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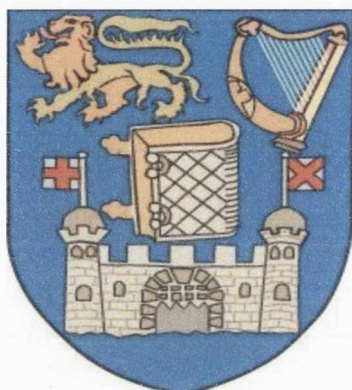
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Immunoregulatory Mechanisms in the Lungs of Mice and Humans

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A thesis submitted to
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Finally, I'd like to dedicate this to the memory of May Regan, the kindest and most knowledgeable person I've ever known.

קרוב יותר אליך, אלוהי

Abstract

The lungs are constantly exposed to inhaled antigen during respiration. The resident immune cells must be hyporesponsive to innocuous inhaled antigen, yet capable of responding appropriately to respiratory pathogens, with minimal immunopathology to the fragile lung tissue. Regulatory T (Treg) cells are crucial for the maintenance of immune tolerance in the lungs. Alveolar macrophages (AM) also contribute to the local immunosuppressive environment of the lungs.

The results of these studies demonstrate that AM-derived retinoic acid (RA) may help to control immunosuppression in the lungs. Human and murine AM promoted conversion of CD4⁺FoxP3⁻ naïve T cells to CD4⁺FoxP3⁺ T cells *in vitro*, which was reversed by inhibiting RA signalling. Furthermore, the majority of CD4⁺FoxP3⁺ T cells in the murine lung lack the activation marker CD25. This phenotype resembles that of the FoxP3⁺ T cells induced by AM *in vitro*. CD4⁺FoxP3⁺CD25⁻ Treg cells must be pre-activated in the presence of IL-2 before they can suppress effector T (Teff) cells *in vitro*, which is mediated by IL-10.

The study also shows that an RA receptor antagonist could overcome the profound immunological non-responsiveness to nasally delivered antigen and adjuvant, and allowed systemic immunity to develop. This strategy might represent a target for immunotherapy in the future. Additionally, the numbers of AM in the lungs increase as a consequence of cigarette smoking, and greater numbers of AM are able to induce more FoxP3⁺ T cells. Smokers who are infected with *Mycobacterium tuberculosis* have greater morbidity and mortality than infected non-smokers. This might be a consequence of the enhanced frequencies of induced FoxP3⁺ T cells in their lungs, which could severely impair protective anti-tuberculosis immune responses and result in persistence of the bacteria.

In a respiratory model of infection with *Bordetella pertussis*, a high frequency of antigen-specific CD4⁺FoxP3⁺CD25⁻IL-10⁺ cells was found in the lungs throughout the infection. Depletion of CD25⁺ cells from wild type mice prior to *B. pertussis* infection did not impair the immunoregulatory response or alter the course of infection, which suggests that an additional method of regulation was present in those mice. However, depletion of CD25⁺ cells in IL-10-deficient (IL-10^{-/-}) mice significantly enhanced bacterial clearance. These results indicate the presence of two Treg cell populations in the lung – CD4⁺FoxP3⁺CD25⁺ nTreg cells and IL-10-secreting CD4⁺FoxP3⁺CD25⁻ Treg cells. It is possible that the CD4⁺FoxP3⁺CD25⁻ Treg cells are induced by AM and comprise a “reservoir” of under-activated Treg cells which will only function optimally if IL-2 is present, i.e. during an adaptive immune response. This illustrates a novel feedback loop in the maintenance of immune tolerance in the lungs, which is induced by AM and executed by FoxP3⁺ T cells.

Publications

- Coleman M. M., Keane J. and Mills K. H. (2010) Tregs and BCG--dangerous liaisons in TB. *J Leukoc Biol* **88**, 1067-9
- Coleman M.M., Finlay C., Moran B., Keane J., Dunne P., and Mills K. (2012) The Immunoregulatory Role of Lung CD4⁺FoxP3⁺CD25⁻ Regulatory T cells in *Bordetella pertussis* Infection. *FEMS Immunology and Medical Microbiology* **64**, 413-424
- Ní Cheallaigh C., Peral de Castro C., Coleman M.M, Hope J.C., Harris J. Macrophages and Tuberculosis. Chapter 1 - *Handbook of Macrophages: Life Cycle, Functions and Diseases* (2012 – in press)

List of Abbreviations

ADC	Albumin-Dextrose-Catalase
AECI	type I Alveolar epithelial cells
AECII	type II Alveolar epithelial cells
AICD	Activation-induced cell death
AIDS	Acquired Immune Deficiency Syndrome
ALDH	Aldehyde dehydrogenase
ALDH1A	Aldehyde dehydrogenase 1A
AM	Alveolar macrophages
AM-CM	Alveolar macrophage-conditioned medium
AMDC	Airway mucosal dendritic cells
AMP	Adenosine monophosphate
APC	Antigen presenting cell
ATP	Adenosine triphosphate
atRA	all- <i>trans</i> Retinoic acid
BAL	Bronchoalveolar lavage
BALF	Bronchoalveolar lavage fluid
BALT	Bronchial-associated lymphoid tissue
BFA	Brefeldin A
BG	Bordet Gengou
<i>B. pertussis</i>	<i>Bordetella pertussis</i>
BSA	Bovine serum albumin
cAMP	cyclic AMP
CD	Cluster of differentiation
cDMEM	complete DMEM
CFU	Colony forming units
CLN	Cervical lymph nodes
CM	Conditioned medium

ConA	Concanavalin A
cRPMI	complete RPMI
CS	Cigarette smoke
CTLA-4	Cytotoxic T-lymphocyte antigen 4
CyaA	Adenylate cyclase from <i>B. pertussis</i>
DC	Dendritic cells
DEAB	Diethylaminobenzaldehyde
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
DP	Double positive
EAE	Experimental Autoimmune Encephalomyelitis
EBAO	Ethidium bromide/acridine orange
EBV	Epstein-Barr Virus
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-Linked Immunosorbent Assay
FACS	Fluorescence-Activated Cell Sorting
FAE	Follicle-associated epithelium
FCS	Foetal calf serum
FHA	Filamentous haemagglutinin
FMO	Fluorescence minus one
FoxP3	Forkhead Box P3
GALT	Gut-associated lymphoid tissue
GFP	Green Fluorescent Protein
GITR	Glucocorticoid-induced Tumor Necrosis Factor receptor
GM-CSF	Granulocyte/Monocyte Colony Stimulating Factor
GVHD	Graft-versus-host Disease
HBSS	Hank's Balanced Salt Solution
HEV	High endothelial venules

HIV	Human Immunodeficiency Virus
HKBP	Heat-killed <i>B. pertussis</i>
HLA	Human leukocyte antigen
HRP	Horseradish peroxidase
IBD	Inflammatory Bowel Disease
ICER	Inducible cAMP early repressor
ICOS	Inducible co-stimulator
IDO	Indoleamine 2, 3-dioxygenase
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
i.n.	Intranasal
iNOS	inducible nitric oxide synthase
i.p.	Intraperitoneal
IPEX	Immunodysregulation Polyendocrinopathy Enteropathy X-linked Syndrome
IPF	Idiopathic Pulmonary Fibrosis
i.t.	Intratracheal
iTreg	inducible Treg cells
LAG	Lymphocyte activation gene
LAP	Latency-associated peptide
LFA	Leukocyte function-associated antigen
LPS	Lipopolysaccharide
LTBI	Latent tuberculosis infection
mAb	Monoclonal antibody
MACS	Magnet-associated cell sorting
MCP	Monocyte chemoattractant protein
MDM	Monocyte-derived macrophages
MFI	Median fluorescence intensity

MHC	Major histocompatibility complex
MMP	Matrix metalloproteinase
MOI	Multiplicity of infection
mRNA	messenger RNA
<i>M. tuberculosis</i>	<i>Mycobacterium tuberculosis</i>
NK cells	Natural killer cells
NLR	Nod-like receptor
NO	Nitric oxide
NOD	Non-obese diabetic
NOD SCID	Non-obese diabetic severe combined immunodeficient
NOS	Nitric oxide synthase
nTreg	natural Treg cells
OPD	O-Phenylenediamine
Ova	Ovalbumin
PAMP	Pathogen-associated molecular pattern
<i>P. aeruginosa</i>	<i>Pseudomonas aeruginosa</i>
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PFA	Paraformaldehyde
PM	Peritoneal macrophages
PMA	Phorbol myristate acetate
PM-CM	Peritoneal macrophage-conditioned medium
PNAd	Peripheral node addressin
PPD	Purified protein derivative
PRR	Pattern recognition receptor
RA	Retinoic acid
RALDH	Retinal dehydrogenase
RAR	Retinoic acid receptor

RARE	Retinoic acid response element
RARi	Retinoic acid receptor inhibitor
RNI	Reactive nitrogen intermediate
ROR γ t	Retinoic acid orphan receptor γ t
rpm	Revolutions per minute
RPMI	Roswell Park Memorial Institute
RXR	Retinoid X Receptor
sCD25	soluble CD25
SCID	Severe Combined Immunodeficient
STAT	Signal Transducer and Activator of Transcription
TB	Tuberculosis
TCR	T cell receptor
Teff	effector T cells
TGF	Transforming growth factor
Th	T helper cells
TLN	Thoracic lymph node
TLR	Toll-like receptor
TMB	Tetramethylbenzidine
TNF	Tumor necrosis factor
Tr1	Inducible regulatory T cells
Trem	regulatory effector/memory-like T cells
Treg	regulatory T cells
VCAM-1	Vascular cellular adhesion molecule-1
WT	Wild-type

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Chapter 1

General Introduction

1.1 Protective immunity against infection

The innate and adaptive immune systems work together to efficiently eradicate harmful pathogens. The innate immune system is the first line of defence and it also provides the signals to induce an appropriate adaptive immune response against the invading pathogen (Murphy *et al.*, 2008). Innate immunity is activated upon binding of pathogen-associated molecular patterns (PAMPs) to pattern recognition receptors (PRRs), which are expressed by dendritic cells (DC), macrophages and other innate immune cells. PRRs include Toll-like receptors (TLRs), Nod-like receptors (NLRs), and lectin receptors (Murphy *et al.*, 2008). PRR engagement with PAMPs initiates intracellular signalling pathways, ultimately activating genes associated with the immune response (Murphy *et al.*, 2008).

Immature dendritic cells are resident in most tissues, where they continuously sample their environment by receptor-mediated and receptor-independent mechanisms of phagocytosis. The majority of antigens they encounter are harmless and do not induce an immune response. However, pathogen-induced TLR signalling activates DC and enhances the processing of pathogen-derived antigens (Murphy *et al.*, 2008). TLR signalling also induces the chemokine receptor CCR7 on the surface of DC. CCR7 influences both the migration of DC into draining lymph nodes and the upregulation of costimulatory molecules and major histocompatibility complex (MHC) class II molecules on the surface of DC (Medzhitov and Janeway, 1997; Moser and Murphy, 2000). The activation of DC also downregulates their phagocytic ability – they no longer take up environmental antigen but instead they function as antigen presenting cells (APC) to activate naïve CD4⁺ and CD8⁺ T cells (Moser and Murphy, 2000).

Priming of naïve T cells in the lymph nodes requires three signals – Signal 1 is the activating signal which involves specific interaction of MHC/antigen complex with the T cell receptor (TCR). Signal 2 is a survival signal, mediated by costimulatory molecules on the surface of DC interacting with specific molecules on the T cells. One example includes the B7 group of

molecules (CD80 and CD86) on the DC which bind to CD28 on adjacent T cells (Bour-Jordan and Blueston, 2002).

Signal 3 results in the differentiation of the naïve CD4⁺ T cells into T helper (Th1, Th2, and Th17) or regulatory T (Treg) cell subsets. This signal also arises from DC and other innate immune cells and requires cytokines, which polarise the differentiation of the activated T cells into the most appropriate subset to eradicate the particular invading pathogen (Kapsenberg, 2003). Different cytokines influence this differentiation – for example interleukin (IL-12) promotes Interferon- γ (IFN- γ)-secreting Th1 cells, characterised by the transcription factor T-bet, whereas IL-4 induces GATA-3-expressing Th2 cells, while IL-6 and transforming growth factor (TGF)- β_1 induce retinoic acid orphan receptor (ROR) γ T-expressing Th17 cells (Mills, 2009). Natural and adaptive Treg cells can also be induced/expanded by IL-10 or TGF- β_1 (Mills, 2009). Natural Treg cells and some adaptive Treg cells express the transcription factor FoxP3.

1.2 Immunological tolerance

Immunological self-tolerance involves the regulation of a damaging immune response that might arise against self-molecules, cells or tissues (Pugliese, 2004). Loss of this self-tolerance is associated with the onset of autoimmune disease. The induction of self-tolerance is reliant on interactions between lymphocytes and APC for both central tolerance and peripheral tolerance (Pugliese, 2004).

1.2.1 Central tolerance

Central tolerance involves mechanisms of positive and negative selection of T cells within the thymus (Pugliese, 2004). Initially, double positive (DP) CD4⁺CD8⁺ thymocytes are exposed to MHC-peptide complexes in the thymic cortex. The majority of DP thymocytes will have no affinity to such complexes. They therefore do not receive survival signals and die by neglect via apoptosis (Fig 1.1) (Romagnani, 2006; Starr *et al.*, 2003). Surviving cells (or “positively selected” cells) migrate to thymic medullary areas to differentiate into single positive CD4⁺ or CD8⁺ thymocytes (Starr *et al.*, 2003). However, this pool of positively selected cells contains those that have affinity

for either foreign antigen or self-antigen. A further process is required to purge the pool of self-reactive cells – this is called negative selection (Fig 1.1).

T cells encounter MHC-self antigen complexes on the medullary epithelial cells. Those that show high avidity for these complexes undergo apoptosis (Fig 1.1) (Romagnani, 2006). Cells which do not react with self antigens and therefore survive negative selection can subsequently migrate from the thymus to peripheral lymphoid organs (Fig 1.1) (Romagnani, 2006).

1.2.2 Peripheral tolerance

Despite the efficiency of central tolerance, not all self-reactive T cells will be deleted in the thymus. An additional mechanism is therefore required to control potentially self-reactive T cell responses in the periphery. This process is known as peripheral tolerance and involves at least four different mechanisms: anergy, deletion and immune suppression (Romagnani, 2006; Taams *et al.*, 2003), and immunological ignorance (Taams *et al.*, 2003).

Anergy occurs when a T cell receives only one activating signal from an APC – i.e. MHC-peptide alone (Signal 1) in the absence of costimulatory signalling (Signal 2). In such cases the T cell is not activated but instead becomes anergic, i.e. unresponsive to antigen even if it later encounters an APC presenting the same MHC-peptide complex with costimulatory molecules (Powell, 2006). Additionally, anergy can occur if a T cell is stimulated with its specific MHC-peptide complex and cytotoxic T-lymphocyte-associated-molecule-4 (CTLA-4), an inhibitory costimulatory molecule (Asnagli and Murphy, 2001). In both cases the T cell does not die but persists in the body in a permanent state of unresponsiveness.

Deletion requires apoptotic cell death of the self-reactive T cells. It usually occurs when T cells encounter high concentrations of specific self antigen and/or are strongly activated (Romagnani, 2006). This is known as activation-induced cell death (AICD) and involves the extrinsic apoptotic pathway, specifically mediated by the “death receptor” Fas and its ligand, FasL (Fas *et al.*, 2006).

Immunological ignorance refers to a situation where reactive T cells in an individual have not encountered their specific antigen and have a naïve phenotype (Ochsenbein, 2005). There is a minimal “threshold” dose of antigen required to induce an immune response to a particular antigen, and is dependent on several factors. For example, an antigen might be exclusively expressed on cells that either do not migrate to secondary lymphoid organs, or do not efficiently migrate there (Ochsenbein, 2005). It is thought that this is true for most self antigens as well as tumour antigens. Tumours arise from the host's own tissues, therefore many of the target antigens are tissue-specific molecules shared by cancer cells and normal cells. These weak antigens therefore do not typically elicit immunity (Perales *et al.*, 2002).

Sequestration, or “walling off” of antigen from the immune system also contributes to immunological ignorance. A well-characterised example is the eye, whose posterior chamber is separated from the immune system early in ontogeny, separated by the blood-retinal barrier (Caspi, 2006). It was believed that ocular antigens therefore remained “unknown” to the immune system, as a result of this relative inaccessibility. However, it is now known that the reality is more complex – in addition to basic sequestration, other mechanisms exist that actively inhibit immune responses within the eye and modulate immune responses to antigens released from the eye (Caspi, 2006).

Immunosuppression is mediated by regulatory T (Treg) cells, which will be described in subsequent sections.

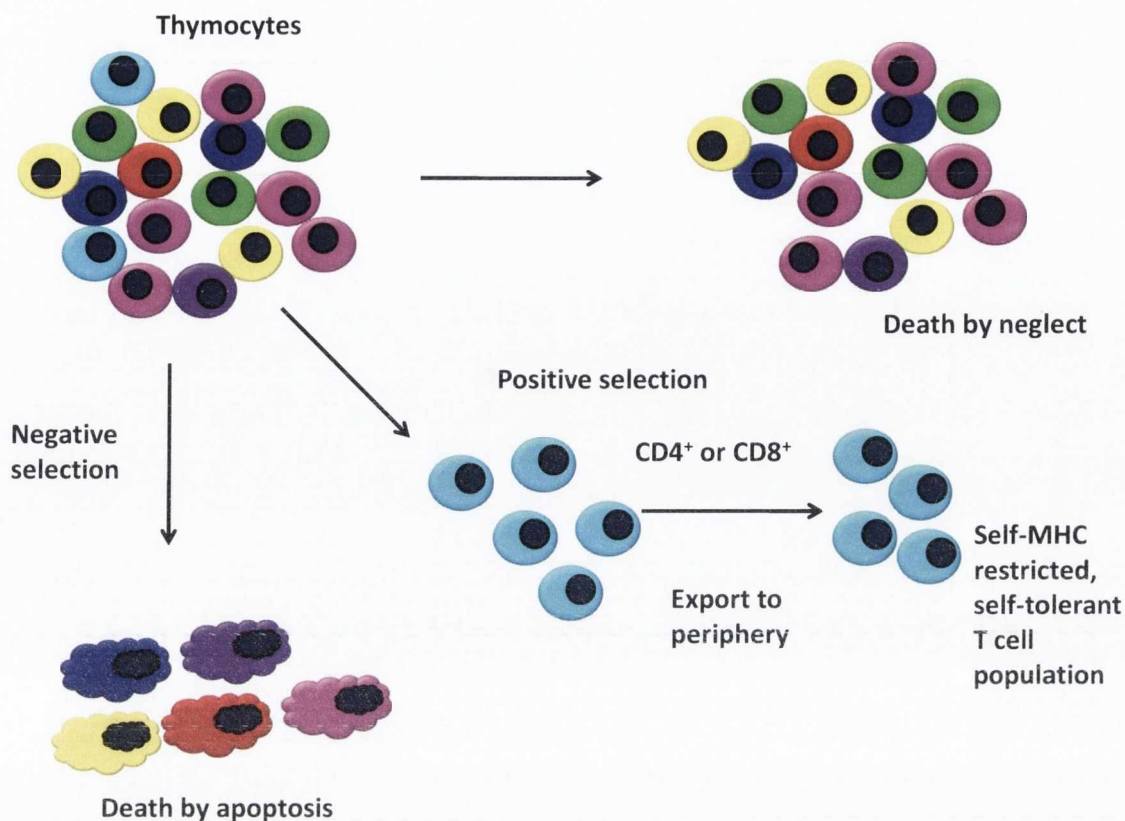


Figure 1.1 Central tolerance
 Adapted from Romagnani (Romagnani, 2006)

In the thymic cortex, double positive (DP) CD4⁺CD8⁺ thymocytes are exposed to MHC-peptide complexes. Those which lack affinity for such complexes (>95%) do not respond and die of neglect via apoptosis. Surviving, positively selected DP cells subsequently migrate to the thymic medulla. Here they differentiate into single positive CD4⁺ or CD8⁺ thymocytes. The pool of positively selected cells has affinity for self antigen as well as foreign antigen. Those which have avidity for self antigen are then “negatively selected”, i.e. they undergo apoptosis. Surviving single positive CD4⁺ or CD8⁺ cells migrate to the secondary lymphoid organs, where they comprise the entire the T cell repertoire, which can respond to foreign antigen presented by self-MHC complexes.

1.3 Natural regulatory T cells

Natural regulatory T (nTreg) cells are a naturally occurring, functionally mature population of CD4⁺ T cells which constitutively express CD25, the alpha chain of the IL-2 receptor (Sakaguchi *et al.*, 1995; Sakaguchi *et al.*, 1996; Suri-Payer *et al.*, 1998). After development in the thymus, nTreg cells enter peripheral tissues and mediate peripheral tolerance by suppressing the activation of self-reactive Th cells (Itoh *et al.*, 1999; Toda and Piccirillo, 2006).

The important role of these cells was first shown by Sakaguchi *et al.* (Sakaguchi *et al.*, 1995). Sakaguchi demonstrated that the transfer of CD4⁺ T cells, from which the CD4⁺CD25⁺ population had been depleted, into immunocompromised mice caused spontaneous autoimmune diseases. This autoimmunity could be prevented by reconstituting the animals with CD4⁺CD25⁺ T cells (Sakaguchi *et al.*, 1995).

Murine CD4⁺CD25⁺ T cells can suppress CD4⁺CD25⁻ responder cells in a cell-contact dependent manner *in vitro*, which involves the inhibition of IL-2 production by the CD4⁺ responder cells (Thornton and Shevach, 1998). However, CD25 is an activation marker for all T cells and is therefore not a definitive marker for nTreg cells (Sakaguchi *et al.*, 1996). This makes it difficult to differentiate between T cells with effector and regulatory function during an immune response. nTreg cells have been identified using a number of other surface markers which include CTLA-4 (Read *et al.*, 2000), glucocorticoid-induced TNF receptor family-related gene (GITR) (Ono *et al.*, 2006), low levels of CD127 (Banham *et al.*, 2006) or low levels of CD45RB (Powrie *et al.*, 1996). Expression of the transcription factor FoxP3 is also used to identify nTreg cells.

1.3.1 FoxP3

The forkhead box protein transcription factor FoxP3 is presently the most definitive marker of nTreg cells in both mice and humans (Fontenot *et al.*, 2003; Khattri *et al.*, 2003). Mutations in FoxP3 result in Scurfy, an X-linked syndrome in male mice, which is a CD4⁺ T cell-mediated lymphoproliferative disease characterised by wasting and multi-organ lymphocytic infiltrates (Clark *et al.*, 1999). In humans the recessive IPEX (immunodysregulation polyendocrinopathy

enteropathy X-linked) syndrome, an aggressive, fatal autoimmune condition is secondary to mutations in the human homologue of *Foxp3* (Bennett *et al.*, 2001). Other studies have shown that transfection of CD4⁺CD25⁻FoxP3⁻ T cells with *FoxP3* confers them with regulatory activity (Hori *et al.*, 2003; Ziegler, 2006). In addition, mice with the Scurfy phenotype could avoid disease when reconstituted with CD4⁺CD25⁺ Treg cells or when the same mice expressed the *FoxP3* transgene (Ziegler, 2006). Moreover, in a conditional deletion system, silencing of the *FoxP3* gene resulted in the rapid expression of multiorgan immunity (Kim *et al.*, 2007).

CD4⁺FoxP3⁺ cells represent approximately 5-8% of the total CD4⁺ population in the spleens of naïve mice (Bochtler *et al.*, 2006). nTreg cells in human peripheral blood represent approximately 3% of total CD4⁺ T cells (Walker *et al.*, 2003). Unlike in mice where there is a distinct correlation between FoxP3 and CD25 expression, almost all activated human CD4⁺ T cells (i.e. CD4⁺CD25^{+/high}) are FoxP3⁺ due to induction of FoxP3 expression via the TCR (Walker *et al.*, 2003). The induced FoxP3⁺ population only expresses FoxP3 transiently however, and these cells do not suppress CD4⁺ T cell responses *in vitro* (Gavin *et al.*, 2006). In addition, the existence of a CD25^{intermediate (int)} population of CD4⁺ T cells further complicates the study of nTreg cells in humans; these cells are also FoxP3⁺ but express it at a lower intensity than CD4⁺CD25^{high} cells.

Since FoxP3 is a nuclear transcription factor it can only be detected by cell fixation and permeabilisation. For this reason FoxP3 cannot be used to purify live Treg cells from murine or human tissue for *in vitro* functional assays. As mentioned in section 1.3, CD45RB and CD127 are surface markers which can distinguish nTreg cells from non-Treg cells. The expression of these markers is inversely correlated with FoxP3 expression, and they are used as surrogate markers to identify FoxP3⁺ and FoxP3⁻ T cells.

1.3.2 CD45RB

The glycoprotein CD45 is a tyrosine phosphatase expressed abundantly on lymphocytes (Alexander, 2000). Its expression is critical for antigen receptor signalling, as shown by the arrested development of CD45^{-/-} T and B cells (Kishihara *et al.*, 1993; Koretzky *et al.*, 1990). Eight

different isoforms of CD45 can be generated by alternative splicing of three consecutive exons termed A, B and C (Rogers *et al.*, 1992). Different stages of T cell differentiation and function are associated with the differential expression of the CD45 isoforms (Lee *et al.*, 1990; Trowbridge and Thomas, 1994). In mice, CD45RB is expressed by CD8⁺ T cells, CD4⁺ helper T cells and most thymocytes (Luke *et al.*, 2001). Its expression decreases as a T cell progresses from naïve to memory states (Lee *et al.*, 1990; Trowbridge and Thomas, 1994).

CD45RB^{high} T cells are associated with organ rejection and inflammation (Powrie *et al.*, 1994a; Powrie *et al.*, 1994b; Read *et al.*, 2000). Notably, T cells with regulatory function express low levels of CD45RB (CD45RB^{low}) and can inhibit allograft rejection (Luke *et al.*, 2001). It has also been shown that the T cells which control intestinal inflammation and autoimmunity express low levels of CD45RB (Read *et al.*, 2000). Anti-CD45RB monoclonal antibody (mAb) administration has been shown to prolong allograft survival (Lazarovits *et al.*, 1996; Parry *et al.*, 1999) and promote immunosuppressive activity in xenografts and preclinical autoimmunity models (Abu-Hadid *et al.*, 2000; Zhang *et al.*, 2000).

Importantly, it has been demonstrated that murine CD4⁺CD45RB^{low} T cells express higher levels of FoxP3 than CD4⁺CD45RB^{high} T cells (Luke *et al.*, 2006). This discovery has made the purification of functional murine Treg cells more straightforward and less contentious than using CD25 alone, due to the potential contamination of CD25⁺ Tregs with non-regulatory activated T cells.

1.3.3 CD45RA and CD45RO in humans

As described in section 1.3.1, almost all activated human CD4⁺CD25^{+/high} T cells are FoxP3⁺ due to induction of FoxP3 expression via the TCR (Walker *et al.*, 2003). Nonetheless, the induced FoxP3⁺ population only transiently expresses FoxP3, and do not suppress CD4⁺ T cell responses *in vitro* (Gavin *et al.*, 2006). Different isoforms of the surface marker CD45 can be generated by alternative splicing of the three consecutive exons A, B and C (Rogers *et al.*, 1992). Studies in humans have reported that the expression of CD45RA or CD45RO can differentiate

naïve T cells and memory T cells, respectively, as expression of these markers is mutually exclusive (Sakaguchi *et al.*, 2010). CD45RO, in conjunction with CD25 and/or FoxP3 expression is a useful human Treg cell marker. CD4⁺CD25^{high}CD45RO⁺ T cells express FoxP3, and were believed to be the human counterpart of murine CD4⁺CD25⁺FoxP3⁺ Treg cells (Baecher-Allan *et al.*, 2001; Levings *et al.*, 2001b; Ng *et al.*, 2001; Taams *et al.*, 2002).

