorski et al., 1994, Yao et al., 2007), which also undergoes the random insertion of non-germline-nucleotides at the V-D and D-J joining junctions (Raaphorst et al., 1994). The potential combinations achievable through both this VDJ rearrangement and CDR3 variability is more than adequate to ensure response to potentially pathogenic antigens that may be encountered in combination with MHC molecules. All of these reactions are catalysed by a group of enzymes encoded by recombination activation genes (RAGs) (Schatz et al., 1989, Wayne et al., 1994). Absence of RAG leads to a deficiency in mature B and T cells within a mouse model (Mombaerts et al., 1992) and has been widely used to study adoptively transferred T cell responses in a lymphopenic host.

1.2.1.1 CD4 and CD8

Binding of the TCR with the MHC-peptide complex alone is insufficient to form a stable interaction between T cell and APC. Other co-receptors are required to stabilize the binding, notably CD4 or CD8. CD4 binds to MHC class II molecules distantly from the peptide binding groove, such that the complex at the TCR is not disrupted, or remains open for TCR engagement to occur (Doyle et al., 1987). Binding of CD4 to MHC leads to interaction of the intracellular portion of CD4 with the tyrosine kinase Lck (Artyomov et al., 2010). Binding of CD8 to MHC class I has a similar stabilizing effect. Expression of CD4 or CD8 on T cells determines their restriction, as $CD4^+$ cells are unable to stabilize interactions with peptide-MHC Class I complexes, so will only undergo TCR stimulation by peptide presented in association with MHC Class II molecules (Gay et al., 1987). Likewise, $CD8^+$ T cells can only stably interact with the peptide-MHC class I complex and initiate their cytotoxic effects upon TCR signaling. The cellular expression of MHC is therefore relevant to the type of response desired and likely pathogens the cells may encounter.

1.2.2 The Major Histocompatibility Complex (MHC)

1.2.2.1 MHC structure and expression

MHC molecules may be Class I or II, dependent on their structure. Class I molecules consist of a transmembrane α chain with three domains noncovalently bound to β_2 microglobulin. The first and second domains fold such that a 'groove' is formed into which peptide processed within the cell can then be presented, forming a TCR ligand (reviewed in Madden, 1995). Class II molecules have two transmembrane glycoprotein chains, α and β, both with two domains, which form a similar folded structure to class I molecules. The peptide

binding groove is formed between the α1 and β1 domains, which is notably shallower than that in class I. The extensive polymorphism amongst MHC molecules is concentrated within the residues that form the peptide binding groove; this determines the repertoire of peptides with which an individual MHC molecule can form stable complexes. Class I molecules can be identified at high expression in most lymphoid tissues and is also expressed on all other nucleated cells. Expression of class II molecules is generally restricted to the myeloid cell lineage, primarily upon dendritic cells, B cells and thymic epithelia – all legitimate antigen presenting cells for T cells (Daar et al., 1984). However class II expression can occur in non-APC mediated by IFN-γ or other stimuli (Steimle et al., 1994). Class II expression has been identified in keratinocytes, endothelium, fibroblasts, mesenchymal stroma, and in pulmonary cells of non-haematopoietic lineage (Kreisel et al., 2010). The role of class II molecules outwith APC may be more related to peripheral tolerance than disease states.

1.2.2.2 Regulation of MHC class II expression

Expression of MHC class II is regulated by MHC class II transactivator (CIITA), which itself has several isoforms with differential cellular expression (Reith et al., 2005). Within immature dendritic cells (DCs), the type I CIITA promoter forms a complex with PU.1, interferon-regulatory factor 8 (IRF-8), nuclear factor-κβ (NF-κβ) and SP1. This promotes CIITA transcription and subsequently class II transcription, leading to high levels of class II. Within mature DCs, CIITA transcription is inhibited by an alternative complex with BLIMP-1 (Piskurich et al., 2000). Further regulation of CIITA expression via transcriptional feedback mechanisms permits lineage-specific expression in health but up-regulation in other cells via chromatin modification and response to extracellular signaling including TGF-β during pro-inflammatory or other states (Reith et al., 2005).

1.2.2.3 Altered peptide ligands

The exquisitely sensitive nature of the MHC groove-peptide interaction can be appreciated when considering the effect of altered peptide ligands (APL). One such example is the acetylated N-terminal nonamer of myelin basic protein, MBPAc1-9 (Anderton, 2001). The strength of the MHC-peptide complex can be indirectly measured via the time taken for the complex to dissociate. Normally, MBPAc1-9 has lysine as the fourth residue, the hydrophilic nature of which antagonizes the hydrophobic binding cleft, leading to a very low affinity (Fairchild et al., 1993). Alteration of this residue to the much more hydrophobic tyrosine (MBPAc1-9 (4Tyr)) forms a peptide-MHC complex with much higher affinity,

readily appreciated *in vitro* by markedly increased proliferation of relevant T cells, and measurably several orders of magnitude higher in direct binding competition assays.

1.2.3 Antigen processing and presentation by MHC

The $CD4^+$ T cell receptor forms a complex with peptide bound within the cleft of a class II MHC molecule. This peptide may be derived from either foreign protein or self. How does the antigen presenting cell turn an external protein into a loaded peptide? Class I and II MHC molecules have different functions and different mechanisms. As this thesis is primarily concerned with $CD4^+$ cells and self-reactivity leading to autoimmunity, only class II processes will be discussed.

Uptake of antigenic material by macrophages or dendritic cells is typically by phagocytosis from the extracellular environment (though intracellular pathogens may induce this process deliberately) (Trombetta et al., 2005). The vesicles thus formed migrate through the cell and bind with intracellular lysosomes containing multiple proteases, of which the cathepsins are most studied, rendered inactive by the neutral pH within the endosome. These are then acidified upon activation of the cell, leading to degradation of the proteins and formation of smaller peptide fragments (reviewed in Jensen et al, 2008).

At synthesis, MHC molecules are translocated to the endoplasmic reticulum (ER). The individual α and β chains remain trapped in the ER until heterodimerisation, a process enhanced by binding non-covalently to the MHC class II-associated invariant chain (Ii) (Busch et al., 2000). This binding occurs in the peptide cleft, preventing binding of other newly manufactured proteins within the ER. The chain complexes with chaperone proteins and enters a dedicated endosome – the MHC class II compartment (MIIC), that transports the complex through the cell (Cresswell, 1996).

The MIIC fuses with early endosomes containing proteases and peptides. These peptides may be of pathogenic origin, but normal cell turnover also provides a rich supply of self-peptide. The fusion of MIIC and early endosomes permits cathepsins to degrade the Ii such that only a small fragment remains behind in the peptide groove – the class II associated Ii peptide (CLIP) (Tolosa et al., 2003). CLIP is then exchanged for antigenic peptides within the MIIC. If binding with peptides does not occur in the endosome, the MHC molecule is rapidly degraded. Once peptide is loaded, the MHC-peptide complex is then returned to the

outer membrane of the MIIC by undefined processes. Whether peptide loading can occur without internal transport is unclear.

Two atypical class II molecules are only identified intracellularly and regulate the peptide loading of MHC class II. HLA-DM is found only within the MIIC, and does not have an open peptide groove. Instead it binds to other MHC class II molecules, catalyzing the emptying of the peptide groove but also binding to peptide-MHC complexes (Kropshofer et al., 1996). In this manner, HLA-DM competitively inhibits very weak binding, removing unstable complexes but being unable to dissociate a stronger peptide-MHC bond. HLA-DO is another atypical class II molecule unable to bind and present peptide, instead binding and inhibiting the action of HLA-DM. This prevents complete removal of the Ii rendering the MHC molecules unable to present or permitting only weakly binding peptides to engage with the MHC (Hornell et al., 2006). This ultimately leads to far fewer effective MHC class II molecules on the cell surface. IFN-γ causes increased expression of HLA-DM and reduced expression of HLA-DO such that within the pro-inflammatory environment, far more class II molecules with strong peptide-MHC complexes appear on the cell surface. Expression of HLA-DO is restricted to B cells and the thymic epithelia.

Once the peptide-MHC complex is formed, this stabilizes the MHC molecule and prevents degradation (Sadegh-Nasseri et al., 1991). The complex is rapidly transported through the cell via microtubule-based motors, dynein transporting inwardly and kinesins to the plasma membrane. This transport is regulated within DCs by maturation signals. Lipopolysaccharide (LPS) causes formation of microtubules by the MIIC itself leading to rapid transport of MHC class II to the cell surface, depleting the intracellular pool (Cella et al., 1997). Thus, maturation promotes a greater number of potential $CD4^+$ T cell interactions.

Degradation of MHC class II is dependent on ubiquitylation via MARCH1, which is increased in the presence of IL-10 (Thibodeau et al., 2008). DC maturation increases the lifespan of the MHC class II molecule, thought to be via expression of CD83, which inhibits this MARCH1 ubiquitylation (Tze et al., 2011). Animal models without MHC class II ubiquitylation still have degradation of these molecules, so there are clearly other undefined processes involved as well (McGeehee et al., 2011).

Though the classical notion of the MHC class II molecule as a ligand for T-cell receptors holds true, signaling also occurs in the other direction with the MHC molecule serving as receptor rather than ligand (Al-Daccak et al., 2004). Lymphocyte activation gene 3 (LAG3)

appears to activate survival pathways to prevent cell death via signaling through MHC (Andreae et al., 2002), whilst conversely ligation of MHC on mature DCs induces caspase-independent cell death (Bertho et al., 2002). More relevant, engagement of T-cell receptors leads to a rapid up-regulation in microtubule formation and enhanced transport of MHC class II to the plasma membrane. This suggests bidirectional signaling occurs, with TCR ligation providing a positive feedback loop that increases the likelihood of further T cell engagement.

1.2.4 T Cell Signaling

T cell-activation is a multi-stage process. Binding of the TCR to the MHC-peptide complex provides 'Signal 1' but other co-stimulatory interactions are required to lead to full cell activation – 'Signal 2', (initially proposed for B-cells; Brestcher et al., 1970). Release of cytokines may then influence other surrounding cells, providing 'Signal 3'.

1.2.4.1 Signal 1

Engagement of the T cell receptor with the peptide-MHC complex commences signaling through the TCR. This is commonly referred to as 'signal 1'. The TCR complexes with CD3, initiating the tyrosine phosphorylation of the immunoreceptor tyrosine-based activation motifs (ITAMs) of four separate components of the complex (Straus et al., 1992). These phosphorylations lead to recruitment via the SH2 domain of ζ-associated protein of 70kDA (ZAP-70) (Wange et al., 1993). ZAP-70 is a protein tyrosine kinase that, on activation, catalyses the phosphorylation of several major signaling molecules, such as linker for activation of T cells (LAT) (Zhang et al., 1998). These reactions occurs rapidly on TCR engagement, leading to downstream activation of Grb2 family proteins, (Zhang et al., 2000), phospholipase C-γ1 (PLCγ1) (Yablonski et al., 1998), and SH2 domain-containing leukocyte phosphoprotein of 76kDa (SLP-76) (Clements et al., 1998). The outcome of these activations is up-regulation of the transcription factors in the nuclear factor for activated T cells (NF-AT) (Peng et al., 2001), nuclear factor–κB (NF-κB) (Kane et al., 2001) and activator protein-1 (AP-1) pathways (Rincon et al., 1994).

Signal 1 alone is inadequate to lead to full T cell activation - clonal expansion and IL-2 production - as defined above. In the absence of further signaling, T cell activation does not occur. Indeed, the T cell is rendered anergic, and will not respond with full activation if both

signal 1 and 2 are subsequently provided (Jenkins et al., 1987). Anergy is discussed further in section 1.7.2.

1.2.4.2 Signal 2

1.2.4.2.1 Co-stimulatory molecules

Additional co-stimulatory molecule interactions are needed to provide 'signal 2'. The commonest interaction to provide this is between CD28 on the T cell, and CD80 or CD86 on the APC, and is mediated via cell survival signaling through bcl-XL (Boise et al., 1995) and phosphatidylinositol-3-kinase (PI3K) pathways (Pages et al., 1994). CD28 is constitutively expressed on most $CD4^+$ T cells and is further up-regulated on T cell activation (Turka et al., 1990). These signals enhance clonal expansion, cell survival, cytokine production and differentiation with effector functions (Lenschow et al., 1996). Despite the nomenclature, the nature of 'signal 2' may be more relevant to how T-cells respond than signal 1 (Wells et al., 1997).

Multiple other co-stimulatory molecules are recognised. OX40 is expressed on activated T cells, and interacts with OX40L on APC (Gramaglia et al., 1998). Prolonged expression of OX40 and OX40L is seen in autoimmunity, including mouse models (Weinberg et al., 1999, Stuber et al., 2000). OX40 signaling promotes T cell proliferation and cytokine production, even in the absence of CD28 (Akiba et al., 1999), though it cannot replace CD28 in providing full co-stimulation. Over- or under-expression of OX40 in mice predominantly affects $CD4^+$ T cell proliferation (Murata et al., 2000).

4-1BB is also expressed on activated T cells though, in contrast to OX40, appears more relevant to $CD8^+$ than $CD4^+$ T cell responses (Taraban et al., 2002). 4-1BBL is expressed on activated DCs, B cells, and macrophages. Interaction between 4-1BB and its ligand leads to T cell proliferation (Gramaglia et al., 2000).

Glucocorticoid-induced T cell receptor (GITR) is up-regulated on activated T cells, and constitutively expressed on Treg (Shimizu et al., 2002), while the ligand (GITRL) undergoes transient up-regulation following TLR ligation by APC (Stephens et al., 2004). GITR/GITRL signaling promotes proliferation and cytokine production by T cells, including in the absence of CD28 (Shimizue et al., 2002), but also has a unique role in Treg, potentially maintaining peripheral Treg numbers (Stephens et al., 2004). GITR signaling in effector T cells renders them relatively resistant to Treg-mediated suppression.

Other members of the TNF superfamily and their ligands may provide costimulation, including CD30, herpes virus entry mediator/LIGHT (lymphotoxin-like, exhibits inducible expression), CD27/CD70, and CD30/CD30L (Watts, 2005). Other molecules may also provide co-stimulation such as CD46 (Astier et al., 2000) or CD55 (Capasso et al., 2006).

1.2.4.2.2. Co-inhibitory molecules

Not all signal 2 interactions provide stimulation. Various co-inhibitory molecules have also been identified, and render T cells less responsive on interaction. Cytotoxic T lymphocyte antigen-4 (CTLA-4) and Programmed Death-1 (PD-1) have both been shown to down-regulate T cell activation and alter the signaling mechanisms activated.

CTLA-4 is robustly confirmed as a co-inhibitory molecule rapidly up-regulated upon cell activation via CD28 (Walunas et al., 1994), an effect proportional to the TCR affinity for the antigen (Allison et al., 1998). CTLA-4 binds to CD80 and CD86, directly competing with CD28 for signaling interactions (Walunas et al., 1996), leading to reduction in IL-2 production, cell proliferation and down-regulation of TCR signaling (Chikuma et al., 2003). Other mechanisms are also suggested, including ligand-independent models (Chikuma et al., 2003a). CTLA-4 deficient mice show spontaneous lymphoproliferative disorders and death within one month of age (Waterhouse et al., 1995), signifying a role in establishing and maintaining peripheral tolerance. CTLA-4 is also highly expressed on the surface of Treg (Tang et al., 2004) and contributes to regulation through APC interactions. The relevance of CTLA-4 is shown by the success of the fusion protein therapy abatacept, discussed later (1.9.4).

PD-1 is a member of the CD28 superfamily and up-regulated on activation in T cells, B cells and some myeloid cells (Agata et al., 1996). PD-1 signaling inhibits IFN-γ production and T cell proliferation (Freeman et al., 2000). Engagement of PD-1 can be via either PDL-1, expressed on T and B cells, DCs, macrophages, and several non-haematopoietic cell lineages (Yamazaki et al., 2002), or PDL-2 which is restricted to DCs and monocytes (Ishida et al., 2002). Both PDL-1 and PDL-2 are up-regulated in response to tissue inflammation (Liang et al., 2003). The development of spontaneous autoimmunity in the PD-1 deficient mouse implies a role for this co-inhibitory molecule in peripheral tolerance (Nishimura et al., 1999), particularly in maintenance of tolerance once re-established (Keir et al., 2006), and appears to be primarily mediated through cell anergy (Fife et al., 2006). Up-regulation of PDL-1 on inflamed tissue may prevent destruction of self (Kinter Jet al., 2008) and the expression of

both PD-1 and PDL-1 on Treg strongly suggests a role in regulation (Francisco et al., 2009). PDL-2 also appears relevant in oral tolerance (Zhang et al., 2006).

1.3 $CD4^+$ effector subsets

Once antigen is processed by an APC and presented within the MHC Class II molecule, it serves as a ligand for the TCR of a $CD4^+$ T cell. Assuming the antigen is a novel entry into the immune repertoire, only naïve T cells will be available to recognize the peptide-MHC complex. Appropriate TCR engagement and co-stimulatory signals from the APC then lead to activation of the T cell and differentiation. The direction of this differentiation depends on the type of co-stimulatory signals, (Signals 1 and 2) but also the surrounding cytokine milieu (Signal 3). Whilst initially this process was thought to be unidirectional and an either/or phenomenon, the potential fates for T cells have expanded whilst the concrete nature of this differentiation is now challenged.

The first segregation of effector T cells came in 1986 when long-term $CD4^+$ cell lines were divided into two groups by their cytokine production – Th1 cells producing IFN-γ and Th2 cells producing IL-4 (Mossman et al., 1986). These two lineages have very different *in vivo* functions, with Th1 cells being crucial for immunity to intracellular micro-organisms whilst Th2 cells co-ordinate immune responses to extracellular pathogens, including parasites (Paul et al., 1994).

1.3.1 Th1 cells

Th1 cells were initially defined by their cytokine profile, with the production of IFN-γ, IL-2, and lymphotoxin-α (LT-α). The importance of IL-12 in inducing a Th1 phenotype was soon identified (Hsieh et al., 1993), with the relevance of IFN-γ providing a positive feedback loop to Th1 differentiation being appreciated later (Lighvani et al., 2001). Up-regulation of the IL-12Rβ2 chain on TCR stimulation is maintained by surrounding IL-12 and IFN-γ, leading to enhanced responses to circulating IL-12 (Szabo et al., 1997), whilst IL-18 may have a synergistic effect (Robinson et al., 1997). Th1 actions are via their cytokines. IFN-γ stimulates macrophages, boosting microbial killing (Schroder et al., 2006), and IL-2 is needed for cellular expansion and memory cell establishment (Darrah et al., 2007). LT-α has

an, as yet, uncertain role though appears to reflect disease activity in autoimmunity (Selmaj et al., 1991).

The Th1 lineage is associated with expression of the master transcription factor, T-box expressed in T cells - Tbet (Szabo et al., 2000). IFN-γ signaling via signal transducer and activator of transcription (STAT)1 up-regulates Tbet expression (Lighvani et al., 2001) whilst STAT4 activation by IL-12 boosts Th1 responses, including the promotion of further Th1 differentiation via IFN-γ (Thierfelder et al., 1996). Tbet-deficient mice are still capable of IFN-γ production via alternate transcription factors. Eomesodermin (Eomes), an equivalent factor triggering IFN-γ in $CD8^+$ T cells, is also up-regulated on Th1 cells (Pearce et al., 2003). IL-21 inhibits the up-regulation of Eomes on Th1 cells but does not affect Tbet (Suto et al., 2006). Other transcription factors highly expressed on Th1 cells are Runx3 (Djuertic et al., 2007) and HIx (Mullen et al., 2002).

Mutations in the IL-12R render individuals at higher risk of intracellular infection (de Jong et al., 1998), as do IFN-γ gene mutations (Newport et al., 1996).

1.3.2 Th2 cells

Th2 cells were the first to be more fully characterized, with the identification of the vital role of IL-4 in differentiation (Swain et al., 1990), and mediating B cell class switching (Kopf et al., 1993). Other Th2 cytokines are IL-5, IL-9, IL-10, IL-13, and IL-25. The actions of IL-4, IL-5 and IL-13 are discussed later (section 1.4.2). IL-9 is involved in allergy (Longphre et al., 1990) whilst IL-25 (also called IL-17E) amplifies Th2 responses (Fallon et al., 2006). Mirroring Th1 development, IL-4Rα is increased during Th2 differentiation to provide positive feedback to production. CD25 and IL-33Rα are also up-regulated (Hwang et al., 2002, Schmitz et al., 2005), potentially increasing survival via greater responsiveness to IL-2. IL-4 activates STAT6 (Kaplan et al., 1996) and induces the Th2 master transcription factor, GATA3 (Zhu et al., 2001). GATA3 deficiency abrogates Th2 differentiation *in vitro* and *in vivo* (Zhu et al., 2004, Pai et al., 2004). IL-2 signals via STAT5 (Cote-Sierra et al., 2004) and is non-redundant for effective Th2 differentiation and expansion.

Absence of GATA3 in humans leads to hyperparathyroidism, sensorineural deafness and renal dysplasia (HDR) syndrome (van Esch et al., 2000) with greatly reduced Th2 cells and IL-4, and diminished IgG4 due to ineffective class switching. GATA3 polymorphisms are implicated in asthma and allergy (Pykalainen et al., 2005). lL-4Rα mutations are also

associated with atopy, both dermatitis and asthma (Hershey et al., 1997, Mitsuyasu et al., 1998).

1.3.3 Th17 cells

The dichotomy of Th1/Th2 differentiation was upset when a Th1 paradox was finally unraveled. IFN-γ deficient mice are more susceptible to experimental autoimmune encephalitis (EAE) – at the time considered to be a prototypic Th1-mediated model. IL-12p40 deficient mice were, conversely, relatively protected. How, then, was the absence of Th1 differentiation protective, but not the absence of the (assumed) key pathogenic cytokine? IL-12, the main inducer of Th1, is composed of two sub-units, p35 and p40, but this latter is also a component of IL-23 – subsequently identified as being necessary for EAE development (Cua et al., 2003). Shortly following the newly appreciated role for IL-23 was a new T cell lineage, identified by its expression of IL-17 in the presence of IL-23 – the Th17 cell (Aggarwal et al., 2003, Harrington et al., 2005).

Induction of Th17 *in vitro* by TCR engagement in the presence of TGF-β and IL-6 (Veldhoen et al., 2006) leads to production of IL-21 (Korn et al., 2007), recapitulating the positive feedback seen in the other two lineages. TGF-β is critical for Th17 differentiation (Yang et al., 2008) whereas IL-6 can be replaced by IL-21. IL-23 appears to be necessary for maintenance of the Th17 lineage (Nurieva et al., 2007). Th17 cells express IL-23R, Il-1R1 and IL-18Rα (Zhou et al., 2007). The actions of Th17 cells are predominantly against extracellular bacteria and fungal pathogens (Weaver et al., 2006), mainly via the recruitment of neutrophils. Activation of STAT3 by either IL-6 or IL-23 leads to IL-22 production by Th17 cells (Zheng et al., 2007), which appears to mediate IL-23 actions as well as having an immunoprotective role (Zenewicz et al., 2007).

The main transcription factor for the Th17 lineage is the retinoic acid orphan receptor RORγt (Ivanov et al., 2006). RORγt deficiency reduces IL-17 production and partly protects against EAE. RORα is also up-regulated (Yang et al., 2008a) and, whilst dispensable in the presence of RORγt, appears to have a synergistic effect.

Mutations in the STAT3 pathway prevent signaling through IL-6, IL-21 and IL-23 i.e. key Th17 cytokines. This has been identified as the cause of hyper-IgE (Job) syndrome, leading to recurrent staphylococcal, streptococcal and fungal infection (Milner et al., 2008). Altered Th17 homeostasis is now being investigated in a wide range of autoimmune disorders.

1.3.4 Other effector T cell subsets

1.3.4.1 Th9 cells

Th9 cells are characterized by induction with IL-4 and TGF-β (Dardalhon et al., 2008, Staudt et al., 2010), and an early and significant production of IL-9 and IL-10. Via STAT6, TGF-β up-regulates expression of the transcription factor PU.1, which appears to be the master factor for Th9 cells (Chang et al., 2010). GATA3 is also up-regulated via STAT6 and IL-4 (Goswami et al., 2012), though not to the levels seen in Th2 cells, and may inhibit TGF-β mediated induction of Foxp3 (Mantel et al., 2007). IL-9 promotes mast cell proliferation and stimulates chemokine release, whilst IL-10 is a major immunoprotective cytokine. Th9 cells appear to be pro-inflammatory in some autoimmune models (Dardalhon et al., 2008, Jager et al., 2009) and allergy (Bullens et al., 2011), but confusingly have protective or Treg-beneficial effects in other reports (Elyaman et al., 2009). Whether this is related to their convergent cytokine production, or is a result of experimental methodology, is one of many questions remaining unanswered on this relatively new subset.

1.3.4.2 Th22 cells

The most recently defined 'distinct' subset is the Th22 lineage (Eyerich et al., 2009). They are induced in the presence of IL-6 and TNF by plasmacytoid dendritic cells (Duhen et al., 2009), and secrete IL-22, IL-26 and IL-13, with IL-22 appearing to be most functionally significant. These cells can be identified via chemokine receptors, being $CCR6^+$, $CCR4^+$ and $CCR10^+$, and have up-regulation of the aryl hydrocarbon receptor (AHR) as their master transcription factor. AHR agonism leads to direct production of IL-22 (Quintana et al., 2008). IL-22 signals through activation of several STAT pathways, including STAT1, STAT3, and STAT5 (Xie et al., 2000), and also directly induces the production of β-defensins within the gastro-intestinal mucosa (Aujla et al., 2008). IL-22 is a relatively pro-inflammatory cytokine implicated in rheumatoid arthritis (Ikeuchi et al., 2005), Crohn's disease (Sekikawa et al., 2010), and psoriasis (Wolk et al., 2009). Th22 cells themselves show dysregulation in rheumatoid arthritis (Zhang et al., 2012) and skin disorders (Cavani et al., 2012).

1.4 Cytokines

Dependent on the signals received and the surrounding environmental milieu, T cells are capable of producing a broad variety of cytokines. Th1 cells classically produce IFN-γ, Th2 cells produce IL-4, IL-5 and IL-13, Th17 cells produce IL-17, and the *in vitro*-generated 'Th-GM-CSF' produce high quantities of GM-CSF. Cytokines recognized as anti-inflammatory include TGF-β and IL-10. Each of these cytokines will be briefly discussed.

1.4.1 IFN-γ

All interferons are glycoproteins, with the nomenclature indicating they 'interfere' with viral replication, their classical function (Isaacs et al., 1957). IFN-γ is the only recognized class II interferon, binding to the IFNG receptor found on many cell subtypes. This receptor is formed by four chains; two IFNGR1 chains responsible for ligand binding, and two IFNGR2 chains involved in signaling transduction (Bach et al., 1997). Neither chain has intrinsic signaling mechanisms. IFNGR1 has binding motifs for both Janus tyrosine kinase (Jak)1 and STAT1. IFNGR2 has a binding motif for Jak2. IFN-γ binds to IFNGR1, then this complex interacts with IFNGR2, leading to full signaling (Kotenko et al., 1995). The Jak-STAT pathway is shared amongst many of the cytokine receptor superfamily, and so will be considered in more detail here (Subramaniam et al., 2001).

Binding of IFN-γ leads to autophosphorylation and activation of Jak2 via IFNGR2. Phosphorylated Jak2 then transphosphorylates Jak1 leading to alteration in IFNGR1 that permits binding and subsequent phosphorylation of STAT1 (Igarashi et al., 1994). Dimerisation of phosphorylated STAT1 (typically homodimerisation with STAT1 but other STATs may be involved) leads to dissociation from the IFNGR-IFN-γ complex (Greenlund et al., 1995), translocation to the nucleus, and binding with promoter elements that then alters transcription of multiple genes, such as up-regulation of Immune Regulatory Factor (IRF-)1, and Tbet (Darnell Jr et al., 1994). STAT1 phosphorylation is a key step in the IFN-γ signaling pathway. STAT1 deficient mice demonstrate a similar phenotype to IFNGR1 deficient mice, in response to IFN-γ (Meraz et al., 1996). STAT1 phosphorylation can also be induced via other stimuli, including LPS, IL-12, IL-2, and TNF, thus providing a means for other cellular responses to modulate IFN-γ production (Schroder et al., 2004).

Production of IFN-γ by $CD4^+$ T cells is mainly associated with the classical Th1 phenotype (i.e. in defence against viral and intracellular infections) and is markedly enhanced in the

presence of IL-12 and IL-18 from APCs (Fukao et al., 2000). IFN-γ also provides positive feedback for its own production, as increased IFN-γ also stimulates further production by Th1 cells (Szabo et al., 2000). IL-4, IL-10, and TGF-β all inhibit the synthesis of IFN- γ. Th17 cells also produce IFN-γ, either singly or in combination with IL-17 (Bettelli et al., 2008). This is seen in animal models frequently several days after initial T-cell activation, and it remains unclear whether IFN-γ production is a natural delayed effect of Th17 stimulation, or if it represents functional plasticity, with re-differentiation to a Th1-like cell. $CD8^+$ T cells and NK cells also produce significant amounts of IFN-γ. B cells, NKT cells and APCs are all also capable of synthesis.

IFN-γ has a multitude of effects. Up-regulation of MHC class I is markedly increased, partly through induction of the 'immunoproteosome', enhancing the APC's efficiency of peptide loading (Groettrup et al., 2001). MHC class II is up-regulated in professional APCs but can also be induced in non-professional APCs. MHC-peptide complexes are also increased in number via an increase in genes and proteins involved in peptide processing and loading, mainly via direct effects on CIITA (Mach et al., 1996). IFN-γ promotes Th1 responses, and stimulates production of IL-12, also boosting cytotoxic effects via APCs (Yoshida et al., 1994). Th2 responses are inhibited via reduction in IL-4 secretion. Macrophages are highly affected by IFN-γ (hence the original name, macrophage-activating factor), with prevention of apoptosis and growth arrest, increased pinocytosis and phagocytosis, and, most importantly, enhanced microbicidal activity. B cells are stimulated to produce immunoglobulins, and undergo class switching to IgG2a. NK cell activity is also enhanced.

Absence of IFN-γ in the mouse does not lead to overt dysmorphology, but does cause increased susceptibility to multiple pathogens, including mycobacteria (Huang et al., 1993). Within autoimmunity models, absence of IFN-γ does not protect against EAE, which highlighted that it was not entirely Th1-mediated (Chu et al., 2000). IFN-γ has been implicated in autoimmune nephritis models. Within humans, IFNGR1 deficiency is a rare genetic disorder marked by high susceptibility to mycobacterial infection (Jouanguy et al., 1997). Naturally occurring mutations within STAT-1 have also been identified, which similarly present with impaired resistance to mycobacterial and viral infection. Complete absence of IFN-γ in humans has not been reported, suggesting it is inherently lethal. IFN-γ is implicated as a key pathogenic cytokine in many disorders, particularly those of autoimmune origin (SLE, type one diabetes mellitus), as might be expected.

1.4.2 'Th2'-associated cytokines – IL-4, IL-5, IL-13

IL-4 is produced by $CD4^+$ Th2 cells, basophils, mast cells, and NKT cells. It has also been reported by γδ-T cells (Ferrick et al., 1995) and eosinophils (Zuany-Amorim et al., 1998). IL-4 and IFN-γ have antagonistic roles in $CD4^+$ T cell differentiation. IFN-γ promotes Th1 responses and inhibits IL-4 production while, conversely, IL-4 inhibits IFN-γ production and stimulates up-regulation of GATA3 in naïve T cells, the master transcription factor for the Th2 phenotype (Zheng et al., 1997). IL-4 is predominant in controlling B cell class switching to the production of IgE and either IgG4 (humans) or IgG1 (mice), and also leads to up-regulation of MHC class II, encourages further B cell growth and prevents B cell apoptosis (Nelms et al., 1999). Eosinophil chemotaxis is partly reliant on IL-4.

The IL-4 receptor is a heterodimeric receptor with wide tissue expression It consists of an IL-4Rα chain (with strong IL-4 affinity), and the common γ chain (CD132) which activates signaling pathways (Letzelter et al., 1998). Signaling occurs via the Jak-STAT mechanism as outlined for IFN-γ, but with Jak1, Jak3, and STAT6 as the main protagonists (Kaplan et al., 1996).

The pathophysiology caused by dysfunctional IL-4 is mainly mediated downstream through IgE. Inappropriate IL-4, and therefore IgE, in response to innocuous antigen is a key pathway leading to Th2-driven allergic airways disease and asthma (Steinke et al., 2001). The effects on B-cell growth are implicated in various lymphomas, including chronic lymphocytic leukaemia and Hodgkin's lymphoma. Naturally occurring mutations of the IL-4Rα chain in humans are associated with allergy (Mitsuyasu et al., 1998). IL4Rα deficient mice have undetectable IgE and clear intestinal nematodal infection less effectively (Barner et al., 1998).

IL-5 is produced by Th2 cells and mast cells. Eosinophil effects are most important, with IL-5 being a key factor in their development, regulating differentiation, proliferation and activation (Foster et al., 1996). IL-5 also causes stimulation of B cell growth and production of immunoglobulins.

The IL-5 receptor is a heterodimer of the IL5Rα chain (CD125) and the common β chain, and is also widely expressed. Signaling through Jak1, Jak3 and STAT5 occurs and is considered the canonical pathway (Mui et al., 1995). There is also activation of the Ras-MAPK pathway. Both have intertwined effects, with elements of crosstalk at several points in both pathways (Adachi et al., 1998).

IL-5 is implicated in allergic airways diseases, along with IL-4. Its main role in eosinophilic development has made IL-5 a target for immunotherapy in disorders such as eosinophilic oesophagitis, and hereditary eosinophilia syndromes (Stein et al., 2006).

IL-13 is produced mainly by Th2 cells and $CD8^+$ T cells. The receptor is a heterodimer of the IL13Rα chain and the IL4Rα chain, and also signals via the Jak STAT pathway using STAT6. Like IL-4 and IL-5, IL-13 promotes B cell growth and class switching. It also down-regulates macrophage activity, and may have a more prominent role than IL-4 in the pathogenesis of asthma (reviewed by Wynn, 2003).

1.4.3 IL-10

IL-10 is a major immunosuppressive cytokine with powerful anti-inflammatory and regulatory effects. Secreted by monocyte-macrophages, DCs, T and B cells, epithelial cells, mast cells and granulocytes, its actions include inhibition of the production of TNF, IL-1, IL-6, and IL-12. Th1 cytokine secretion is diminished, macrophages are deactivated, and multiple lineages of lymphoid cells are prevented from differentiating and proliferating. Production of IL-10 is particularly marked in two groups of regulatory T cells, the gut-resident $Foxp3^+$ natural Treg (nTreg) (Maynard et al., 2007) and the Type 1 regulatory cell (Tr1). IL-10 is considered a key mechanism by which these two cell types effect suppression (Shevach et al., 2009, Roncarolo et al., 2001). Th2 cells also produce significant quantities, IL-10 initially being considered a Th2 cytokine (Fiorentino et al., 1989), and this likely partly mediates the ability of Th2 cells to inhibit a Th1 response. Th1 cells produce IL-10 in co-expression with IFN-γ and appear to self-regulate their own activation in this manner (Meyaard et al., 1996). Th17 cells may also produce IL-10 in a TGF-β stimulated manner (Ghoreschi et al., 2010).

Regulation of IL-10 transcription is predominantly via STAT3, leading to downstream induction of the transcription factor c-Maf, crucial for T-cell expression of IL-10. The effects of TGF-β, ICOS and IL-27 are all via c-Maf induction. C-Maf directly activates IL-10 synthesis by binding to the IL-10 promoter at a Maf recognition element (MARE) motif. IL-21 is also stimulated by c-Maf, and permits expansion and maintenance of IL-10 producing cells, especially Tr1 cells (Xu et al., 2009). Originally considered a major player in Th2 cells (Kim et al., 1999), c-Maf now appears to be a master transcription factor for IL-10. Th2 production of IL-10 is regulated via STAT6 and requires the presence of GATA3.

IL-10 expression by Th1 cells may be induced by IL-12 signaling through STAT4, but continuous activation of STAT4 is required to maintain production.

Notch and aryl hydrocarbon receptor (AhR) pathway signaling also increases IL-10 secretion. Up-regulation of AhR by IL-27 causes binding with c-Maf to promote the differentiation of Tr1 cells. STAT4 may synergise with Notch, leading to marked increase in IL-10 production by Th1 cells as a means of self-regulation (Rutz et al., 2008). More relevantly, Notch ligand Delta-like-4 is expressed on steady state plasmacytoid DCs but rapidly up-regulated on all DCs following toll-like receptor ligation, explaining the ability of DCs to modulate IL-10 production in T cells (Kassner et al., 2010). TLR-2 stimulation *in vitro* strongly promotes IL-10 production by APCs (Agrawal et al., 2003) with correlates *in vivo* (Netea et al., 2004). Other TLRs have also been implicated in innate cell IL-10 production (Higgins et al., 2003, Fujita et al., 2006).

Binding of dimerised IL-10 to a receptor, constituting two IL-10R1 chains and two IL-10R2 chains, leads to activation of Jak1 and tyrosine kinase 2 (Tyk2), causing phosphorylation of IL-10R1. This permits docking of STAT3, allowing phosphorylation and then nuclear translocation with up-regulation of the target genes. Absence of STAT3 in *in vivo* models completely prevents IL-10 effects on macrophages and neutrophils (Takeda et al., 1999). Expression of IL-10R1 is virtually restricted to leucocytes whilst IL-10R2 is widely expressed.

IL-10 is an evolutionarily early attempt by the immune system to limit detrimental effects from immune responses. As such, pathogens have evolved means to abuse its immunosuppressive potential, either through stimulation of IL-10 production (Jang et al., 2004, Sing et al., 2002, Gabrysova et al., 2009) or mimicry (Hsu et al., 1990). Therapeutic blockade of IL-10 has been considered to assist in the clearance of chronic infection (Brooks et al., 2008). As a key immunosuppressive molecule, the role of IL-10 in autoimmunity has been extensively studied. IL-10 is vital in gastro-intestinal homeostasis and modulating immune reactions within the resident commensal population. Loss-of-function mutations in either IL-10 (Kuhn et al., 1993) or IL-10R (Spencer et al., 1998) genes lead to a severe colitis in mice, and similar early-onset inflammatory bowel diseases (IBD) have now been identified as monogenic in humans as well. Genome-wide assocation studies identify IL-10 as a susceptibility factor for more common forms of IBD (Franke et al., 2008). IL-10 also has a protective role in murine models of hepatitis (Di Marco et al., 1999).

1.4.4 IL-17

IL-17 was first identified in the mid-1990s (Yao et al., 1995) but was considered a minor player in immunology until the identification of Th17 cells (Aggarwal et al., 2002) and their potential pathogenicity upset the entire Th1/Th2 axis and forced the re-examination of much of the dogma, particularly relating to 'Th1-mediated diseases'.

IL-17 has two main isoforms, IL-17A and IL-17F, with the former considered the more potent (Ishigame et al., 2009). It is produced by many different cells, including both adaptive cells (e.g. Th17) and cells of the innate system – γδ-T cells, NKT cells, and macrophages. Actions of IL-17 are targeted towards structural cells such as stroma, epithelia, and endothelia, with up-regulation of genes important in the activation of neutrophils and the acute phase response, plus anti-microbial proteins.

The genes for IL-17A and IL-17F lie closely together. RORγt and RUNX1 bind upstream of the IL-17A promoter, with binding of RUNX1 acting to directly inhibit Foxp3 expression. The presence of IL-17-skewing cytokines, such as IL-6, IL-21, or IL-23, leads to activation and phosphorylation of STAT3, which then also interacts with the IL-17A promoter. STAT3 is countered by STAT5 binding (promoted by IL-2) (Laurence et al., 2007, Yang et al., 2011). TGFβ is also beneficial for IL-17 production, as it suppresses eomesodermin via 'single mothers against decapentoplegic' (SMAD), leading to up-regulation of both the IL17A promoter and RORγt.

The IL-17 receptor is a heterodimer of IL-17RA and IL-17RC chains. Binding of IL-17 to one chain increases affinity for the other chain, though there are species-specific differences. Human IL-17RA binds IL-17A with high affinity and IL-17F with very low affinity, whilst IL-17RC has the opposite pattern. In mice, IL-17RC similarly binds IL-17F with high affinity and IL-17A with low affinity, but there is no discernible difference in affinity between the two isoforms for IL-17RA (Kuestner et al., 2007). Signaling of IL-17 is rather different from other cytokines, mediated through Act1 (a U-box E3 ubiquitin ligase) (Qian et al., 2007), though the subsequent downstream targets are poorly defined. TNFR-associated factor 6 (TRAF6) and the NF-κB pathway (Ruddy et al., 2004, Schwandner et al., 2000) can also both be activated through IL-17. Thus, IL-17 has an effect upon pathways traditionally related to both innate and adaptive immunity. IL-17 signaling rarely leads to drastic cellular changes alone, but synergistic action with IFN-γ, TNF and IL-1β have all been observed.

Absence of IL-17 or a reduction in Th17 cells mainly leads to an increased susceptibility to fungal infections, and subsequently chronic mucocutaneous candidiasis (Puel et al., 2011).

Mice deficient in IL-17 succumb to disseminated candidiasis (Huang et al., 2004). IL-17 is highly relevant as a pathogenic cytokine, implicated in autoimmunity in multiple disorders previously considered Th1-mediated, such as rheumatoid arthritis and psoriasis. The role of IL-17 and Th17 cells is not completely clear-cut. In some models, e.g. EAE, the potential pathogenicity of Th17 cells is disputed, and may not be related to the production of IL-17 itself.

1.4.5 TGF-β

TGF-β is a highly pleiotropic cytokine with multiple effects throughout the body on virtually every cell lineage. First described as a fibroblast growth factor (de Larco et al., 1978), then in wound repair (Sporn et al., 1983), its vital role in immunology was amongst the last to be recognized (Kerhl et al., 1986). Even within the immune system, it has directly contrasting effects. Induction and maintenance of adaptive Treg requires the presence of TGF-β (Chen et al., 2003), and it is thus rightly considered a major player in immunosuppression. Conversely, TGF-β is also an important signal for the differentiation of Th17 cells (Veldhoen et al., 2006, Mangan et al., 2006), and so also pro-inflammatory.

TGF-β is produced in an inactive form, pre-pro-TGF-β. The pro-protein component is also known as latency-associated peptide (LAP) (Dubois et al., 1995). The LAP-TGF-β pro-protein complexes with latent TGF-β binding protein (LTBP) (Miyazono et al., 1991), and this large latent complex (LLC) is the substance released by cells (Rifkin, 2005). Processing of LTBP-LAP-TGF-β to release the active TGF-β takes place outwith the cell, or using reactions at the cell membrane. Only the TGF-β itself was initially considered active, though this is now being challenged (Peterson et al., 2012). *In vitro* release of active TGF-β is typically by brief acidification of the sample (Lawrence et al., 1985). *In vivo* metalloproteinases may act to release the complex from the tissue matrix (Dallas et al., 2002), and then the remaining complex can be further processed to release free active TGF-β. Notably, one such enzyme is plasmin, the active enzyme formed from plasminogen during platelet activation (Godar et al., 1999). As such, serum can be anticipated to contain relatively large quantities of free TGF-β, making *in vitro* effects difficult to distinguish.

Activation of TGF-β by cells remains incompletely understood, but a key interaction is via integrins expressed on the cell surface (Munger et al., 1999). Binding of αvβ6 integrin with TGF-β-LAP is reported to be required for extracellular activation. That these integrins are relevant immunologically is evident by the phenotype of autoimmunity and colitis seen in

mice with a conditional deficiency of αv (Lacy-Hulbert et al., 2007). The αv-deficient mice developed a reduction in Treg numbers within the colon, presumably due to an inability to induce new Foxp3 expression in the absence of activated TGF-β.

Two groups of TGF-β receptor exist, binding either with active TGF-β only, or with the LAP-TGF-β complex through LAP. The active TGF-β receptors are further sub-classified into three types (Lin et al., 1992). The type III receptors are not associated with any signaling mechanisms, and appear to be a means of controlling the concentration of free TGF-β available for use by the cells. More importantly, these type III receptors can solubilise and compete for TGF-β with type II receptors, thus preventing its effects. When bound to the cell surface, type III receptors assist in signaling, 'handing over' the TGF-β to the type II receptors. Binding of TGF-β to a type II receptor leads to recruitment of type I receptors and a tetramer is formed of two of each type, each dimer binding to a chain of TGF-β (Massague, 2008). The formation of this complex phosphorylates and activates the Type I receptor triggering the Smad pathway.

Three different Smads may be activated; Smad 2 and Smad3 are receptor-associated whilst Smad4 is common to all. Binding of TGF-β leads to phosphorylation of the Smad 2 or 3 which then binds with Smad4 leading to translocation to the nucleus and subsequent genetic regulation (He et al., 2006). Various other pathways have also been implicated but have not yet been fully delineated (Derynck et al., 2003).

The alternate receptors bind to LAP 'trapping' the TGF-β in complex with the cell surface and allowing direct delivery to another cell, or indeed autocrine signaling (Chong et al., 1999). Activation and delivery of TGF-β by this means uses a soluble factor but requires cell contact for delivery, and has been suggested as one mechanism of regulatory T cell suppression (Chen et al., 2008). TGF-β suppresses the production of IL-2 (Brabletz et al., 1993), causing a direct anti-proliferative effect on most T cells. There is also an effect on cell cycle transcription factors, such as down-regulation of c-myc (Polyak et al., 1994) and up-regulation of kinase inhibitors (Hannon et al., 1994), seen more profoundly on naïve than activated cells (Wolfraim et al., 2004). TGF-β is also essential in the development and maturation of certain types of APC, particularly Langerhans cells within the dermis (Zhang et al., 1999), and assumes great importance in maintaining a tolerogenic phenotype in many DCs (Belladonna et al., 2008, Werner et al., 2000). Suppression is also seen in other innate and adaptive cell lineages (Li et al., 2006).

One of the most notable features of TGF-β is the role alluded to earlier in T cell differentiation (1.3). TGF-β inhibits both Th1 and Th2 differentiation via, respectively, down-regulation of Tbet (and hence IL-12 receptor expression) (Gorelik et al., 2002), and GATA3- and IL-4-triggered phosphorylation of STAT6 (Heath et al., 2000). Suppression of both Th1 and Th2 cytokines will influence activated T cells towards a Th17 phenotype, as in the absence of both of these, IL-6 is sufficient to induce Th17 cells (Das et al., 2009). TGF-β clearly has a more direct role as it up-regulates the master transcription factor for Th17, RORγt (Icihyama et al., 2008). TGF-β can also inhibit cytokine production from fully differentiated Th1 cells, though has no impact on Th2 cells (Ludviksson et al., 2000).

TGF-β is vital in the induction of Foxp3, the master transcription factor for Treg. Stimulation of naïve T cells in the presence of TGF-β and IL-2 *in vitro* leads to up-regulation of Foxp3 and differentiation towards induced Treg (Chen et al., 2003). Similarly, stimulation with DCs can lead to iTreg with proven immunosuppressive capability (Yamazaki et al., 2006). The FOXP3 promoter contains three highly conserved non coding sequences, which have been selectively knocked out in murine models. This demonstrated that TGF-β signaling through NF-AT was redundant in the thymic development of nTreg but essential for the formation of gastro-intestinal resident Treg (Zheng et al., 2010). Both Smad2 and Smad3 are needed to induce Treg (Takimoto et al., 2010), as is IL-2 through phosphorylation and activation of STAT5 (Davidson et al., 2007).

Absence of TGF-β1 signaling in mice leads to lethal multi-organ autoimmunity within weeks of birth (Shull et al., 1992), whilst signaling blockade permits the development of autoimmunity in older mice (Gorelik et al., 2000). Multiple mutations in either receptors or signaling pathways have been identified in human disease, particularly within various cancers. Other mutations show a predilection for structural malformations with chondrodysplasia, craniosynostosis, and abnormal vasculature (Loeys et al., 2005).

1.4.6 Tumour necrosis factor (TNF)

In 1984, a soluble factor released from macrophages which had cytotoxic effects was identified and designated TNF-α to distinguish it from a partly homologous cytokine, lymphotoxin (TNF-β) (Aggarwal et al., 1985). Many authorities now simply refer to the cytokine as 'TNF' and that is the nomenclature adopted herein (Clark et al., 2007). Since the original identification, the TNF family has expanded to contain 19 different ligands, and 29 different receptors. They are predominantly pro-inflammatory but with wide-ranging

activity on many cells, particularly in proliferation, apoptosis and morphogenesis (reviewed in Aggarwal et al., 2011). For the purposes of this thesis, the most relevant TNF superfamily (TNFSF) members are TNF, CD40L, FasL, OX40L, TNF-related apoptosis-inducing ligand (TRAIL), receptor activator of NF-κB ligand (RANKL), and GITR ligand. Many of these ligand-receptor interactions are discussed later (section 1.7.1); here TNF will be the focus.

TNF interacts with two of the 29 receptors, TNFR1 and TNFR2. TNFR1 contains a death domain within its intracellular region, and is the main receptor to which trimerised TNF will bind. TNFR1 is widely expressed on virtually every cell type, whilst TNFR2 is restricted to immune, endothelial, and nerve cells. TNF may be in a soluble form or expressed as a transmembrane protein on the cell surface, with the effects of ligand binding partly dependent on how TNF is presented. Reverse signaling has also been demonstrated, with transmembrane TNF signaling back into the host cell on binding with an antibody (Harashima et al., 2001).

Binding of TNF with its receptor leads to activation of various pathways. Common to all members of the TNFSF is usage of the NF-κB pathway. TNF also signals via p38 mitogen-activated protein kinase (p38MAPK), extracellular signal-regulated kinase (ERK), and c-Jun N-terminal kinase (JNK). Apoptosis may be triggered by recruitment of the TNFR-associated death domain stimulating (TRADD) on TNFR1 binding with the Fas-associated protein with death domain (FADD) and activation induced cell death (AICD) as discussed later (Section 1.7) (Hsu et al., 1996). TNF also uses the intrinsic pathway for apoptosis, via mitochondrial activation and caspase-9/caspase-3 release. Activation of the NF-κB pathway leads to a large number of mainly pro-inflammatory downstream effects, including the release of IL-6, IL-8, IL-18, cyclooxygenase-2, inducible nitric oxide synthase, and more TNF, as well as inducing cell proliferation. This is also enhanced via up-regulation of AP-1, another transcription factor controlling cell cycle proteins (Natoli et al., 1997). TNFR2 lacks the death domain TRADD common to activation of all pathways by TNFR1-TNF interaction, but is able to bind directly to the downstream molecule TNFR-associated factor 2 (TRAF2) and hence signal via both MAPK and NF-κB. Unlike other TNFSF members, TNF activates NF-κB in a non-selective manner and is potently pro-inflammatory. AICD is avoided as TNF alone is insufficient to causes apoptosis, though the mechanisms preventing this are ill-defined (Sugarman et al., 1985).

Due to its promiscuous nature, TNF is widely implicated in many disease states, from Alzheimer's disease (Swardfager et al., 2010) to heart failure (Feldman et al., 2000) to obesity (Tzanavari et al., 2010). Relevant here is the well recognized role of TNF in

autoimmunity. Dysregulation of TNF is implicated in systemic lupus erythematosus (SLE), MS, arthritis, and IBD. Indeed, the development of successful therapies utilizing anti-TNF biologics is a landmark in the treatment of several disorders (Lin et al., 2007).

Given the universal impact of TNF, its absence in a knock-out mouse is curiously without morphological changes with only increased infective susceptibility a major part of the phenotype (Marino et al., 1997). TNF mutations in humans have been implicated in cerebral infarction (Um et al., 2003), whilst receptor mutations are found in periodic fever syndromes (TNFR1-associated periodic syndrome (TRAPS)) (McDermott et al., 1999) and Crohn's disease (Waschke et al., 2005).

1.4.7 Granulocyte monocyte-colony stimulating factor (GM-CSF)

As the name suggests, GM-CSF was first described in the context of granulocyte and monocyte maturation from bone marrow precursors (Burgess et al., 1980). Later recognition in both innate and adaptive immunity consolidated GM-CSF as a relevant cytokine in health and disease. More recently, GM-CSF has been implicated as the essential pathogenic cytokine in EAE, the murine model of MS, and may yet emerge to have a key role in other disorders (Codarri et al., 2011, El-Behi et al., 2011).

GM-CSF is not plentiful in the steady state, but production by many cell lineages increases rapidly in association with stimulus. The receptor is a heterodimer of a binding chain (CSFR2α) and a signaling chain (CSFR2β), and is expressed widely within the myeloid cell lineage, but is not expressed on T cells (Rosas et al., 2007). The receptor complex for GM-CSF is notably complicated – two GM-CSF molecules bind to two chains each of CSFR2α and CSFR2β forming a hexamer, but this is insufficient for full signaling. Two of these hexamers then interact with one another, forming a dodecameric complex that permits signaling through the Jak-STAT pathway, MAPK pathway, and the PI3K pathway (Fleetwood et al., 2005). Actions of GM-CSF include the maturation of dendritic cells (a well established *in vitro* phenomenon) (Inaba et al., 1992), activation and survival signals for macrophages, eosinophils and neutrophils, and differentiation for alveolar macrophages and invariant NK T cells.

Given its pro-inflammatory nature, it is perhaps not surprising that elevated serum concentrations of GM-CSF have been identified in a number of autoimmune disorders. A network model proposes that GM-CSF, and other CSFs, act as intermediates between

myeloid cells and activated T or B cells in chronic inflammation (Hamilton, 1993). Following this, GM-CSF blockade was proposed as a therapeutic modality for some chronic inflammatory disorders, supported by a murine model of collagen-induced arthritis (Campbell et al., 1998, Cook et al., 2001). A clinical trial suggested benefit from blockade in Crohn's disease (Korzenik et al., 2005) though this particular study was faulted due to undeclared competing interests in the study investigators. The initial observation that GM-CSF deficiency was protective in EAE (McQualter et al., 2001) was borne out by the discovery that GM-CSF production is non-redundant for the establishment of disease in that model (Codarri et al., 2011). GM-CSF is also allocated a pathogenic role in experimental glomerulonephritis (Kitching et al., 2002), and allergic airways disease (Yamashita et al., 2002). GM-CSF deficient mice have specific defects in alveolar macrophages and invariant NK T cells, (Stanley et al., 1994, Bezbradica et al., 2006). GM-CSF is widely used clinically post-radiotherapy to boost mobilization of granulocytes and monocytes and hasten immune reconstitution.

1.5 Effector T cell plasticity

The dichotomy proposed by Mossman et al of Th1 and Th2 has been blown apart by recent developments in the characterization of T cell subsets. One key aspect initially proposed was that the Th1/Th2 decision was irreversible – cells terminally differentiated to one lineage or the other (reviewed in Zhou et al., 2009). The plasticity of these cells is now being investigated. Much evidence for plasticity arose in the context of Th17 and iTreg differentiation. Both Foxp3 and RORγt are induced by TGF-β, but Foxp3 inhibits function of the latter (Zhou et al., 2008). IL-6 prevents the induction of Foxp3 (Korn et al., 2008), leading to skewing towards Th17, while IL-2 and retinoic acid further promote towards iTreg (Benson et al., 2007). Thus, the two subsets are intricately balanced and the final fate of the cell is likely to rely on multiple factors. Even the well-established Th1/Th2 paradigm is challenged by the presence of $GATA3^{+}Tbet^{+}$ Th2 cells secreting Th1 cytokines (Hegazy et al., 2010). One proposed model suggests that some differentiated states are more 'stable' (Murphy et al., 2010) such that de-differentiation is more difficult to achieve. 'All T cell subsets are differentiated, but some are more differentiated than others'.

Characterisation of T cell subsets was originally by cytokine profile, but has matured to a combination of cytokines released, inducing conditions, and transcription factors – each T cell subset originally appeared to have a uniquely highly expressed transcription factor.

There is a great deal of overlay with no cytokine being unique to a single subset, nor any transcription factor uniquely up-regulated in only one group. For example, IL-10 was initially described as a Th2 cytokine, but is now known to be secreted by Th1, Tr1, Th17, and Treg, plus other non-$CD4^+$ cells (Saraiva at al., 2010). To complicate matters further, the cytokine profile of a cell may change over time e.g. Th17 cells classed by their IL-17 production often acquire IFN-γ production (Hirota et al., 2011). Transcription factor expression is also not a useful discriminator. $ROR\gamma t^+$ Th17 cells can also co-express either Tbet (Ghoreschi et al., 2010) or GATA3 (Wang et al., 2010). Equivalent to this, loss of the defining transcription factor does not necessarily remove the associated traits of that subset. Absence of Tbet does not completely remove Th1 differentiation (Usui et al., 2006). A similar degree of plasticity is also seen in $Foxp3^+$ Treg (section 1.10.4.3.2).

Epigenetic modifications, previously thought to cement the fate of a cell to a given lineage, have now emerged as contributing, rather than detracting, to plasticity, particularly in Th17 cells (Lee et al., 2009). The Tbx21 gene encoding Tbet, for example, has epigenetic modifications promoting expression in Th1 and repressing in Th2 and Th17. The same promoting modification is also seen within the Th2 and Th17 cells suggesting that removal of the repressing influence could rapidly lead to up-regulation of Tbet (Wei et al., 2009).

To conclude, $CD4^+$ cells have been categorized into a number of effector subsets based on their expression of various cytokines and transcription factors, and their effects *in vivo*. Though initially a useful discriminator, the burgeoning number of subsets, and the inter-relationships between them all, implies that a degree of functional plasticity is almost certainly present; T cells are more functionally fluid than previously acknowledged.

1.6 Central tolerance

There is no fundamental biology preventing the processing and presentation of proteins derived from self, and indeed cells reactive to self have been demonstrated for various antigens in healthy individuals (Yang et al., 2006). The concept of 'self' and 'non-self' as distinct determinants requiring different immune reactions was first raised by Sir Frank Macfarlane Burnet in 1949 (Burnet et al., 1949).

T cell development occurs in the thymus. One crucial requirement is to efficiently prevent the export of self-reactive T cells capable of mounting an unwarranted immune reaction. This is the main principle behind central tolerance – deletion of self-reactive cells at their

point of origin. Precursor T cells migrate from the bone marrow to the thymus as double negative $CD4^-$ $CD8^-$ cells. Within the thymus, the majority develop α and β chains of the T cell receptor (a minority progressing to γδ lineage) (Washburn et al., 1997). The TCR chains then undergo rearrangement along with up-regulation of both CD4 and CD8, so that the double positive cells have a functional heterodimeric TCR (Fehling et al., 1995). Ongoing development and cell survival then requires signaling through this TCR via peptide-MHC complexes within the thymic cortex (Starr et al., 2003). In the absence of a functional TCR or adequate signal strength, cells undergo apoptosis. Those cells that are positively selected then migrate to the thymic medulla.

Medullary thymic epithelial cells (mTECs) are notable for high gene expression of tissue-specific genes and their high expression of the gene 'Autoimmune Regulator' (Aire) (Derbinski et al., 2001). Though the exact mechanisms remain to be clarified, Aire regulates the transcription of multiple tissue-specific antigens (TSA), leading to their expression and presentation on mTECs (Anderson et al., 2002). Indeed, there is a 'dose-dependent' relationship, with increased Aire activity enhancing negative selection of Aire-driven antigens. These cells also express high levels of CD80 and MHC class I and II molecules, and may be responsible for antigen presentation to the developing T cell (Derbinski et al., 2005). Alternatively, mTECs may function as a large self-antigen reservoir for dendritic cells resident within the thymic medulla, which then undertake processing and antigen presentation (Koble et al., 2009). These two mechanisms likely co-exist, with evidence that mTECs are superior in transcription but DCs present more effectively, suggesting a co-operative and partly redundant approach to self-antigen presentation. Irrespective of the presenting cell, should a developing T cell receive a strong TCR signal within the thymic medulla from a TSA-MHC complex, this indicates high potential for self-reactivity and the cell undergoes apoptosis (reviewed in Starr et al., 2003).

Defects in either early positive selection or later negative selection can cause escape from central tolerance. Proof of this is seen in mutations affecting TCR signaling. Several ZAP-70 mutations have been identified in models of spontaneous autoimmunity (Sakaguchi et al., 2003). If initial basal signaling during positive selection is defective, then a stronger self-signal is needed for progression into the medulla. These more strongly self-reactive T cells are still unable to respond sufficiently to self-antigen to then undergo apoptosis, and are released into the periphery. There, the abundance of antigen overcomes TCR signal weakness and causes progression to autoimmunity (Hsu et al., 2009).

The mechanisms of negative selection and the crucial role of Aire were highlighted in 1997, when Aire was identified as the gene dysregulated in autoimmune polyglandular syndrome type 1 (APS1), the hallmarks of which are summarised in the alternate name, autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) (Aaltonen et al., 1997). In the absence of Aire expression within the thymus, presentation of self-antigen is less effective leading to an escape from negative selection for self-reactive T cells, in particular those with reactivity to endocrine organs.

Thymic development of T cells thus requires a two stage survival process, with sufficient positive signaling at the double positive stage within the cortex to survive to the medulla, then insufficient signaling as single positives to self-antigen MHC complexes, permitting escape from negative selection. For this process to be entirely infallible, T cells with even weak self-reactivity would need to undergo deletion. Such stringency would not permit an adequate T cell pool for response to pathogens, and so some self-reactive T cells do enter the periphery. Identification of such potentially pathogenic cells in both humans and animal models confirms this is indeed the case and indicates the need for a second level of regulation – peripheral tolerance.

1.7 Peripheral Tolerance

Having taken a necessarily 'relaxed' approach to central tolerance to ensure sufficient promiscuity of the T cell receptor repertoire, additional mechanisms are needed to control self-reactive T cells when they exit the thymus. This is the broad concept of peripheral tolerance, and may be loosely considered as three connected but disparate mechanisms; death, anergy, and regulation.