However, FoxP3 expression and suppressive function have also been described in naïve human CD4⁺ T cells which express CD45RA, but not CD45RO (Fritzsching *et al.*, 2006; Miyara *et al.*, 2009; Seddiki *et al.*, 2006b; Valmori *et al.*, 2005). These cells have been designated as “naïve” Treg cells, and are characterised by their surface expression of CD45RA and their low levels of FoxP3 (Fritzsching *et al.*, 2006; Ito *et al.*, 2008; Miyara *et al.*, 2009). This population of Treg cells are probably not strictly “naïve”, given that Treg cells must be stimulated with their cognate antigen in order to be maintained in the periphery (Fisson *et al.*, 2003). However, it has been known for some time that activated CD4⁺ T cells can express CD45RA (Brod *et al.*, 1989). Nonetheless, most of these CD45RA⁺FoxP3^{low} “naïve” Treg cells express CD32, which identifies recent thymic emigrants, while CD45RO⁺ Treg cells lack this marker (Haas *et al.*, 2007; Miyara *et al.*, 2009). Once they are activated, “naïve” Treg cells proliferate, upregulate FoxP3 expression and convert to a CD45RO⁺CD25^{high}FoxP3⁺ phenotype (Miyara *et al.*, 2009).

1.3.4 CD127

CD127, the alpha chain of the IL-7 receptor, is also routinely used to discriminate FoxP3⁺ regulatory T cells from FoxP3⁺CD25⁺ activated CD4⁺ T cells, as Treg cells are generally CD127^{low} (Banham, 2006). Two studies have illustrated the inverse correlation between FoxP3 and CD127 on Treg cells (Liu *et al.*, 2006; Seddiki *et al.*, 2006a). It was also shown in these reports that CD127 was a more specific marker for Treg cells than CD25 in mice as most CD127^{low} cells were FoxP3⁺ (Liu *et al.*, 2006; Seddiki *et al.*, 2006a). Importantly, both studies found functionally suppressive CD127^{low} human Treg cell populations irrespective of their CD25 expression. In the same way that CD45RB can be used to distinguish murine Treg cells from non-Treg cells, using CD127 negativity

as a surrogate marker for human and murine Treg cells has facilitated the purification of uncontaminated Treg cell populations.

1.3.5 Helios

Helios is a member of the Ikaros transcription factor family (Thornton *et al.*, 2010). Its expression appears to be restricted to the T lymphocyte lineage (Hahm *et al.*, 1998). It is thought that Ikaros family members regulate gene transcription via chromatin remodelling (Thornton *et al.*, 2010). Helios is thought to be a potential FoxP3 target gene, and it has been shown that Helios is selectively expressed in FoxP3⁺ T cells (Hill *et al.*, 2007; Kang *et al.*, 2007a; Sugimoto *et al.*, 2006). It has been subsequently reported that Helios is exclusively expressed by thymically-derived FoxP3⁺ nTreg cells and can therefore differentiate these cells from those which acquire FoxP3 expression in the periphery in mice and humans (Thornton *et al.*, 2010). Helios expression does not seem to be required for Treg function (Thornton *et al.*, 2010).

However, more recent studies have challenged the idea that Helios can distinguish thymically-derived and peripherally induced FoxP3⁺ Treg cells, as it has been identified in inducible FoxP3⁺ Treg cells *in vitro* and *in vivo* (Gottschalk *et al.*, 2011). Furthermore, Helios expression is apparently induced in human and murine T cells during proliferation and activation, but can regress in the same cells when they are rested (Akimova *et al.*, 2011). It would appear that Helios expression is associated with T cell activation and cellular division, regardless of the cell subset involved. Therefore it might not be suitable as a marker to distinguish natural and induced Treg cells.

1.3.6 Additional surface markers used to identify Treg cells

Depletion of total CD4⁺CD25⁺ T cells either by thymectomy (Day 3 thymectomy model) or the adoptive transfer of CD4⁺CD25⁻ T cells into athymic nude mice induces gastritis, thyroiditis and either oophoritis or prostatitis/orchitis, depending on the gender of the mice (Sakaguchi *et al.*, 2006; Sakaguchi *et al.*, 1995). As stated in section 1.3, CD25 is an imperfect phenotypic marker of Treg cells, and it has been reported that FoxP3⁺CD25⁻ Treg cells exist. However, it is not clear

whether they are nTreg cells which have lost CD25 expression or whether they represent inducible FoxP3⁺ Treg cells.

Furthermore, there are diverse subsets within the CD25⁺ Treg cell population, which express or lack particular surface markers. Investigation into organ-specific autoimmune disease has demonstrated that distinct diseases are evident depending on the type of Treg cells that are depleted. CD25⁺ nTreg cells prevent gastritis and late onset diabetes in NOD SCID (non-obese diabetic, severe combined immunodeficient) mice (Alyanakian *et al.*, 2003), however CD25⁻ CD62L⁺ (L-selectin) cells protect against fulminant diabetes, and CD4⁺CD45RB^{low} cells inhibit colitis with wasting disease but have little or no effect on gastritis or diabetes (Alyanakian *et al.*, 2003). However, only CD4⁺CD25⁺ Treg cells could efficiently suppress proliferation of target T effector (Teff) cells *in vitro*. Furthermore, the cytokine profile varies significantly for each subset analysed (Alyanakian *et al.*, 2003). This data suggests distinct subsets of CD4⁺ Treg cells prevent induction of certain autoimmune diseases *in vivo* and these subsets are not necessarily confined to the CD4⁺CD25⁺ population.

Marker	Function	Human/Mouse	Evidence
FoxP3	Transcription factor, governs expression of regulatory genes	Human and mouse	CD4 ⁺ FoxP3 ⁺ T cells have regulatory function <i>in vitro</i> and <i>in vivo</i> (Fontenot <i>et al.</i> , 2003). Defects in <i>foxp3</i> gene are associated with IPEX (humans) and scurfy (mice) (Bennett <i>et al.</i> , 2001).
CD25	Alpha chain of IL-2R, T cell activation marker	Human and mouse	Thymically-derived nTreg cells are CD4 ⁺ FoxP3 ⁺ CD25 ⁺ (Sakaguchi <i>et al.</i> , 1995). CD25 expression may not be essential to identify functional Treg cells, but may discriminate activated from inactive Treg cells (Zelenay <i>et al.</i> , 2005).
GITR	member of the TNFR superfamily, Costimulatory molecule	Human and mouse	CD4 ⁺ FoxP3 ⁺ CD25 ⁺ GITR ^{high} T cells have suppressive function in murine organ transplantation (Nishimura <i>et al.</i> , 2004), inflammatory bowel disease (Uraushihara <i>et al.</i> , 2003) and autoimmune myocarditis in mice (Ono <i>et al.</i> , 2006)
CTLA-4	Inhibitory costimulatory molecule	Human and mouse	Constitutive expression of CTLA-4 has been reported in Treg cells (Read <i>et al.</i> , 2000). Anti-CTLA-4 blocking experiments and experiments using CTLA-4-deficient Treg cells have suggested a role for CTLA-4 in Treg cell-mediated suppression via DC (Oderup <i>et al.</i> , 2006; Serra <i>et al.</i> , 2003).
ICOS	Costimulatory molecule, stimulates IL-10 production	Human and mouse	ICOS plays an essential and specific role in mucosal tolerance (Miyamoto <i>et al.</i> , 2005). ICOS is crucial for the induction of peripheral tolerance (Akbari <i>et al.</i> , 2002; Tuettenberg <i>et al.</i> , 2009)
CD45RB	CD45 is a tyrosine phosphatase. CD45RB isoform is a naïve T cell marker	Mouse	FoxP3 ⁺ cells are generally CD45RB ^{low} (Luke <i>et al.</i> , 2001). Regulatory activity has been described for CD4 ⁺ CD45RB ^{low} CD25 ⁺ T cells in rodents (Hori <i>et al.</i> , 2003; Powrie <i>et al.</i> , 1994a)
CD127	Alpha chain of IL-7R	Human and mouse	FoxP3 ⁺ cells are generally CD127 ^{low} (Banham, 2006; Liu <i>et al.</i> , 2006; Seddiki <i>et al.</i> , 2006a).
CD45RO	Tyrosine phosphatase. CD45RO isoform is a memory T cell marker	Human	CD45RO can be used in conjunction with FoxP3 and CD25 expression to identify human Treg cells (Baecher-Allan <i>et al.</i> , 2001; Taams <i>et al.</i> , 2002).
CD45RA	Tyrosine phosphatase. CD45RA isoform is a naïve T cell marker	Human	Most CD45RA ⁺ human T cells are FoxP3 ⁺ , however a “naïve” Treg cell population has been described – these cells are CD45RA ⁺ FoxP3 ^{low} and become CD45RO ⁺ FoxP3 ^{high} CD25 ⁺ upon activation (Miyara <i>et al.</i> , 2009)
Helios	Transcription factor	Human and mouse	May differentiate thymically-derived FoxP3 ⁺ Treg cells from peripherally induced FoxP3 ⁺ Treg cells (Thornton <i>et al.</i> , 2010)
CD62L	Adhesion molecule	Human and mouse	CD25 ⁺ CD62L ⁺ T cells protect against fulminant diabetes in mice (Alyanakian <i>et al.</i> , 2003)

Table 1.1 Summary of important markers for Treg cells

1.4 Adaptive Treg cells

Adaptive Treg cells differ from nTreg cells as they do not develop in the thymus. Instead they are generated from mature CD4⁺ T cells in the periphery under particular conditions of sub-optimal antigenic stimulation and/or costimulation. The cytokine milieu also plays an important role in their induction (Roncarolo *et al.*, 2006). Adaptive Treg cells are generally FoxP3⁺ and are defined by their cytokine production profile rather than their cell surface markers (Taams and Akbar, 2005). They are also weaker suppressors than nTreg cells and are less stable (Gupta *et al.*, 2008). Furthermore, there is highly variable expression of CD25 among such Treg cell populations (Bluestone and Abbas, 2003).

Adaptive Treg cells are specific for antigens which are not encountered in the thymus during clonal selection, such as food antigens, commensal microorganisms, and certain pathogens (Curotto de Lafaille and Lafaille, 2009). They are especially important in mucosal immunology, as the mucosal environments, such as the lungs and intestines, encounter substantial amounts of foreign antigen.

In the event of infection or injury, the generation of adaptive Treg cells facilitates a dynamic immune response. Activated T cells can recruit other inflammatory cells to the site, but as the response progresses and matures, adaptive Treg cells can develop locally in response to the inflammatory environment. These Treg cells can allow inflammation to resolve by regulating the magnitude of the T cell response. They can also limit bystander immune responses directed against self-antigen that might have become exposed during the inflammatory response (Buckner and Ziegler, 2004).

It has also been hypothesised that the Treg cells might restrict the number of surviving T cells and therefore memory cells, in order to avoid overwhelming secondary immune responses upon repeated exposure to the same antigen. The importance of Treg cells in this context is highlighted by studies on *Leishmania major* infection, whereby the absence of Treg cells or IL-10

allows the infection to be cleared more quickly in mice, but the animals do not develop resistance to a second infection (Belkaid *et al.*, 2002). On the other hand, the presence of Treg cells leads to low-level persistence of the pathogen but facilitates the development of long-term immunity (Belkaid *et al.*, 2002).

1.4.1 Tr1 cells

CD4⁺FoxP3⁻ Treg cells that secrete predominantly IL-10 are called Tr1 cells (Groux *et al.*, 1997). They can be generated *in vitro* from naïve human or mouse CD4⁺ T cells via antigenic stimulation in the presence of IL-10 (Chen *et al.*, 1994; Groux *et al.*, 1997). IFN- α in conjunction with IL-10 has also been shown to induce human Tr1 cells *in vitro* (Levings *et al.*, 2001a). Despite being FoxP3⁻, Tr1 cells are functionally similar to FoxP3⁺ nTreg cells as they are hypo-proliferative when stimulated with antigen (Vieira *et al.*, 2004). Tr1 cells can also be induced *in vitro* by repeated stimulation of the TCR by immature DC or IL-10-producing DC in the presence of high concentrations of IL-10 (McGuirk *et al.*, 2002). These cells can also be generated by culture with vitamin D3 and dexamethasone (Barrat *et al.*, 2002).

It has been demonstrated that antigen-specific Tr1 cells can be generated *in vivo*, in mice infected with the gram negative bacterium *Bordetella pertussis*, and these cells can suppress Th1 cells (McGuirk *et al.*, 2002). It is thought that these cells function to limit immunopathology as a result of the Th1 response against *B. pertussis*. Pathogens are also able to induce regulatory cells as a mechanism of immune evasion, such as Epstein Barr virus (EBV) which has been reported to induce IL-10-producing Tr1 cells (Marshall *et al.*, 2003).

1.4.2 Th3 Cells

TGF- β_1 -producing adaptive Treg cells have been called Th3 cells. In the gut the generation of Th3 cells is associated with the induction of oral tolerance to exogenous ingested antigens (Chen *et al.*, 1994). Oral tolerance is a continuous immunologic process established in the gut-associated lymphoid tissue (GALT) (Weiner, 2001b). It is presumed that oral tolerance evolved to avert unwarranted immune responses against commensal flora in the gut and of course

innocuous food antigens from the diet (Weiner, 2001b). Research has shown that there are two mechanisms of oral tolerance – induction of clonal anergy or clonal deletion and induction of Treg cells which actively suppress effector immune responses (Fig 1.2). The dose of antigen appears to be the deciding factor for the type of oral tolerance induced (Weiner, 2001b).

Stimulation with low doses of antigen generates Th2 cells as well as antigen-specific Th3 cells (Chen *et al.*, 1994). Th3 cells are believed to be induced by TGF- β_1 , which along with IL-10 and IL-4 is constitutively expressed at high levels in the gut (Inobe *et al.*, 1998; Seder *et al.*, 1998). After feeding oral antigen, their expression is further upregulated (Gonnella *et al.*, 1998). Th3 cells are distinct from Th2 cells as they secrete little or no IL-4 or IL-10 (Weiner, 2001a). Notably, these cells can transfer tolerance *in vivo* and are also able to suppress *in vitro* (Miller *et al.*, 1992). In contrast, hyporesponsiveness to oral antigen is generated after administration of high dose of antigen and is mediated by anergy or deletion, as demonstrated by a study in which the development of experimental autoimmune encephalomyelitis (EAE) in rats could be inhibited by feeding high doses of antigen known to induce EAE (Whitacre *et al.*, 1991).

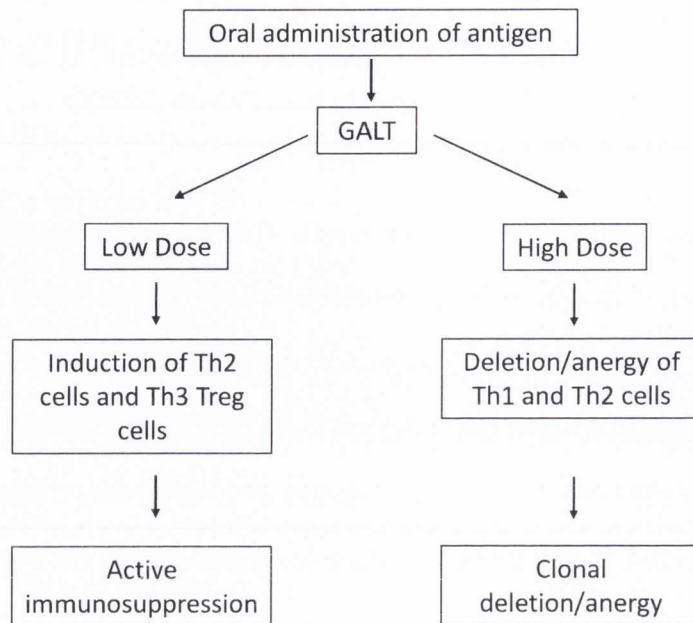


Figure 1.2 Different mechanisms of oral tolerance

Adapted from H.L. Weiner (Weiner, 2001b)

The different mechanisms of oral tolerance are determined by the dose of fed antigen. Stimulation with low doses of antigen generates Th2 cells as well as antigen-specific Th3 cells. Th3 cells can actively transfer tolerance *in vivo* and are also able to suppress *in vitro*. In contrast, hyporesponsiveness to oral antigen is generated after administration of high dose of antigen and is mediated by clonal anergy or clonal deletion.

1.4.3 Inducible FoxP3⁺ Treg cells

In contrast to FoxP3⁻ Tr1 and Th3 cells, adaptive or inducible FoxP3⁺ Treg (iTreg) cells have also been reported. Like Tr1 and Th3 cells, iTreg cells arise in the periphery and therefore differ from thymically-derived nTreg cells (Bluestone and Abbas, 2003). *In vitro* and *in vivo* studies support the existence of FoxP3⁺ iTreg cells, and it appears that TGF- β_1 is important for their induction, at least in mice.

In vitro, TGF- β_1 converts naïve CD4⁺FoxP3⁻ T cells to CD4⁺FoxP3⁺CD25⁺ iTreg cells (Chen *et al.*, 2003; Fantini *et al.*, 2004). These cells were able to suppress normal T cell proliferation *in vitro* and could also inhibit allergic responses upon adoptive transfer *in vivo* (Chen *et al.*, 2003). DC have been reported to play a role in the TGF- β_1 -dependent induction of FoxP3⁺ iTreg cells. Studies in a diabetes model have demonstrated that DC pulsed with beta-cell peptide from NOD mice can convert naïve CD4⁺ T cells into CD4⁺FoxP3⁺CD25⁺ iTreg cells in the presence of TGF- β_1 . The iTreg cells could inhibit T cell proliferation *in vitro* and prevented autoimmune diabetes *in vivo* (Luo *et al.*, 2007). The induction of Treg cells might be an important strategy for maintaining tolerance at mucosal interfaces. Indeed, the conversion of naïve CD4⁺ T cells to antigen-specific CD4⁺FoxP3⁺ iTreg cells has been shown to take place in the intestine (Coombes *et al.*, 2007). CD103⁺ DC from the mesenteric lymph nodes promoted this conversion, via TGF- β_1 and the vitamin A metabolite retinoic acid (RA) (Coombes *et al.*, 2007).

It has been reported that up to 90% of naïve CD4⁺ T can be converted to FoxP3⁺ iTreg cells in the presence of TGF- β_1 (Selvaraj and Geiger, 2007). Conversion only occurs if TGF- β_1 is added to T cell culture within 2-3 days of activation *in vitro*, and it is reversible, as removal of the cytokine resulted in loss of FoxP3 expression after approximately 4 days (Selvaraj and Geiger, 2007). In addition, FoxP3 expression was lost from most of the iTreg cells upon adoptive transfer into wild-type mice (Selvaraj and Geiger, 2007). This suggests that *in vitro* conversion is transient, and depends on TGF- β_1 .

IL-2 may promote the TGF- β_1 -mediated conversion of iTreg cells (Zheng *et al.*, 2007). Both cytokines are essential for the maintenance of nTreg cells (Khattar *et al.*, 2009). However, normal Treg cell function has been reported in the absence of TGF- β_1 or in mice unresponsive to TGF- β_1 (Piccirillo *et al.*, 2002). In humans TGF- β_1 might not be required for suppression of immune responses, as suggested by a study showing cell-contact dependent iTreg cell function (Walker *et al.*, 2003). Nonetheless, it seems that activation of the T cells by antigen or superantigen is required for the generation of iTreg cells (Buckner and Ziegler, 2004; Duthoit *et al.*, 2004; Thorstenson and Khoruts, 2001; Zheng *et al.*, 2002).

In vivo Treg cell conversion has also been reported. When CD4⁺CD25⁻ T cells from CD45.1⁺ mice were adoptively transferred into congenic CD45.2⁺ mice, 5-12% of the transferred cells converted to CD4⁺FoxP3⁺CD25⁺ iTreg cells within 6 weeks (Liang *et al.*, 2005). The iTreg cells were refractory to stimulation and could suppress Teff cell proliferation *in vitro* (Liang *et al.*, 2005). The administration of very low doses of antigen in conjunction with suboptimal DC activation can also induce conversion of FoxP3⁺ iTreg cells *in vivo*, which can be further enhanced by TGF- β_1 but does not require IL-2 (Kretschmer *et al.*, 2005). Furthermore, blocking TGF- β_1 during feeding of Ovalbumin (Ova) inhibited both the induction of oral tolerance and the peripheral conversion of Ova-specific Treg cells (Mucida *et al.*, 2005).

It has been reported that CD4⁺FoxP3⁻CD25⁻ T cells can convert to CD4⁺FoxP3⁺CD25⁻ iTreg cells. Kretschmer *et al* demonstrated the existence of antigen-specific CD4⁺FoxP3⁺CD25⁻ iTreg *in vivo* (Kretschmer *et al.*, 2005). Malaria infection can also drive the conversion of CD4⁺FoxP3⁻CD25⁻ T cells to CD4⁺FoxP3⁺CD25⁻ iTreg cells (Couper *et al.*, 2007).

1.4.4 Retinoic Acid

The induction of Treg cells and Th17 cells are reciprocally regulated by cytokines (Bettelli *et al.*, 2006). When activated CD4⁺ T cells are exposed to TGF- β_1 alone, they can convert to FoxP3⁺ iTreg cells, but if IL-6, IL-1 β and IL-23 or IL-21 are also present FoxP3 induction is blocked and Th17 cells are induced (Bettelli *et al.*, 2006; Manel *et al.*, 2008; Mangan *et al.*, 2006; 34

Veldhoen *et al.*, 2006). The induction of iTreg cells by TGF- β_1 is concomitant with the inhibition of Th1 and Th2 polarisation (Li *et al.*, 2006; Mucida *et al.*, 2005; Wahl *et al.*, 2006).

This reciprocal relationship suggests that the TGF- β_1 polarisation of Treg or Th17 differentiation is highly controlled. Retinoic acid (RA) has been shown to modulate the pleiotropic effects of TGF- β_1 and promote induction of FoxP3⁺ Treg cells (Benson *et al.*, 2007; Coombes *et al.*, 2007; Kang *et al.*, 2007b; Mucida *et al.*, 2007; Sun *et al.*, 2007). If CD4⁺ T cells are activated in the presence of TGF- β_1 and RA, FoxP3⁺ iTreg cell induction is enhanced. Even if IL-6 is also present, Th17 cell differentiation is inhibited because RA blocks the IL-6-dependent expression of ROR γ t, the key Th17-polarising transcription factor (Ivanov *et al.*, 2006; Mucida and Cheroutre, 2007; Mucida *et al.*, 2007; Zhou *et al.*, 2008).

RA, or all-trans retinoic acid (atRA), is a biologically active metabolite of vitamin A. Vitamin A is obtained from the diet, in the form of all-trans retinol, retinyl esters or β -carotene (Blomhoff and Blomhoff, 2006; Moise *et al.*, 2007). The oxidation of all-trans retinol or β -carotene by alcohol dehydrogenases or short-chain dehydrogenase reductases results in the synthesis of all-trans retinal. All-trans retinal is the precursor of RA. RA is formed when all-trans retinal is irreversibly oxidised by retinal dehydrogenases (RALDH) (Blomhoff and Blomhoff, 2006; Moise *et al.*, 2007). The expression of RALDH is tightly controlled and is restricted to specific cells and tissues; therefore RA production is restricted to these cells and tissues, for example the gut, where RA is found in abundance. RALDH is expressed by certain gut-associated cells such as intestinal epithelial cells (Iwata *et al.*, 2004; Lampen *et al.*, 2000) and gut-associated DC (Coombes *et al.*, 2007; Germain *et al.*, 2006).

It is well known that vitamins play a role in the immune system, in particular vitamins A and D (Mora *et al.*, 2008). Both promote Th2 responses and inhibit Th1 and Th17 responses. In addition, they promote the induction of FoxP3⁺ iTreg cells (Cantorna *et al.*, 1994; Mora *et al.*, 2008). The importance of vitamin A in T cell function is exemplified by the fact that vitamin A-

deficient mice have defective T cell activity (Carman *et al.*, 1989), and in vitamin A-deficient rats, where deficiency is associated with decreased Th2 responses (Wiedermann *et al.*, 1993). In a mouse model of asthma, vitamin A supplementation led to increased disease severity, while vitamin A deficiency has the opposite effect, due to decreased Th2 cytokines (Schuster *et al.*, 2008). Vitamin A deficiency also correlates with impaired intestinal immune responses (Mora and von Andrian, 2008; Sirisinha *et al.*, 1980; Wiedermann *et al.*, 1993) and increased mortality, associated with gastrointestinal and respiratory infections (Sommer *et al.*, 1983).

As the biologically active metabolite of vitamin A, the presence or absence of RA is likely to be responsible for these effects. Supplementation of vitamin A blocks the production of Th1 cytokines *in vitro* and *in vivo* (Iwata *et al.*, 2003; Nikawa *et al.*, 1999). RA is directly responsible for these effects on T cells. The *IL4* gene is induced by RA, and thus promotes Th2 differentiation (Lovett-Racke and Racke, 2002). Furthermore, RA blocks T-bet, the Th1-cell promoter, and induces Th2-cell promoting factors such as GATA3 and STAT6 (Dawson *et al.*, 2006; Iwata *et al.*, 2003).

In addition, RA can enhance the induction of FoxP3⁺ iTreg cells by TGF- β ₁ in the periphery (Benson *et al.*, 2007; Mucida *et al.*, 2007). While it has been reported that gut DC from GALT and small intestinal lamina propria use RA to induce iTreg cells (Coombes *et al.*, 2007; Sun *et al.*, 2007), this is contentious since there is evidence that macrophages from lamina propria induce iTreg cells, while DC induce Th17 cells (Denning *et al.*, 2007).

In vivo studies have demonstrated that the administration of exogenous RA inhibited the induction of Th17 cells, while injection of retinoic acid receptor (RAR) antagonists decreased the frequencies of lamina propria FoxP3⁺ cells (Mucida *et al.*, 2007). RA treatment has proven to be beneficial in an arthritis model. Arthritis is associated with enhanced IL-17 production and treatment with RA improved clinical symptoms by reducing the levels of IL-6, tumor necrosis factor (TNF)- α and IFN- γ (Hirota *et al.*, 2007; Tabata *et al.*, 2006).

Modulation of the immune response by RA also affects B cells. Activated B cells undergo immunoglobulin (Ig) class-switching, which gives rise to different antibody isotypes. Class-switching is differentially influenced by Th1 and Th2 cytokines – IFN- γ (Th1) promotes switching to IgG_{2a} and IgG₃, whereas IL-4 (Th2) induces IgG₁ and IgE production and suppresses IgG_{2b} and IgG₃ (Snapper *et al.*, 1997). The promotion of Treg/Th2 immunity by RA and its inhibition of Th1 responses therefore affects both humoral and cellular immune responses. With respect to the role of RA in mucosal immunology and homeostasis, its abundant production by intestinal DC, epithelial cells and macrophages results in dominance of RA over IL-6 in terms of modulating the effects of TGF- β_1 . This might explain the dominance of FoxP3⁺ Treg cells in the intestine, which facilitate a tolerogenic environment in spite of a large burden of commensal microbes and regular encounters with food antigens (Mucida and Cheroutre, 2007).

RA functions in a paracrine manner, by binding to nuclear hormone receptors (Mora *et al.*, 2008). These receptors belong to the RAR family, which has three isoforms – α , β and γ . Unlike RALDH, RAR proteins are ubiquitously expressed (Blomhoff and Blomhoff, 2006; Moise *et al.*, 2007). They form heterodimers with nuclear receptors of the retinoic X receptor (RXR) family. RAR-RXR heterodimers can interact with retinoic acid responsive elements (RAREs), which are found within the promoters of RA-responsive genes (Blomhoff and Blomhoff, 2006; Moise *et al.*, 2007).

STAT5 and STAT3 are the transcription factors which are important for the transcription of FoxP3 and IL-17, respectively (Ivanov *et al.*, 2006; Laurence *et al.*, 2007; Yang *et al.*, 2007). The enhancement of FoxP3 expression by RA suggests a relationship between RAR and STAT5, in a similar manner to the relationship between ROR γ t and STAT3. It should also be noted that RARs and ROR γ t are very homologous (Winoto and Littman, 2002). Indeed, interaction between STAT5 and RARs has been demonstrated *in vivo*, which promotes RAR-mediated transcription (Si and Collins, 2002). The STAT5 consensus binding site overlaps with a RARE, and therefore facilitates

coordinated transcriptional activity in response to RA signalling, rather than competing for the same binding site (Si and Collins, 2002).