1.7.1 Cell death

1.7.1.1 Active cell death

A key aspect of a self-reactive T cell is its ongoing exposure to the cognate antigen. Unlike pathogen-responsive cells, which may spend their entire lifetime in ignorance of their antigen, an autoreactive cell is guaranteed to have a plentiful supply (unless the antigen is immunologically sequestered). Constant signaling through the TCR in the absence of any associated danger signal may lead to activation of an alternate pathway. The discovery of

the Fs7-associated cell surface antigen (Fas) in 1989 (Yonehara et al., 1989) and subsequently its interaction with Fas ligand (FasL) opened up the mechanisms that lead to activation-induced cell death (AICD) (Brunner et al., 1995). Binding of Fas with Fas-ligand leads to trimerisation of the Fas receptor, bringing together three death domains that can interact with Fas-associated death domain protein (FADD) (Kischkel et al., 1995). Pro-caspase-8 (also known as FLICE) is recruited and forms a complex with FADD and the Fas receptor – the death inducing signaling complex (DISC) cleaving pro-caspase 8 to caspase 8 (Medema et al., 1997). This then activates a multiple caspase pathway, ultimately leading to cell death (Lavrik et al., 2005). A key cleavage at this point is of the BH3-only protein, Bid, to truncated Bid (tBid) (Korsmeyer et al., 2000). This then translocates and inhibits the Bcl-2 pathway in mitochondria, leading to up-regulation of kinases and formation of the apoptosome which serves to amplify the apoptotic signal to the cell. In many cells (including lymphocytes), Fas-FasL signaling is sufficient, but amplification via the apoptosome is necessary for others e.g. pancreatic β cells, tumour lines (Scaffidi et al., 1998).

Regulation of AICD is predominantly through expression of FasL. Both Fas and FasL are expressed on repetitively stimulated T cells such that AICD may be triggered amongst a relatively homogeneous population, or even on the same cell (Li-Weber et al., 2003). Transcription of FasL is initially promoted by activation of the NF-AT pathway, which is up-regulated on TCR stimulation (Li-Weber et al., 1998). The NF-κB pathway also regulates FasL expression, with NF-κB inhibitors rendering cells more susceptible to AICD (Micheau et al., 2001). Other factors also influence FasL expression. Transcription factors c-myc (via TGFβ), IRF1 and IRF2 all have regulatory roles on FasL (Kavurma et al., 2003). Activation of STAT5 leads to up-regulation of FasL and down-regulation of FLICE/caspase-8 inhibitory protein (FLIP), rendering cells more liable to AICD. Multiple other signaling pathways also feed into the Fas-FasL pathway.

The Fas–FasL pathway is not the only signaling trigger for apoptosis. Other TNF family members are able to cause cell death through the alternative ligand, TRAIL, which is expressed on a variety of immune cells, including activated T cells (Martinez-Lorenzo et al., 1998). The signaling mechanism is similar to Fas-FasL, with binding of TRAIL to either TRAIL-R1 or TRAIL-R2, trimerisation of the receptor permitting binding of FADD and a subsequent DISC. The TRAIL pathway is sensitive to pro-inflammatory signals with up-regulation of TRAIL on various innate cells in the presence of LPS or IFN-γ (Griffith et al.,

1999). TRAIL interactions appear particularly relevant in the regulation of NK function upon tumour cells (Takeda et al., 2001).

Though sharing a common outcome, Fas and TRAIL are preferentially used by different cell lineages. On *in* vitro activation with anti-CD3, Th1 cells up-regulate Fas whereas Th2 cells up-regulate TRAIL (Zhang et al., 2003). Despite this, Th1 cells are more sensitive to TRAIL-induced cell death and absence of TRAIL signaling in knock-out mice leads to a reduction in Th2-mediated allergic airways disease (Weckmann et al., 2007).

Is activation-induced cell death relevant to autoimmunity? Impairments in Fas-FasL signaling lead to lymphoproliferative disorders, specifically autoimmune lymphoproliferative syndrome (ALPS) in humans, with corollaries in mice (Rieux-Laucat et al., 2003). More subtle defects have been identified in other autoimmune disorders. Reduced apoptosis of myelin-reactive T cells has been shown in MS patients (Zang et al., 1999). Conversely, up-regulation of inhibitors of the Fas-FasL such as FLIP has also been seen in MS patients (Semra et al., 2001) whilst forced overexpression led to widespread autoimmunity in a mouse model (Djerbi et al., 2003). Absence of TRAIL or TRAILR in mice does not lead to overt autoimmunity though does appear to increase susceptibility. Absence of TRAIL in the EAE model of MS caused worsening of disease though apoptosis of the inflammatory cells was unaffected, whilst direct administration of a TRAIL blocking Fc into the central nervous system (CNS) had a protective effect by limiting neuronal apoptosis during ongoing inflammation (Cretney et al., 2005, Aktas et al., 2005). Unlike Fas-FasL, TRAIL appears to have no role within the thymus in central tolerance.

1.7.1.2 Death by neglect

Death is not always an active process. Cells may also undergo passive death through neglect, characteristically through the deprivation of growth factors such as IL-2. This is a common mechanism used for early thymocytes that have failed to achieve a useful TCR rearrangement, and so do not receive adequate survival signals in the thymus (Von Boehmer, 1994). As opposed to the extrinsic signals required for AICD, passive cell death is considered an 'intrinsic' pathway, though there is interaction between the two (Igney et al., 2002). The default outcome for a cell is thought to be programmed cell death, with the provision of survival signals essential to prevent this from occurring. Alternatively, the cell may be damaged by a variety of noxious stimuli, all of which lead to the loss of apoptotic suppression by bcl, as occurs in AICD.

That passive cell death centered on the mitchondria has been known for more than half a century (Ashwell et al., 1952). The initial event in passive death is release of pro-apoptotic proteins from the mitochondria, via the mitochondrial permeability transition (MPT) pore. This pore is formed by oligomerisation of Bcl-2 family proteins, such as Bid and the related peptide Bax, within the outer mitochondrial membrane (Kuwana et al., 2002). Bax may also complex with other ligands to form similarly large pores (Marzo et al., 1998). The proteins thereby released include cytochrome c, endonuclease G, and apoptosis-inducing factor (AIF), Smac/DIABLO, HtrA2/Omi and others (Saelens et al., 2004).

In conjunction with expression of apoptotic protease-activating factor-1 (Apaf-1), cytochrome c promotes the formation of the apoptosome that leads to a similar caspase cascade as occurs in AICD (Zou et al., 1997). Cytochrome c binding to Apaf-1 allows association with deoxyadenosine-5-triphosphate (dATP), permitting oligomerisation and thence caspase 9 activation (Adrain et al., 1999). Smac/DIABLO and HtrA2/Omi promote this by inhibiting 'inhibitor of apoptosis proteins'. The nucleases released contribute to cell death later in the process, contributing to nuclear degradation.

Regulation of these mitochondrial events is predominantly through Bcl-2 family proteins, which may be either pro- or anti-apoptotic (Cory et al., 2002). As might be anticipated, mutations within Bcl-2 proteins can have significant clinical consequences. Dysregulation of passive cell death is a major mechanism leading to malignancy (Kerr et al., 1994). Insufficient passive cell death may lead to the survival of self-reactive T cells or the development of chronic autoimmunity. This has been suggested in animal models, including EAE (Issazadeh et al., 2000). Though most identified defects in cell death leading to human autoimmunity involve AICD, or common pathways, it is likely that intrinsic pathway defects cause similar pathology (Worth et al., 2006)

Thus, activation-induced cell death via either FasL or TRAIL signaling occurs on repetitive stimulation by the same (self) antigen as a means of peripheral tolerance, whilst passive death occurs through neglect of cells. Cell death is an expensive outcome and clearly just moving the site of AICD and neglect from the thymus to the periphery would not be a rational evolutionary decision. Other regulatory mechanisms are also in play.

1.7.2 Anergy and adaptive tolerance

First described in B cells as the absence of clonal proliferation, the ability of cross-linked APC to anergise T cells was reported in 1987 (Jenkins et al., 1987). The strictest definition of anergy is the absence of clonal proliferation or IL-2 production in an *in vitro* system, and relates to the inactivation of T cells responding to cognate antigen in the absence of inflammatory or 'danger' signals. *In vivo*, this is often termed adaptation.

As discussed earlier (section 1.2.4.2), engagement of the TCR with its antigen is insufficient stimulus to cause full T cell activation, with a requirement for other co-stimulatory signals such as CD28 (Thompson et al., 1989). Co-stimulation on initial TCR engagement leads to full activation of the phospholipase Cγ, Ras and protein kinase Cϕ pathways which, via various intracellular kinases, cause mobilisation of calcium and activation of the NF-AT, AP-1, CREB, and NF-κB systems, all involved in up-regulation of IL-2 transcription (Linsley et al., 1991). In the absence of CD28 co-stimulation, activation of AP-1 does not occur, leading to uncoupling of NF-AT from AP-1 and activation of alternate NF-AT downstream signals (Macian et al., 2002). This restricts IL-2 production and clonal expansion does not occur. Importantly, these cells remain less responsive to further stimulation even when full co-stimulatory signals are subsequently provided (Harding et al., 1992), through active repression of IL-2 expression (Telander et al., 1999). This situation can be replicated by the addition of adenosine to the *in vitro* culture. Stimulation through the A_2a receptor leads to mobilisation of intracellular cyclic adenosine monophosphate (cAMP), antagonistic to the CD28 signaling pathways (Zarek et al., 2008). Transcription and translation of IL-2 is a key element in avoiding anergy, as addition of IL-2 to *in vitro* cultures without CD28 co-stimulation will break anergy (Beverly et al., 1992), whilst IL-2 neutralisation in the presence of both signals will still anergise the T cells. IL-2 signals through the IL-2 receptor which downstream leads to activation of the mammalian target of rapamycin (mTOR) pathway, inhibition of which leads to anergy (Powell et al., 1999). Further factors are also required for the induction of anergy. Inhibition of the PI3K pathway via PTEN (phosphatase and tensin homologue deleted on chromosome ten) (Buckler et al., 2006), and downstream of mTOR via $p27^{kip1}$, promotes anergy (Rowell et al., 2005). TCR engagement without CD28 enhances expression of $p27^{kip1}$ whilst CD28 signaling leads to its degradation (Boussiotis et al., 2000).

The *in vitro* phenomenon of anergy does not fully reflect the processes *in vivo* in adaptive tolerance. Significantly, transfer of *in vivo* tolerised T cells is able to restore reactivity i.e. the profound inability to be re-activated is lost if ongoing antigenic stimulation is removed

(Tanchot et al., 2001). This implies that antigen must be frequently encountered, if not constantly presented, for adaptive tolerance to develop. One proposed model (Grossman et al., 2001) is that T cells 'tune' their responsiveness according to the persistence of antigen (Singh et al., 2003). If an antigen is always present, the cells slowly become desensitised and need greater levels of antigen presentation to evoke any response; somewhat analogous to the increasing dosage of opiates needed in those with drug dependency. If the T cells then receive no stimulation (i.e. 'cold turkey'), a much smaller subsequent exposure may elicit a strong response (Anderton et al., 2002).

The specifics of adaptive tolerance are, in many ways, unclear. The $CD4^+$ T-cell response includes clonal proliferation but also differentiation, typically to a T-helper phenotype. Differentiation, and thus the ability to produce effector cytokines, may be maintained in a 'tolerant' cell (Malvey et al., 1998), though in that report antigen was not persistent, but rather triggered signaling in the absence of inflammation. Both $CD8^+$ and $CD4^+$ T cells have been tolerised using murine models of chronic lymphocytic choriomeningitis virus (LCMV) infection (Tanchot et al., 1998, Oxenius et al., 1998), though $CD8^+$ cells retained their cytotoxic capabilities. Transfer of naïve T cells into a T-cell deficient host transgenically expressing a foreign antigen led to tolerance in the transferred cells without clonal deletion or the development of a classically 'regulatory' phenotype (Singh et al., 2006).

Thus, adaptive tolerance serves as another means to control self-reactive cells within the periphery, as these cells will have a ready supply of self-antigen.

1.7.3 Regulation

The third tenet of peripheral tolerance is the presence of cells dedicated to a regulatory, rather than effector, function. Various subsets of regulatory cell are now known to exist. Regulatory T cells can be broadly categorised by the presence or absence of the master transcription factor, forkhead box protein 3 (Foxp3), constituting naturally occurring thymically derived regulatory T cells (nTreg) and those where Foxp3 has been induced in the periphery by antigenic stimulus under appropriate cytokine conditions (adaptive, aTreg). These are the main cells of interest for this thesis, and, as such, are discussed in much greater detail shortly (section 1.10).

1.7.3.1 Tr1 cells

Other regulatory cells are also present. $CD4^+$ Type 1 regulatory cells (Tr1) are defined by their cytokine production, particularly their high production of IL-10 (Groux et al., 1997). Differentiation towards Tr1 is stimulated by IL-10 (Levings et al., 2001), and can be enhanced in the presence of vitamin D (Vieira et al., 2004). Suppression is mediated mainly via the local production of IL-10 and TGF-β (Bacchetta et al., 1994, Barrat et al., 2002). $CD8^+$ Tr1-like cells also produce large quantities of IL-10 (Gilliet et al., 2002), and are similarly generated in an IL-10-dependent manner (Steinbrink et al., 1999). Tr1 cells expand in the presence of IL-2 and IL-15 (Bacchetta et al., 2002) and are stimulated in an antigen-specific manner via their TCR. Once stimulated, the production of IL-10 and TGFβ likely accounts for their suppressive potential (Groux, 2003). As they use soluble mediators, the effects of regulation are not targeted towards a single cell, so suppression of nearby uninvolved effectors, potentially reacting to a different antigen, may also occur. Thus, these cells effect bystander suppression.

1.7.3.2 Th3 cells

Th3 cells are thought to play a pivotal role in the maintenance of tolerance towards oral antigens within the gastro-intestinal tract. First identified in 1994 (Chen et al., 1994), experimentally they are induced by the administration of oral antigens, and suppress in a TGF-β dependent manner (Miller et al., 1992). TGF-β within the gut is produced by epithelial cells, along with IL-10 (Weiner et al., 2001), and this combination of antigen exposure in an anti-inflammatory cytokine milieu promotes the formation both of Th3 cells and $Foxp3^+$ Treg. Th3 cells are particularly characterised by large quantities of latency-associated peptide (LAP) complexed with TGF-β on the cell surface (Oida et al., 2003). These LAP^+ cells are particularly inducible by oral anti-CD3 (Ochi et al., 2006) and have also been identified in human gut (Gandhi et al., 2010). One model proposes that Th3 LAP^+ cells are the orchestrators of the entire gut immune response. $CD103^+$ dendritic cells seem relevant to the formation of gut Treg (Coombes et al., 2007), with the combined action of TGF-β and retinoic acid promoting a large localised population of Foxp3 cells (Mucida et al., 2009). Oral antigen stimulates Th3 cells to produce TGF-β, which then inhibits effector T cell differentiation, sustains resident Treg, and induces adaptive Treg all within the gut (Weiner et al., 2011).

1.7.3.3 $CD8^+$ regulatory T cells

$CD8^+$ T cells with a suppressive effect upon B cell help have been identified, and are restricted to a subset expressing a non-classical MHC Class I molecule, HLA-E (Qa-1 in mice) (Cantor et al., 1975, Noble et al., 1998). A role for CD8 regulatory cells was suggested by early studies in which infusion of an irradiated T cell line specific for MBP attenuated EAE caused by that antigen, but this effect was lost if the cell line was initially depleted of $CD8^+$ cells (Koh et al., 1992, Jiang et al., 1992). This effect was subsequently isolated to only those cells expressing Qa-1 (Hu et al., 2004). Qa-1 deficient mice do not develop spontaneous autoimmunity but have excessive $CD4^+$ responses following relevant self-peptide immunisation or viral infection. $CD8^+$ Treg appear to be more relevant later in the immune response, mirroring the cytolytic effects of $CD8^+$ T cells, as it requires previous antigen encounter. Similarly to Tr1 cells, $CD8^+$ Treg have an absolute requirement for IL-15 (Kim et al., 2010). The exact role of $CD8^+$ regulatory T cells is not yet fully determined.

1.7.3.4 Regulatory B cells and miscellaneous

Other cell lineages may also have dedicated regulatory equivalents. IL-10 is produced by a subset of $CD1d^{high}$ B cells that accumulate in the gastro-intestinal tract, and may be suppressive (Mizoguchi et al., 2002). Potential evidence for regulatory B cells has been reported in models of arthritis (Mauri et al., 2003) and in EAE (Mann et al., 2007, Fillatreau et al., 2002, Matsushita et al., 2008), but to date no dedicated 'Breg' has been identified *in vivo*. Regulatory NK cells (Deniz et al., 2008), and regulatory γδ T cells (Wu et al., 2004) have both been described.

The periphery therefore has a wide range of cell lineages (almost as wide as the effector population), which are able to maintain tolerance and prevent excessive immune reactions using a number of mechanisms.

1.8 Loss of tolerance

In order to cope with the vast array of potential pathogens, T cells require a broad repertoire of receptor recognition to provide adequate coverage and initiation of an immune response. Equally, self-reactivity must be curbed to prevent overwhelming immunity. Permissible release of 'moderately' self-reactive cells from the thymus to ensure maximal pathogenic coverage demands the systems of peripheral tolerance delineated above. With the

triumvirate of activation-induced cell death, induction of adaptive tolerance (anergy), and dedicated regulatory populations, the immune system has evolved highly efficiently with virtually no pathogens going unrecognized, without every individual succumbing at some point in life to autoimmunity.

That this system is imperfect is evidenced by the burden of autoimmune disease in the population. With a prevalence of common autoimmune disease in the UK of 3% (Boelaert et al., 2010), a breakdown in tolerance is a common occurrence. A small proportion of those affected will have monogenic disorders affecting the regulatory systems (e.g. the disorders listed in section 1.7). However the vast majority have diseases with a multifactorial aetiology – insulin dependent diabetes mellitus, rheumatoid arthritis, IBD, etc. Genetic associations abound, particularly with the major histocompatibility complex (e.g. HLA-DR3 and HLA-DR4 in Caucasians with type I diabetes mellitus) (Weber et al., 2010) and the increasing use of genome-wide assays is likely to boost our knowledge of these risk factors. Environmental factors leading to breaks in tolerance are much more troublesome to investigate and prove, typically requiring large-scale epidemiological studies, with a variety of confounding factors. The influence of cigarette smoking, associated with multiple examples both protective and detrimental, is virtually impossible to separate from social class, housing issues including overcrowding, diet, stress, etc.

1.8.1 Goodpasture's disease

1.8.1.1 History and clinical presentation

To consider breakdown in tolerance, an example of human autoimmunity – Goodpasture's disease – will be discussed. Goodpasture's is a rare autoimmune disorder characterised by rapid onset glomerulonephritis and pulmonary haemorrhage associated with the detection of pathogenic auto-antibodies to the non-collagenous domain of the α3-chain of type IV collagen (α3(IV)NC1). The clinical correlate of glomerulonephritis and pulmonary haemorrhage was first described by Ernest Goodpasture in a young solider in the WWI trenches (Goodpasture, 1919). Forty years later, the combination was eponymously granted to him by Stanton and Tange describing the linkage (Stanton et al., 1958). Early experiments demonstrated the pathogenicity of the auto-antibody, through elution of immunoglobulin from sick patients and administration of this serum causing an identical disease in previously healthy rhesus monkeys (Lerner et al., 1967).

Why does Goodpasture's represent a good example of an autoimmune disease? The disease itself is reasonably homogeneous and clinically definable (Phelps et al., 1999). This ensures that patient studies are less encumbered with false positive or false negative cases being included. Importantly, the natural history of the disorder is well defined. Spontaneous emergence of auto-antibody causes the disease state, which is treated with broad immunosuppression and plasmapheresis to remove the pathogenic antibody. What makes Goodpasture's most relevant is that the autoimmunity spontaneously resolves (Wilson et al., 1973). The levels of auto-antibody decrease over time irrespective of whether this is hastened by therapeutic means. It therefore demonstrates both loss and re-establishment of tolerance. Understanding the mechanisms behind this re-emergence of tolerance has implications for both the pathogenesis of autoimmunity, and therapeutic manipulations that could lead to the long-term reversal of auto-immune disease rather than the current 'fire-fighting' approach of immunosuppression and management of ensuing complications.

1.8.1.2 Loss of tolerance in Goodpasture's

Examining the specifics of the development of Goodpasture's can contribute to knowledge of how tolerance is lost. Epitope mapping has suggested that the 71-90 and 131-150 regions are able to stimulate T cells in the peripheral blood, responsive T cells being identified in 100% of patients in one study (Cairns et al., 2003). Both of these epitopes are degraded early in peptide processing by cathepsins, suggesting that they may not be presented by APCs (Zou et al., 2007). This may contribute to the break from tolerance.

How is peripheral tolerance subverted? Various strands of evidence point to a role for environmental pollutants in the development of the disease, particularly cases with pulmonary involvement. Reports have associated cigarette smoking and hydrocarbons, and clustering of cases has occurred (Kalluri et al., 2000). The disease also has a very strong genetic component, particularly HLADRB. Individuals with DRB*1501 have a greater susceptibility to development of Goodpasture's (indeed, 80% of patients in one study were DRB*1501 positive) whilst DRB*0701 and DRB*0101 are protective (Phelps et al., 1999). Molecular binding studies have shown that these MHC class II variations impact on the formation of the peptide-MHC complex, but the protective alleles form a stronger affinity complex than the detrimental ones. This mirrors the effects seen with altered peptide ligands, whereby a greater binding affinity is associated with induction of tolerance rather than disease (Anderton, 2001).

One report has suggested that the re-establishment of tolerance is associated with a change in T cell populations. By limiting dilutional analysis, patients showing spontaneous remission all demonstrated an increase in the number of IL-10^+ T cells and a reduction in IFN-γ^+ T cells (Cairns et al., 2003). Flow cytometry showed that the number of $CD25^+$ T cells also increases as the disease resolves. Depletion of these regulatory cells in *in vitro* cultures led to a re-emergence of IFN-γ^+ cells (Salama et al., 2003).

Thus, Goodpasture's serves as a model to show how tolerance may be broken. Central tolerance is avoided due to early processing of the key antigenic residues preventing their presentation on thymic epithelia. Thus self-reactive T-cells are not negatively selected for. Due to some environmental or infective process, not yet elucidated, the antigen is then differently processed in such a way that presentation to T-cells does occur, thereby triggering activation with B-cell recruitment and antibody production. That T-cells are vital is seen in mice deficient in immunoglobulin, which still develop the model correlate, experimental autoimmune glomerulonephritis (Dean et al., 2005). Blockade of T-cell co-stimulatory molecules in rat models is protective for the disease (Reynolds et al., 2000).

How do these mechanisms correlate with other autoimmune diseases? Loss of central tolerance but maintenance of peripheral tolerance suggests that autoreactive T cells to various antigens should persist in the peripheral blood. This is indeed the case for Goodpasture antigen (Zou et al., 2008). Glutamic acid decarboxylase 65 (GAD-65) associated with type one diabetes mellitus, melanocyte differentiation antigen tyrosinase, and the tumour antigen NY-EOS-1 were all found to stimulate human peripheral blood mononuclear cells (PBMCs) (identified via tetramer staining) that had been depleted of their regulatory population (Danke et al., 2004).

1.8.2 EAE as a model of autoimmunity

Animal models have contributed significantly to our knowledge of Treg biology. Proof of concept that Treg may be useful therapeutically is available in several disease models. Pre-clinical models have shown promise in inflammatory bowel disease (Mottet et al., 2003) and in a humanised mouse model of skin graft tolerance (Sagoo et al., 2011). Several references have already been made to a long-established mouse model, EAE. This model is used to mimic the demyelinating lesions seen in multiple sclerosis through the administration of a central nervous system auto-antigen together with adjuvant, leading to an ascending paralysis (McGeachy et al., 2005). The outcome of this paralysis is partly strain-dependent

and may resolve completely, follow a relapsing-remitting pattern, or persist. In this way, the model serves to emulate the different clinical presentations of MS. Several antigens are used to initiate EAE. The dominant epitope in myelin oligodendrocyte glycoprotein (MOG) is found within residues 35-55 ($pMOG_{35-55}$) in mice on the C57BL/6 background (i.e. I-A^b restricted). 2D2 mice with TCR specificity for pMOG have been developed (Bettelli et al., 2003). MBP is also used to initiate EAE, with the acetylated nonameric N-terminus Ac1-9 being the dominant epitope in I-A^u restricted mice. Tg4 mice have TCR specificity for MBPAc1-9 (Liu et al., 1995). Both transgenic strains are used extensively in this thesis. Other encephalitogenic proteins such as proteolipid protein (PLP) may contain several immunodominant epitopes, partly accounting for the phenomenon of epitope spreading.

The regulatory equivalent of epitope spreading is linked suppression, also seen within the EAE model (Anderton et al., 1998). Tolerance to $PLP_{139-151}$ was able to promote tolerance to MBPAc1-9 and MBP_{89-101} in that report. Whether that linked suppression represents bystander suppression by Treg is open for debate and may be dependent on the initial stimulating antigen. Reports that antigen-specific iTreg are unable to suppress responses caused by other antigens (Zhang et al., 2010) are counterbalanced by multiple accounts of bystander suppression in other models (Homann et al., 1999, Weiner et al., 1994 Bayrak et al., 1998, Nicholson et al., 1997, Karim et al., 2005)

1.9 Treatment of autoimmunity

1.9.1 Corticosteroids

For many years, the mainstay of therapy was not to restore or induce tolerance, but simply to limit the inflammatory process. This was achieved most frequently through the use of systemic corticosteroids, particularly prednisolone. Corticosteroid was first used successfully in 1948 in a patient with rheumatoid arthritis by Hench, who shared the Nobel Prize in Physiology or Medicine in 1950 for the work on corticosteroids. Prednisolone entered clinical usage in 1955. The combined anti-inflammatory and immunosuppressive effects had profound results upon previously progressive conditions, and steroid usage rapidly gained acceptance (British Medical Journal, 1969). How corticosteroids exert their immunomodulatory effects is still under investigation over half a century later (Xu et al., 2009). The adverse effect profile of long-term steroid usage, plus the almost inevitable relapse on stopping therapy, renders them unsuitable for life-long use. The search for superior medications continued.

1.9.2 Chemotherapy agents

Following corticosteroids, immunologists' attention was diverted to the world of oncology. The broad anti-proliferative effects of 6-mercaptopurine (6-MP) and its derivative, azathioprine was then superceded by the use of cyclosporine A – a calcineurin inhibitor which blocks the NF-AT pathway (though of course this was not appreciated at the time!). Treatment of type one diabetes mellitus was a success regards extending the honeymoon period of persisting β-cell function (Assan et al., 1985) but adverse effects, particularly nephrotoxicity, limited usage. Drug withdrawal led to immediate relapse proving that the break in tolerance was held in check, but not resolved. Use of cyclosporine A and the newer equivalent, tacrolimus (FK506) remains ongoing for immunosuppression for organ transplantation. The propensity for FK506 to cause autoimmune diabetes has limited its use within autoimmunity (Lohmann et al., 2000) but cyclosporine remains in use for several autoimmune disorders (O'Grady et al., 2011, Manno et al., 2010).

Alkylkating agents, such as cyclophosphamide, are also used to block DNA synthesis. Intriguingly, the mechanism of action of cyclophosphamide is antagonistic to formation of the enzyme retinaldehyde dehydrogenase 1 (ALDH1A1) (Duester et al., 2011) that converts retinaldehyde to retinoic acid (Jones et al., 1995). Cells with high levels of ALDH1A1, such as haematopoietic stem cells, are relatively resistant whilst lymphocytes are more susceptible. Targeting a pathway that involves reduction in retinoic acid may also have specific effects upon the induction of Treg, though limited studies show conflicting effects (Audia et al., 2007, Ghiringhelli et al., 2007). High dosage cyclophosphamide is used successfully in several severe autoimmunities, including aplastic anaemia (Baran et al., 1976), SLE (Dolff et al., 2010), and as second-line therapy in MS (Gladstone et al., 2006).

Ultimately corticosteroids, 6-MP and derivatives, the calcineurin inhibitors and alkylating agents all share the common pathway of limiting proliferation by immune cells in a relatively non-selective manner. Targeting the pathogenic cells or molecules whilst sparing the irrelevant aspects of the immune system would limit adverse effects and permit more efficacious eradication. This has led to the use of molecular-targeted therapies known collectively as 'biologics' that are typically specific for a single molecule, be that soluble cytokine, cell surface receptor, or intracellular signaling pathway.

1.9.3 OKT3 and derivatives

The first biologic to specifically deplete T cells was the murine monoclonal antibody to human CD3ε, OKT3 (Cosimi et al., 1981). Targeting CD3 effectively depletes all T cells, allowing a 'reset' of the immune system. Reconstitution of the immune system occurs with a higher proportion of regulatory cells initially. The ability of the murine anti-CD3 to activate T cells also led to massive cytokine release (a cytokine storm) with the potential for fatality (Charpentier et al., 1992). Modifications of OKT3 to limit its ability to activate (by preventing FcR-binding) lead to the newer anti-CD3 biologics such as teplizumab and otelixizumab. These are less effective at depleting the entire T cell population at equivalent dosage, but show more specificity for pathogenic cells via multiple effects including the induction of anergy (Smith et al., 1997), blockade of antigen recognition (Herold et al., 2005), and selective sparing of regulatory cells (Chatenoud et al., 2007). Importantly, the problematic cytokine storm is also ameliorated, making these biologics more appealing for clinical use. Clinical trials for anti-CD3 show potential promise (Utset et al., 2002, Friend et al., 1999, Bresson et al., 2006).

1.9.4 Alemtuzumab

Alemtuzumab (Campath-1H) is another depleting agent, being a monoclonal antibody directed against CD52, found on the cell surface of mature lymphocytes. Administration of alemtuzumab leads to rapid depletion of $CD52^+$ cells via activation induced cell death (Coles et al., 2008). Co-stimulation through CD52 also appears to preferentially induce Treg (Watanabe et al., 2006). Alemtuzumab has been used successfully in multiple sclerosis for over 20 years (Moreau et al., 1994), particularly in relapsing-remitting MS, with effects above and beyond those expected from CD52 depletion alone (Jones et al., 2010). The most notable adverse effect is the development of other autoimmune disease, particularly thyroiditis in up to 30% of patients (Jones et al., 2009), but also immune thrombocytopenic purpura, Goodpasture's disease, and several other autoantibody-mediated diseases. The causes for these novel autoimmunities to arise in the context of relative immunosuppression remain under investigation (Jones et al., 2009a).

1.9.5 CTLA-4 and abatacept

Targeting other signaling molecules does not lead to depletion but may permit a more specific effect. The CD28-related CTLA-4 is expressed on activated cells, competitively inhibits CD80/CD86-CD28 interaction, and thereby prevents further TCR signaling. The extracellular part of CTLA-4 and human constant domain IgG1 are fused to create the biologic, abatacept. This has proven efficacy in rheumatoid arthritis (Kremer et al., 2008), and importantly may have direct effects on the pathology itself (Choy, 2009). A Phase I trial in MS was successful in reducing *in vitro* parameters but had no impact on clinical disease (Viglietta, 2008). It is not efficacious in inflammatory bowel disease (Sandborn et al., 2012). Post-transplant lymphoproliferative disease has been reported following treatment with abatacept (Vincenti et al., 2008). Why there should be such discrepancy amongst autoimmune diseases is likely related to the mechanisms causing the initial loss of tolerance.

1.9.6 Lymphocyte trafficking manipulation

Fingolimod (FTY720) is analogous to sphingosine-1-phosphate, a molecule that promotes the exit of lymphocytes from the thymus and lymphoid organs into the peripheral circulation. Binding of fingolimod to the receptor prevents lymphocyte trafficking (Mandala et al., 2002) leading to a marked reduction in peripheral white cell counts (Brinkmann et al., 2001). Aside from physically preventing egress, fingolimod binding also promotes Treg activity whilst inhibiting Th1 activity (Daniel et al., 2007, Mehling et al., 2008). Through these actions, fingolimod has proven efficacy both in transplantation and MS (Kappos et al., 2006).

Preventing lymphocyte accumulation in the area of pathology can be achieved by alternate means. Rather than trapping them within the lymphoid system, affecting adhesion markers can render lymphocytes unable to leave the periphery. Natalizumab is a monoclonal directed against α_4 integrin, thereby preventing interactions by CD49d and LPAM-1 – both containing α_4 chains and crucial in adherence to the vascular endothelium. Both Th1 and Th17 cells express CD49d but LPAM-1 appears specific to Th17 cells. (Cox et al., 2008). Efficacious in disease (Lanzillo et al., 2012, Kane et al., 2012), serious adverse effects have dogged use of natalizumab. An association with progressive multifocal leucoencephalopathy (PML) following reactivation of latent JC viral infection (Tur et al., 2012) led to initial withdrawal. Since reintroduction, several lymphomas have been reported (Matzke et al., 2012, Schowinsky et al., 2012). More concerning, withdrawal of the drug is

associated with an immune reconstitution inflammatory syndrome that may have equally devastating effects (Gheuens et al., 2012). PML has been associated with a number of biologics including rituximab (anti CD20 monoclonal) and efalizumab (Tavazzi et al., 2011) (anti-CD11a, now withdrawn).

1.9.7 TNF blockade

Given that much of the pathogenesis of disease is attributable to cytokines, blocking the receptors or incapacitating the molecules themselves appears a logical step. Herein is one of the true success stories of the last 20 years. Blockade of TNF with anti-TNF (infliximab, adalimumab) or 'mopping up' with a non-signaling soluble receptor fusion protein (etanercept) has become a regular therapy in rheumatoid arthritis (Thalayasingam et al., 2011), IBD (Nattiv et al., 2012), and latterly severe psoriasis (Brezinski et al., 2012). Other therapeutic applications are under consideration (Catanoso et al., 2011), including some very different pathological processes (Austin et al., 2012). Part of the success of TNF blockade is related to the profound symptomatic improvement seen in patients, but it is also partly attributable to the safety profile. First used in 1998, more than a million patient experiences (Lin et al., 2008) in the first decade reassured that complications were mainly related to infections, hardly unique to TNF blockade. Since then, the recognition that hepatosplenic T cell lymphoma is increased in IBD patients on TNF blockade has led to greater caution, particularly where multiple immunomodulatory therapies are in use (Parakkal et al., 2011).

1.9.8 Antigen-specific therapy

1.9.8.1 Peptide therapy

Attempts to induce tolerance through the administration of peptides have been ongoing for over a century (Noon, 1911). Provision of the immunodominant epitopes to T cells, in the absence of danger signals, leads to the establishment of tolerance to that antigen. This was demonstrated in mouse models, using peptides relevant to EAE (Bitar et al., 1988) and arthritis (Nagler-Anderson et al., 1986). Oral administration provoked regulatory populations capable of bystander suppression. One group reported efficacy of an MBP epitope in preventing the development of EAE in rats initiated by a different MBP epitope (MBP_{21-40} and MBP_{71-90} respectively (Miller et al., 1993). The mechanisms at this time were felt related to TGF-β production (Chen et al., 1994). This successful establishment of

tolerance was repeated in several different animal models of autoimmunity (Weiner et al., 1994).

Oral tolerance is partly mediated through the induction of Treg. $CD103^+$ gut DCs, in the presence of retinoic acid (which they also produce), are highly efficient at generating $Foxp3^+$ iTreg (Coombes et al., 2007), though all recognised subsets of Treg have been identified as relevant in oral tolerance (Weiner et al., 2011). High doses of oral antigen lead to T cell anergy, with one report demonstrating this was due to an inability to stabilise APC interactions (Ise et al., 2005). Other interactions are clearly crucial, as oral tolerance is not possible in CTLA-4-deficient mice (Samoilova et al., 1998).

Despite an increased understanding of the mechanisms used and the success of animal models, full translation of peptide therapy into clinical use has not yet occurred beyond phase II trials (Weiner et al., 2011). Most promising was use of oral insulin that appeared to delay progression to clinically evident diabetes mellitus in a subgroup analysis of a larger trial (Skyler et al., 2005). Thus far, peptide therapy remains a work in progress.

1.9.8.2 Other antigen-specific therapies

A recent phase I trial evaluated the use of a recombinant TCR ligand (RTL) consisting of the α1 and β1 chains of MHC Class II molecule DR2, covalently bound to the dominant antigenic epitope, $pMOG_{35-55}$ (Offner et al., 2011). Mouse models demonstrated partial agonism of the TCR (Wang et al., 2003) with either protection from, or resolution of, EAE (Huan et al., 2004, Vandenbark et al., 2003). RTL therapy was also able to ameliorate disease caused by a cocktail of multiple antigens provided $pMOG_{35-55}$ was included, suggesting bystander suppression and providing a therapeutic modality that could counter epitope spreading. Completion of the phase I trial in human MS patients demonstrated no adverse effects within a reasonable dose range equivalent to that used in murine models. There was a suggestion of a reduction in contrast-enhancing cerebral lesions, which was dose-dependent.

Various other biologics exist and to discuss them all would not enlighten any further. The vast majority of the biologics have one vital drawback shared with the use of prednisolone - the underlying defect in tolerance persists and withdrawal of therapy leads to disease relapse, requiring lifelong therapy with attendant risks.

What then is the solution? An ideal therapy would target only those cells actively causing inflammation, have minimal adverse effects, and, most importantly, would lead to

restoration of tolerance so that patients could resume a normal drug-free life. Obviously, this is the ideal outcome for any therapeutic intervention for any chronic disease – but are immunologists closer than most? The presence of autoreactive cells in healthy individuals controlled by peripheral tolerance signifies that there are natural mechanisms to restore tolerance. Harnessing the cells responsible for this regulation could prove the answer.

1.10 Foxp3$^+$ regulatory T cells

1.10.1 Natural Treg

The existence of cells that suppressed immune responses, rather than stimulated them, was first postulated in the 1970s as suppressor T cells (Gershon, 1975) residing within the CD8$^+$ population. Subsequent experimentation failed to convincingly demonstrate existence of this lineage and the concept was largely abandoned. Some groups persisted, however, given the undeniable suppressive effect of T cell transfer in some animal models (Penhale et al., 1976). Identification of CD4$^+$ T cells with suppressive function was made in two overlapping populations. Sakaguchi's lab made the vitally important finding in 1995 that depletion of a CD4$^+$CD25$^+$ population in mice led to autoimmunity, and reconstitution of that population after neonatal thymectomy prevented it (Sakaguchi et al., 1995). At a similar time, a second report demonstrated the ability of CD4$^+$CD45RB^{hi} cells to induce colitis, whereas co-transfer with CD4$^+$CD45RB^{low} cells prevented this (Powrie et al, 1993). Finer discrimination using CD4$^+$CD45RB^{low}CD25^{high} identified a highly suppressive cell population, able to prevent colitis when co-transferred at 1:8 with effector cells (Read et al., 2000). Further characterization confirmed that this sub-population of CD4$^+$CD25$^+$ cells had regulatory properties and were defined by the master transcription factor forkhead box protein 3 (Foxp3) (Fontenot et al., 2003). The importance of Foxp3 is evidenced by the severe and spontaneous autoimmunity seen in its absence, both in the *scurfy* mouse (Brunkow et al., 2001), and in the human syndrome immune dysregulation, polyendocrinopathy, enterocolitis, X-linked (IPEX) (Bennett et al., 2001, Wildin et al., 2001). Similarly, forced expression of Foxp3 on T cells through viral transduction confers a regulatory phenotype (Hori et al., 2003) whilst suppressing IL-2 production. The Foxp3 transcription factor directly interacts with NF-κB and NF-AT leading to suppression of IL-2, IL-4 and IFN-γ transcription (Bettelli et al., 2005). Foxp3 complexes with NF-AT (Wu et al., 2006), leading to up-regulation of both the IL-2 receptor α chain (CD25) and CTLA-4, whilst interactions with Runx provide a positive feedback loop to maintain Foxp3 expression (Rudra et al.,

2009). Genome-wide assays have identified hundreds of molecules either up- or down-regulated by Foxp3, earning its status as a 'master' transcription factor.

$Foxp3^+$ cells can be broadly grouped into two populations. Natural Treg (nTreg) are thymically-derived and enter the peripheral circulation as mature nTreg (Sakaguchi et al., 2005). Expression of Foxp3 in the thymic developing cells is seen at the double positive $CD4^+$ $CD8^+$ stage (Liston et al., 2008). These nTreg are traditionally anergic, with suppressive capacity *in vitro* though highly proliferative *in vivo* (Fisson et al., 2003). The TCR repertoire of nTreg can be considered as broad as that of naïve cells with a greater affinity for self (Hsieh et al., 2006).

The human Foxp3 population can be further characterised by the expression of CD45RA (Miyara et al., 2009), into three groups. As CD25 is typically up-regulated on activated cells whilst CD45RA is down-regulated, high expression of both suggests a Treg. These were classed as 'resting' Treg with relatively low Foxp3 expression. $CD45RA^-Foxp3^{high}$ Treg ('activated' Treg) were more liable to lose Foxp3 expression on repeated *in vitro* stimulation, though both groups were suppressive *in vitro*. Finally, $CD45RA^-Foxp3^{low}$ Treg were identified as producing cytokines but were non-suppressive.

1.10.2 Induced Treg

Adaptive, or induced, Treg develop in the periphery from naïve T cells on TCR stimulation in the presence of IL-2 and TGF-β (Chen et al., 2003). This induction is antagonized and dominated by IL-6, leading to differentiation of Th17 cells instead (Bettelli et al., 2006, Veldhoen et al., 2006, Korn et al., 2008). Development of $Foxp3^+$ cells from naïve precursors seems particularly relevant in the gastro-intestinal tract with retinoic acid (RA), a vitamin A metabolite, promoting the induction of Treg (Sun et al., 2007) and inhibiting the actions of IL-6 (Mucida et al., 2009). Indeed, RA appears to be able to replace IL-2 in the generation of Treg (Coombes et al., 2007). The ability of human iTreg to suppress remains partly equivocal (Tran et al, 2007) but, certainly in mice *in vitro* and *in vivo*, is now well established (O'Connor et al., 2010). The stability of the iTreg phenotype has been questioned, with apparent conversion to effector cells reported (Yang et al., 2008a, Deknuydt et al., 2009, Koenen et al., 2008, Zhou et al., 2009a). Stability of iTreg has been related to epigenetic modification at the Foxp3 locus, the Treg specific demethylated region (TSDR). This region is fully demethylated in nTreg whilst the methylation status of iTreg is more variable (Baron et al., 2007, Floess et al., 2007). iTreg and nTreg are also functionally

different (Horwitz et al., 2004). Generation of iTreg requires co-stimulation partly through low levels of CD28, but also CTLA-4, as TGF-β cannot induce Treg in CTLA-4 deficient mice (Kretschmer et al., 2005, Zheng et al., 2006). Induced Treg are less susceptible to IL-6 mediated blockade of suppression (Zheng et al., 2008), and are more likely to have a foreign antigen TCR.

Treg are partly characterized by having a high requirement for IL-2. The up-regulated expression of CD25 identifies them as having high affinity for this cytokine (Furtado et al., 2002). Treg numbers are reduced in mice deficient in IL-2 (Fontenot et al., 2005) whilst IL-2 blockade replicates the phenotype of Treg depletion (Setoguchi et al., 2005, Suzuki et al., 1995). Administration of exogenous IL-2 to patients induces Treg proliferation and Foxp3 expression via STAT3 and STAT5 (Zorn et al., 2006).

Aside from policing immune self-tolerance, Treg are also important in controlling responses to allergens (Chatila, 2005), allogeneic transplants (Battaglia et al., 2006), and maintaining fetomaternal tolerance in pregnancy (Zenclussen, 2006).

1.10.3 Suppression by Treg

Treg are able to suppress both $CD4^+$ and $CD8^+$ T cells (Piccirillo et al., 2001), B cell proliferation, class switching and immunoglobulin synthesis (Lim et al., 2005), NK and NKT cytotoxicity (Azuma et al., 2003), and maturation of dendritic cells (Misra et al., 2004). Suppression of T cells is not restricted to the naïve cell pool as both effectors and memory cells are also susceptible to the machinations of Treg (Levings et al., 2001a). Suppression by nTreg *in vitro* appears to be contact-dependent as experiments using transwell systems demonstrated the abolition of any regulatory effect (Takahashi et al., 1998).

A major aspect of nTreg suppression is that they exert bystander suppression. Once activated by their cognate antigen, nTreg are able to suppress T cells responding to different antigens (Thornton et al., 2000). The bystander suppression phenomenon has been demonstrated in a number of instances, and will be discussed in greater detail in Chapter 3.

How is suppression mediated? The classical view is that natural Treg suppress in a contact-dependent manner whilst adaptive Treg suppress in a contact-independent means via the production of immunoregulatory cytokines such as IL-10, TGF-β and IL-35. The seminal demonstration of *in vitro* suppression by Shevach (Thornton et al., 1998) proposed this is via deprivation of IL-2, an essential survival cytokine. The high expression of CD25 on Treg

supported this model, as do the *in vitro* effects of further depriving IL-2 (Pandiyan et al., 2007). Given the essential requirement for IL-2 by Treg, this mechanism would also serve as a balance to limit the regulatory response. Curiously though, IL-2 receptor deficient Treg suppress equally efficiently *in vitro* (de la Rosa et al., 2004).

It is not just IL-2 that may be scavenged. Adenosine triphosphate (ATP) promotes inflammation on its release from damaged cells (Yip et al., 2009), partly via TCR-mediated NF-AT activation and promotion of IL-2. Treg express both CD39 and CD73 on their surface (Deaglio et al., 2007), which degrades ATP to AMP, and AMP to nucleosides respectively, thereby depriving inflammatory cells of this additional stimulus.

Full signaling through the peptide-MHC complex and the TCR requires engagement over several hours. *In vivo,* two-photon microscopy has demonstrated fleeting interactions between APC and irrelevant T cells, which become enduring with an antigen-specific cell (Tang et al., 2006). This stable interaction is inhibited in the presence of Treg, which are able to engage with APC even more rapidly than the memory cell population (Onishi et al., 2008). This strong reaction is mediated partly through the high expression by Treg of several molecules, including ICAM-1, neuropillin-1 (Sarris et al., 2008), and LAG-3 (Huang et al., 2004a, Liang et al., 2008). Thus Treg may physically prevent any stable interaction between naïve/effector cells and APC.

Other 'inhibitory' molecules are expressed on the surface of nTreg which may account for the contact dependence of suppression, including CTLA-4 and glucocorticoid-induced TNFR (GITR). CTLA-4 is constitutively expressed on nTreg (Takahashi et al., 2000), and up-regulated even further on TCR engagement. CTLA-4 interaction with DCs leads to induction of indoleamine 2,3-dioxygenase (IDO), which catalyses tryptophan into immunosuppressive cytotoxic metabolites (Fallarino et al., 2006), including kynurenine which acts to induce further Treg (Mezrich et al., 2010). IDO may also directly influence DCs towards a tolerogenic phenotype (Pallotta et al., 2011). Competition between Treg CTLA-4 (which has greater affinity) and naïve/effector cell CD28 may further limit T cell activation (Yokosuka et al., 2010), whilst binding of Treg to CD80 or CD86 on effector cells may lead to a direct regulatory effect (Paust et al., 2004). Treg have been shown to remove CD80 and CD86 from the APC surface by trans-endocytosis and degradation, preventing APCs from providing co-stimulatory signals to other naïve T cells (Qureshi et al., 2011). This may partly explain why mature DCs expressing high levels of CD80 and CD86 are able to overcome suppression (Zheng et al., 2004). Absence of CTLA-4 does not remove the suppressive capacity of Treg *in vitro* (Tang et al., 2004), entailing other mechanisms. In

contrast, depletion of CTLA-4 using monoclonal antibodies leads to similar autoimmune defects to mice undergoing Treg depletion, and prevents protection in colitis (Read et al., 2000).

Suppression by secretion of cytokines is well recognized. IL-10 blockade accelerates allogeneic skin graft loss in mice (Kingsley et al., 2002), prevents tolerance induction in a model of liver injury (Erhardt et al., 2007), and prevents regulation of Th17 responses leading to colitis (Chaudhry et al., 2011), whilst IL-10 deficient Treg cannot control colitis or lymphoproliferation in a Rag-deficient model (Annacker et al., 2001), though they remain competent in suppressing other autoimmune disease (Suri-Payer et al., 2001). IL-10 secretion by Treg is seen in inflamed tissues (McGeachy et al., 2005, Uhlig et al., 2006) and contributes to disease resolution. IL-10 induces a tolerogenic phenotype in DCs and differentiation towards Tr1 cells by naïve T cells (Steinbrink et al., 1997, Groux et al., 1997). TGF-β is evident both as a soluble cytokine and in its membrane-bound form on Treg (Nakamura et al., 2004), enabled partly by their high expression of GARP (Tran et al., 2009). TGF-β may partly act in Treg via the activation of the Notch pathway (Ostroukhova et al., 2006), but nTreg lacking TGF-β (from knockout mice) still display suppressive capacity both *in vitro* (Piccirillo JEM 2002) and *in vivo* (Fahlen et al., 2005). The crucial role of TGF-β in induction of Treg will contribute to an overall suppressive milieu. IL-35 (Niedbala et al., 2007) appears to be mainly produced by Treg (Collison et al., 2007), and contributes to efficacy against allergy (Whitehead et al., 2012) and colitis (Wirtz et al., 2011). Production of IL-35 by human Treg appears non-redundant for contact-independent suppression, unlike both IL-10 and TGF-β (Chaturvedi et al., 2011), and mediates further induction of Treg (Collison et al., 2010).

Treg may have direct cytotoxic effects as they have been shown to be capable of perforin and granzyme release (Gondek et al., 2005, Grossman et al., 2004). This is unlikely to be a major mechanism given the demonstrations of induction of anergy. A role for galectin proteins has been suggested (Kubach et al., 2007). Cyclic AMP has a detrimental effect on T cell proliferation via activation of the inducible cAMP early repressor (ICER) (Bodor et al., 2001), with one group proposing Treg are able to transfer cAMP directly into effector cells via gap junctions (Bopp et al., 2007). Clearly Treg have multiple mechanisms at their disposal, with a degree of redundancy and flexibility that may be dependent on the inflammatory context.

Given that Treg constitute 5-10% of $CD4^+$ cells in a mouse and an even smaller 1-3% in human subjects (Baecher-Allan et al., 2001), how does this minority population regulate the

much vaster population of potential effectors? Treg require stimulation via the TCR to effect regulation but once activated, these suppressive mechanisms are not antigen-specific (Thornton et al., 2000) and this permits bystander suppression to occur (Karim et al., 2005). More importantly, one effect of Treg is to induce a suppressive phenotype in surrounding cells as a means of infectious tolerance (Dieckmann et al., 2002, Jonuleit et al., 2002).

The regulators themselves require regulation. An uncontrolled Treg response would prevent effective pathogen clearance or destruction of aberrant self cells. Indeed, limiting the Treg population is now a major therapeutic focus in oncology in the hope that instilling immunity to the semi-foreign malignant tissue will be far less toxic than current chemotherapeutic regimens (Baechar-Allan et al., 2006). Strong co-stimulatory signals through the TCR (Takahashi et al., 1998), or provision of exogenous IL-2, TNF (Valencia et al., 2006), or IL-6 (Pasare et al., 2003) all render responding cells more resistant to suppression. Treg also express toll-like receptors (TLRs) 2 and 8 (Sutmuller et al., 2006, Peng et al., 2005), suggesting a mechanism by which pathogens may directly suppress Treg responses.

1.10.4 Therapeutic application of Treg

The presence of a pool of cells dedicated to down-regulating inflammatory responses has obvious implications for therapy. As all autoimmunity can be considered a break in tolerance due to an ineffective regulatory response, restoring the balance through the administration of Treg makes theoretical and physiological sense. There are complicating factors, however. Aside from the stringent requirements that any cell-based therapy must meet, and the likely heavy financial costs (more than compensated for if a therapy is curative rather than merely delaying), the nature of Treg themselves causes issues. Overwhelming the immune system with Treg would equate to immunosuppression whilst dysregulated Treg are implicated in many malignancies. Identification of a 'pure' Treg population remains impossible in the clinical setting, due to the overlap of cell surface markers with other activated cells. Infusing effector cells into a patient would be unlikely to achieve optimal outcome! There is also the issue of antigen specificity, as antigen-specific Treg are able to suppress more effectively than polyclonal Treg (Tang et al., 2004). nTreg are at low numbers in the peripheral circulation and though protocols for expansion of these cells have now been generated (Peters et al., 2008), selecting for antigen specificity may limit the practicality of this approach significantly. The much larger pool of naïve cells offers the use of iTreg – removing the patient's own naïve cells and converting them to a regulatory

phenotype would avoid any allogeneic reactions. However, the stability of iTreg cannot be assured, with the attendant danger that de-differentiation into effector cells with pathogenic antigen specificity could well be clinically devastating. Despite these caveats, Treg remain an appealing option, and attempts to translate them into a feasible therapy are well underway.

1.10.4.1 Diabetes mellitus

Type I diabetes mellitus offers a highly appealing prospect for Treg therapy. As a disease it is relatively common, auto-antibodies can be detected before clinical disease in susceptible people, clinical onset occurs while there is still functioning tissue, and patients are frequently free of other diseases (Bach et al., 2011). Several antigens are identified as relevant and the financial burden of lifelong treatment of both diabetes and its sequelae greatly exceeds the presumed cost of any cell-based therapy, were it to prove curative or simply delay the onset by several years. A phase one trial using polyclonal ex vivo expanded $CD4^{+}CD25^{+}CD127^{low}$ nTreg (using the methods of (Putnam et al., 2009)) is currently recruiting (study end date 2016) [NCT01210664]. Animal models have demonstrated an ability to both prevent disease onset (a non-obese diabetogenic mouse) but also reverse established disease (Tang et al., 2004) with a more marked effect from treatment with antigen-specific Treg than polyclonal.

1.10.4.2 Graft versus host disease

Another area of interest is in the prevention of graft versus host disease (GVHD). Infusion of donor Treg after bone marrow transplantation prevented the development of GVHD whilst maintaining graft versus leukaemia effects in a murine system (Edinger et al., 2003). There are now several reports in the literature of the usage of Treg as a modality in human GVHD. A phase I trial using umbilical cord blood (UCB)-derived Treg in patients with advanced haematologic malignancy receiving a double UCB transplant demonstrated no safety concerns and showed a positive, if modest, reduction in the incidence of serious GVHD (Brunstein et al., 2011). A second phase I trial infusing polyclonal Treg after completion of GVHD prophylaxis also demonstrated no safety or feasibility issues, with no incidence of GVHD in the treated group (Edinger et al., 2011). Another group used expanded polyclonal Treg as therapy for established GVHD with efficacy in chronic, but not acute, onset GVHD (Trzonkowskin et al., 2009, 2011). Finally, a very brave approach was taken in administering Treg as the sole immunosuppressive therapy in patients receiving a haploidentical stem cell graft. The Treg expanded *in vivo* and only a small number of

patients developed any significant GVHD, suggesting a high efficacy of the Treg therapy compared to controls (Di Ianni et al., 2011).

Another application in transplantation is in solid organ transplants. An intriguing trial is evaluating subcutaneous infusion of expanded polyclonal Treg shortly following living-related donor kidney transplant in paediatric patients [NCT01446484]. A much larger consortium of institutions is also commencing a trial evaluating the use of Treg in renal transplantation, though the exact details of patient selection and cellular therapy are not yet finalised (the ONE study).

1.10.4.3 Therapeutic hurdles

1.10.4.3.1 Identifying 'pure' Treg

Foxp3 is not a perfect marker for human Treg. As a nuclear transcription factor, its intracellular location renders it unsuitable for cell isolation by flow cytometry. The Foxp3 promoter gene contains binding sites for both NF-AT and AP-1, both of which are up-regulated on TCR stimulation (Mantel et al., 2006), leading to transient expression of Foxp3 on T cell activation which does not equate to regulatory status (Miyara et al., 2009). Other markers such as low expression of the IL7-Rα (CD127) (Liu et al., 2006) and high glycoproteins A repetitions predominant (GARP) expression (Stockis et al., 2009) have allowed more specific identification but a unique marker has yet to be identified.

In the stem cell transplant setting, transfer of a small number of non-Treg is likely to be positively beneficial in mediating the graft versus leukaemia effect (Edinger et al., 2003). As therapy for autoimmunity, as alluded to above, a pure Treg population would likely be essential. Various manipulations have been attempted to derive a 'pure' Treg yield.

Most promising is the addition of the mTOR inhibitor, rapamycin, which inhibits cell proliferation in most cells but permits the proliferation of Treg through their ability to activate an alternative pathway (Battaglia et al., 2005). Rapamycin is particularly appealing as a therapeutic agent already licensed for use in the transplant setting. Clinician familiarity can be a major advantage in acceptance of a new indication for an agent. Rapamycin also induces Foxp3 expression in naïve T cells, while inhibiting the expression of other lineage transcription factors (Long et al., 2008, Delgoffe et al., 2009). This may be considered a positive as it would skew contaminating cells to the regulatory phenotype but the stability of these cells once transferred *in vivo* cannot be guaranteed. One might expect, however, that

the ratio of Treg to effector cells would be sufficient to prevent an escalating inflammatory response.

1.10.4.3.2 Plasticity of Treg

A major concern for Treg therapy is that Treg will display a similar plasticity to that seen by effectors (section 1.5), particularly in the pro-inflammatory environment found *in vivo* in ongoing disease, and revert to an effector phenotype with obvious therapeutic disadvantage. That this could feasibly occur was lent weight by demonstrations that nTreg could lose expression of Foxp3 following TLR2 stimulation and MyD88 signaling (Lal et al., 2011).

Confirmation that Foxp3 expression was not irreversible is provided by one report showing loss of Foxp3 in approximately 20% of cells previously having expression (Zhou et al, 2009a). In contrast, a similarly designed study showed nTreg to have high stability of Foxp3 expression, even when infection promoted an inflammatory milieu (Rubstov et al., 2010). A recent report confirmed a subgroup of 'Foxp3^{+}' cells which had only transient expression, but also that true Treg were again remarkably stable (Miyao et al., 2012). Loss of Foxp3 expression by Treg has been observed in other more specialized circumstances, including within the gut (Tsuji et al., 2009), and lymphopenia (Duarte et al., 2009). Similar plasticity of nTreg has been seen in the context of human autoimmunity, with acquisition of Th1-like characteristics (Dominguez-Villar et al., 2011).

In contrast, iTreg are known to rapidly lose expression of Foxp3 *in vitro* (Selvaraj et al, 2007) and *in vivo* on antigen stimulation, though this can be stabilised by IL-2 (Chen et al., 2011). Foxp3 expression is also maintained in iTreg by exogenous TGF-β (Marie et al., 2005). Loss of Foxp3 expression does not necessarily equate to a loss of regulatory function, however (O'Connor et al., 2010, Lin et al., 2007).

Plasticity of Treg extends beyond de-differentiation. Numerous reports have proposed that Treg mature to resemble the nearby effectors i.e. in Th1 conditions, Treg will develop Th1-like characteristics. This has been demonstrated for Th1 (Oldenhove et al., 2009, O'Connor et al., 2010), Th2 (Zheng et al., 2009) and Th17 (Voo et al., 2009) phenotypes. *In vitro* exposure to IL-6 was reported to skew both nTreg and iTreg towards a Th17-like phenotype with production of IL-17 (Yang et al., 2008a), which confirmed previous reports that nTreg could develop IL-17 production (Xu et al., 2007). Foxp3^{+} Treg have been demonstrated to co-express Tbet (Koch et al., 2009), GATA3 (Wang et al., 2011), RORγt (Voo et al., 2009), and Bcl6 (Chung et al., 2011), with one model proposing that Treg up-regulate the relevant

transcription factors to match the targets of regulation. *In silico* modeling has also suggested there is a high degree of plasticity amongst Foxp3$^+$ Treg (Naldi et al., 2010).

Many reports suggested that iTreg were less stable than nTreg, and related the stability to the methylation status of the TSDR (Zhou et al., 2009a). This was supported by the apparent finding that loss of Foxp3 on adoptively transfer was greater in iTreg than nTreg (Selvaraj et al., 2007). Subsequent reports, however, have demonstrated that iTreg are less susceptible to IL-17 production following exposure to IL-6 (Zheng et al., 2008) and that iTreg may indeed represent a superior therapy to nTreg (Huter et al., 2008).

Thus, iTreg represent a potential therapy for autoimmunity. The exact mechanisms of suppression by both nTreg and iTreg remain under investigation, but the bulk of the literature has centred on nTreg.

1.11 Hypothesis and aims

The central tenet of this thesis is that *in vitro* and *in vivo* iTreg are able to suppress in a bystander fashion. This is achieved via a soluble factor released upon their activation, which may affect the effector cell population, the antigen-presenting cells, or both.

Through the course of this thesis, I aim to:-

- Demonstrate that iTreg effect bystander both *in vitro* and *in vivo*, and show the requirement for MHC class II *in vitro*
- Clarify the kinetics of the *in vitro* suppression assay in a peptide-stimulated model
- Clarify the soluble cytokines produced by *in vitro* generated iTreg
- Determine which, if any, of the above are non-redundant in effecting suppression

2 Materials and Methods

2.1 Mice

C57BL/6 (I-A^b), B10.PL (I-A^u), C57BL/6xB10.PL, Tg4 (Liu et al., 1995) (I-A^u, CD90.1 or CD45.1), Tg4xFoxp3LuciDTR-4 (CD45.1) (O'Connor et al., 2010), 2D2 (I-A^b , CD90.1 or CD90.2) (Bettelli et al., 2003), OT-II (I-A^b, CD45.1) (Barnden et al., 1998), and Foxp3GFP (I-A^b) (Fontenot et al., 2005a) mice were bred under specific pathogen-free conditions at the University of Edinburgh (Edinburgh, U.K.). The 2D2 founder mice were a generous gift from Dr V.K. Kuchroo (Harvard University, Boston, MA) and the Foxp3gfp mice were originally provided by Dr A. Rudensky (University of Washington, Seattle, WA). Tg4 CD45.1 IFN-γKO were generated at the University of Edinburgh (Edinburgh, U.K.) by crossing IFN-γKO mice (Dalton et al., 1993) with Tg4 CD45.1 mice and backcrossing for ten generations. All mice were age and sex matched for experiments and used between the ages of 6-16 weeks. Experiments received University of Edinburgh ethical approval and were performed under U.K. legislation.