More recent studies have indicated that RA may also have a pro-inflammatory role in immunity, rather than exclusively promote anti-inflammatory responses (Hall *et al.*, 2011). Mice were orally infected with *Toxoplasma gondii*, an intracellular protozoan which induces strong Th1 immunity. Compared with control mice, those on a vitamin A-deficient diet had reduced Th1 cell numbers in their spleens and intestines, and had a higher parasite load (Hall *et al.*, 2011), suggesting that vitamin A/RA is required for an optimal Th1 immune response against *T. gondii*. Feeding of RA to the vitamin A-deficient mice restored CD4⁺ T cell immunity to *T. gondii* (Hall *et al.*, 2011).

The same study also investigated the role of RA in T cell responses to vaccination. Using ovalbumin as antigen, the oral vaccination of vitamin A-deficient mice failed to induce Th1 and Th17 responses, which occur in response to ovalbumin in mice which are fed a normal diet (Hall *et al.*, 2011). As with *T. gondii* challenge, feeding RA to vitamin A-deficient mice allowed them to respond to orally administered ovalbumin (Hall *et al.*, 2011). It has been proposed that the concentration of RA is crucial in determining whether the induction of Th17 cells occurs or not. Lower RA concentrations (1-10 nM) seem to promote, while higher concentrations (>100 nM) suppress Th17 responses (Hall *et al.*, 2011). Furthermore, the presence or absence of microbial antigens may be important in determining the outcome of RA stimulation of naïve T cells. Flagellin is a TLR5 ligand, and it stimulates intestinal DC to both produce RA and induce Th17 cell differentiation (Uematsu *et al.*, 2008). Finally, the source of RA might be important in terms of dictating whether pro- or anti-inflammatory immune responses are invoked. While intestinal DC can produce RA and promote Treg cell induction, it is not clear whether systemic DC can produce RA.

It might be the case that the cytokine milieu determines the outcome of RA signalling. Certainly, RA and TGF- β_1 synergise to induce FoxP3⁺ Treg cells, and TGF- β_1 is abundant in the

gut. However, in the absence of TGF- β_1 , and/or abundant production of other cytokines, perhaps RA can promote pro-inflammatory rather than anti-inflammatory immune responses.

1.5 CD25⁻ Treg cells

It has been reported that Treg cells are not exclusive to the CD25⁺ population (Annacker *et al.*, 2001; Lehmann *et al.*, 2002). Subsets of CD4⁺ T cells which lack CD25 expression have been shown to have suppressive function, such as CD4⁺CD25⁻CD62L⁺ cells discussed in section 1.3.6 (Alyanakian *et al.*, 2003) and CD4⁺FoxP3⁺CD25⁻GITR⁺ cells which contribute to suppression of several autoimmune diseases (Ono *et al.*, 2006). Conversely, FoxP3 positivity is proving to be a more reliable indicator of regulatory function, irrespective of cell surface marker expression. A recent study in mice has shown that CD25⁻FoxP3⁺ cells can acquire CD25 expression in lymphopenic conditions to allow them to maintain homeostasis and that CD25 surface expression on Treg cells is labile, with cells able to lose and regain CD25 on the surface without affecting their function (Zelenay *et al.*, 2005). However, the number of CD25⁻ cells that are able to convert to CD25⁺ nTreg cells *in vivo* is limited (Curotto de Lafaille *et al.*, 2004).

It is not clear whether expression of CD25 on the cell surface is absolutely required for effective regulatory function. However, it has been shown that activation (i.e. upregulation of CD25) confers suppressive ability on CD4⁺CD45RB^{low}CD25⁻ cells, which otherwise do not suppress (Caramalho *et al.*, 2003). It has also been shown that nTreg cells can “shed” soluble CD25 (sCD25) upon activation, thereby acting as an IL-2 sink to prevent survival of Teff cells (von Bergwelt-Baildon *et al.*, 2006). However, while a recent study has demonstrated that activated human CD4⁺CD25⁺ Treg cells can secrete sCD25, they also showed that sCD25 does not inhibit IL-2-dependent T cell function (Pedersen and Lauritsen, 2009). In addition, a significant increase in the frequency of CD4⁺FoxP3⁺CD25⁻ T cells has been observed in aged mice compared with young mice (Nishioka *et al.*, 2006) and it has been postulated that this population might be responsible for the age-related decline in T cell-mediated immunity rather than to any quantitative or qualitative enhancement of the suppressive capacity of CD4⁺CD25⁺ nTreg (Nishioka *et al.*, 2006).

Regulatory activity has also been described for CD4⁺CD45RB^{low}CD25⁻ T cells in rodents (Hori *et al.*, 2003) and the corresponding CD4⁺CD45RO⁺CD25⁻ T cell subset in humans (Yagi *et al.*, 2004). The suppressive activity of CD4⁺CD25⁻ Treg cells is generally weaker than nTreg cells (Annacker *et al.*, 2001; Lehmann *et al.*, 2002; Stephens and Mason, 2000), although CD4⁺FoxP3⁺CD25⁻GITR^{high} T cells have been shown to have suppressive function in murine organ transplantation (Nishimura *et al.*, 2004) and inflammatory bowel disease (Uraushihara *et al.*, 2003). Furthermore, a role has been proposed for CD4⁺CD25⁻GITR^{high} cells in preventing autoimmune myocarditis in mice (Ono *et al.*, 2006). Further studies demonstrated that CD25⁻ Treg cells can also prevent colitis, myositis, hepatitis and glomerulonephritis upon transfer into NOD SCID mice, but not gastritis, thyroiditis, oophoritis, sialoadenitis, adrenalitis or insulinitis; all of which can be prevented by transfer of CD4⁺CD25⁺ cells (Alyanakian *et al.*, 2003). This finding is consistent with the suggestion of an organ-specific role for different subsets of Treg cells.

Further evidence that Treg cells are not confined to the CD4⁺CD25⁺ population comes from a study on heart transplantation (Degauque *et al.*, 2007). The transfer of CD3⁺CD25⁻ T cells from rats which had received a heart graft into naïve hosts could convey acceptance to a subsequent heart graft. In addition, CD3⁺CD25⁻ T cells from tolerant rats have high expression of FoxP3, IL-10 and IFN- γ mRNA in response to the graft when compared to control rats.

Studies in the rat lung have illustrated the downregulation of CD25 by adoptively transferred pre-activated CD3⁺CD25⁺ T cells (Strickland *et al.*, 1996b). This phenomenon has been attributed to “active inhibition” in the lung environment and may result from alveolar macrophage (AM)-derived inhibitory molecules such as nitric oxide (NO) (Holt *et al.*, 1993; Strickland *et al.*, 1993). Such organ-specific modulation of T cells might explain how different Treg cell subsets can modulate distinct autoimmune diseases (Alyanakian *et al.*, 2003; Ono *et al.*, 2006).

While there is a large body of evidence regarding suppressive function in CD4⁺FoxP3⁺CD25⁻ T cells, it is not clear whether these cells represent nTreg cells that have

somehow downregulated CD25 expression, or whether FoxP3 expression was induced in the periphery.

1.6 Mechanisms of suppression by Treg cells

It has been widely reported that nTreg cells exert their suppressive effects through direct cell-contact with target cell(s). Evidence supporting this theory has come from experiments using transwell inserts, where the Treg cells and Teff cells are separated by a permeable membrane. These studies have shown that Treg cells are unable to suppress responder cells when both populations are separated (Takahashi *et al.*, 1998; Thornton and Shevach, 1998). However, suppressive mechanisms requiring direct cell-contact between populations are still unclear.

FoxP3⁺ nTreg cells do not produce the T cell growth factor IL-2, a feature attributed to transcriptional repression by FoxP3 (Fontenot *et al.*, 2003; Ono *et al.*, 2007). However, nTreg cells still require IL-2 for survival (Maloy and Powrie, 2005). It has therefore been proposed that nTreg cells may suppress by depriving Teff cells of IL-2, thereby preventing their proliferation (Fig 1.3). A recent study reported that nTreg cells can induce cytokine deprivation-mediated apoptosis (Pandiyani *et al.*, 2007). However, this hypothesis has been compounded by conflicting data showing efficient suppression mediated by CD25^{-/-} (IL-2R α ^{-/-}) Treg cells (Fontenot *et al.*, 2005a), and experiments detailing a defect in IL-2 production by responder cells in culture with Treg cells (Duthoit *et al.*, 2005).

Other studies have illustrated that Treg cells can directly lyse target cells using a perforin/granzyme B-dependent mechanism (Fig 1.3) (Cao *et al.*, 2007; Gondek *et al.*, 2005; Zhao *et al.*, 2006). Additionally, it has been proposed that Treg cells may induce the release of adenosine nucleotides (Fig 1.3) (Bopp *et al.*, 2007; Deaglio *et al.*, 2007). Adenosine, generated by the degradation of adenosine nucleotides by the ectonucleotidases CD39 and CD73 on Treg cells, can suppress Teff cell function through its interaction with the A2A receptor on Teff cells (Deaglio *et al.*, 2007). CD39 converts ATP directly to AMP (Wang and Guidotti, 1996). Extracellular ATP is released from damaged cells as a result of trauma or cell death and it acts as a danger signal to alert

the immune system to tissue destruction (Borsellino *et al.*, 2007; Matzinger, 2002). Extracellular ATP predominantly induces proinflammatory responses (Borsellino *et al.*, 2007; Ferrari *et al.*, 1997; Idzko *et al.*, 2002; MacKenzie *et al.*, 2001). CD39 is expressed on a wide range of cell types including activated lymphocytes (Kansas *et al.*, 1991; Maliszewski *et al.*, 1994), vascular endothelial cells (Kansas *et al.*, 1991) and neurons (Wang and Guidotti, 1998; Wang *et al.*, 1997).

Notably, murine CD4⁺FoxP3⁺CD25⁺ Treg cells express CD39 (Borsellino *et al.*, 2007), and it has also been shown that FoxP3 induces CD39 expression (Borsellino *et al.*, 2007). CD39 appears to be a marker for human Treg cells, but unlike murine cells its expression is restricted to a population of FoxP3⁺ regulatory effector/memory-like T (Trem) cells (Borsellino *et al.*, 2007). It is believed that the removal of extracellular ATP by CD39 could enable Treg cells to enter inflamed tissues and suppress immune responses. Alternatively, CD39⁺ Treg cells could function to inhibit ATP-induced IL-1 β secretion by endothelial cells or monocytes (Imai *et al.*, 2000). Additionally, the generation of the suppressive molecule adenosine from extracellular ATP through CD39 and CD73 could further amplify these anti-inflammatory effects (Ferrari *et al.*, 1997).

Similarly to CD39, CD73 is expressed on a wide range of murine cells including lymphocytes (Thomson *et al.*, 1990), but it does not appear to have a role in human cells. CD73 catalyses the conversion of AMP to adenosine (Bours *et al.*, 2006), and may also play a role in cell adhesion (Airas *et al.*, 1995). Adenosine has potent anti-inflammatory properties, such as inhibition of neutrophil function and prevention of collateral tissue damage (Cronstein, 1994; Ohta and Sitkovsky, 2001; Sitkovsky and Ohta, 2005; Trevethick *et al.*, 2008).

Furthermore, Treg-derived cAMP, the inhibitory second messenger, was shown to enter Teff cells via gap junctions in their cell membrane (Bopp *et al.*, 2007). cAMP has a multitude of functions, including potent inhibition of T cell growth, differentiation and proliferation (Bodor *et al.*, 2001). cAMP can directly attenuate IL-2 production in CD4⁺ T cells by inducing ICER (Inducible cAMP Early Repressor) (Hoeffler *et al.*, 1988; Molina *et al.*, 1993).

The mechanisms described above all suggest that Treg cells have direct contact or close association with their target T cell(s), however other mechanisms have suggested that Treg cells may act on APC to suppress their maturation and/or function (Bluestone and Tang, 2005). It has been shown that Treg cells contact DC more frequently than Teff cells (Tang *et al.*, 2006). In this respect it has been hypothesised that Treg cells might manipulate the maturation and/or function of DC which is required for Teff cell activation (Fig 1.3) (Bluestone and Tang, 2005). Indeed, it has recently been reported that this direct Treg cell:DC interaction results in attenuation of Teff activation (Tadokoro *et al.*, 2006; Tang *et al.*, 2006).

CTLA-4, which is highly expressed on Treg cells, could be responsible for Treg cell suppression via DC. Data from anti-CTLA-4 blocking experiments and experiments using CTLA-4-deficient Treg cells have suggested a role for CTLA-4 in Treg cell-mediated suppression via DC (Oderup *et al.*, 2006; Serra *et al.*, 2003). Other studies have reported a CTLA-4-dependent increase in indoleamine 2,3-dioxygenase (IDO) in DC (Fallarino *et al.*, 2003). IDO is a potent regulatory molecule which catabolises tryptophan into pro-apoptotic metabolites. These metabolites can subsequently suppress Teff cells (Mellor and Munn, 2004) (Fig 1.3). A recent study has added weight to the argument that Treg cells mediated suppression via DC. The experiments showed that Treg cells can form aggregates on DC and can subsequently downregulate expression of CD80 and CD86, but not CD40 or MHC class II molecules (Onishi *et al.*, 2008). Formation of aggregates appears to be leukocyte function-associated antigen-1 (LFA-1) dependent, but the resulting downmodulation relies on both LFA-1 and CTLA-4 (Onishi *et al.*, 2008).

Lymphocyte activation gene 3 (LAG3) may also influence the suppressive effect of Treg cells on DC. LAG3 is induced upon CD4⁺ T cell activation, binds MHC class II molecules with high affinity and is required for optimal Treg cell function (Huang *et al.*, 2004; Workman *et al.*, 2004). The binding of LAG3 to MHC class II molecules on DC prevents DC maturation (Liang *et al.*, 2008).

The cytokines IL-10 and TGF- β_1 play a role in suppression by Tr1 and Th3 cells; the suppressive effects of Tr1 and Th3 cells can be reversed by anti-IL-10 or anti-TGF- β_1 antibodies, respectively (Asseman *et al.*, 1999; Powrie *et al.*, 1996). However, the relative contributions of IL-10 and TGF- β_1 to nTreg function is still unclear (Shevach, 2006). IL-10 is a key anti-inflammatory molecule which inhibits the production of the proinflammatory cytokines TNF- α and IL-12 by DC and macrophages (Hawrylowicz, 2005; Hawrylowicz and O'Garra, 2005; Moore *et al.*, 2001). However, there is conflicting evidence about its role in nTreg cell-mediated suppression. For example, adoptive transfer of allergen-specific Treg cells led to enhanced IL-10 production by CD4⁺ Teff cell upon allergen challenge (Kearley *et al.*, 2005). IL-10 was essential for the suppression of airway hyperreactivity and allergic inflammation. Interestingly, however, Treg cell-derived IL-10 was not crucial for this suppression, as demonstrated by the transfer of IL-10-deficient Treg cells (Kearley *et al.*, 2005). Conversely, IL-10 production by Treg cells is crucial for the prevention of colitis in inflammatory bowel disease (IBD) models (Asseman *et al.*, 1999).

With respect to TGF- β_1 , studies have indeed shown that it is required for suppression, in particular in the gut (Fig 1.3) (Fahlen *et al.*, 2005), however, it is likely that this cytokine is not secreted by nTreg but is instead generated locally by Th3 cells in the gut and could also be involved in inducing Treg cells in conjunction with RA (Benson *et al.*, 2007; Coombes *et al.*, 2007; Lucas *et al.*, 2000).

IL-35 is a more recently identified cytokine which has been shown to have a role in Treg cell function (Fig 1.3). It is a heterodimer comprised of IL-12p35 and Ebi3 subunits and is expressed by murine nTreg cells (Collison *et al.*, 2007; Gavin *et al.*, 2007). Treg from Ebi3- and IL-12p35-deficient mice have reduced regulatory activity *in vitro* and cannot prevent IBD *in vivo*. Additionally, ectopic expression of IL-35 conferred regulatory abilities on naïve T cells, and exogenous IL-35 could suppress T cell proliferation (Collison *et al.*, 2007).

The Treg cell suppressive mechanisms can be interpreted in different ways. The first is “hierarchical” – i.e. only one or two of the mechanisms are really crucial, with the others relevant only when Treg cells encounter a substantial inhibitory challenge (Vignali, 2008). The second is “contextual”, whereby the different mechanisms are more or less important depending on the context in which the Treg cell resides and the type of target it must suppress (Vignali, 2008). The contextual interpretation also takes into account the different compartments in which immune responses occur and different disease situations.

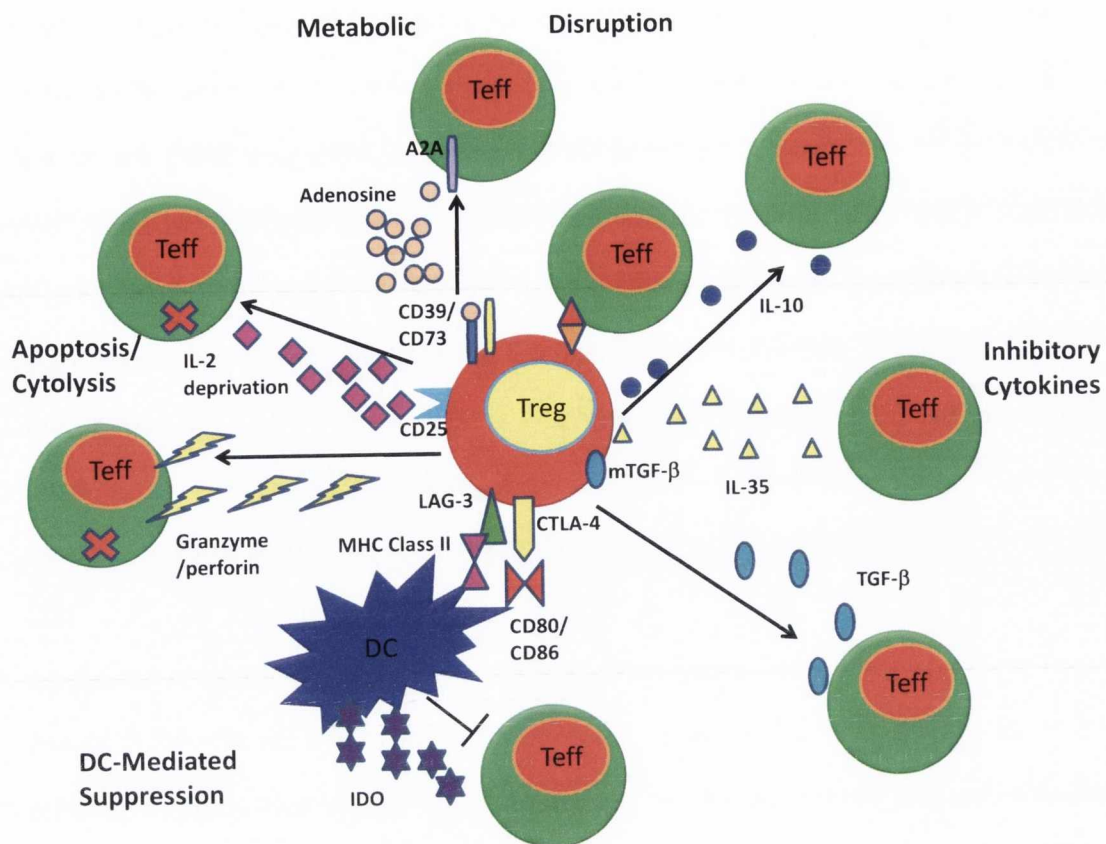


Figure 1.3 Mechanisms of Treg cell-mediated suppression

Adapted from Vignali (Vignali, 2008)

Several theories have been proposed by which Treg cells mediate suppression. These include metabolic disruption, the production of inhibitory cytokines, promotion of suppressive functions by DC, and apoptosis or cytolysis of target cells. Metabolic disruption can be mediated by the ectonucleotidases CD39 and CD73, expressed on the surface of Treg cells. These enzymes degrade the adenosine nucleotides ATP and AMP to adenosine. Adenosine can interact with the A2A receptor on Teff cells and exert anti-inflammatory effects.

The cytokines IL-10 and TGF- β_1 play a role in suppression by Tr1 and Th3 cells. IL-10 inhibits the production of the proinflammatory cytokines TNF- α and IL-12 by DC and macrophages. Studies of TGF- β_1 have shown that it is required for suppression, in particular in the gut. IL-35 may also have suppressive function, as ectopic expression of IL-35 can confer regulatory abilities on naïve T cells, and exogenous IL-35 can suppress T cell proliferation.

It is possible that Treg cells can manipulate the maturation and/or function of DC and affect Teff cell activation. Treg cells express high levels of CTLA-4, which can inhibit the function of DC and prevent optimal Teff activation. CTLA-4 signalling might also cause and increase in the regulatory molecule indoleamine 2,3-dioxygenase (IDO) in DC. Furthermore, it has been demonstrated that Treg cells can form aggregates on DC, leading to the downregulated expression of CD80 and CD86, but not CD40 or MHC class II molecules. Lymphocyte activation gene 3 (LAG3) may also influence the suppressive effect of Treg cells on DC. LAG3 is induced upon CD4⁺ T cell activation, binds MHC class II molecules with high affinity and is required for optimal Treg cell function (Huang *et al.*, 2004; Workman *et al.*, 2004). The binding of LAG3 to MHC class II molecules on DC prevents DC maturation.

With respect to the apoptotic and cytolytic effects of Treg cells, it has been reported that Treg cells can deprive Teff cells of IL-2, causing apoptosis of Teff cells. Additionally, they might cause direct cytolysis of target cells by a perforin/granzyme B-dependent mechanism.

1.7 Lung

The respiratory tract requires T cell-mediated immunity to protect the lung from inhaled pathogens and foreign environmental antigens. However this must be strictly controlled to prevent tissue damage as a consequence of chronic inflammation, for a number of reasons; Firstly, the epithelium at the blood/air interface is very delicate and secondly, the lung is constantly exposed to the external environment and therefore a high frequency of environmental antigens are inhaled, most of which are innocuous and should not induce an immune response (Strickland *et al.*, 1996b). The maintenance of immunological homeostasis in the lung and accordingly, the integrity of the respiratory surfaces is a constant challenge to the immune system. A delicate balance must be struck to discriminate between harmless antigens and much rarer pathogen-derived antigens. Excessive or inappropriate T cell responses in normal lungs are associated with the pathogenesis of a number of respiratory diseases, including sarcoidosis, bronchitis and asthma (Pinkston *et al.*, 1983; Poulter *et al.*, 1994; Reynolds, 1987).

The lung is an important site for accumulation of lymphocytes, and the number of T cells localised within the lung at any time has been estimated to exceed the numbers in peripheral blood. However, most of these cells are only temporarily sequestered in this organ as they transit to lymph nodes (Pabst *et al.*, 1987). It is believed that the unique physiological structure of the peripheral lung vascular bed facilitates this sequestration, as the vessels are ultrafine, there is low net perfusion pressure and the vessels are repeatedly flattened during the respiratory cycle (Strickland *et al.*, 1996b). This maximises physical contact between circulating lymphocytes and the endothelial cells of blood vessels, thus increasing the potential of cell-cell contact (Strickland *et al.*, 1996b).

The lungs comprise two functionally distinct compartments: the conducting airways which are overlayed by mucosal tissue and the lung parenchyma, which consist of alveoli, specialised thin-walled sites of gas exchange (Holt *et al.*, 2008).

1.7.1 Conducting airways

Ciliated cells and secretory goblet cells make up the respiratory epithelium of the airways. In conjunction with local IgA secretion, these cells facilitate clearance of inhaled antigens (Holt *et al.*, 2008). The airway mucosa is enriched with DC and macrophages (Nelson *et al.*, 1994). The DC pool is dominated by myeloid DC but also contains plasmacytoid DC (Jahnsen *et al.*, 2001). Airway mucosal DC (AMDC) which are resident in the airway mucosa are specialised for uptake of antigen due to their strategic position within and directly beneath the surface epithelium (Stumbles *et al.*, 1998). They can also extend protrusions into the airway lumen, indicating they sample antigen directly from the airway luminal surfaces through intact epithelium (Jahnsen *et al.*, 2006). However they are unable to efficiently present antigen as they are functionally immature, MHC class II^{low}, and preferentially stimulate Th2 immunity as a default. However, when AMDC are exposed to cytokine signals such as GM-CSF, they can induce Th1 immunity (Stumbles *et al.*, 1998).

1.7.2 Lung Parenchyma

Immune cells in lung parenchyma are found above the alveolar epithelium in the terminal airways and also in the underlying parenchyma (Holt *et al.*, 2008). In a normal lung, the dominant immune cell (>90%) in the alveolar space is the alveolar macrophage (AM) (Holt *et al.*, 2008). T cells are sequestered in the vascular bed of the lung parenchyma (Holt *et al.*, 2008).

It is believed that primary immune responses to inhaled antigens are initiated in the draining lymph nodes of the lung and that memory T cells subsequently localise to lung tissue where they can mediate secondary immune responses to their cognate antigens (Bice and Muggenburg, 1988).

T cells from human lungs are generally of the memory/activated phenotype and activation of a number of these cells appears to be a recent event (Marathias *et al.*, 1991). For example the lung T cells show increased levels of human leukocyte antigen (HLA)-DR and reduced CD3 expression compared with T cells derived from peripheral blood (Becker *et al.*, 1990; Nylen *et al.*,

1990; Yamaguchi *et al.*, 1990). Additionally, both CD4⁺ and CD8⁺ T cells from human lung express the memory marker CD45RO. CD4⁺ and CD8⁺ T cells are found in distinct locations in the lung – there is a high frequency of CD4⁺ T cells in the lamina propria, whereas CD8⁺ cells are intraepithelial (Holt *et al.*, 2008).

Human and rat studies have shown that T cells from lung interstitium and bronchoalveolar lavage (BAL) differ from T cells in blood, showing reduced cloning efficiency and cytokine production (Garlepp *et al.*, 1992; Holt *et al.*, 1988; Nelson *et al.*, 1990). However, lung T cells produce high levels of IFN- γ (Holt *et al.*, 1988). The high frequency of memory cells in the lung has been proposed to account for the poor proliferation of lung T cells in response to mitogens in culture and their poor cloning efficiency, as memory T cells do not respond as readily as naïve cells to these stimuli (Becker *et al.*, 1990).

The lung environment itself also affects the function of T cells. It has been suggested that local T cells in the lung are maintained in a state of “partial hyporesponsiveness”, and the lung can employ various immunoregulatory mechanisms to accomplish this. These mechanisms include components of the surfactant lining (Wilsher *et al.*, 1988), alveolar macrophages (Strickland *et al.*, 1996a) and secreted products of type II alveolar epithelial cells (AECIIs) (Paine *et al.*, 1992). Further support for an “active inhibition” model comes from a study of surface phenotypic markers on lung T cells in the rat (Strickland *et al.*, 1996b). While T cells displayed an activated, memory phenotype, they were found to be “locked in” to the G₀/G₁ resting phase of the cell cycle as opposed to actively cycling G₂-S/M as suggested by both the surface marker expression and the observation that they have reduced calcium flux in response to calcium ionophore stimulation (Strickland *et al.*, 1996b). Additionally, transfer of pre-activated T cells into congenic rats resulted in downregulation of CD25 (Strickland *et al.*, 1996b). The data indicates a mechanism of active inhibition in the lung environment which prevents the cells from progressing normally through the cell cycle into cell division. Of note, several of these observations in lung T cells have also been reported for gut tissue-derived T cells (Jarry *et al.*, 1990), suggesting mucosal organ-specific functions.