2.2 Peptides

Myelin oligodendrocyte glycoprotein peptide 35-55 ($pMOG_{35-55}$ MEVGWYRSPFSRVVHLYRNGK) and myelin basic protein acetylated peptide 1-9 (MBPAc1-9 (4Lys); ASQKRPSQR and MBPAc1-9 (4Tyr); ASQYRPSQR) were synthesized by Cambridge Research Biomedicals (Cambridge, U.K). Ovalbumin peptide 323-339 ($pOVA_{323-339}$; ISQAVHAAHAEINEAGR) was synthesised by PepLogic, Essex, UK. Chromatographically purified chicken ovalbumin (Wothington Biochemical Corporation, Lakewood, USA) was used for intratracheal airway challenges (section 2.5.3). All commercially available peptides were provided by the manufacturer at purities consistently greater than 95%. Recombinant myelin oligodendrocyte glycoprotein (rMOG) was synthesised at the University of Edinburgh by Claire Sweenie. Lipopolysaccharide contamination was assessed using the limulus amoebocyte lysate reaction (Neun et al., 2011). Though the solubility of all peptides was not formally evaluated, all were readily soluble in either phosphate-buffered saline or the culture medium used at the required concentrations.

2.3 General reagents

2.3.1 Wash buffer

RPMI 1640 medium containing 25mM Hepes (Gibco, Life Technologies, Paisley, U.K.).

2.3.2 RPMI-10

RPMI 1640 medium containing 25mM Hepes (Gibco) supplemented with 10% heat-inactivated fetal calf serum (FCS; Sigma-Aldrich, Poole, U.K.), 2mM L-glutamine (PAA Laboratories Ltd, Somerset, UK), 100U/ml penicillin (PAA), 100μg/ml streptomycin (PAA) and 50 μM 2-β-mercaptoethanol (Gibco).

2.3.3 MACS buffer

Hanks Balanced Salt Solution (PAA) supplemented with 2% heat-inactivated FCS (Sigma).

2.3.4 FACS buffer

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

2.4 T cell purification

2.4.1. Preparation of single cell suspensions from spleen and lymph nodes

Spleens and peripheral lymph nodes were harvested from mice and disaggregated mechanically through gauze (Sefar, Heiden, Switzerland) to obtain a single cell suspension. Cells were washed by resuspending in wash buffer (10-15ml) and centrifuging at 300g for five minutes. Red blood cells (RBC) were lysed using two ml Red Blood Cell Lysis buffer (Sigma) for two minutes at room temperature. Cells were then washed with RPMI-10 and the number of live cells determined by trypan blue (Sigma) exclusion. Samples were then used for flow cytometric analysis or were further purified as outlined below.

2.4.2 $CD4^+$ T cell isolation: Positive selection by magnetic separation

Following RBC lysis and counting, cells were resuspended in 45μl MACS buffer per 10^7 cells and 5μl CD4 (L3T4) Microbeads (Miltenyi-Biotec, Bergisch Gladbach, Germany) per 10^7 cells. Cells were incubated at 4°C for 15 minutes, and then washed in MACS buffer. Where cells were intended for subsequent purification by fluorescence activated cell sorting

(FACS), 0.5µl of anti-CD4-efluor450 (eBioscience, Hatfield, U.K., table 2.1) or anti-CD4-AlexaFluor700 (BD Pharmingen, Oxford, U.K.) per 10^7 cells were added for the final ten minutes of incubation. Cells were resuspended in 500µl MACS buffer per 10^8 cells then positively selected using an AutoMACS Pro (Miltenyi) as per the manufacturer's instructions. Following positive selection, cells were washed and resuspended in MACS buffer and counted. The post-sort purity of $CD4^+$ cells (analysed by flow cytometry) was routinely 95% ± 5%.

2.4.3 $CD4^+$ T cell isolation: Negative selection by magnetic separation

Cells were prepared as in 2.4.1, then resuspended in 40µl MACS buffer per 10^7 cells and 10µl $CD4^+$ T Cell Biotin-Antibody Cocktail II (Miltenyi) (containing biotin-conjugated antibodies against CD8a, CD45R, CD49b, CD11b, and Ter-119) per 10^7 cells. Cells were incubated at 4°C for 10 minutes. A further 30µl MACS buffer per 10^7 cells was then added to cells, plus 20µl Anti-Biotin Microbeads (Miltenyi) (also including antibodies directed against CD25 and TCRγ/δ) per 10^7 cells. Cells were incubated at 4°C for 15 minutes and then washed in MACS buffer. Cells were resuspended in 500µl MACS buffer per 10^8 cells and $CD4^+$ T cells were isolated by negative selection using an autoMACS Pro.

2.4.4 Isolation of $CD4^+$ $CD62L^{high}$ cells by magnetic separation

$CD4^+$ T cells were isolated as in 2.4.3 and then washed and resuspended in 80µl MACS buffer and 20µl CD62L (L-selectin) Microbeads (Miltenyi) per 10^7 cells. Cells were incubated at 4°C for 15 minutes then washed in MACS buffer, and resuspended in 500µl MACS buffer per 10^8 cells. $CD4^+CD62L^{high}$ cells were then isolated using positive selection on an AutoMACS Pro. Purity was routinely >90%.

2.4.5 Isolation of $CD4^+$ $Foxp3gfp^-$ cells from mice with the Foxp3gfp reporter

Foxp3gfp and Tg4xFoxp3LuciDTR-4 mice both express gfp in association with Foxp3. FACS can therefore be used to isolate $Foxp3^+$ cells without a need for cell permeabilisation. $CD4^+$ cells were isolated as per 2.4.2. These cells were subsequently resuspended in MACS buffer at $3x10^7$ cells per ml and filtered through a CellTrics 50µM filter (Partec, Münster, Germany). $CD4^+Foxp3^-$ cells were purified using a FACS Aria II (BD Biosciences, Franklin Lakes, NJ). Purity of the $CD4^+Foxp3^-$ population was routinely >99%.

2.4.6. Isolation of $CD4^+CD62L^{high}V\alpha3.2^+$ cells from 2D2 mice

Cells were positively selected for $CD4^+$ as above (2.4.2). During $CD4^+$ T cell isolation, cells were incubated with anti-CD4-efluor 450, anti-CD62L-PE, and anti-Vα3.2-FITC (Table 2.1). After $CD4^+$ T cells were isolated, cells were washed and resuspended in MACS buffer at $3x10^7$ per ml and filtered. The $CD4^+CD62L^{high}V\alpha3.2^+$ population was then sorted on the FACS Aria II. Purity was >99%.

2.4.7 CFSE labeling of $CD4^+$ T cells

$CD4^+$ cells were isolated as in 2.4.2, then washed and resuspended in wash buffer at $5x10^7$ cells per ml. 5μM carboxyfluorescein succinimidyl ester (CFSE) was added to the cells which were then incubated at 37°C for 7 minutes. Cold RPMI-10 was added to halt the reaction, and the cells were washed twice in RPMI-10. A small aliquot of cells was taken for confirmation of CFSE staining using flow cytometry and the remaining cells were counted.

2.5 Generation and purification of iTreg

2.5.1 iTreg generation

Using Tg4xFoxp3LuciDTR-4 or Foxp3gfp mice, naïve $CD4^+$ T cell populations were isolated as in 2.4.2 and purified as in 2.4.5. Alternatively, for mice without the Foxp3gfp reporter, naïve $CD4^+$ T cells were isolated as per 2.4.3 and 2.4.4, with additional purification as described in 2.4.6 for 2D2 mice. Once obtained, these naïve $CD4^+$ T cells were resuspended in RPMI-10 at $4x10^5$/ml. 24 well plates were coated with anti-mouse-CD3e (clone 145.2C11, eBioscience) and anti-mouse-CD28 (clone 37.51, eBioscience), both at 2μg/ml in PBS (PAA). Plates were incubated at 37°C for ≥ two hours then washed three times in wash buffer. Cells were added to wells at a final concentration of $4x10^5$ per well, in the presence of IL-2 100U/ml (purified from the X63-IL-2 hybridoma, a kind gift from David Gray, University of Edinburgh) and recombinant human TGF-β1 (R&D Systems, Minneapolis, MN) 5ng/ml. Plates were incubated for five days at 37°C in 5% CO_2. This protocol was derived from that reported by Davidson (Davidson et al., 2007), with reagent concentration optimization performed by Dr Richard O'Connor to maximize induction of Foxp3 expression and cell survival. Shorter incubation times did not permit as much cell

proliferation, whilst longer times led to excess cell death. Cell numbers were typically expanded 4-10 fold.

2.5.2 Purification of iTreg cells

At the end of five days iTreg generation culture, cells were washed in MACS buffer and resuspended after counting in MACS buffer at $3x10^7$ cells per ml. Where iTreg were generated from Foxp3gfp reporter mice, cells were filtered through 50μM CellTrics filters then the $Foxp3^+$ population was purified according to Foxp3gfp expression on the FACS Aria II. When iTreg were generated from mice lacking the Foxp3gfp reporter, cells were washed in MACS buffer and resuspended, after counting, in 200μl MACS buffer and 2μl anti-CD25-PE (eBioscience) per 10^7 cells. Cells were incubated at 4°C for 15 minutes and washed in MACS buffer. After filtering, the $CD25^+$ population was purified using the FACS Aria II. Post-sort purities were consistently >95%.

2.6 *In vitro* manipulations

2.6.1. Depletion of iTreg

After generation (2.5.1) and sorting for purity (2.5.2), Tg4xFoxp3LuciDTR-4 iTreg were resuspended in RPMI-10 at $1x10^6$/ml and cultured in 24 well plates in two ml per well. Diphtheria toxin (Sigma) was added at varying final concentrations from 0-1500ng/ml. Cells were counted daily by trypan blue exclusion and analysed by flow cytometry for Foxp3 expression.

2.6.2 iTreg response to antigen

A top concentration of 100μM MBP Ac1-9 (4Lys) was diluted twofold across a 96 well round-bottomed plate in RPMI-10, with the final wells left without antigenic stimulation. iTreg were generated as per 2.5.1, purified as in 2.5.2, and resuspended in RPMI-10 at $4x10^5$ cells/ml. $2x10^4$ cells were added per well to the plate. To provide APC, Tg4 CD90.1 $CD4^-$ cells were irradiated at 30Gray using a source of ^{137}Cesium (University of Edinburgh, U.K.). These irradiated cells were resuspended at $2x10^6$ cells/ml in RPMI-10 and $1x10^5$ added to each well to a final volume of 200μl RPMI-10. Plates were incubated for 48 hours at 37°C in 5% CO_2, then supernatants were taken for analysis by ELISA.

2.6.3 Re-stimulation of iTreg

48 well plates were coated with anti-CD3 and anti-CD28 (both 2μg/ml) diluted in PBS and incubated for ≥ two hours at 37°C. Plates were then washed three times in wash buffer. iTreg were purified as in 2.5.2 and resuspended in RPMI-10 at $1x10^6$ per ml, then $1x10^6$ cells were added per well. The following cytokines were added individually to triplicate wells, as described in chapter 5: IL-12 25ng/ml (R&D), IL-27 10ng/ml (R&D), IL-6 30ng/ml (Miltenyi), IL-23 30ng/ml (R&D), IL-1β 10ng/ml (R&D), IFN-γ 100ng/ml (BD), anti-IFN-γ 10μg/ml (clone XMG1.2, Bioxcell), TGF-β 10ng/ml (R&D), IL-2 100U/ml. Cells were cultured for 72 hours at 37°C in 5% CO_2 and subsequently analysed by flow cytometry (section 2.8).

2.6.4 Suppression assays

iTreg were purified as in 2.5.2 and resuspended at $4x10^5$ cells/ml in RPMI-10 then $2x10^4$ cells were added to several wells of a round-bottomed 96 well plate. iTreg were then diluted twofold along the plate in RPMI-10. Naïve $CD4^+$ cells were prepared from Tg4 CD90.1, 2D2 CD90.1, or OT-II CD45.1 mice as described (2.4.1-4, 2.4.6). These naïve $CD4^+$ cells were resuspended in RPMI-10 at $4x10^5$ cells/ml then added to each well at a final concentration of $2x10^4$ cells per well. To provide APC, $CD4^-$ cells from magnetic separations (2.4.2) or whole splenocyte populations from C57BL/6xB10.PL (for experiments assessing bystander suppression) were irradiated as in 2.6.2. Irradiated cells were resuspended at $4x10^6$/ml in RPMI-10 and $2x10^5$ cells added to each well of the round-bottomed 96 well plate. For experiments investigating antibody blockade of MHC, irradiated APC were incubated with 10μg/ml of anti-I-A^u (clone OX-6, BD), or isotype (IgG_1, κ), for three hours at 37°C and washed twice in wash buffer before being added to co-culture.

Cells were stimulated with 10μM MBP Ac1-9 (4Lys), 10μM $pMOG_{35-55}$, or 8μM $pOVA_{323-339}$ as indicated. As controls, naïve $CD4^+$ T cells were cultured in the absence of iTreg, iTreg cells were cultured in the absence of naïve $CD4^+$ T cells, and both naïve and iTreg cells were co-cultured without the addition of antigen. All experiments were performed with triplicate wells.

Plates were incubated at 37°C in 5% CO_2. After 48 hours of culture, supernatants from replicate plates were taken for subsequent analysis by enzyme-linked immunosorbent assays (ELISA) for production of IL-2 and GM-CSF. Similarly, after 72 hours of culture, supernatants from replicate plates were taken for subsequent analysis by ELISA for

production of GM-CSF, IFN-γ, and TNF. After 80 hours of culture (unless otherwise specifically indicated), 0.5μCi of [^{3}H] thymidine ribose (Amersham Biosciences, Amersham, U.K.) was added to each well. Sixteen hours later, plates were harvested and incorporation of ^{3}H-thymidine was measured using a β-scintillation counter (Wallac, Turku, Finland).

2.6.4.1 Additions to suppression assays

Additional cytokines and antibodies were added in various experiments at the onset of the assay culture, unless otherwise specifically indicated in the text. The following were used:

- Rat Anti-mouse GM-CSF (clone MP1-22E9, BD Pharmingen) at 10μg/ml.
- Recombinant GM-CSF (BD Pharmingen) at 0,5, 10, 25, 50 ng/ml.
- Rat Anti-mouse TNF (clone G281-2626, BD Pharmingen) at 10μg/ml.
- Rat Anti-mouse IL-10 (clone JES5-2A5, BD Pharmingen) at 10μg/ml.
- Recombinant IFN-γ (BD) at 100ng/ml
- Mouse Anti-IFNγ (clone XMG1.2, Bioxcell, West Lebanon, NH) at 10μg/ml.
- Diphtheria toxin (Sigma) at 300ng/ml.
- SB431542 (Sigma) at 10μM.

2.6.4.2 Quantification of suppression

The efficacy of suppression of ^{3}H-thymidine by iTreg was quantified using the calculation below. The reduction in IL-2 concentration was similarly quantified, using '[IL-2] pg/ml' in place of 'mean cpm'.

$$100*\frac{\text{Mean cpm of naïve cells alone} - (\text{Mean cpm of naïve cells} + \text{iTreg at indicated ratio})}{\text{Mean cpm of naïve cells alone}}$$

2.6.5 T cell polarisations

Single cell suspensions were isolated from Tg4 CD90.1 mice as in 2.4.1. Cells were resuspended in RPMI-10 at $4x10^6$ per ml and cultured in six or 24 well plates in the presence of MBP Ac1-9 (4Lys) 10μg/ml. Cells were polarised towards a Th1 phenotype by the addition of 25ng/ml rIL-12 (R&D), 25ng/ml rIL-18 (MBL, Nagayo, Japan), and 10U/ml rIL-2. After 48 hours, the concentration of rIL-2 was increased to 20U/ml for the final 24 hours of culture. For polarisation towards the Th2 phenotype, cells were cultured in the presence of 4ng/ml rIL-4 (Peprotech), 5μg/ml anti-IL-12 (clone C17.8, BioXcell), 5μg/ml anti-IFN-γ (clone XMG1.2, BioXcell), and 40U/ml IL-2. Th2 cells were cultured for a total of 4-5

days, with all cytokines and antibodies being replenished at day three. Cells were polarised to a Th17 phenotype by the addition of 20ng/ml rIL-6 (Miltenyi), 10ng/ml rIL-1β (R&D), 20ng/ml rIL-23 (R&D), and 3ng/ml rhTGF-β1 (R&D) being cultured for three days in total. Finally 'ThGM-CSF' polarisation was achieved with the addition of 10μg/ml anti-IL-12, 10μg/ml anti-IFN-γ, and 3μg/ml soluble anti-CD28 (clone 37.51 eBioscience) for three days of culture. In all cases, cultures were incubated at 37°C in 5% CO_2.

The polarisation of T cells was assessed by detection of cytokine production using flow cytometric analysis and ELISA of supernatant. For subsequent use in *in vitro* assays, polarised cells were purified for $CD4^+$ expression as described in 2.4.2.

2.7 *In vivo* manipulations

2.7.1 Immunisations

Mice were immunized with 10μg MBP Ac1-9 (4Tyr) emulsified in complete Freund's adjuvant (CFA), containing 50μg heat-killed *Mycobacterium tuberculosis* H37Ra, (Sigma) at a final volume of 100μl. 50μl was injected subcutaneously (s.c.) into each hind leg. Where indicated, MBP Ac1-9 (4Tyr) was resuspended in PBS rather than CFA, as a control lacking pro-inflammatory signals.

2.7.2 Cell transfer

Cells were polarised towards the Th2 phenotype (2.6.5) or towards iTreg (2.5.1). Polarised or naïve cells were sorted for $CD4^+$ as in 2.4.2, iTreg were purified as in 2.5.2. Cells were washed three times in serum free PBS (PAA) and resuspended in sterile PBS at stated concentrations. Cells were then transferred intravenously (i.v.) via the tail vein in a total volume of 200μl PBS per mouse.

2.7.3 Intratracheal airway challenge

Mice were anaesthetised with intraperitoneal (i.p.) medetomidine hydrochloride 1μg per gram body weight (Pfizer Ltd, Surrey, U.K.) and ketamine (Fort Dodge Animal Health Ltd, Hampshire, U.K.) 75μg/g body weight. Mice were supported by a frame with the mouth held open. Visualisation of the glottis and vocal cords was aided using a cold lamp near the neck. Antigen (50μg OVA and/or 50μg recombinant MOG) was introduced directly into the trachea via a blunt needle attached to a syringe. Reversal of anaesthesia was achieved with

atipamezole hydrochloride (Pfizer Ltd, Surrey, UK) 20µg/g body weight. All airway challenges were performed by Dr Karen Mackenzie or Dominika Nowakowska.

2.8 Flow cytometry

2.8.1 Antibodies

Antibodies used were from eBioscience unless otherwise indicated. Table 2.1 provides a summary of antibody clones, conjugates, and concentrations used.

2.8.2 Live/dead staining

Cells were washed twice in serum free PBS and resuspended in 500µl PBS per 10^7 cells containing fixable viability dye (efluor780, eBioscience) at 1µl/ml. Cells were incubated for 30 minutes at 4°C in the dark and then washed twice in FACS buffer.

2.8.3 Surface staining

Cell samples containing 1-10x10^6 cells were washed in FACS buffer and resuspended in 25µl FACS buffer containing the relevant antibody cocktail. Cells were incubated for 10-15 minutes at 4°C in the dark and subsequently washed in FACS buffer. Cells were resuspended in 300µl FACS buffer for immediate analysis, or in 300µl 1% paraformaldehyde (PFA, Sigma) for storage at 4°C until analysis was possible.

2.8.4 Intracellular cytokine staining (ICCS)

For the final four hours of culture, cells were incubated at 37°C in the presence of 50ng/ml phorbol 12-myristate 13-acetate (PMA), 1µg/ml ionomycin (Sigma), and 3µg/ml brefeldin A (eBioscience). Cells were washed in FACS buffer and live/dead staining and surface staining carried out as above (2.8.2, 2.83). After surface staining, cells were resuspended in 250µl Fixation/Permeabilisation Solution (BD) per sample and incubated at 4°C for 20 minutes. Samples were washed twice in Perm/Wash buffer (BD, diluted 1:10 with distilled water) and resuspended in 50µl Perm/Wash buffer containing the appropriate anti-cytokine antibody cocktail, or isotype controls. Staining of transcription factors (i.e. Foxp3) was also conducted simultaneously where indicated. Samples were incubated with the antibody mix for 30 minutes at 4°C in the dark then washed twice in Perm/Wash buffer. Samples were resuspended in 300µl FACS buffer and stored at 4°C until they could be acquired as soon as practical.

2.8.5 Transcription factor staining in the absence of ICCS

Cells were washed in FACS buffer and surface stained as per 2.8.3. Samples were resuspended in 400µl Fixation/Permeabilisation Buffer (eBioscience) and incubated overnight at 4°C in the dark. Cells were washed twice in Permeabilisation Buffer (diluted 1:10 with distilled water, eBioscience) then resuspended in 50µl Permeabilisation Buffer containing anti-transcription factor antibodies or appropriate isotype controls. Samples were incubated for 30 minutes at 4°C in the dark, and washed twice in Permeabilisation Buffer. Cells were resuspended in 300µl 2% PFA and stored at 4°C until acquisition. There was no difference in the proportion of cells staining $Foxp3^+$ when the BD and eBioscience protocols were compared on identical samples [data not shown].

2.9 Flow cytometric data analysis

Samples were acquired using an LSR Fortessa II (BD Biosciences). Gates for ICCS and transcription factor staining were determined using isotype controls. Gating strategies used are illustrated in the relevant results. Following acquisition, analysis was performed using FlowJo software (Treestar version 3.2.1).

2.10 Cytokine quantification by enzyme-linked immunosorbent assay

The presence of IL-2, IL-17, IL-5, IL-13 and IFN-γ in the supernatant of cultures was determined using an enzyme-linked immunosorbent assay (ELISA). Maxisorp microtiter plates (Nunc International, NY) were coated with 50µl of the appropriate cytokine-capture antibody in bicarbonate buffer and incubated overnight at 4°C. Antibodies used are listed in Table 2.2 and ELISA reagents are listed in Table 2.3. Plates were washed twice in PBS-0.1% Tween (Sigma) (PBS-T) then 200µl of 1% bovine serum albumin (BSA, Sigma) in PBS (1% BSA/PBS) was added to each well and incubated for one hour at 37°C. Plates were washed four times in PBS-T then a doubling dilution of cytokine standards was set up at the top of each plate. 100µl of supernatants from the relevant cultures were added in duplicate or triplicate, and incubated at room temperature for two hours. Plates were washed six times in PBS-T and 100µl of the relevant biotinylated detection antibody (table 2.2) was added to each well and incubated for a further hour at room temperature. Plates were washed eight times in PBS-T and100µl of extravadin peroxidase (Sigma) diluted 1:1000 in

1% BSA/PBS was added to each well followed by 30 minutes incubation at room temperature. After a final eight washes with PBS-T, the ELISA was developed by adding 100µl of TMB solution (table 2.3) per well for 5-15 minutes then 100µl 2M H_2SO_4 was added to terminate the reaction. Plates were read at 450nM using an 'Anthos HT' plate reader and Stingray (v1.5, Dazdaq Ltd.).

The presence of GM-CSF and TNF in supernatants was quantified using ELISA kits (Ready-SET-Go! ELISA kits, eBioscience), performed according to the manufacturer's instructions. Maxisorp plates were used with the supplied reagents.

2.11 Assessment of disease following allergic airways inflammation (kindly performed by Dr Karen Mackenzie and Dominika Nowakowska)

2.11.1 Bronchoalveolar lavage (BAL)

Mice were culled and the trachea exposed. The lungs were lavaged with 1ml sterile PBS via a tracheal cannula. BAL cells were counted and cytospins prepared with a Shandon Cytospin 3 centrifuge at 300rpm for three minutes. Slides were left to air dry for 60 minutes then fixed in 100% methanol for 20 minutes. Slides were stained with Quick-Diff red stain for 90 seconds followed by Quick-Diff blue stain for 15 seconds (both Gamidor Technical Services, U.K.). Slides were rinsed in non-sterile water and air dried. Differential cell counts were performed by light microscopy by researchers blinded to the experimental conditions. Three hundred cells were counted per cytospin.

2.11.2 Isolation of cells from lung

Following perfusion, the left lung was removed and placed into sterile PBS. The tissue was finely chopped and incubated in collagenase (Type I-AS Sigma) solution (0.23mg/ml collagenase) for 45-60 minutes at 37°C. Tissue was flushed through a 20G needle to prepare a single cell suspension and washed twice in PBS by centrifugation at 300g for five minutes. RBC lysis was performed as in 2.4.1 with a further wash in PBS following this. Cells were then resuspended in PBS and filtered through a 40µM strainer (BD) prior to counting.

2.12 Statistical analyses

Data was analysed using GraphPad Prism (v.4.0a for Macintosh). For comparisons between replicate wells in suppression assays, a two-tailed unpaired t test with Welch's correction was used. For comparisons between groups of mice in *in vivo* experiments, data were treated as non-parametric non-normally distributed and analysed using Mann-Whitney U tests. The Kruskal-Wallis test with Dunn's multiple comparison post-test was used when comparing three or more groups. Statistical advice was provided by Dr Margo Chase-Topping, who kindly performed a meta-analysis of the iTreg cytokine production data from the *in vivo* experiments. $P<0.05$ was considered significant for all tests.

Table 1: Antibodies used in flow cytometry

Antibody	Conjugate	Clone	Dilution	Concentration
CD4	-eF450 -AF700 (BD)	RM4-5	1:100 1:100	0.5mg/ml 0.2mg/ml
CD44	-FITC	IM7	1:100	0.5mg/ml
CD62L	-PE	Mel-14	1:100	0.5mg/ml
CD25	PE PerCPCy5.5 (Biolegend)	PC61.5 3C7	1:100 1:100	0.2mg/ml 0.2mg/ml
CD90.1	-APC -FITC	HIS51	1:100	0.2mg/ml
CD45.1	-PerCPCy5.5	A20	1:100	0.2mg/ml
CD126	-PE	D7715A7	1:200	0.2mg/ml
gp130	-APC	KGP130	1:200	0.2mg/ml
Foxp3	-eF450 -FITC -APC	FJK-16s	1:100	0.2mg/ml
IFN-γ	-FITC -APC	XMG1.2	1:100	0.2mg/ml
GM-CSF	-PE (BD)	MP1-22E9	1:25	0.2mg/ml
TNF	-eF450	MP6-XT22	1:100	0.2mg/ml
IL-17	-PercpCy5.5	eBio17B7	1:100	0.2mg/ml
IL-5	-PE (BD)	TRFK5	1:100	0.2mg/ml
IL-13	-AF647	eBio13A	1:100	0.2mg/ml
CD11b	-eF450	M1/70	1:100	0.2mg/ml
Vα3.2	-FITC (BD)	RR3-16	1:100	0.5mg/ml
CD3	-PerCPCy5.5	145-2C11	1:200	0.2mg/ml
IgG1 isotype	-FITC -eF450 -APC -PE -AF647 (BD)	eBRG1 R3-34	1:100 1:100	0.2mg/ml 0.2mg/ml
IgG2a isotype	-PE (BD) -eF450 -APC -FITC -PerCPCy5.5 (Biolegend)	R35-95 eBR2a eBM2a MOPC-173	1:100 1:100 1:100 1:100	0.2mg/ml 0.2mg/ml 0.2mg/ml 0.2mg/ml
IgG2b isotype	-APC	eB149/10H5	1:100	0.2mg/ml

All antibodies manufactured by ebioscience, unless otherwise indicated.

Table 2: Antibodies used in ELISAs

Cytokine	Capture antibody		Maximum concentration	Detection antibody	
	Concentration	Clone		Concentration	Clone
IL-2	2µg/ml	JES6-1A12	5ng/ml	0.5µg/ml	JES6-5H4
IL-4	2µg/ml	11B11	5ng/ml	0.5µg/ml	BVD6-24G2
IL-13	1µg/ml	eBio13A	10ng/ml	5µg/ml	eBio1316H
IL-17	2µg/ml	TC11-18H10	10ng/ml	0.25µg/ml	TC11-8H4.1
IFN-γ	0.5µg/ml	R4-6A2	100ng/ml	0.5µg/ml	XMG1.2

Table 3: – Reagents used in ELISAs

10x bicarbonate buffer	6.36g Na_2CO_3 11.72g $NaHCO_3$ 400ml distilled H_2O pH 9.6
Phosphate citrate buffer	25.7 ml 0.2M Na_2HPO_4 24.3 ml 0.1M citrate (anhydrous) Made up to 100ml with dH_2) pH 5.0
TMB solution	100µl TMB 9.9ml phosphate citrate buffer 3µl H_2O_2

3 *In vitro* bystander suppression by iTreg

3.1 Introduction

Early in the rediscovery of the regulatory T cell populations, *in vitro* experiments demonstrated that nTreg ($CD4^+$ $CD25^+$ T cells) were able to effect suppression of responding cells in a non-antigen specific manner, once stimulated by their antigen (Thornton et al., 2000). Bystander suppression is thus a feature of nTreg function. The first demonstration of this *in vitro* suppressive effect used a simple co-culture assay, with a diluting effect seen when nTreg were added at lower ratios to responding cells (Thornton et al., 1998). The initial conclusions were that nTreg suppressed via a contact-dependent APC-independent manner but the outcome was antigen non-specific. Subsequent experiments used expanded nTreg from a TCR transgenic mouse (specific for an immunogenic epitope of myelin proteolipid protein, $PLP_{139-151}$) in an *in vivo* EAE model (Yu et al., 2005). Those expanded nTreg were able to suppress disease caused by a CNS homogenate (containing a mix of self antigens) and disease caused by immunization with $PLP_{139-151}$ which nTreg responded to. EAE induced by other antigens was not suppressed by the nTreg, unless they had been pre-activated *in vitro* by $PLP_{139-151}$. This suggested linked but not bystander suppression.

Linked suppression may also refer to a proposed mechanism of suppression in which both the Treg and effector T cell are directly interacting with the same APC. iTreg-APC interaction then influences the signaling between APC and the effector cell (Frasca et al., 1997). This was demonstrated in a model of GVH disease in mice, using OVA-specific iTreg to effectively suppress GVHD caused by polyclonal effectors in OVA^+ recipients, whilst in OVA^- recipients, no suppressive effect was seen (Semple et al., 2011).

In contrast to the EAE model reported above, a different group expanded myelin-responsive Tg4 nTreg and demonstrated that administration of nTreg at the time of initiation of EAE with the antigen $PLP_{139-151}$ led to a less severe and briefer disease course, though the initial onset of disease was not affected (Stephens et al., 2009). Further evidence for the bystander suppressive effect of nTreg has emerged in various models (Homann et al., 1999, Jun et al., 2012).

Given the multiple differences between nTreg and TGF-β induced Treg (iTreg) (Horwitz et al., 2008), and the relative ease of generating iTreg for therapeutic use, determining whether they also exhibit bystander suppression is of great interest.

A recent study of iTreg failed to demonstrate bystander suppression in the EAE model using immunogenic epitopes associated with the induction of EAE (Zhang et al., 2010). $PLP_{139-151}$ specific iTreg were able to suppress EAE and delayed-type hypersensitivity induced with $PLP_{139-151}$, but had no impact upon responsiveness to unrelated antigens (MOG or ovalbumin epitopes), nor even upon response to a distinct epitope within the same protein ($PLP_{178-191}$).

3.1.1 Experimental aims

The experiments described in this chapter were designed to determine whether iTreg generated from one TCR transgenic mouse were able to suppress effector cells from a different TCR transgenic i.e. to test the ability of iTreg to effect bystander suppression. Subsequent experiments aimed to identify essential requirements for bystander suppression to occur *in vitro*.

3.1.2 Experimental approach

The ability of iTreg to suppress naïve T cells that respond to a different antigen was investigated using TCR transgenic mice. The Tg4 strain has a TCR that recognizes the nonameric immunodominant epitope MBPAc1-9 and is restricted to $I\text{-}A^u$. The 2D2 strain has a TCR repertoire limited mainly to $pMOG_{35-55}$ and is on the C57BL/6 background restricted to $I\text{-}A^b$. F_1 mice from B10.PL crossed with C57BL/6 were used as antigen presenting cells, being able to present both A^u and A^b peptide complexes. Thus, Tg4 iTreg could be generated and co-cultured with naïve 2D2 cells in the presence of F_1 APC, or *vice versa* 2D2 iTreg co-cultured with naïve Tg4 cells.

3.2 Results

3.2.1 Cross-reactivity does not occur between peptides

To test bystander suppression, both peptides needed to be added to co-culture. It was therefore necessary to ensure that cross-reactivity did not occur, nor was there an 'interfering' element whereby the presence of the alternative peptide would somehow limit presentation to the responding naïve cells. Culture of naïve 2D2 $CD4^+$ cells with F_1 APC and either $pMOG_{35-55,}$ MBPAc1-9, or both antigens, was performed and incorporation of ^{3}H-thymidine measured at 72 hours (Fig 3.1A). Naïve 2D2 $CD4^+$ cells responded robustly to $pMOG_{35-55}$ and this response was unaffected by the presence of MBPAc1-9 (Fig 3.1B). No response was seen by 2D2 $CD4^+$ cells to MBPAc1-9 alone. Naïve Tg4 $CD4^+$ cells were cultured in the same manner (Fig 3.1C). Similarly, incorporation of ^{3}H-thymidine by Tg4 $CD4^+$ cells was unaffected by the presence of $pMOG_{35-55}$, nor did the Tg4 cells respond to $pMOG_{35-55}$ alone (Fig 3.1D). Thus, there was no cross-reactivity by transgenic TCR cells to the alternative peptide used in bystander suppression assays, nor was there any influence of the alternate peptide on presentation of the cognate antigen.

3.2.2 Generation of iTreg

iTreg were generated from TCR transgenic mice as described in Materials and Methods (Davidson et al., 2007). Where mice expressing a Foxp3-gfp reporter were available, $CD4^+$ cells were isolated by autoMACS then further sorted using flow cytometry for the absence of gfp expression (Fig 3.2A,B). After five days culture with plate-bound anti-CD3 and anti-CD28 (both at 2μg/ml) in the presence of 100U/ml IL-2 and 5ng/ml TGF-β, cells were further sorted for the presence of Foxp3 by gfp expression (Fig 3.2C). Where mice lacked a Foxp3 reporter, $CD4^+$ cells were initially sorted by autoMACS using negative selection, followed by further magnetic positive selection for $CD62L^{high}$ (Fig 3.2D). Representative flow cytometry plots are shown (Fig 3.2E). At the end of the iTreg culture in conditions identical to those above, these iTreg were immediately used in experiments without further sorting. Induction of Foxp3 was consistently greater than 85% of live cells (Fig 3.2F).

3.2.3 The generated iTreg are suppressive *in vitro*

The conventional assay first designed by Shevachs' group (Thornton et al., 1998) entails co-culture of iTreg at varying ratios with a fixed number of naïve $CD4^+$ T cells, in the presence of irradiated splenocytes and soluble anti-CD3. This demonstrated suppression of incorporation of ^{3}H-thymidine at high ratios of Treg:naïve T cells, but loss of the suppressive effect as iTreg were diluted out. In order to investigate bystander suppression, the ability to stimulate the naïve T cells without further stimulating the iTreg was crucial. Thus, assays were performed using irradiated APC and peptide rather than anti-CD3 (Fig 3.3A). Similarly to the pattern seen in other reports, incorporation of ^{3}H-thymidine at 96 hours showed a suppressive effect at high ratios of iTreg:naïve T cells with a gradient of effect as numbers of iTreg diminished (Fig 3.3B). The suppressive 'capacity' of iTreg can then be demonstrated, as calculated in Materials and Methods (Section 2.6.4.2) (Fig 3.3C) (Collison et al., 2011).

Assuming that greater than 50% suppression represents a substantial impact, it can be seen in the experiment represented that iTreg were effective down to ratio of 1:8 with loss of suppressive capacity beyond this (Fig 3.3C).

The assay is very sensitive and can be performed with a small number of cells though problems with it have been highlighted (Collison et al., 2011). One such issue is the insensitivity of ^{3}H-thymidine and high degree of variability, even between replicate wells. A superior alternative would be CFSE staining of naïve T cells but due to the high experimental turnover, this was not feasible. T cell activation leads to both clonal expansion and production of IL-2. Whilst incorporation of ^{3}H-thymidine gives an indirect estimate of expansion, IL-2 can be measured by ELISA analysis of assay supernatants. IL-2 production was assayed at 48 hours of culture and demonstrated a similar pattern of effect to ^{3}H-thymidine incorporation (Fig 3.3D). An equivalent calculation of the reduction in IL-2 was also performed (Fig 3.3E). It is notable that the reduction in IL-2 was often more pronounced than the effect on ^{3}H-thymidine incorporation, with suppressive effects maintained to lower ratios of Treg:naïve T cells. Whether IL-2 production was arrested in the naïve T cells, or if IL-2 produced was rapidly scavenged and used by the iTreg cannot be determined from this experimental set-up.

3.2.4 Bystander Suppression by iTreg

3.2.4.1 iTreg can suppress the response of naïve cells to a different antigen

iTreg generated from 2D2 cells were cultured with naïve $CD4^+$-sorted Tg4 T cells and irradiated C57BL/6xB10.PL splenocytes (hereafter referred to as F_1 APC) with exogenously added MBPAc1-9 and $pMOG_{35-55}$, both at 10μM (Fig 3.4A). Proliferation was estimated by the incorporation of ^{3}H-thymidine at 96 hours. Naïve T cells cultured without iTreg had a robust response (Fig 3.4B) whilst iTreg cultured without naïve T cells had virtually no incorporation of ^{3}H-thymidine. Co-culture of both iTreg and naïve T cells in the absence of antigen (labeled as 'Unstim') also gave very little response. ^{3}H-thymidine incorporation by naïve T cells was suppressed in the presence of iTreg effectively (greater than 50% suppression) maintained down to a Treg:naïve T cell ratio of 1:8, similar to the effect seen with homologous suppression (Fig 3.4C). IL-2 was also similarly reduced in the presence of iTreg, with loss of the inhibitory effect seen at a similar ratio of Treg:naive (Fig 3.4D,E). The iTreg were able to suppress in a bystander fashion in this tightly controlled *in vitro* system. The ensuing experiments were designed to elucidate what features of the *in vitro* assay contributed to bystander suppression – APC interaction, the need for MHC, and both antigens.

3.2.4.2 Linked suppression

One postulated mechanism of iTreg action is that of linked suppression. Peptide is presented on the surface of APC such that a single APC may interact with both Treg and responding naïve T cells. It has been suggested (Walsh et al., 2004) that Treg may mediate their effects via the APC as it interacts with both Treg and naïve T cells i.e. the APC is acting as a conduit for suppressive signals between Treg and responding cells. This would explain the necessity for cell contact seen in Treg cultures.

This hypothesis can be tested relatively easily using the above suppression assay. Replacing F_1 APC with the $CD4^-$ fraction of the two strains (which have different MHC restrictions) meant it was not possible for the Treg to form a stable peptide-MHC complex with the APC stimulating the naïve T cells. Tg4 iTreg were co-cultured with the naïve $CD4^+$ 2D2 cells and either F_1 APC or a combination of the $CD4^-$ fractions of both Tg4 and 2D2 sorts. These fractions were mixed equally so there was not a preponderance of one MHC (Fig 3.5A). Comparing the ability of iTreg to suppress naïve T cells stimulated by either mixed APC or F_1 APC, suppression of ^{3}H-thymidine incorporation was seen in both circumstances (Fig 3.5B) and was no more readily suppressed in the cultures with F_1 APC than in mixed APC

cultures. The reduction in IL-2 also appeared equivalent between cultures. Thus, interaction between iTreg and naïve T cells on the same APC is not a requirement for suppression in this *in vitro* system.

3.2.4.3 Preventing iTreg interaction with APC

Whether iTreg need to interact with APC via the peptide-MHC complex to effect suppression can be tested using two approaches. Antibodies against I-A^u will prevent any stable interaction between the APC and a T cell (either iTreg or naïve). To confirm this effect, Tg4 $CD4^-$ cells used as APC were incubated for 3 hours in the presence of 10ng/ml anti-I-A^u. These cells were then washed to remove any excess antibody. Naïve Tg4 $CD4^+$ cells were cultured with 10μM MBPAc1-9 with either the treated or untreated APC. Incorporation of ^{3}H-thymidine by naïve T cells with antigen presented by 'blocked' APC was significantly reduced compared to unblocked APC (Fig 3.6A). 'Blocked' Tg4 $CD4^-$ cells were then mixed with the $CD4^-$ 2D2 cells in a bystander suppression assay as described above in the presence of both MBPAc1-9 and $pMOG_{35-55}$ (Fig 3.6B). Binding of I-A^u with antibody resulted in abolition of suppression of ^{3}H-thymidine incorporation (Fig 3.6C,D).

The second approach removed iTreg 'parental' APC completely. Tg4 iTreg were co-cultured with naïve 2D2 $CD4^+$ cells and $pMOG_{35-55}$ in the presence of irradiated $CD4^-$ 2D2 cells (Fig 3.7A). Suppression of the incorporation of ^{3}H-thymidine was again not evident at all in these cultures (Fig 3.7B), with no difference between naïve T cells alone or those cultured with iTreg.. Thus, some form of interaction with APC and the MHC restriction element is clearly required in this *in vitro* system.

3.2.4.4 iTreg can effect bystander suppression in the absence of cognate antigen

A defining feature of nTreg bystander suppression is that once activated, they are able to effectively suppress regardless of the surrounding antigenic stimuli (Thornton et al., 2000). As the generation of iTreg requires TCR stimulation via anti-CD3 and anti-CD28, these cells are inherently activated. Was the persistence of their antigen necessary for their suppressive effects?

2D2 iTreg were co-cultured with naïve Tg4 $CD4^+$ T cells with irradiated F_1 APC and 10μM MBPAc1-9 only (Fig 3.8A). Suppression of ^{3}H-thymidine incorporation was seen at high ratios of Treg:naïve T cells though it was generally less effective than in the presence of both antigens (Fig 3.8B, C). IL-2 production was similarly reduced almost equivalently in the

presence or absence of iTreg cognate antigen (Fig 3.8D,E). The IL-2 data suggest that in the absence of cognate antigen, iTreg are able to suppress, but less efficiently, with twice as many iTreg required to exert the same suppressive effect as when both antigens were present in co-culture.

The converse experiment was also performed. Tg4 iTreg were co-cultured with naïve 2D2 $CD4^+$ T cells with irradiated F_1 APC and either both antigens, or $pMOG_{35-55}$ alone (Fig 3.9A). In this experiment, suppression of ^{3}H-thymidine incorporation was maintained in the presence of both antigens down to low numbers of iTreg, still being evident at 1:64 (Fig 3.9B, C). In the presence of $pMOG_{35-55}$ alone, suppression was maintained down to a Treg:naïve T cell ratio of 1:8. Similarly, IL-2 production was inhibited at ratios of Treg:naïve of 1:16 (Fig 3.9D, E), though the overall production of IL-2 from the naïve 2D2 T cells was much lower than from Tg4 cells (Fig 3.8D). Whether the quantity of IL-2 produced represents a property of the transgenic cells used is unclear.

These experiments demonstrated that, similar to the situation described for nTreg, iTreg were able to suppress ^{3}H-thymidine incorporation, possibly via effects on IL-2, in the absence of ongoing stimulatory signals through their TCR. This suppressive effect can be seen even at low proportions of iTreg, in some experiments being evident even when iTreg form less than 2% of the *in vitro* population.

3.2.4.5 Stronger peptide-MHC interactions cannot overcome suppression by iTreg

If the strength of the peptide-MHC interaction is relevant, this is open to investigation using the altered peptide ligand, MBPAc1-9 (4Tyr). The MBPAc1-9 (4Lys)–A^u complex is notable for a very poor binding affinity. Though the complex is formed, it is very weak and rapidly dissociates (Mason et al., 1995). It is a curiosity that such a poorly bound peptide is able to stimulate disease in *in vivo* systems (Anderton et al., 2001). Alteration of the fourth residue from lysine to tyrosine increases the binding affinity such that a highly stable peptide-MHC complex is formed. Returning to the simple 'homologous' suppression assay and culturing Tg4 iTreg with naïve Tg4 $CD4^+$ T cells, co-culture with soluble MBPAc1-9(4Tyr) (Fig 3.10A) led to greater ^{3}H-thymidine incorporation by the naïve T cells but the efficacy of iTreg in suppressing this was equivalent (Fig 3.10B,C). Furthermore examining IL-2 production, use of MBPAc1-9 (4Tyr) greatly increased IL-2 production by naïve T cells (Fig 3.10D). The proportionate reduction in IL-2 was maintained similarly between cultures stimulated with MBPAc1-9 (4Lys) or (4Tyr) (Fig 3.10E). This implies that the magnitude of

the IL-2 production by naïve T cells does not affect the ability of the iTreg to effect suppression. Importantly, this suggests that iTreg do not simply act by consuming all IL-2 in the culture, as it might then be expected that in the presence of 5000pg/ml of IL-2, suppression by this mechanism alone would be broken. For comparison, iTreg are generated in cultures containing 400pg/ml IL-2, an order of magnitude lower than in this experiment. Thus, the affinity of MBPAc1-9 for the MHC does not affect suppression. This contrasts with previous reports suggesting a quantitative effect of nTreg with peptide concentration (Stephens et al., 2005) but may be related to differences between nTreg and iTreg.

A curiosity of the cells stimulated by MBPAc1-9 (4Tyr) is the production by the iTreg group alone of a small but definite quantity of IL-2. The iTreg were sorted for Foxp3gfp expression at the end of primary culture, with a purity of >98%. Thus the number of contaminating non-Foxp3$^+$ cells within the wells would be fewer than 400 cells. This suggests that iTreg may be capable of IL-2 production themselves, possibly related to the strength of antigenic stimulus.

3.2.5 Kinetics of Suppression

The exact nature of suppressive mechanisms used by iTreg remains elusive. It is likely that both nTreg and iTreg use a number of mechanisms with multiple compensatory pathways. One clue to the mechanisms involved may be in the kinetics of the response. An interaction between iTreg and naïve T cells occurring within several hours of cell culture might suggest direct cell-mediated cytotoxicity for example, whereas if the suppressive effect was only seen after 2-3 days this might imply transcriptional/translational changes in at least one cell type within the co-culture. As the following experiments concern fundamental features of iTreg suppression, all experiments were conducted with a 'homologous' *in vitro* suppression system i.e. Tg4 iTreg suppressing naïve Tg4 CD4$^+$ T cells.

3.2.5.1 Assessment of proliferation by ^{3}H-thymidine

To assess the kinetics of suppression, highly pure Tg4xFoxp3LuciDTR-4 iTreg were generated and sorted for Foxp3gfp expression, then co-cultured with naïve Tg4 CD4$^+$ T cells with the irradiated CD4$^-$ fraction in the presence of 10μM MBPAc1-9 (4Lys). ^{3}H-thymidine was then added to cultures at the onset, or after 24, 48, or 72 hours. Plates were harvested 16-18 hours following the addition of ^{3}H-thymidine.

The ongoing activation status of iTreg was clearly evident in this experiment as demonstrated by their high incorporation of ^{3}H-thymidine within 24 hours of culture, including the cells cultured in the absence of antigen (Fig 3.11A). The incorporation of ^{3}H-thymidine by iTreg diminished over time with the lowest counts recorded at 96 hours. This may be related to death of the iTreg prior to harvesting of the cultures, though this was not ascertained from this experiment.

The naïve T cells alone showed a predictable course of increasing ^{3}H-thymidine incorporation from a low level at 24 hours of culture to a maximum at 96 hours, with the caveat that cultures were not extended beyond this point. Thus much of the ^{3}H-thymidine incorporation in the first 72 hours in the co-culture wells can be readily attributed to either iTreg or naïve T cells. The high counts of ^{3}H-thymidine incorporation at 1:1 (and lesser ratios) after 72 hours of culture suggests both cell populations are proliferating together. The final timepoint of 96 hours shows a dramatic reversal in the incorporation of ^{3}H-thymidine by naïve T cells in the presence of iTreg with the previously seen suppressive picture emerging. Analysis of the kinetics at different ratios of Treg:naïve T cells in parallel showed a clear divergence at higher iTreg proportions occurred between 72 and 96 hours (Fig 3.11B).

This represents one of the major disadvantages of this *in vitro* system, in that a reduction in the incorporation of ^{3}H-thymidine when iTreg and responding T cells were co-cultured may reflect either reduced proliferation of responding T cells, or increased cell death, potentially of both cell populations. Distinguishing between these is not possible using ^{3}H-thymidine alone.

This showed that the reduction in ^{3}H-thymidine incorporation by naïve T cells appeared to occur late in the *in vitro* cultures. This could be attributable to consumption of cell survival factors within the medium being used up by the iTreg simply as a function of being activated cells. To ensure suppression was indeed a true effect, naïve Tg4 CD4^{+} T cells were cultured for five days in the presence of anti-CD3 and anti-CD28, then used in co-culture with fresh naïve Tg4 CD4^{+} T cells in a suppression assay. Again, incorporation of ^{3}H-thymidine was assessed at 24, 48, 72, and 96 hours. The pattern of incorporation was very similar for the first 72 hours of co-culture, but no suppression of naïve T cell incorporation of ^{3}H-thymidine was seen at the 96 hour time point (Fig 3.11C). Thus, iTreg may have a specific suppressive function, rather than simply consuming cell growth factors in the medium. However, an alternative explanation may be that activated cells are simply more robust than iTreg, and that the ^{3}H-thymidine incorporation seen at 1:1 ratios represents continued incorporation by

the activated cells as well as the responding cells. If this were the case, the 'suppression' seen with iTreg would indeed simply represent consumption of essential growth factos early in co-culture, leading to cell death at 96 hours of both cell types.

In summary, early in this *in vitro* system, proliferation by naïve T cells accelerated over time (as estimated by ^{3}H-thymidine incorporation) as might be expected (though there was some impact of co-culture at the earlier points). Thus, the impact of iTreg suppression was not seen until a late time point in this *in vitro* system, nor was it simply a function of iTreg being activated cells, though clarification of the viability of iTreg versus activated cells would be necessary to consolidate this finding.

3.2.5.2 Assessment by CFSE dilution

That incorporation of ^{3}H-thymidine by the naïve T cells was not immediately inhibited can also be shown through the use of cell labeling. Naïve Tg4 CD4^{+} T cells were labeled with CFSE and co-cultured with iTreg in the same *in vitro* system as above. At 72 hours, some effect of iTreg was evident, though most cells appeared to have diluted CFSE (Fig 3.12A). At 96 hours, virtually all CFSE-labeling had been lost in all stimulated conditions except at 1:1 (Fig 3.12B), implying that suppression of proliferation is not an early effect (correlating with the ^{3}H-thymidine data shown above). This contrasts with much literature reporting inhibition of CFSE dilution (and hence proliferation) in the presence of Treg. One important distinction was that only live CD4^{+} cells were assessed for CFSE dilution here. If all CD4^{+} cells were then analysed, a stronger peak of CFSE was seen in all conditions (only a single example is shown for clarity, Fig 3.12C).

If proliferation is occurring early in culture, and suppression is not seen until after 4 days, do iTreg need this long to exert their effects?

3.2.6 Depletion of iTreg

The low incorporation of ^{3}H-thymidine by the iTreg after 96 hours of culture might imply that a large proportion of iTreg had died at the point when suppression was assessed. This was confirmed by viability staining of iTreg after 72 hours of re-stimulation (discussed further in Chapter 4). What then would be the effect of removing Treg earlier in this culture system? This can be achieved taking advantage of the Tg4xFoxp3LuciDTR-4 transgenic in which expression of the diphtheria toxin receptor (DTR) is under the Foxp3 promoter (Suffner et al., 2010, O'Connor et al., 2010).

3.2.6.1 Diphtheria toxin can deplete iTreg *in vitro*

Validation of the ability of diphtheria toxin (DTX) to remove iTreg *in vitro* was performed. Tg4xFoxp3LuciDTR-4 iTreg were generated as previously with a five day culture in the presence of IL-2 and TGF-β, then sorted for Foxp3gfp expression and re-cultured in the presence of DTX at increasing concentrations over three days. This demonstrated that iTreg could be effectively depleted to negligible numbers of cells within 24 hours at an *in vitro* DTX concentration of 300ng/ml (Fig 3.13A,B). This provided a useful adjunct to this *in vitro* system, in which iTreg can be co-cultured with naïve cells and then removed from the culture without impacting on the naïve cell/APC interactions.

3.2.6.2 Depletion of iTreg in suppression

The need for cell contact has been demonstrated previously using transwell inserts in various systems (Kong et al., 2012, Gondek et al., 2005), but whether this contact must be maintained is unknown. The ability to deplete iTreg within 24 hours of co-culture was used to determine whether persistence of the cells was necessary to exert their suppressive effect.

Sorted Tg4xFoxp3LuciDTR-4 iTreg were co-cultured with naïve Tg4 CD90.1 $CD4^+$ T cells (which do not express the diphtheria toxin receptor) with irradiated APC and 10μM MBPAc1-9 (4Lys). DTX was added at the onset of culture or after 48 hours. Plates were then pulsed with ^{3}H-thymidine at 80 hours of culture and harvested at 96 hours.

Depletion of iTreg at the onset of culture (i.e. addition of DTX at the beginning leading to complete depletion of iTreg within 24 hours) completely prevented suppression whilst depletion at the 48 hour time point did not affect the suppression of incorporation of ^{3}H-thymidine at all (Fig 3.13C). Repeating the experiment with addition of DTX at 24 hours also had minimal impact on the ability of iTreg to suppress. This implied that the effects of iTreg in this *in vitro* system were within the first 24-48 hours and that persistence of the iTreg thereafter was not required.

3.2.6.3 Diphtheria toxin is not intrinsically cytotoxic

Though unlikely, it was possible that DTX had a direct cytotoxic effect upon iTreg regardless of their receptor expression. To eliminate this possibility, iTreg were generated from either Tg4xFoxp3LuciDTR-4 mice or Tg4 CD45.1 mice. These iTreg were then co-cultured with naïve Tg4 CD90.1 $CD4^+$ T cells with DTX added at the onset of culture. As expected, depletion of the Tg4xFoxp3LuciDTR-4 iTreg led to the abrogation of suppression while the Tg4 CD45.1 iTreg were unaffected and suppressed normally (Fig 3.13D).

These experiments demonstrated that the physical presence of iTreg was required at the onset of co-culture, but that whatever effects are mediated do not require the ongoing persistence of iTreg. As addition of DTX at 24 hours maintained suppression, this effect must occur early in the *in vitro* system, even though the subsequent impact on naïve cells was not seen until the 96 hour time point.

3.3 Discussion

3.3.1 Generation of iTreg

The low number of nTreg occurring in the peripheral bloodstream and ongoing difficulties in identifying a truly 'pure' marker for human nTreg have complicated extension of Treg therapy into human trials, though newer protocols for nTreg expansion have led to recent advances in this area (Pahwa et al., 2010). The improved efficacy of antigen-specific Treg therapy over polyclonal therapy, now confirmed in several models (Tang et al., 2004, Stephens et al., 2009, Huter et al., 2008), combined with the much greater pool of 'potential' iTreg obtainable from a patient make iTreg an appealing therapeutic prospect. However, most mechanistic research into Treg has concentrated on nTreg, predominantly due to concerns over the plasticity of iTreg and potential to differentiate into effector cells (Zhou et al., 2009b).

Generation of iTreg is now long established as being a TGF-β dependent process (Chen et al., 2003) with high levels of IL-2 necessary to promote Treg proliferation. *In vitro* the combination of IL-2 and TGF-β leads to an efficient conversion of $CD4^+$ $CD25^-$ cells into $CD4^+$ $CD25^+$ $Foxp3^+$ cells. Within the gastro-intestinal tract, the conversion of cells into iTreg is enhanced by the presence of retinoic acid (RA), and this has also been reported to 'boost' conversion to iTreg *in vitro* (Mucida et al., 2009). Using the protocol established within the laboratory, conversion of naïve cells to $Foxp3^+$ iTreg was highly efficient with purities consistently of >85% and in some experiments greater than 95% $Foxp3^+$ prior to sorting (Figure 3.2). The exact specifics of iTreg generation have considerable variation in the concentrations of cytokines and strength of stimulation used across different reports. Notably, the high conversion here is greater than that seen by many other groups. The addition of retinoic acid had no further beneficial effect on the conversion rate to iTreg [data not shown]. The ability to sort on the basis of Foxp3gfp expression gave highly pure iTreg populations. In experiments where cells were sorted based on naïve markers alone, final Foxp3 expression at the end of the five day iTreg culture was always greater than 85%.

3.3.2 Bystander suppression *in vitro*

In this chapter, an *in vitro* system has been developed with cells derived from two different TCR transgenic mouse strains. The Tg4 T cells respond particularly robustly to MBPAc1-9, whilst 2D2 T cells respond to $pMOG_{35-55}$. That this holds true is seen in figure 3.1, in which

no incorporation of ^{3}H-thymidine was evident from naïve CD4^{+} T cells of either strain cultured in the presence of the alternative peptide (Fig 3.1B,D). Importantly, there was also no evidence of a mixed lymphocyte reaction in any of the *in vitro* cultures of Tg4 and 2D2 cells together.

Previous experiments by Dr Sarju Patel (Patel, 2008) demonstrated an inability of transferred 2D2 cells to persist in a F_1 mouse. This was presumed due to deletion of the 2D2 cells by the host immune system, potentially due to the absence of H-2^{u} encoded MHC molecules on the 2D2 cell surface. Attempts were made to cross the 2D2 strain onto the B10.PL background to then permit co-transfer of both 2D2 and Tg4 cells into a F_1 mouse, and allow a true test of *in vivo* bystander suppression using the EAE model (e.g. naïve Tg4 cells and 2D2 iTreg in EAE induced by MBPAc1-9). Crossing of the 2D2 strain with the B10.PL line led to the unexpected complication that cells inheriting the pMOG$_{35-55}$ TCR, identifiable as Vα3.2^{+} cells, were deleted at the double negative stage of T cell development in the thymus [data not shown]. In adult mice with a normal CD4^{+} complement, persistence of the pMOG$_{35-55}$ TCR was almost absent. In mice with a significant number of Vα3.2^{+} cells, a marked reduction in CD4^{+} count was apparent. As the purpose of these mice was to provide TCR transgenic cells for subsequent experiments after sufficient cross-breeding and the effect of the relative lymphopenia was unclear, these breeding efforts were abandoned. Thus 2D2 and Tg4 cells could not be used in the same *in vivo* host.

This chapter shows that iTreg are able to suppress proliferative responses and reduce the IL-2 content of co-cultures with naïve CD4^{+} T cells recognizing either the same antigen ('homologous' suppression) or a completely different antigen (bystander suppression). iTreg were able to effect bystander suppression *in vitro* in the presence or absence of their cognate antigen (Figs 3.4, 3.8, 3.9). This is consistent with the similar bystander effects seen using nTreg *in vitro*. That nTreg suppress in an antigen-non-specific manner following activation is well established *in vitro* (Thornton et al., 2000). As the iTreg receive TCR stimulation during their initial generation, they begin the co-culture in an activated state. The ability of both nTreg and iTreg to effect bystander suppression *in vivo* remains controversial.

A recent paper (Oh et al., 2012) reported that administration of TCR-transgenic nTreg with specificity for the PR8 haemagglutinin of influenza (HA) was unable to prevent spontaneous development of arthritis in an *in vivo* model whereby the host mouse normally develops inflammatory arthritis due to expression of the PR8 HA as a self antigen i.e. antigen-specific nTreg were unable to prevent disease development presumably initiated by that antigen. Polyclonal nTreg modulated disease onset, however, by preventing a Th17 response within

the relevant lymph nodes. The failure of the antigen-specific Treg was not related to their ability to suppress proliferation or IFN-γ production, nor was it differentiation to an effector phenotype, but rather an inability to suppress the non-clonotypic population of responding $CD4^+$ T cells magnifying the inflammatory response. In this model using nTreg, direct but not bystander suppression was observed.

In contrast, a different paper showed that nTreg expanded for antigen-specificity to $HA_{111-119}$ or $HA_{126-138}$ in the presence of $CD8^+$ dendritic cells were able to effectively suppress the development of diabetes in a novel transgenic mouse expressing HA under the control of the insulin promoter (Ins-HA mice)**.** Importantly, the $HA_{126-138}$ expanded nTreg were able to prevent disease onset from $HA_{111-119}$ conventional cells, even though no effect was seen in *in vitro* culture (Fisson et al., 2006). This suggests that bystander suppression can occur in expanded nTreg, though the two epitopes were very closely related.

Closely related to this thesis was the use of iTreg specific for myelin proteolipid protein $(PLP)_{139-151}$ in *in vivo* models of EAE triggered by various peptides (Zhang et al., 2010). Adoptively-transferred expanded antigen-specific iTreg were able to effectively prevent the development of EAE following immunization with the same antigen in adjuvant. However those $PLP_{139-151}$ iTreg were unable to modulate disease caused by $PLP_{178-191}$ or a combination of both peptides. This was further extended into a model of delayed type hypersensitivity in which the $PLP_{139-151}$ -specific iTreg could only suppress a response to the same peptide, but not to one of several other peptides, even when $PLP_{139-151}$ was included in the original sensitizing mix. Notably the same $PLP_{139-151}$ iTreg were able to effectively suppress proliferation of $pMOG_{35-55}$ specific naïve cells in *in vitro* culture. Is the bystander suppression effect then just a curiosity of *in vitro* methodology? Certainly the behaviour of Treg is known to differ greatly between *in vitro* and *in vivo* systems (Klein et al., 2003).

Examples of successful bystander suppression *in vivo* by nTreg are present within the literature. $CD4^+CD25^+CD62L^{high}$ T cells were expanded from Tg4 mice using CD3/CD28-coated beads in the presence of high dose IL-2 (Stephens et al., 2009). Those expanded pre-activated MBP-responsive nTreg were then transferred into B10.PLxSJL F_1 mice, in which EAE can be induced using either MBPAc1-9 or $PLP_{139-151}$. The nTreg were protective against EAE induced with MBPAc1-9, with a very delayed onset of disease and much less severe clinical course. Though the same nTreg had no impact on the onset of EAE caused by administration of $PLP_{139-151}$, or both MBPAc1-9 and $PLP_{139-151}$, the subsequent disease course was less severe with more rapid resolution and fewer relapses. Thus, those expanded

MBPAc1-9 responsive nTreg were able to limit the disease caused by host T cells responding to $PLP_{139-151}$.

Bystander suppression has also been demonstrated in other models. Antigen-specific nTreg directed towards an islet autoantigen were able to effectively prevent the development of diabetes upon transfer of polyclonal effector cells and even to reverse established diabetes *in vivo* (Tang et al., 2004).

Examining the ability of iTreg to suppress GVHD, $pOVA_{323-339}$ specific iTreg were generated from OT-II mice and adoptively transferred into either OVA^+ or OVA^- recipients. Those mice on a B6 background received B6 x bm12 marrow, causing activation of donor $CD4^+$ cells due to mismatch in MHC class II H2. Subsequent transfer of polyclonal effector cells led to GVHD in the OVA^- recipients but not where the iTreg had exposure to their cognate antigen (Semple et al., 2011). As OVA in this context served as a self antigen, GVHD developed due to donor effectors proliferating and responding to the host antigen (i.e. alloantigen $H2^{bm12}$). Thus, OVA-specific iTreg were suppressing a disease process stimulated by a different antigen *in vivo*, albeit in a highly manufactured system.

Other more circumstantial evidence for bystander suppression exists. Recent papers suggest that iTreg may be of more benefit in established disease rather than at onset. In a direct comparison of nTreg and iTreg (of polyclonal specificities), iTreg were superior in ameliorating histological changes in a mouse model of arthritis (Kong et al., 2012) with the added tolerising effect of skewing responding cells within the local lymph nodes to a Treg-predominant population. Treatment of rats with a nasal bystander epitope lessened the severity of subsequent experimental arthritis, an effect that was transferable via $CD4^+$ T cells (Zonneveld-Juijssoon et al., 2011). Generation of antigen-specific iTreg and administration during acute viral infection was able to reduce the severity of subsequent lesions in models of herpetic stromal keratitis (Sehrawat et al., 2008). Given this was infection with whole virus rather than relevant epitopes, it is plausible that bystander suppression would play a role in this protection.

3.3.3 Kinetics of suppression

These experiments have demonstrated that iTreg are able to exert their suppressive effect within 24 hours of culture onset but the effects upon naïve cell incorporation of ^3H-thymidine are not appreciable until at least 96 hours of culture (Fig 3.11, 3.12). Persistence

of the iTreg is also not required for this suppressive effect to be maintained (Fig 3.13). There are two broad theories that could account for this.

3.3.3.1 IL-2 deprivation

Early reports of apoptosis caused by growth factor deprivation identified IL-2 as a key cytokine, the absence of which is able to cause apoptosis (Duke et al., 1986). This process occurs over 24-48 hours following removal of IL-2, and fits well with the timescale offered above. IL-2 deprivation mediated apoptosis was suggested as a mechanism of Treg function in mouse models of colitis (Pandiyan et al., 2007), though the concept of 'mopping up' excess IL-2 appeared much earlier (Thornton et al., 1998). Further evidence for this is that the addition of IL-2 to suppression assays is able to wholly break the iTreg suppressive effect (Takahashi et al., 1998). All Treg cells are obligate users of IL-2, hence the high expression of the IL-2Rα. Experiments depleting IL-2 may therefore lead to Treg cell death, rendering any subsequent suppression or lack thereof difficult to interpret. Clearly then, IL-2 deprivation is likely to be a major mechanism used by these cells in this *in vitro* model. Indeed, IL-2 deprivation has been proposed to be the major non-redundant mechanism of Treg suppression (Wang et al., 2010a).

How achievable is IL-2 deprivation *in vivo?* An *in vitro* system with a limited number of cells and a defined timespan is a very different beast to an inflammatory process in disease, during which there is ongoing recruitment of new cells to the process, and a multitude of sources of IL-2. Can all of the effects of iTreg be explained as due to this alone? Certainly administration of a monoclonal anti-IL-2 antibody may have a profound immunosuppressive effect (Kelley et al., 1986, Kirkman et al., 1985). Conversely, IL-2 deprivation has been suggested as a means of initially suppressing the regulatory response during an initial infection (Benson et al., 2012). How plausible is it that Treg are both regulated and regulate by exactly the same mechanism? The development of autoimmunity in IL-2 deficient mice supports the absolute requirement for IL-2 for persistence of Treg, with comparison between IL-2 deficient and CD25 deficient adoptively transferred $CD4^+$ cells in protection from EAE demonstrating that IL-2 signaling is most vital to Treg function (Furtado et al., 2002). Opposing this, scurfy mice with a complete absence of Treg suffer mortality typically within 4 weeks of birth due to overwhelming autoimmunity. Scurfy mice that are also IL-2 deficient survive longer with inflammation predominantly in the colon, rather than the skin and lung seen in IL-2 sufficient mice (Zheng et al., 2007). Thus IL-2 has a crucial role in both survival of the regulatory population, but also in effector cell proliferation within autoimmunity.

3.3.3.2 Effects on APC

The need for cell contact and the requirement for Treg only at the onset of culture but not when the effects of suppression are evident may suggest that regulation is mediated through another cell population, the most obvious candidate of which is the APC. Whether interaction with the APC is non-redundant remains unclear, but clearly in the presence of an APC that permits interaction, suppression is more effectively performed.

Suppression assays have been reported in the absence of APC, using anti-CD3 and anti-CD28 *in vitro*. Stimulation of $V\beta8^+$ effector cells using anti-Vβ8-coated microbeads did not alter the ability of nTreg to suppress in one report (Szymczak-Workman et al., 2009). That group also found that MHC restriction was irrelevant to suppression, in contrast to the data shown here, and that TCR stimulation of Treg was not necessary for suppression to occur i.e. Treg had a constitutive suppressive effect. Investigation of the roles of IL-35 and IL-10 using *in vitro* suppression assays also stimulated both Treg and conventional cells with anti-CD3 and anti-CD28 (Collison et al., 2009). In that report, contact with conventional cells was found to enhance the suppressive capacity of Treg, but the Treg could then effect suppression of proliferation of other cells across a semi-permeable membrane through the actions of IL-35 and IL-10. As this differs somewhat from other reports, it is plausible, indeed highly likely, that Treg suppress with a variety of redundant mechanisms, which may be influenced by the presence or absence of APC in co-culture. Thus Treg may have an APC-independent mechanism of suppression (such as IL-35, IL-10 production) that is down-regulated in the presence of APC. This may explain the discrepancy between that report and the absence of suppression seen when APC interaction with iTreg is prohibited through the peptide-MHC complex (Fig 3.6, 3.7), as the APC are still present within the culture and may be influencing iTreg through soluble factors as well. This merits further study, though cannot be determined from the data presented.

The ability of Treg to affect APC is documented however. Using a murine model of autoimmune gastritis, antigen-specific iTreg responding to an immunodominant peptide in the H^+/K^+ ATPase were demonstrated to prevent the onset of disease (Di Paolo et al., 2007). Following culture with iTreg, dendritic cells were less able to stimulate antigen-responsive naïve T cells and had reduced co-stimulatory markers, CD80 and CD86, suggesting that iTreg rendered the DC less able to prime the immune response.

Down-regulation of CD80 and CD86 on APC by Treg was reported to be mediated through CTLA-4 expression, via the process of trans-endocytosis (Qureshi et al., 2011). Indeed,

CTLA-4 expression was demonstrated to confer suppressive function, rather than expression of Foxp3. In that system, suppression was antigen-dependent. Whether the bystander suppression in the absence of cognate antigen by the iTreg generated here is dependent upon CTLA-4 expression would merit further investigation. Certainly, there is increasing evidence that CTLA-4 plays a crucial role in Treg function (Sansom et al., 2006).

Various reports of Treg influencing APC through other mechanisms exist, suggesting that nTreg are able to limit APC interactions with responding cells (Tadokoro et al., 2006), affect their maturation (Misra et al., 2004, Veldhoen et al., 2006), or molecular expression of co-stimulatory and inhibitory molecules (Cederbom et al., 2000, Kryczek et al., 2006). However all of this evidence relates to nTreg rather than iTreg.

3.3.3.3 Alternative mechanisms

Other mechanisms may also be at play. The role of the responding effector cells on the Treg initially was investigated in the report already alluded to above (Collison et al., 2009) examining cytokine production by nTreg (defined as $CD^{+}CD25^{+}CD45RB^{low}$) and found that IL-10 and IL-35 secretion was potentiated in the presence of conventional cells. More relevantly in a transwell system, nTreg alone in an upper well were unable to suppress conventional cells in the bottom well. When conventional T cells were also added to the upper transwell, suppression of the cells in the lower half was also seen – suggesting that the cell contact required for suppression is an initiating factor but not required for suppression of all cells. The need for interaction between Treg and the effector cells has been supported by similar reports from other groups (Grinberg-Bleyer et al., 2010).

Another proposed mechanism of suppression is via expression of co-inhibitory molecules on the surface of Treg, e.g. CTLA-4. *In vivo,* nTreg (identified as $CD4^{+}$ $CD25^{+}$ $CD45RB^{low}$) were able to inhibit the development of colitis in mice when co-transferred with $CD45RB^{high}$ cells, but this protective effect was lost in mice receiving anti-CTLA-4 monoclonal antibody treatment (Read et al., 2000). This was contradicted by *in vitro* data showing Treg from CTLA-4 deficient mice demonstrate full suppressive potential (Read et al., 2006). However this was potentially explained by the compensatory up-regulation in TGF-β seen in CTLA-4 deficient Treg compared to CTLA-4 sufficient Treg (Tang et al., 2004). Thus a further mechanism of Treg action may be via co-inhibitory signals, which would require cell contact, certainly in the early stages of naïve cell activation. In concert with many other aspects of Treg biology, most studies of CTLA-4 have concentrated on nTreg rather than iTreg. CTLA-4 appears essential for iTreg generation, with a lack of adaptive Treg in

CTLA-4 deficient mice (Zheng et al., 2006), and has even been suggested as a more potent marker of suppressive activity than Foxp3 in humans (Zheng et al., 2008a).

The need for Treg to have contact with cells they are suppressing can be harmonized with the depletion data shown here (Fig 3.13). If the early effects of co-culturing Treg with naïve cells triggered the suppressive effects, further cell contact may be unnecessary, and suppression may subsequently occur regardless of the need for ongoing cell contact. An important caveat is that much of the reported literature examines nTreg rather than iTreg, and these are increasingly recognized as disparate cell populations with differing mechanisms (Horwitz et al., 2008).

Investigation of the requirement of iTreg for contact with APC could feasibly be performed using transwell systems. Having demonstrated that iTreg do not need presentation of their cognate peptide to effect suppression, a vital experiment to perform would be *in vitro* transwell culture with iTreg separated from naïve T cells, with APC either present or absent in the iTreg section of the transwell (obviously APC are non-redundant in the naïve T cell compartment, as peptide presentation is required to effect proliferation). This may conceivably provide a definitive answer as to whether APC are a crucial requirement for the suppression of ^{3}H-thymidine incorporation within naïve T cells by iTreg, or they may enhance suppressive function. Parallels can be drawn to the transwell experiments demonstrating greater suppressive function when iTreg are co-cultured with some naïve T cells (Grinberg-Bleyer et al., 2010). Unfortunately, transwell experiements were not performed during the course of this thesis, due to financial limitations. They represent an obvious next step in clarifying the role of APC and the need for MHC interaction.

How exactly are the iTreg exerting their effects? It is highly likely that no one single mechanism will account for the suppressive potential of Treg in different *in vitro* and *in vivo* systems. Indeed, the up-regulation of TGF-β in the absence of CTLA-4 implies multiple compensatory mechanisms that can be directly influenced by the experimental system used. The fact that the physical presence of iTreg is not required at the time that suppression of ^{3}H-thymidine incorporation occurs suggests there may be a soluble factor released during early co-culture having a later influence. Investigation of the cytokines released by iTreg thus became the next focus.

3.3.3.4 Experimental Caveats

The crude nature of the ^{3}H-thymidine incorporation assay has already been alluded to (section 3.2.3) and the disadvantages of the system are well-recognised (Collison et al.,

2011). Absence of ^{3}H-thymidine incorporation may represent either an absence of proliferation, or cell death occurring earlier than the timepoint interrogated. The 'non-proliferative' nature of iTreg is shown not to be evident until 96 hours (Fig 3.11A) strongly suggesting that it is cell death responsible for the absence of ^{3}H-thymidine incorporation, rather than a truly anergic response. Similarly, the co-cultured wells cannot distinguish between iTreg exerting a suppressive effect on naïve T cells, or actively causing cell death. Congenic markers are available to distinguish between iTreg and naïve T cells, so co-cultured wells could be interrogated by flow cytometry to confirm the viability of cells at the end of co-culture. Usage of CFSE-dilution by naïve T cells could also clarify whether naïve T cell proliferation is truly suppressed, or if cell death plays a significant role. Given the demonstration above that the patterns of CFSE-dilution is partly influenced by cell viability (Fig 3.12), a thorough investigation of the kinetics of this reponse would also be required.

3.3.4 Concluding remarks

iTreg are able to suppress naïve cell proliferation in response to an unrelated antigen, in the absence of ongoing stimulation themselves. This effect appears partly mediated via the APC, in particular through MHC interaction though peptide is not required. The suppressive effect is not on an individual APC interacting with both iTreg and naïve T cells, nor is it wholly related to the APC alone. A proposed requirement for cognate MHC is shown (Figure 3.14). Suppression of ^{3}H-thymidine incorporation is not seen until late in the culture but at this point there is no requirement for the iTreg to still be present.

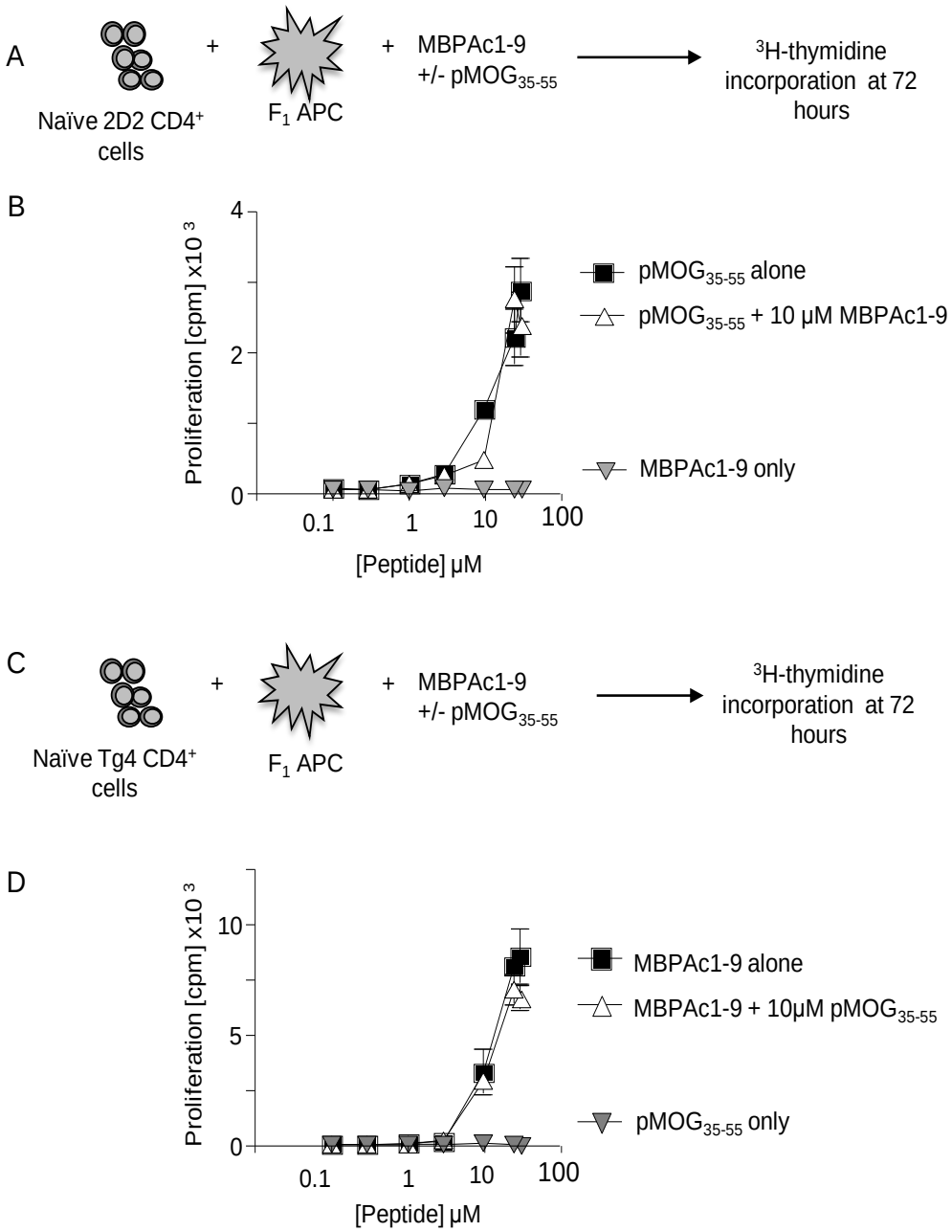


Figure 3.1 Cross-reactivity of antigens does not occur, nor is there any effect of the alternate peptide on the cognate response.

A) Experimental scheme. Naïve 2D2 cells were cultured with F_1 APC and $pMOG_{35-55}$ at varying concentrations, with or without 10µM MBPAc1-9. B) Incorporation of ^{3}H-thymidine after 72 hours is shown. C) Experimental scheme. The same approach was used for Tg4 cells. D) ^{3}H-thymidine incorporation after 72 hours of culture is shown. Data are shown from a single experiment.

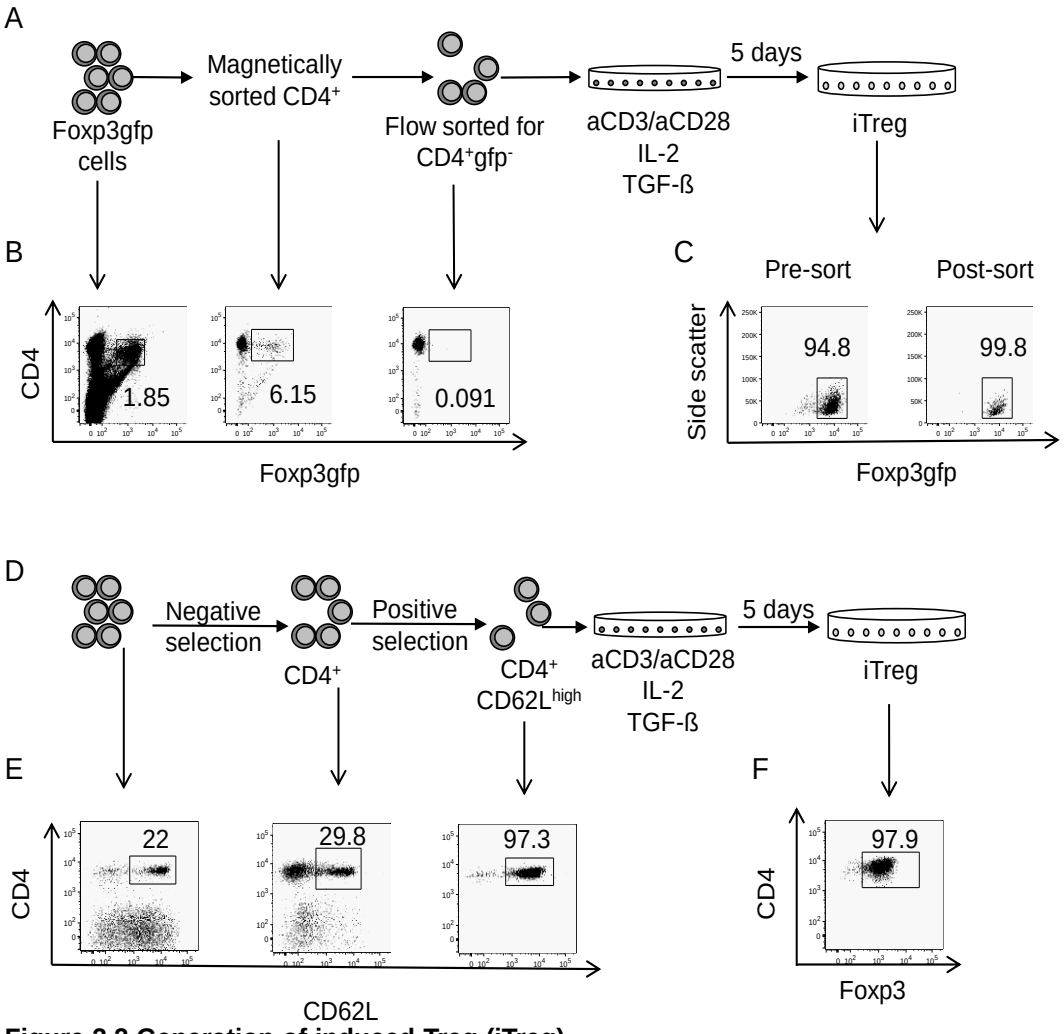


Figure 3.2 Generation of induced Treg (iTreg)

A) Protocol for iTreg generation from Foxp3gfp reporter mice. Splenocytes and lymph node tissues from mice expressing gfp on the Foxp3 promoter were sorted by positive selection using magnetic beads for the expression of CD4. The $CD4^+$ fraction was then sorted by flow cytometry for the absence of gfp expression, yielding a highly pure $CD4^+$ $Foxp3gfp^-$ population. These cells were cultured with plate-bound anti-CD3 and anti-CD28 (both at 2µg/ml) with 100U/ml IL-2 and 5ng/ml TGF-β for 5 days. B) Representative flow plots of Foxp3gfp expression, gated on all cells, are shown pre- and post-sorting at the beginning of iTreg culture. Numbers represent the percentage of $CD4^+$ $Foxp3gfp^+$ cells. C) Representative flow plots of Foxp3 expression pre- and post-sorting at the end of iTreg culture. Plots are gated on live cells. Numbers represent percentage of $Foxp3gfp^+$ cells. D) Protocol for iTreg generation from mice without the Foxp3gfp reporter. Cells were negatively selected for CD4 expression using magnetic beads then further magnetic positive selection for $CD62L^{high}$ expression. $CD4^+CD62L^{high}$ cells were cultured as in A). E) Representative flow plots gated on all cells before iTreg culture. Numbers represent percentage of $CD4^+$ $CD62L^{high}$ cells. F) Representative flow plot of Foxp3 expression gated on live cells at the end of iTreg culture. Numbers indicate percentage of $CD4^+Foxp3^+$ cells. Data are from one of >five experiments giving consistent results.

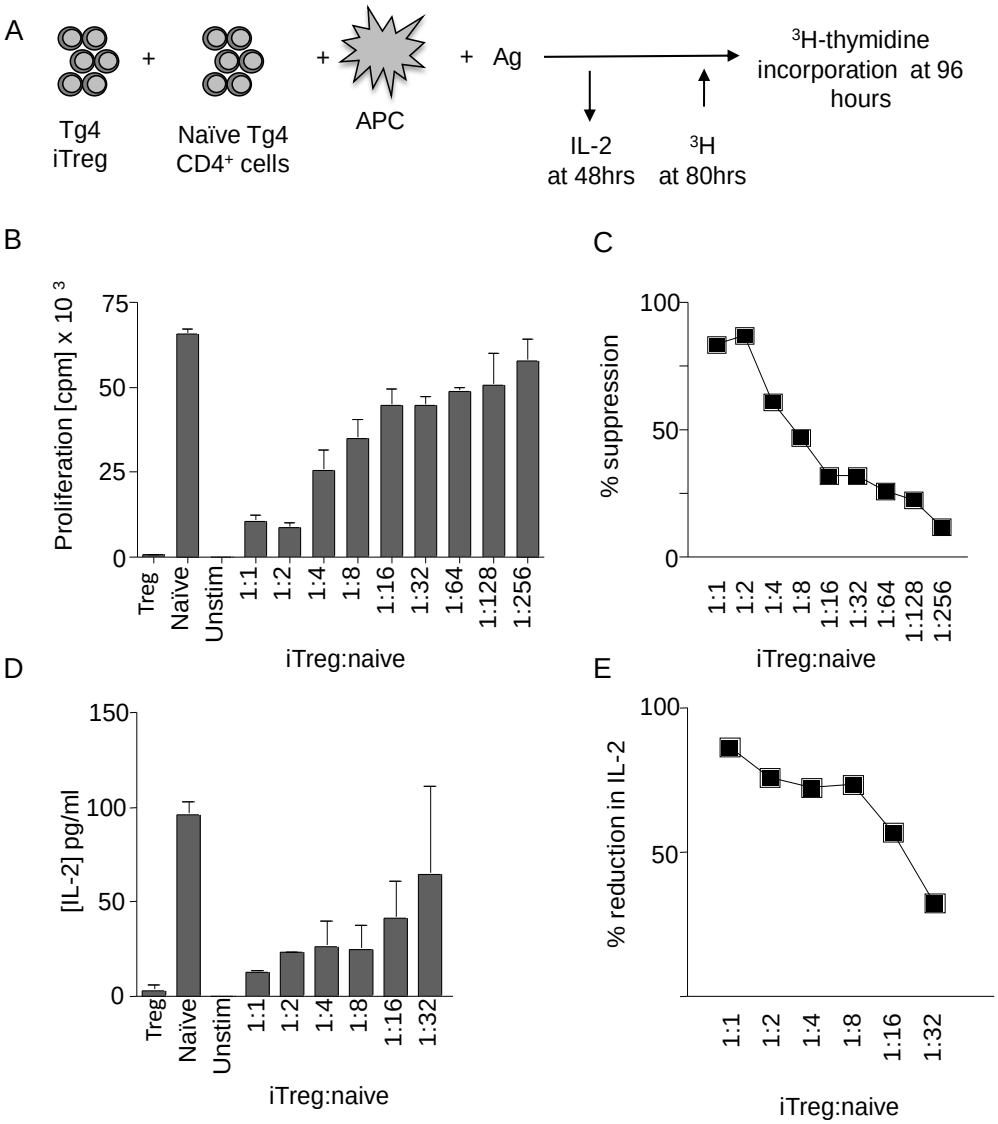


Figure 3.3 iTreg suppress ^{3}H-thymidine incorporation and IL-2 is reduced *in vitro.*
A) Experimental scheme. Tg4xFoxp3LuciDTR-4 iTreg were co-cultured with naïve Tg4 CD90.1 CD4^{+} T cells, irradiated APC and 10μM MBPAc1-9 at varying ratios of Treg:naïve cells. Duplicate supernatants were frozen at 48 hours for subsequent analysis by ELISA. ^{3}H-thymidine was added for the final 16-18 hours of culture and then plates were harvested. iTreg and naïve cells were also cultured in isolation with APC and antigen, as controls. B) Proliferation was estimated by the incorporation of ^{3}H-thymidine at 96 hours of culture. C) Quantification of the ability to suppress was calculated (as described in Chapter 2) comparing incorporation of 3H-thymidine of naïve cells co-cultured with decreasing numbers of iTreg to that of naïve cells alone. D) IL-2 production was measured at 48 hours of culture by ELISA. E) Reduction in IL-2 concentration was similarly quantified. Data are from one of >five experiments giving consistent results.

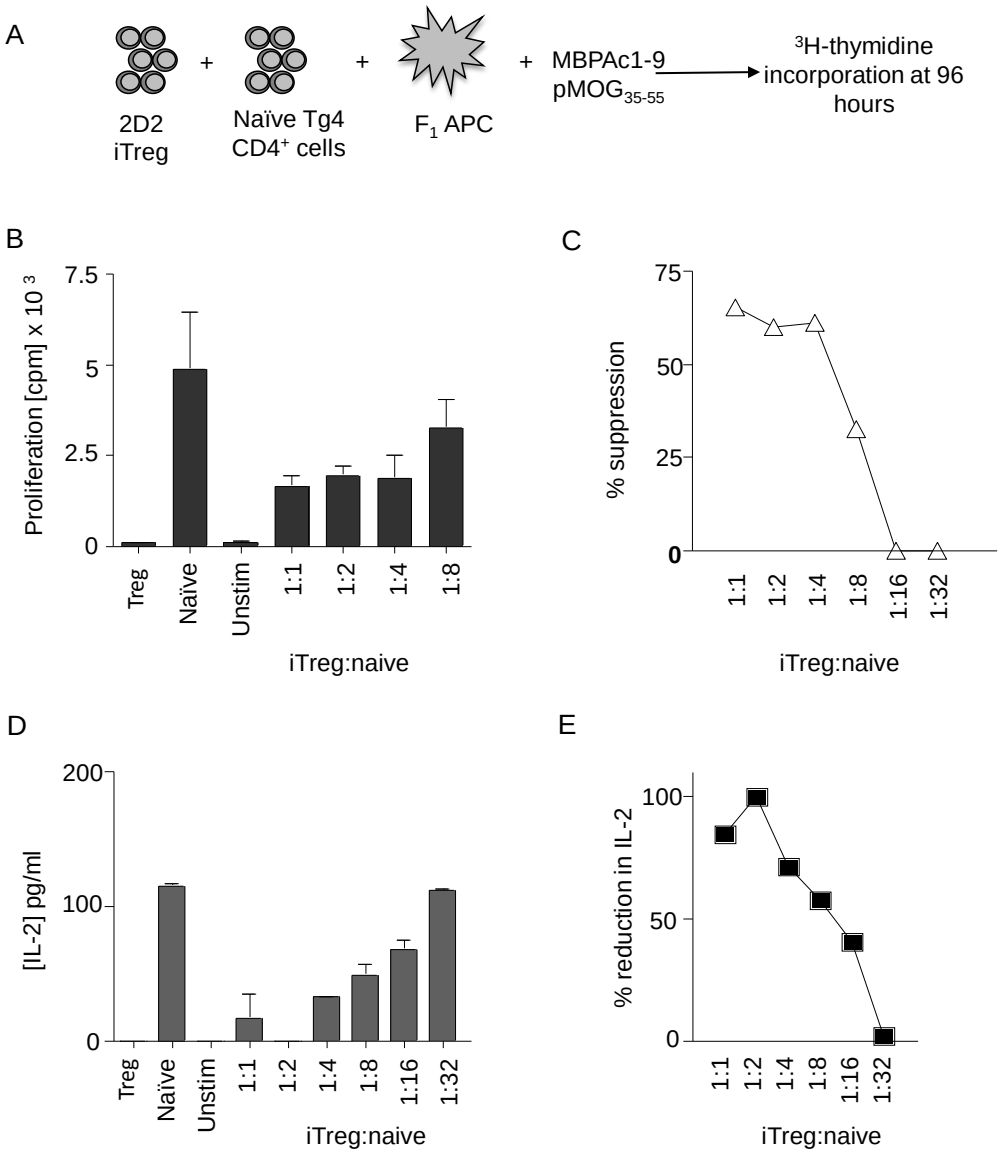


Figure 3.4 2D2 iTreg suppress naïve Tg4 cells in the presence of both antigens.
A) Experimental scheme. 2D2 iTreg were co-cultured with naïve Tg4 CD4+ cells in the presence of F_1 APC and both 10μM MBPAc1-9 and 10μM $pMOG_{35-55}$. B) Incorporation of ^{3}H-thymidine at 96hours. C) Quantification of suppression. D) IL-2 production was measured at 48 hours by ELISA. E) Quantification of the effect on IL-2. Data are from one of three experiments giving consistent results..

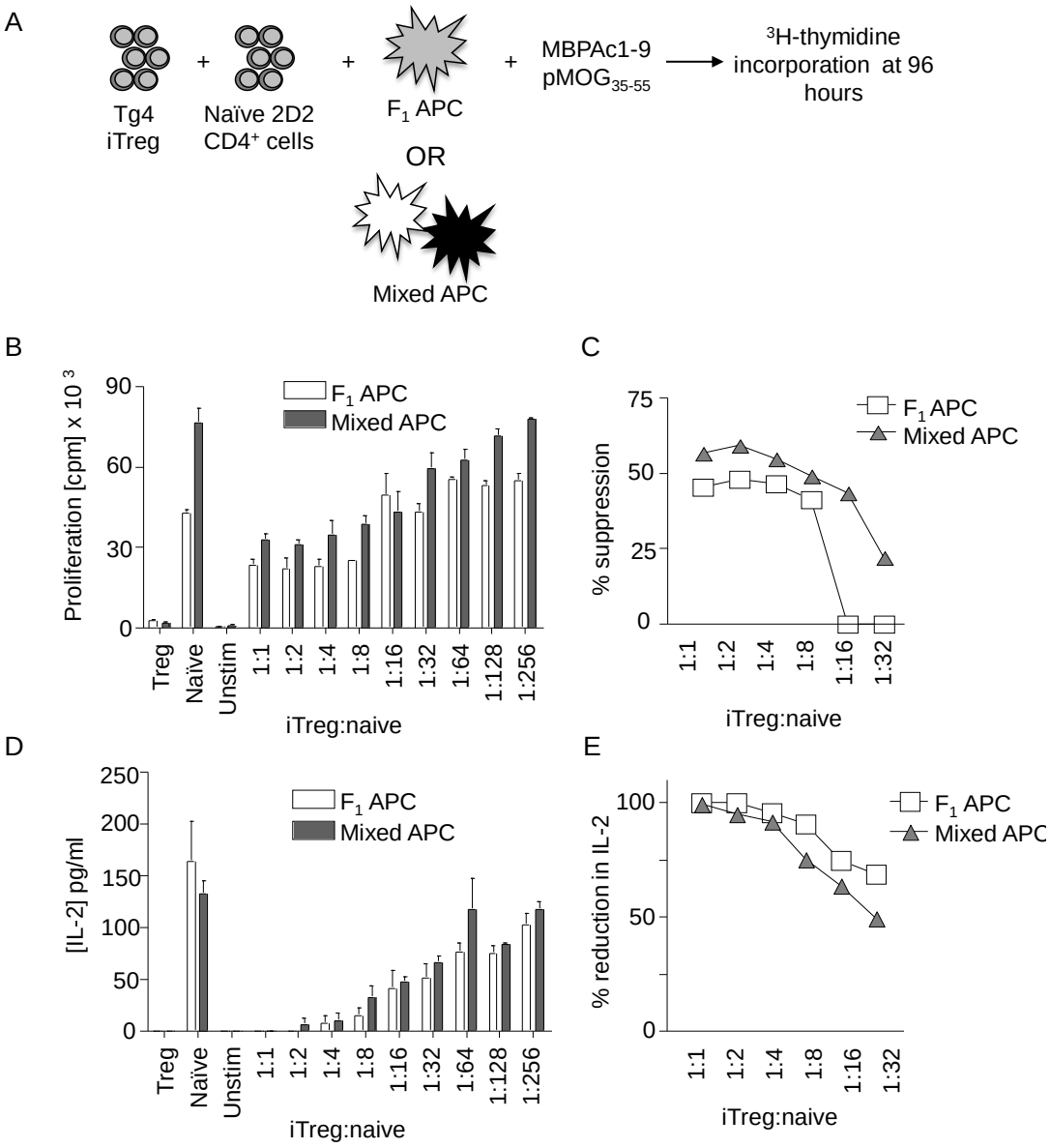


Figure 3.5 Co-presentation of antigens on a single APC is not required for suppression.

A) Experimental scheme. Tg4 iTreg were co-cultured with naïve 2D2 cells with both MBPAc1-9 and $pMOG_{35-55}$ with either F_1 APC or mixed Tg4 and 2D2 $CD4^-$ fractions, giving the same total number of APC in each group. B) Incorporation of ^{3}H-thymidine at 96 hours of culture is shown. C) Quantification of suppression is shown. D) Production of IL-2 at 48 hours measured by ELISA. E) Quantification of the reduction in IL-2. Data are from one of three experiments giving consistent results.

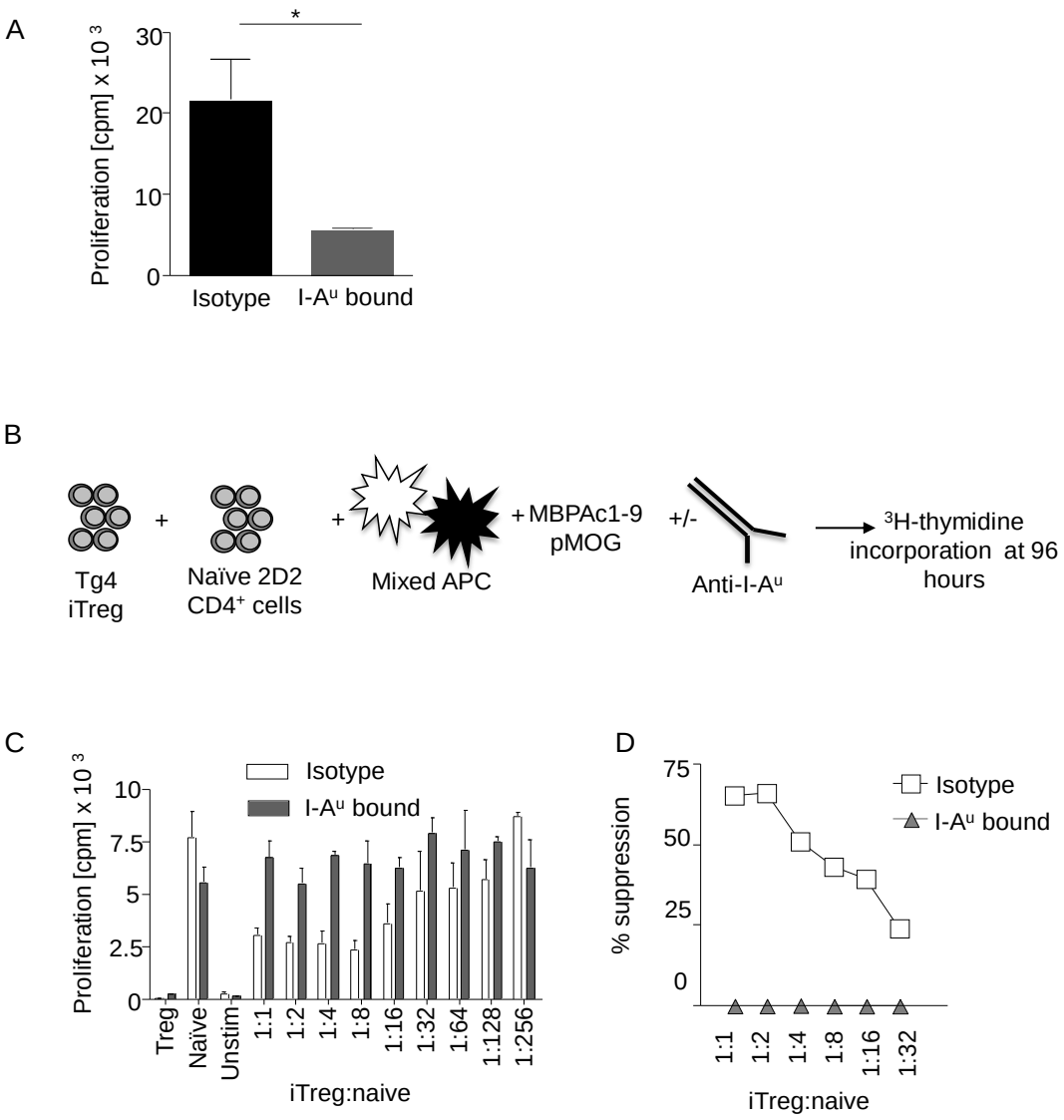
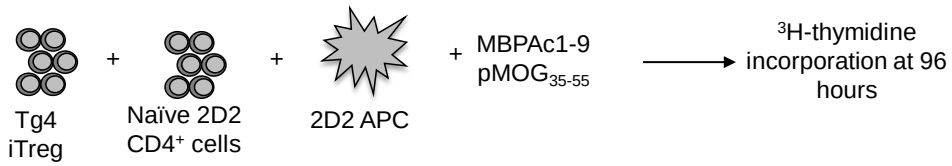


Fig 3.6. Binding of MHC I-A^u with exogenous antibody reduces the efficacy of suppression.

A) Naïve Tg4 CD4$^+$ cells were cultured with 10μM MBPAc1-9 and irradiated APC pre-incubated with Anti-I-A^u or isotype for 3 hours. The incorporation of ^{3}H-thymidine was measured at 72 hours. Data are shown from a single experiment. *p=<0.05 (unpaired t-test) B) Experimental scheme. Mixed APC were pre-incubated with antibody directed to I-A^u for 3 hours then washed prior to use in a suppression assay. C) Incorporation of ^{3}H-thymidine at 96 hours. D) Quantification of suppression. Data are from one of two experiments giving consistent results.

A



B

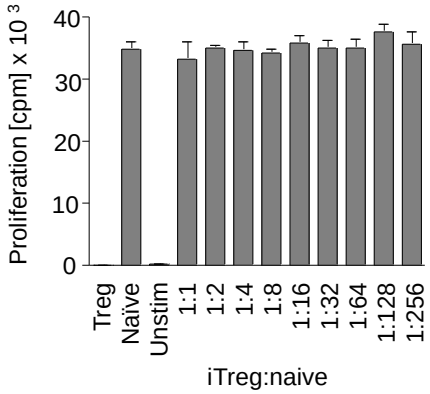


Fig 3.7. Absence of 'parental' APC prevents suppression
A) Experimental scheme. Naïve 2D2 $CD4^+$ cells were cultured with Tg4xFoxp3LuciDTR-4 iTreg in the presence of MBPAc1-9, $pMOG_{35-55}$ and irradiated 2D2 $CD4^-$ cells but not Tg4 APC. B) The incorporation of ^{3}H-thymidine was measured at 96 hours. Data are from one of four experiments giving consistent results..

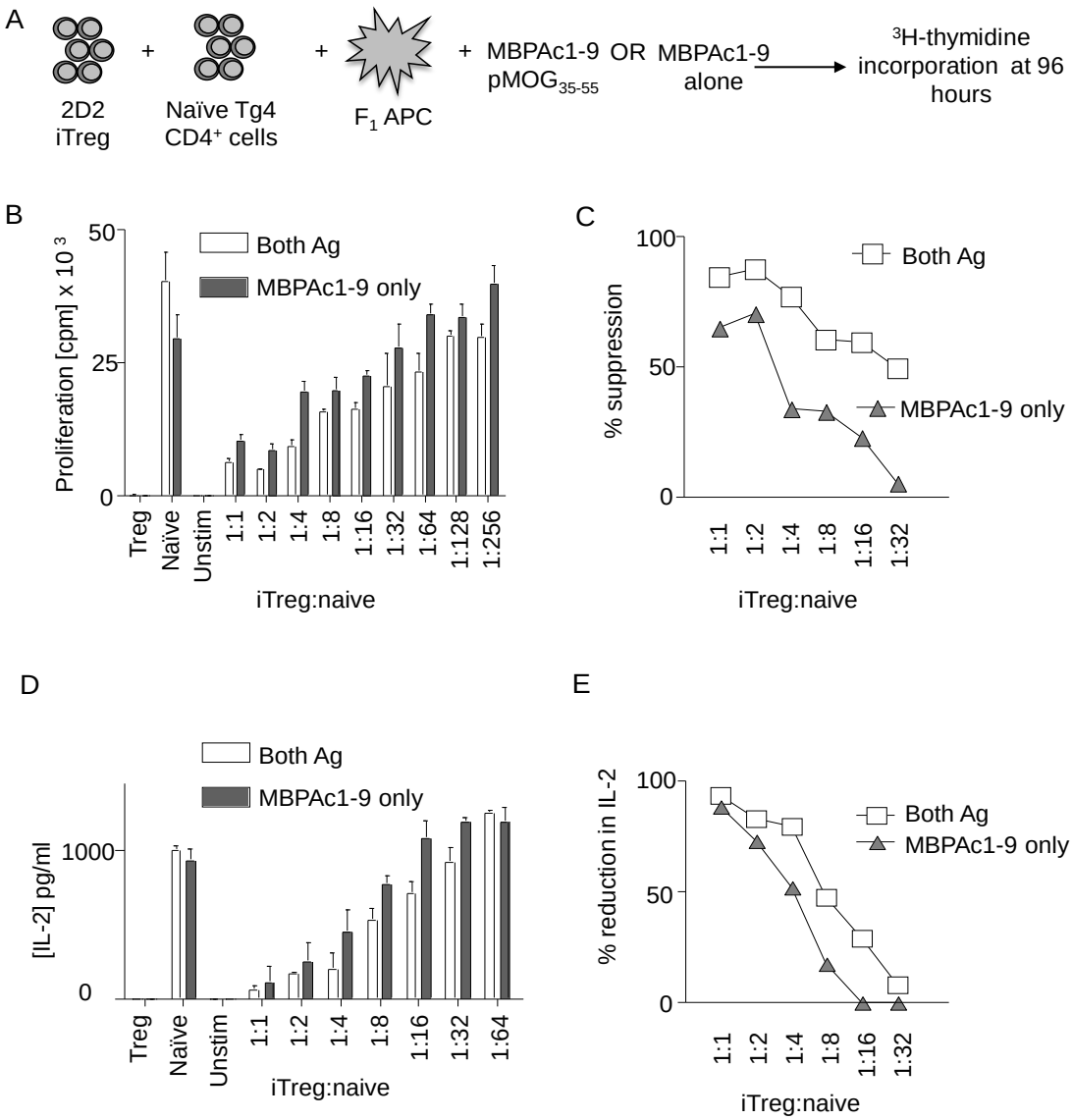


Figure 3.8 2D2 iTreg do not require their cognate antigen to effect bystander suppression.

A) Experimental scheme. 2D2 iTreg were cultured with naïve Tg4 $CD4^+$ cells, F_1 APC and both 10μM MBPAc1-9 and $pMOG_{35-55}$, or 10μM MBPAc1-9 alone. B) Proliferation was measured at 96 hours by ^{3}H-thymidine incorporation. C) Quantification of suppression. D) Measurement of IL-2 by ELISA at 48 hours of culture. E) Quantification of IL-2 reduction. Data are from one of two experiments giving consistent results.

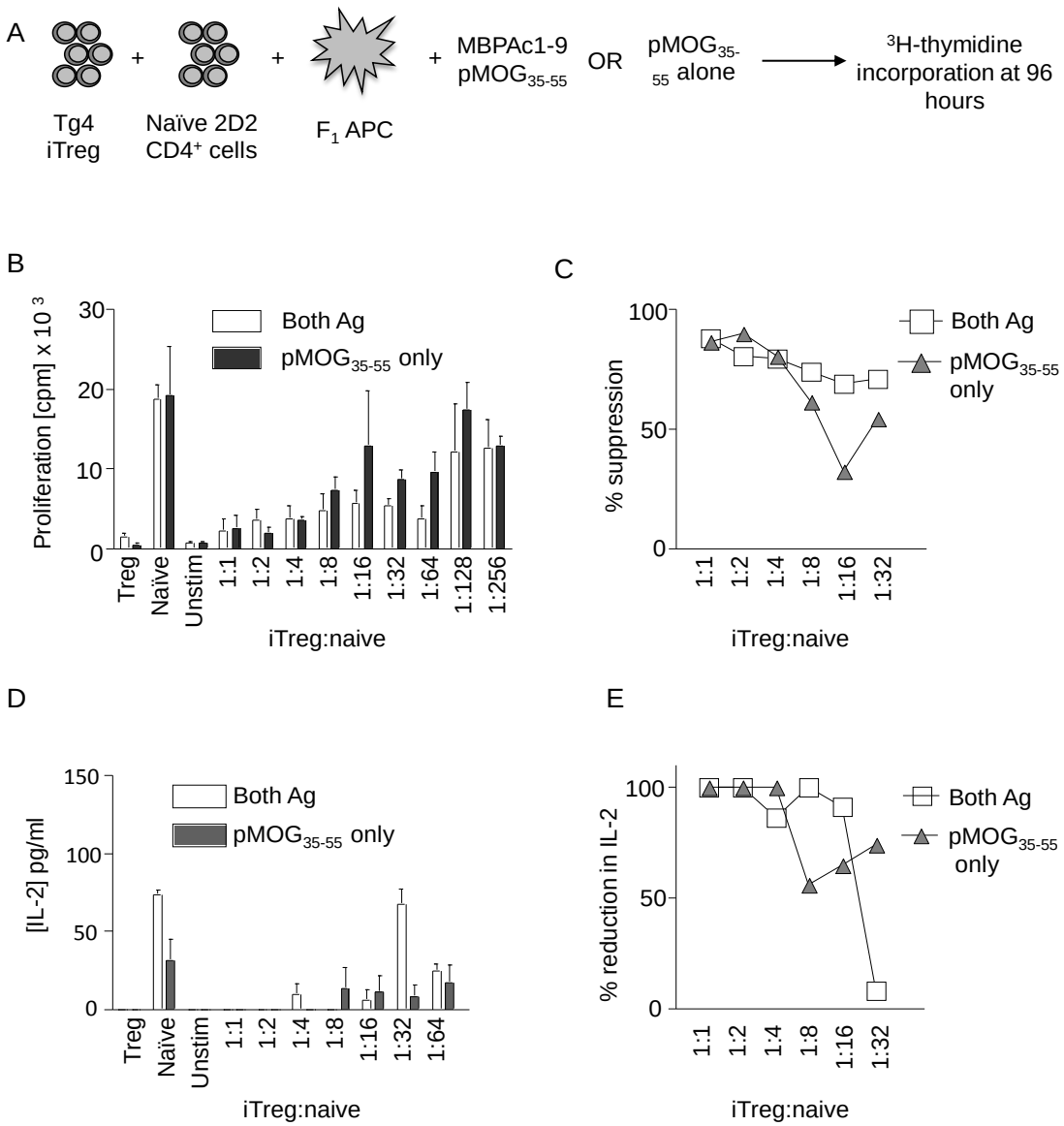


Figure 3.9 Tg4 iTreg are also able to suppress in the absence of cognate antigen
A) Experimental scheme. Tg4 iTreg were co-cultured with naïve 2D2 cells with F_1 APC and both 10μM MBPAc1-9 and $pMOG_{35-55}$ or 10μM $pMOG_{35-55}$ alone. B) Proliferation as assessed by incorporation of ^{3}H-thymidine. C) Summary of proliferation data. D) IL-2 production at 48 hours as measured by ELISA. E) Summary of IL-2 data. Data are from one of three experiments giving consistent results.

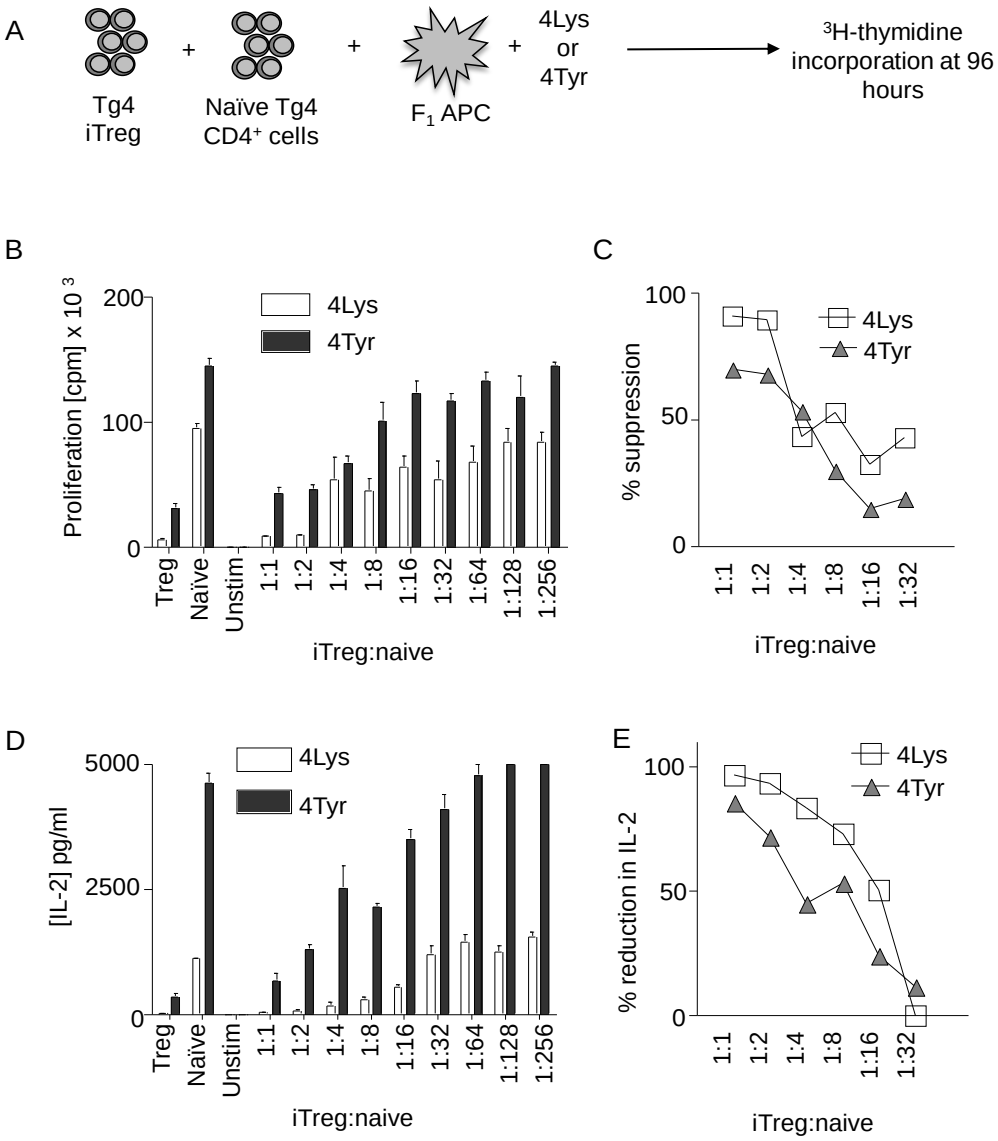


Figure 3.10 Peptide affinity for MHC does not affect iTreg suppression
A) Experimental scheme. Tg4 iTreg were cultured with naïve Tg4 CD4+ cells with F_1 APC and either wild-type MBPAc1-9 (4Lys) or the altered peptide ligand, MBPAc1-9 (4Tyr). B) Proliferation at 96 hours as measured by ^{3}H-thymidine incorporation. C) Quantification of suppression. D) IL-2 production measured at 48 hours by ELISA. E) Quantification of the reduction in IL-2. Data are shown from a single experiment.

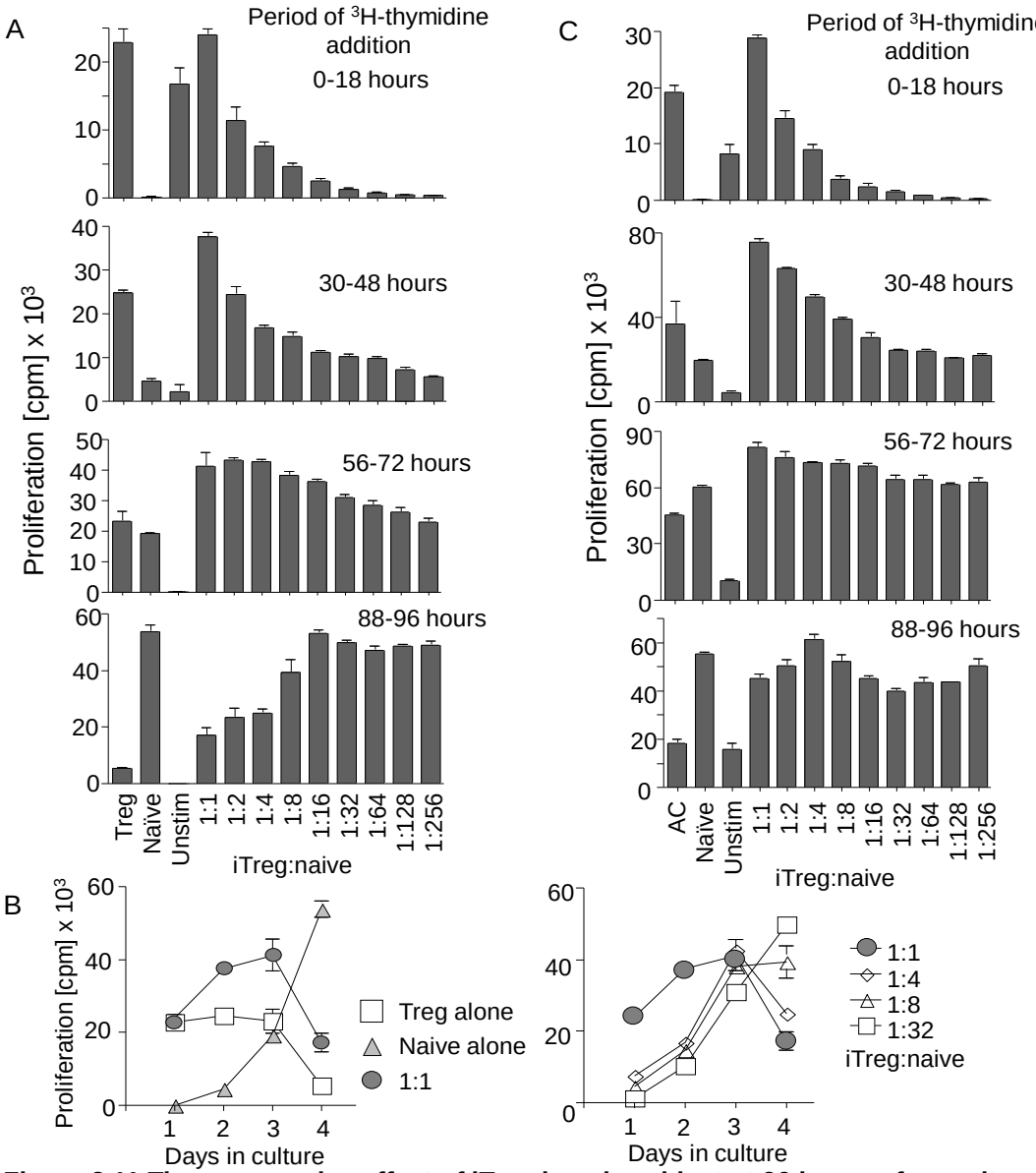


Figure 3.11 The suppressive effect of iTreg is only evident at 96 hours of co-culture.
A) Tg4xFoxp3LuciDTR-4 iTreg were cultured with naïve Tg4 $CD4^+$ cells, irradiated $CD4^-$ cells as APC, and 10μM MBPAc1-9. ^{3}H-thymidine was added immediately, or after one, two, or three days of culture. Plates were then harvested 16-18 hours after thymidine addition. B) Plots demonstrating kinetics of cell proliferation at varying ratios of Treg:naïve cells. C) Naïve $CD4^+$ cells were activated for 5 days with anti-CD3 and anti-CD28, then co-cultured with fresh naïve $CD4^+$ cells . ^{3}H-thymidine was added immediately, or after one, two, or three days of culture. Plates were then harvested 16-18 hours after ^{3}H-thymidine addition. AC = activated cells. Data are shown from a single experiment.

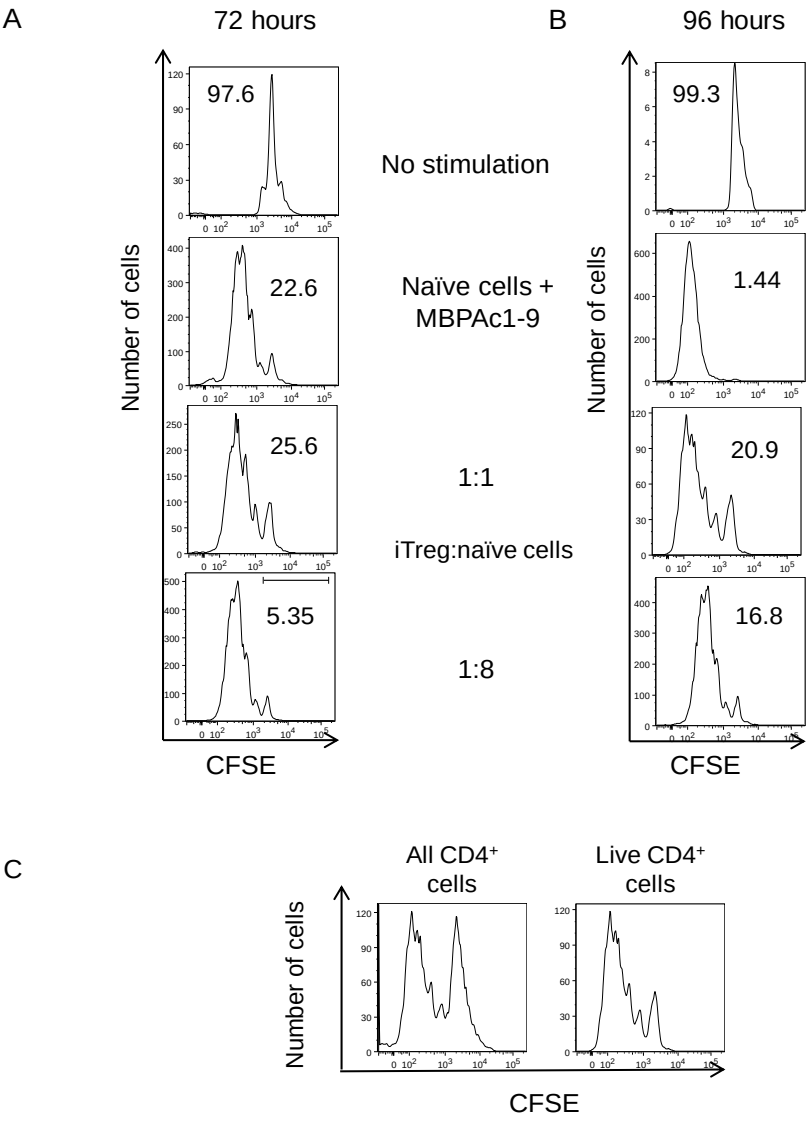


Fig 3.12 iTreg suppress proliferation by some naïve cells; many 'non-proliferating' naïve cells are not viable.