1.7.3 Bronchial-Associated Lymphoid Tissue

The airway mucosa contains potential inductive sites called bronchial-associated lymphoid tissue (BALT), composed of discrete organised lymphoid cell aggregates which underlie a specialised epithelium (Holt *et al.*, 2008). This epithelium is comprised of specialised microfold or M cells, which can take up antigen, and follicle-associated epithelium (FAE) (Bienenstock and McDermott, 2005). BALT is distinct from secondary lymphoid tissues, because afferent lymphatics do not transport to it (Bienenstock and McDermott, 2005). Instead antigen enters directly via M cells, and post-capillary high endothelial venules (HEVs) allow lymphocyte migration into BALT (Bienenstock and McDermott, 2005). Lymphocytes in BALT form a follicle, which consists of a large central dome of B cells that is surrounded by a smaller number of T cells (Murphy *et al.*, 2008). Lymphocytes can bind to vascular cellular adhesion molecule-1 (VCAM-1) and peripheral node addressin (PNAd) on HEVs via L-selectin (CD62L) to gain access to BALT.

BALT is common in young children but its role in adults is unclear, and its existence differs between species (Pabst and Tschernig, 1995). Notable findings have shown that inducible BALT in mice, which lack other organised lymphoid tissue, can protect against pathogens (Moyron-Quiroz *et al.*, 2004). This suggests BALT may be important with respect to respiratory immune homeostasis early in life when central lymphoid structures are functionally immature.

In humans, it seems there is a direct correlation between the amount of BALT and the extent of antigenic stimulation (Delventhal *et al.*, 1992; Meuwissen and Hussain, 1982; Pabst and Gehrke, 1990). This suggests that BALT is the result of an adaptation in response to the degree of antigen load and this is compliant with findings in the GALT (Bienenstock and McDermott, 2005).

BALT is part of a common mucosal immune system, as demonstrated by the migration of cells from GALT to other mucosal sites via the bronchus (Scicchitano *et al.*, 1984). Additionally, it has been shown that lymphocytes from GALT can populate the bronchial mucosa and vice versa (Rudzik *et al.*, 1975). The fact that cells can be sensitised at one mucosal site and subsequently

provide primed cells selectively to other mucosal sites, has facilitated to the development of an oral vaccine against acute bronchitis (Clancy *et al.*, 1985).

1.7.4 Immune cell dynamics in the lung

A non-inflammatory Th2 response is the default mechanism in the lung in response to ubiquitous environmental stimuli (Stumbles *et al.*, 1998). Furthermore, it is believed that T cell-mediated immunological tolerance also contributes to sustaining an immunosuppressive environment in the lung, similar to gastric mucosa (Umetsu and DeKruyff, 2006; Weiner *et al.*, 1994). DC appear to dictate this local anti-inflammatory state (de Heer *et al.*, 2004; Strickland *et al.*, 2006). Unwelcome memory T cell responses, which are not controlled by DC, can be regulated by alternative mechanisms including AM (Bilyk and Holt, 1993; Eriksson *et al.*, 2005; Gereke *et al.*, 2009; Lambrecht, 2006; Thepen *et al.*, 1989).

Pathogen sensing is facilitated by PRRs, such as TLRs. These highly conserved components of the innate immune system are expressed by AMDC in the lung. Their engagement circumvents the lung's Th2-dominant phenotype and results in effective Th1 cell-mediated immunity along with the generation of memory T cells against harmful pathogens (Holt *et al.*, 2008). Innocuous inhaled antigens can also trigger PRRs, but it is thought that they can be distinguished qualitatively and quantitatively from pathogen-derived immunomodulatory molecules, which induce a much stronger immune response by virtue of engaging multiple types of PRR and being present at a high enough frequency to induce an appropriate immune response (Eisenbarth *et al.*, 2002).

1.7.5 Alveolar Macrophages

Macrophages are specialised haematopoietic cells which are distributed throughout different tissues of the body. They have diverse functions, including homeostatic tissue remodelling and host defence (Lambrecht, 2006). Macrophage function can be further modified by their particular anatomical location, the environmental factors they encounter there and the stimuli they are exposed to, in order to function most appropriately and effectively (Lambrecht, 2006).

The lung is an obvious gateway for many pathogens to enter the body, and it is also exposed to other environmental challenges, such as toxic gases and particulate matter in the air (Lambrecht, 2006). The main physiological function of the lung is gas exchange, which occurs in the alveoli. Alveoli are comprised of thin type I AECs (AECIs) and thicker AECIIs. To allow gases to diffuse easily, the capillaries of the lung are in close proximity to AECIs. They are only separated by a very thin (0.2 μ M) basement membrane. Inhaled pathogens and toxic substances can easily gain access to the bloodstream via this delicate alveolar-capillary membrane. As a result, there must be strong defences in place at this interface, but at the same time such defence mechanisms need to be tightly controlled, because excessive oedema, inflammation and cellular recruitment would lead to the thickening of the alveolar wall, which would interfere with gas exchange (Lambrecht, 2006).

AM are the most common immune cell in the airways of the lower respiratory tract – they comprise greater than 90% of the immune cells in a normal lung (Holt *et al.*, 2008). AM are highly phagocytic (Thepen *et al.*, 1993), but they are normally quiescent, to prevent damage to the alveolar wall (Lambrecht, 2006). Their quiescent state is characterised by low levels of inflammatory cytokine production and reduced migration to draining lymph nodes following the uptake of nasally administered particulate antigen (Jakubzick *et al.*, 2006). They are also poorly phagocytic when quiescent, and downregulate the phagocytic receptor CD11b (Holt, 1978).

Specific and nonspecific immune defence mechanisms are utilised by the lung. Larger particles can be removed from the upper respiratory tract by coughing and sneezing, as well as the secretion of mucus. In the lower respiratory tract, where AM are located, innate immunity is well developed. AM express numerous PRRs for foreign PAMPs. Nonspecific humoral immunity in the lower respiratory tract involves lactoferrins, lysozyme, surfactant proteins, mannose binding lectin and defensins (Lambrecht, 2006).

If such non-specific innate mechanisms fail, the adaptive immune response is instigated by epithelial and alveolar DC (Lambrecht, 2006). Nonetheless, AM are extremely efficient in

phagocytosing large numbers of pathogens and it has been estimated that they can internalise up to 1×10^9 intratracheally (i.t.) inhaled bacteria before there is a “spillover” of bacteria to DC and the induction of adaptive immunity (MacLean *et al.*, 1996).

T cells in the lungs bear little phenotypic or functional resemblance to those in blood. They are a heterogeneous population, at various postactivation stages (Strickland *et al.*, 1996b). T cells from lung interstitium and bronchoalveolar lavage fluid (BALF) show strikingly reduced cloning efficiency and a predisposition for IFN- γ production in comparison to peripheral blood T cells (Garlepp *et al.*, 1992; Holt *et al.*, 1988; Nelson *et al.*, 1990). Due to the large numbers of T cells, and the high frequency of inhaled antigen from the environment, the overall level of steady-state T cell responses must be strictly controlled to prevent tissue damage and interference with gas exchange (Strickland *et al.*, 1996a).

AM actively suppress the induction of adaptive immune responses by exerting regulatory effects on alveolar and interstitial DC and T cells. Their robust suppression of adaptive immunity is illustrated by studies which eliminated AM *in vivo*. Elimination of AM in mice is achieved using a cytotoxic drug – dichloromethylene diphosphate (Cl_2MDP). It is targeted to AM by encapsulation in liposomes and administered i.t. into the lung, and completely removes AM but not interstitial macrophages or other immune cells. The elimination of AM resulted in overwhelming inflammation to otherwise innocuous particulate and soluble antigen (Strickland *et al.*, 1993; Thepen *et al.*, 1992; Thepen *et al.*, 1991; Thepen *et al.*, 1989), and it seems to be T cell-dependent (Strickland *et al.*, 1993; Thepen *et al.*, 1991). Studies across several species have demonstrated that AM can suppress the *in vitro* proliferation of T cells in response to mitogenic stimuli (Holt, 1993). The downregulation of T cell responses in peripheral tissues such as the lung is believed to contribute to peripheral tolerance to exogenous antigen and the control of self-reactive lymphocytes (Schwartz, 1989).

AM can suppress T cell proliferation in response to the mitogen Concanavilin A (ConA) *in vitro*, and they exhibited even stronger suppression of antigen-specific responses to Ova protein

(Strickland *et al.*, 1996a). Of note, the suppression of T cell proliferation could only be achieved if AM were added to the T cell culture within 24 hours of activation (Strickland *et al.*, 1996a). Furthermore, AM-mediated suppression of T cell proliferation is reversible, as the removal of AM from ConA- or Ova-activated cultures after 24 hours resulted in increased T cell proliferation despite the absence of additional stimulation (Strickland *et al.*, 1996a; Strickland *et al.*, 1996b).

It appears that AM interfere with T cell progression through the cell cycle. T cells stimulated with ConA progressed to S/G₂M phases, while those stimulated with ConA in the presence of AM did not. They remained in G₀/G₁ phases, similar to unstimulated T cells and also T cells derived from the lung (Strickland *et al.*, 1996b). The removal of AM from culture after 24 hours allowed some progression to S phase (Strickland *et al.*, 1996a). It seems that suppression of T cell responses is only restricted to proliferation, which is the final step in the activation process (Upham *et al.*, 1995). S phase begins after 24 hours of T cells becoming activated, which might explain why AM must be present within a narrow 24 hour window to exert suppression, and why their effect is reversible.

Earlier activation events including CD3 downmodulation, IL-2 secretion and expression of the IL-2R proceed normally, however (Strickland *et al.*, 1996a; Upham *et al.*, 1995). Addition of exogenous IL-2 to AM/T cell cultures had no impact on T cell proliferation (Upham *et al.*, 1995). This finding distinguishes these T cells from “classically anergic” T cells, whose hyporesponsiveness is associated with the failure to produce IL-2, and which is reversible upon the addition of IL-2 to culture (Schwartz, 1989). Loss of CD28 mRNA has been linked to induction of classical T cell anergy (Lake *et al.*, 1993), but the T cells rendered hyporesponsive by AM have normal CD28 expression (Strickland *et al.*, 1996a; Upham *et al.*, 1995). Interestingly, while *ex vivo* analysis has shown that lung T cells are “locked” into G₀/G₁ phase like those cultured with AM *in vitro*, lung T cells had lower IL-2R expression (Strickland *et al.*, 1996b). This might reflect the effect of more prolonged exposure to AM, as occurs *in vivo*, or additional factors that were not reproducible *in vitro*. Further evidence for this *in vivo* phenomenon is the observation that

postactivated T cells lose IL-2R expression, but not other markers, when they traffic to the lung after adoptive transfer (Strickland *et al.*, 1996b).

The fact that only the final stage of T cell activation is reversibly inhibited by AM suggests a subtle and sophisticated mechanism to constrain local tissue damage *in vivo*, yet allows the “suppressed” T cells to retain their proliferative ability should they migrate from the lung, for instance to the draining lymph nodes. Essentially the capacity to generate T cell memory against commonly encountered lung antigens is not abolished, but tight control of potentially damaging T cell immunity is preserved at the original site of antigen exposure (Strickland *et al.*, 1996a).

Across different species, AM efficiently suppress T cell responses, but it would appear that while they all prevent T cell proliferation, AM from rodents and humans use different mechanism to achieve this (Upham *et al.*, 1995). AM supernatants from several species can suppress T cell proliferation, indicating a role for a diffusible factor (Pennline *et al.*, 1979; Pennline and Herscowitz, 1981; Strickland *et al.*, 1996a). Indeed, it has been proposed that rat and mouse AM use the nitric oxide synthase (NOS) pathway to regulate T cell proliferation, but human AM suppression does not involve NOS activity (Upham *et al.*, 1995). Rodent macrophages use the NOS pathway to kill microorganisms, but human macrophages can kill the same microorganisms via NO-independent mechanisms (Bermudez, 1993; Cameron *et al.*, 1990; Schneemann *et al.*, 1993).

It has been proposed that human AM might use direct cell-cell contact as a mechanism of T cell suppression, based on experiments in which AM supernatant transfer did not inhibit T cell proliferation (Rich *et al.*, 1991). Nonetheless, given that human AM can strongly suppress antigen-specific T cell proliferation at ratios of 0.05:1 AM:T cells (Upham *et al.*, 1995), cell contact is unlikely to be the only mechanism.

AM adhere closely to AECs at the alveolar wall (Lambrecht, 2006). It is believed that the integrin $\alpha\text{v}\beta 6$ exerts control over AM activation. $\alpha\text{v}\beta 6$ binds to the latency associated peptide (LAP), an inactivating fragment of TGF- β_1 and activates latent TGF- β_1 (Morris *et al.*, 2003). AM

in $\alpha\text{v}\beta 6$ -deficient mice are constitutively activated, presumably due to the absence of active TGF- β_1 , and its associated immunosuppressive effects (Morris *et al.*, 2003). It has also been reported that the $\alpha\text{v}\beta 6$ /TGF- β_1 axis can influence immune homeostasis in the lung (Takabayshi *et al.*, 2006). Under steady-state conditions, AM closely adhere to AECs, which induces the expression of $\alpha\text{v}\beta 6$ on AECs, in a TGF- β_1 -dependent manner. This leads to localised TGF- β_1 activation in the immediate AM environment. Binding of TGF- β_1 to its receptors on AM induces phosphorylation of SMAD-2 and SMAD-3, which suppresses AM phagocytosis and cytokine production. This mechanism appears to be unique to the lung (Takabayshi *et al.*, 2006).

Infectious agents are able to overcome this robust inhibition of AM function. For example, when TLRs are triggered on AM, they lose contact with AECs. Consequently, $\alpha\text{v}\beta 6$ expression is downregulated in AECs. Accordingly, TGF- β_1 is no longer activated and so cannot exert an inhibitory effect on AM. AM become primed to secrete TNF- α and IL-6 and they can phagocytose particulate matter. Once AM are activated they can eliminate the infectious agent on their own (Takabayshi *et al.*, 2006).

Many pathogens also induce the recruitment of CCR2⁺ inflammatory monocytes to the alveolar space (Warmington *et al.*, 1999). Such cells are proinflammatory and promote T cell and DC activation, and they do not acquire the suppressive phenotype of AM for a few days. Therefore this “lag” allows for the initiation of innate and adaptive immune responses in the lung (Bilyk and Holt, 1995).

Ultimately, collateral damage to the lung tissue must be avoided and gas exchange needs to be restored as quickly as possible. Therefore AM activation must be constrained and there is an efficient and rapid mechanism to achieve this. IFN- γ -producing lymphocytes stimulate the production of matrix metalloproteinase 9 (MMP-9) by AM (Takabayshi *et al.*, 2006). MMP-9 can activate latent TGF- β_1 , and subsequently inhibit AM function. AM re-adhere to AECs and the expression of $\alpha\text{v}\beta 6$ by AECs is restored (Takabayshi *et al.*, 2006). A further advantage of

enhanced TGF- β_1 production in this situation would be its stimulation of collagen synthesis in interstitial fibroblasts, which is crucial for the repair of alveolar wall structure (Lambrecht, 2006).

These findings have wider implications for lung fibrosis, a condition that can occur after severe lung infections but also as an idiopathic disease (Gross and Hunninghake, 2001). Fibrosis results in a loss of AECIs along with hyperplasia of AECIIs. Despite enhanced levels of activated TGF- β_1 , AM are persistently activated and produce excessive TNF- α , IL-1 β and IL-6 (Lambrecht, 2006). It is likely that the loss of AECIs results in a loss of anchoring points for AM and a loss of $\alpha v \beta 6$. In this way, AM miss an important feedback mechanism and they continuously produce toxic products that can cause tissue damage.

1.7.6 Lymphocyte homing to lungs

The lung is a unique organ in the context that it can recruit cells from two circulatory systems – the systemic circulation via the bronchial arteries and the pulmonary circulation with circulates the lung parenchyma (Holt *et al.*, 2008). Lung-homing T cells have a distinct phenotype from gut-homing T cells, as they express the chemokine receptors CCR5 and CXCR3, but not CCR9 (Campbell *et al.*, 2001). Lung homing T cells can therefore bind the chemokines CCL3, CCL4, CCL5, CXCL9, CXCL10 and CXCL11. However, production of these chemokines is not exclusive to the lung. No lung-specific chemokine has been identified, although disease-specific profiles of chemokine receptors have been defined on T cells from patients with various pulmonary disorders (Medoff *et al.*, 2005).

1.7.7 Treg cells in the lung

Treg cells have been shown to be beneficial in lung disease by controlling lung injury and local inflammation. A study of mice infected with the fungus *Pneumocystis* demonstrated the crucial role for CD4⁺FoxP3⁺CD25⁺ nTreg cells, as animals which were depleted of CD25⁺ Treg cells had exacerbated Th2 responses (increased levels of IL-4, IL-5 and IL-13) as well as enhanced lung injury (McKinley *et al.*, 2006). Of note, *Pneumocystis*-infected SCID mice reconstituted with CD4⁺CD25⁻ T cells showed enhanced clearance of the pathogen but developed even worse lung

injury and significantly increased levels of proinflammatory Th1 and Th2 cytokines and chemokines were detected in their lungs (McKinley *et al.*, 2006). However, *Pneumocystis*-infected SCID mice which were reconstituted with CD4⁺CD25⁺ T cells had similar levels of lung inflammation and damage as non-reconstituted control mice although they had a higher fungal burden (McKinley *et al.*, 2006). This study corroborates widely held hypotheses about Treg cells in that they impair or delay efficient clearance of pathogens but actually benefit the host by preventing collateral damage to host tissue. This is especially important for the lung which is composed of delicate tissue yet is at the environmental interface of the body and so encounters high frequencies of antigen.

In this regard, Treg cells also play a role in controlling allergy. Depletion of CD4⁺CD25⁺ Treg cells prior to allergen challenge results in increased eosinophil recruitment to the lungs and a strong T cell response to the allergen (Boudousquie *et al.*, 2009). Interestingly, both CD4⁺CD25⁺ and CD4⁺CD25⁻ lung-derived T cells have been shown to suppress allergic T cell responses (Boudousquie *et al.*, 2009).

A novel mechanism of lung-specific immunosuppression involving AECIIs has been proposed (Gereke *et al.*, 2009). AECIIs can present MHC-restricted self antigen to CD4⁺ T cells. Self reactive T cells which recognise endogenous antigen become activated but acquire FoxP3, converting to Treg cells (Gereke *et al.*, 2009). The release of soluble anti-inflammatory factors, such as surfactant proteins and TGF-β₁ by AECIIs has been attributed to this induction of FoxP3⁺ CD4⁺ T cells (Gereke *et al.*, 2009). Treg cells have also been associated with prevention of fibrosis. Idiopathic pulmonary fibrosis (IPF) patients have fewer Treg cells in blood and in the lung than controls (Kotsianidis *et al.*, 2009). Furthermore, Treg cells from IPF patients are also functionally deficient, as shown by defective antiproliferative activity and impaired suppression of Th1 and Th2 cytokines by responder T cells (Kotsianidis *et al.*, 2009).

IL-10 is essential for Treg cell-mediated airway immunosuppression, as demonstrated by a study involving mutant IL-10-deficient mice, which had enhanced lung inflammation in response

to antigen challenge, as measured by enhanced eosinophil and total cell infiltrate, increased levels of mucus and airway oedema and higher percentages of goblet cells than controls (Rubtsov *et al.*, 2008). IL-10 has also been shown to have a functional role in preventing rejection of transplanted lungs. Autoimmune responses against the self protein collagen V are associated with decreased levels of Treg cells and lung rejection in transplant recipients (Haque *et al.*, 2002). A novel subset of collagen V-specific IL-10-secreting T cells has been reported in humans. They are induced by CD4⁺CD25⁺ Treg cells and can suppress collagen V-specific Th1 cells, thus facilitating graft survival (Bharat *et al.*, 2006).

A wide variety of mechanisms have been proposed to explain how Treg cells function (Section 1.6). Regardless of specific mechanisms utilised, Treg cells must be in close proximity to their targets and therefore must localise with these targets. The migration of Treg cells, in particular the role of chemokines and chemokine receptors, is therefore the focus of much research. It has been shown that CCR4⁺ Treg cells are enriched in the lung and other nonlymphoid tissues (Sather *et al.*, 2007). Interestingly, CCR4-deficiency results in significantly impaired Treg cell accumulation in the lungs and skin with increased pulmonary inflammation and lymphocytic infiltration (Sather *et al.*, 2007). This data implies that recruitment of immune cells to the lung and other nonlymphoid organs is strictly controlled and that specific factors allow cells to home to distinct compartments. Such mechanisms of migration can be manipulated to benefit the host, for example in graft-versus-host disease (GVHD). In a murine model where GVHD was induced, high levels of the chemokines CXCL9, CXCL10 and CXCL11 were detected in liver, lungs and intestine (Hasegawa *et al.*, 2008). CXCR3 is the receptor for these chemokines, and mice which received nTreg cells that had been transfected with CXCR3 showed enhanced inhibition of GVHD-associated changes in lung, liver and intestine, compared with mice that received either non-transfected or nTreg cells (Hasegawa *et al.*, 2008).

1.8 Tuberculosis

Tuberculosis (TB) is a bacterial disease caused by *Mycobacterium tuberculosis*. *M. tuberculosis* is the single biggest bacterial cause of death globally (Raja, 2004; WHO, 1999). *M.*

tuberculosis has persisted for centuries, and is a global health emergency despite the development of chemotherapy and preventative vaccines (Tufariello *et al.*, 2003). TB is a communicable disease, transmitted mainly by the respiratory route (Flynn and Chan, 2001; Lee *et al.*, 2009). After infection, it can spread to most organs of the body (Raja, 2004). Initial infection is known as primary TB. Reactivation TB occurs when latent TB progresses to cause disease after an interval.

Patients whose lungs are the source of *M. tuberculosis* infection are often infectious due to the ease of transmission by coughing and the creation of infectious aerosols (Ahmad, 2010). However, infection with *M. tuberculosis* leads to active disease in only 10% of individuals (Styblo, 1980), so anti-TB immunity is sufficient to prevent active disease in most cases. In the vast majority of infected individuals, the immune response only succeeds in containing the infection rather than eradicating it completely (Flynn and Chan, 2001). This is because *M. tuberculosis* has evolved strategies to subvert the microbicidal effects of immune cells (Ahmad, 2010). Consequentially, the pathogen remains in a nonreplicating, dormant or latent state in the host. This is known as latent tuberculosis infection (LTBI). The dormant bacteria can resuscitate and cause reactivation TB to develop if the immune response is somehow disrupted, for example in the case of co-infection with human immunodeficiency virus (HIV) (Ahmad, 2010; Chan and Flynn, 2004).

Individuals with LTBI are non-infectious because the bacilli are contained, and they are asymptomatic (Tufariello *et al.*, 2003). The risk of latently infected individuals developing active TB in their lifetime is 5%-10% (Dye *et al.*, 1999). These different manifestations of disease reflect the balance of the bacillus and host mechanisms, and the outcome reflects the quality of the host immune response (van Crevel *et al.*, 2002).

The World Health Organisation estimates that 1/3 of the world's population is latently infected with *M. tuberculosis* (WHO, 2009; WHO, 2010). TB was declared a global health emergency by the WHO in 1993, and part of this resurgence is associated with the AIDS epidemic (De Cock and Chaisson, 1999) and the increasing resistance of *M. tuberculosis* strains to the most effective first-line anti-TB drugs (Pablos-Mendez *et al.*, 1998; Snider and Castro, 1998). TB is

preventable and curable in most instances, but these measures are confounded by demographic and socioeconomic factors such as population expansion, poor clinical detection and cure rates in developing countries, active transmission of infection in overcrowded hospitals, prisons and other public places, migration of infected individuals from high-incidence countries, drug abuse, social decay and homelessness (Ahmad, 2010; Tufariello *et al.*, 2003).

1.8.1 Transmission of *M. tuberculosis*

M. tuberculosis is transmitted when infectious droplet nuclei are inhaled. They are present in the air when expectorated by patients with active TB. The primary route of infection is via the lungs, because the tiny droplet nuclei can avoid nonspecific bronchial defences due to their small size and they ultimately diffuse to the lower airways. AM and DC can phagocytose the bacilli-containing droplets and initiate the innate immune response, which is coordinated by the signalling of PAMPs through PRRs (Akira *et al.*, 2006). AM provide an intracellular niche that is crucial for *M. tuberculosis* to establish infection in the host, and they are also the main effector cell for its killing (Lee *et al.*, 2009).

Once *M. tuberculosis* has been internalised, it replicates within the host cell. Infected cells can cross the thin alveolar wall to enter the bloodstream and cause systemic dissemination (Bermudez *et al.*, 2002; Teitelbaum *et al.*, 1999). Both the intracellular replication and dissemination of *M. tuberculosis* occur prior to the generation of adaptive immune responses against the pathogen (Chackerian *et al.*, 2002). Once they have phagocytosed *M. tuberculosis*, AM produce inflammatory cytokines and chemokines to recruit monocytes, neutrophils and lymphocytes to the site of infection (Ahmad, 2010).

Furthermore, *M. tuberculosis* has evolved ways to dampen the innate immune response and promote its survival. For example, intracellular bacilli can resist killing by AM by preventing the fusion of phagosomes with lysosomes. Lysosomes are acidic environments and cause death of the bacilli, but *M. tuberculosis* can prevent phagolysosomal fusion, and avoid death. They multiply in the phagosome and cause necrosis of the host AM (Chen *et al.*, 2006). Necrosis allows *M.*

tuberculosis to escape and multiply extracellularly, and they can be phagocytosed by other AM, which similarly fail to control bacterial growth and die by necrosis.

1.8.2 Granuloma

DC which have phagocytosed *M. tuberculosis* mature and migrate to the regional lymph nodes where they prime antigen-specific CD4⁺ and CD8⁺ T cells (Bodnar *et al.*, 2001). In most infected individuals, cell-mediated immunity develops 2-8 weeks after initial infection and it prevents further replication of the bacilli in host cells (Ahmad, 2010). Primed T cells migrate to the site of infection. The accumulation of macrophages, T cells, DC and other cells such as fibroblasts and endothelial cells facilitates the formation of a granuloma at the site of infection (Gonzalez-Juarrero *et al.*, 2001).

The granuloma sequesters the infected cells and tissue and it serves to limit the replication and spread of *M. tuberculosis* (Ahmad, 2010). The granulomatous environment has low O₂ levels which limits the aerobic respiration of the bacteria (Wayne and Sohaskey, 2001; Zahrt, 2003). Importantly, the granuloma shields the surrounding lung tissue from chronic inflammation, therefore limiting the immunopathological damage caused by the infection and the subsequent aggressive immune response (Co *et al.*, 2004b). Non-infected macrophages comprise the majority of cells in a granuloma, and they include activated epitheloid macrophages and multinucleated giant cells which result from the fusion of macrophages (Ma *et al.*, 2003).

LTBI is a result of this containment of the pathogen within a granuloma in the infected lungs. Ultimately the host is able to contain but not eradicate the pathogen, even though most of the *M. tuberculosis* bacilli are killed. This is the hallmark of *M. tuberculosis* infection in humans. *M. tuberculosis* has evolved effective immune evasion strategies which results in the survival and persistence of some bacilli in a dormant, nonreplicating state in the host (Frieden *et al.*, 2003; Hingley-Wilson *et al.*, 2003; Tufariello *et al.*, 2003). The granuloma microenvironment is hypoxic, with a low pH. Carbon monoxide and nitric oxide are also present, and these extreme and stressful conditions increase the expression of several dormancy genes by *M. tuberculosis* (Rustad *et al.*,

2009; Shiloh and DiGiuseppe Champion, 2010; Ulrichs and Kaufmann, 2006). Dormant bacilli can survive in the granuloma during the lifetime of the host, as evidenced from the detection of *M. tuberculosis* DNA in the lung tissue of individuals who died from other diseases and who did not exhibit any pathological sign of TB disease (Hernandez-Pando *et al.*, 2000). The dormant bacilli can resuscitate and cause active disease in the event of local immunosuppression (Ahmad, 2010).