Naïve $CD4^+$ cells were labelled with CFSE and co-cultured with iTreg and 10 μM MBPAc1-9 at the indicated ratios for A) 72 and B) 96 hours. CFSE profiles are shown gated on live $CD4^+$ cells, with numbers indicating the percentage of cells with no reduction in CFSE dilution compared to unstimulated cells. B) CFSE profiles from stimulated naïve cells at a 1:1 ratio with iTreg at 96 hours, gated on all $CD4^+$ cells or just live CD4+ cells. Data are shown from a single experiment.

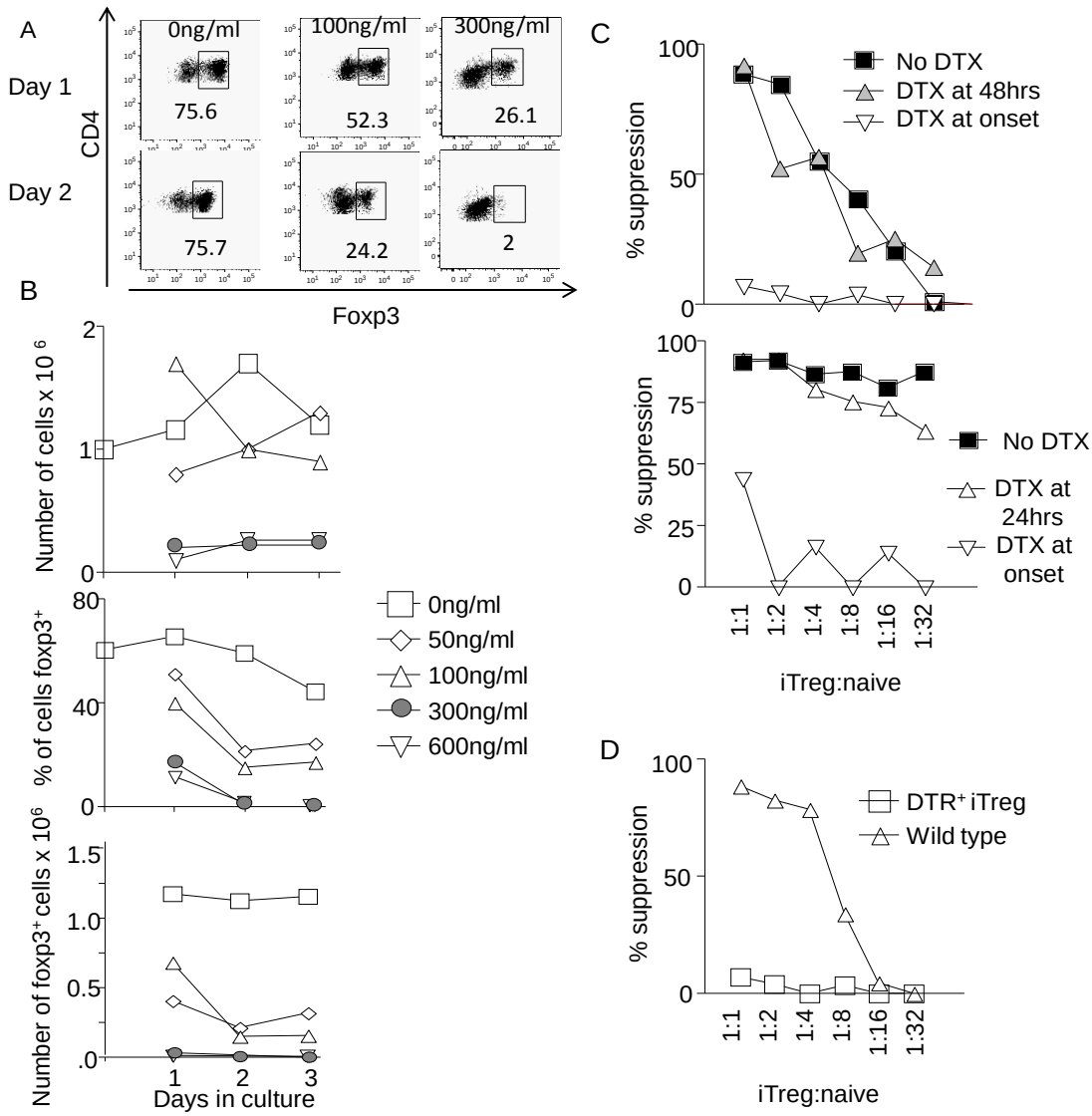


Figure 3.13 iTreg can be depleted *in vitro.* Suppression does not require persistence of iTreg

A) Tg4xFoxp3LuciDTR-4 iTreg were generated over 5 days then resuspended in medium containing 0-600ng/ml of diphtheria toxin. Representative flow cytometry plots gated on $CD4^+$ cells after 24 and 48 hours in medium containing DTX at the indicated concentrations. B) Cells were counted and analysed by flow cytometry for Foxp3 expression. C) Tg4xFoxp3LuciDTR-4 iTreg were cultured with naïve Tg4 cells and 10μM MBPAc1-9 with the addition of DTX at the onset of culture, or after 24 or 48 hours. Quantitative results of suppression of proliferation are shown. Representative of four experiments. D) Naïve Tg4 $CD4^+$ cells were cultured with either Tg4 Foxp3LuciDTR-4 iTreg or Tg4Ly5.1 iTreg, with the addition of DTX at the onset of culture. Quantitative results of suppression are shown. Data are shown from a single experiment.

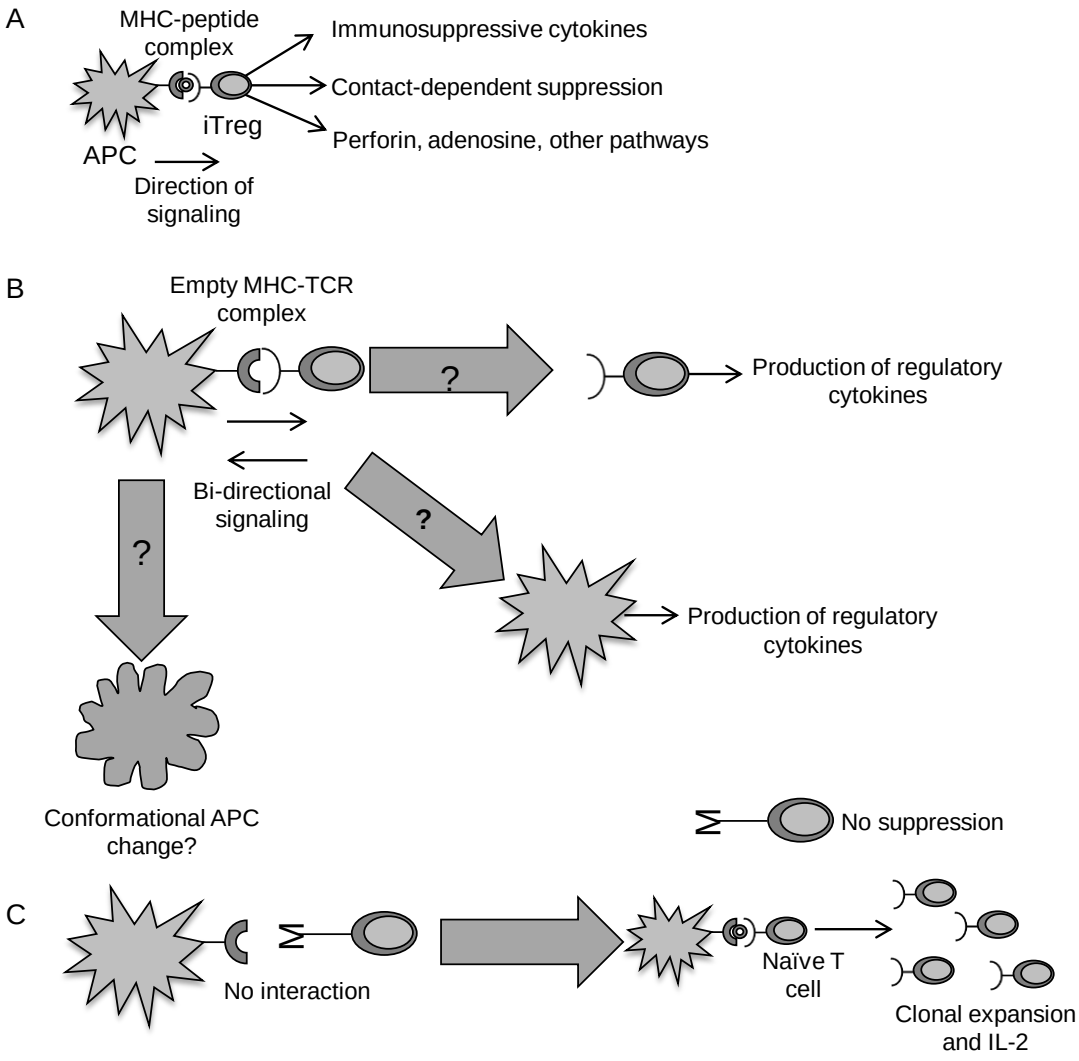


Figure 3.14 Hypothetical model of iTreg suppression
A) The 'traditional' view ofiTreg suppression is that signaling through the TCR-MHC-peptide complex leads to suppression via Treg through a variety of mechanisms. Data presented in this chapter show that iTreg suppression is dependent on a low-affinity interaction with the appropriate MHC on an APC. B) Whilst peptide presentation by the MHC leads to TCR stimulation and iTreg activation remains a prerequisite for iTreg induction, activated cells may form brief unstable complexes with the empty MHC. Bidirectional signaling may then occur, triggering either release of suppressive cytokines (from either the APC or the iTreg themselves), or altering the conformation of the APC such that it is less likely to trigger a pro-inflammatory response e.g. removal of CD80/CD86, or interaction with CTLA-4. C) If this weak interaction is prevented from occurring, or cannot occur due to MHC restriction, APC's remain pro-inflammatory, and suppression does not take place.

4 iTreg produce pro-inflammatory cytokines but this does not impact upon *in vitro* suppression

4.1 Introduction

A major hurdle for the future use of iTreg as a cellular therapy is their potential to lose expression of Foxp3 and convert to effector cells under the influence of pro-inflammatory cytokines. If antigen-specific iTreg were infused into a patient, the possibility these could all rapidly convert to pathogenic effector cells and worsen disease would render this therapy a highly risky strategy, consigning it to 'last-ditch' therapeutic efforts rather than a mainstay of treatment. How plausible is this 'conversion' to an effector phenotype?

The defining feature of the iTreg used in this thesis is the presence of Foxp3 expression at the end of five days of culture in iTreg-generating conditions. The expression of Foxp3 in murine T cells confers regulatory ability (Hori et al., 2003). The loss of Foxp3 from iTreg has been noted previously (O'Connor et al., 2010) with factors influencing Foxp3 stability under ongoing investigation (Marie et al., 2005, Chen et al., 2011). Cytokines released by iTreg may be used to mediate effects on target cells distant to the responding iTreg but may also have an autocrine effect and alter the iTreg itself. Arguably the most important aspect of an iTreg is its expression of Foxp3. Do iTreg maintain their own regulatory status when re-stimulated?

An important paper addressing this issue (O'Connor et al., 2010) demonstrated that iTreg produce IFN-γ with an associated upregulation of Tbet. Despite this, those iTreg remained suppressive both *in vitro* and *in vivo* in limiting the development of EAE, even when "re-conditioned" with IL-12 to maximize IFN-γ production. Transfer of high numbers of those IL-12-conditioned iTreg alone caused only mild short-lived disease. This reduction in pathogenicity despite IFN-γ production was then potentially explicable by the revelation that, in EAE, the primary pathogenic cytokine causing disease appears to be GM-CSF (Codarri et al., 2011).

Is the cytokine profile of iTreg relevant? One of the proposed differences between nTreg and iTreg is that of the mechanism of action. nTreg have been demonstrated to suppress via cell-cell contact (Thornton et al., 1998, Nakamura et al., 2001) with a potential pivotal role

for CTLA-4 (Sakaguchi et al., 2004). Other reports have postulated roles for soluble mediators such as IL-10 (Annacker et al., 2001), IL-35 (Collison et al., 2007) and TGF-β (Huber et al., 2004). The mechanisms of iTreg suppression are similarly ill-defined. Though a majority view is that iTreg exert their suppression (at least *in vivo*) mainly through the action of soluble cytokines, especially IL-10 (Nguyen et al., 2011), the exact mechanisms are controversial (Yamagiwa et al., 2001). Some groups have demonstrated that cell contact is not necessary for iTreg action (Maganto-Garcia et al., 2011), whilst others have shown a requirement for this for iTreg suppression (Chen et al., 2003, Zhang et al., 2009). Thus the role of soluble mediators is not yet established.

One explanation for some of these conflicting results is the role of TGF-β, which has been implicated in suppression by both nTreg (Peng et al., 2004) and iTreg (Li et al., 2006a). The difficulty in distinguishing between the effects of membrane-bound and free TGF-β has complicated investigation, but may explain how cell contact is necessary to exert suppression via what might be considered a soluble factor. The ability of Treg to release large amounts of TGF-β, or indeed to signal via TGF-β which remains membrane-bound, is still a subject of debate (Piccirillo et al., 2002, Nakamura et al., 2001).

The previous chapter demonstrated that, *in vitro*, the ongoing presence of iTreg was not required beyond 24 hours, but the effects of suppression were not seen until 72 hours or greater. Interaction with APC appeared necessary and this appeared to be MHC-restricted. The influence of a soluble factor may be responsible for these effects.

4.1.1 Experimental aims

This chapter thus concentrates on determining the nature of cytokines that are produced by iTreg, and how these may be influenced by the surrounding cytokine milieu. Further experiments aimed to establish the impact of these cytokines on the ability of iTreg to suppress *in vitro*.

4.1.2 Experimental approach

Following iTreg generation, cells were sorted by FACS on the basis of Foxp3gfp expression to ensure highly pure iTreg populations (routinely >99%). These iTreg were then re-stimulated *in vitro*, either using plate-bound anti-CD3 and anti-CD28 where Foxp3gfp mice were used, or MBPAc1-9 and irradiated APC in those experiments using cells from Tg4xFoxp3LuciDTR-4 mice. Cytokine production by iTreg was initially screened using cytokine bead arrays then the cytokines of interest were further analysed via a combination of ELISA and intracellular cytokine staining.

4.2 Results

4.2.1 Foxp3 expression by iTreg is not stable

Whilst performing the suppression assays detailed in chapter 3, flow cytometry of cells at the end of the assay had demonstrated very few $Foxp3^+$ cells, implying either death of the iTreg or loss of Foxp3 [data not shown]. Though a considerable number of cells seemed non-viable on trypan blue exclusion, there was still an appreciable proportion surviving that did not express Foxp3. In order to investigate the relative stability of the iTreg generated, cells were re-sorted at the end of iTreg culture on the basis of Foxp3gfp expression to ensure a pure population of $Foxp3^+$ iTreg and then re-stimulated with plate-bound anti-CD3 and anti-CD28 for 72 hours (Fig 4.1.A). Viability of cells could be assessed using a fixable live/dead stain as described in Chapter 2 (2.8.2). Cells were then permeabilised and stained for Foxp3.

4.2.1.1 TGF-β improves iTreg stability and survival

To assess the contribution of cytokines relevant to the initial generation of iTreg, 100U/ml IL-2 with or without 5ng/ml TGF-β was also added to some wells at the time of re-stimulation. The survival of the iTreg on re-stimulation was generally poor. Only 6% of cells were alive after re-stimulation in medium alone, whilst more than ⅔ of the surviving $CD4^+$ iTreg had lost Foxp3 expression within 72 hours (Fig 4.1B). The addition of 100U/ml IL-2 did not affect the stability of Foxp3 in these stimulated iTreg (in contrast to previous reports *in vivo* (Chen et al., 2011)), nor did it affect survival. In contrast, the addition of TGF-β to the re-stimulation culture had a marked effect both on the maintenance of Foxp3 expression but also on the survival of these cells (Fig 4.1C) with the cumulative effect that the number of $Foxp3^+$ cells remaining at the end of culture was greatly enhanced. The combination of IL-2 and TGF-β was significantly more effective in promoting cell survival compared to medium alone, consistent with the absolute requirement that Treg have for IL-2. TGF-β is well recognised as being essential for the initial development of iTreg (Chen et al., 2003). In the *in vivo* setting it is unlikely that iTreg would develop or be maintained without sufficient TGF-β in the surrounding milieu.

4.2.2 Cytokine production by iTreg

So the cytokine milieu is able to influence Foxp3 expression and iTreg survival. How do the iTreg themselves contribute to this mix? The demonstration that iTreg produced IFN-γ proved they were capable of production of cytokines traditionally considered pro-inflammatory. Occurring at the time of these experiments was the recognition that GM-CSF was a major pro-inflammatory influence in the development of EAE and therefore had a pathogenic role as well (Codarri et al., 2011).

In order to determine what other cytokines were produced by iTreg, supernatants from a standard suppression assay of iTreg, naïve $CD4^+$ cells, and the two together at a 1:1 ratio were interrogated by flow cytokine bead arrays [kindly performed by Dr Richard O'Connor] (Fig 4.2A). This confirmed the production of IFN-γ, but also identified GM-CSF as being produced by iTreg. Subsequent bead arrays of iTreg supernatant also identified TNF production [data not shown]. Previous assays performed within the laboratory had confirmed that nTreg produced neither GM-CSF nor TNF in appreciable quantities.

4.2.2.1 iTreg produce IFN-γ, GM-CSF and TNF

The cytokine bead arrays were performed on supernatants from suppression assays. The culture conditions included irradiated cells as APC that could feasibly be the source of the pro-inflammatory cytokines. To ensure that cytokine detection was truly from iTreg, Foxp3gfp mice were used to generate iTreg. At the end of iTreg culture, cells were sorted by FACS for $Foxp3gfp^+$ expression to a high purity (Fig 4.2B). These highly pure iTreg were re-stimulated using plate-bound anti-CD3 and anti-CD28 at 2μg/ml in medium alone (Fig 4.2B). After three days in culture, cells were further stimulated with PMA and ionomycin in the presence of brefeldin A for a further four hours to ensure maximal cytokine production, as described (2.8.4). Cells were then permeabilised and stained for Foxp3 expression and intracellular cytokine production.

After 72 hours of culture, iTreg had lost Foxp3 expression in the majority of cells, as seen previously (Fig 4.1B, 4.2C). The intracellular staining confirmed the cytokine bead array data. IFN-γ was produced by approximately 30-40% of cells by both $Foxp3^+$ and $Foxp3^-$ cells. Staining was of a greater intensity within the $Foxp3^-$ population however. GM-CSF was produced in a very similar fashion with both $Foxp3^+$ and $Foxp3^-$ cells contributing but

again with a higher number and greater intensity of staining seen in Foxp3$^-$ cells. TNF production was extremely marked with almost all cells showing some expression. Very little difference between Foxp3$^+$ and Foxp3$^-$ cells was evident in the proportion producing TNF. lL-17 production could not be detected, consistent with previous reports [data not shown] (O'Connor et al., 2010). Though production of TNF was seen in virtually all cells, production of GM-CSF and IFN-γ was more restricted. Of the iTreg producing GM-CSF, approximately half of them were also producing IFN-γ (Fig 4.2D). Thus, approximately 25% of the cells were producing all three cytokines following stimulation with PMA and ionomcyin.

Therefore, these iTreg with proven suppressive capacity in *in vitro* culture (Fig 3.2) were producing three cytokines that are all considered to be pro-inflammatory.

4.2.2.2 Pro-inflammatory cytokines are produced during primary iTreg generation

In the experiments above, most cytokine expression was by the Foxp3$^-$ cells (with the exception of TNF). It was conceivable that iTreg do not produce any of these cytokines whilst expressing Foxp3 and that it was only on re-stimulation - with the associated loss of Foxp3 expression - that this occurs. To clarify, cytokine production by iTreg was investigated during their generation.

Foxp3gfp$^-$ CD4$^+$ cells were cultured for five days with plate-bound anti-CD3, anti-CD28, IL-2, and TGF-β, in standard iTreg conditions. On each day, cells were permeabilised and stained for Foxp3 expression. At the same timepoints, supernatants were sampled for quantification of TNF, GM-CSF and IFN-γ by ELISA.

Upregulation of Foxp3 expression occurred rapidly, with almost 30% of cells expressing Foxp3 after only 48 hours in culture (Fig 4.3A). By 72-96 hours, almost full conversion to an iTreg phenotype had occurred but importantly this was also maintained at a high level to the end of the culture at day five. This implies that any cytokine production between these timepoints was from cells expressing Foxp3.

The production of all three cytokines measured followed an upward trend from day three onwards (Fig 4.3B) when nearly all cells were Foxp3$^+$, strongly suggesting it was the iTreg

responsible for production. Thus iTreg were producing a variety of pro-inflammatory cytokines whilst co-expressing Foxp3. To further confirm this, cells were taken at the end of iTreg culture (day five) and stained for intracellular cytokines. Production of IFN-γ, GM-CSF and TNF was evident from the $Foxp3^+$ population (Fig 4.3C).

4.2.2.3 Cytokine production by iTreg during secondary stimulation

Whilst demonstration of cytokine expression by flow cytometry confirms the production by the iTreg population, it cannot quantify the levels being produced, or whether they are related to T cell stimulation. To clarify the above, sorted Tg4xFoxp3LuciDTR-4 $Foxp3^+$ iTreg were cultured with irradiated splenocytes in the presence of varying concentrations of MBPAc1-9 from 100μM to 0.3μM. After 48 hours, supernatants were then analysed by ELISA for the production of IFN-γ, GM-CSF, and TNF.

iTreg responded to peptide stimulation with the production of all three cytokines analysed (Fig 4.4). Production of IFN-γ was in the 1-10ng/ml range across experiments. Far more potent stimulation using the altered peptide ligand, MBPAc1-9 (4Tyr), at a range of concentrations did not lead to greater quantities detectable of IFN-γ [data not shown]. Conversely GM-CSF was strongly produced at low levels of antigen stimulation such that 10μM was sufficient to reach the upper limit of detection by ELISA, 500pg/ml. TNF production also demonstrated a clear dose response with no evidence of a maximal amount reached, even at very high levels of stimulation (100μM).

Thus iTreg are capable of producing these pro-inflammatory cytokines on encounter with antigen *in vitro*.

4.2.3 Cytokine production by iTreg in *in vitro* suppression assays

Having established that the iTreg do produce IFN-γ, GM-CSF and TNF, the next logical step was to determine whether the cytokine profile of iTreg had any influence on their ability to suppress naïve T cell responses to peptide stimulation. Utilising the *in vitro* suppression assays first visited in Chapter 3, the production of cytokines was assessed by ELISA. The relative contribution of naïve T cells and iTreg to the cytokine production was discerned using conditions with either naïve T cells or iTreg alone. Further extending the use of the *in vitro* system from chapter 3, utilizing the two transgenic strains with different TCR

reactivities allowed the TCR of naïve T cells to be stimulated without further stimulation of the iTreg. In this manner, it was possible to distinguish the relative contributions of naïve T cells and iTreg by comparing the cytokine production between assays where both cell types were stimulated, or just the naïve T cells.

4.2.3.1 IFN-γ is produced by iTreg

IFN-γ was produced by iTreg in the 0.5-1ng/ml range in the *in* vitro suppression assays. The reduction in IFN-γ concentration as the proportion of iTreg was decreased strongly suggested that the iTreg were the main source (Fig 4.5A). Naïve T cells alone produced undetectable levels of IFN-γ. This may relate to the low level of cells ($2x10^4$ cells per well) or that at 72 hours, production was only just commencing from naïve T cells.

4.2.3.2 TNF production is not specific to iTreg

TNF production was also analysed at 72 hours. Consistent with the intracellular cytokine staining data, iTreg and naïve T cells produced roughly equivalent amounts of TNF when cultured alone or in co-culture, with no evidence of an increase or decrease in concentration as iTreg numbers dwindled (Fig 4.5B).

4.2.3.3 GM-CSF production by iTreg occurs early in *in vitro* suppression

Given the recent importance attached to GM-CSF as a key pro-inflammatory cytokine in EAE (El-Behi et al., 2011), GM-CSF production was investigated in greater detail. Sorted Tg4xFoxp3LuciDTR-4 iTreg were co-cultured with naïve Tg4 $CD4^+$ cells with irradiated APC and 10μM MBPAc1-9. After 24, 48, 72, and 96 hours of culture, levels of GM-CSF were analysed by ELISA. At 48 hours, iTreg were producing greater quantities of GM-CSF than naïve T cells, an effect borne out by the reduction in concentration seen as iTreg were diluted out of the co-culture (Fig 4.5C). After 72 hours of culture, the situation was reversed; naïve T cells were now producing greater quantities of GM-CSF than iTreg, with no distinguishable impact of co-culture at any ratio of iTreg (Fig 4.5D). Production of GM-CSF thus appeared to 'switch' from iTreg at the beginning of culture to the naive T cells at the later time points. Levels of GM-CSF at 24 hours were very similar to 48 hours, whilst levels at 96 hours were similar to 72 hours [data not shown].

4.2.3.4 IFN-γ production by iTreg was independent of ongoing TCR stimulation

Naïve 2D2 $CD4^+$ cells were co-cultured with Tg4 iTreg and stimulated with $pMOG_{35-55}$ with, or without, Tg4 iTreg cognate antigen (MBPAc1-9). The absence of iTreg stimulation did not particularly impact upon the pattern of production (Fig 4.6A,B). Notably, production of IFN-γ by either iTreg alone and unstimulated cells was similar regardless of whether one or both antigens were present. This suggests that the IFN-γ production was not related to ongoing stimulation by iTreg antigen (where present), but rather that it was a residual response to the initial TCR stimulation required for iTreg generation.

4.2.3.5 GM-CSF production by iTreg required ongoing TCR stimulation

To compare the need for ongoing TCR stimulaton for IFN-γ production with GM-CSF production, Tg4xFoxp3LuciDTR-4 iTreg were co-cultured with naïve OT-II $CD4^+$ cells in the presence of both MBPAc1-9 and $pOVA_{323-339}$ or with $pOVA_{323-339}$ alone. iTreg alone produced similar quantities of GM-CSF after 48 hours to that seen in the direct suppression assay (Fig 4.6C) whilst naïve OT-II T cells had undetectable production. Again, the level of GM-CSF in co-culture reduced with fewer iTreg. Culture in the absence of MBPAc1-9 completely removed GM-CSF production within the range detectable (lower limit of ELISA 4pg/ml) (Fig 4.6D). Why GM-CSF production was absent from naïve OT-II T cells but present when naïve Tg4 T cells were used is unclear, and cannot be ascertained from the data available. Thus GM-CSF was produced by iTreg only when they received ongoing TCR stimulation, in contrast to IFN-γ production.

Neither IL-10 nor TGF-β could be detected using commercially available ELISA kits [data not shown]. Further attempts to assess TGF-β using cytokine bead arrays were also unsuccessful [data not shown]. There is not yet a commercially available means to reliably distinguish IL-35 production, either by ELISA or by flow cytometry, and so this cytokine was not investigated.

4.2.4 Cytokines and suppression

4.2.4.1 IFN-γ and suppression

Production of IFN-γ by iTreg regardless of the presence of ongoing TCR stimulation (Fig 4.6B) may suggest a role for IFN-γ in suppression. To assess this, three approaches were taken: supplementation with, or blockade of, IFN-γ, and the use of IFNγ$^{-/-}$ iTreg. Tg4Foxp3LuciDTR-4 iTreg were co-cultured with naïve Tg4 CD4^{+} cells. The addition of 100ng/ml IFN-γ to the culture medium appeared to reduce ^{3}H-thymidine incorporation of naïve cells (Fig 4.7A) but after this effect was taken into account, no significant effect on suppressive activity was evident (Fig 4.7B). Addition of anti-IFN-γ at 10ng/ml to suppression assays did not significantly alter the ^{3}H-thymidine incorporation in naïve cells (Fig 4.7C) and the suppressive capacity of iTreg was again unaffected (Fig 4.7D). Furthermore, Tg4 IFN-γ $^{-/-}$ iTreg were able to suppress as efficiently as Tg4 IFN-γ $^{+/+}$ iTreg (Fig 4.7E, F). There was therefore no apparent role for IFN-γ in this model of *in vitro* suppression.

4.2.4.2 GM-CSF and suppression

The presence of GM-CSF boosted the incorporation of ^{3}H-thymidine by naïve cells significantly (Fig 4.8A), whereas anti-GM-CSF had the reverse effect (Fig 4.8C). However, naïve cells were equally suppressed regardless of the presence of additional GM-CSF (Fig 4.8B), or anti-GM-CSF (Fig 4.8D) demonstrating that not only is GM-CSF production unlikely to mediate suppression, but also that more responsive cells are not more resistant to suppression (as previously investigated in 3.2.3.5). Various concentrations of GM-CSF were tested to assess whether there was a dose-response effect but suppressive capacity was equivalent across all concentrations tested [data not shown]. So despite the evident production of GM-CSF by iTreg on re-stimulation, there did not appear to be any evidence of effect in *in vitro* suppression.

4.2.4.3 TNF and suppression

Given the approximately equal production of TNF by iTreg and naïve cells as measured by ELISA, it was unlikely that TNF would contribute significantly to suppression. Addition of anti-TNF at 1μg/ml to suppression assays had no impact on ^{3}H-thymidine incorporation by

either iTreg or naïve cells (Fig 4.9A), nor was there an effect on suppressive capacity (Fig 4.9B).

4.2.4.4 IL-10 and TGF-β and suppression

Despite the production of the pro-inflammatory cytokines IFN-γ, GM-CSF, and TNF, iTreg remained suppressive and neutralization of these cytokines did not impact at all upon suppression *in vitro*. Though unable to detect either IL-10 or TGF-β by ELISA, these cytokines could be contributing to suppressive function.

To determine the effect of IL-10 potentially produced by the iTreg, suppression assays were performed in the presence or absence of 25μg/ml anti-IL-10R. No effect on the incorporation of ^{3}H-thymidine by either iTreg or naïve cells was evident (Fig 4.10A) nor was there any significant difference between the two groups (Fig 4.10B).

TGF-β can be either free or membrane bound (Miyazono et al., 1993), so using neutralizing anti-TGF-β may not prevent signaling through the TGF-β receptors where cell contact is required. As an alternative, TGF-β signaling can be blocked by inhibiting activin receptor-like kinase (ALK)5 (a domain of the Type I TGF-β receptor) (Callahan et al., 2002). To confirm the functionality of the ALK5 inhibitor, SB431542 (Imman et al., 2002), naïve Tg4xFoxp3LuciDTR-4 CD4^{+} Foxp3gfp^{-} cells were cultured in IL-2 and TGF-β as per the standard iTreg generation protocol. After two days, Foxp3^{+} expression was over 40% in the control wells containing vehicle (DMSO), but effective inhibition of Foxp3 expression was apparent using 10μM of SB431542 (Fig 4.11A). At the end of the iTreg culture, nearly all cells were Foxp3^{+} in the absence of the inhibitor, whilst only a small proportion (<10%) had any degree of Foxp3 staining in the treated wells. Thus, SB431542 effectively prevented functional TGF-β signaling from generating iTreg.

Suppression assays were then carried out with the addition of either DMSO (as a vehicle control) or 10μM SB431542 to all wells. No effect on the incorporation of ^{3}H-thymidine by either naïve cells or iTreg alone was seen (Fig 4.11B), but suppression was less effective in the ALK5 inhibited group, with loss of a suppressive effect at a higher ratio of iTreg:naïve cells (Fig 4.11C). Measurement of IL-2 showed that production of IL-2 was greater by naïve cells in the treated wells (Fig 4.11D) with the reduction in IL-2 concentration mirroring the suppressive pattern (Fig 4.11E). The reduced efficacy of suppression may be due to the

greater production of IL-2 by naïve cells, implying that there may be no direct requirement for iTreg-derived TGF-β other than its effect on IL-2.

To further clarify the role of TGF-β, the inhibitor was added at the onset of the suppression assay, or at 24 and 48 hours. Inhibition of TGF-β signaling from the onset of culture reduced the ability of iTreg to suppress the incorporation of ^{3}H-thymidine as seen previously (Fig 4.11B, Fig 4.12A). This effect was lost if addition of SB431542 was delayed until 24 hours into the co-culture, when suppression by iTreg was as effective as in cultures without the inhibitor (Fig 4.12B). This kinetic is similar to that seen in Chapter 3 with the depletion of iTreg using diphtheria toxin (Fig 3.13) and implies that, whilst TGF-β signaling by iTreg is very important early in *in vitro* suppression, both are dispensable beyond 24 hours. This hints at an immediate effect in the co-culture system that persists beyond the removal of the iTreg or the signaling mechanism that initiated this change.

4.2.5 *In vitro* suppression of effector T cells by iTreg

Having demonstrated that iTreg are efficient at suppressing naïve cells (^{3}H-thymidine incorporation and IL-2 production), it was essential to determine whether this was also true for cells already differentiated towards an effector phenotype (as described in 2.6.5). Naïve CD4^{+} Tg4 cells were cultured *in vitro* for 3-5 days in appropriate cytokine cocktails to polarise them towards a Th1, Th2, or Th17 phenotype, or towards GM-CSF secreting T cells (so-called 'ThGM-CSF', (Codarri et al., 2011). The production of IFN-γ, IL-17 and GM-CSF by Th1, Th17 and ThGM-CSF was assessed by intracellular staining for flow cytometry, and ELISA of supernatants. The production of IL-4 and IL-13 by Th2 cells was assessed by ELISA alone. Polarised cells were then co-cultured with iTreg at the same proportions as naïve cells and ^{3}H-thymidine incorporation assessed at 96 hours (Fig 4.13A). Suppression of the incorporation of ^{3}H-thymidine was evident for naïve cells as seen previously. Th1, Th2, and Th17 polarisation did not affect the absolute ability of iTreg to suppress though in all cases this was less efficient at lower ratios of Treg:responder cell than with naïve responders (Fig 4.13B). Incorporation of ^{3}H-thymidine by 'ThGM-CSF' cells was unaffected by the presence of iTreg, even at a high Treg:ThGMCSF ratio.

As one of the hallmarks of effector T cell differentiation is the production of certain 'signature' cytokines, the ability of iTreg to impact on these was also investigated. Supernatants from suppression assays at 72 hours were analysed by ELISA (Fig 4.14). The

production of IFN-γ by Th1 cells was not suppressed (Fig 4.14A), nor was the production of IL-17 by Th17 cells (Fig 4.14B) or GM-CSF by ThGM-CSF (Fig 4.14D). IL-13 production by Th2 cells was also unaffected. The only cytokine obviously suppressed by iTreg was IL-4 production by Th2 cells (Fig 4.14C). This has potential implications for the optimal usage of iTreg in therapeutic applications. One important caveat is that in both the IFN-γ and GM-CSF ELISA, multiple wells contained cytokine concentrations in excess of the maximum detectable. The production of IFN-γ by naïve cells alone fell within the range detectable in some wells, so it is likely that the absence of suppression of IFN-γ is a true finding. The production of GM-CSF by naïve cells alone exceeded the level detectable except where Treg:ThGMCSF was 1:1. It is feasible that a suppressive pattern may have been seen if the true concentrations could be determined. The observed absence of suppression of ^{3}H-thymidine incorporation by ThGM-CSF makes this less likely.

4.3 Discussion

4.3.1 iTreg stability and cytokine influence

This chapter begins by examining the stability of the Foxp3$^+$ iTreg generated in this thesis. These iTreg were shown to rapidly lose Foxp3 expression on TCR re-stimulation with anti-CD3 and anti-CD28, but this effect was reduced in the presence of TGF-β. Importantly, cell survival was also enhanced in the presence of TGF-β such that the overall number of Foxp3$^+$ cells was markedly increased (Fig 4.1C). This stabilizing effect of TGF-β has been described (Selvaraj et al., 2007, Marie et al., 2005) though the beneficial effect on cell survival was not highlighted. Re-stimulation with antigen has also been shown to hasten loss of Foxp3 expression *in vivo* though IL-2 signaling was proposed to influence Foxp3 stability (Chen et al., 2011). In these *in vitro* experiments, IL-2 alone was insufficient to maintain Foxp3 expression when cells were re-stimulated, showing no demonstrable improvement over unsupplemented medium. The loss in Foxp3 expression was rapid with only 30% of sorted iTreg remaining Foxp3$^+$ after 72 hours of culture, unless TGF-β was present.

Epigenetic modifications have a significant effect on the stability of Foxp3 expression. Notably the Treg-specific demethylation region (TSDR) is completely demethylated in nTreg but has variable methylation in iTreg. In iTreg generated in the presence of TGF-β and anti-CD3 (i.e. a similar protocol to the one used here), the TSDR was almost fully methylated (Lal et al., 2009). An alternate means of iTreg generation in the absence of any pro-inflammatory signals showed almost complete demethylation and much greater stability of Foxp3 expression (Polansky et al., 2008). The epigenetic modifications of the iTreg generated in this thesis were not analysed, but this is an area currently undergoing investigation.

One group found that iTreg rapidly lost Foxp3 expression in an *in vivo* model of GVHD (Beres et al., 2010). Those iTreg were generated using a three day protocol with 60-70% Foxp3 expression at the end of culture, but the iTreg produced no IFN-γ at the end of primary generation. The infused iTreg did not persist *in vivo* beyond 14 days but did convert to a Foxp3$^-$ IFN-γ$^+$ phenotype. Despite describing these cells as pro-inflammatory, they were unable to provoke GVH disease when infused alone. That same group also investigated the role of retinoic acid in iTreg generation and found no positive effect on the

maintenance of Foxp3 expression *in vivo*, or impact on conversion to IFN-γ production. The absence of effect in that model was presumed due to the magnitude of inflammation caused by total body irradiation. IL-6 was suggested as a factor reducing Foxp3 stability, and the addition of anti-IL-6 appeared to promote iTreg induction *in vivo*.

A second group also demonstrated rapid loss of Foxp3 expression and inability to prevent murine GVHD (Koenecke et al., 2009) though the iTreg generation protocol used a novel method of 'cluster disrupted' dendritic cells, with Foxp3 expression in only 12% of $CD4^+$ T cells at the end of six days culture. Given the low yield and long culture (despite the presence of RA as well), it is questionable how similar those 'iTreg' are to these generated herein.

The inability of iTreg to persist *in vivo* suggested a relatively short lifespan (Selvaraj et al., 2007) though a subset of those iTreg persisted, maintained their Foxp3 expression, and appeared to migrate to the bone marrow. On investigating the iTreg generated in this thesis, donor Treg accounted for 2-3% of the $CD4^+$ population within the bone marrow though Foxp3 expression was not analysed [data not shown]. This implies that there are functional similarities between these iTreg and those described by other researchers.

In any iTreg investigation, the protocol used to induce expression of Foxp3 is likely to highly influence the cells that are subsequently produced. Though it is generally accepted that sub-optimal co-stimulation through the TCR in the presence of IL-2 and TGF-β leads to iTreg generation, the exact concentrations used vary between almost every laboratory. This is an important caveat when comparing the behaviour of the iTreg generated here to those of other groups. The very high induction level of Foxp3 after as little as 72 hours compares favourably to that reported in the original description of the protocol used (Davidson et al. 2007), but the rapid loss of Foxp3 following the removal of TGF-β suggests this may be at the expense of stability.

4.3.2 Cytokine Production by iTreg

The ability of iTreg to produce pro-inflammatory cytokines obviously has significant therapeutic implications. The assumption is that a cell producing such mediators must therefore contribute to the ongoing pathology. That this is not always true can be seen in

some clinical scenarios e.g. the worsening of MS in a subset of patients on anti-TNF medication (Lenercept MS Study Group, 1999).

4.3.2.1 The role of IFN-γ in suppression

The iTreg generated here were capable of IFN-γ production, which was evident during their primary generation (Fig 4.3B). Re-stimulation with antigen produced IFN-γ in the 5-20ng/ml range (Fig 4.4) whilst production appeared to be completely dispensable in the suppression of ^{3}H-thymidine incorporation and reduction in IL-2 concentration. IFN-γ $^{-/-}$ iTreg were functionally equivalent to IFN-γ replete iTreg *in vitro*.

That iTreg were capable of pro-inflammatory cytokine production as IFN-γ was demonstrated initially on *in vitro* secondary stimulation of iTreg (O'Connor et al., 2010), and subsequently confirmed as occurring during initial iTreg generation as well. The production of IFN-γ was associated with upregulation of Tbet, though this did not occur in IFN-γ deficient iTreg unless an additional source of IFN-γ was provided. Despite their production of IFN-γ, those iTreg were still suppressive in *in vitro* co-culture with naïve T cells, and in an *in vivo* model of EAE involving the transfer of naïve myelin-responsive Tg4 cells (though they could not suppress a passive model of EAE whereby transfer of polarised Tg4 Th1 cells caused disease). Importantly, the iTreg were not as pathogenic as effector cells in the same model when transferred alone, requiring much higher numbers and producing only mild disease. The lack of pathogenicity of the IFN-γ producing 'Treg' has now been shown in several reports. Indeed, a necessity for IFN-γ to exert suppressive function has also been reported (Sawitzki et al., 2005) with anti-IFN-γ treatment leading to a rapid rejection of skin grafts in tolerised mice with circulating alloantigen specific Treg. A similar graft loss was seen using IFN-γ deficient mice implying that IFN-γ production plays a vital role in maintaining graft tolerance.

Other evidence for an anti-inflammatory role for IFN-γ has been suggested by an immunoregulatory effect at low concentration (Flaishon et al., 2002) and the impact of STAT1 deficiency (through which IFN-γ signals) in reducing the nTreg population (Nishibori et al., 2004). A different group demonstrated that the development of iTreg (or adaptive Treg) was enhanced in the absence of IFN-γ, particularly if autocrine secretion was abrogated (Chang et al., 2009). The induction of Treg in wild-type mice was very poor

there, however (only 8%). Those same authors did not demonstrate the abnormality in the nTreg population.

IFN-γ^+ Treg have also been reported in the clinical setting, and related to regulatory function (Daniel et al., 2008) as higher numbers were identified in post-renal transplant patients with good graft function than in those with poor graft function. Similarly, suppressive IFN-γ^+ Treg have been identified in patients with autoimmune diabetes mellitus (McClymont et al., 2011) and identified as adaptive Treg.

Thus, the production of IFN-γ by Treg is not in doubt, though whether it is crucial for suppression seems controversial. The data presented here confirm the production of IFN-γ both during initial iTreg generation (Fig 4.3) and on re-stimulation (Fig 4.4). The quantities of IFN-γ produced on re-stimulation are an order of magnitude lower than produced by polarised Th1 cells (Fig 4.14A) and may influence the regulatory effect of 'low-dose' IFN-γ (Flaishon et al., 2002). However, in the *in vitro* system examined, the effects of IFN-γ did not appear relevant. Supplementing the low concentration of IFN-γ to 100ng/ml had no effect on the suppression of ^{3}H-thymidine incorporation, nor did neutralization of IFN-γ through the use of antibodies (Fig 4.7). More convincingly, iTreg generated from IFN-$\gamma^{-/-}$ mice were as suppressive as iTreg from IFN-γ replete mice. Clearly IFN-γ is dispensable in this assay tested, but may have a relevance in other models.

4.3.2.2 The role of TNF in suppression

The production of TNF by iTreg has also convincingly been shown. Stimulation *in vitro* with PMA and ionomycin led to over 80% of cells staining positive for TNF (Fig 4.2C). Production was shown in primary generation (Fig 4.3B) while re-stimulation with cognate antigen also demonstrated TNF production (Fig 4.4) with no evidence of a maximal concentration even with high doses of antigen (up to 100μM). Neutralisation of this TNF did not impact on the suppression of the incorporation of ^{3}H-thymidine, nor did there appear to be any effect on the production of TNF by naïve T cells (Fig 4.5B, 4.9). What reported role, if any, does TNF have in suppression by Treg?

Production of TNF has been reported in the context of human Foxp3$^+$ cells producing pro-inflammatory cytokines in patients with Chagas disease (de Araujo et al., 2012) though it is notable that those 'Treg' produced a wide variety of other cytokines, including IL-6. A role

for TNF production by Treg has not been identified nor has it been extensively investigated, but multiple examples of Treg being influenced by TNF appear in the literature.

As discussed in the introduction (section 1.4.6), TNF signals through two receptors, with expression of TNFR2 limited to lymphoid cells. Expression of TNFR2 has been related to suppressive function (Chen et al., 2008). Release of the TNFR2 receptor by nTreg has been suggested as a mechanism of suppression through binding free TNF and thereby limiting its pro-inflammatory potential (van Mierlo et al., 2008). TNF has also been proposed as the mechanism by which the presence of effector cells is paradoxically beneficial to Treg function (Grinberg-Bleyer et al., 2010).

Using TNFR2-deficient mice, TNF was shown to be necessary for *in vivo* suppression by nTreg of the development of colitis in $RAG^{-/-}$ mice receiving wild-type effector cells (Housley et al., 2011). In contrast, iTreg from TNFR2-deficient mice were able to suppress colitis as efficiently as wild type iTreg, implying that TNF signaling was redundant for this effect. Of note, stimulation of the TNFR2 deficient nTreg in the presence of TGF-β (i.e. partially replicating the conditions needed for iTreg generation) restored their ability to suppress colitis.

The converse has been suggested in humans, with TNF inhibiting *in vitro* nTreg (Valencia et al., 2006) and anti-TNF therapy leading to an increase in *in vivo* generated Treg (Nadkarni et al., 2007). Treg from patients treated with anti-TNF antibody appeared to be more effective in suppressing Th17 responses through IL-6 inhibition than Treg from untreated patients (or healthy controls) (McGovern et al., 2012) though no effect was seen on Th1 responses, or in patients treated with the TNF receptor blocker, etanercept. All of these reports are tempered by the difficulty in identifying and isolating human Treg, in which Foxp3 is not a definitive marker.

Thus, TNF has a definite impact on Treg function, though its influence appears both positive and detrimental, depending on the circumstance and outcome measure. The presence of TNFR2 on Treg implies that TNF would have a stimulatory function, whilst observed differences between nTreg and iTreg may reflect the different *in vivo* localizations of these cells. Some of the conflicts in reports have been attributed to the quantity of TNF used in assays. Removal of suppression by Treg was not seen at 5ng/ml but was present at 50ng/ml TNF (Ehrenstein et al., 2004). In the re-stimulation experiments here (Fig 4.4), up to

300pg/ml of TNF were produced, suggesting the production of TNF by iTreg would have no impact on suppression.

That TNF boosts and promotes nTreg in the murine system (Chen et al., 2007) may imply that the production of TNF by the generated iTreg is a means of promoting nTreg, thereby expanding the regulatory population at the site of inflammation. The absence of any differential effect between $Foxp3^+$ and $Foxp3^-$ cells may simply reflect activation of the cells, with expression of Foxp3 *per se* not influencing TNF production.

The above findings may also explain the lack of effect of anti-TNF in the *in vitro* suppression assay. If the beneficial effect of TNF *in vivo* is to enhance the nTreg population, then the addition of antibody to an *in vitro* system lacking nTreg would be expected to show no effect. Clearly, in these assays, nTreg play no role in the mediation of suppression but this is unlikely to be the case *in vivo*.

Finally, TNF can be either soluble or in a transmembrane form (Black et al., 1997). One study involving tolerogenic DC-driven Treg demonstrated that Treg responded to the transmembrane form but not soluble TNF (Kleijwegt et al., 2010). This would further imply that the secretion of TNF by iTreg has no autocrine effect. Whether nTreg would still show an expansion remains unknown. Similarly, the very high secretion of TNF by iTreg may be sufficient to provoke the same response in the absence of the transmembrane form, as the TNFR2 binds with a lower affinity. Investigating the presence of transmembrane TNF on iTreg and modulating the quantities of TNF in nTreg assays may clarify the role, if any, played by TNF in iTreg-mediated suppression.

4.3.2.3 The role of GM-CSF in suppression

The recent identification of GM-CSF as potentially the key pathogenic T-cell derived cytokine in EAE (Codarri et al., 2011) led to extensive investigation of the significance of GM-CSF in the *in vitro* iTreg suppression assay. Initial experiments were designed to assess whether iTreg suppressed GM-CSF production by naïve T cells. Instead they demonstrated that not only was suppression of GM-CSF absent (Fig 4.5C,D) but iTreg preceded naïve T cells in their GM-CSF production, with appreciable quantities after 48 hours of stimulation. Clarifying this, intracellular staining confirmed the production of GM-CSF by iTreg (Fig 4.2), as did ELISA analysis during primary and secondary stimulation (Fig 4.3, 4.4).

Addition of GM-CSF to suppression assays had a significant impact on the initial proliferation of naïve T cells, possibly mediated via an effect on the APC used in the cultures, whilst the addition of anti-GMCSF had the opposite effect. No impact was seen of supplementation or blockade of GM-CSF on the ability of iTreg to suppress either ^{3}H-thymidine incorporation or IL-2 (Fig 4.8). Thus the absolute level of GM-CSF in culture did not appear relevant to either readout of iTreg suppression. This further supports the findings in Chapter 3 (Fig 3.10) that the ability of iTreg to suppress is not diminished if the responding T cells are proliferating at a high level. The production of GM-CSF by iTreg appears to be a short-lived phenomenon, occurring early during re-stimulation of these cells and diminishing within 72 hours (Fig4.5C, D), with no clear impact on the suppressive capacity of iTreg certainly *in vitro*. What contribution might the presence of GM-CSF make otherwise?

As discussed in the introduction (section 1.4.7) GM-CSF is a relatively pleiotropic cytokine with multiple actions on different cell lineages. Two of these actions are of particular relevance here – the description of GM-CSF as the vital pro-inflammatory cytokine in EAE (Codarri et al., 2011) and its role in the maturation and activation status of DCs. This latter is of crucial importance in considering how iTreg may influence the pro-inflammatory response, and themselves be influenced by APC.

In vitro-generated mature bone marrow-derived DCs have been demonstrated to reverse nTreg suppression in assays similar to those performed here, with TLR-activation of the DCs prior to the assay also causing proliferation within the nTreg (Kubo et al., 2004). Notably, the established *in vitro* protocol for maturation of the DC uses GM-CSF (Lutz et al., 1999). A simple role for iTreg produced GM-CSF could be in the maturation of DC. However the last decade or so has witnessed a burgeoning in the classification of sub-types of DCs, identifiable by various markers. Multiple subsets are now recognized, broadly classed as resident, migratory, and monocyte-derived (Zhan et al., 2012). Within these, multiple different types of DC can be recognized. For the sake of brevity, only a few examples of the influence of GM-CSF will be considered.

GM-CSF likely has an impact on the ability of $CD8^{+}$ DC to co-present antigen on both MHC Class I and II (Sathe et al., 2011), with cross-presentation greatly reduced in GM-CSF deficient mice. Again, this is partly related to GM-CSF having a maturation role for DC.

Using a murine model of myasthenia gravis, one group demonstrated that GM-CSF-treated mice had a reduced production of TNF, IL-1β and IL-12 by splenic DC, and an expansion of $Foxp3^+$ cells within the spleens of treated animals (Sheng et al., 2008). Conversely, GM-CSF production by Th17 cells was associated with pro-inflammatory DC (Campbell et al., 2011).

Similarly to the paradox of IFN-γ, GM-CSF may have a 'dose effect'. High concentrations of GM-CSF in *in vitro* DC culture generate pro-inflammatory mature DCs whilst lower concentrations may lead to an immature DC phenotype with regulatory properties (Lutz et al., 2000). Thus the low-level production of GM-CSF by iTreg may permit development of this immature and more tolerogenic DC phenotype.

The proposed model, then, is that $Foxp3^+$ cells are induced from naïve cells in the presence of TCR stimulation (via antigen) and TGF-β, in the presence of tolerogenic DCs. The low level production of both IFN-γ and GM-CSF then maintain these DCs with a tolerogenic phenotype. This situation can be disturbed by antigen presentation in the context of 'danger signals' (i.e. pathogen associated molecular patterns, PAMPs) leading to differentiation of naïve T cells into effectors and a subsequent shift in the concentrations of these pro-inflammatory cytokines. This leads to maturation of other DCs 'breaking' the tolerogenic phenotype and triggering a full inflammatory response. The short-lived nature of the iTreg cytokine production may prevent high pro-inflammatory concentrations from being reached in the absence of further pathogenic signaling. This could feasibly be modeled *in vitro* with supplementation of exogenous cytokines to DC cultures in the presence or absence of Treg, and the subsequent ability of those DC to stimulate naïve or effector cells.

4.3.2.4 The role of 'regulatory' cytokines in suppression

Investigation of the regulatory cytokines produced by iTreg was more complex. Very little IL-10 was detected on intracellular cytokine staining [data not shown] neither was a cytokine bead array for IL-10 convincing of any production (Fig 4.2A). Similar problems were encountered in trying to analyse the production of TGF-β. Both ELISA and cytokine bead arrays were unsuccessful in identifying TGF-β above quantities found in unsupplemented medium. Antibodies for detecting IL-35 are not yet available and so detection of this by flow cytometry was also impossible.

With regards effects in the suppression assays, IL-35 was not analysed for its effects in this *in vitro* model for the same reasons as outlined above. This would be an obvious and essential experiment to perform if IL-35 neutralisation becomes available. Neutralisation of IL-10 had no effect.

As discussed above, TGF-β is a source of many conflicting reports, with no clear consensus on whether it is dispensable for Treg function. Using an inhibitor of TGF-β signaling pathways in the *in vitro* suppression assay indicated that TGF-β signaling was not absolutely required at a Treg:naïve ratio of 1:1 (Fig 4.11). However the suppressive capacity of the iTreg was greatly reduced with loss of suppression at Treg:naïve T cell ratios as high as 1:2. It appears that here, TGF-β plays either a major supporting, or amplifying role for suppression by the iTreg.

One of the unexpected results from the addition of the inhibitor SB431542 to the suppression assays was the observation that naïve $CD4^+$ cells produced more IL-2. As one of the proposed mechanisms of Treg function is via the high affinity IL-2 receptor, CD25, 'mopping up' IL-2 and thus preventing its use by clonally expanding cells, any additional factors that influence IL-2 production could impact upon suppression. This observation that ALK5 inhibition enhances IL-2 could partly explain the 'soluble mediator' experiments in which TGF-β was considered to be the key element. Against this as a mechanism is the absence of impact upon suppression when IL-2 production was much greater (section 3.2.3.5). The demonstration using CFSE staining that naïve T cells still initially proliferate (section 3.2.4.2) but then die may actually support the IL-2 theory. If the production of IL-2 occurs early, and so does scavenging of this IL-2 by iTreg, naïve T cells would initially proliferate but then be deprived and subsequently die. Though increases in IL-2 production by naïve T cells may not overcome the consumption by iTreg, interfering with TGF-β signaling could feasibly also impact on the ability of iTreg to utilize IL-2 given the highly pleiotropic nature of the cytokine. Teasing apart these differing effects would be another point meriting further study.

It is noteworthy that the *in vitro* assay is very time-sensitive to the addition of the ALK5 inhibitor. Preventing signaling through TGF-β had no impact on the assay if it was delayed by as little as twenty-four hours (Fig 4.12). The parallel with the limited need for iTreg to be physically resident within the co-culture demonstrated in depletion experiments (Fig 3.13) implies that iTreg have their effect early in culture, likely to require TGF-β signaling.

4.3.3 *In vitro* suppression of polarised effector T cells

To be of true therapeutic utility, Treg must be able to suppress ongoing inflammation from fully differentiated effector cells, rather than naïve T cells undergoing primary TCR stimulation as the iTreg are re-stimulated. The ability of the iTreg to suppress different effector cells was investigated using the same suppression assay as previously. Incorporation of ^{3}H-thymidine was suppressed in Th1, Th2, Th17 and naïve T cells. Cells polarised towards the production of GM-CSF, so-called 'Th-GM-CSF' were more resistant to suppression. Given the production of pro-inflammatory cytokines by the iTreg, and the signature cytokines known for the various effector sub-types, the reduction in signature cytokine was then measured for each cell type. As iTreg also secrete IFN-γ even without further antigen stimulation (Fig 4.5), suppression of IFN-γ production by Th1 cells seemed unlikely and this was indeed the case (Fig 4.14A). IL-17 production by Th17 cells was not notably reduced at even the higher concentrations of iTreg, while GM-CSF secretion by 'ThGM-CSF' cells was only affected at a 1:1 ratio. Of all the effector-specific cytokines, a pattern of suppression was only seen in the production of IL-4 by Th2 cells, whilst IL-13 production was unaffected. Thus the only pro-inflammatory effector cytokine in which iTreg had a clear and suppressive effect was IL-4. Given the production of IFN-γ by these iTreg, they display a 'Th1-like' cytokine profile and so might be expected to be more proficient against Th2 cells. Indeed, previous reports have demonstrated superiority of Treg against Th2-mediated disease (Josefowicz et al., 2012). This pattern fits with the theory that nTreg are directed towards self-antigen (by definition as they arise in the thymus with self-reactive TCR) whereas iTreg can arise in response to foreign innocent antigens i.e. allergens. The low concentrations of IFN-γ and TNF produced by iTreg would be unlikely to promote a true 'Th1 response' but may be sufficient to inhibit Th2 differentiation by responding naïve T cells.

4.3.4 Concluding remarks

The stability of Foxp3 expression by iTreg is dependent on the surrounding cytokine environment (Fig 4.15). The persistence of TGF-β is required to maintain Foxp3 at high levels. iTreg produced the pro-inflammatory cytokines IFN-γ, GM-CSF and TNF. IFN-γ and GM-CSF were reduced on secondary stimulation if the re-stimulation medium contained TGF-β. Despite production of these cytokines, iTreg remained suppressive of ^{3}H-thymidine incorporation and reduced IL-2 in *in vitro* cultures with both naïve and effector T cells. Surplus or neutralization of any of the pro-inflammatory cytokines has no effect on suppression, nor did neutralization of IL-10. Inhibiting TGF-β signaling through the ALK5 inhibitor, SB431532, did reduce suppressive capacity though did not abrogate it completely. This effect only occurred if inhibition was in the first 24 hours of culture. Together with the data from chapter 3 depleting iTreg with diphtheria toxin, this suggests iTreg have an almost immediate effect dependent upon TGF-β signaling in the co-culture assay, though the exact target of this effect has not yet been identified.