1.8.3 Immune cells and cytokines in *M. tuberculosis* infection

The microenvironment of the granuloma promotes interactions among macrophages and other immune cells and the cytokines they produce (Ahmad, 2010). Of particular importance is IFN- γ , which is produced mainly by CD4⁺ T cells, CD8⁺ T cells and natural killer (NK) cells. Humans and mice defective in IFN- γ or IFN- γ R genes are more susceptible to *M. tuberculosis* infection (Chan and Flynn, 2004; Cooper, 2009; Flynn *et al.*, 1993). IFN- γ synergises with TNF- α to activate murine macrophages to kill intracellular *M. tuberculosis* (Ahmad, 2010; Co *et al.*, 2004b; Wolf *et al.*, 2008) and stimulate the production of antimicrobial nitric oxide and other reactive nitrogen intermediates (RNI) via inducible NOS (iNOS) (Scanga *et al.*, 2001). IFN- γ also enhances antigen presentation, which results in the recruitment of CD4⁺ and/or CD8⁺ T cells which function in bacterial killing and prevent the exhaustion of memory T cells (Chan and Flynn, 2004; Russell *et al.*, 2009).

M. tuberculosis infection of CD4⁺ T cell-deficient mice has shown that the early production of IFN- γ by CD4⁺ T cells and the ensuing activation of macrophages determine the outcomes of infection (Caruso *et al.*, 1999). CD4⁺ T cells are also crucial for granuloma initiation and construction (Co *et al.*, 2004a). *M. tuberculosis*-infected mice which lack CD4⁺ T cells form anomalous lesions that cannot control bacilli numbers and allow dissemination of the pathogen (Mogues *et al.*, 2001; Saunders *et al.*, 2002). In humans, evidence for a requirement for CD4⁺ T cells in the maintenance of the granuloma comes from HIV co-infected patients, where LTBI becomes reactivated due to the depletion of CD4⁺ T cells by HIV (Barnes *et al.*, 1991; Scanga *et al.*, 2000; Waxman *et al.*, 1995). The loss of CD4⁺ T cells results in decreased levels of IFN- γ and consequently a reduction in macrophage activation and killing of *M. tuberculosis* (Raja, 2004).

Additionally, CD4⁺ T cells induce apoptosis of infected macrophages via Fas/Fas ligand interaction (Ashany *et al.*, 1995; Kagi *et al.*, 1994; Oddo *et al.*, 1998; Stalder *et al.*, 1994). This is particularly important, because the intracellular bacilli are also killed and therefore do not spread, unlike necrosis. Apoptotic vesicles are phagocytosed by DC. They contribute to host defence against *M. tuberculosis* because they contain mycobacterial antigens, and can prime CD8⁺ T cells by antigen cross-presentation.

CD8⁺ T cells are present in similar numbers to CD4⁺ T cells in the *M. tuberculosis*-induced granuloma (Randhawa, 1990). It has been reported that they directly kill *M. tuberculosis* via granulysin (Grotzke and Lewinsohn, 2005; Stenger *et al.*, 1998; Stenger *et al.*, 1999). *M. tuberculosis*-specific CD8⁺ T cells are present in abundance in individuals with LTBI, suggesting they have a role in controlling latent infection. Indeed, the depletion of CD8⁺ T cells results in reactivation in a mouse model of LTBI (van Pinxteren *et al.*, 2000). It is thought that CD8⁺ T cells are protective in the later stages of infection, as their accumulation at the granuloma is somewhat delayed relative to CD4⁺ T cell accumulation (Gonzalez-Juarrero *et al.*, 2001). CD8⁺ T cells also produce IFN- γ during *M. tuberculosis* infection.

TNF- α , produced by macrophages, DC and T cells, has an important role in protection against *M. tuberculosis* infection in mice and humans (Bean *et al.*, 1999; Keane, 2005). Paradoxically, TNF- α is also responsible for much of the pathology associated with TB (Flynn and Chan, 2005). Like IFN- γ , mice which lack TNF- α or its receptor are more susceptible to mycobacterial infections and have aberrant or delayed granuloma formation (Bean *et al.*, 1999; Flynn *et al.*, 1995). TNF- α synergises with IFN- γ to enhance iNOS expression and the antimycobacterial activity of macrophages (Chan and Flynn, 2004; Scanga *et al.*, 2001). Impairment of TNF- α responses results in overgrowth of mycobacteria (Chan and Flynn, 2004; Cooper, 2009). TNF- α also drives phagolysosomal maturation and autophagy events (Harris *et al.*, 2008; Harris and Keane, 2010).

TNF- α also promotes cell migration and the formation of granulomas because it induces the production of chemokines such as IL-8, MCP-1 and RANTES by infected macrophages. These chemokines stimulate the migration of immune cells to the focus of infection (Algood *et al.*, 2003). T cell- and macrophage-derived TNF- α is essential for effective long-term protection against *M. tuberculosis* infection (Saunders *et al.*, 2005). It is required for maintenance of the granuloma, as it has been shown that neutralisation of TNF- α in mice with chronic *M. tuberculosis* infection results in the loss of granuloma organisation, abnormal pathology and is ultimately fatal (Mohan *et al.*, 2001). In humans, the blockade of TNF- α as a treatment for inflammatory conditions such as rheumatoid arthritis results in enhanced susceptibility to *M. tuberculosis* (Keane *et al.*, 2001).

IL-12 is also important in the immune response against *M. tuberculosis*. As with TNF- α and IFN- γ , mice and humans deficient in IL-12 or IL-12R are more susceptible to mycobacterial infections (Jouanguy *et al.*, 1999). Furthermore, IL-12- or IL-12R-defective individuals have increased susceptibility to active TB disease (Lichtenauer-Kaligis *et al.*, 2003). IL-12 is crucial because it influences CD4⁺ T cells to produce both TNF- α and IFN- γ .

1.9 Treg cells in health and disease

Treg cells which are induced during infection can be both beneficial and detrimental to the host. Treg cells which are generated in response to pathogens may limit inflammation and therefore limit damage to the host tissue (Belkaid, 2007). However, such Treg cell induction can lead to persistent infection with the pathogen by preventing complete pathogen eradication by the adaptive immune system (Belkaid, 2007; Joosten and Ottenhoff, 2008). Despite this, persistent infections may be kept under control by effective immune dynamics but there is always the possibility of disease reactivation as a result of immune dysfunction later in life (Joosten and Ottenhoff, 2008).

Since pathogens fall into a number of distinct categories (i.e. bacteria, viruses, fungi) and within these categories there are diverse populations, they also have many interactions with the host. Accordingly, Treg cells have a variable influence in the context of different infections. For

instance, in infections which resolve rapidly, inducible or adaptive Treg cells may not be generated in time, and as most nTreg cells are self-reactive they might not respond to acute infections. As a result, most knowledge of Treg cells in the context of infectious disease comes from studies of chronic infections (Belkaid, 2007; Joosten and Ottenhoff, 2008).

1.9.1 Infection

1.9.1.1 Treg cells in Tuberculosis

In general, Treg cells are associated with active tuberculosis – they are found at the site of infection and it is thought they act locally to downregulate protective immunity against *M. tuberculosis* antigens (Joosten and Ottenhoff, 2008). Such impairment of the immune response against the pathogen may lead to its persistence. IL-10-secreting cells have been reported in patients infected with TB but who are anergic, i.e. they do not react to intradermal injection with purified protein derivative (PPD) from *M. tuberculosis*. These patients comprise approximately 15% of those with active disease. They show decreased responses against *M. tuberculosis* but not to other pathogens, indicating that the suppression observed is antigen-specific (Boussiotis *et al.*, 2000; Delgado *et al.*, 2002). It has been reported that patients with active TB have increased frequencies of nTreg cells – i.e. CD25^{+/high} and/or FoxP3⁺ cells - which normalise with treatment (Guyot-Revol *et al.*, 2006). Importantly the frequency is highest at the site of infection compared with peripheral blood (Chen *et al.*, 2007; Guyot-Revol *et al.*, 2006; Ribeiro-Rodrigues *et al.*, 2006; Scott-Browne *et al.*, 2007).

Moreover, Treg cells are found at a high frequency in granulomas and as such they might function to help contain local infection by preventing excessive immunopathology, or they might dampen protective Th1 immunity (Chen *et al.*, 2007). Indeed, depletion of Treg cells *in vitro* enhances the Th1 response against mycobacterial antigens as measured by increased IFN- γ (Chen *et al.*, 2007; Guyot-Revol *et al.*, 2006; Ribeiro-Rodrigues *et al.*, 2006). Furthermore, the depletion of Treg cells prior to *M. tuberculosis* infection reduced bacterial growth in mice (Kursar *et al.*, 2007; Scott-Browne *et al.*, 2007).

With respect to FoxP3, its expression in whole blood correlates well with the kinetics of Treg cell induction and with active disease (Burl *et al.*, 2007; Roberts *et al.*, 2007). Expression of FoxP3 mRNA in combination with IL-8 and IL-12 mRNA can be used to differentiate LTBI from active disease, as patients with active TB has enhanced expression of these three genes in PBMC when compared with those with LTBI (Wu *et al.*, 2007).

1.9.1.2 Whooping cough

B. pertussis is the bacterium responsible for whooping cough, or pertussis. This respiratory disease is very severe in infants and young children worldwide and remains an endemic disease despite widespread availability of vaccines (Cherry, 2006). *B. pertussis* is a Gram-negative bacterium which colonises ciliated epithelial cells of the upper respiratory tract and bronchial tree of the lungs and can also survive inside macrophages (Dunne *et al.*, 2009).

Humans are the only natural host for *B. pertussis*, however numerous animal models have been developed to study immune responses against the pathogen, including mouse, rat and rabbit models (Mills, 2001). It was first demonstrated that T cells played a role in immunity to *B. pertussis* by experiments with athymic mice which failed to clear the bacterium, whereas wild type mice cleared the infection after approximately 35 days (Mills *et al.*, 1993). Adoptive transfer experiments have shown that *B. pertussis*-specific CD4⁺ T cells can confer protection to athymic or sublethally irradiated mice (Mills *et al.*, 1993). Additionally, depletion of CD4⁺ T cells prior to immunisation with killed *B. pertussis* abrogated protection (Leef *et al.*, 2000).

B. pertussis infection of naïve mice results in infiltration of neutrophils, macrophages and lymphocytes into the lung. Eradication of *B. pertussis* is IFN- γ -dependent, the source of which are infiltrating NK cells early in infection and Th1 cells later in the course of disease (Mahon *et al.*, 1997; Mills *et al.*, 1993; Ryan *et al.*, 1997). IFN- γ acts to contain the infection in the respiratory compartment (Mahon *et al.*, 1997). Antigen-specific Th17 cells and IL-10-producing Treg cells are also generated during infection (Fennelly *et al.*, 2008; McGuirk *et al.*, 2002).

B. pertussis is able to utilise certain strategies which enables it to evade host protective immunity. For example, the virulence factor filamentous haemagglutinin (FHA) acts directly on DC to promote IL-10 and therefore inhibit lipopolysaccharide (LPS)-induced IL-12 (McGuirk *et al.*, 2002). In addition, IL-10-producing Tr1 cells are induced by FHA-stimulated DC, permitting the bacterium to further avoid eradication (McGuirk *et al.*, 2002).

The toxic effects of *B. pertussis* adenylate cyclase toxin (CyaA) are mediated by the enhancement of intracellular [cAMP] (Ladant, 1988). CyaA binds to the $\alpha M\beta 2$ integrin CD11b/CD18, expressed on DC and macrophages (Guermonprez *et al.*, 2001). Binding inhibits DC chemotaxis, phagocytosis and superoxide production, resulting in bacterial colonisation and persistence (Njamkepo *et al.*, 2000; Pearson *et al.*, 1987). CyaA also enhances IL-10 production and independently inhibits IL-12, consequently promoting Tr1 cell induction (Boyd *et al.*, 2005; Hickey *et al.*, 2008; Ross *et al.*, 2004). While the induction of antigen-specific IL-10-secreting Tr1 cells during infection with *B. pertussis* suppresses the protective host immune response mediated by IFN- γ , Tr1 cells also restrict immunopathology (Higgins *et al.*, 2003).

1.10 Summary

Treg cells are important for regulating self-tolerance and preventing autoimmune diseases. They are also crucial for limiting the inflammation and tissue damage, which can result from immune responses. However, the role of CD25⁻ Treg cells is still unclear, particularly in lung disease. The lung is a key site of antigen entry into the body, yet the induction of immune responses is stringently controlled in this organ to generate protective immunity against pathogens, but simultaneously prevent aberrant reactions to environmental antigens. Therefore I will investigate the role of CD4⁺FoxP3⁺CD25⁻ Treg cells in regulating immune responses in mouse lungs, with particular emphasis on their role in *B. pertussis* infection. I will study the immunosuppressive function of CD4⁺FoxP3⁺CD25⁻ Treg cells, the mechanisms by which they suppress, and investigate how they influence other cell populations to regulate protective immunity to *B. pertussis* infection *in vivo*.

I will also explore how these lung CD25⁻ Treg cells arise and how the broader lung environment influences their phenotype and function. In particular, I will examine whether AM control the induction of these cells in mice and humans and whether the relationship can be targeted for immunotherapy. I will also consider whether lung Treg cells represent a risk factor for the progression of LTBI to active TB disease in smokers.

1.11 Aims

The overall aims of this project were:

1. To examine the distribution and phenotype of CD4⁺FoxP3⁺CD25⁻ Treg cells in mouse tissue, and to elucidate the mechanism(s) by which they suppress target cells *in vitro* and in response to *B. pertussis* infection *in vivo*
2. To investigate whether human and murine AM can induce FoxP3⁺ T cells *in vitro*, and determine the mechanism(s) they use to achieve this
3. To establish
 - a. whether the suppressive function of AM represents a target for immunotherapy and
 - b. if induced FoxP3⁺ T cells in the lung contribute to the increased risk of developing active TB disease in smokers with LTBI

Chapter 2

Materials and Methods

2.1 Materials

Ammonium Chloride Lysis Buffer

0.829 g NH_4Cl (Sigma)

0.109 g KHCO_3 (Sigma)

0.037 g Disodium Ethylenediaminetetraacetic acid (EDTA, Sigma)

100 ml dH_2O

pH to 7.3 to 7.4.

Cell Culture Medium

Roswell Park Memorial Institute (RPMI)-1640 medium (Biosera) was supplemented with 10% heat inactivated (56°C for 30 mins) foetal calf serum (FCS, Biosera), 100mM L-Glutamine (Gibco), 100 $\mu\text{g/ml}$ penicillin/streptomycin (Biowest). Complete RPMI (cRPMI) was used to culture murine T cells.

RPMI-1640 medium supplemented with 100mM L-Glutamine and 100 $\mu\text{g/ml}$ penicillin/streptomycin was used to culture murine alveolar macrophages.

RPMI-1640 supplemented with 10% AB human serum (Sigma), 50 $\mu\text{g/ml}$ cefotaxime (Melford Laboratories), and 50 $\mu\text{g/ml}$ fungizone (Gibco) was used to culture human monocyte-derived macrophages, primary human AM and human T cells.

Dulbeccos Modified Eagle Medium (DMEM, Sigma) was supplemented with 2.5%, 5% or 10% ultralow IgG FCS (Invitrogen), 100 mM L-Glutamine, 100 mM penicillin/streptomycin and 0.05 M β -mercaptoethanol (Sigma). Supplemented DMEM was used to culture PC61 hybridoma cells.

Carbonate Buffer (0.05 M)

2.93 g NaHCO₃ (Sigma)

1.5 g Na₂CO₃ (Sigma)

Dissolved in 1L dH₂O

Collagenase D (Roche)**DNase (Sigma)****EDTA (25 mM)**

0.93 g EDTA (Sigma)

Dissolved in 50 ml Baxter's Water

EDTA (0.5 M)

20.8 g EDTA (Sigma)

Dissolved in 100 ml Phosphate Buffered Saline (PBS, Biosera) and filter sterilised

MACS Rinsing/Wash Buffer (PBS + 2 mM EDTA)

2 ml 0.5 M EDTA

500 ml sterile Dulbecco's Phosphate Buffered Saline (PBS, Gibco)

MACS Buffer (PBS + 2 mM EDTA + 5% FCS)

2 ml 0.5 M EDTA

2.5 ml sterile FCS

500 ml sterile PBS

FACS Buffer

500 ml Dulbecco's PBS (Biosera)

0.5 g Sodium Azide (Sigma)

5 g Bovine Serum Albumin (BSA, Sigma)

FoxP3 Staining Kit (eBioscience)

Fix/Perm Solution = 1 part Fix/Perm Concentrate to 3 parts Fix/Perm Diluent

Permeabilisation Buffer = 1 part 10X Permeabilisation Buffer to 9 parts dH₂O.

2% Paraformaldehyde

100 ml dH₂O

2.0 g Paraformaldehyde (Sigma)

Heat to 70°C to allow powder to fully dissolve. Store at 4°C

20X PBS

800 g NaCl, 1.4 M (Aldrich)

92 g Na₂HPO₄, 0.08 M (Sigma)

20 g KH₂PO₄, 0.01 M (Merck)

20 g KCl, 0.03 M (Riedel de Hahn)

Dissolved in 5L dH₂O, pH 7.0

ELISA Wash Buffer (1X PBS + 0.05% Tween)

500 ml 20X PBS

9.5 L dH₂O

5ml Tween (Sigma)

1% BSA in PBS

5 g BSA (Sigma)

500 ml PBS (Biosera)

5% Milk in PBS

25 g Dried Skimmed Milk (Fluka)

500 ml PBS (Biosera)

10% Milk in PBS

50 g Dried Skimmed Milk (Fluka)

500 ml PBS (Biosera)

10% FCS in PBS

50 ml FCS (Biosera)

450 ml PBS (Biosera)

Phosphate Citrate Buffer

10.19 g Anhydrous Citric Acid (Sigma)

36.96 g $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ (Sigma)

Made up in 1L dH_2O , pH 5.0

ELISA O-Phenylenediamine (OPD) Developing Solution

1 OPD tablet (Sigma)

25 ml Phosphate Citrate Buffer

15 μl H_2O_2 (Sigma)

ELISA 3,3',5,5'-Tetramethylbenzidine (TMB) Developing Solution (Sigma)

ELISA Stop Solution

25 µl 1M H₂SO₄ (Fluka)

TGF-β₁ Activation

Incubate samples at 80°C for 10 mins and allow to cool before adding to ELISA plate

1% Casein

6.0 g NaCl (Aldrich)

10.0 g Casamino acid (Difco)

Adjust pH to 7.0-7.2 with 1 N NaOH (Fluka).

Autoclave at 115°C.

Stainer and Scholte Liquid Medium

10.72 g L-Glutamic acid (monosodium salt) (Sigma)

0.24 g L-Proline (Sigma)

2.5 g NaCl (Aldrich)

0.5 g KH₂PO₄ (Merck)

0.2 g KCl (Riedel de Hahn)

0.1 g MgCl₆.6H₂O (Sigma)

0.02 g CaCl₂.2H₂O (Sigma)

1.525 g Tris (Riedel de Hahn)

Dissolve in 1 L ddH₂O, adjust pH to 7.3-7.4 with 6 N HCl.

Autoclave and store at 4°C.

Supplement for Stainer and Scholte Liquid Medium

0.4 g L-cystine (Sigma)

Dissolve L-cystine in 1 ml conc. HCl, and then add:

0.1 g FeSO₄·7H₂O (Sigma)

0.2 g Ascorbic acid (Sigma)

0.04 g Nicotinic acid (Sigma)

1.0 g Glutathione (reduced) (Sigma)

100 ml ddH₂O, sterilize by membrane filtration

Cephalexin (10 mg/ml)

0.1 g Cephalexin (Sigma)

10 ml dH₂O

Filter sterilise

Bordet Gengou (BG) Blood Agar Plates

500 ml ddH₂O

5 ml Glycerol (Sigma)

14 g BG broth (Difco)

Autoclave with tops slightly loose. Allow to cool to 37°C.

Add 1 ml of Cephalexin (10 mg/ml stock) and 100 ml of pre-warmed sterile horse blood (Cruinn), swirling continuously.

Pipette approximately 23 ml into each Petri dish, avoiding any bubbles.

Allow the plates to dry in the hood overnight.

Store the plates inverted at 4°C. Use within 5 weeks

Middlebrook 7H9 broth

6.47g Middlebrook 7H9 broth (Difco)

50 µl Tween 80 (Difco)

90 ml Low-endotoxin H₂O (Sigma)

Autoclaved 120°C

10 ml Albumin-Dextrose-Catalase (ADC) (Becton Dickinson)

2.2 Methods

2.2.1 Animals

Specific pathogen-free female BALB/c and C57Bl/6 mice were purchased from Harlan UK Ltd (Bicester, UK) and IL-10 knockout mice were bred in-house. All mice were maintained according to the regulations and guidelines of the European Union and the Irish Department of Health. All experiments were conducted under university ethical approval and under license from the Department of Health and Children. Mice were 6-8 weeks old at the initiation of each experiment.

2.2.2 Cell counts

EBAO

Cell counting was performed by diluting 20 μ l cells in 20 μ l of Ethidium Bromide/Acridine Orange (EBAO). A 20 μ l volume of the cell suspension was loaded onto a disposable haemocytometer (Hycor Biomedical). The number of viable cells (green) and dead cells (orange) were counted using a fluorescent microscope. The number of cells per ml was calculated by multiplying the cell numbers in 9 squares, within 1 large square, by 10^4 , by the dilution factor:

$$\text{Number of cells/ml} = \text{cell count} \times \text{dilution factor} \times 10^4$$

Trypan Blue

Cell counting was performed by diluting 10 μ l cells in 90 μ l Trypan blue (Sigma). A 10 μ l volume of the cell suspension was loaded onto a haemocytometer (Nunc). The number of viable cells (white) and dead cells (blue) were counted using a light microscope. The number of cells per ml was calculated by multiplying the cell numbers in 9 squares, within 1 large square, by 10^4 , by the dilution factor:

$$\text{Number of cells/ml} = \text{cell count} \times \text{dilution factor} \times 10^4$$

2.2.3 Isolation of cells from lung, liver, colon, spleen, cervical and thoracic lymph nodes

Mice were euthanised by cervical dislocation, lungs were removed and digested in RPMI containing 1 mg/ml Collagenase D (Roche) for 1 hour at 37°C on a rotary mixer. Cells were isolated by homogenising lung tissue and passing it through a 40 µm filter. The same procedure was used to isolate cells from liver tissue with the exception that Collagenase IV (Sigma) was used instead of Collagenase D.

Colon tissue was incubated in Hank's Balanced Salt Solution (HBSS, Biosera) containing 5 mM EDTA (Sigma) and 1 mM dithiothreitol (DTT, Sigma) for 20 minutes at 37°C and passed through a 100 µm cell strainer. The tissue was then digested in 0.05 g of Collagenase D (Roche), 0.05 g of DNase I (Sigma) and 0.3 g of Dispase II (Roche) digestion solution in 100 ml of PBS for 20 minutes at 37°C.

Spleens and lymph nodes were homogenised and passed through a 40 µm filter.

For all tissues, homogenate was centrifuged at 1800 rpm for 5 mins; erythrocytes were lysed by addition of 3 ml ammonium chloride lysis buffer and incubating at 37°C for 5 mins. cRPMI was added, and a cell count was performed and suspension was centrifuged at 1800 rpm for 5 mins.

2.2.4 Flow cytometry analysis for T cell surface markers

Cells were isolated from tissues as described in section 2.2.3 and resuspended at 1×10^7 cells/ml. Cells were stained with various fluorescently labelled antibodies against surface markers. After 15 minutes, cells were washed with FACS buffer and pelleted by centrifugation at 1800 rpm for 5 mins.

Cells were either fixed in 2% paraformaldehyde, or if intracellular markers were to be analysed, they were permeabilised in Fix/Perm Buffer (eBioscience) for 30 mins, washed in

permeabilisation buffer (eBioscience) and stained with relevant fluorescently labelled antibodies for 30 mins. After washing in permeabilisation buffer cells were either analysed immediately or fixed in 2% paraformaldehyde and stored in the dark at 4°C and analysed within 24 hours of staining.

2.2.5 Flow cytometry analysis for intracellular cytokines

Cells were isolated as described in section 2.2.3 and added at 5×10^5 cells/well to 96-well round-bottom tissue culture plates (Greiner). 2 µg/ml Brefeldin A (BFA, Sigma) was added to each well and cells were incubated overnight at 37°C, 5% CO₂. Cells were stained as described in section 2.2.4.

2.2.6 Cell sorting

Cell pellet was resuspended at 50×10^6 cells/ml in cRPMI and stained with rat anti-mouse CD4-PE (eBioscience), CD45RB-FITC (eBioscience), CD25-PE-Cy5 (eBioscience) and sorted using a MoFlo cell sorter (DakoCytomation). Sorted populations were CD4⁺CD25⁻CD45RB^{low} Treg cells from mouse lungs and CD4⁺CD25⁻CD45RB^{high} naïve T effector cells from mouse spleens.

2.2.7 Suppression assay

Purified CD4⁺CD25⁻CD45RB^{low} (1×10^4) lung cells were activated with 1 µg/ml immobilised anti-CD3 (BD Pharmingen) and irradiated spleen cells (APC: 5×10^5) in the presence of 20 U/ml IL-2 (Immunotools) for 5 days, then washed and re-activated in the presence of CD4⁺CD25⁻CD45RB^{high} effector T cells (1×10^4) purified from spleen, using 10 µg/ml immobilised anti-CD3 and irradiated APC (5×10^5). IL-10, IFN-γ, TGF-β₁ and TNF-α concentrations were determined in supernatants 48 hours later by enzyme-linked immunosorbent assay (ELISA) (R&D systems) while proliferation was determined by ³H-thymidine (Amersham) incorporation after 72 hours.

2.2.8 ^3H -thymidine incorporation

Supernatant was removed from suppression assay culture after 48h and retained for ELISA analysis. Wells were replenished with fresh medium containing ^3H -thymidine and cells were incubated overnight at 37°C, 5% CO_2 . Cells were harvested using a Tomtec Harvester 96 and radioactive counts were determined using a WALLAC beta plate counter.

2.2.9 CFSE dilution

Suppression by carboxyfluorescein diacetate succinimidyl ester (CFSE) loss was determined by labelling freshly isolated $\text{CD4}^+\text{CD25}^-\text{CD45RB}^{\text{high}}$ effector T cells with 0.5 μM CFSE (Molecular Probes). Pre-activated CD25^- Treg cells were co-cultured with CFSE-labelled effector T cells (1:1) and activated in the presence of 10 $\mu\text{g/ml}$ immobilised anti-CD3 and 5×10^5 irradiated APC for 5 days. Cells were subsequently stained with antibodies directed against surface, CD4 and CD25, and CFSE loss was determined by flow cytometry.

2.2.10 IL-10 receptor blocking

$\text{CD4}^+\text{CD25}^-\text{CD45RB}^{\text{low}}$ (1×10^4) cells were pre-activated and cultured either separately or together with effector T cells as described in section 2.2.7, either in the presence or absence of 10 $\mu\text{g/ml}$ anti-IL-10R blocking antibody (BD Pharmingen). Cytokine concentrations and proliferation were both determined as described in sections 2.2.7 and 2.2.8.

2.2.11 Transwell assays

$\text{CD4}^+\text{CD25}^-\text{CD45RB}^{\text{low}}$ (1×10^4) cells were activated as described in section 2.2.7 but re-activated in a Transwell plate (Millipore) in a separate chamber from the effector cells. Cytokine concentrations and proliferation were both determined as described in sections 2.2.7 and 2.2.8.

2.2.12 Isolation of murine alveolar macrophages – bronchoalveolar lavage

Mice were euthanized by cervical dislocation and a bronchoalveolar lavage (BAL) was performed. This method involves the insertion of a cannula into the exposed trachea. The cannula was connected to a double syringe system; one syringe was filled with cold PBS and the second

syringe acted as a connecting vial. 5 ml total of cold PBS was used in each individual BAL. 1 ml of PBS was injected at a time into the lungs before being removed and kept on ice.

BAL fluid from each mouse was pooled and AM were counted as described in section 2.2.2. AM are noticeably bigger than other cells isolated from the lung (e.g. lymphocytes), therefore smaller cells can be excluded from the cell count. AM were subsequently plated in a flat-bottomed 96 well plate at a concentration of 5×10^4 cells/well in serum-free RPMI and incubated at 37°C, 5% CO₂ for three hours, to allow the AM to adhere. Non-adherent cells were removed by gentle pipetting, fresh serum-free RPMI was added to the AM cultures, and cells were incubated at 37°C, 5% CO₂ for 24 hours prior to the initiation of each experiment.

2.2.13 AM and T cell co-culture

Supernatant was removed from the AM culture and retained. Purified splenic CD4⁺CD25⁻CD45RB^{high} cells (6×10^3) were cultured with AM alone or in the presence of 2 µg/ml soluble anti-CD3. Inhibition studies were performed by the addition of an inhibitor against the retinoic acid receptor alpha-chain (RAR α , Biomol; final concentration 5 µM), and/or a neutralising antibody against TGF- β_1 (50 µg/ml; antibody was a gift from Dr. L. Boon, Utrecht, the Netherlands and was produced by 1D11.16.8 hybridoma cells). IL-10, IFN- γ , IL-4, IL-5, TGF- β_1 and TNF- α concentrations were determined in supernatants 24 hours later by ELISA (R&D Systems), while the induction of FoxP3⁺ T cells and cell proliferation were determined as described in sections 2.2.4 and 2.2.8, respectively.

2.2.14 AM supernatant and T cell co-culture

Supernatants from AM which had been cultured *in vitro* for 24 hours were harvested and diluted twofold for *in vitro* studies. Undiluted, 1:2 and 1:4 dilutions were used. Purified CD4⁺CD25⁻CD45RB^{high} T cells were activated with 2 µg/ml soluble anti-CD3 and 5×10^5 irradiated splenic APC, either in the presence or absence of 20 µl of diluted AM supernatants. This was a 1:10 dilution of the AM-CM, so final dilutions of AM-CM in culture were therefore 1:10, 1:20 and 1:40. Inhibition studies were performed by the addition of an inhibitor against RAR α (final

concentration 5 μ M), and/or a neutralising antibody against TGF- β_1 (50 μ g/ml; a gift from Dr. L. Boon, Utrecht, the Netherlands). IL-10, IFN- γ , IL-4, TGF- β_1 and TNF- α concentrations were determined in supernatants 24 hours later by ELISA (R&D Systems), while the induction of FoxP3⁺ T cells and cell proliferation were determined as described in sections 2.2.4 and 2.2.8, respectively.

2.2.15 Proliferation assay

Cells were isolated from the lungs or spleens of naïve Balb/c mice, resuspended at 2 x 10⁶/ml and stimulated with 2 μ g/ml anti-CD3 and irradiated APC from either spleen or lung. To remove AM, lung cells were cultured in a Petri dish for 3 hours at 37°C, 5% CO₂ for 3 hours prior to purification, to allow AM to adhere to the dish. Non-adherent lung cells were harvested, resuspended at 2 x 10⁶/ml and stimulated under the same conditions as total spleen and total lung cells.

Cells were cultured in the presence or absence of 5 μ M RARi. IL-10, IFN- γ , IL-17, TGF- β_1 and TNF- α concentrations were determined in supernatants 48 hours later by ELISA (R&D Systems), cells were replenished with either fresh medium or medium containing ³H-thymidine for the final 18 hours of culture, and FoxP3 expression and cell proliferation were determined, as described in sections 2.2.4 and 2.2.8, respectively.

2.2.16 Ovalbumin intranasal immunisation

Naïve female Balb/c mice were given two intranasal vaccine combinations (22 μ l), 21 days apart. The individual components of the vaccine were 25 μ g (10 μ l) Ova protein (Hyglos), 5 μ g (5 μ l) CpG (CpG 1668, Sigma) as adjuvant, and 100 μ g (7 μ l) RARi. Stock Ova protein was dissolved in PBS, CpG was dissolved in LAL water and RARi was dissolved in 90% ethanol. Therefore, the specific nasal vaccine combinations per mouse were:

Vehicle:	10 μ l PBS + 5 μ l LAL water + 7 μ l ethanol
Ova protein:	10 μ l Ova protein + 5 μ l LAL water + 7 μ l ethanol
Ova + CpG:	10 μ l Ova protein + 5 μ l CpG + 7 μ l ethanol

Ova + RARi: 10 µl Ova protein + 5 µl LAL water + 7 µl RARi

Ova + CpG + RARi: 10 µl Ova protein + 5 µl CpG + 7 µl RARi

Positive control mice were vaccinated subcutaneously with 25 µg Ova and 5 µg CpG, in 100 µl PBS.

2.2.17 *Bordetella pertussis* culture

Frozen BP W338 was thawed and 100 µl was streaked on pre-warmed blood agar plate which was incubated at 37°C for 5 days. A virulent colony was removed and added to an Erlenmeyer flask (Corning) containing pre-warmed complete Stainer and Scholte (S&S) medium (99 ml plus 1 ml supplement). The culture was incubated on a shaker for 24 hours at 37°C.

This culture was diluted 1/10 – i.e. 10 ml was added to each of 8 Erlenmeyer flasks containing pre-warmed S&S medium (89 ml + 1 ml supplement). Cultures were incubated on a shaker for 48 hours at 37°C. These suspensions were added to 4 autoclaved Kendro Sorvall bottles (200 ml per bottle) and centrifuged for 20 mins at 11,000 rpm (Sorvall R45C Plus ultracentrifuge). Pellets were combined and resuspended in 100 ml 1% casein.

Bacterial concentration was determined – 20 and 50 µl were removed and added to cuvettes containing 980 and 950 µl of 1% casein, respectively. Absorbance was read at 600 nm (blank; 1% casein plus 1 in 50 and 1 in 20 dilutions of bacterial suspension).

Calculation: Multiply the absorbance by 224.1 and by the appropriate dilution factor i.e. 50 or 20. Therefore – the number of bacteria in the solution = 224.1 x dilution factor x absorbance. Solution was adjusted to 3×10^{10} bacteria in 150 ml of 1% casein.

2.2.18 Exposure of mice to aerosol containing *B. pertussis*

A nebuliser was attached to the Kendro Sorvall bottle containing 3×10^{10} BP W338 in a Class 2 Laminar Flow Hood. Mice were placed in a cage within a Perspex box, the nebuliser was inserted into a hole in the box and the nebuliser pump was switched on. Mice were exposed to the

aerosol for 15 minutes, then pump was switched off and mice left to rest for a further 10 minutes. The nebuliser delivery rate was 0.5 ml per minute.

2.2.19 Production of heat killed *B. pertussis* (HKBP)

Frozen BP W338 was thawed and cultured as described in section 2.2.18. When the bacterial concentration was determined, the solution was adjusted to 1×10^{10} bacteria per ml of 1% casein. This solution was heated to 65°C for 30 mins to kill the bacteria and was stored in 1 ml (1×10^{10} heat killed bacteria) aliquots at -20°C.

2.2.20 *Ex vivo* T cell responses in the lungs of *B. pertussis*-infected mice

Mice were infected with *B. pertussis* by exposure to an aerosol, as described in section 2.2.18. 7, 14, 21 and 29 days post-challenge, mice were injected with 4×10^9 (400 µl) heat-killed *B. pertussis* 4 hours prior to removal of lungs, which were digested with 1 mg/ml of Collagenase D (Sigma) for 1 hour at 37°C. Lung were homogenized, isolated cells were added at 5×10^5 /well to a 96-well plate and incubated with 2 µg/ml BFA overnight at 37°C 5% CO₂. Cells were stained as described in section 2.2.4.

2.2.21 PC61 Hybridoma cell culture

Frozen cells (European Collection of Cell Cultures, Health Protection Agency, UK) were thawed and added to 10 ml complete DMEM (containing 10% low IgG FCS, Penicillin/Streptomycin, L/Glutamine and β-mercaptoethanol). Cells were washed twice in 10 ml cDMEM (1000 rpm, 5 mins), added to a T25 flask containing pre-warmed cDMEM, and were incubated for 48 hours at 37°C, 5% CO₂.

Cells were subcultured in a T75 flask containing 40 ml pre-warmed cDMEM (supplemented with 5% low IgG FCS) and were incubated for 48 hours at 37°C, 5% CO₂. Cells were subsequently subcultured in a T175 flask containing pre-warmed 50 ml cDMEM (supplemented with 2.5% low IgG FCS) and were incubated for 48 hours at 37°C, 5% CO₂.

Cells were centrifuged at 2000 rpm for 5 mins and resuspended in cDMEM (supplemented with 2.5% low IgG FCS). Cell suspension was split between 4 T175 flasks and cells were incubated for 48h at 37°C, 5% CO₂. Cells were split 1/4 or 1/5 every 2-3 days until a sufficient volume of supernatant was obtained. Cell suspension was centrifuged at 2000 rpm for 5 mins; the supernatant was harvested and pooled, and frozen at -20°C until needed.

2.2.22 Purification of anti-CD25 antibody from hybridoma cell supernatant

Supernatant was thawed, filtered (Stericup filter, Millipore) and degassed prior to purification using protein G chromatography (mAB Trap affinity chromatography kit, Amersham Bioscience). The optimal concentration of antibody required to deplete greater than 95% of the relevant target cells was determined by titration. Mice were injected i.p. with increasing concentrations of depleting antibody. The spleens of these mice were examined for CD25 and FoxP3 expression by flow cytometric analysis on 24, 48 h and 7 d post injection. A rat horse radish peroxidase (HRPN) IgG₁ was used as the isotype matched control (Bioxcell). An alternative anti-CD25 antibody specific for a different epitope on the CD25 molecule (7D4; eBioscience) was used to examine CD25 depletion.

2.2.23 Depletion of CD25⁺ T cells in *B. pertussis* infection

150 µg of either PC61 anti-CD25 depleting antibody or rat IgG₁ control antibody were injected into mice 24 hours prior to infection with *B. pertussis*. Mice were exposed to *B. pertussis* aerosol as described in section 2.2.18. After 4 hours and 7, 14 and 21 days, lungs were removed and were homogenised in 1 ml 1% casein and plated onto blood agar (with cephalixin) for colony forming unit (CFU) counting. CFUs were determined after 5 days' incubation at 37°C. The lung homogenate was also analysed for the presence of IFN-γ, IL-10, IL-17, TNF-α and IL-1β, as described in section 2.2.35.

2.2.24 *B. pertussis*-specific cytokine production by spleen and lymph node cells

Spleens and cervical lymph nodes from infected mice were removed and homogenised by passing through a 40 µM filter. Cells were resuspended at 2 x 10⁶/ml or 1 x 10⁶/ml, respectively

and were stimulated for four days with 4 µg/ml filamentous haemagglutinin (FHA, Kaketsuken, Japan), 1×10^5 /ml heat-killed *B. pertussis* (HKBP), 0.5 µg/ml anti-CD3 and 25 ng/ml phorbol myristate acetate (PMA, Sigma), or medium. Supernatants were removed and IFN-γ concentrations were quantified by ELISA, as described in section 2.2.35. Cells were replenished with fresh medium containing PMA (10 ng/ml) ionomycin (100 ng/ml, Sigma) and BFA (2 µg/ml) for four hours and intracellular staining was performed as described in section 2.2.4.

2.2.25 *B. pertussis* antigen-specific proliferation and cytokine production by purified T cells from spleen or lung

Mice were infected with *B. pertussis* by exposure to an aerosol, as described in section 2.2.18. 21 days post-challenge, cells were isolated from lungs and spleens as described in section 2.2.3. CD3⁺ T cells were purified from both of these tissues using the Pan T cell II isolation kit (Miltenyi Biotec). Prior to purification lung cells were cultured in a Petri dish for 3 hours at 37°C, 5% CO₂ for 3 hours to allow AM to adhere to the dish and prevent them from contaminating the purified cell population. Non-adherent lung cells were harvested and counted as described in section 2.2.2.

Briefly, spleen cells and non-adherent lung cells were resuspended in 40 µl of MACS buffer per 10^7 cells. 10 µl of biotin-antibody cocktail was added per 10^7 cells, and the suspension was incubated at 4°C for 10 minutes. 30 µl of buffer and 20 µl of anti-biotin microbeads were added and the suspension was incubated at 4°C for a further 15 minutes. Cells were washed by adding 2 ml buffer to the suspension and centrifuging at 1200 rpm for 10 minutes. Supernatant was aspirated and cells were resuspended at a concentration of 1×10^8 cells/500 µl buffer. T cells were purified from magnetically labelled unwanted cell using the DepleteS programme on the AutoMACS Separator (Miltenyi Biotec).

Purified T cells from lung and spleen were counted, washed and resuspended in cRPMI at a concentration of 2×10^5 /ml. Cells were stimulated for 4 days with FHA (4 µg/ml), HKBP ($1 \times$

10⁵/ml), anti-CD3 and PMA (5 µg/ml and 250 µg/ml) or medium, in the presence of irradiated syngeneic lung and/or spleen mononuclear cells as a source of APC (2 x 10⁶/ml) and in the presence or absence of 5 µM RARi. Supernatants were removed and IL-10, TNF-α, TGF-β₁, IL-17 and IFN-γ concentrations were quantified by ELISA, as described in section 2.2.35. Cells were replenished with either fresh medium or medium containing ³H-thymidine for the final 18 hours of culture, and FoxP3 expression and cell proliferation were determined, as described in sections 2.2.4 and 2.2.8, respectively.

2.2.26 Nasal delivery of RARi to *B. pertussis*-infected mice

Mice were infected with *B. pertussis* by exposure to an aerosol, as described in section 2.2.18. 24 hours post-challenge, and every 48 hours thereafter, mice were given 22 µl of treatment, delivered nasally. Treatment consisted of vehicle (PBS + 90% ethanol), 75 µg RARi in PBS, or 150 µg RARi in PBS. After 4 hours and 7, 14 and 21 days, lungs were removed and were either homogenised in 1 ml 1% casein and plated onto blood agar (with cephalixin) for colony forming unit (CFU) counting, or were digested and cells were stained with rat anti-mouse antibodies against CD4 and FoxP3 and analysed by flow cytometry. CFUs were determined after 5 days' incubation at 37°C.

2.2.27 Human alveolar macrophages

Human alveolar macrophages were obtained at bronchoscopy, after written consent, under a protocol approved by the St. James's Hospital/ AMNCH ethics board. The letter of protocol approval is available on request. All regulations regarding patient confidentiality were adhered to and the data is fully anonymised. In each case the reason for bronchoscopy was documented.

Normal human AM: These were harvested from bronchoscopies that were performed on patients who were non-smokers, and who were subsequently found to be free of much pulmonary disease. The airway was examined to rule out the presence of any unusual luminal obstruction (e.g. rare endobronchial neoplasms)

Smokers' AM: These were harvested from bronchoscopies that were done on patient who were smokers. The indication varied, but was often to rule out lung cancer. Specifically, these patients did not have sarcoidosis, or pneumonia, though they may have had COPD. In this regard, they are typical of smokers' pulmonary conditions.

Lavage fluid was filtered through a 100 μ M membrane and centrifuged at 1400 rpm for 15 mins. Cells were then resuspended at 5×10^5 cells/ml in RPMI 1640 culture medium. The medium was supplemented with 10% pooled type AB human serum (Sigma), 50 μ g/ml fungizone and 50 μ g/ml cefotaxime. Cells were incubated for 3 hours at 37°C, 5% CO₂. Nonadherent cells were removed and retained for phenotypic analysis by flow cytometry. Fresh medium was added to adherent cells. These cells were ready for infection after 24 hours.

2.2.28 Peripheral blood mononuclear cell isolation

Peripheral blood mononuclear cells (PBMC) were isolated from the buffy coat of healthy donors (provided, with permission, by the Irish Blood Transfusion Service) by centrifugation (400 g for 25 min) on a Lymphoprep gradient (Axis-Shield), washed, and resuspended in RPMI 1640 culture medium. The medium was supplemented with 10% pooled type AB human serum (Sigma), 50 μ g/ml cefotaxime and 50 μ g/ml fungizone (Gibco).

2.2.29 Monocyte-derived macrophages (MDM)

PBMC were seeded onto two-well LabTek glass chamber slides (Nunc, Inc., Naperville, IL) and 24-well plates. Nonadherent cells were removed by washing the wells with cRPMI after 24 h, and fresh medium was added. The medium was replaced, with washing to remove any remaining nonadherent cells, every 2 to 3 days. Macrophages were cultured for 7 to 10 days before infection with *M. tuberculosis*.

2.2.30 *Mycobacterium tuberculosis* culture

M. tuberculosis H37Ra was obtained from the American Type Culture Collection (ATCC 25177; Manassas, VA) and was grown to log phase in Middlebrook 7H9 broth (Difco)

supplemented with albumin-dextrose-catalase (ADC, Becton Dickinson) and 0.05% Tween 80 (Difco) and made up in low-endotoxin water (Sigma). Aliquots were stored at -80°C, thawed, and propagated in Middlebrook 7H9 medium to log phase prior to infection.

2.2.31 Infection of alveolar macrophages with *M. tuberculosis*

Log-phase mycobacteria were centrifuged (3800 rpm) for 10 min, and the pellet was resuspended in complete RPMI 1640 medium. Prior to inoculation of macrophages, mycobacteria were aspirated through a 25-gauge needle 10 times, followed by centrifugation (800 rpm) for 3 min. The supernatant was transferred to a fresh tube and used to infect macrophages.

To ensure an optimal infection ratio, multiplicities of infection (MOIs) were determined by adding dilutions of prepared bacilli to macrophages growing in two-well LabTek glass chamber slides (Nunc, Inc., Naperville, IL). After 3 h of incubation at 37°C in the presence of 5% CO₂, extracellular mycobacteria were washed away, and the cells were fixed in 2% paraformaldehyde (PFA) in phosphate-buffered saline for 5 min.

Cells were then stained using a TB Modified Auramine O stain kit (Scientific Device Laboratory, IL) according to the manufacturer's instructions. Briefly, slides were immersed in Modified Auramine O for 1 min, washed gently in running water, and decolourised with Modified Auramine O Decolorizer for 1 min. This was followed by another wash step and Hoechst 33358 (10 g/ml; Molecular Probes) staining for 5 min in the dark. The slides were finally washed gently with water, and cover slips were added using Dako fluorescent embedding medium (Dakocytomation). The MOI was determined by fluorescent microscopy using a Leica photomicroscope (Leica Microscopy Systems, Heerbrugg, Switzerland).

Macrophages were infected with a high MOI – this required that most cells were infected, ideally with 5-10 bacilli/cell. After 4 h of incubation at 37°C in the presence of 5% CO₂, extracellular mycobacteria were washed away, and fresh medium was added. Macrophages were infected for 24 hours prior to co-culture with allogeneic naïve T cells.

2.2.32 Purification of CD4⁺CD25⁻CD45RO⁻ naïve T cells using EasySep® magnetic separation

PBMC were resuspended at 5×10^7 cells/ml in buffer (2% FCS in PBS) and 1-1.5 ml of cell suspension was added to a sterile 5 ml FACS tube. 50 µl anti-CD45RO antibody was added per ml of cells and incubated for 15 mins at room temperature. 50 µl EasySep® negative selection human naïve CD4⁺ T Cell enrichment cocktail was added per ml of cells and incubated for a further 10 mins at room temperature. 100 µl EasySep® magnetic nanoparticles was added per ml of cells and suspension was incubated for 10 mins at room temperature.

The sample was brought to a final volume of 2.5 ml with buffer and the tube was placed in the EasySep® magnet for 10 mins at room temperature. The magnet and tube were inverted and the purified fraction was poured off into a new sterile 5ml FACS tube. The magnetically labelled unwanted cells remain bound inside the original tube, held by the magnetic field of the EasySep® magnet. For increased purity, the new tube was placed in the magnet for an additional 10 mins. The enriched population was then ready for use.

2.2.33 Culture of CD4⁺CD25⁻ naïve T cells with normal AM and AM infected with *M. tuberculosis* H37Ra

6×10^3 purified CD4⁺CD25⁻CD45RO⁻ naïve T cells were activated with 2 µg/ml soluble anti-CD3 in the presence of 5×10^4 uninfected AM or AM infected with low, medium and high MOIs of *M. tuberculosis* H37Ra. Inhibition studies were performed by the addition of RARi (5 µM or 20 µM). After 24 hours, the T cell phenotype was determined by flow cytometry, as described in section 2.2.4

2.2.34 Culture of CD4⁺CD25⁻ naïve T cells with macrophage supernatant

Supernatants from AM which had been cultured *in vitro* for 24 hours were harvested and diluted twofold for *in vitro* studies. Undiluted, 1:2 and 1:4 dilutions of uninfected supernatants were used. Supernatants from infected AM were not diluted. Purified CD4⁺CD25⁻CD45RO⁻ naïve

T cells (6×10^3) were activated with 2 $\mu\text{g/ml}$ soluble anti-CD3 and irradiated PBMC (APC: 5×10^5) in the presence of 50 μl of conditioned medium from uninfected AM or AM infected with low, medium and high MOIs of *M. tuberculosis* H37Ra. This was a 1:4 dilution of the AM-CM, so final dilutions of uninfected AM-CM in culture were therefore 1:4, 1:8 and 1:16, while all AM-CM from infected AM was just 1:4. Inhibition studies were performed by the addition of RARi (5 μM or 20 μM). After 24 hours, the T cell phenotype was determined by flow cytometry, as described in section 2.2.4 or cells were replenished with medium containing ^3H -thymidine for the final 18 hours of culture, cell proliferation were determined, as described in section 2.2.8

2.2.35 Standard cytokine ELISA

The concentrations of murine IL-10, IL-4, IL-5, IL-17, IL-1 β , TGF- β_1 , IFN- γ and TNF- α were quantified using pairs of antibodies (Table 2.1). High-binding 96-well microtiter plates (Greiner Bio-one) were coated overnight at 4°C with 50 $\mu\text{l/well}$ of rat anti-mouse capture antibody in coating buffer (Table 2.2). After washing in 1X PBS-Tween, non-specific binding sites were blocked by the addition of 200 $\mu\text{l/well}$ of blocking solution (Table 2.2) for 2 hours at room temperature. After blocking, plates were washed and 50 $\mu\text{l/well}$ of sample supernatant or serially diluted recombinant cytokine (0-2000 pg/ml) in assay diluent (Table 2.2) were added to plates and incubated overnight at 4°C. After washing, 50 μl of biotinylated goat anti-mouse (R&D) or rat anti-mouse (BD Pharmingen) detection antibody was added per well and incubated for 2 hours at room temperature. Plates were washed and cytokine was detected by incubating plates in the dark for 20 mins with 50 $\mu\text{l/well}$ of horseradish-peroxidase (HRP)-conjugated streptavidin followed by washing and addition of 50 $\mu\text{l/well}$ developing solution (OPD substrate in phosphate citrate buffer).

ELISA plates were developed in the dark after which the enzyme reaction was stopped by addition of 25 $\mu\text{l/well}$ of 1M H_2SO_4 . The OD values were determined by measuring absorbance at 492 nm using a microtiter plate reader and cytokine concentration for supernatant samples were calculated using the standard curve prepared using recombinant mouse cytokine.

2.2.36 Serum IgG_{2a} ELISA

Medium binding 96-well microtiter plates (Greiner Bio-one) were coated overnight at 4°C with 50 µl/well of HKBP (1×10^7 /ml). After washing in 1X PBS-Tween, non-specific binding sites were blocked by the addition of 200 µl/well of blocking solution (10% milk in PBS) for 2 hours at room temperature. After blocking, plates were washed, and samples were serially diluted in PBS. 50 µl/well of serially diluted serum samples were added to the plates and incubated overnight at 4°C.

After washing, 50 µl of biotinylated rat anti-mouse detection antibody (BD Pharmingen) was added per well and incubated for 1 hour at room temperature. Plates were washed and antigen-specific IgG_{2a} was detected by incubating plates in the dark for 20 mins with 50 µl/well of horseradish-peroxidase (HRP)-conjugated streptavidin followed by washing and addition of 50 µl/well of TMB substrate (Sigma). ELISA plates were developed in the dark after which the enzyme reaction was stopped by addition of 25 µl/well of 1 M H₂SO₄. The OD values were determined by measuring absorbance at 450 nm using a microtiter plate reader and LogEC₅₀ for serum samples were calculated using nonlinear regression analysis (sigmoidal dose response curve fit).

2.2.37 Serum IgE and IgG₁ ELISA

The concentrations of murine IgE and IgG₁ were quantified using pairs of antibodies (Table 2.1). Medium binding 96-well microtiter plates (Greiner Bio-one) were coated overnight at 4°C with 50 µl/well of Ovalbumin protein (10 µg/ml) or rat anti-mouse capture antibody in coating buffer (0.5M Carbonate buffer) (Table 2.2). After washing in 1X PBS-Tween, non-specific binding sites were blocked by the addition of 200 µl/well of blocking solution (10% FCS in PBS for IgE; 1% BSA in PBS for IgG₁) for 2 hours at room temperature. After blocking, plates were washed and 50 µl/well of serum sample, or serially diluted recombinant IgE or IgG₁ (0-500 ng/ml) in assay buffer were added to the plates and incubated overnight at 4°C.

After washing, 50 μ l of biotinylated rat anti-mouse detection antibody was added per well and incubated for 1 hour at room temperature. Plates were washed and total and antigen-specific immunoglobulin was detected by incubating plates in the dark for 20 mins with 50 μ l/well of horseradish-peroxidase (HRP)-conjugated streptavidin followed by washing and addition of 50 μ l/well developing solution (OPD substrate in phosphate citrate buffer). ELISA plates were developed in the dark after which the enzyme reaction was stopped by addition of 25 μ l/well of 1M H_2SO_4 . The OD values were determined by measuring absorbance at 492 nm using a microtiter plate reader and immunoglobulin concentration for serum samples was calculated using the standard curve prepared using recombinant mouse IgE or IgG₁.

2.2.38 Aldefluor assay

Aldehyde dehydrogenase activity in individual cells was estimated using Aldefluor® staining kits (StemCell Technologies) according to the manufacturer's protocol. Briefly, 1×10^6 cells were suspended in 1ml in Aldefluor® assay buffer containing activated Aldefluor® substrate (150 nM), in the presence or absence of the ALDH inhibitor diethylaminobenzaldehyde (DEAB; 100 μ M) for 45 minutes at 37°C. Cell viability was determined by flow cytometry with propidium iodide exclusion. ALDH1A activity was measured by flow cytometric analysis. Alternatively, cells were also stained with Hoechst 33358 (10 g/ml; Molecular Probes) and ALDH1A activity was determined by fluorescent microscopy using a Leica photomicroscope (Leica Microscopy Systems, Heerbrugg, Switzerland).

2.2.39 Statistical analysis

All statistical analysis was performed using GraphPad Prism software (Version 5.0a). Functionality was compared between study groups using nonparametric tests: Mann-Whitney for comparing only 2 groups and Kruskal-Wallis followed by a Dunn test for multiple comparisons comparing 3 or more groups.