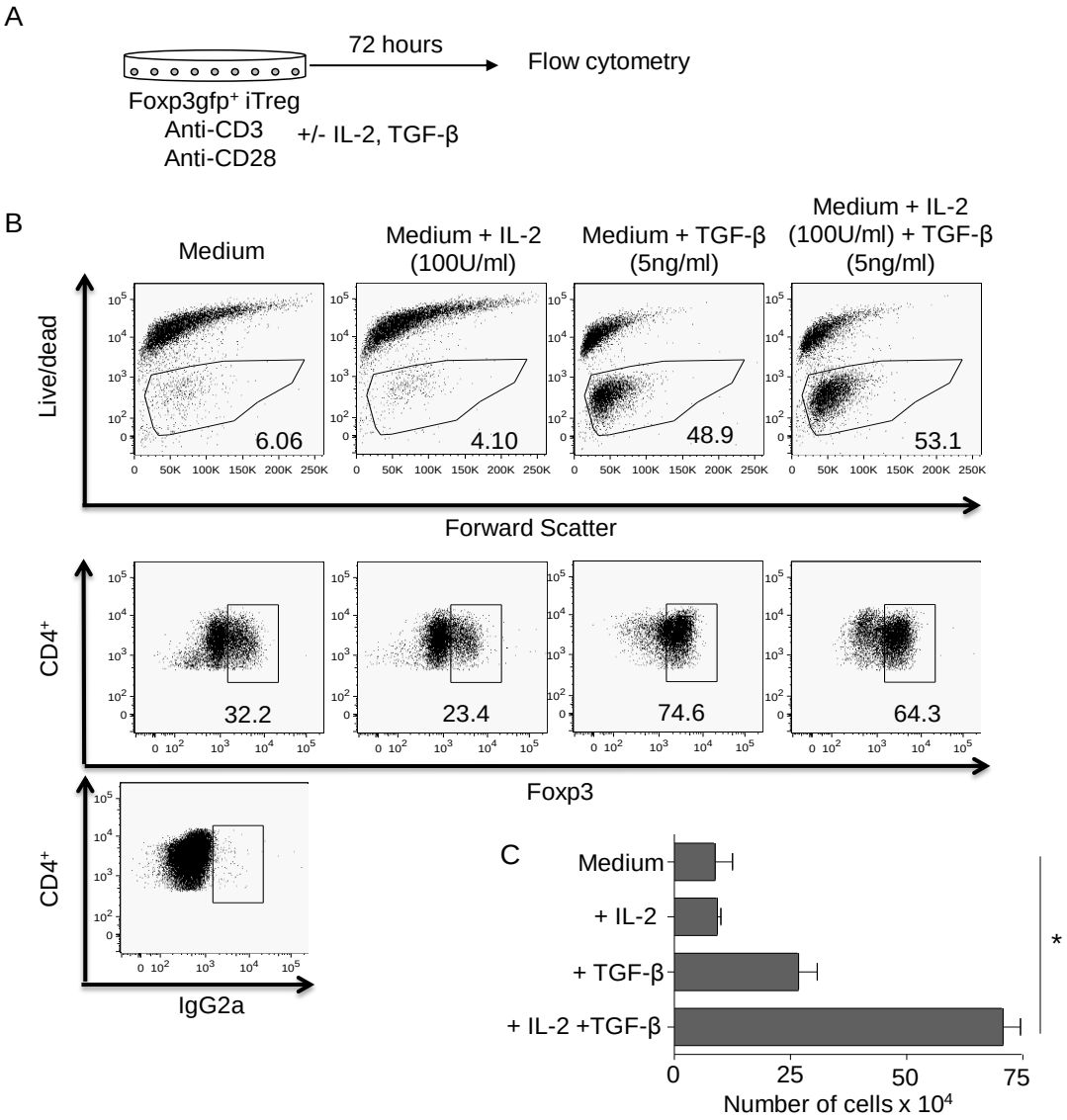


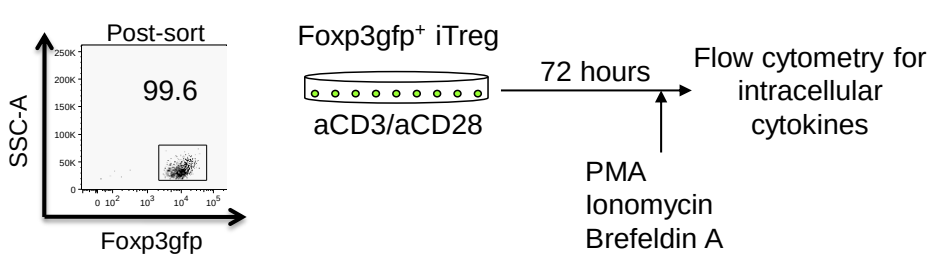
Figure 4.1 Maintenance of Foxp3 expression in iTreg is improved in the presence of TGF-β.

A) Experimental scheme. iTreg were generated as previously described in Materials and Methods. iTreg were then recultured at 0.5 x 10^6 cells/well with plate-bound anti-CD3 and anti-CD28 at 2μg/ml for 72 hours. Cells were cultured in medium alone or with additional IL-2 (100U/ml) and/or TGF-β (5ng/ml) as indicated. B) Representative flow cytometry plots showing the proportion of live cells (numbers indicate live cells as a percentage of total cells), then Foxp3 expression gated on $CD4^+$ cells (numbers indicate percentage of cells expressing Foxp3). C) Number of cells in the indicated culture conditions. Data are from one of four experiments giving consistent results. *=p<0.05 by Mann-Whitney U test.

A

Cytokine (pg/ml)	IFN-γ	GM-CSF	IL-10	IL-2	IL-4
iTreg	27.51	37.98	0	0	0
Naïve cells	0	5.83	0	176.13	68.17
1:1	12.92	72.26	0	0	0

B



C D

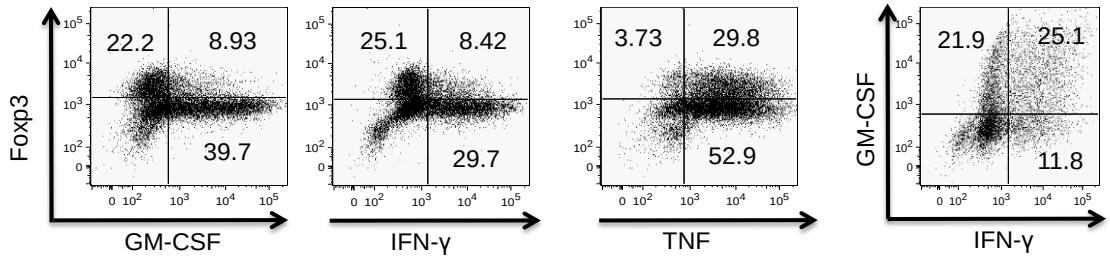


Figure 4.2 iTreg produce IFN-γ, GM-CSF and TNF.
A) A cytokine bead array was performed (by Dr R O'Connor) on supernatants from a standard suppression assay as described in Chapter 3. Numbers indicate the cytokine concentration in pg/ml. Data are from a single experiment. B) iTreg were sorted for Foxp3gfp expression at the end of generation (>99% purity). The flow cytometry plot is gated on all cells. The iTreg were re-stimulated with plate-bound anti-CD3 and CD28 for 72 hours. Brefeldin A, PMA and ionomycin were added for the final 4 hours of culture, then samples were stained for intracellular cytokines. C) Representative flow cytometry plots gated on live $CD4^+$ cells demonstrating the production of GM-CSF, IFN-γ, and TNF in relation to Foxp3 expression. D) Flow cytometry plot gated on live $CD4^+$ cells demonstrating co-production of GM-CSF and IFN-γ. Data are from one of three experiments giving consistent results.

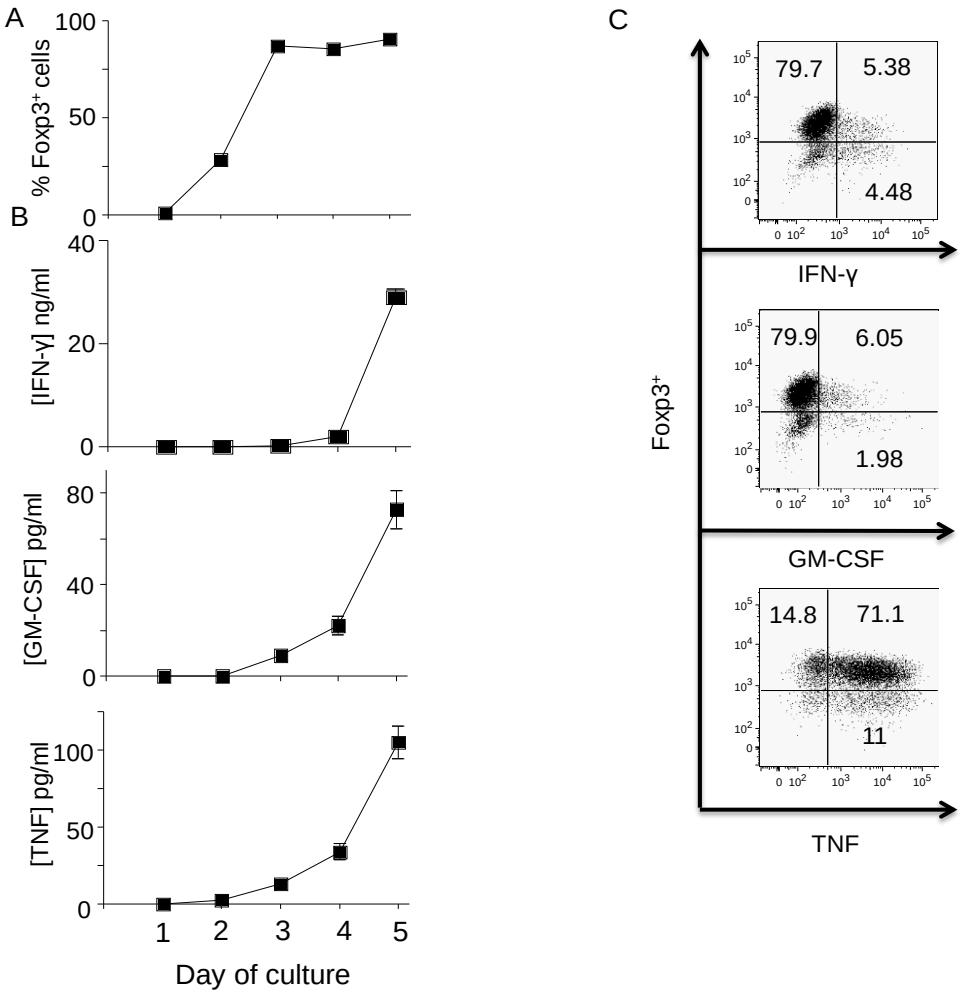


Figure 4.3 IFN-γ, GM-CSF and TNF are produced during iTreg generation.
Naïve Tg4xFoxp3LuciDTR-4 CD4+ cells were cultured for five days in IL-2 and TGF-β with plate-bound anti-CD3 and anti-CD28. Supernatants were removed on a daily basis and frozen at -20°C. Foxp3 expression was analysed by intracellular staining and flow cytometry. A) Proportion of cells expressing Foxp3 determined by flow cytometry. B) Supernatants were analysed by ELISA for GM-CSF, IFN-γ and TNF. Data are from one of three experiments giving consistent results. C) Intracellular staining at the end of iTreg culture showing Foxp3 expression and cytokine production, gated on live CD4+ cells. Data are shown from a single experiment.

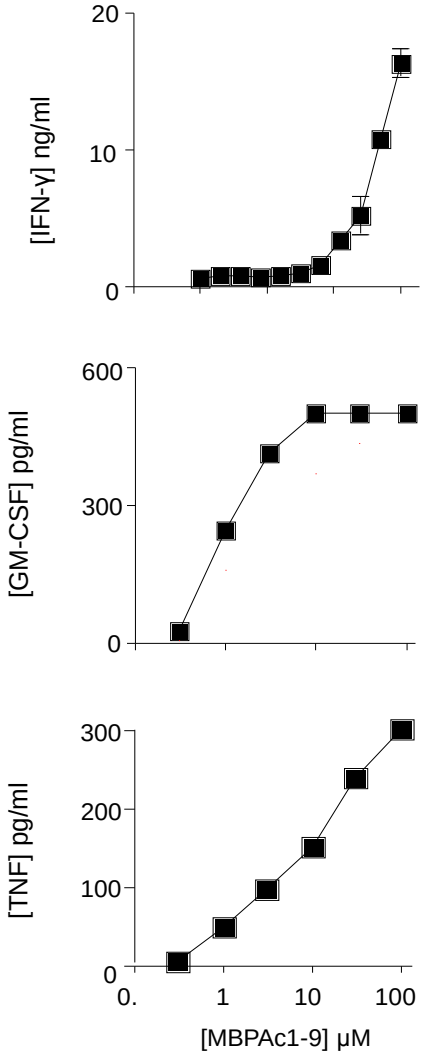


Figure 4.4 iTreg produce IFN-γ, GM-CSF and TNF on re-stimulation.
Tg4xFoxp3LuciDTR-4 Foxp3^{+} iTreg (sorted to >98% purity) were re-stimulated for 48 hours with various concentrations of MBPAc1-9. Supernatants were then analysed by ELISA. Data are from one of three experiments giving consistent results.

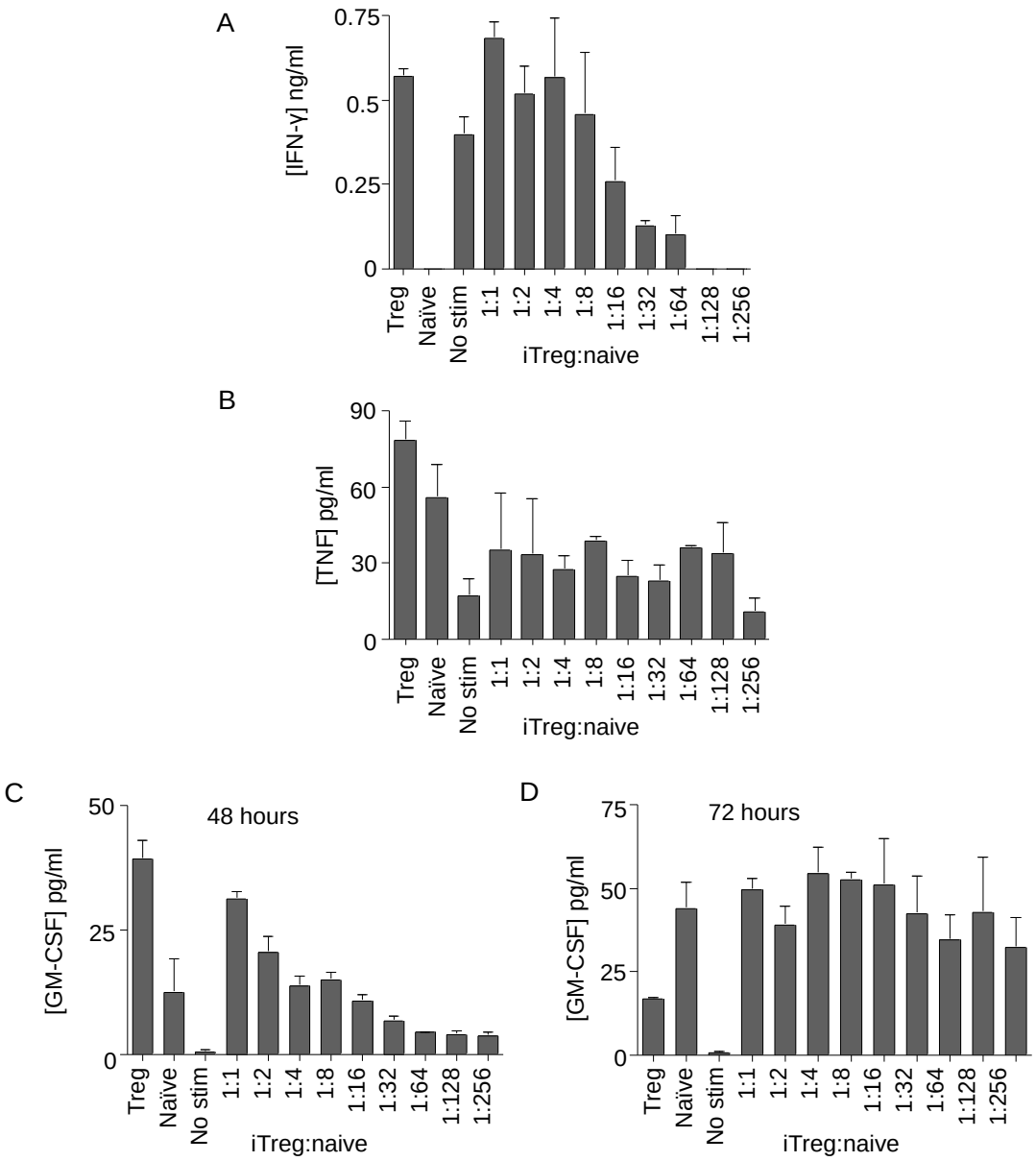


Figure 4.5 IFN-γ, GM-CSF and TNF are produced by iTreg co-cultured with naïve CD4⁺ T cells but no suppressive effect is seen.

Tg4xFoxp3LuciDTR-4 iTreg were co-cultured with naïve Tg4 CD4⁺ cells, irradiated APC, and 10μM MBPAc1-9. A) IFN-γ production was measured by ELISA at 72 hours. Data are from one of three experiments giving consistent results. B) TNF production was measured at 72 hours. Data are from one of two experiments giving consistent results. GM-CSF production was measured by ELISA at C) 48 and D) 72 hours. Data are from one of four experiments giving consistent results.

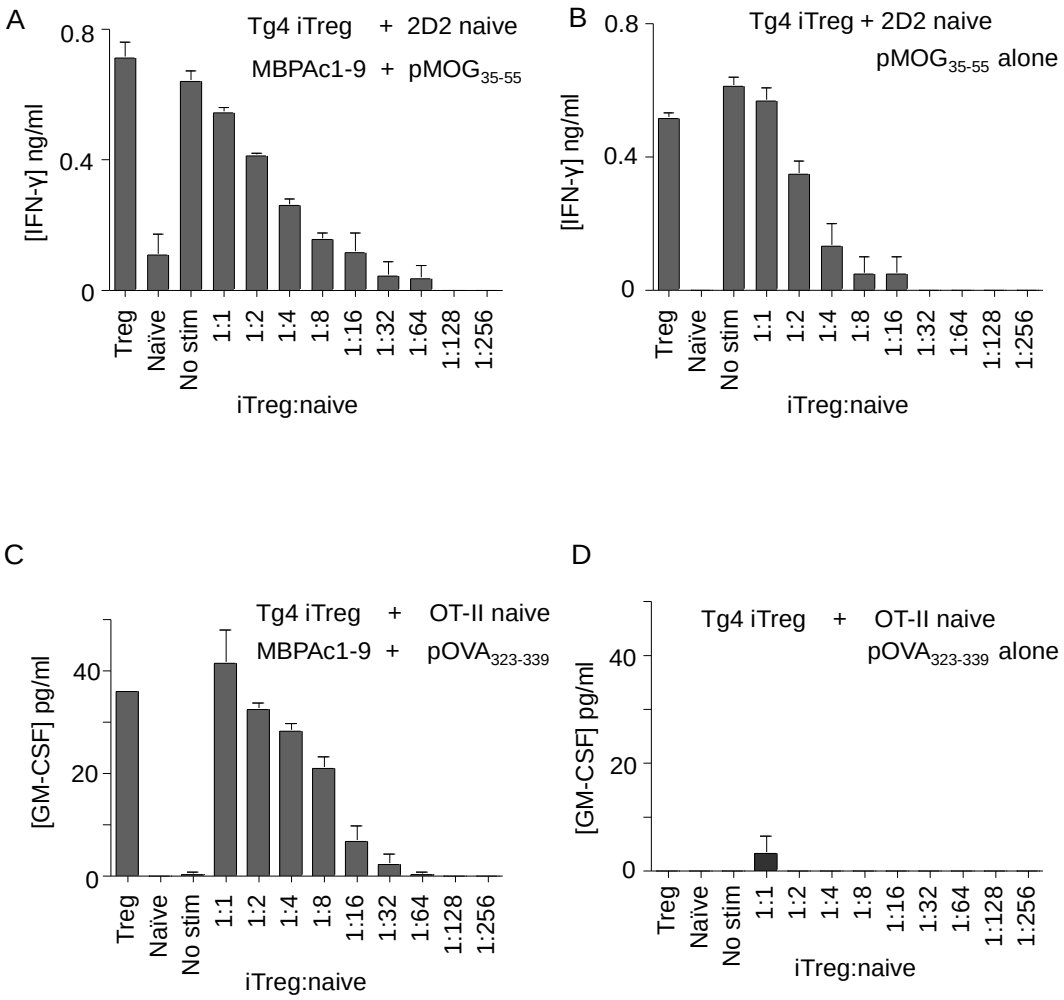


Figure 4.6 IFN-γ production is independent of ongoing stimulation, while GM-CSF production requires ongoing antigenic stimulation of iTreg.
A) Tg4xFoxp3LuciDTR-4 iTreg were co-cultured with naïve 2D2 $CD4^+$ cells and 10μM MBPAc1-9 and 10μM $pMOG_{35-55}$ or B) with 10μM $pMOG_{35-55}$ alone. IFN-γ production at 72 hours was measured by ELISA. Data are from one of two experiments giving consistent results. C) Tg4xFoxp3LuciDTR iTreg were co-cultured with naïve OT-II $CD4^+$ cells with 10μM MBPAc1-9 and 8μM $pOVA_{323-339}$ or D) with 8μM $pOVA_{3323-339}$ alone. GM-CSF production was measured at 48 hours. Data are from one of two experiments giving consistent results.

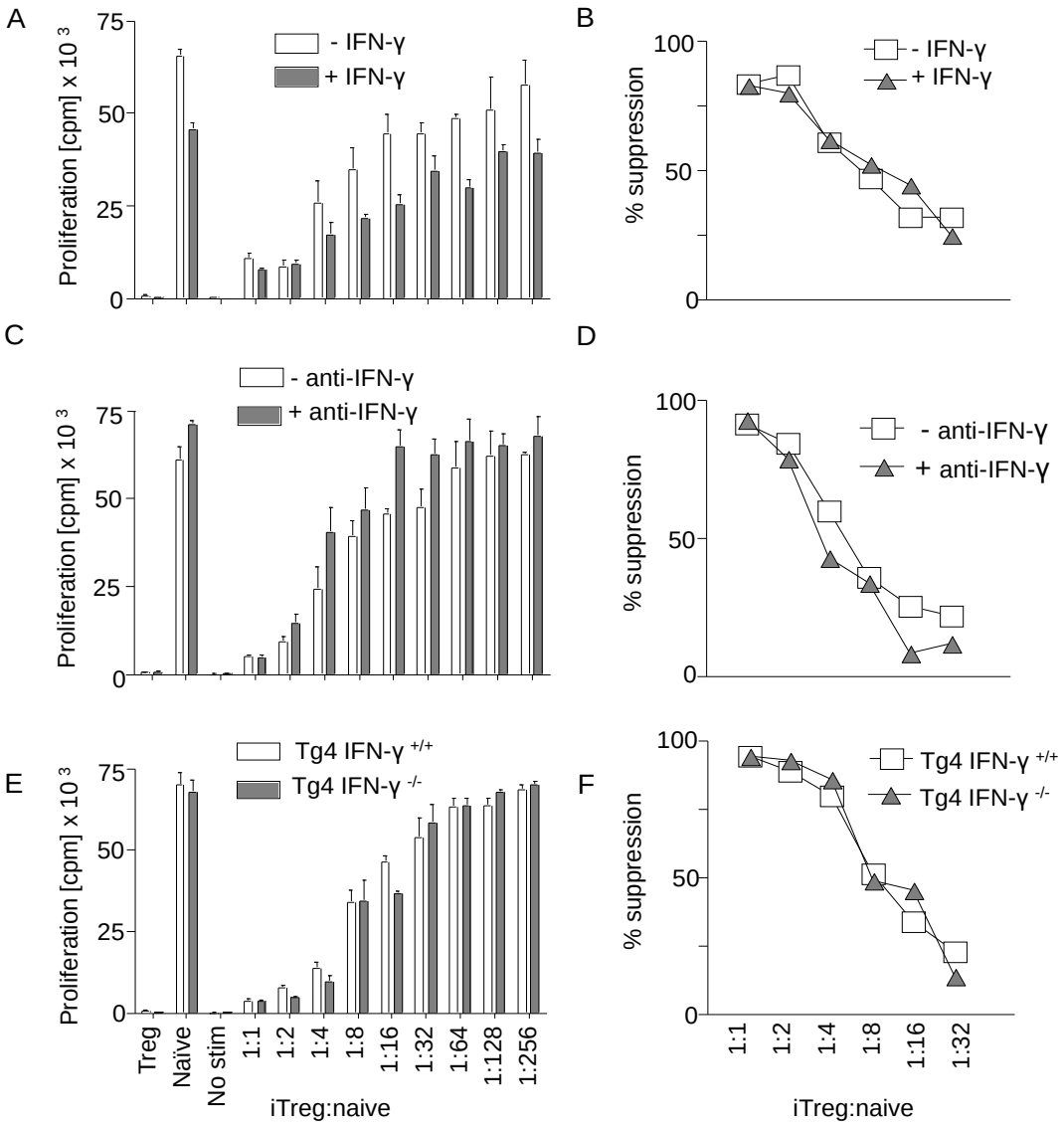


Figure 4.7 IFN-γ does not affect *in vitro* suppression.
A) IFN-γ (100ng/ml) was added at the onset of culture to suppression assays. Proliferation was measured at 96 hours by the incorporation of ^{3}H-thymidine. B) Quantification of suppression. C) Anti-IFN-γ (10ng/ml) was added at onset to suppression assays. Incorporation of ^{3}H-thymidine at 96 hours is shown. D) Quantification of suppression. E) iTreg generated from Tg4 IFN-γ deficient mice were compared with iTreg from Tg4 CD90.1 IFN-γ sufficient mice. ^{3}H-thymidine incorporation was measured at 96 hours. F) Quantification of suppression. Data are from one of two experiments giving consistent results.

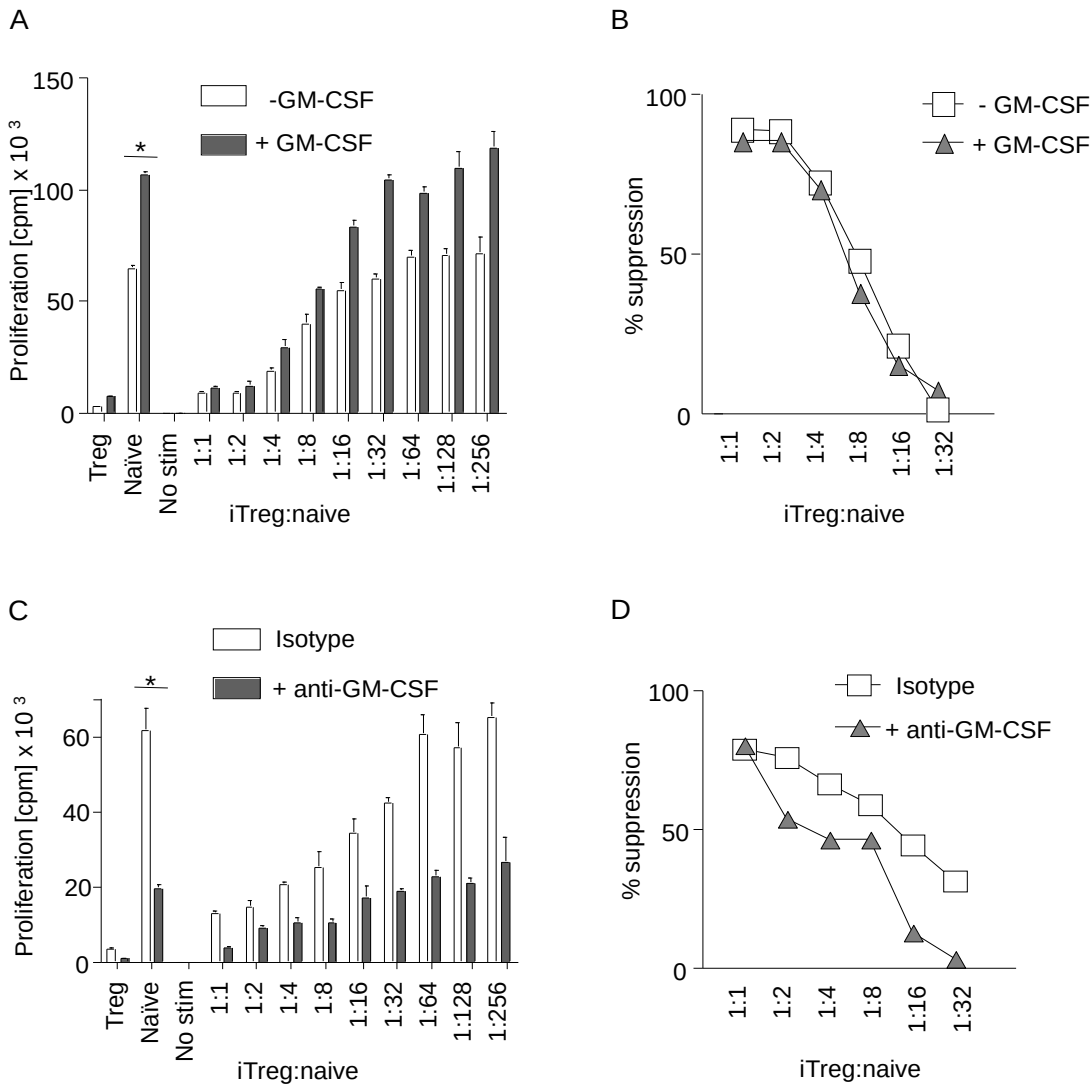


Figure 4.8 GM-CSF does not affect *in vitro* suppression.
Recombinant GM-CSF (5ng/ml) was added at onset to suppression assays. A) Proliferation was measured at 96 hours by the incorporation of 3H-thymidine. B) Quantification of suppression. C) Anti-GM-CSF was added at 10ng/ml at onset of culture. Incorporation of 3H-thymidine was measured at 96 hours. D) Quantification of suppression. Data are from one of two experiments giving consistent results. p=<0.05 (by unpaired t-test with Welch's correction).

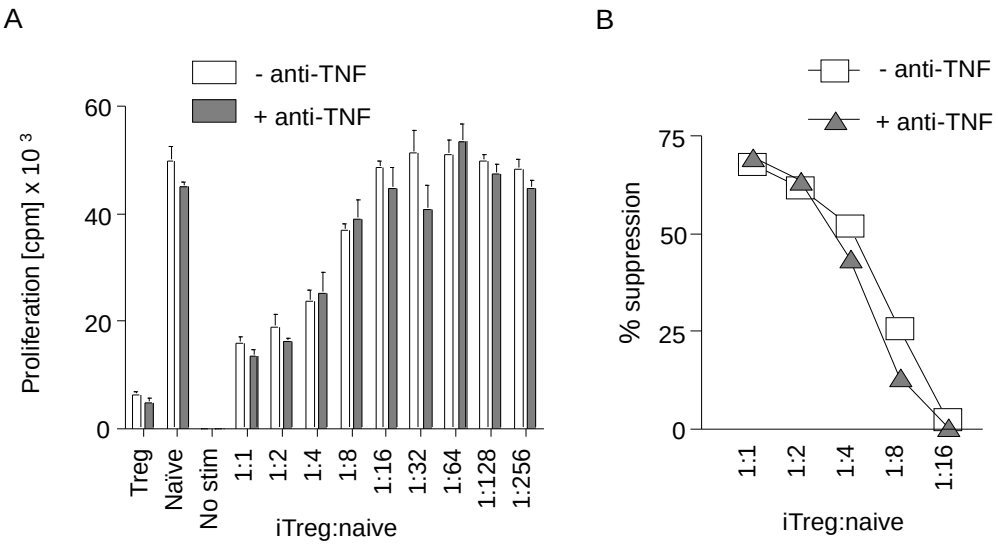


Figure 4.9 TNF neutralisation does not affect *in vitro* suppression.
Anti-TNF (1µg/ml) was added at onset of culture to suppression assays. A) Proliferation was measured at 96 hours by the incorporation of ^{3}H-thymidine. B) Quantification of suppression. Data are shown from a single experiment.

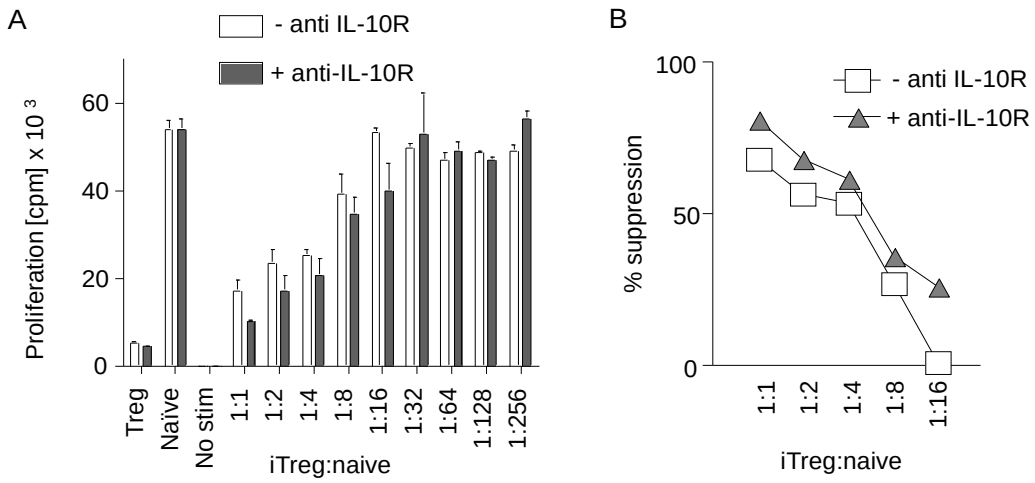


Figure 4.10 IL-10 neutralisation does not affect *in vitro* suppression.
Anti-IL-10R (25µg/ml) was added to suppression assays at the onset of culture. A) Proliferation was measured at 96 hours by incorporation of ^{3}H-thymidine. B) Quantification of suppression. Data are from one of two experiments giving consistent results.

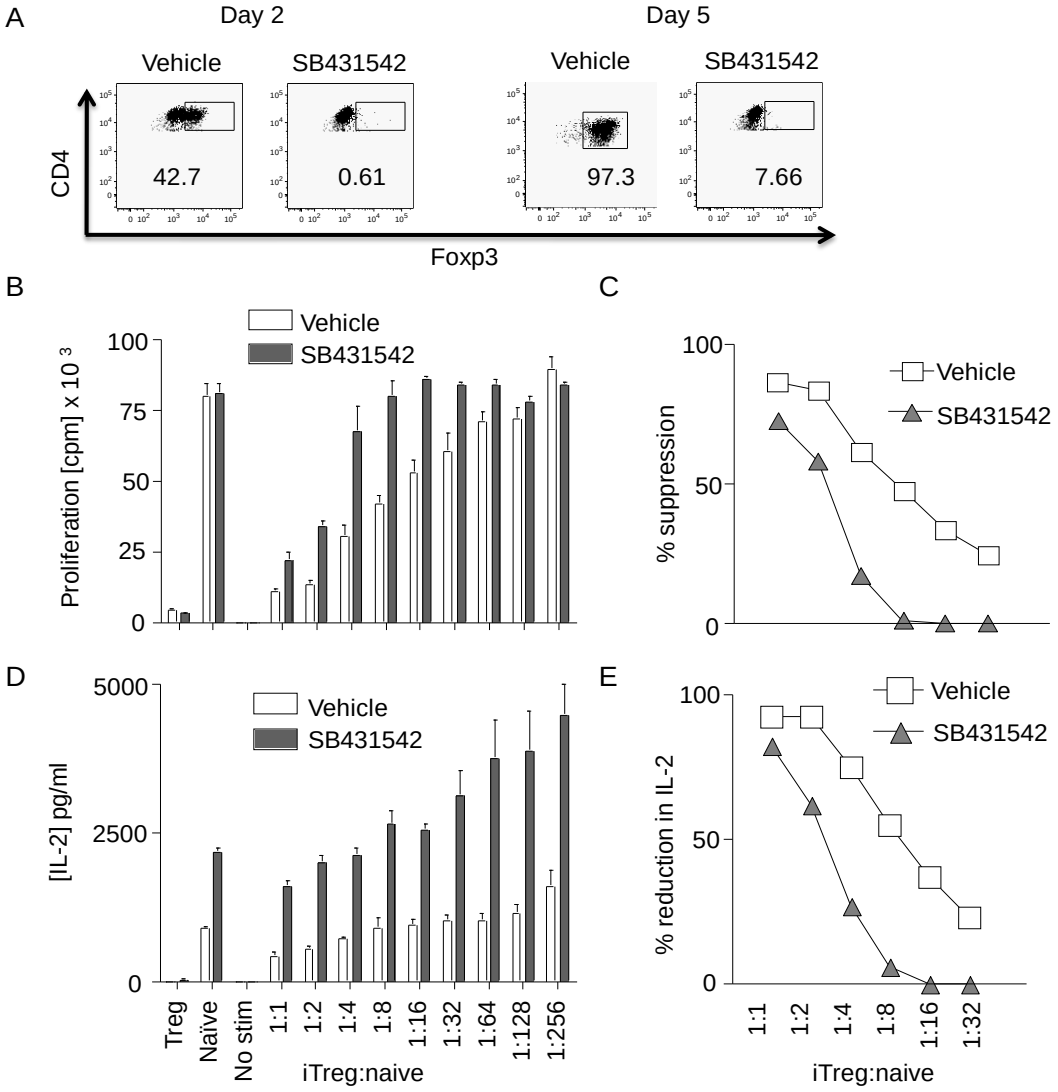


Figure 4.11 Inhibition of TGF-β signalling reduces the efficacy of suppression by iTreg *in vitro*.

A) Naïve Tg4xFoxp3LuciDTR-4 CD4$^+$ cells were cultured in IL-2 and TGF-β, with or without the addition of the ALK5 inhibitor, SB431542 at 10μM. After 5 days of culture, Foxp3 expression was assessed by flow cytometry. Foxp3 expression gated on CD4$^+$ cells is shown. B) 10μM SB431542 or vehicle control (DMSO) was added at the onset of culture to suppression assays. Proliferation was measured at 96 hours by the incorporation of ^{3}H-thymidine. C) Quantification of suppression. D) Measurement of IL-2 at 48 hours by ELISA. E) Quantification of reduction in IL-2. Data are from one of four experiments giving consistent results.

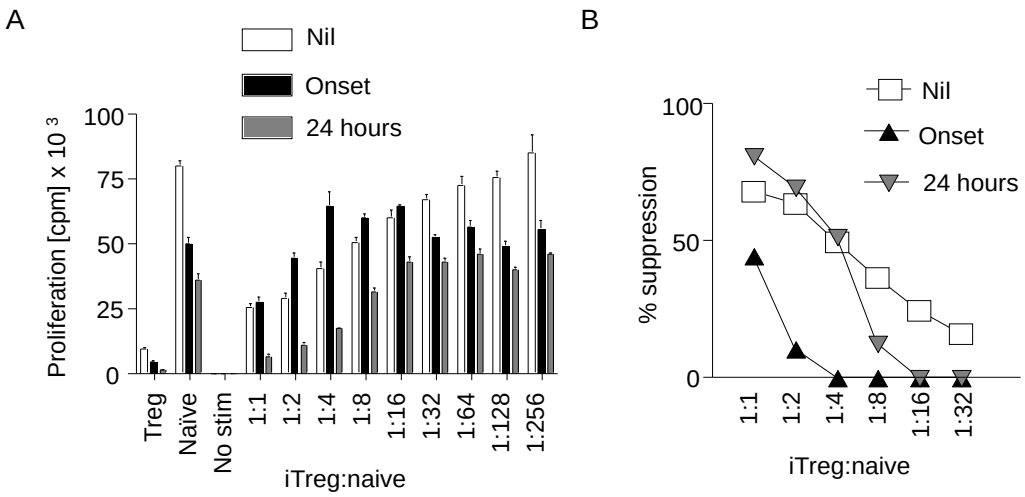


Figure 4.12 Inhibition of TGF-β signalling only affects suppression by iTreg early in co-culture.

A) Tg4xFoxp3LuciDTR-4 iTreg were co-cultured with naïve Tg4 $CD4^+$ cells in a standard suppression assay. 10μM SB431542 was added at the onset of culture, after 24 hours, or not at all. Proliferation was assessed by the incorporation of ^{3}H-thymidine at 96 hours. B) Quantification of suppression. Data are shown from a single experiment.

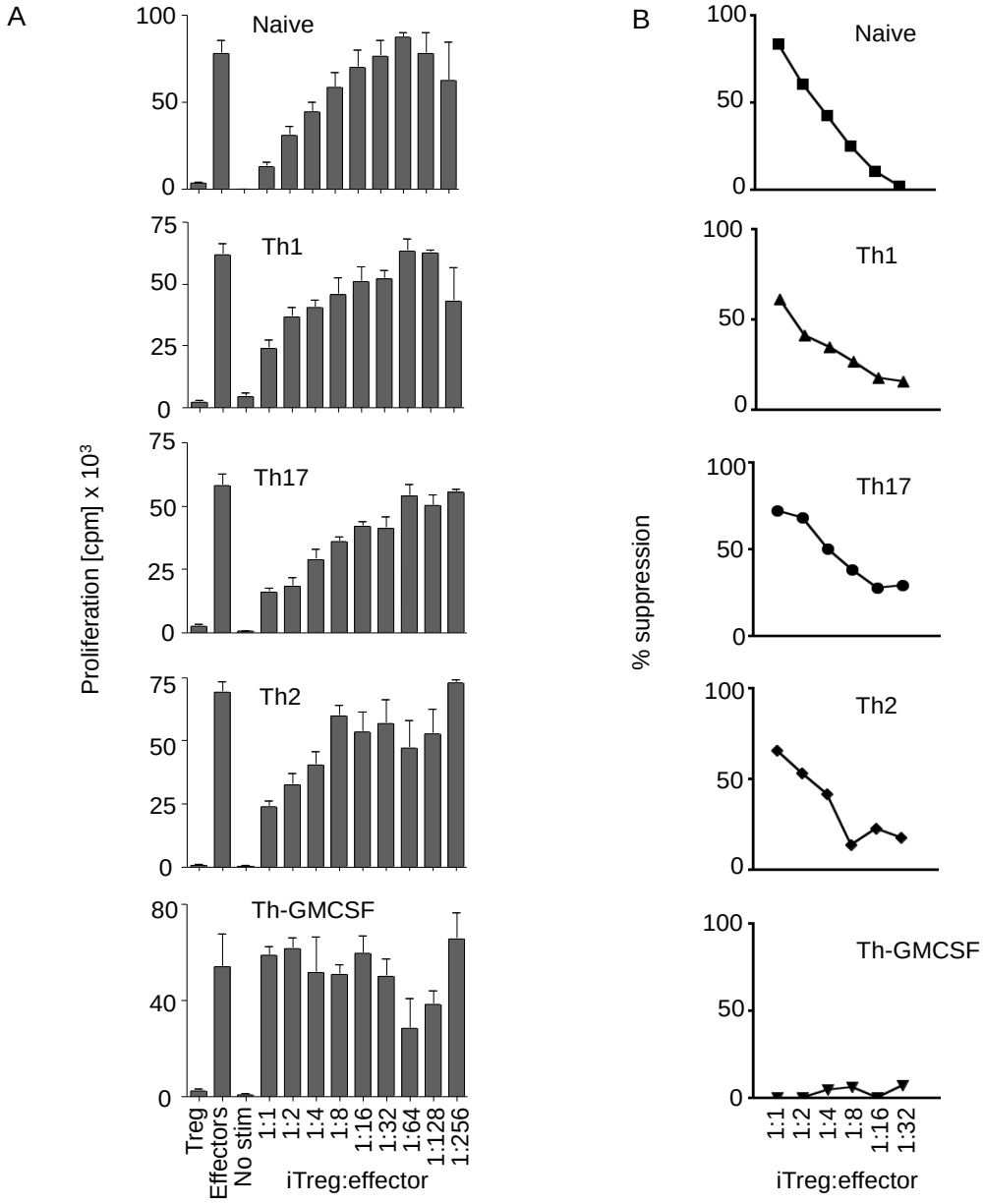


Figure 4.13 iTreg can suppress effector cells polarised to Th1, Th2, and Th17 but not 'ThGM-CSF'.

Tg4xFoxp3LuciDTR-4 iTreg were co-cultured with Tg4 CD90.1 $CD4^+$ cells polarised towards Th1, Th2, Th17, or 'Th-GM-CSF' phenotypes. A) Proliferation was measured at 96 hours via the incorporation of ^{3}H-thymidine. B) Quantification of suppression. Data are from one of three experiments giving consistent results.

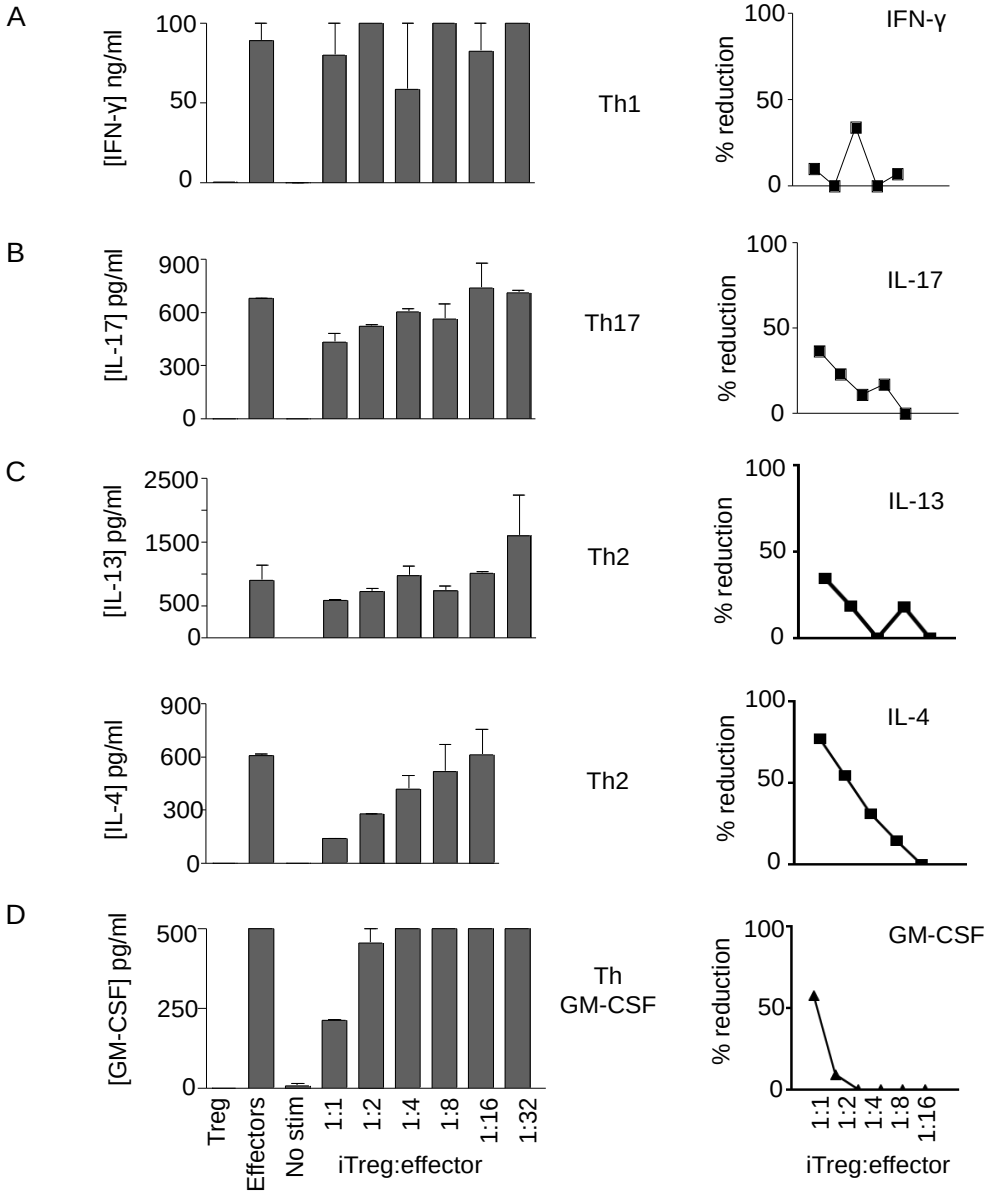


Figure 4.14 iTreg do not suppress cytokine production by effector cells, except for IL-4 by Th2 cells.

'Signature' cytokine production was analysed by ELISA of supernatants taken from the suppression assays featured in Fig 4.13, at 72 hours. A) IFN-γ production by Th1 cells. B) Production of IL-17 by Th17 cells. C) Production of IL-4 and IL-13 by Th2 cells D) Production of GM-CSF by ThGM-CSF. Graphs on the right represent quantification of suppression of the indicated cytokine. Data are shown from a single experiment.

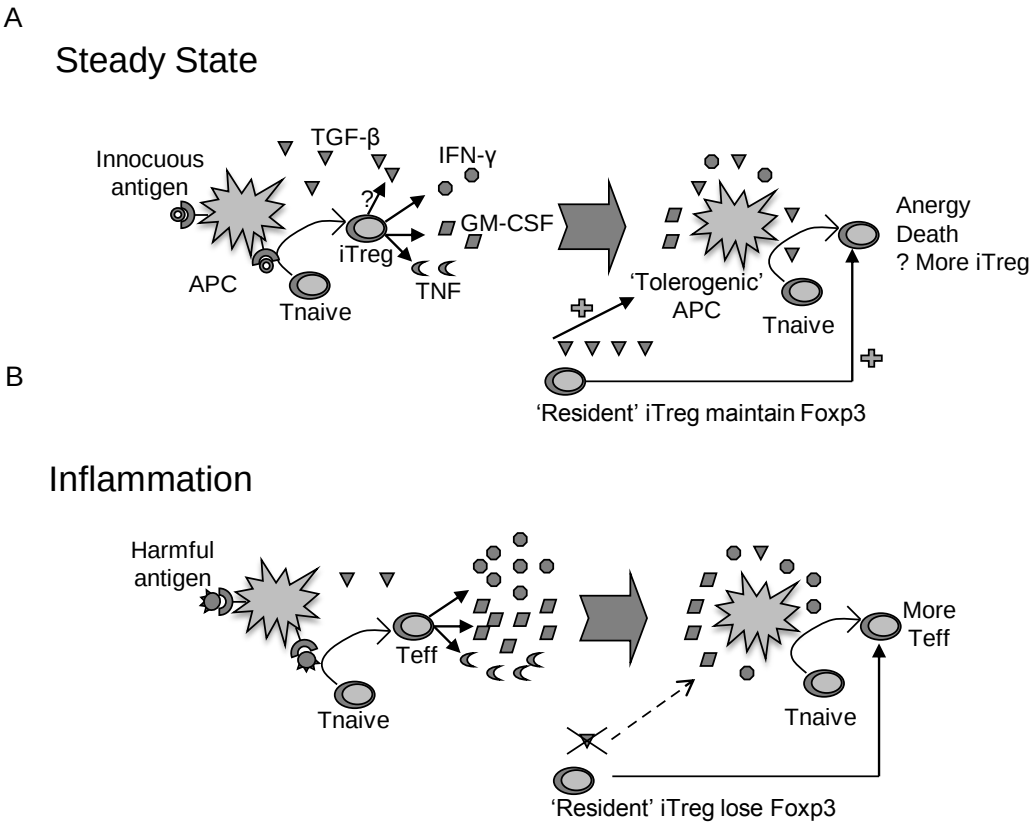


Figure 4.15 Hypothetical model for maintenance of steady state by iTreg and loss of Foxp3 in pro-inflammatory states.
A) In the steady state, innocuous antigen is presented by APC in the absence of danger signals, in a TGF-β-rich environment. This promotes induction of Foxp3 in naïve cells, leading to a high number of 'resident' iTreg. High TGF-β concentrations maintain Foxp3 expression. Low-level production of IFN-γ, GM-CSF and TNF promote an immature tolerogenic state in APC, such that ongoing antigen presentation promotes more regulation, and prevents a pro-inflammatory response by other naïve T cells. B) Presentation of harmful antigen with concomitant danger signals leads to differentiation of naïve T cells to effector phenoytypes, with production of high concentrations of cytokines. This alters the milieu, promoting maturation of APCs to a pro-inflammatory state, and promoting further effector T cell polarisation. The relative reduction in TGF-β leads to loss of Foxp3 and reduces the induction of new iTreg. Foxp3$^-$ iTreg may not be able to influence APC, but retain suppressive function and so are able to contribute to resolution of the inflammatory state.

5 Direct and bystander suppression by iTreg *in vivo*

5.1 Introduction

The *in vivo* efficacy of both nTreg and iTreg in either preventing onset or ameliorating ongoing disease has been established in several models, including EAE (Stephens et al., 2009, Kohm et al., 2002, Selvaraj et al., 2008), diabetes (Tang et al., 2004, Weber et al., 2006), and colitis (Liu et al., 2003, Fantini et al., 2006). The exact mechanisms of action of Treg remain unclear though the assumption often made was that proliferation of effector cells was inhibited (Thornton et al., 1998, Piccirillo et al., 2001). Given that this did not appear to be the case *in vitro* (section 3.2.4), the impact of iTreg on proliferation of naïve T cells (as measured by CFSE dilution) deserved study. Similarly, the pathogenic nature of effector T cells is thought related to cytokine production (Dardalhon et al., 2008a) so analyzing the cytokines produced by naïve T cells following *in vivo* stimulation in the presence or absence of iTreg may elucidate a further mechanism able to limit disease progression.

The behaviour of Treg *in vivo* is known to be different to that *in vitro.* For example, the 'anergic' nature of nTreg *in vitro* contrasts with their rapid proliferation *in vivo* (Walker et al., 2003). iTreg are known to lose Foxp3 expression *in vivo,* which is countered in the presence of additional IL-2 (Chen et al., 2011) unlike *in vitro* where IL-2 has no effect (Fig 4.1). Having demonstrated the pro-inflammatory cytokine profile of iTreg on *in vitro* stimulation, the impact of stimulating iTreg in an *in vivo* environment merited investigation.

Chapter 4 established that iTreg produce the pro-inflammatory cytokines, IFN-γ, GM-CSF and TNF, with an associated loss of Foxp3 expression over time unless TGF-β was maintained in *in vitro* culture. The potential for iTreg to convert to an effector-like phenotype has complicated translation into the therapeutic setting (Zhou et al., 2009a). Identification of Th17 cells as a source of pathology (Stockinger et al., 2007) and the similarity between Th17 and iTreg differentiation (Bettelli et al., 2006) magnified these concerns. Though established iTreg appear to be stable in the face of conditions favouring Th17 polarisation (Zheng et al., 2008), the effects of other pro-inflammatory cytokines are unclear. IL-12-conditioned iTreg were shown to maintain efficacy even with loss of Foxp3 expression (O'Connor et al., 2010) and to have very limited pathogenic potential despite IFN-γ production. The impact of other cytokines relevant to effector cell polarisation has been less studied.

The effect of TGF-β on the maintenance of Foxp3 and iTreg survival was demonstrated in Chapter 4 (Fig 4.1). TGF-β is known to be a highly pleiotropic cytokine (Tran, 2012). That its effect on cells is dependent on the surrounding milieu is clearly demonstrated in the dichotomy of polarisation by naïve T cells towards either Th17 or Treg, influenced by the presence of IL-6 (Bettelli et al., 2006, Korn et al., 2008). How other polarizing cytokines may alter the stabilizing effect of TGF-β has been poorly studied.

5.1.1 Experimental aims

The first aim of this chapter was to ascertain whether iTreg were suppressive *in vivo*, and whether this impacted on cytokine production by naïve T cells, followed by assessment of the proinflammatory environment on the cytokine profile of iTreg. Secondly, various cytokines were investigated for their influence on the stability of Foxp3 expression and the iTreg cytokine profile *in vitro*, to determine if the *in vivo* situation could be replicated. Finally, a model of allergic airways inflammation was used to investigate whether bystander suppression by iTreg could be demonstrated *in vivo*.

5.1.2. Experimental approach

Previous studies have demonstrated superior efficacy of antigen-specific Treg compared to polyclonal Treg (Tang et al., 2004, Stephens et al., 2009). Myelin-responsive Tg4 iTreg were therefore utilized for most *in vivo* experiments. The functional impact of these iTreg was assessed by co-administering CFSE-labelled naïve Tg4 $CD4^+$ cells at the same time as the iTreg to host mice. These mice were then primed with MBPAc1-9 (4Tyr) emulsified in CFA (s.c. into the hind legs) to initiate an inflammatory response. In some experiments, an alternate priming using MBPAc1-9 (4Tyr) in PBS was used to distinguish the effect of pro-inflammatory signaling. Cells were subsequently analysed using flow cytometry. In subsequent experiments, mice were infused with iTreg alone, followed by priming with MPBAc1-9 (4Tyr), then changes in iTreg cytokine profile were analysed by intracellular staining.

In vitro experiments were used to test individual cytokines known to be relevant in the *in vitro* polarization of naïve T cells to either Th1 or Th17 phenotypes i.e. cytokines likely to be present early in an inflammatory process. In all cases, iTreg were generated from Foxp3gfp

or Tg4xFoxp3LuciDTR-4 reporter mice and subsequently sorted for Foxp3gfp^{+} expression to provide highly pure iTreg for investigation.

Finally, a model of allergic airways inflammation was used to assess bystander suppression *in vivo*. Mice were administered 2D2 iTreg and OT-II Th2 cells, then received an airway antigen challenge with rMOG and OVA over several days. The ability of the 2D2 iTreg to effect bystander suppression was assessed by examining the number of donor Th2 cells in spleen, mediastinal lymph nodes, and the lungs, with intracellular cytokine staining of Th2 cells, and using cytospins and histology to assess the degree of allergic airways inflammation.

5.2 Results

5.2.1 iTreg suppress naïve T cells *in vivo*

That iTreg have potential applicability in a clinical setting is supported by various successes in animal models of disease, including EAE and diabetes (Selvaraj et al., 2008, Tonkin et al., 2008). However, the iTreg generated here could produce three pro-inflammatory cytokines. Despite this, they could suppress *in vitro*. Were these iTreg able to effect suppression of naïve T cells stimulated *in vivo*?

Naïve Tg4 CD90.1 $CD4^+$ cells were administered to C57BL/6xB10.PL F_1 mice, either alone or in equal numbers with Tg4xFoxp3LuciDTR-4 iTreg. One day following infusion of cells, mice received a subcutaneous injection of MBPAc1-9 (4Tyr) emulsified in CFA (Fig 5.1A). After one week, mice were culled, spleens and inguinal lymph nodes harvested, and cells analysed by flow cytometry. Donor naïve T cells could be distinguished by expression of the congenic marker CD90.1, whilst donor iTreg expressed CD45.1. For measurement of intracellular cytokines, cells were additionally stimulated with PMA and ionomycin in the presence of brefeldin A (Fig 5.1A,B).

5.2.1.1 iTreg reduce the persistence of naïve T cells *in vivo*

An effect of co-administration of iTreg was immediately apparent upon harvesting the mice. The spleens of mice receiving both iTreg and naïve T cells (i.e. double the number of donor cells in total) were significantly smaller than the spleens from mice receiving naïve T cells alone (Fig 5.1C). The proportion of donor naïve T cells within the $CD4^+$ population was also reduced in those mice receiving iTreg, leading to a significant reduction in the total number of donor naïve T cells (Fig 5.1D). The same pattern was seen within the lymph nodes (though the difference in total cellularity was not statistically significant) (Fig 5.1E). Thus, the presence of iTreg led to a reduction in the detectable number of donor naïve T cells capable of responding to the antigen.

5.2.1.2 iTreg do not affect the proliferation of naïve T cells *in vivo*

Data presented in chapter 3 demonstrated that iTreg give the appearance of suppressing incorporation of ^{3}H-thymidine at 96 hours *in vitro*, but both CFSE and earlier kinetic data demonstrated that the majority of naïve T cells in the assay had already proliferated (Fig 3.11,3.12). The lower number of naïve T cells seen *in vivo* (Fig 5.1D) could be accounted

for by suppression of proliferation, or increased death of those cells. Proliferation of the naïve T cells was therefore assessed using CFSE-labelling.

As previously, naïve Tg4 CD90.1 $CD4^+$ cells were administered to F_1 mice with or without Tg4xFoxp3LuciDTR-4 iTreg. The naïve T cells were labeled with CFSE prior to administration. One day following cell administration, mice received MBPAc1-9 (4Tyr) emulsified in CFA, or in PBS, subcutaneously. One week later, spleens and inguinal lymph nodes were harvested and analysed by flow cytometry (Fig 5.2A).

Dilution of CFSE staining was evident in all groups in both spleen and lymph nodes (Fig 5.2B). In those mice receiving only naïve T cells and MBPAc1-9 (4Tyr) in PBS, approximately 50% of cells had retained CFSE stain within the spleen, and 80% within the lymph node. Loss of CFSE in this group is attributed to the presence of the antigen in the absence of the pro-inflammatory signals. The addition of CFA led to CFSE staining being completely lost, implying robust proliferation of all stained T naïve cells. Mirroring the situation *in vitro*, dilution of CFSE occurred regardless of the presence of iTreg. A tiny number of cells retaining CFSE staining (accounting for less than 5% of the total) could be identified when iTreg were co-transferred, but the majority of the donor naïve T cells lost CFSE expression completely. This implies that the reduction in numbers is not related to inhibition of proliferation of the naïve T cells.

5.2.1.3 iTreg suppress IFN-γ by naïve T cells in the lymph nodes *in vivo,* but not GM-CSF or TNF

Following PMA and ionomycin stimulation, cells were assessed for the production of IFN-γ, GM-CSF, and TNF. Staining for all cytokines was evident from the donor naïve T cells in both spleen and lymph nodes, confirming that the cells had undergone some activation. No difference was seen in the spleen in production of any of the cytokines investigated in the presence of iTreg (Fig 5.3A). Within the lymph nodes, cytokine production was similarly unaffected except for IFN-γ where a significant reduction was seen when iTreg were co-administered (Fig 5.3B). On meta-analysis of all experiments, IFN-γ production by naïve T cells was significantly reduced in the presence of Treg in the inguinal lymph nodes, but neither GM-CSF nor TNF production was significantly altered.

Thus, iTreg reduce the number of donor naïve T cells present in the spleen and lymph nodes, but the remaining cells are still potentially pathogenic given their production of IFN-γ, GM-CSF and TNF. The total number of donor cells producing cytokines is very small however, being less than 1000 cells in all cases. Proliferation of the naïve T cells was unaffected,

suggesting that iTreg prevent survival of the donor cells, rather than limiting expansion. Whether this is mediated through active cytotoxicity or passive processes cannot be determined from these data.

5.2.2 Effects of a pro-inflammatory environment on iTreg *in vivo*

Given that iTreg clearly have a suppressive effect overall *in vivo*, does the more complex inflammatory environment caused by CFA alter the stability of Foxp3 expression, and cytokine production by iTreg compared to that seen *in vitro*?

5.2.2.1 iTreg survive but lose Foxp3 expression *in vivo*

Tg4xFoxp3LuciDTR-4 iTreg ($2x10^6$ cells) were transferred into B10.PL mice, followed one day later by a subcutaneous injection of 10μg MBPAc1-9 (4Tyr) emulsified in CFA, or in PBS. After 48 hours spleen and inguinal lymph nodes were harvested then cultured overnight with 10μM MBPAc1-9 (4Lys) followed by PMA, ionomycin and brefeldin A for the final four hours. Cells were then stained for surface markers and intracellular cytokines (Fig 5.4A), with donor iTreg identifiable by the expression of the congenic marker, CD45.1 (Fig 5.4B).