Table 2.1 Origin of Antibodies and Recombinant Cytokines used in Mouse ELISA

Cytokine	Supplier	Antibody	Supplier
IL-10	R&D	IgE	BD Pharmingen
TGF- β_1	R&D	IgG ₁	BD Pharmingen
TNF- α	R&D	IgG _{2a}	BD Pharmingen
IL-17	R&D		
IL-1 β	R&D		
IFN- γ	BD Pharmingen		
IL-4	BD Pharmingen		
IL-5	BD Pharmingen		

Table 2.2 Mouse ELISA Specificities

Cytokine	Capture 50 µl well	Blocking 200 µl/well	Detection 50 µl/well
IL-10	4 µg/ml in PBS	1% BSA in PBS	400 ng/ml in Reagent diluent (1% BSA in PBS)
TGF-β ₁	4 µg/ml in PBS	1% BSA in PBS	200 ng/ml in Reagent diluent (1% BSA in PBS)
TNF-α	4 µg/ml in PBS	1% BSA in PBS	200 ng/ml in Reagent diluent (1% BSA in PBS)
IL-17	4 µg/ml in PBS	1% BSA in PBS	400 ng/ml in Reagent diluent (1% BSA in PBS)
IL-1β	4 µg/ml in PBS	1% BSA in PBS	400 ng/ml in Reagent diluent (1% BSA in PBS)
IFN-γ	0.5 µg/ml in PBS	5% milk in PBS	1 µg/ml in PBS
IL-4	1 µg/ml in PBS	5% milk in PBS	1 µg/ml in PBS
IL-5	1 µg/ml in PBS	5% milk in PBS	1 µg/ml in PBS
Antibody	Capture 50 µl well	Blocking 200 µl/well	Detection 50 µl/well
IgE	2 µg/ml in coating buffer	10% FCS in PBS	2 µg/ml in PBS
IgG ₁	2 µg/ml in coating buffer	1% BSA in PBS	2 µg/ml in PBS
IgG _{2a}	n/a	10% milk in PBS	2 µg/ml in PBS

Chapter 3

**Identification of a novel population of hyporesponsive
CD4⁺FoxP3⁺CD25⁻ Treg cells in the lungs of naïve mice**

3.1 Introduction

Treg cells can be divided into two general categories – thymically-derived nTreg cells (Sakaguchi *et al.*, 1995) and iTreg cells (Hafler *et al.*, 1997; McGuirk *et al.*, 2002; Roncarolo *et al.*, 2001; Tran *et al.*, 2007). iTreg cells include Tr1 and Th3 cells as well as inducible FoxP3⁺ Treg cells. Depletion of or defects in nTreg cells can lead to the emergence of a wide variety of autoimmune disease and debilitating tissue destruction as exemplified in *Scurfy* mutant mice (Ono *et al.*, 2006) and the IPEX syndrome in humans (Patel, 2001). Both these phenotypes are the consequence of a mutation in the gene encoding the transcription factor FoxP3. Intracellular FoxP3 expression is currently regarded as the definitive marker for cells with regulatory function in mice and humans (Fontenot *et al.*, 2003; Fontenot *et al.*, 2005b).

The use of FoxP3 to identify Treg cells has its drawbacks. It cannot be used to purify live Treg cells from mice or humans for *in vitro* functional assays for instance, as it can only be detected by cell fixation and permeabilisation. Furthermore, human T cells transiently express FoxP3 when activated, which makes it difficult to distinguish between Treg cells and activated T cells during an immune response (Allan *et al.*, 2007). The search for a common, stable Treg cell marker has proved elusive, forcing researchers to use a battery of surface and intracellular markers to identify a growing number of Treg subsets. Chief of these surface markers has been CD25, the alpha chain of the IL-2 receptor (Asano *et al.*, 1996; Sakaguchi *et al.*, 1995; Suri-Payer *et al.*, 1998). CD25 is constitutively expressed on nTreg cells (Sakaguchi *et al.*, 1995). However, CD25 is essentially an activation marker for both murine and human CD4⁺ T cells and therefore does not represent a definitive marker for Treg cells (Asano *et al.*, 1996). Nonetheless, a combination of FoxP3 and CD25 is widely used to detect, analyse and purify Treg cells.

In spite of the fact that the majority of Treg cells express surface CD25 (particularly nTreg cells), subsets of CD4⁺FoxP3⁺ cells that lack CD25 expression have also been shown to have suppressive function *in vitro* and *in vivo* (Alyanakian *et al.*, 2003; Ono *et al.*, 2006). Furthermore, it has been postulated that Treg cells lacking CD25 might accumulate in aged mice and humans

within memory T cell subsets (Nishioka *et al.*, 2006). Regulatory activity has been detected in CD4⁺CD45RB^{low}CD25⁻ memory T cells in rodents (Hori *et al.*, 2003) and the corresponding CD4⁺CD45RO⁺CD25⁻ memory T cell subset in humans (Yagi *et al.*, 2004). CD4⁺FoxP3⁺CD25⁻ GITR^{high} T cells have been shown to have suppressive function in murine organ transplantation (Nishimura *et al.*, 2004), inflammatory bowel disease (Uraushihara *et al.*, 2003) and in the prevention of autoimmune myocarditis in mice (Ono *et al.*, 2006). Further investigation has revealed that CD25⁻ Treg cells can also prevent colitis, myositis, hepatitis and glomerulonephritis following transfer into NOD-SCID mice (Alyanakian *et al.*, 2003).

With respect to the lung, it has been demonstrated that AM can render infiltrating CD4⁺ T cells anergic, which is accompanied by impaired IL-2 signalling (Strickland *et al.*, 1996a; Strickland *et al.*, 1993). It has also been shown, using FoxP3^{gfp}⁺ mice, that CD4⁺FoxP3^{gfp}⁺CD25^{low} T cells accumulated in the lungs of uninfected as well as chronically *M. tuberculosis*-infected mice (Fontenot *et al.*, 2005b). Furthermore, it was demonstrated that Treg cells from the lung parenchyma of mice infected with respiratory syncytial virus express less CD25 than their counterparts in the draining lymph nodes (Fulton *et al.*, 2010).

The aim of this study was to examine the role of CD4⁺FoxP3⁺CD25⁻ Treg cells in maintaining tolerance and immunity to a pathogen in the lungs, using a murine model of respiratory infection with *B. pertussis*. I demonstrate that the majority of FoxP3⁺ T cells in the lungs of naïve mice do not express surface CD25. CD4⁺FoxP3⁺CD25⁻ T cells isolated from naïve murine lungs must be pre-activated in order to suppress CD4⁺ effector T cells *in vitro* and this suppression is not dependent on cell contact, but does require IL-10. In addition, the numbers of IL-10-secreting CD4⁺FoxP3⁺CD25⁻ T cells significantly increase in the lungs throughout the course of infection with *B. pertussis*. Depletion of CD25⁺ cells prior to challenge with *B. pertussis* significantly accelerated the rate of bacterial clearance, but only in IL-10^{-/-} mice.

3.2 Results

3.2.1 CD4⁺FoxP3⁺CD25⁻ cells are the dominant Treg cell population in the lungs of naïve mice

A number of studies have reported suppressive activity in CD4⁺CD25⁻ T cells (Annacker *et al.*, 2001; Lehmann *et al.*, 2002; Ono *et al.*, 2006); however the role of such cells in the lung has not been investigated. The present study sought to determine the role of CD4⁺FoxP3⁺CD25⁻ Treg cells in the lungs of mice, based on preliminary novel findings that CD4⁺FoxP3⁺ cells in the lung are predominantly CD25⁻. Murine cells isolated from lung were stained with antibodies specific for CD4, CD25 and FoxP3, and analysed by flow cytometry.

Approximately 6% of CD4⁺ T cells in the lung express FoxP3 (Fig. 3.1A) and the majority (62%) of these CD4⁺FoxP3⁺ cells do not express CD25 (Fig. 3.1B). However, in the spleen, the majority of CD4⁺FoxP3⁺ cells expressed CD25 (approximately 80%) (Fig. 3.1B). CD25 expression in the lung CD4⁺ population was approximately 7% (Fig. 3.1C). The percentage of CD4⁺FoxP3⁺CD25⁻ T cells is significantly ($p < 0.01$) greater in the lungs of naïve mice when compared with the spleen (Fig. 3.1D). I next investigated whether other mucosal tissues, especially those which are associated with tolerance induction and immune homeostasis (Crispe, 2009; Hill and Artis, 2010), also contained a higher frequency of CD4⁺FoxP3⁺CD25⁻ T cells, when compared with peripheral lymphoid tissues. When analysed alongside the data in Fig. 3.1D, it was revealed that the percentage of CD4⁺FoxP3⁺CD25⁻ T cells is significantly greater in the liver, colon and lungs of naïve mice when compared with the spleen and the cervical and thoracic lymph nodes (CLN and TLN) (Fig. 3.2A and 3.2B). These results identify a previously uncharacterised population of CD4⁺FoxP3⁺ T cells resident in mucosal tissues, which are characterised by lack of CD25 expression.

3.2.2 Surface phenotype of CD4⁺FoxP3⁺CD25⁻ and nTreg cells isolated from the lungs and spleens of naïve mice

Several studies have used different surface markers to distinguish nTreg cells from other CD4⁺ populations. For example, CD4⁺ T cells expressing low levels of CD45RB have been shown

to have regulatory function (Luke *et al.*, 2001) and CD45RB and FoxP3 expression are inversely correlated (Luke *et al.*, 2006). A similar inverse relationship is observed between FoxP3 and CD127 expression (Liu *et al.*, 2006; Seddiki *et al.*, 2006a). In addition, studies have confirmed that nTreg cells express CD39 and CD73, which are nucleotidases involved in converting ATP to AMP and AMP to adenosine, respectively (Bours *et al.*, 2006; Wang and Guidotti, 1996). The present study investigated the phenotype of lung-derived CD4⁺FoxP3⁺CD25⁻ Treg cells and whether the observed phenotype may predict their function *in vivo*. Cells isolated from murine lung and spleen were stained with antibodies against a variety of phenotypic and functional markers (Fig. 3.3).

CD4⁺FoxP3⁺CD25⁻ Treg cells isolated from lungs and spleens of naïve mice expressed the activation markers CD44 and CD69, however, they did not express the cell surface marker CD45RB, and did not express high levels of CD40L (CD154) (Fig. 3.3A, Fig. 3.3B). As T cells divide they lose expression of CD40L. Lack of CD45RB expression is also an indicator of murine memory cells (Trowbridge and Thomas, 1994). The results indicate that the CD4⁺FoxP3⁺CD25⁻ T cell subset are not naïve T cells, but instead represents a population of activated, antigen-experienced memory T cells. CD127 expression was also very low in this population of cells (Fig. 3.3A, Fig. 3.3B), in keeping with data showing an inverse relationship between levels of FoxP3 and CD127 (Banham, 2006).

Both CD39 and CD73 were expressed by CD4⁺FoxP3⁺CD25⁻ T cells (Fig. 3.3). These results suggest that CD4⁺FoxP3⁺CD25⁻ Treg cells may have a functional role in maintaining the immunosuppressive environment in the lung and preventing tissue damage. Expression of other markers associated with Treg cells was examined. In the lung, CD4⁺FoxP3⁺CD25⁻ T cells had high levels of inducible co-stimulator (ICOS), GITR and CTLA-4, however, GITR expression was still significantly lower than that on CD4⁺FoxP3⁺CD25⁺ T cells (Fig. 3.3B). In the spleen, CD4⁺FoxP3⁺CD25⁻ T cells expressed very significant amounts of ICOS compared with nTreg cells, and the expression of CTLA-4 was significantly lower than on nTreg cells (Fig. 3.3B). Representative dot plots and average values of the median fluorescence intensities of the various surface markers are detailed in Fig. 3.3C and Fig. 3.3D, respectively.

FoxP3 remains the “gold standard” for identification of functional, naturally occurring Treg cells in mice. Indeed, it has been shown that CD4⁺FoxP3⁺ T cells are not exclusively CD25⁺, and that the frequency of CD4⁺FoxP3⁺CD25⁻ T cells increases with age in mice (Nishioka *et al.*, 2006). It has been demonstrated elsewhere that CD25 expression is labile on Treg cells, and the loss and regain of this surface marker does not affect their function (Zelenay *et al.*, 2005). It has been postulated that Treg cells lose or gain CD25 to maintain homeostasis (Zelenay *et al.*, 2005). However, this mechanism appears to be strictly regulated as it has also been shown that only a limited number of CD25⁻ T cells can convert to a nTreg cell phenotype *in vivo* (Curotto de Lafaille *et al.*, 2004).

In summary, this study suggests that lung-derived CD4⁺FoxP3⁺CD25⁻ Treg cells are a mature, activated cell population, which express several markers indicative of a regulatory cell population, and have a very similar phenotypic profile to CD4⁺FoxP3⁺CD25⁺ nTreg. The next logical step was to investigate their suppressive function against effector T cells *in vitro*.

3.2.3 CD4⁺FoxP3⁺CD25⁻ Treg cells must be pre-activated in order to suppress responder cells

CD4⁺FoxP3⁺CD25⁻GITR⁺ have been reported to suppress in murine organ transplantation (Nishimura *et al.*, 2004) and inflammatory bowel disease (Uraushihara *et al.*, 2003). However, while it appears that CD25 expression is not required for Treg cells to function, it has also been shown that the *in vitro* suppressive activity of CD4⁺FoxP3⁺CD25⁻ Treg cells is weaker than that of nTreg cells (Annacker *et al.*, 2001; Lehmann *et al.*, 2002; Stephens and Mason, 2000).

As mentioned in section 3.2.2, CD4⁺ T cells which express low levels of CD45RB have been reported to have regulatory function (Luke *et al.*, 2001) and CD45RB and FoxP3 expression are inversely correlated (Luke *et al.*, 2006). CD4⁺CD45RB^{low} T cells have been shown to be mainly comprised of FoxP3⁺ nTreg cells in naïve mice and can be used to ameliorate colitis in a mouse model, while CD4⁺CD45RB^{high} T cells represent FoxP3⁻ effector cells capable of inducing colitis in immune compromised mice. In order to examine the ability of CD4⁺FoxP3⁺CD25⁻ cells to suppress

effector T cell responses *in vitro*, lung-derived CD4⁺CD45RB^{low}CD25⁻ Treg cells were used as a source of CD4⁺FoxP3⁺CD25⁻ Treg cells (Appendix, Fig 8.1).

As CD45RB expression is progressively lost each time a cell divides, it is difficult to differentiate between “positive” and “negative”, as can be seen in Figures 8.1 and 8.2. Rather, expression of CD45RB on cells is classed as “low/negative” or “high”. Strict gating based on fluorescence minus one (FMO) staining controls was applied when purifying CD4⁺CD45RB^{low}CD25⁻ T cells (Appendix, Fig 8.1). This resulted in low cell yields, but we were confident that the population of interest was as pure as possible, based on such strict gating strategies. A proportion of the sorted CD4⁺CD45RB^{low}CD25⁻ cells were tested for FoxP3 expression after each sort; these cells were routinely greater than 98% FoxP3 (Fig. 3.4B).

CD4⁺CD45RB^{high}CD25⁻ naïve effector T (Teff) cells were purified from the spleens of naïve mice and activated with anti-CD3 (2 µg/ml) and irradiated splenic APC (5 x 10⁵), in the presence or absence of equal numbers of lung-derived CD4⁺CD45RB^{low}CD25⁻ Treg cells. Again, strict gating based on FMO staining controls was applied when purifying CD4⁺CD45RB^{high}CD25⁻ T cells (Appendix, Fig 8.2). After 48 hours of culture, cells were replenished with fresh medium containing ³H-thymidine and cells were harvested 24 hours later. The results showed that Treg cells were unable to suppress the proliferation of Teff cells at a 1:1 ratio (Fig. 3.4A).

Studies have shown that CD25⁻ Treg cells must be activated before they can exert suppressive effects *in vitro* (Caramalho *et al.*, 2003; Zelenay *et al.*, 2005). It is thought that CD25⁻ Treg cells might have a higher threshold of activation than nTreg cells and therefore require stronger activation stimuli. Zelenay and colleagues have postulated that CD4⁺FoxP3⁺CD25⁻ cells are part of a “reservoir” of Treg cells at the steady state which can upregulate CD25 when required to maintain homeostasis (Zelenay *et al.*, 2005). It was shown that these cells are selectable by IL-2 *in vitro*, presumably upregulate CD25 in response to this cytokine and consequently gain effective suppressor function. Studies in humans have demonstrated that IL-2 promotes survival and the

suppressive function of Treg cells *in vitro*, and enhances expression of FoxP3 and CD25 (Yates *et al.*, 2007).

In order to examine the ability of pre-activated CD4⁺FoxP3⁺CD25⁻ cells to suppress effector T cell responses *in vitro*, CD4⁺CD45RB^{low}CD25⁻ Treg cells were purified from naïve murine lungs and activated for 5 days with immobilised anti-CD3 (1 µg/ml), recombinant IL-2 (20 U/ml) and irradiated splenic APC (5 x 10⁵). After 5 days CD4⁺CD45RB^{high}CD25⁻ Teff cells were sorted from naïve murine spleens and activated with immobilised anti-CD3 (10 µg/ml) and irradiated splenic APC (5 x 10⁵) in the presence or absence of pre-activated CD4⁺CD45RB⁻CD25⁻ Treg cells. After 48 hours supernatants were removed and the concentrations of IL-10, IFN-γ and TNF-α were quantified by ELISA. Cell proliferation was determined after 72 hours by ³H-thymidine incorporation. CD4⁺CD45RB^{low}CD25⁻ Treg cells significantly suppressed (p<0.001) proliferation of Teff cells when cultured at a 1:1 ratio (Fig. 3.5A). In addition, co-culture of Treg and Teff cells resulted in significantly reduced IFN-γ (p<0.01) (Fig. 3.5A). TNF-α production was not affected (Fig. 3.5A). Activated Treg cells produced slightly more IL-10 than activated Teff cells, and IL-10 production increased when Treg cells were cultured with T eff cells, but was not significant. TGF-β₁ was not detected in these cultures.

The suppressive capacity of lung CD4⁺CD25⁻CD45RB^{low} Treg cells was confirmed by examining CFSE loss on Teff cells *in vitro* (Fig. 3.5B). Freshly isolated CD4⁺CD25⁻CD45RB^{high} Teff cells were labelled with 0.5 µM CFSE and subsequently co-cultured with pre-activated CD25⁻ Treg cells (1:1) and activated in the presence of 10 µg/ml immobilised anti-CD3 and 5 x 10⁵ irradiated APC for 5 days. Cells were subsequently stained with antibodies directed against surface CD4 and CD25, and CFSE loss was determined by flow cytometry. As expected, non-activated control effector T cells did not proliferate to any great extent, whereas activated effector T cells alone did (Fig. 3.5B). However, pre-activated CD25⁻ Treg cells suppressed the proliferation of activated effector T cells (solid line) when compared with activated control cells (Fig. 3.5B).

In these experiments, FoxP3⁺ cell contamination of sorted CD4⁺CD45RB^{high}CD25⁻ Teff cells was less than 2% (Fig. 3.4B). While co-culture and activation of Teff cells in the presence of pre-activated CD4⁺CD45RB^{low}CD25⁻ Treg cells at a 1:1 ratio resulted in reduced proliferation, the suppressive capacity of these cells was not as potent as nTreg cells. CD25⁺ nTreg cells have been shown to suppress Teff cells *in vitro* at a ratio of 1:4 or less (Takahashi *et al.*, 1998). Therefore, the suppression observed when sorted CD4⁺CD45RB^{low}CD25⁻ Treg cells were activated in the presence of Teff cells was unlikely to be due to contaminating nTreg cells.

It was unlikely that the reduced suppressive capacity was due to contaminating Teff cells during pre-activation either. The majority of sorted cells were routinely >98% FoxP3⁺ (Fig. 3.4B). Nonetheless, the effect of “spiking” the pre-activation culture with Teff cells was investigated. CD4⁺CD25⁻CD45RB^{high} Teff cells were purified at the same time as CD4⁺CD25⁻CD45RB^{low} Treg cells. Treg cells were activated for 3 days with immobilised anti-CD3 (1 µg/ml), recombinant IL-2 (20 U/ml) and irradiated splenic APC (5 x 10⁵), in the presence or absence of different ratios of Teff cells. Addition of Teff cells at ratios of 0.5:99.5, 1:99, 5:95 and 10:90 Teff:Treg did not increase proliferation levels above that of Treg alone (Fig. 3.6).

Zelenay *et al* have proposed that pre-activation results in upregulation of CD25 on CD4⁺FoxP3⁺CD25⁻ Treg cells (Zelenay *et al.*, 2005). Phenotypic analysis of the cells 24 hours post-stimulation confirmed that approximately 50% of the cells now express CD25 (Fig. 3.7). Some of the cells appeared to be FoxP3⁻; however this might be explained by the presence of irradiated APC in the culture.

The results show that pre-activated CD4⁺FoxP3⁺CD25⁻ Treg cells upregulate CD25, and can suppress proliferation of effector T cells *in vitro*. IL-10 may be responsible for this suppression, as activated Treg cells could produce this suppressive cytokine.

3.2.4 Suppression by pre-activated CD25⁻ Treg cells is cell-contact independent

There are a number of possible mechanisms whereby Treg cells can suppress responder T cells, including direct cell-cell contact and soluble factors (Miyara and Sakaguchi, 2007; Vignali, 2008). The failure of Treg cells to inhibit the suppression of target cells when they are separated by a semi-permeable membrane usually indicates a requirement for *in vitro* cell-cell contact in order for Treg cells to function (Takahashi *et al.*, 1998; Thornton and Shevach, 1998).

To examine whether the suppression of Teff cells seen in Section 3.2.3 was mediated by cell-contact or soluble factors, CD4⁺CD45RB⁻CD25⁻ Treg cells were sorted from lung tissue and pre-activated with immobilised anti-CD3 (1 µg/ml), recombinant IL-2 (20 U/ml) and irradiated splenic APC (5 x 10⁵). Treg and Teff cells were co-cultured but separated via a semi-permeable membrane. Each population above and below the semi-permeable membrane was activated with immobilised anti-CD3 (10 µg/ml) and irradiated splenic APC (5 x 10⁵). While Treg cells and Teff could not directly contact each other, soluble factors were able to cross the 0.4 µm membrane. As a positive control, Treg and Teff were also activated and cultured together. Supernatants were harvested after 48 hours to measure cytokine production, and proliferation was measured by ³H-thymidine incorporation after 72 hours.

The results show that Treg cells were able to significantly suppress ($p < 0.001$) proliferation of Teff cells *in vitro* in the absence of cell-cell contact (Fig. 3.8). Furthermore, the observed suppression was similar to that when Treg and Teff cells were activated and cultured together. IFN- γ production was also significantly suppressed despite the absence of cell-cell contact ($p < 0.01$) compared with activated Teff cells alone (Fig. 3.8). No change was observed in TNF- α or IL-10 concentrations when the two populations were separated (Fig. 3.8). TGF- β_1 was not detected. The data suggests that CD4⁺FoxP3⁺CD25⁻ Treg cells are a population of Treg cells which can suppress target T cells without the absolute requirement for direct cell-cell contact.

3.2.5 IL-10 is required for CD25⁻-induced suppression

As shown in Section 3.2.4, the CD4⁺FoxP3⁺CD25⁻ Treg cells are able to suppress their target cells in the absence of cell contact. This suggests they use a soluble mediator to suppress proliferation of effector T cells. IL-10 and TGF- β_1 are well-characterised cytokines with anti-inflammatory properties and have been associated with suppressive functions of both natural Treg cells and adaptive Treg cell populations (Gorelik *et al.*, 2002; Gorham *et al.*, 1998; Hawrylowicz, 2005; Moore *et al.*, 2001). As TGF- β_1 was not detected in activated CD4⁺FoxP3⁺CD25⁻ Treg cells, and pre-activated Treg cells produced IL-10 *in vitro*, this study investigated the effect of blocking the IL-10 receptor (IL-10R), thus preventing IL-10 from signalling via APC, Treg cells or Teff cells.

CD4⁺CD25⁻CD45RB^{low} Treg cells were sorted from lung tissue and pre-activated for five days with immobilised anti-CD3 (1 μ g/ml), recombinant IL-2 (20 U/ml) and irradiated splenic APC (5×10^5). Pre-activated Treg cells and freshly isolated Teff cells were co-activated with immobilised anti-CD3 (10 μ g/ml) and irradiated splenic APC (5×10^5) in the absence or presence of anti-IL-10 receptor (IL-10R; 10 μ g/ml) antibody. Blocking the IL-10R significantly restored the proliferative capacity of Teff cells ($p < 0.0001$) co-cultured with Treg cells when compared with cells incubated with an irrelevant isotype-matched antibody (Fig. 3.9). There was no significant difference in IL-10 concentrations in the supernatant in the presence of the blocking antibody (Fig. 3.9), but the concentration of IFN- γ and TNF- α were significantly enhanced ($p < 0.0001$ and $p < 0.01$, respectively) (Fig. 3.9).

These results indicate that CD4⁺FoxP3⁺CD25⁻ Treg cells might use IL-10 to suppress effector T cell responses *in vitro*. Collectively, the results of the *in vitro* studies so far have indicated a suppressive role for lung CD4⁺FoxP3⁺CD25⁻ Treg cells, which appear to have a higher threshold of activation than nTreg cells. However, once they are activated, they upregulate CD25 and can suppress Teff cells, and this suppression appears to be mediated in part by IL-10. Nonetheless, they do not suppress as effectively as nTreg cells, as they suppressed at a 1:1 ratio of

Treg cells: T_H17 cells, while it has been reported that freshly isolated nTreg cells can suppress T_H17 cells at a 1:4 ratio or less (Takahashi *et al.*, 1998). I next examined the possible role of these cells in infection.

3.2.6 Kinetics of CD25⁺ Treg cells in *B. pertussis* infection

The *B. pertussis* respiratory model of infection was used to investigate the role of CD4⁺FoxP3⁺CD25⁺ T cells *in vivo*. Mice were infected with *B. pertussis* aerosol, and lung cells were examined at weekly intervals post-challenge. In order to quantify the frequencies of antigen-specific Treg cells, mice were injected i.p. with 4×10^9 HKBP at the relevant time-points post-challenge. This induces a recall response to the bacterial antigens *in vivo*. 4 hours after injection of HKBP, cells were isolated from lung tissue and the phenotype and intracellular cytokine expression profile in CD4⁺FoxP3⁺CD25⁺ T cells was determined.

The percentages of CD4⁺CD25⁺ or CD4⁺FoxP3⁺ cells remained relatively constant during the course of *B. pertussis* infection (Fig. 3.10A and B). The percentage of CD4⁺FoxP3⁺CD25⁺ cells was low in the lungs of naïve mice. 7 days post challenge the percentage increased to 21%, peaking at 24% 14 days post challenge. The frequencies decreased to pre-infection levels at subsequent time points (Fig. 3.10C)

Both CD4⁺FoxP3⁺CD25⁺ and CD4⁺FoxP3⁺CD25⁻ cells produced IL-10 during *B. pertussis* infection (Fig. 3.11A); with its intracellular expression peaking in the CD25⁻ cells and CD25⁺ cells 14 days post-challenge (Fig. 3.11B). These results suggest that CD4⁺FoxP3⁺CD25⁻ cells are able to produce IL-10 upon challenge with *B. pertussis*. The frequencies of CD4⁺FoxP3⁺CD25⁺ cells increased during the infection, peaking at 24% 14 days post-challenge. These cells may be derived from CD4⁺FoxP3⁺CD25⁻ which have upregulated CD25 upon stimulation, or they may be infiltrating nTreg cells. It has already been shown that CD25⁻ Treg cells upregulate CD25 upon activation *in vitro* (Fig. 3.7). However, it is still difficult to tell whether the enhanced levels of CD4⁺FoxP3⁺CD25⁺ Treg cells in this experiment are a consequence of CD25 upregulation by resident CD4⁺FoxP3⁺CD25⁻ Treg cells, or are infiltrating cells.

The next question regarding the role of CD4⁺FoxP3⁺CD25⁻ Treg cells *in vivo* is whether they can control the immune response to *B. pertussis* when nTreg cells are removed.

3.2.7 Depleting CD25⁺ T cells in IL-10-deficient mice enhances immunity to *B. pertussis* *in vivo*

The aim of this study was to remove CD25⁺ nTreg cells, leaving the CD25⁻ Treg cells and determine whether they can control infection in the absence of nTreg cells. Mice received a depleting antibody against CD25 i.p. 24 hours prior to aerosol challenge with *B. pertussis*, which reduces the frequency of CD4⁺CD25⁺ T cells in spleen from >5% to <1% (Fig 3.12). It has been reported that this treatment eliminates CD25⁺ T cells *in vivo* for at least a week (Couper *et al.*, 2007). The peak of activated (CD25⁺) Teff infiltration into the lungs during *B. pertussis* infection is between 7 and 14 days post-challenge, so to avoid the possibility of removing the activated Teff cells, only one injection was administered prior to bacterial challenge.