No difference in overall total cellularity of the organs was seen between mice receiving CFA or PBS (Fig 5.4C and Fig 5.5A). Donor iTreg accounted for a higher percentage of the $CD4^+$ population in the spleen (Fig 5.4D) in those mice receiving pro-inflammatory signals via CFA, though this did not reach significance. Only very small numbers of donor iTreg were identifiable in the mice receiving Ac1-9 (4Tyr) in PBS. Donor iTreg numbers appeared higher in the mice receiving Ac1-9 (4Tyr) emulsified in CFA (though this did not reach statistical significance) with approximately $15x10^4$ iTreg detectable in the spleen, and $1x10^4$ within the lymph nodes (Fig 5.4D, 5.5B). Approaching 10% of the injected iTreg could therefore be recovered from these two organs 48 hours after antigen administration. Foxp3 expression was poorly preserved, with less than 10% of the cells remaining $Foxp3^+$ after only 48 hours in the mice receiving antigen in CFA. In contrast, maintenance of Foxp3 expression was significantly higher in the mice that received Ac1-9 (4Tyr) in PBS, at 50-60% in both spleen and LN (Fig 5.4E and Fig 5.5C). The greater persistence of Foxp3 in the Ac1-9 (4Tyr)/PBS group led to numbers of $Foxp3^+$ donor iTreg being similar between the Ac1-9 (4Tyr)/CFA and Ac1-9 (4Tyr)/PBS treated mice within the spleen (Fig 5.4E, Fig 5.5C).

Foxp3 expression was >99% prior to administration to mice. Thus, following administration of antigen, iTreg lost some Foxp3 expression (down to 50-60% in the PBS group) but this was exacerbated in the presence of inflammatory signals caused by CFA. Total donor cell numbers were also greater in mice receiving antigen in CFA, leading to an increase in $Foxp3^-$ donor cells. This suggests that pro-inflammatory signals from CFA contribute to a loss of Foxp3 expression in iTreg.

5.2.2.2 iTreg produce IFN-γ and TNF but very little GM-CSF *ex vivo*

What effect was seen on the iTreg cytokine profile *in vivo*? Assessing the production of cytokines by intracellular staining following additional stimulation with PMA and ionomycin, no difference was seen in the proportions of cytokines produced between donor iTreg cells from spleen or lymph nodes. IFN-γ production was seen in approximately 20% of cells and TNF production in 60% (Fig 5.6A). The major difference from the *in vitro* setting was the absence of any GM-CSF production by donor iTreg, which was indistinguishable from isotype. There was no difference between mice treated with Ac1-9 (4Tyr) in CFA, and those treated with Ac1-9 (4Tyr) in PBS (Fig 5.6B). In comparison, production of the same cytokines by host $CD4^+$ cells (following PMA and ionomycin stimulation) demonstrated that host $Foxp3^+$ cells make no GM-CSF either, or IFN-γ, but again appreciable staining of TNF^+ cells was seen (Fig 5.7). Host $Foxp3^-$ cell production of GM-CSF confirmed that the absence of iTreg GM-CSF staining was not due to technical issues with the assay. The donor iTreg cytokine profile did not differ between mice treated with CFA or PBS. Insufficient donor iTreg were detectable in lymph nodes in mice treated with PBS to allow meaningful comparisons of cytokine production with donor iTreg from lymph nodes in CFA-treated mice.

5.2.3 The effects of polarising cytokines on iTreg *in vitro*

Thus, after exposure to antigen in an inflammatory environment (CFA immunization), iTreg maintained their ability to make IFN-γ and TNF but their capacity to produce GM-CSF seemed to be lost. To be of utility therapeutically, iTreg would be administered into such a pro-inflammatory environment. Thus, cytokines known to be relevant to the development of either Th1 or Th17 cells following CFA immunization were investigated for their effects on iTreg *in vitro* i.e. IL-12, IL-6, IL-23, IL-1β, and IL-27. Similar to the approach in chapter 4 (4.2.1.1), iTreg were re-stimulated using plate-bound anti-CD3 and anti-CD28 (both at 2μg/ml) with the exogenous addition of each individual cytokine to the culture medium (Fig 5.8A). Cytokines were added at the following concentrations: IL-27 (10ng/ml), IL-12

(25ng/ml), IL-6 (30ng/ml), IL-1β (10ng/ml), and IL-23 (30ng/ml). To further assess the role of IFN-γ, additional IFN-γ (100ng/ml) was also added to these cultures, as was a neutralizing antibody to IFN-γ (10ng/ml).

5.2.3.1 IL-27, IL-12 and IL-6 exacerbate the loss of Foxp3 expression by iTreg

The most profound effects were seen with the addition of **IL-12.** Though cell survival was increased on live/dead staining (Fig 5.8B) there was no significant difference in total cell numbers compared to medium (Fig 5.8D). IL-12 did, however, lead to a notable reduction in the expression of Foxp3 compared to medium alone (Fig 5.8C). **IL-27** and **IL-6** had similar though less marked effects on the proportion of cells staining with the live/dead marker, and on the loss of Foxp3 expression. These cytokines thus increased the number of $Foxp3^-$ cells with the potential to reverse any regulatory effect. As both IL-12 and IL-27 are relevant in Th1 differentiation, the possibility of conversion to effector status must be considered. The increased proportion of cells identified on flow cytometry as alive in the presence of IL-12 or IL-27 did translate to a greater number of cells counted, though this did not reach statistical significance (Fig 5.8.D).

The other cytokines investigated had minimal effects on both cell survival and Foxp3 expression. **IL-1β** (Fig5.8B-D), **IL-23**, **IFN-γ**, and **anti-IFN-γ** (Appendix 1) all had no impact on Foxp3 expression or cell viability.

5.2.3.2 IL-27, IL-12 and IL-6 reduce the proportion of GM-CSF^+ iTreg on *in vitro* restimulation

As with Foxp3 expression, the most notable effects on cytokine production by iTreg were seen with **IL-27**, and **IL-12**, and less markedly with **IL-6**. Cells staining positive for GM-CSF production were reduced in the presence of IL-27, IL-12, or IL-6 (Fig 5.9A,B). IL-12 had a particularly marked effect on cells staining positive for IFN-γ, with a lesser effect of IL-27 whilst IL-6 had virtually no effect on the proportions of cells that were IFN-γ^+. TNF production was also somewhat reduced by any of IL-27, IL-12 or IL-6 (Fig 6.2B) though none reached statistical significance. **IL-1β** had no impact on IFN-γ or GM-CSF production compared to medium alone, in contrast to its reported effects of boosting GM-CSF production by naïve cells (Lukens et al., 2012). The other cytokines investigated – IL-23, IFN-γ and anti-IFNγ – did not affect the iTreg cytokine profile (Appendix 1).

5.2.3.3 iTreg express IL-6 receptor chains CD126 and gp130

The ability of IL-6 to influence Foxp3 expression, iTreg survival and cytokine production is at odds with other reports in the literature. iTreg are reported to be resistant to conversion to a Th17 phenotype (Zheng et al., 2008) due to very low expression of IL-6Rα (CD126), and nTreg recovered from the inflamed CNS do not produce IL-17 when isolated from an inflammatory environment (O'Connor et al., 2012), suggesting both nTreg and iTreg lose the ability to respond to IL-6 in the face of pro-inflammatory signals, with loss of CD126 in each case. The data shown here suggest that iTreg can still respond to IL-6, albeit in a limited fashion. Flow cytometry of freshly generated iTreg for the IL-6 receptor α-chain (CD126, specific to IL-6) and gp130, a signaling chain common to several cytokines including IL-6 and IL-27, demonstrated that iTreg express CD126 at lower levels than naïve $CD4^+$ T cells, but expression was still apparent (Fig 5.10). Staining of gp130 was also higher than isotype, and of a similar intensity to naïve T cells. This implies that the iTreg used here were able to respond to IL-6, most likely through direct interaction with the IL-6R, although trans-signaling through soluble IL-6R and gp130 also remained possible.

Thus, the presence of several cytokines relevant to the differentiation of Th1 and Th17 cells are able to exacerbate the loss of Foxp3 expression from iTreg. Some pro-inflammatory cytokines - IL-6, IL-12, and IL-27 - exacerbate the loss of Foxp3 expression and this was associated with an increase in the proportion of iTreg producing IFN-γ, but a reduction in the number of $GM\text{-}CSF^+$ cells. TNF production was somewhat reduced by these cytokine manipulations, though no clear discriminative effect could be seen between $Foxp3^+$ and $Foxp3^-$ cells.

5.2.3.4 TGF-β has a dominant effect on Foxp3 expression and iTreg survival

Chapter 4 described the stabilizing effect of TGF-β on iTreg Foxp3 expression (Fig 4.1). This was in the absence of other cytokines that may skew iTreg towards a different phenotype. Though the inability of IL-6 to 'convert' iTreg to Th17 cells in the presence of TGF-β is well documented (Zheng et al., 2008), the effect of other polarising cytokines is less clear. In the above report, though TGF-β is considered as essential for Th17 polarisation, in this circumstance it appeared to be stabilizing Foxp3 expression. This suggested that TGF-β could also maintain Foxp3 expression in the presence of other cytokines. To investigate this further, iTreg were again re-stimulated *in vitro* as above with

anti-CD3, anti-CD28, and exogenous cytokines as previously. In addition, each culture condition was supplemented either with extra IL-2 (100U/ml), or extra IL-2 (100U/ml) and TGF-β (5ng/ml).

Provision of additional IL-2 did not affect the cytokine profile with any of the other cytokines, nor did it alter the proportion of cells staining with the live/dead marker. Trypan blue exclusion demonstrated no change in viable cell number between wells with, or without IL-2, in addition to the individual cytokines (Fig 5.11A). In contrast, addition of TGF-β with the IL-2 had a notable effect in all circumstances. Cell counts were increased in all circumstances where TGF-β was included within the culture medium, suggesting a survival benefit (Fig 5.11A), with a significant effect seen with IL-6 and IL-1β. Addition of TGF-β alone was previously demonstrated to improve survival of iTreg (Fig 4.1) but this effect was enhanced in the presence of exogenous IL-2. The exact mechanism by which TGF-β improves survival of the re-stimulated iTreg is not clear, but may relate to the maintenance of Foxp3 and CD25, providing survival signals in the presence of the exogenous IL-2.

Intriguingly, addition of IL-2 and TGF-β with the other polarizing cytokines had a stabilizing influence on the production of pro-inflammatory cytokines by iTreg. IL-6, IL-12 and IL-27 all had the overall effect of reducing the proportion of iTreg being GM-CSF$^+$, with an increase in IFN-γ^+ cells with IL-12 (and, less so, with IL-27) (Fig 5.11B). The addition of IL-2 and TGF-β led to an increase in the proportion of iTreg producing GM-CSF and a reduction in IFN-γ^+ iTreg re-stimulated in the presence of IL-12 or IL-27. The effect of IL-2 and TGF-β on iTreg re-stimulated in the presence of IL-6 was very minor. TNF production was broadly unaffected.

Also notable was the impact of TGF-β addition in the cultures with IL-1β. IL-1β has been reported to increase GM-CSF production by naïve T cells (Lukens et al., 2012). Not only was no significant rise in the proportion of GM-CSF$^+$ iTreg seen with the addition of IL-1β (compared to iTreg re-stimulated in medium alone), but the addition of IL-2 and TGF-β reduced the proportion of iTreg staining GM-CSF$^+$ rather than increasing it.

Thus, TGF-β appeared to have a dominant effect on the cytokine profile of re-stimulated iTreg. Addition of TGF-β reduced the proportion of cells producing GM-CSF by an unknown mechanism, but the addition of other cytokines that had a similar effect (i.e. IL-27, IL-12) did not reduce the proportion of cells staining GM-CSF$^+$ further. Conversely, cytokines such as IL-1β are not able to 'restore' the proportion of cells producing GM-CSF back to that seen in medium. This suggests that TGF-β has an antagonistic effect to the other

cytokines, preventing them from influencing iTreg further. In these experiments, TGF-β was added in conjunction with IL-2 so a combined effect cannot be disregarded. However the addition of IL-2 alone did not alter the effects of other exogenous cytokines (Fig 5.11B). If the presumptive model has the induction of Treg in an environment with high levels of TGF-β, then clearly continuation of this is necessary for iTreg to maintain both Foxp3 expression and their cytokine profile.

Thus, TGF-β plays a crucial role in all aspects of iTreg. It is needed for induction (Chen et al., 2003), and maintenance of Foxp3 (Selvaraj et al., 2007). Persistence of TGF-β is required to prevent iTreg from altering their cytokine productions in the presence of other cytokines. Finally, TGF-β signaling appears vital for some aspects of iTreg suppression, certainly *in vitro* (Fig 4.11). The pattern of cytokine production by iTreg *in vivo* closely resembles the cytokine profile seen *in vitro* by iTreg cultured in the presence of IL-6, IL-12 or IL-27, suggesting that *in vivo* these cytokines may predominate.

5.2.4 Bystander suppression by iTreg *in vivo*

The efficacy of the iTreg in effecting bystander suppression of naïve cells *in vitro* was shown in Chapter 3 (Fig 3.4), and their ability to directly suppress polarised effectors in chapter 4 (Fig 4.13). That iTreg can suppress naïve cells *in vivo* has been shown here, and previous reports have demonstrated the potential for iTreg to suppress either disease onset (Selvaraj et al., 2008) or ameliorate ongoing disease (Tang et al., 2004). The initial experimental plan was to utilize the well-established EAE model, using Tg4 iTreg to suppress MOG-driven disease by 2D2 cells, or 2D2 iTreg to suppress MBP-driven disease by Tg4 cells. As discussed in chapter 3 (section 3.3.2), this was not feasible due to an inability of the donor cells to persist in the C57BL/6xB10.PL mouse.

Work done by Dr Karen Mackenzie and Dominika Nowakowska has established a model of short-term allergic airways inflammation, entailing administration of Th2-polarised effectors followed by three separate intra-tracheal airway challenges with the relevant antigen. This model has been successful in causing disease using OT-II Th2 cells and ovalbumin (OVA) as the initiating antigen, or 2D2 Th2 cells with recombinant MOG (rMOG) as the antigen. Direct suppression of disease, using 2D2 iTreg co-administered with 2D2 Th2 cells, has been demonstrated by Dominika Nowakowska (PhD thesis in preparation). This model provides a means to assess bystander suppression – are 2D2 iTreg capable of suppressing allergic airways inflammation initiated by OT-II cells?

The following work was done in collaboration with Dominika Nowakowska and Karen Mackenzie. 2D2 iTreg were generated from FACS sorted $CD4^+CD25^-V\alpha3.2^+$ cells as described in Chapter 2, then sorted as $CD25^{high}$ at the end of culture. Naïve OT-II $CD4^+$ cells were polarised towards the Th2 phenotype. C57BL/6 mice received $4x10^6$ 2D2 iTreg, then 24 hours later $2.5x10^6$ OT-II Th2 cells. Mice were then challenged one, four, and seven days following Th2 administration, with intratracheal rMOG and OVA (both 50μg) (Fig 5.12A). Two days after the last airway challenge, mice were culled. The lungs underwent bronchoalveolar lavage (BAL) as described in Section 2.11.1, and then spleen, mediastinal lymph nodes and lungs were harvested. One lung was processed for histological analysis, the other lung provided cells for flow cytometry analysis.

The experiment also included a group of mice that received 2D2 iTreg and OT-II Th2 cells, with airway challenges using OVA only. In that group, no differences were distinguishable in any end-point from mice that did not receive iTreg. On flow cytometry, very few iTreg were identifiable in spleen, lymph nodes, or lung from the mice receiving OVA intratracheally only, whilst mice receiving OVA and rMOG had significantly higher numbers of iTreg in all three organs (Fig 5.12B). For clarity, the data from the mice receiving OVA alone has not been included.

5.2.4.1 iTreg reduce the number of eosinophils in BAL fluid

Analysis of the BAL fluid used cytospins, and was calculated per ml of fluid in order to standardise the results between individual mice. The total number of cells in the BAL fluid was significantly reduced in the mice receiving 2D2 iTreg, suggesting either less severe disease, or more rapid resolution (Fig 5.12C). On classifying cell type within the BAL fluid, no difference was seen between PBS-treated and iTreg-treated mice in the numbers of macrophages or lymphocytes. The number of eosinophils was significantly reduced in the mice receiving iTreg, however (Fig 5.12C). Given this was a model of Th2-mediated disease, with eosinophils expected to be the main inflammatory cell type responsible for clinical disease, this reduction might be expected to translate to a disease-relevant difference in a clinical setting.

5.2.4.2 iTreg do not affect the number of donor OT-II cells in spleen, lymph nodes, or lungs

Following harvest, cells from each organ were analysed by flow cytometry for the proportions of donor OT-II cells. There was no difference in overall cellularity of the spleen (Fig 5.13A), mediastinal lymph nodes (Fig 5.13B), or lungs (Fig 5.13C), following

administration of OT-II Th2 cells and 2D2 iTreg, compared to mice receiving OT-II Th2 cells alone. Similarly, no difference was seen in the proportion of $CD4^+$ cells within each organ that were donor OT-II Th2 cells in the presence or absence of iTreg, implying no survival disadvantage for the donor cells in the presence of iTreg. Notably, a high proportion of $CD4^+$ cells within the lung were donor OT-II Th2 cells, both confirming the ability of the donor Th2 cells to traffic to the lung and also demonstrating their potential pathogenicity. Given the absence of difference between total numbers and proportions of donor cells, unsurprisingly there was no difference in the calculated total number of donor OT-II Th2 cells in each organ. Thus, 2D2 iTreg were not able to alter the total number of donor OT-II cells in any of the sites investigated, in contrast to the effect on naïve T cells seen previously (Fig 5.1). Importantly, work by Dominika Nowakowska had also demonstrated a significant reduction in the number of effector Th2 cells within the lung when 'direct' suppression was investigated i.e. 2D2 iTreg suppressing 2D2 Th2 cells (manuscript in preparation).

5.2.4.3 iTreg reduce the proportion of cells producing IL-5 and IL-13 in both the spleen and mediastinal lymph nodes

Cells from spleen and mediastinal lymph nodes were stimulated overnight in the presence of 20μg/ml rMOG and OVA. Brefeldin A was added for the final four hours of culture, then cells were stained for intracellular cytokine production. Insufficient cells were recovered from the lungs to permit meaningful comparisons between mice. Cells were stained for production of IFN-γ, TNF, IL-5, and IL-13 (Fig 5.14A).

No difference was seen in the proportion of donor OT-II Th2 cells producing either IFN-γ or TNF in the presence or absence of iTreg in either the spleen (Fig 5.14B) or the mediastinal lymph nodes (Fig 5.14C). The proportion of cells producing IL-5 in the spleen was also not significantly different between the two groups, though there were fewer $IL\text{-}5^+$ donor Th2 cells in the mice receiving 2D2 iTreg as well. In contrast, the proportion of $IL\text{-}13^+$ donor Th2 cells in the spleen was significantly reduced in the mice that had iTreg co-administered. Within the mediastinal lymph nodes, significant reductions in the proportion of both $IL\text{-}5^+$ and $IL\text{-}13^+$ donor OT-II Th2 cells were seen in the presence of 2D2 iTreg.

Thus, 2D2 iTreg had a definable effect upon OT-II Th2 cells within a C57BL/6 host in the presence of both relevant antigens. Though no difference in the number of donor Th2 cells was observed, there were fewer eosinophils/ml in the BAL fluid, and fewer donor Th2 cells were $IL\text{-}5^+$ and $IL\text{-}13^+$ in the mice that had also received iTreg. Histology did not

demonstrate any difference in disease severity between the groups. This may be related to the timing of assessment as resolution occurs rapidly in this model regardless of any additional interventions. The disease induced by OT-II cells in the presence of OVA is also more severe than that by 2D2 cells with rMOG (Dominika Nowakowska, personal communication). Thus, any histological difference may be too mild to be recognised.

5.3 Discussion

5.3.1 Suppression by iTreg *in vivo*

Efficacy of iTreg in preventing the onset of disease has been shown in numerous models, including EAE. One report using polyclonal iTreg generated from Foxp3gfp reporter mice demonstrated marked reduction in the development of EAE if given prior to immunization (Selvaraj et al., 2008). That same report also demonstrated an effect of iTreg given four days following priming, implying that iTreg could exert an impact on T cells already responding. Reduced numbers of effector cells were identified in the CNS, with a greater proportion of $CD4^+$ cells being $Foxp3^+$, a well-characterized finding (O'Connor et al., 2007). Selvaraj et al suggested the reduction in effector cell number was due to the induction of anergy and infectious tolerance. Analysis of lymph node tissue revealed loss of Foxp3 expression in the donor iTreg (recapitulating the data presented here), but when depleted of donor iTreg, those same lymph node cells were then protective for EAE when transferred into new host mice. Anergy was proposed because the response of host T cells to $pMOG_{35-55}$ was markedly reduced in mice receiving iTreg, but this response was restored in the presence of exogenous IL-2. The data presented here showed an overall reduction in donor effector number, which would account for the protective effect of iTreg. However the donor naïve T cells recovered here were able to produce IFN-γ, TNF and GM-CSF in response to subsequent PMA and ionomycin stimulation. As IL-2 production and proliferation were not assessed on donor naïve T cells recovered from either spleen or lymph nodes, anergy cannot be confirmed as a mechanism but the production of the other cytokines render this possibility less likely. The loss of CFSE staining would suggest however that if anergy is indeed induced, this may not occur immediately, but only after several cell cycle divisions have already taken place.

Suppression of proliferation as measured by CFSE dilution has been demonstrated previously. Use of $PLP_{139-151}$-specific iTreg in an EAE model with SJL mice limited dilution of CFSE in naïve $CD4^+$ T cells primed with $PLP_{139-151,}$ but not of control 2D2 $CD4^+$ T cells primed with $pMOG_{35-55}$ within the same mouse (Zhang et al., 2010). In those experiments, CFSE dilution was assessed at three time points - days 1, 3 and 6. Of note, suppression of CFSE dilution was seen at day three, but extensive proliferation following immunization led to limited analysis at day six. Thus, rather than contradicting the data presented here, that report supports the finding that naïve T cell proliferation at a later time point (day six in that report, day seven in this thesis) is not prevented in the presence of iTreg. That report suggests that iTreg can suppress proliferation only in the first few days following

administration. That delay in proliferation, rather than complete suppression, may be sufficient to effect the delayed onset of EAE reported in the mice receiving iTreg.

In a different model, H^+/K^+ ATPase specific iTreg were able to suppress the development of autoimmune gastritis (AIG) (DiPaolo et al., 2007). The model used transfer of TCR transgenic thymocytes (recognizing a peptide of the H^+/K^+/ATPase autoantigen) which leads to AIG in host mice. Mice receiving iTreg together with transferred donor thymocytes had reduced cellularity of the gastric lymph nodes, and very few donor thymocytes detectable. In that report, Foxp3 expression by gastric lymph node resident iTreg was stable *in vivo* (97% $Foxp3^+$ after 7 days). There, CFSE dilution was assessed five days after cell transfer, representing an intermediate time between the report by Zhang et al. and the data shown here. As might be predicted, CFSE dilution was affected in the presence of iTreg with 14% of donor thymocytes not having undergone division at that time point. This compares to 36% undivided cells in the EAE model at 3 days and less than 1% in the data here. That study, thus, further supported that iTreg delay the proliferation of naïve T cells, rather than suppressing it completely. The proposed mechanism was that iTreg prevent stimulation of thymocytes by the gastric lymph node DC, partly through downregulation of expression of the co-stimulatory molecules CD80 and CD86.

How the iTreg mediate the reduction in donor effector number was suggested by an early study using TGF-β-generated 'Treg' in non-obese diabetogenic mice (Weber et al., 2006). Those iTreg were able to suppress the development of diabetes and greatly reduced the number of effector Th1 cells in the pancreatic lymph nodes. *In vitro* assays suggested direct cytotoxicity by iTreg may be responsible for the loss of effector Th1 cells. This was supported by *in vivo* blockade of Fas-FasL interactions at the time of administration of donor Th1 cells restoring the number of effector cells detectable in the lymph nodes two days later.

Thus, the data presented here concord with much of the published literature. The ability of iTreg to reduce the number of recoverable cells (both naïve cells and Th1 polarised effectors) after only a short time *in vivo* is witnessed in several models, and has been suggested to be a direct cytotoxic effect of the iTreg, or related to changes in the phenotype of the priming DCs.

5.3.1.1 Survival by iTreg *in vivo*

The recovery of iTreg from mice receiving antigen and CFA was approximately 15% of infused cells within the spleen and LN (Fig 5.4D, 5.5B) though the vast majority of these cells no longer expressed Foxp3 (Fig 5.4E, 5.5C). The fate of the remaining infused iTreg is

unclear. Previous reports have demonstrated migration of Treg to the bone marrow (Selvaraj et al., 2007) and examination of the bone marrow of mice receiving iTreg with congenic markers confirmed that 2-3% of the $CD4^+$ population within the marrow were donor iTreg [data not shown]. Though isolation of the marrow involved multiple wash steps prior to extraction, contamination with peripheral blood cannot be wholly excluded. Thus, iTreg also migrated to an organ not regularly assessed in the majority of experiments. The subsequent allergic airway inflammation model demonstrated that iTreg were also able to migrate to the lung, though this was highly dependent on the presence of cognate antigen (Fig 5.12B). Similarly, migration of nTreg to the CNS is recognized in EAE models (O'Connor et al., 2007). Other organs, such as the liver and skin, may also serve as migratory targets for iTreg, but none were specifically examined in the *in vivo* experiments described here. Clarifying the chemokine markers on the iTreg may elucidate potential organ targets, as may multi-organ harvests of mice receiving iTreg. In the absence of antigen, however, it is likely that the majority would remain within the haematogenous and lymphatic compartments.

The reduced numbers of recoverable iTreg may also reflect their survival *in vivo*. It is near impossible to determine whether the absent iTreg are resident elsewhere or have not survived. However, the survival *in vitro* of these iTreg is very limited, with poor viability on re-stimulation as described in Chapter 4. Other reports similarly demonstrate a very limited lifespan when transferred *in vivo* (Selvaraj et al., 2007) though this is contrasted by others who have reported persistence of iTreg several weeks post-transfer (Chen et al., 2011) admittedly with experimental manipulation to enhance survival. Kinetic experiments may have utility here, if the number of recoverable iTreg is shown to diminish rapidly over several days *in vivo*. Such an experiment would also require harvesting of multiple organs to ensure that diminuition of iTreg was not due to migration elsewhere.

The loss of Foxp3 expression also occurs rapidly, and is consistent with previous descriptions of Treg kinetics (Selvaraj et al., 2007). Again, this partly replicates the situation seen *in vitro* in the absence of high concentrations of TGF-β. It is notable that mice that did not receive pro-inflammatory signals via CFA had a higher expression of Foxp3 though overall iTreg recovery was lower (Fig 5.4E, 5.5C). This may imply that TCR stimulation in the presence of pro-inflammatory signalling enhances loss of Foxp3 expression but also promotes cell survival. An alternative explanation is that there is expansion of contaminating $Foxp3^-$ cells. However the high yield of $Foxp3^+$ cells at the end of iTreg primary culture, followed by subsequent FACS prior to infusion to purities consistently

greater than 99% make this possibility unlikely. Additionally, other experimental models within the laboratory have utilized highly pure iTreg sorted by flow cytometry to 100% with similar loss of Foxp3 expression when then recovered *ex vivo* (Richard O'Connor, personal communication). Importantly, despite the loss of Foxp3 expression and limited survival *in vivo*, the suppressive effect of these iTreg is maintained when interrogating the number of naïve T cells recoverable (Fig 5.1) and their cytokine production (Fig 5.3).

5.3.2 iTreg and cytokine production

The production of IFN-γ and TNF by the transferred naïve T cells was not affected within the spleen, though IFN-γ^+ donor naïve T cells were significantly lower in inguinal lymph nodes on meta-analysis of all experiments. GM-CSF^+ donor naïve T cells were consistently found at a lower frequency in mice receiving both naïve and iTreg cells, though this effect did not reach significance. An alteration in cytokine production by these naïve T cells may indicate a change in their differentiation towards an effector phenotype. This has been proposed as a mechanism of action of nTreg (Sarween et al., 2004). Indeed, nTreg may alter the cytokine profile of $CD8^+$ T cells without changing the differentiation program (Mempel et al., 2006). This has also been suggested to occur in $CD4^+$ T cells, for example a reduction in IFN-γ production by Th1 cells without influencing Tbet expression (Sojka et al., 2011). Less literature exists on the influence of iTreg on cytokine production by effector cells.

In the reports examining the *in vivo* efficacy of iTreg, the polyclonal iTreg used in EAE appeared to alter the cytokine profile of the $pMOG_{35-55}$ primed T cells subsequently obtained from the lymph nodes, with a reduction in IFN-γ and IL-17 plus an increase in IL-10 (Selvaraj et al., 2008). In the model of autoimmune gastritis, iTreg treatment reduced the secretion by the gastric mucosal cells of IFN-γ and IL-17, again with a concomitant increase in IL-10 production (Nguyen et al., 2011). The ability of Treg to suppress GM-CSF production by naïve or effector T cells has not been reported to date. Most literature surrounding GM-CSF is in relation to the maturation effect on dendritic cells and promotion of the pro-inflammatory state. If iTreg do indeed reduce the proportion of effector T cells secreting GM-CSF, this would support another mechanism by which they can both prevent the onset of inflammation, and limit ongoing disease.

Whilst various reports exist of the need for TNF in regulatory responses by Treg (discussed in chapter 4.3.2.2), no impact on TNF production by effector cells has been reported, consistent with the data shown here.

Thus, the alteration in the cytokine profile of antigen-stimulated naive T cells seen in these *in vivo* experiments is consistent with the literature, showing that IFN-γ production by either naïve or effector T cells is reduced in the presence of iTreg. This is intriguing given the production of IFN-γ by the iTreg themselves, and the recognised phenomenon that IFN-γ promotes its own production by effector T cells (O'Connor et al., 2010). This suggests that, whilst iTreg may influence differentiation by responding cells, the predominant effect *in vivo* is to reduce the number of potentially pathogenic cells.

5.3.2.1 IL-6

IL-6 hastened loss of Foxp3 expression in the *in vitro* restimulation experiments (Fig 5.8C), as did IL-27 and IL-12. The IL-6/TGF-β combination plays a crucial role in the generation of Th17 cells (Veldhoen et al., 2006). Thus, IL-6 is a key cytokine driving naïve cell differentiation when TGF-β is already present, preventing induction of the iTreg phenotype and promoting Th17 differentiation (Bettelli et al., 2006). The data presented here suggest that IL-6 can have similar effects on Foxp3 expression if introduced later into the milieu. IL-17 production was not seen (data not shown), replicating other reports (O'Connor et al., 2010). A change to IL-17 production has been previously described in iTreg (Xu et al., 2007, Osorio et al., 2008) and indeed in nTreg (Yang et al., 2008a) though iTreg production of IL-17 has been contradicted by other reports (Zheng et al., 2008). Notably, IL-17 production is not seen in cells isolated from the CNS in an EAE model, suggesting organ-specificity may play a role (O'Connor et al., 2012). Thus, IL-6 is likely to promote loss of Foxp3 expression though cannot complete 'conversion' to a Th17-like cell. This is supported by the increase in $Foxp3^+$ Treg populations seen in mice unable to respond to IL-6 due to T-cell restricted deletion of gp130 (Korn et al., 2008).

That this can feasibly occur is confirmed by the presence of both IL-6 receptor chains on iTreg, including persistence of the gp130 signaling subunit at levels similar to naïve T cells (Fig 5.10). This implies that iTreg can respond to IL-6 through *trans*-signaling as well as conventional IL-6 signals. IL-6 is able to bind to the soluble IL6Rα (CD126) and then interact directly with gp130 on the cell, negating the need for surface expression of CD126. *Trans*-signaling by IL-6 in naïve cells at the initiation of the inflammatory response has been demonstrated to prevent the development of iTreg *in vivo* (Dominitzki et al., 2007). Downregulation of both gp130 and CD126 occurs with TCR stimulation (Betz et al., 1998) in all $CD4^+$ cells. Though expression of both CD126 and gp130 is often closely correlated, differential expression has been reported in human nTreg which are $CD126^+$ but with

minimal gp130 detectable (Oberg et al., 2006). That same report also suggested that nTreg have more stable CD126 expression, relatively resistant to down-regulation on TCR stimulation. Given that Foxp3 expression is induced in naïve T cells by TCR stimulation, reduced expression of both CD126 and gp130 would be the expected outcome. Contrasting murine nTreg and iTreg, the different response to IL-6 was attributed to receptor expression in one report (Zheng et al., 2008). There, iTreg re-stimulated in the presence of IL-6 maintained Foxp3 expression at high levels (over 80%) in contrast to the rapid loss of Foxp3 seen in the experiments performed here. The differential response to IL-6 between nTreg and iTreg also correlated with suppressive function; IL-6 re-conditioned iTreg remained suppressive in both *in vitro* and *in vivo* models. Of note, gp130 expression by iTreg was not included in that report, and CD126 expression was still evident (albeit at a low level) in 21% of the iTreg generated there. The data here support the assertion of that paper that CD126 expression is lower following TCR stimulation in the presence of IL-2 and TGF-β, but conversely also shows that gp130 expression is maintained on iTreg. Thus IL-6 responses may still occur through *trans*-signaling and are evident through effects of IL-6 addition *in vitro* on GM-CSF production by iTreg.

IL-6 has received most attention within the literature due to its role in the dichotomous differentiation of iTreg or Th17. What of the other cytokines recognized to be relevant in Th1 and Th17 differentiation?

5.3.2.2 IL-12

IL-12 is primarily important in Th1 differentiation, and promotes production of IFN-γ. The presence of IL-12 in the iTreg re-stimulation cultures led to an increased loss of Foxp3 expression, a reduction in GM-CSF$^+$ and an increase in IFN-γ$^+$ cells (Fig5.8B, C, 5.9A, B). Other reports examining IL-12 in Treg stability have had similar results. A role for conversion of Treg to IFN-γ producing cells in an *in vivo* colitis model demonstrated the importance of intestinal IL-12 (Feng et al., 2011) though not all of those cells lost Foxp3 expression or suppressive potential. The same report confirmed an effect of IL-12 on Foxp3 expression *in vitro* though to a far less impressive extent than seen in the data presented here. Those iTreg also retained Foxp3 expression when cultured in medium alone, compared to the 40% of iTreg maintaining Foxp3 expression shown here (Fig 5.8B). This may suggest that the protocol used by those investigators (antigen-pulsed APC and TGF-β though not

exogenous IL-2) may lead to a more stable Foxp3 phenotype, though this cannot be confirmed from the published report.

IL-12 was also implicated in conversion of Treg to IFN-γ^+ cells in a model of *Toxoplasma gondii* infection (Oldenhove et al., 2009). There, nTreg were shown to express Tbet and produce IFN-γ in response to APC from *T.gondii* infected mice, with a similar effect on iTreg when IL-12 was added to primary cultures. There was no investigation of the effect of IL-12 on iTreg after generation in that report.

Finally, and highly relevant to the data presented here, re-stimulating iTreg in the presence of IL-12 led to loss of Foxp3 expression but those "ex-iTreg" were still able to inhibit proliferation (as measured by CFSE dilution) of naïve T cells in co-culture (O'Connor et al., 2010). In a model of EAE, IL-12 conditioned iTreg reduced both the incidence and the severity of the disease that developed, even with almost undetectable Foxp3 expression. Those IL-12 conditioned iTreg were also able to produce clinically detectable EAE when administered alone, though much higher cell numbers were needed, and the disease was both brief and mild. The data presented here provides one plausible mechanism for this, as IL-12 exposure to these iTreg leads to a reduction in GM-CSF production (Fig 5.9) and GM-CSF is reported to be a key encephalitogenic cytokine in EAE (Codarri et al, 2011).

Curiously, given the major role of IL-12 in Th1 differentiation, very little other literature on the effect of IL-12 on Treg is identifiable. Given the presumed plasticity of iTreg, IL-12 here appears to promote loss of Foxp3 expression and the production of IFN-γ (Fig 5.8C, 5.9A), corroborating the reports above.

5.3.2.3 IL-27

In the data presented here, IL-27 had a similar effect to IL-12 on both Foxp3 expression and cytokine production by iTreg on restimulation (Fig 5.8, Fig 5.9). IL-27 is a more recently described member of the IL-12 family (and equally has similarities to IL-6) (Hunter et al., 2005), and was first thought to promote Th1 responses. Subsequently, reports have demonstrated a protective role in models of EAE (Stumhofer et al., 2006) through the inhibition of Th17 development, partly through antagonism of the effects of IL-6. IL-27 is not required for nTreg function, nor do IL-27 deficient mice have an abnormal nTreg population (Batten et al., 2006). IL-27 does promote the generation of Tr1 cells, however,

leading to an increase in IL-10 and thus has an anti-inflammatory role (Pot et al., 2009). In support of the data shown here, IL-27 is reported to limit iTreg generation when present initially (Neufert et al., 2007), mainly through effects via the STAT3 pathway (Huber et al., 2008), and may affect iTreg survival through effects on IL-2 (Tait Wojno et al., 2011). IL-27 is not reported to antagonize Foxp3 expression of already differentiated iTreg, however (in contrast to IL-6), so the direct effect presented here on re-stimulation is a novel finding. This may relate to preferential survival of non-Foxp3$^+$ cells in the presence of IL-2, though supplementing additional IL-2 in these re-stimulation cultures did not affect the loss of Foxp3 (data not shown). The effect of IL-27 on the cytokine production by iTreg has not been previously reported. A recent paper demonstrated that IL-27 suppressed GM-CSF production by naïve CD4$^+$ and CD8$^+$ T cells and cells polarized towards the Th1 phenotype, in both mice and humans (Young et al., 2012). These data support that report, implying that IL-27 may have a broader remit in suppressing GM-CSF from a variety of different cellular sources.

5.3.2.4 TGF-β

The crucial role of TGF-β in the maintenance of iTreg is again reiterated. In the presence of ongoing TGF-β, Foxp3 expression was consistently higher after 72 hours of re-stimulation, as seen in chapter 4 (Fig 4.1). The presence of exogenous TGF-β in culture medium also had the same effect on cell survival (as indicated by cell counts using trypan blue exclusion) as reported in chapter 4. Where the other cytokines of interest were being investigated, TGF-β had a clearly dominant role in promoting both cell survival (Fig 5.11A), an effect not seen when IL-2 was the only exogenous addition, and Foxp3 expression.

In one report, the impact of TGF-β on the survival of re-stimulated iTreg was attributed to a differential use of cell death pathways by iTreg (Tischner et al., 2012). There, iTreg were resistant to Fas/FasL mediated killing due to downregulation of Fas and FasL but were highly susceptible to activated cell autonomous death (ACAD) in the absence of IL-2. TGF-β in restimulated iTreg upregulated components of the BH3 signaling pathway used in ACAD, suggesting that TGF-β actually enhanced cell death in iTreg. A balance between IL-2 and TGF-β was identified in that paper, whereby the presence of IL-2 counteracted the BH3 pathway in iTreg, and TGF-β counteracted the Fas/FasL upregulation that occurred when iTreg were re-stimulated. Thus, in the presence of both IL-2 and TGF-β, iTreg had maximal survival.

That report further confirms the findings of this thesis that Foxp3 expression is lost from iTreg on secondary TCR stimulation, with the addition of IL-2 and TGF-β partially rescuing expression.

The influence of TGF-β on cytokine production by iTreg has been scarcely investigated. Much of the literature concentrates on the IL-6/TGF-β axis for iTreg/Th17 differentiation, whilst the influence of TGF-β on already-established iTreg is relatively neglected. The impact of TGF-β on pro-inflammatory cytokines by iTreg has not been reported. One group demonstrated that nTreg increased IL-6 production by mast cells via membrane-bound TGF-β signaling through SMAD2/3 pathways (Ganeshan et al., 2012).

Cytokine production by these re-stimulated iTreg was also stabilized in the presence of TGF-β. In all cases, the provision of TGF-β in addition to the other exogenous cytokine led to the proportion of GM-CSF^{+} cells being similar to that of TGF-β alone. TGF-β thus appeared to have a dominant effect on the GM-CSF production by iTreg.

5.3.2.5. GM-CSF production by iTreg was affected by other cytokines

GM-CSF production by iTreg appeared most susceptible to cytokine manipulations, compared to production of IFN-γ or TNF. In the presence of additional TGF-β and maintenance of Foxp3 expression, iTreg production of both IFN-γ and GM-CSF was reduced (Fig 5.11B). Conversely, in the presence of Th1-relevant cytokines, IL-12, IL-27, and the Th17-relevant cytokine, IL-6, GM-CSF production was the only cytokine reduced in production (Fig 5.9). This pattern of cytokine production by iTreg is similar to that seen when donor iTreg were stimulated *in vivo* (Fig 5.6), in which pro-inflammatory cytokines might be expected to predominate in the presence of antigen with CFA. Despite the finding by others that the production of GM-CSF by Th17 cells is promoted by IL-23 (El-Behi et al., 2011) and IL-1β (Lukens et al., 2012), addition of either cytokine alone to re-stimulation cultures did not alter the production of GM-CSF (Fig 5.9, Appendix 1), most likely due to the absence of the relevant receptors (certainly the case for IL-23) (Zhou et al., 2007). Combined with the absence of IL-17 production in response to these various cytokine manipulations, this may suggest that upregulation of Foxp3 expression renders cells less sensitive to environments promoting a 'Th17-like' response, the correlate of that reported for Th17 cells in a regulatory milieu (Korn et al., 2008).

5.3.3 Bystander suppression by iTreg in vivo

As previously discussed in chapter 3, the evidence for bystander suppression *in vivo* is conflicting. In this chapter, a model of allergic airways inflammation (AAI) was used to assess whether iTreg could suppress in a bystander fashion. A different model of AAI demonstrated that nTreg (defined as $CD4^+$ $CD25^+$ cells) were able to reduce the number of lung-resident effectors, as well as the proportion of cells producing IL-5 and IL-13 (Kearley et al., 2005). In that report, the suppression was dependent upon IL-10. This has been postulated to contribute to the effect of nTreg in AAI in suppressing the differentiation of cells towards the Th2 phenotype (Jaffar et al., 2004). A role for TGF-β has also been proposed (Joetham et al., 2007). The use of 'iTreg' in AAI was demonstrated to be effective in a model utilising cockroach antigen to cause airway hyperresponsiveness, though these were Tr1 cells subsequently expressing Foxp3, rather than TGF-β-induced iTreg (McGee et al., 2009). An important role for iTreg generated *in vivo* in controlling allergic airways inflammation was suggested (de LaFaille et al., 2008).

Most directly applicable to the experiments performed here is a report demonstrating that the administration of $5x10^6$ polyclonal TGF-β-induced iTreg was able to reduce the development of AAI, and reduce both IL-5 and IL-13 production, in an OVA-sensitised model (Xu et al., 2012). Given the well-established superiority of antigen-specific iTreg over polyclonal iTreg in Th1/Th17 mediated disease (Weber et al., 2006) one might expect even more impressive results had OVA-specific iTreg been used. Importantly, that report also demonstrated an impact of iTreg administered during airway challenges, though this was less effective than iTreg given prior to challenge. This has particular relevance to a clinical setting, when therapy prior to disease onset is much less feasible.

Here, 2D2 iTreg have been shown to have a significant impact on the development of eosinophilia within the BAL fluid, and reduce the proportion of donor Th2 cells producing IL-5 and IL-13. No impact was seen on disease histology, however. Thus, bystander suppression has been seen *in vivo* in a model of AAI, but appeared less effective than 'direct' suppression. What might account for this?

2D2 iTreg were able to effectively suppress disease by donor 2D2 Th2 cells (work by D Nowakowksa, manuscript in preparation). The severity of disease caused using 2D2 Th2 and rMOG was notably less than that caused by OT-II Th2 and OVA. Thus, the disease baseline for this bystander suppression was greater. Though iTreg are able to impact on ongoing disease, this may have affected the ability of the experimental model to demonstrate

statistically significant differences. Secondly, the iTreg were sorted at the end of initial culture by expression of CD25. The fluorochrome-antibody combination used for this was antiCD25-PE, clone PC61.5 – which may have had a depleting or functional effect on the iTreg themselves. Finally, bystander suppression may simply be less effective than direct suppression, though the reasoning for this requires a fuller understanding of the exact suppressive mechanisms used.

Further experiments could increase the number of iTreg administered or reduce the number of donor Th2 cells infused, though this may then impact on development of a disease readout. Alternatively, the protocol could be reversed, using OT-II iTreg to suppress 2D2-Th2-mediated inflammation. However the level of inflammation caused by 2D2 Th2 with rMOG is much milder such that demonstrating significant improvement with Treg may be problematic.

Importantly, the data presented here offer the first demonstration of iTreg suppressing in a bystander fashion in a model of AAI. As the antigens responsible for clinical disease are often multitude, these experiments provide proof of principle that iTreg could influence the development of disease in an antigen-non-specific manner without causing generalised immunosuppression. How then do iTreg exert their suppressive effects *in vivo*? The data presented herein suggest it is not via inhibiting proliferation of responding Th2 cells, nor is it consistently via an effect on cytokine production by responders. The production of pro-inflammatory cytokines by iTreg may be altered in an inflammatory milieu, but this does not appear to affect the subsequent suppression of naïve T cells. The *in vitro* data from Chapter 4 demonstrate no significant role for any of the three cytokines identified in suppression. The *in vivo* data here cannot contribute directly to refuting any role for these cytokines in iTreg function, but the maintenance of IFN-γ and TNF *ex vivo* in the face of effective suppression strongly implies they do not have a detrimental impact. Conversely, the loss of GM-CSF production in the presence of inflammatory signals may well have functional significance, and this merits further study.

A recent report proposed that iTreg may be more efficient in controlling Th2 responses than Th1 responses, and this may be partly attributable to their production of IFN-ɣ. The presence of IFN-ɣ is known to inhibit polarization towards a Th2 phenotype (as discussed in section 1.4.1) and promotes Th1 polarisation. Thus, production of IFN-γ by iTreg may be relevant in suppressing Th2-mediated disease. This is clearly not the only means of suppression however, as iTreg generated from IFN-γ deplete mice were equally suppressive

in vitro (Fig 4.7E, F) and also suppressed the persistence of naïve T cells *in vivo*, in identical experiments to those described in Fig 5.1 [data not shown]. It is likely that iTreg utilize a variety of mechanisms to suppress, but a further informative experiment would entail using iTreg generated from IFN-γ-deplete mice in the allergic airways inflammation model. If IFN-γ production is relevant to suppression of Th2-mediated disease, one may expect a reduction in the efficacy of suppression in that scenario. Similarly, it may be anticipated that iTreg would be more effective in the suppression of polarization of responding T cells towards a Th2 phenotype, rather than in suppressing the differentiated Th2 cells used here.

As suggested earlier, it is likely that iTreg, much like nTreg, have a variety of immunosuppressive mechanisms available to them, and utilization of any given means of suppression may be determined by a variety of factors, including the stimuli and cytokine environment leading to Treg induction, whether the target of suppression is a naïve or already differentiated cell and the nature of that differentiation, the presence or absence of ongoing pro-inflammatory signals, and indeed where suppression is actively mediated, whether in the draining lymph nodes as seems to be the case for transferred naïve T cells, or the inflamed organ itself as may be suggested in the AAI model. Clearly, a great deal of work remains in elucidating the very basic nature of iTreg suppression.

Clarification of the role of the various cytokines produced by iTreg could be provided by using various transgenic models (GM-CSF-deplete mice are available), or the *in vivo* administration of antibodies directed to those cytokines. Further *in vivo* depletion experiments could also partly determine the kinetics of the *in vivo* response – are iTreg required to physically persist to prevent organ-specific inflammation, or is their effect early in the inflammatory process, mirroring the findings in the *in vitro* assay? Another necessary experiment would be to replicate the AAI data in mice that had been depleted of their own nTreg population. An absence of effect may indicate that iTreg 'recruit' nTreg to assist in suppression, or indeed that nTreg were wholly responsible for suppression with the presence of iTreg being an incidental finding. Depletion experiments with subsequent restoration of nTreg would therefore expand upon these data.

5.3.4 Concluding remarks

iTreg are functional *in vivo* by reducing the number of transferred naïve T cells able to respond in a pro-inflammatory manner. Antigen stimulation of iTreg *in vivo* hastens loss of

Foxp3 expression but this does not appear to impact on their suppressive potential. Cytokine production by iTreg is altered *in vivo*, and can be replicated *in vitro* by the addition of either IL-27, IL-12, or to a lesser extent, IL-6 to *in vitro* restimulation cultures. Bystander suppression by iTreg occurs *in vivo* though is less efficacious than direct suppression, and appears to predominantly affect cytokine production by effector Th2 cells, rather than effector cell number. These findings have implications for translation of iTreg therapy into the clinical setting, both positive – bystander suppression does not require foreknowledge of the disease initiating antigen – and negative – proof that these iTreg do not become pathogenic in the *in vivo* pro-inflammatory environment is now even more crucial.

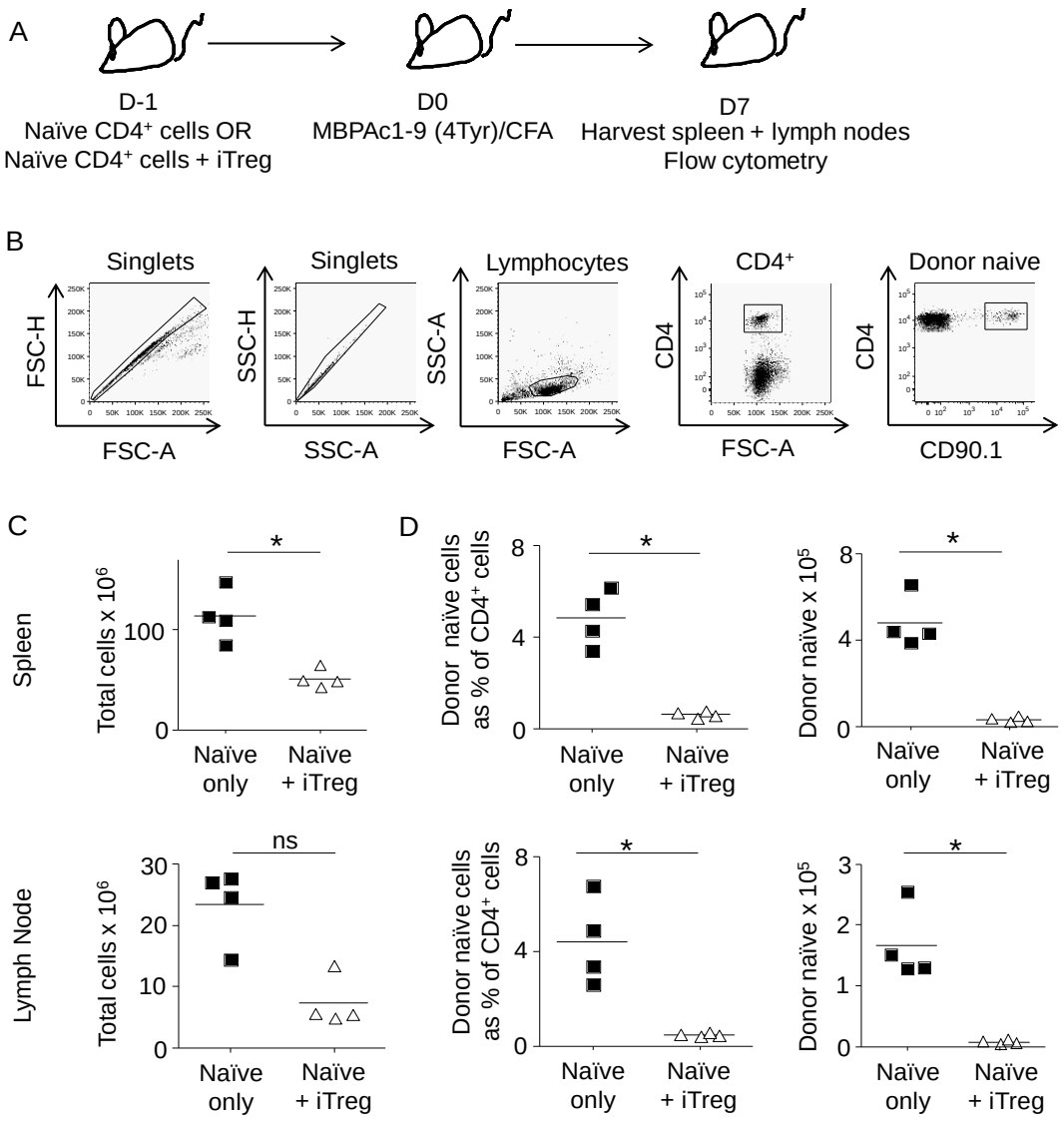


Figure 5.1 iTreg reduce persistence of co-transferred naïve cells in the spleen and lymph nodes (LN).

A) Experimental scheme. Either $2x10^6$ Tg4xFoxp3LuciDTR-4 iTreg and $2x10^6$ naïve CD4+ Tg4 CD90.1 cells, or $2x10^6$ naïve cells alone were transferred into C57BL/6xB10.PL hosts. One day later, 10µg MBPAc1-9 (4Tyr) emulsified in CFA was injected subcutaneously. After one week, mice were culled, spleens and inguinal lymph nodes harvested and analysed by flow cytometry. B) Gating strategy. C) Total cellularity of spleens and lymph nodes, assessed by trypan blue exclusion. D) Percentage and total number of donor naïve cells within the CD4+ population in both spleen and lymph nodes. Data are from one of three experiments giving consistent results. *=$p<0.05$ as determined by Mann-Whitney U test. ns= not significant

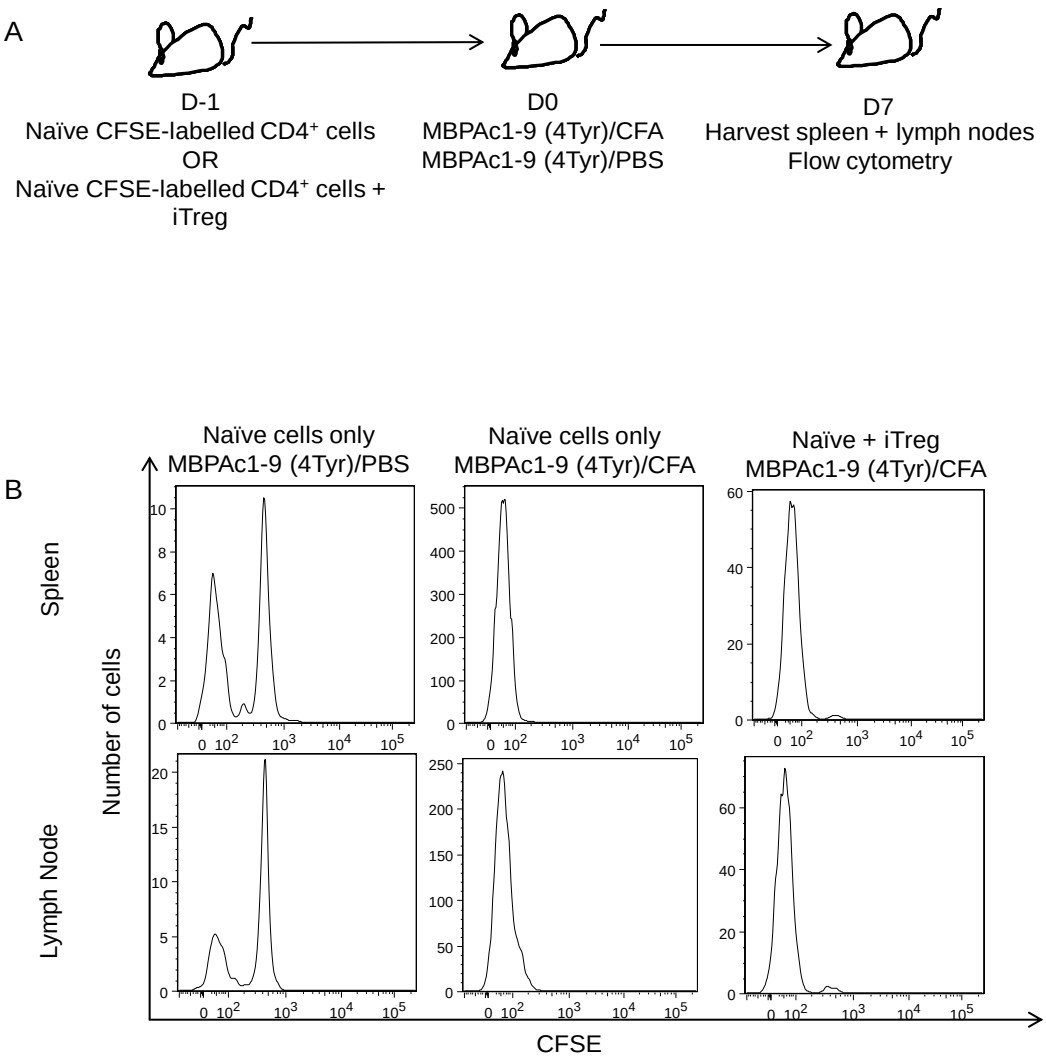


Figure 5.2 iTreg do not suppress proliferation by naïve cells *in vivo.*
A) Experimental scheme. Either 2 x 10^6 Tg4xFoxp3LuciDTR-4 iTreg and 2 x 10^6 naïve CD4$^+$ Tg4 CD90.1 cells, or naïve cells alone were transferred into C57BL/6xB10.PL hosts. One day later, 10µg MBPAc1-9 (4Tyr) emulsified in CFA, or in PBS, was injected subcutaneously. After one week, mice were culled, spleens and inguinal lymph nodes harvested, and analysed by flow cytometry. B) Cells were gated on live CD4$^+$ cells. Representative histograms of CFSE from mice receiving 10µg MBPAc1-9 (4Tyr) in either CFA or PBS, in the presence of absence of iTreg. Plots from spleen and inguinal lymph nodes are shown. Data are from one of two experiments giving consistent results.

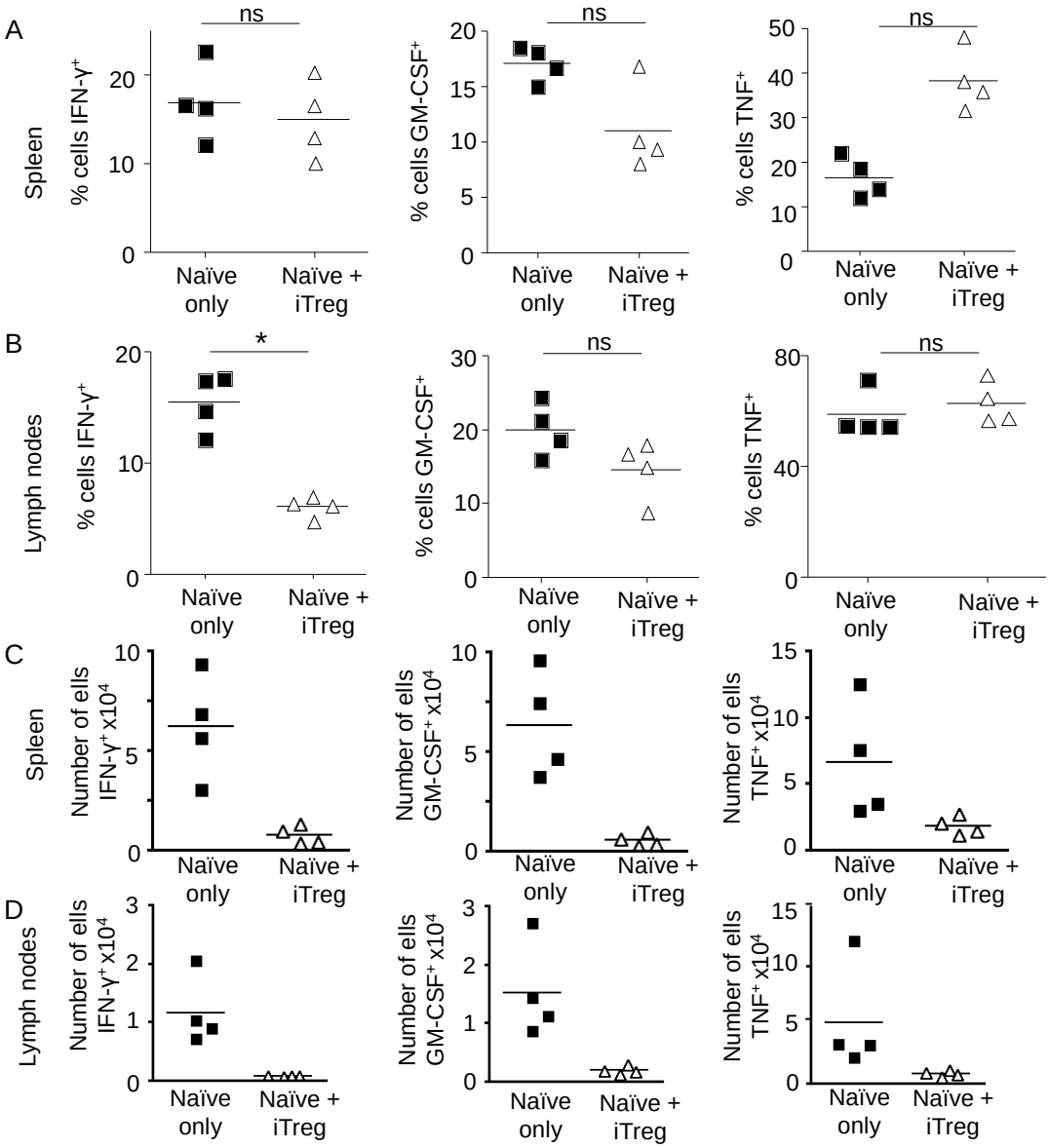


Figure 5.3 iTreg reduce the proportion of naïve donor cells producing IFN-γ in LN, but not GM-CSF or TNF.

As detailed in Fig 5.1, mice received naïve cells with or without iTreg, then MBPAc1-9 (4Tyr) emulsified in CFA, then were culled one week later. Cells were stimulated overnight with 10μM MBPAc1-9 (4Lys) then for a final four hours with PMA and ionomycin in the presence of brefeldin A. Intracellular cytokine staining of the donor naïve cells gated on $CD4^+$ $CD90.1^+$ cells is shown from spleen (A) and LN (B). Calculated numbers of cells producing each cytokine are shown from spleen (C) and LN (D). Data are from one of three experiments giving consistent results. *=p<0.05 as determined by meta-analysis of all experiments. ns=not significant.

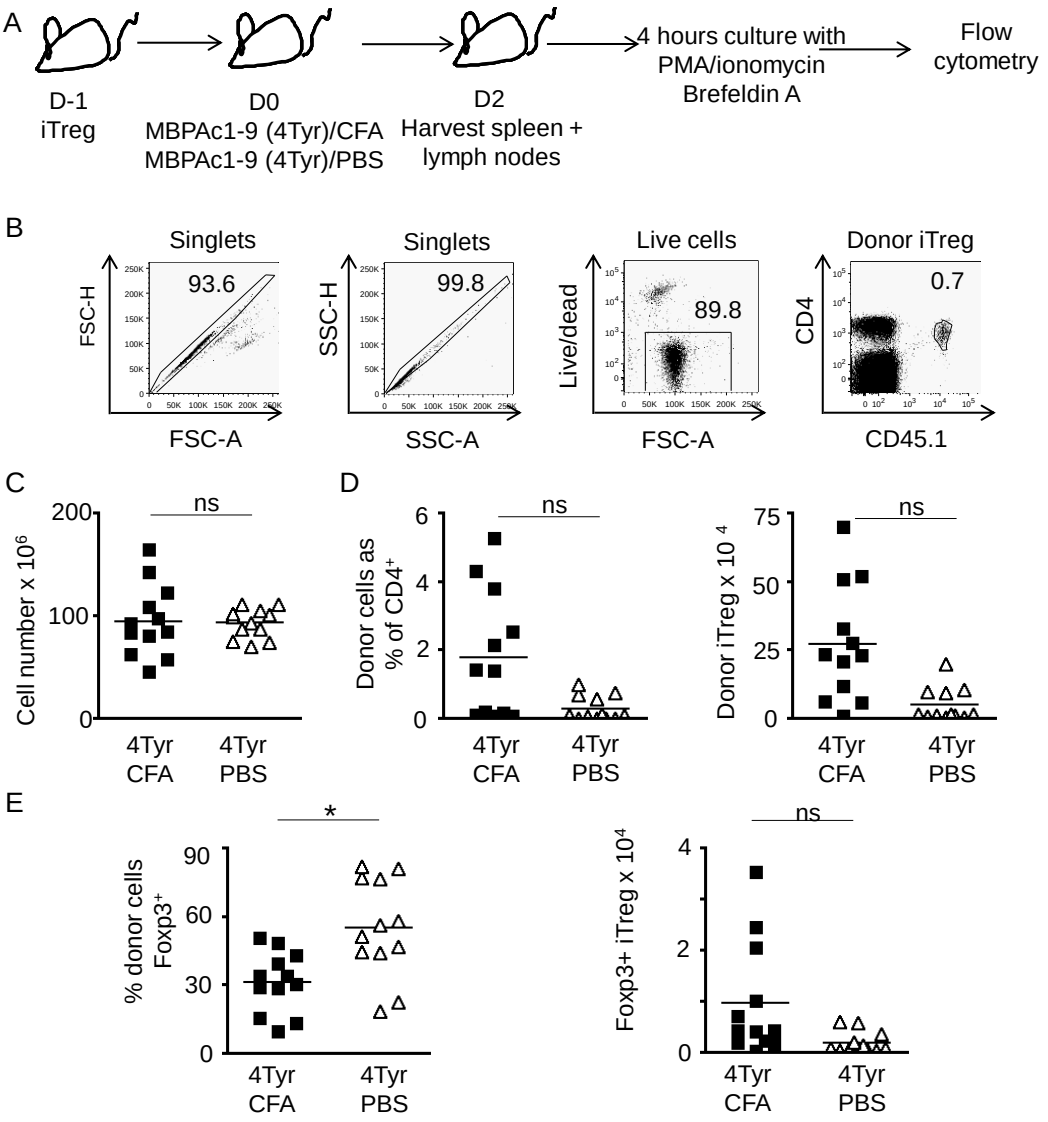


Figure 5.4 CFA-derived inflammatory signals enhance iTreg numbers in the spleen, but Foxp3 expression is reduced.

A) Experimental scheme. B10.PL mice intravenously received $2x10^6$ Foxp3gfp$^+$ Tg4xFoxp3LuciDTR-4 iTreg (FACS sorted for purity, >99% Foxp3gfp$^+$), then 24 hours later 10µg MBPAc1-9 (4Tyr) subcutaneously in either CFA or PBS. 48 hours later, mice were culled, spleens (shown above) and inguinal lymph nodes (shown in Fig 5.5) were harvested, and stained for surface and intracellular markers. B) Gating strategy for flow cytometry. C) Summary data combined from three experiments are shown. Total cellularity of spleens of mice treated with MBPAc1-9 in either CFA or PBS. D) Percentage and total number of donor iTreg in spleen. E) Percentage and total number of donor iTreg retaining Foxp3 expression in the spleen. *=p<0.05 as determined by Mann-Whitney U test, ns= not significant

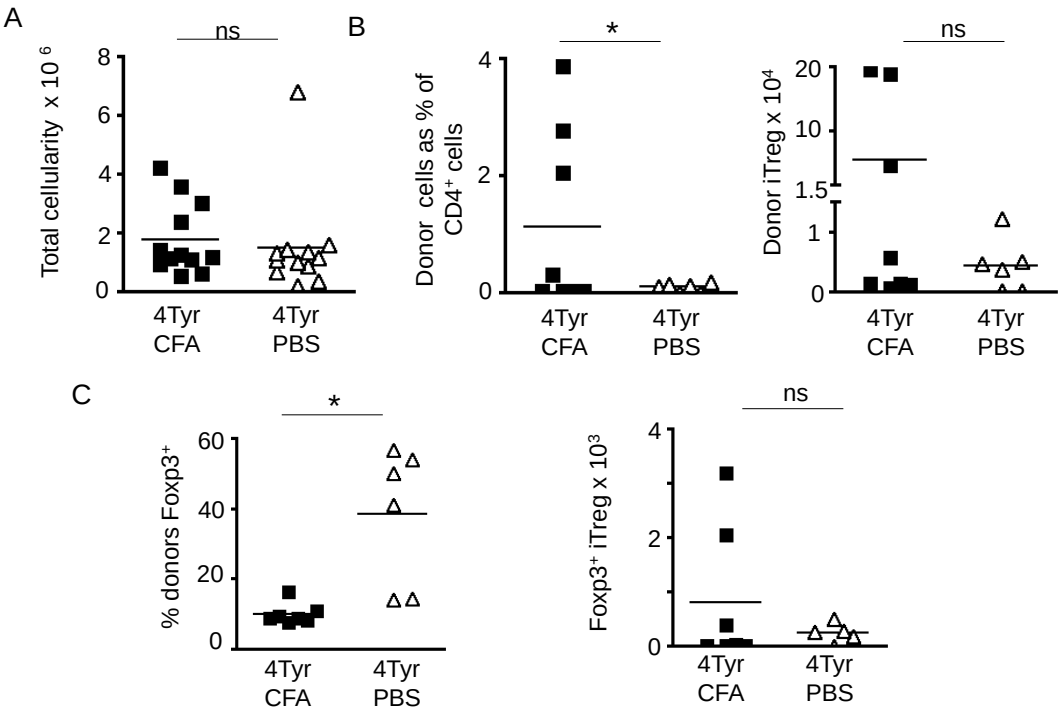


Figure 5.5 CFA-derived Inflammatory signals enhance iTreg numbers in the inguinal LN but Foxp3 expression is reduced.

Summary data from two experiments of donor iTreg within the lymph nodes from the experiment outlined in Fig 5.4 A) Total cellularity of lymph nodes. B) Percentage and total number of donor iTreg in lymph nodes. C) Percentage and total number of iTreg retaining Foxp3 expression. *=p<0.05 as determined by Mann-Whitney U test. ns=not significant.

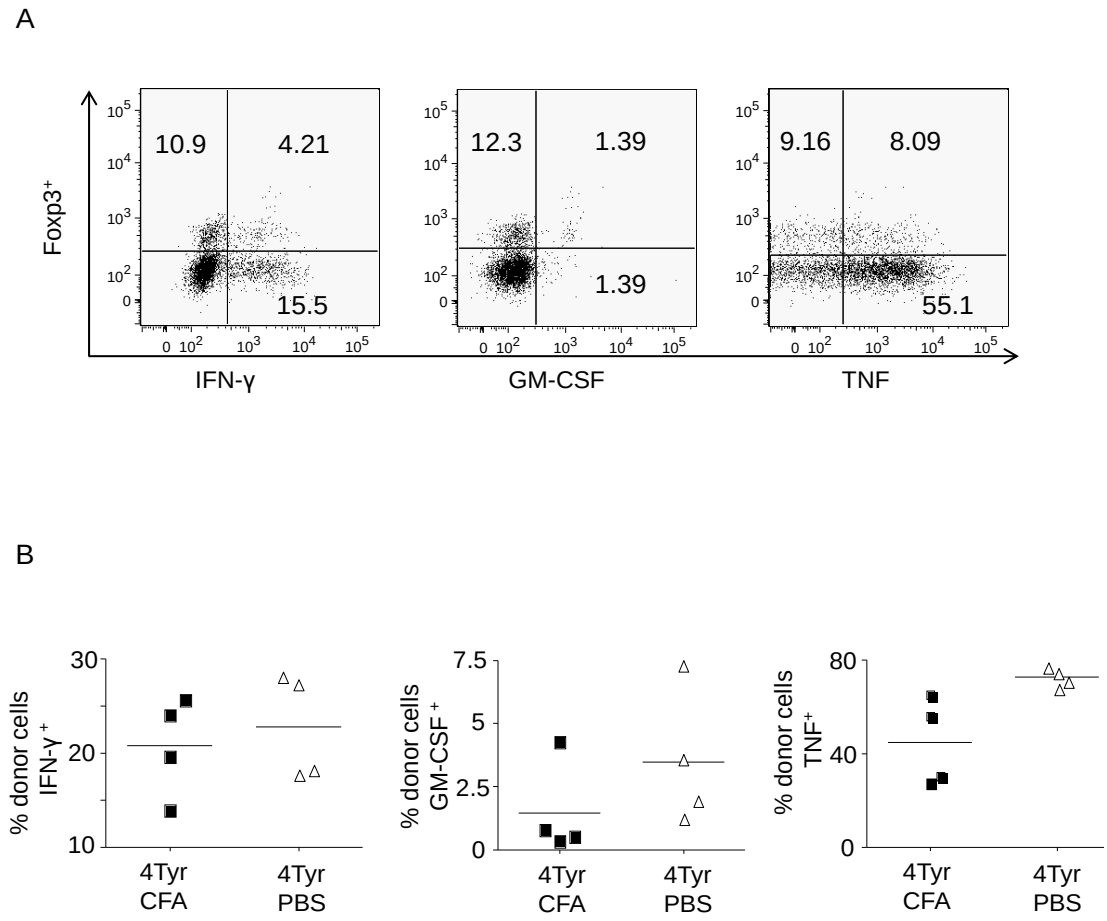


Figure 5.6 *Ex vivo*, iTreg produce IFN-γ and TNF but very little GM-CSF.
As detailed in Fig 5.4, mice received iTreg then, 24 hours later, sucbcutaneous MBPAc1-9 (4Tyr) emulsified in CFA. After one week, mice were culled and splenocytes were re-stimulated overnight with 10μM MBPAc1-9 (4Lys), then for the final four hours of culture with PMA and ionomycin in the presence of brefeldin A. Cells were stained for intracellular cytokines – IFN-γ, GM-CSF, and TNF - and Foxp3 expression. A) Representative flow cytometry plots gated on $CD4^+$ $CD45.1^+$ donor iTreg showing intracellular cytokine staining. B) Summary data of cytokine staining. Data are from one of three experiments giving consistent results.

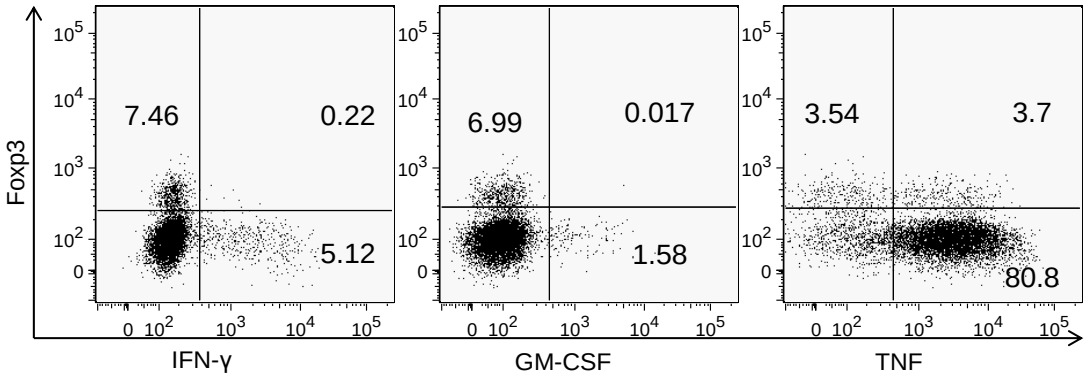


Figure 5.7 Host Foxp3$^+$ cells within the spleen do not produce GM-CSF or IFN-γ following PMA and ionomycin stimulation.
The flow cytometry data from the experiment detailed in Fig 5.6 were also gated on host CD4$^+$ cells from the spleen (i.e. CD45.1$^-$ cells). Staining of all three cytokines is shown. Data are from one of three experiments giving consistent results.

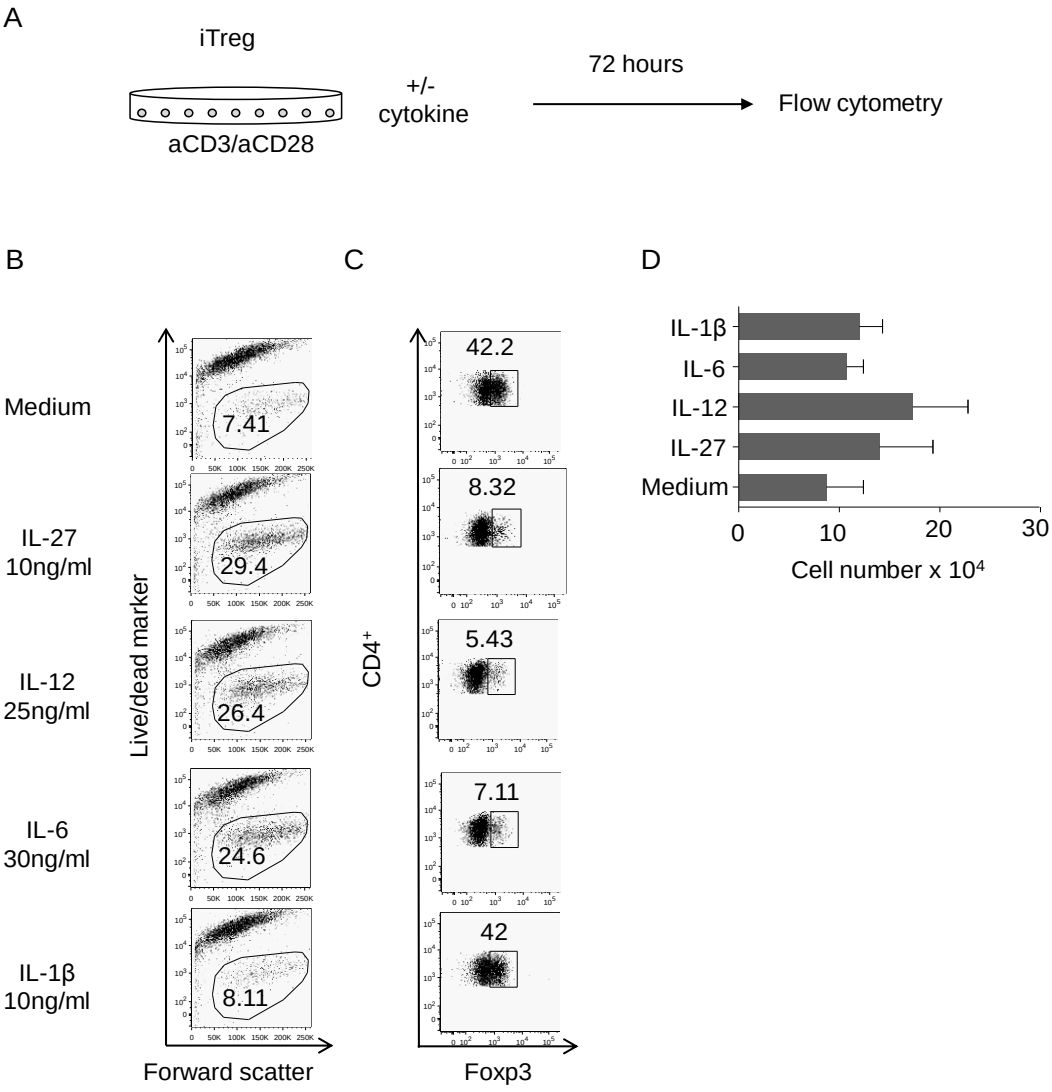


Figure 5.8 IL-27, IL-21 and IL-6 exacerbate Foxp3 loss from re-stimulated iTreg *in vitro*.

A) Experimental scheme. Foxp3GFP iTreg sorted by FACS for Foxp3gfp$^+$ (99% purity) were re-stimulated with plate-bound anti-CD3 and anti-CD28 (both 2μg/ml) for 72 hours with the addition of IL-12 (25ng/ml), IL-27 (10ng/ml), IL-6 (30ng/ml), or IL-1β (10ng/ml). B) Flow cytometry plots show the proportion of cells staining with live dead marker. Numbers indicate percentage of surviving cells. C) Representative plots of Foxp3 expression gated on CD4$^+$ cells. Numbers indicate percentage of Foxp3$^+$ cells. D) Total cell numbers (viability assessed by trypan blue exclusion) in each condition after 72 hours of culture. Data are from one of four experiments giving consistent results.

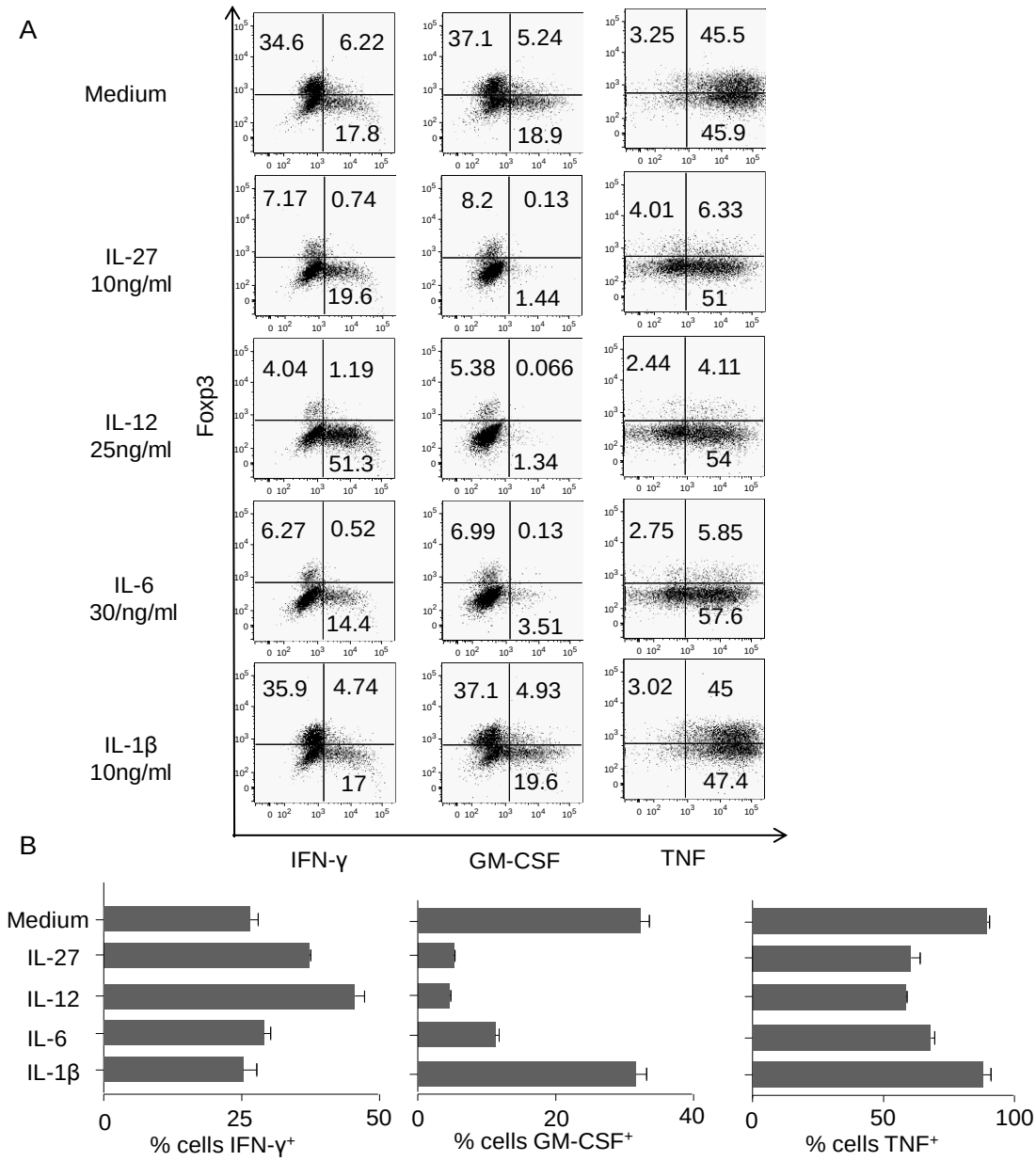


Figure 5.9 IL-27, IL-12 and IL-6 reduce GM-CSF and increase the proportion of iTreg producing IFN-γ.

iTreg, re-stimulated as described in Fig 5.8, were further stimulated with PMA and ionomycin in the presence of brefeldin A for the final 4 hours of culture. Cytokine production was then assessed by intracellular staining. A) Representative flow cytometry plots gated on live $CD4^+$ cells are shown. B) Summary data of triplicate wells. Data are from one of three experiments giving consistent results.

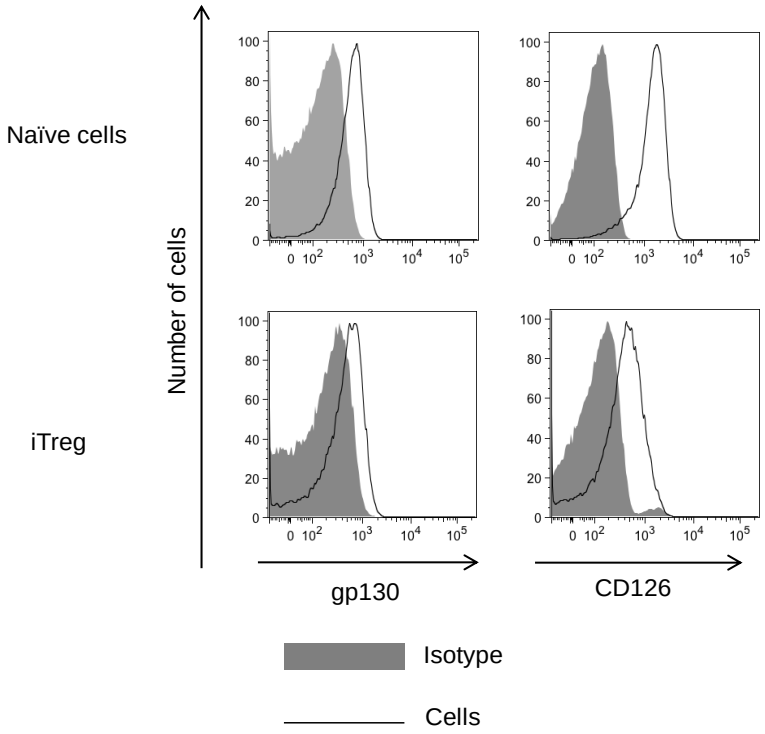


Figure 5.10 iTreg express gp130 and CD126.
Tg4xFoxp3LuciDTR-4 iTreg were sorted for Foxp3gfp$^+$ expression then stained for CD126 and gp130. Naïve Tg4 CD4$^+$ cells were similarly stained. Histograms of gp130 and CD126 expression compared to isotype are shown, gated on CD4$^+$ cells. Data are shown from a single experiment.

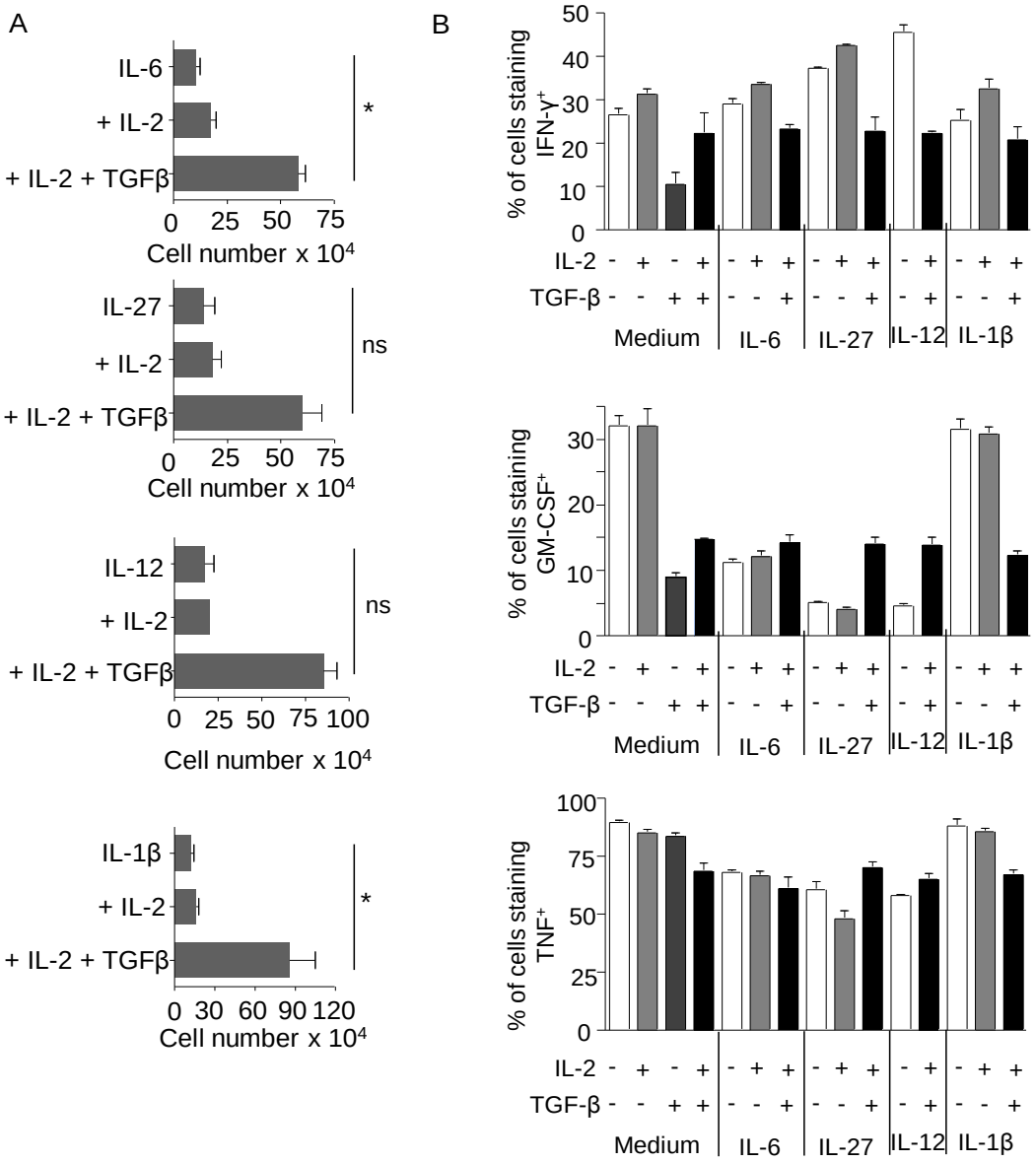


Figure 5.11 TGF-β has a dominant effect on iTreg survival and production of GM-CSF.

1x10^6 Foxp3GFP iTreg were re-stimulated as previously in the presence of IL-6,IL-27, IL-12, or IL-1β, plus either IL-2 (100U/ml) or IL-2 and TGF-β (5ng/ml). Cells were stained as previously. A) Effect of IL-2 and TGF-β on cell numbers after culture in the cytokine indicated after 72 hours. B) Summary data of staining of iTreg for IFN-γ, GM-CSF, and TNF after culture for 72 hours with the indicated cytokine in the presence or absence of IL-2 (100U/ml) and TGF-β (5ng/ml). Data are from one of three experiments giving consistent results. *=p<0.05 as determined by Mann-Whitney U test. ns=not significant.

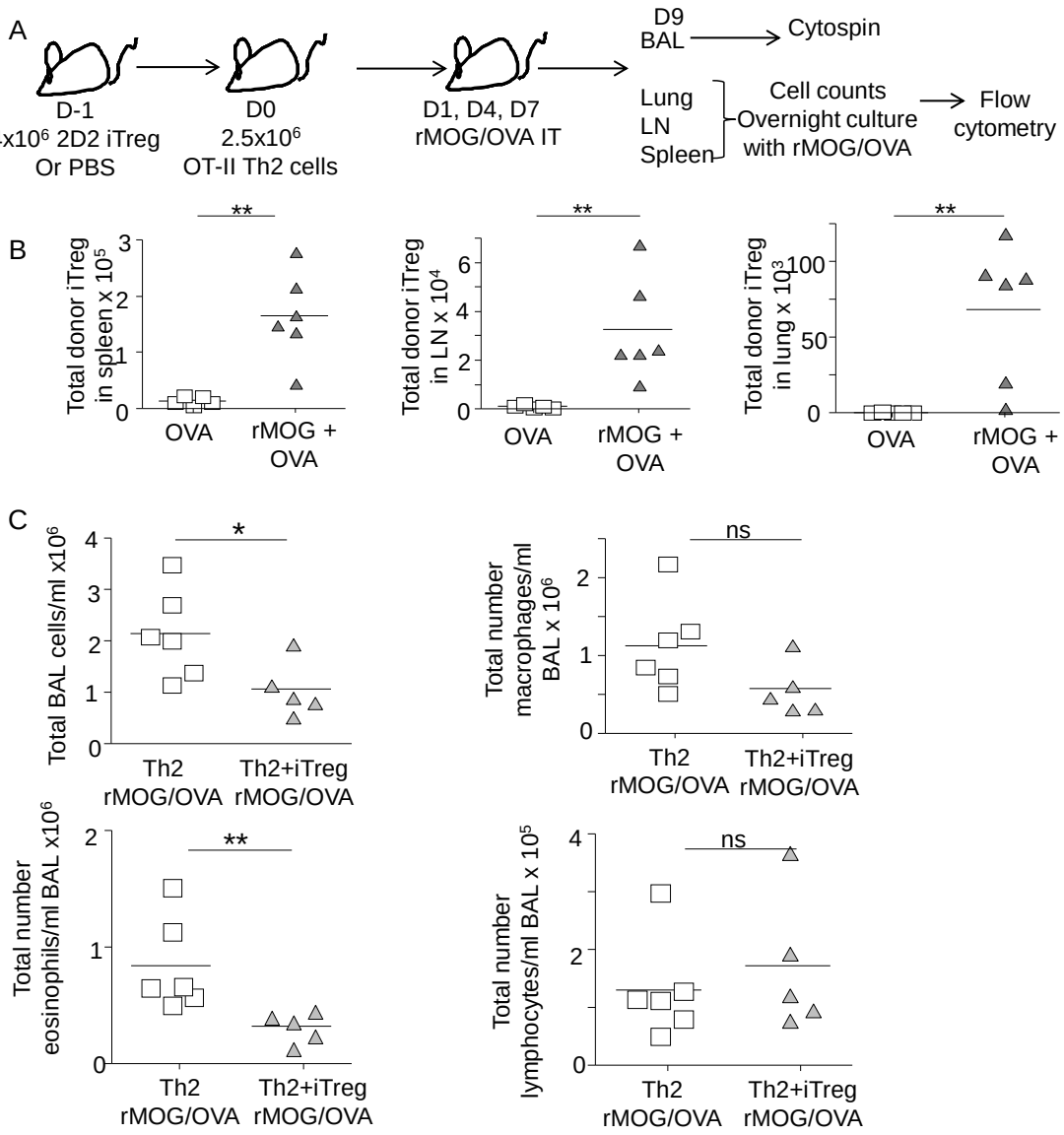


Figure 5.12 iTreg reduce lung eosinophils in airway inflammation induced by effectors responding to a different antigen.

A) Experimental scheme. C57BL/6 mice received 4x10^6 2D2 iTreg (sorted for CD25^{high}) or PBS control, then one day later 2.5x10^6 OT-II Th2 cells. 50µg rMOG and 50µg OVA were instilled in combination intratracheally (IT) one, four, and seven days later. Two days following the final airway challenge, mice were culled, lungs, spleen and lymph nodes harvested. Lungs were lavaged prior to harvesting. B) The number of all cells, and subtypes in the bronchoalveolar lavage were quantified from cytospins. Data are shown from a single experiment. *=p<0.05. **=p<0.01 as determined by Mann-Whitney U tests. ns=not significant.

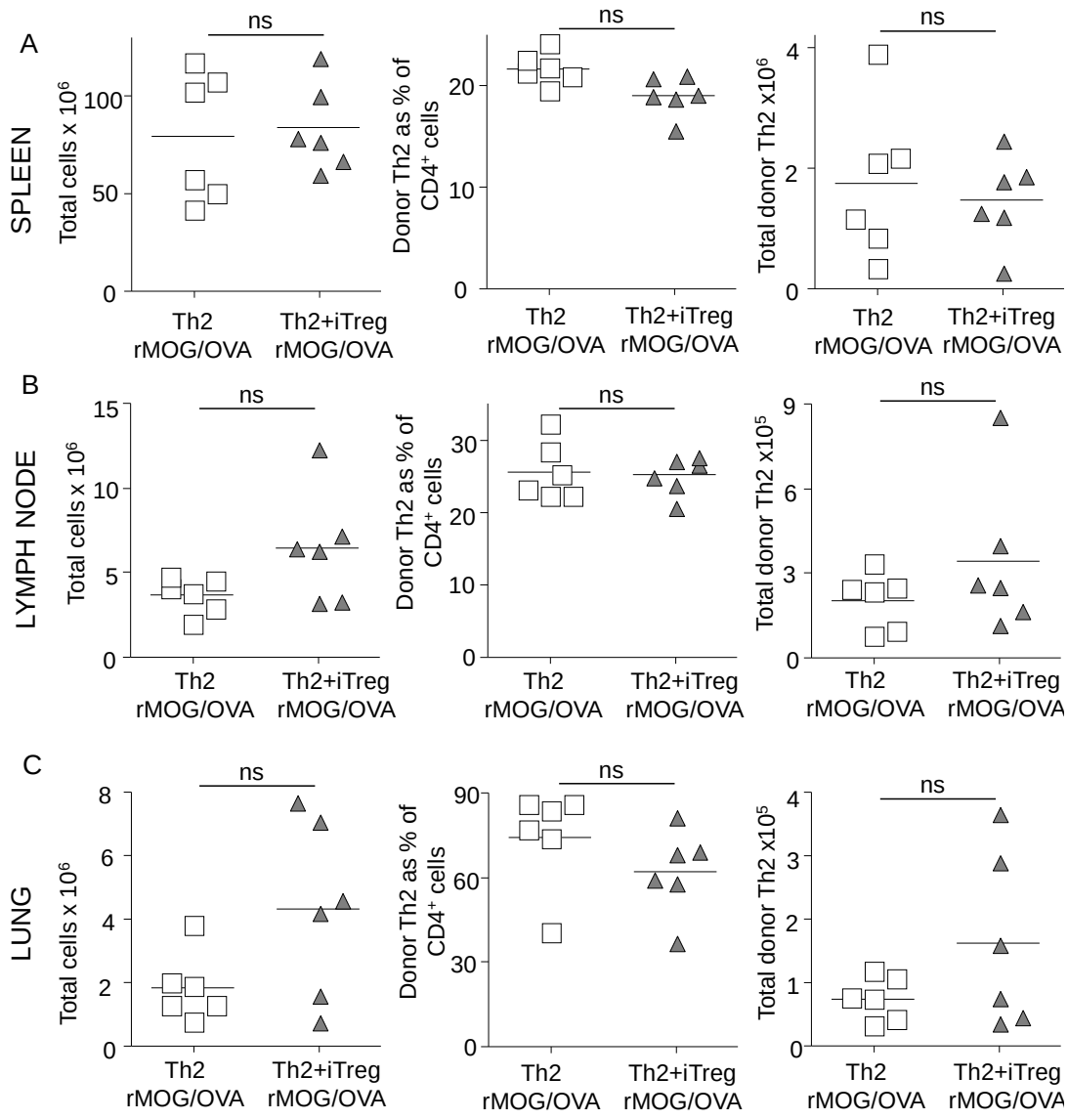


Figure 5.13 2D2 iTreg do not affect the survival of donor OT-II Th2 cells
As detailed in Fig 5.12, spleen, mediastinal lymph nodes, and lungs were harvested. Cells were counted by trypan blue exclusion, and proportions of donor Th2 cells and iTreg assessed by flow cytometry. Summary data from all mice are shown for total cell number, the percentage of $CD4^+$ cells that were donor Th2, and calculated numbers of donor Th2 cells in the A) spleen B) lymph nodes and C) lung. Data are shown from a single experiment. Groups were conpared using the Mann-Whitney U test. ns=not signficant.

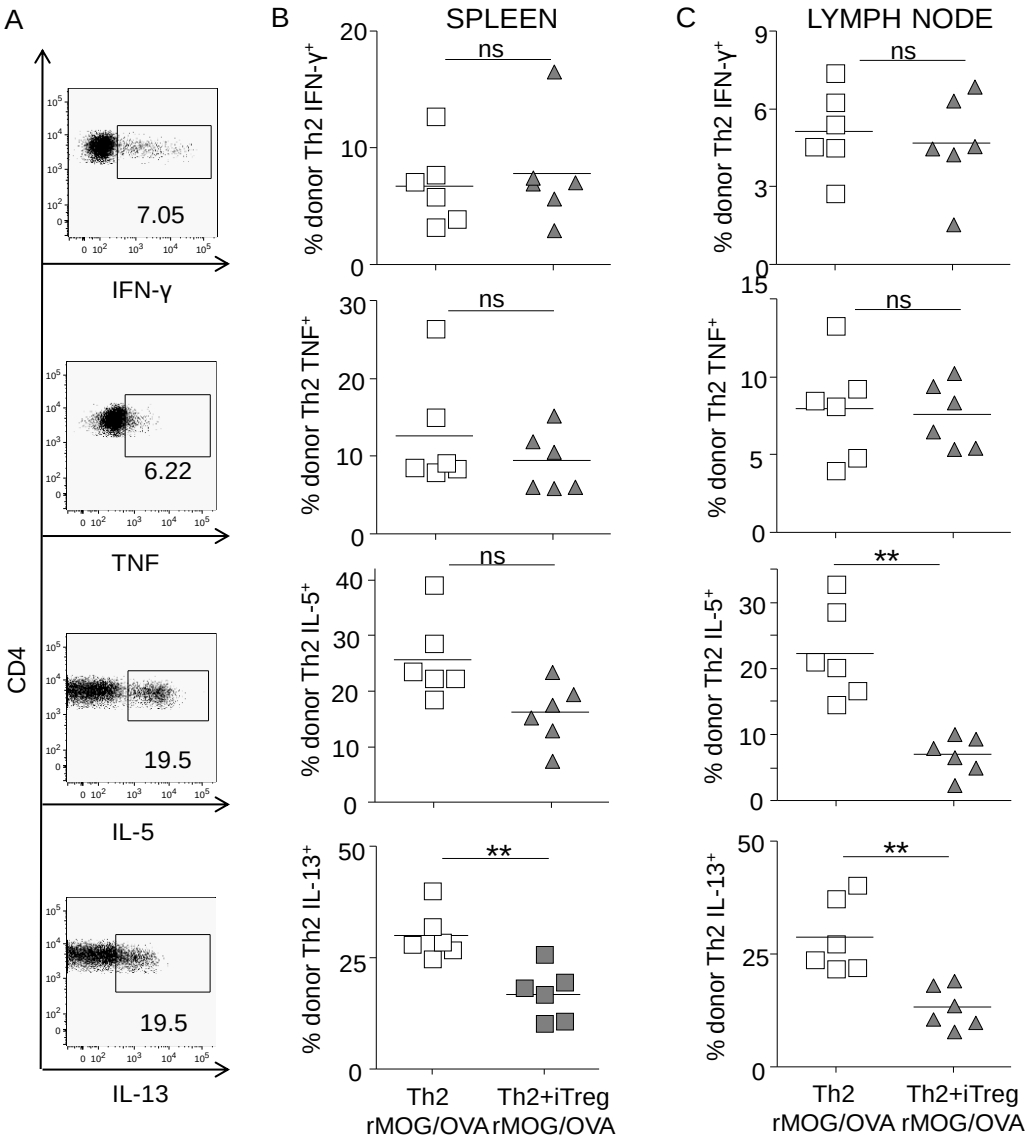


Fig 5.14 2D2 iTreg reduce the percentage of donor OT-II cells producing IL-13 and IL-5 in the spleen and LN.

As detailed in Fig 5.12, lymph nodes and spleens were harvested. Cells were then stimulated overnight in the presence of rMOG and OVA with brefeldin A for the final four hours. Cells were then stained for intracellular cytokines: IFN-γ, TNF, IL-13, and IL-5. A) Representative flow cytometry plots gated on live $CD4^+$ cells. Numbers indicate percentage of $cytokine^+$ cells. B) Summary data for cytokine staining of donor Th2 cells in the spleen. C) Summary data for cytokine staining of donor Th2 cells in the mediastinal lymph nodes. Data are shown from a single experiment. *=p<0.05. **=p<0.01 as determined by Mann-Whitney U tests. ns=not significant.

6 Discussion

6.1 Pro-inflammatory cytokine production by iTreg

An important finding of the work presented herein is the identification of pro-inflammatory cytokine production by iTreg, namely IFN-γ, GM-CSF, and TNF. The production of GM-CSF by iTreg is a novel finding, not previously reported. Though none of these cytokines appeared relevant in *in vitro* suppression assays, the cytokine profile of iTreg changed *in vivo*, most notably with a reduction in the number of cells producing GM-CSF. This profile could be emulated *in vitro* by re-stimulating iTreg in the presence of IL-6, IL-27, or IL-12. This suggests that cytokines produced in the inflammatory environment can directly influence the cytokine output of iTreg, and this may be relevant to how they exert their effects *in vivo*.

The production of IFN-γ and IL-17 has been reported in human nTreg subpopulations. Production of IL-17 by nTreg in the presence of IL-6 (Xu et al., 2007) showed that this lineage is susceptible to plasticity. Culture of nTreg in Th1 polarising conditions led to upregulation of Tbet and IFN-γ production (Zeng et al., 2009). Using $CD4^{+}CD45RO^{+}CD25^{+}CD127^{low}$ to identify human memory Treg, subtypes were shown expressing either RORγt or Tbet, associated with production of IL-17 and IFN-γ with co-production of IL-10 (Duhen et al., 2012). However, this may be dependent upon the location of the nTreg, as a report from O'Connor et al. demonstrated that nTreg isolated from the CNS during EAE (i.e. Treg resident within the target organ of inflammation) were resistant to IL-6 and did not produce IL-17 (O'Connor et al., 2012).

The production of IFN-γ, but resistance to IL-17 production, by iTreg in cytokine conditions promoting Th17 development further emphasized that nTreg and iTreg are not equivalent (O'Connor et al., 2010). IFN-γ production by iTreg was found in that report to occur on secondary TCR stimulation regardless of additional exogenous cytokines, though was markedly increased in the presence of additional IL-12. Further stimulating the iTreg in IL-12 to create 'IL-12 conditioned Treg' led to loss of Foxp3 expression and increased IFN-γ production, but did not affect the ability of these cells to suppress proliferation by naïve T cells both *in vitro* and *in vivo*. Those iTreg were mildly pathogenic when transferred into mice at high numbers, and were unable to prevent disease caused by already-activated Th1

cells. The production of IFN-γ by iTreg may actually be relevant to their suppressive function (Willenborg et al., 1996).

Other reports show no production of these cytokines by iTreg. Using $CD4^{+}CD8^{-}$ $CD25^{-}$ thymocytes as precursors in almost identical iTreg generation conditions, one group reported minimal production of IFN-γ and only 18% iTreg producing TNF on anti-CD3/anti-CD28 re-stimulation (Di Paolo et al., 2007). This may relate to using thymic cells rather than naive T cells derived from spleen and lymph nodes. A second group generated iTreg using splenocytes and demonstrated no IFN-γ production on re-stimulation for 24 hours with islet cells as antigen (Tonkin et al., 2008). That group included anti-IFN-γ in initial iTreg generating conditions however, which was very likely to have influenced their subsequent cytokine production. It is well recognized that the presence of IFN-γ stimulates further IFN-γ production by other cells (O'Connor et al., 2010).

To summarise, this thesis has demonstrated that iTreg generated using a well established *in vitro* protocol (Davidson et al., 2007) are capable of producing IFN-γ, GM-CSF and TNF. The *in vivo* environment reduces production of GM-CSF compared to iTreg re-stimulated *in vitro*. Though other groups have demonstrated an absence of IFN-γ from iTreg, those were always generated in a different manner. What might the relevance of the cytokine production by these cells be? Though all three cytokines have been implicated in pathogenesis, protective effects have also been attributed to them all as well. The increased severity of various autoimmune models in mice lacking IFN-γ, including EAE (Chu et al., 2000) and experimental glomerulonephritis (Kitching et al., 2004), highlights that IFN-γ clearly has an immunoprotective role in some circumstances. Similarly, administration of recombinant TNF is protective in mouse models of lupus (Gordon et al., 1989) and diabetes (Satoh et al, 1989). Anti-TNF therapy has been associated in patients with worsening/development of MS (Sicotte et al., 2001) and lupus (Lin et al., 2008).

The wide-ranging effects of GM-CSF in multiple cell systems renders it more difficult to ascribe a positive or negative immunomodulatory effect, though its role in promoting autoimmunity has been recognized for many years (Campbell et al., 1998). The immunoprotective effects of GM-CSF have been best described in the context of immune evasion by malignant cells (Graner et al., 2000).

Attributing either a pro- or anti- inflammatory characteristic to any given cytokine therefore appears somewhat simplistic and misleading, as clearly the response to cytokines depends upon a multitude of other factors, including the other components of the inflammatory

milieu, the types of cells responding, and potentially the pathogenic stage of the disease under investigation.

How does this relate to iTreg production of these cytokines? It is highly likely, indeed almost certain, that iTreg will only be generated *in vivo* in the context of high quantities of TGF-β. The interplay between this and the other cytokines is therefore a crucial one to investigate. Does TGF-β influence the signaling pathways triggered by IFN-γ, or impair the maturation of DCs in the presence of GM-CSF? These questions remain unanswered, though merit further study.

For an individual cell, cytokine production is a relatively expensive task, requiring energy-demanding processes. It is unlikely that the production of these cytokines has no relevance at all to the function of the iTreg, even though this appears to be suggested by the absence of effect in *in vitro* suppression assays (Chapter 4). Some reports have indicated that Treg have a more important role in diseases where Th2 cells are the primary protagonists (Josefowicz et al., 2012), correlating with the suppression of 'signature' cytokines presented in Fig 4.14. Given the reciprocal inhibition of Th1 and Th2 differentiation, partly mediated by the relative cytokines, it is plausible that production of IFN-γ by iTreg is relevant in their suppression of Th2-mediated processes, perhaps by interrupting the positive feedback effect on trafficking naïve T cells towards the area of inflammation.

Another area worthy of investigation is whether the initial generating conditions influence the subsequent cytokine production by iTreg. The absence of IFN-γ production by iTreg generated in identical circumstances to the protocol used here and by others (O'Connor et al., 2010), with the exception of the addition of anti-IFN-γ, implies iTreg may be influenced by the environment in which they are generated. Much of the data presented in chapter 5 shows that the cytokine environment can influence production of cytokines by iTreg after generation, but this may be subject to manipulation during primary generation as well.

The alteration in cytokine profile *ex vivo* in the presence of inflammatory signals also suggests that iTreg can modulate their cytokine production according to the environment in which they are resident. In the healthy steady state where iTreg are generated in the absence of inflammation e.g. at the gut mucosal level with foreign antigens derived from food, the low production of these cytokines may maintain the gut-resident DCs in their tolerogenic state. Conversely, the presence of inflammatory signals may alter the iTreg cytokine profile. The presence of IL-6 reduced GM-CSF and increased IFN-γ; this may lead to iTreg having a differing effect on the DCs. Alternately, though in most circumstances IFN-γ promotes

production of IFN-γ by other cells, perhaps a negative feedback loop exists within iTreg, with increasing IFN-γ concentrations stimulating a reduction in other pro-inflammatory cytokines. These hypotheses could be tested utilizing the well-established models of colitis and assessing the cytokine production of iTreg in these circumstances, compared to those within a healthy gut.

6.2 Bystander suppression

This thesis has presented data demonstrating the ability of iTreg to suppress in a bystander fashion, using two different transgenic mouse strains to clearly show that persistence of the iTreg cognate antigen is not required, but that interaction with the MHC is necessary for suppression to occur. Depletion experiments led to the unexpected finding that iTreg do not need to physically persist in *in vitro* culture beyond 24 hours for suppression to occur. This *in vitro* bystander suppression model was partially reliant on TGF-β signaling, as inhibition of ALK5 reduced the efficacy of iTreg.

Two different patterns of suppression were seen using iTreg *in vivo*. Where naïve T cells sharing the same transgenic TCR were transferred and stimulated with antigen in the presence of CFA, iTreg reduced the total number of naïve T cells surviving in both the spleen and the lymph nodes, with a significant reduction in the production of IFN-γ by naive T cells in the LN also evident. Thus, these iTreg were able to reduce the number of potential effector cells.

In the *in vivo* bystander suppression experiments, a different pattern of suppression was seen. In experiments of 'direct' suppression with 2D2 iTreg administered to mice receiving 2D2 Th2 cells and rMOG-induced AAI, the total number of effector cells in the lung was reduced, as was production of IL-13 (work by Dominika Nowakowska). In the bystander suppression experiments presented here, administration of 2D2 iTreg was unable to reduce the number of OT-II Th2 cells present in the lung following challenge with either OVA, or both rMOG and OVA. The numerical difference in effectors following iTreg administration was lost. Cytokine production was significantly affected however, with a reduction in OT-II Th2 cell production of IL-5 and IL-13 evident within the spleen and LN. How this related to the site of inflammation remains to be investigated. Thus, *in vivo*, two potential mechanisms by which iTreg may ameliorate disease have been demonstrated. A reduction in the number of donor cells (either naïve or Th2) would limit the pool of potential effectors, and they had demonstrably lower production of IFN-γ, or IL-13 and IL-5 respectively. Bystander

suppression by iTreg was less effective in reducing the number of effector cells, but cytokine production by Th2 cells was still affected.

These differing effects may be related to the type of cell undergoing suppression. The initial *in vivo* experiments used naïve T cells and CFA immunization whereas the bystander suppression experiment involved polarised Th2 cells. Not only are iTreg able to suppress polarised effectors *in vitro* (Fig 4.13), they are also efficacious in treating established disease as well as preventing its onset (Nguyen et al., 2011), and appear to be more efficient than nTreg in several disease models (Kong et al., 2012). Previous groups have failed to demonstrate any evidence for bystander suppression *in vivo*, including a convincing absence of effect in EAE (Zhang et al., 2010). This may relate to the models used or the end-points chosen. The data presented in Chapter 5 imply that iTreg can exert a bystander suppressive effect but in the model used this was predominantly on cytokine production rather than the total number of effectors. If a longer-term Th1/Th17-mediated disease model is used, the iTreg generated by current protocols may be less efficient (particularly given their intrinsic production of IFN-γ and GM-CSF) than if a Th2-mediated model is examined. Against this is the consistent finding that iTreg generated following an identical protocol are able to ameliorate EAE induced by immunisation with antigen/CFA and transfer of naïve cells (O'Connor et al., 2010). The already-differentiated effector status of the pathogenic cells may thus also be relevant.

In the bystander suppression experiments here, the reduction in the proportion of cells producing IL-5 and IL-13 is the main finding. This provides proof of principle that iTreg are able to exert a suppressive effect on effector cells responding to a different antigen *in vivo* as well as *in vitro*. Though no effect was seen on the number of effectors present in the lung in the bystander model, there was an effect when Th2 and iTreg shared the same cognate antigen. This could be related to the milder severity of disease, but may also be related to the signals permitting trafficking of the iTreg to the lungs. That the iTreg do traffic there is clearly evident, but could this be less efficient in a bystander suppression model? To determine whether the effect of iTreg was simply delayed, a longer model of AAI could be employed. In the model used, the resolution of the airways inflammation occurs rapidly (K Mackenzie, personal communication). A further experimental question, permitted by the rapid resolution of inflammation, is the potential for re-challenge with both antigens once inflammation has resolved. The ability of Treg to form a 'memory' population has been suggested (Rosenblum et al., 2011). Whether iTreg are able to contribute to this regulatory

memory is unclear, but could feasibly be tested using the model above. Unknowns include the lifespan of iTreg within the mice.

If the bystander suppression effect is limited to an effect on cytokine production, but not cell number, this may actually increase the utility of iTreg therapeutically. Many of the concerns regarding bystander effects were that that the adverse effects would replicate those of generalized immunosuppression, removing the benefits of an antigen-specific therapy. The results above may also suggest that iTreg would be of most utility in those diseases where Th2 cells are the protagonists.

6.3 Concluding remarks

This thesis has presented data showing the ability of iTreg to produce pro-inflammatory cytokines, and effect bystander suppression both *in vitro* – when cognate antigen is unnecessary but MHC interaction is required – and *in vivo*. The ability of these iTreg to suppress regardless of their cytokine profile implies that some concerns re the plasticity of iTreg may be unfounded, as they remain suppressive despite the production of pro-inflammatory cytokines and loss of their 'master' transcription factor, Foxp3. How the cytokine production of iTreg is altered in different disease models is unknown, and deserves additional study. Conversely, deliberately altering the cytokine profile of iTreg through the use of exogenous cytokines during generation or re-stimulation may provide new insights into their suppressive mechanisms. Further investigation of the efficacy *in vivo* of iTreg in Th2-mediated disease is also warranted.

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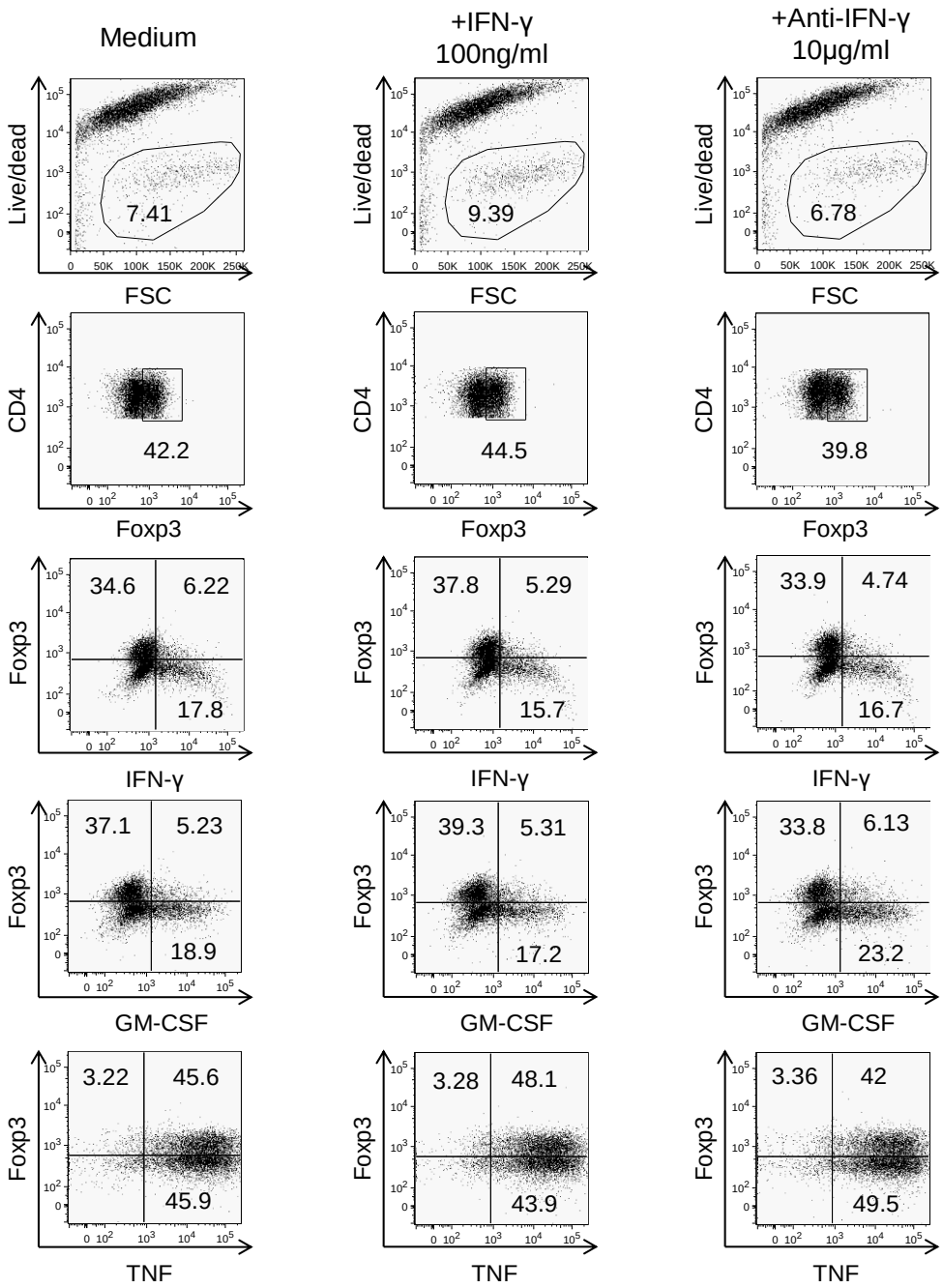
Appendix 1


Medium
+IFN-γ 100ng/ml
+Anti-IFN-γ 10μg/ml
Live/dead
FSC
7.41
9.39
6.78
CD4
Foxp3
42.2
44.5
39.8
Foxp3
IFN-γ
34.6
6.22
17.8
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4.74
16.7
GM-CSF
37.1
5.23
18.9
39.3
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TNF
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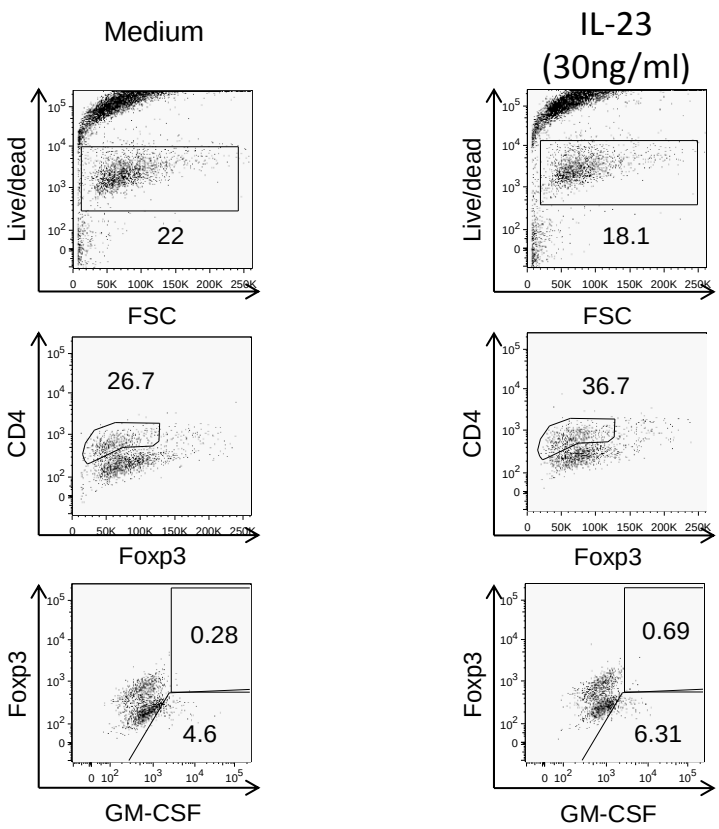
42
49.5



IFN-γ, anti-IFN-γ, or IL-23 have no effects on cytokine production by re-stimulated iTreg *in vitro*

Sorted iTreg were re-stimulated as described in Fig 5.8. Medium was supplemented with IFN-γ (100ng/ml) , anti-IFN-γ (10μg/ml), or IL-23 (30ng/ml). After 72 hours of culture, cells were analysed by flow cytometry for viability, expression of Foxp3, and production of the indicated cytokines. Data are shown from single experiments. Numbers indicate the percentage of cells within the gate.