4 hours, 7, 14 and 21 days post-challenge bacterial levels in the lungs were determined by performing CFU counts. There was no difference in the CFU counts post-challenge in mice treated with anti-CD25 depleting antibody compared with mice that received an isotype control antibody (Fig. 3.13A). The results suggest that the CD4⁺FoxP3⁺CD25⁻ cell population is able to regulate immunity to infection in the lung and can function effectively in the absence of nTreg cells.

The *in vitro* assays (Figs. 3.5, 3.8 and 3.9) and *in vivo* kinetics studies (Fig. 3.10) have suggested CD4⁺FoxP3⁺CD25⁻ Treg cells suppress via IL-10. Therefore, I examined the effect of depleting CD25⁺ nTreg cells in IL-10-deficient mice. It was hypothesised that mice lacking both nTreg cells and IL-10-producing Treg cells would have completely impaired immune regulation and would therefore eradicate the pathogen more quickly than wild-type (WT) mice or control IL-10^{-/-} mice. Indeed, depletion of CD25⁺ cells in IL-10^{-/-} mice significantly enhanced ($p < 0.01$) bacterial clearance compared with controls (Fig. 3.13C). These mice also had detectable IgG_{2a} in serum from as early as 7 days post-challenge, suggesting an enhanced Th1 immune response was functioning early to eradicate the infection (Fig. 3.14).

7, 14 and 21 days post challenge splenocytes (2×10^6 /ml) from infected mice were cultured *in vitro* with FHA from *B. pertussis* (4 μ g/ml), HKBP (1 $\times 10^5$ /ml), anti-CD3 and PMA (5 μ g/ml and 25 ng/ml) or medium for four days. Culture with HKBP resulted in significant production of IFN- γ by cells from IL-10 $^{-/-}$ mice as early as 7 days post-challenge (Fig. 3.15A), and was this was sustained at days 14 and 21 post-challenge. 7 days post-challenge, IFN- γ was slightly enhanced in cells from CD25-depleted WT mice, but was not significant (Fig. 3.15A). However, splenocytes from these mice did produce significantly higher levels of IFN- γ in response to HKBP at the later time points compared to unstimulated controls. Enhanced FHA-specific IFN- γ production was observed in cells from CD25-depleted WT mice (Fig. 3.15B, C). Antigen-specific IFN- γ was produced by spleen cells from undepleted WT mice from day 14 post-challenge (Fig. 3.15B, C). Anti-CD3 and PMA stimulation promoted strong IFN- γ production in mice from all experimental groups at all time points post-challenge (Fig. 3.15).

Cells from CLN were also cultured with *B. pertussis* antigen, but no antigen-specific IFN- γ was detected in any group at any time point (Fig. 3.16). However, strong IFN- γ production was detected in response to anti-CD3 and PMA stimulation (Fig. 3.16).

Flow cytometry analysis of the restimulated splenocytes found a higher frequency of antigen specific IFN- γ^+ lymphocytes in the spleens of CD25-depleted IL-10 $^{-/-}$ mice (Figs. 3.17A, B) compared with isotype controls 21 days post-challenge, and was significantly higher than controls in response to HKBP stimulation ($p < 0.05$). Cells from CLN did not show a difference in antigen-specific IFN- γ^+ cells (Fig. 3.18B), although stimulation with PMA and anti-CD3 (Fig. 3.18A) revealed significantly higher frequencies of IFN- γ^+ lymphocytes in the CD25-depleted IL-10 $^{-/-}$ mice ($p < 0.01$).

These results indicate that two immunomodulatory arms might be involved in regulating immunity to *B. pertussis* in the lung, namely CD25 $^+$ nTreg and IL-10-secreting Treg cells, which include CD4 $^+$ FoxP3 $^+$ CD25 $^-$ Treg cells. Depletion of both nTreg and IL-10-secreting Treg cells

completely impairs immunoregulation in *B. pertussis* infection in the lung, resulting in significantly accelerated Th1-mediated clearance of the pathogen.

Further evidence for two immunoregulatory arms in the lung was revealed through analysis of cytokines in the lung homogenate of infected mice. Since significant differences in CFU were observed at day 7 post-challenge, it was decided to investigate whether the CD25-depleted IL-10^{-/-} mice produced increased pro-inflammatory cytokines at this time point due to lack of immune regulation. Only IL-10^{-/-} mice had detectable lung IFN- γ , and it was slightly elevated in the CD25-depleted population (Fig. 3.19A). CD25-depleted WT mice produced significantly more IL-10 ($p < 0.001$) than controls (Fig. 3.19B), suggesting that in the absence of CD25⁺ Treg cells, the IL-10-producing CD25⁻ population might compensate for this and prevent excessive Th1 immunity, as no IFN- γ was detected in the lungs of these mice. Interestingly, CD25-depleted WT mice had significantly higher concentrations of the pro-inflammatory cytokines IL-17 ($p < 0.001$), TNF- α ($p < 0.01$) and IL-1 β ($p < 0.05$) in lungs 7 days post-challenge (Fig. 3.20).

Presumably the CD25⁺ Treg cells in undepleted IL-10^{-/-} mice are responsible for regulating immunity to *B. pertussis*, as despite having detectable IFN- γ in their lungs they cleared the infection at the same rate as WT controls. In the same vein, removing nTreg cells from WT mice results in enhanced production of IL-10, which can regulate the protective Th1 response. However, removing CD25⁺ Treg cells in IL-10^{-/-} mice disables both regulatory mechanisms and allows an excessive IFN- γ -mediated Th1 immune response to proceed, resulting in accelerated bacterial eradication.

3.3 Discussion

The present study has identified a novel population of Treg cells, which are resident in the lungs of naïve mice and are distinguished from nTreg cells by their lack of CD25 expression. These CD4⁺FoxP3⁺CD25⁻ Treg cells have regulatory capacity *in vitro* but are not as effective as nTreg cells at suppressing effector T cell proliferation. They also have a higher threshold of activation than nTreg cells, and are less potent suppressors than nTreg cells, as they suppressed at a 1:1 ratio of Treg cells:Teff cells, while it has been reported that freshly isolated nTreg cells can suppress Teff cells at a 1:4 ratio or less (Takahashi *et al.*, 1998). *In vitro*, CD4⁺FoxP3⁺CD25⁻ Treg cells appear to suppress via IL-10. These cells have a mature phenotype which suggests that they are antigen-experienced memory cells. CD4⁺FoxP3⁺CD25⁻ Treg cells are also present in the liver and gut, so there might be a physiological reason for the accumulation of these cells in classically immunoregulatory environments. *In vivo* experiments using the *B. pertussis* model of infection have also shown that CD4⁺FoxP3⁺CD25⁻ Treg cells produce IL-10 in response to bacterial challenge. Furthermore, removal of CD25⁺ nTreg cells prior to infection with *B. pertussis* does not affect the bacterial load in mice. These findings suggest that CD4⁺FoxP3⁺CD25⁻ Treg cells function in lung immunology in addition to resident nTreg cells.

The results show that the dominant regulatory T cell population in the naïve lung lacks CD25 expression, which is in direct contrast to those in the spleen. As the lung encounters a wide variety of antigen from the environment (Holt *et al.*, 2008), it is reasonable to expect a tightly regulated immunoregulatory system in order to avoid unnecessary tissue damage. The results of the present study are therefore surprising, as CD4⁺FoxP3⁺CD25⁺ nTreg cells were the minority FoxP3⁺ population in the lung, and the CD4⁺FoxP3⁺CD25⁻ Treg cells are weaker suppressor than nTreg cells, suppressing at a 1:1 ratio compared with a 1:4 ratio or less, as reported in the literature (Takahashi *et al.*, 1998; Zelenay *et al.*, 2005). However, CD4⁺ effector T cells are constantly recruited to the lung and are subsequently exposed to antigen, so it might be possible that the lung microenvironment induces Treg cells from these infiltrating effector T cells, which are CD25⁻.

It is possible that the dominant CD4⁺FoxP3⁺CD25⁻ Treg cell population could be unique to mucosal surfaces. In order to investigate this possibility, cells from the liver and colon were examined. Like the lung, these organs are associated with active immunosuppression (Boden and Snapper, 2008; Tiegs and Lohse, 2009). Indeed, CD4⁺FoxP3⁺ cells from lung, liver and colon had significantly higher percentages of CD4⁺FoxP3⁺CD25⁻ T cells than in the spleen. While CLN and TLN also contained higher frequencies of these cells than the spleen, the percentages were lower than in the mucosal tissues, and might be attributed to their lung-draining locations.

CD4⁺FoxP3⁺CD25⁻ cells isolated from the lungs of naïve mice bear a memory/activated phenotype associated with high expression of both CD44 and CD69. The expression levels of these markers were similar for both CD25⁻ and CD25⁺ cells, indicating that both subsets are antigen experienced, with nTreg cells encountering antigen in the thymus. The origin of CD4⁺FoxP3⁺CD25⁻ Treg cells remains to be identified. In keeping with published data on Treg cells (Banham, 2006; Powrie *et al.*, 1994a), CD4⁺FoxP3⁺CD25⁻ cells isolated from naïve mouse lung and spleen expressed little or no CD45RB or CD127. Both CD25⁺ and CD25⁻ cells in lung and spleen also expressed similar levels of CD40L, but this was not especially high (<50%), which indicates that these cells are not naïve and have undergone cell division. Again this correlates with published data on Treg cells (Kasprowicz *et al.*, 2003).

CD4⁺FoxP3⁺CD25⁻ Treg cells isolated from the lung had reduced GITR and CTLA-4 surface and intracellular expression than their CD4⁺FoxP3⁺CD25⁺ counterparts. Expression of these markers on CD4⁺CD25⁺ Treg cells has been widely reported as being crucial for their function (McHugh *et al.*, 2002; Sansom and Walker, 2006; Shevach and Stephens, 2006; Shimizu *et al.*, 2002). The data is consistent with studies in patients with untreated new-onset lupus. CD4⁺FoxP3⁺CD25⁻ Treg cells from lupus patients have decreased GITR and CTLA-4 on their surface compared with CD4⁺FoxP3⁺CD25⁺ Treg cells (Yang *et al.*, 2009). Lupus is associated with an abnormal increase in the percentage of CD4⁺FoxP3⁺CD25⁻ Treg cells (Zhang *et al.*, 2008). The percentage of these cells correlates with disease activity, but treatment with glucocorticoids and cyclophosphamide causes a decrease in their frequency (Zhang *et al.*, 2008). Furthermore,

investigation of CD4⁺CD25⁻CD127⁻ Treg cells from children with type 1 diabetes demonstrated a decrease in the mRNA expression of GITR and CTLA-4, but no change in overall frequencies of Treg cells. It is possible that these markers are important for optimal nTreg cell function but less crucial for the function of CD4⁺FoxP3⁺CD25⁻ Treg cells, at least in humans.

CD4⁺FoxP3⁺CD25⁻ and CD4⁺FoxP3⁺CD25⁺ Treg cells isolated from the lung express more ICOS than their counterparts isolated from the spleen. An inverse relationship between ICOS and CTLA-4 was evident in CD25⁻ and CD25⁺ Treg populations from spleen, as has been previously reported (Riley *et al.*, 2001). However, this relationship was not observed in the lung Treg cells, as both populations strongly expressed ICOS and CTLA-4. Studies have shown that ICOS is crucial for the induction of peripheral tolerance (Akbari *et al.*, 2002; Tuettenberg *et al.*, 2009). Specifically, ICOS appears to have an important role in mucosal tolerance (Akbari *et al.*, 2002; Bonhagen *et al.*, 2003; Miyamoto *et al.*, 2005), which might explain why this study detected such high expression of the molecule in the lung but not the spleen.

The expression of ICOS on CD4⁺FoxP3⁺CD25⁻ Treg cells but not on CD4⁺FoxP3⁺CD25⁺ Treg cells in the spleen provides further evidence that these two Treg cell populations might function in different ways. Indeed, it has been previously reported that human FoxP3⁺ cells in both the thymus and periphery can be either ICOS⁺ or ICOS⁻, and that both of these populations have distinct functions, with FoxP3⁺ICOS⁺ cells using IL-10 and TGF- β_1 to suppress DCs and T cells, respectively, and FoxP3⁺ICOS⁻ cells using TGF- β_1 only (Ito *et al.*, 2008). Furthermore, it has recently been reported that IL-10 acts on Treg cells to maintain FoxP3 expression in the gut (Murai *et al.*, 2009). The IL-10 secreted here is derived from lamina propria macrophages. Given that IL-10 is constitutively present in healthy lungs (Bonfield *et al.*, 1995; Fernandez *et al.*, 2004), and ICOS expression is associated with IL-10 production, this provides further support for the hypothesis that ICOS might be required for Treg cells to function in the lung.

The expression of the ectonucleotidases CD39 and CD73 on the surface of CD4⁺FoxP3⁺CD25⁻ Treg cells and CD4⁺FoxP3⁺CD25⁺ Treg cells in the lung and spleen was also

investigated in this study. Both enzymes were strongly expressed by each population in both lung and spleen, in keeping with published data (Deaglio *et al.*, 2007; Eckle *et al.*, 2007). Overall, a similar pattern of expression was observed, with CD39 being more strongly expressed than CD73 in all conditions. Additionally, CD4⁺FoxP3⁺CD25⁺ Treg cells expressed enhanced levels of both enzymes in lung and spleen compared with CD4⁺FoxP3⁺CD25⁻ Treg cells. Except for CD73 expression in the lung, these differences between the two populations were not significant.

It has been previously reported that freshly isolated CD4⁺CD25⁻CD45RB^{low} Treg cells do not suppress responder T cells *in vitro* (Caramalho *et al.*, 2003). However, the Treg cells in that study were purified from spleen and lymph node, not lungs. Nonetheless, our studies on freshly isolated lung-derived CD4⁺CD25⁻CD45RB^{low} cells confirm that they cannot suppress responder T cells *in vitro*. It was hypothesised that CD4⁺FoxP3⁺CD25⁻ Treg cells from the lung must be activated before they can suppress the proliferation of responder T cells. Indeed, Zelenay and colleagues have also shown that CD4⁺CD25⁻CD45RB^{low} Treg cells isolated from the lymph nodes of naïve mice must be activated with anti-CD3 and IL-2 before they can suppress (Zelenay *et al.*, 2005). However, the suppressive effect of these cells is still weaker than that observed with freshly isolated nTreg cells (Zelenay *et al.*, 2005). Our experiments demonstrated that purified lung-derived CD4⁺CD25⁻CD45RB^{low} Treg cells, which had been activated for 5 days with anti-CD3 and cultured with IL-2, were able to significantly suppress the proliferation and IFN- γ production of responder cells at a 1:1 ratio. Unfortunately, it was not possible to perform a titration to test whether these Treg cells could suppress at other ratios of Treg cells:Teff cells, as Treg cell numbers were a limiting factor. These assays contained 1×10^4 Treg cells and 1×10^4 Teff cells, which explains why the observed cpm values were relatively low. For these reasons, it was decided to use a 1:1 ratio of Treg cells:Teff cells for subsequent experiments.

It was also shown that activated CD4⁺CD25⁻CD45RB^{low} Treg cells can produce the anti-inflammatory cytokine IL-10, which is consistent with the findings of this study (Zelenay *et al.*, 2005).

CD4⁺CD25⁻CD45RB^{low} cells isolated from the lungs of naïve mice upregulated CD25 *in vitro* in response to the pre-activation stimuli. This finding is in accordance with those of Zelenay *et al*, who have proposed that CD4⁺FoxP3⁺CD25⁻ cells comprise a “reservoir” of Treg cells which can upregulate CD25 to maintain homeostasis (Zelenay *et al.*, 2005). These cells can also lose and regain CD25 expression with no apparent functional implications (Zelenay *et al.*, 2005). With regard to the lung, such a “reservoir” of under-activated cells might represent a practical way to maintain immune homeostasis. Such Treg cells would be present in the lung, but would be unable to function in the absence of IL-2 – that is, in the absence of a T-cell mediated adaptive immune response. However, when such a response does occur, then the lung has a resident population of regulatory cells which can be “switched on” by the very cells they are required to regulate, and the result is tightly regulated protective immunity – with minimal tissue pathology. Studies on human Treg cells have highlighted the importance of IL-2 for their survival and function *in vitro* (Yates *et al.*, 2007). IL-2 prevents apoptosis of Treg cells and is crucial for maintaining their suppressive phenotype *in vitro* (Yates *et al.*, 2007).

A number of different mechanisms have been proposed regarding how Treg cells suppress immune responses (Miyara and Sakaguchi, 2007; Vignali, 2008). Direct cell-cell contact with responder cells in conjunction with secretion of anti-inflammatory mediators has been reported as the modus operandi of nTreg cells. To investigate whether the observed suppression mediated by CD4⁺CD25⁻CD45RB^{low} Treg cells was cell-contact dependent or due to the production of anti-inflammatory cytokines, activated CD4⁺CD25⁻CD45RB^{low} Treg cells and Teff cells were separated by a semi-permeable membrane. Despite the absence of cell-cell contact, the proliferation of responder cells and the production of IFN- γ were significantly inhibited by Treg cells, to the same extent as when the two populations were in direct contact with each other.

Blocking the IL-10 receptor and therefore IL-10 signalling completely abrogated the suppression of Teff cells by pre-activated Treg cells and significantly enhanced the production of IFN- γ and TNF- α . This suggests that pre-activated CD4⁺CD25⁻CD45RB^{low} Treg cells suppress proliferation of Teff cells via IL-10. These *in vitro* suppression assays demonstrate that lung

CD4⁺CD25⁻CD45RB^{low} Treg cells can suppress, but only when they are sufficiently activated. Upon activation, they upregulate CD25 and use IL-10 to inhibit proliferation of Teff cells. However, they are not as potent as nTreg cells at suppressing T cell responses.

Infection with *B. pertussis* increased the percentages of CD4⁺FoxP3⁺CD25⁺ Treg cells in the lung, which peaked 14 days post-challenge. Interestingly, the percentages of CD4⁺FoxP3⁺CD25⁺ Treg cells decreased thereafter and ultimately returned to pre-infection levels. Consistent with the present study, others have shown that CD4⁺FoxP3⁺CD25⁻ Treg cells can upregulate CD25 upon activation (Zelenay *et al.*, 2005), but whether the increased frequency of CD4⁺FoxP3⁺CD25⁺ Treg cells is a result of activation, or whether it is due to increased percentages of resident and/or infiltrating CD4⁺FoxP3⁺CD25⁺ Treg cells proliferating in response to *B. pertussis* infection was not determined. Subsequent experiments have confirmed that CD4⁺FoxP3⁺CD25⁻ Treg cells can upregulate CD25 and produce IL-10 *in vivo*, as determined by tracking PKH67-labelled Treg cells during infection (Coleman *et al.*, 2012). Notably, only some of the labelled cells expressed CD25, indicating that only those with antigen specificity are able to convert to a CD25⁺ phenotype *in vivo* (Coleman *et al.*, 2012). This is in agreement with the findings by Curotto de Lafaille *et al.*, who report that the number of CD25⁻ T cells which can express CD25 *in vivo* is limited (Curotto de Lafaille *et al.*, 2004), and also corroborates the hypothesis that CD25 expression on the surface of Treg cells is labile, with cells able to lose and regain CD25 on the surface without affecting their function (Zelenay *et al.*, 2005).

Analysis of Treg cell cytokine production demonstrated a role for IL-10 in regulating the immune response to *B. pertussis*. Both CD4⁺FoxP3⁺CD25⁻ and CD4⁺FoxP3⁺CD25⁺ Treg cells produced IL-10. This data is consistent with the *in vitro* studies which showed that CD4⁺FoxP3⁺CD25⁻ Treg cells suppress proliferation of Teff cells by an IL-10-dependent mechanism. By 28 days post-challenge, both populations produced only background levels of IL-10, suggesting that not only had the frequencies of both cell populations returned to pre-infection levels but that they were in a state of unresponsiveness.

The fact that the frequencies of CD4⁺FoxP3⁺CD25⁻ and CD4⁺FoxP3⁺CD25⁺ Treg cells return to pre-infection levels suggests that the lung environment might actively keep resident Treg cells in a quiescent state under normal conditions. Indeed, there is evidence for this from studies in rats which found that, like in mice, lung T cells display an activated phenotype, yet have downmodulated CD25 expression and are “trapped” in the resting G₀/G₁ phase of the cell cycle (Strickland *et al.*, 1996b). Furthermore, these properties are very similar to the properties of T cells activated in the presence of AM (Strickland *et al.*, 1996a). However, this AM-induced T cell anergy is reversible (Strickland *et al.*, 1996a), and studies where AM have been depleted *in vivo* have demonstrated the restoration of lung T cell immunocompetence (Strickland *et al.*, 1993; Thepen *et al.*, 1989). Additionally, it has been demonstrated that the stimulation of AM via TLRs, in particular TLR2, TLR4 and TLR9, overcomes their inhibitory functions and allows an appropriate immune response to develop (Fernandez *et al.*, 2004). Since LPS, a TLR4 agonist is a component of the Gram-negative *B. pertussis* outer membrane, and *B. pertussis* can infect AM, it is therefore very likely that AM respond in this manner in response to infection with *B. pertussis*.

Furthermore, it has been reported that *B. pertussis* can infect AM along with ciliated epithelial cells of the upper respiratory tract (Hellwig *et al.*, 1999). Once the pathogen has been cleared, it is likely that the immunosuppressive environment of the normal lung serves to limit the local clonal expansion of T cells after activation, presumably to limit collateral damage to the delicate lung tissue. This could be made possible by restoring the inhibitory capacity of AM, which in turn suppress T cell populations in the lung and keep them in a resting, quiescent state. IL-10 has been shown to keep alveolar macrophages in an inhibitory state (Fernandez *et al.*, 2004). Our lab has also demonstrated the induction of FoxP3⁺ Tr1 regulatory cells in response to *B. pertussis* (McGuirk *et al.*, 2002). These Tr1 cells produce IL-10, and this IL-10 may promote the return to an immunosuppressive environment once the infection has been eradicated.

As this novel population of CD4⁺FoxP3⁺CD25⁻ Treg cells appears to have an important role in regulating the immune response to *B. pertussis*, particularly by producing IL-10, the ability of these cells to function in the absence of CD4⁺FoxP3⁺CD25⁺ nTreg cells was examined by

administering a depleting antibody against CD25 to remove all nTreg cells prior to challenge with *B. pertussis*. Only one dose of depleting antibody was administered, to avoid the possibility of depleting any infiltrating CD25⁺ activated CD4⁺ Teff cells during the peak of *B. pertussis* infection, which occurs 7-14 days after the initial challenge.

I hypothesised that if CD4⁺FoxP3⁺CD25⁻ and CD4⁺FoxP3⁺CD25⁺ Treg cells represented distinct Treg cell populations in the lung, then the removal of nTreg cells would not affect bacterial clearance. Indeed, no change in CFU was observed between CD25-depleted mice and controls. Since depleting CD25⁺ nTreg cells does not remove CD4⁺FoxP3⁺CD25⁻ Treg cells, it is likely that these cells were able to compensate for the lack of nTreg cells in this study. The effect of antigen-specific Tr1 cells must also be considered, as they are induced during *B. pertussis* infection and allow the pathogen to evade protective immunity (McGuirk *et al.*, 2002). Since IL-10 appears to be the main mechanism by which CD4⁺FoxP3⁺CD25⁻ Treg cells might suppress Teff proliferation - the effect of depleting CD4⁺FoxP3⁺CD25⁺ Treg cells in IL-10-deficient mice was investigated, as this would impair both types of immune regulation in the lung - namely, removal of CD4⁺FoxP3⁺CD25⁺ nTreg cells and functionally impaired CD4⁺FoxP3⁺CD25⁻ Treg cells. While the bacterial burden of undepleted IL-10^{-/-} mice had was similar to WT mice, analysis of bacterial CFU showed that IL-10^{-/-} mice depleted of CD25⁺ cells cleared the pathogen faster than control mice. This was significant early on in the infection, at 7 days post-challenge.

The accelerated bacterial clearance in CD25-depleted IL-10^{-/-} mice appears to be due to enhanced Th1 immunity to *B. pertussis*, as demonstrated by the presence of antigen-specific IgG_{2a} in their serum from as early as 7 days post-challenge, compared to 21 days for undepleted IL-10^{-/-} and CD25-depleted WT mice. Furthermore, cells from the spleens of depleted and undepleted IL-10^{-/-} mice produced extremely significant concentrations of antigen-specific IFN- γ early in infection and higher frequencies of IFN- γ ⁺ lymphocytes were present in the spleens of CD25-depleted IL-10^{-/-} mice 21 days post-challenge. While CD25-depleted WT mice also produced some early antigen-specific IFN- γ , the concentrations were lower than that produced by the IL-10^{-/-} mice.

Examination of cytokines and chemokines in the lung homogenate 7 days post-challenge revealed detectable IFN- γ in IL-10^{-/-} mice only, albeit at low concentrations. Interestingly, the CD25-depleted WT mice had significantly elevated concentrations of IL-10 in their lungs, compared with undepleted WT mice. The elevated IL-10 concentrations possibly compensates for the lack of nTreg cells and might explain why those mice did not clear the infection more quickly than controls, despite the presence of enhanced pro-inflammatory cytokines such as IL-17, TNF- α and IL-1 β in their lung homogenate. There is evidence that IL-17 and IFN- γ can be antagonistic, so it is possible that IL-17 prevents extreme Th1-mediated immune responses to *B. pertussis* in these mice.

IL-17 has a protective role in *B. pertussis* infection in mice (Dunne *et al.*, 2010; Higgins *et al.*, 2003). It has been reported that IL-17-deficient mice infected with *B. pertussis* have a greater bacterial burden than WT mice (Dunne *et al.*, 2010). Furthermore, CyaA has been shown to promote Th17 cells in *B. pertussis*-infected WT, but not IL-1RI-deficient mice (Dunne *et al.*, 2010). These observations are linked, as CyaA promotes innate IL-1 β production via activation of the NALP3 inflammasome, thereby promoting a Th17 protective immune response to *B. pertussis* (Dunne *et al.*, 2010). Although it is somewhat counterintuitive that the WT CD25-depleted mice had such a high concentration of pro-inflammatory cytokines in their lungs, it is likely that the effects of these cytokines can be regulated by the high levels of IL-10 produced, hence their bacterial burden is the same as undepleted controls.

While the CD25-depleted IL-10^{-/-} mice did not produce such high levels of these pro-inflammatory cytokines, perhaps the production of IFN- γ in the absence of functional Treg cells is sufficient to mediate the significant eradication of *B. pertussis*. IL-10 and IL-17 are co-regulated, so it is also likely that the IL-10^{-/-} mice lack appropriate Th17 immunity, which in the CD25-depleted IL-10^{-/-} mice would further facilitate uncontrolled Th1 responses. The CD25⁺ nTreg cells in undepleted IL-10^{-/-} mice probably regulate the immune response to *B. pertussis* and most likely prevent accelerated clearance of the infection. However, depleting CD25⁺ nTreg cells in IL-10^{-/-}

mice clearly disables both immunoregulatory mechanisms in the lung, enabling the mice to eradicate the infection more quickly, but unable to prevent dysregulated inflammation.

These results suggest that Treg cells prevent the optimal clearance of the pathogen, but it is very likely that they function to limit collateral damage in the lung as a result of an overzealous immune response which might otherwise irreversibly harm the delicate lung tissue. It is also clear that eliminating $CD4^+FoxP3^+CD25^+$ Treg cells alone is not sufficient to impair the lung's immunoregulatory mechanisms. Rather, removal of both $CD4^+FoxP3^+CD25^+$ Treg cells and IL-10-producing $CD4^+FoxP3^+CD25^-$ Treg cells completely abolishes immune regulation in the lung - suggesting that inducible $FoxP3^-$ Tr1 cells as well as $CD4^+FoxP3^+CD25^-$ IL-10 $^+$ cells might play redundant roles in *B. pertussis* infection. However, it is unclear whether this is unique to *B. pertussis* infection.

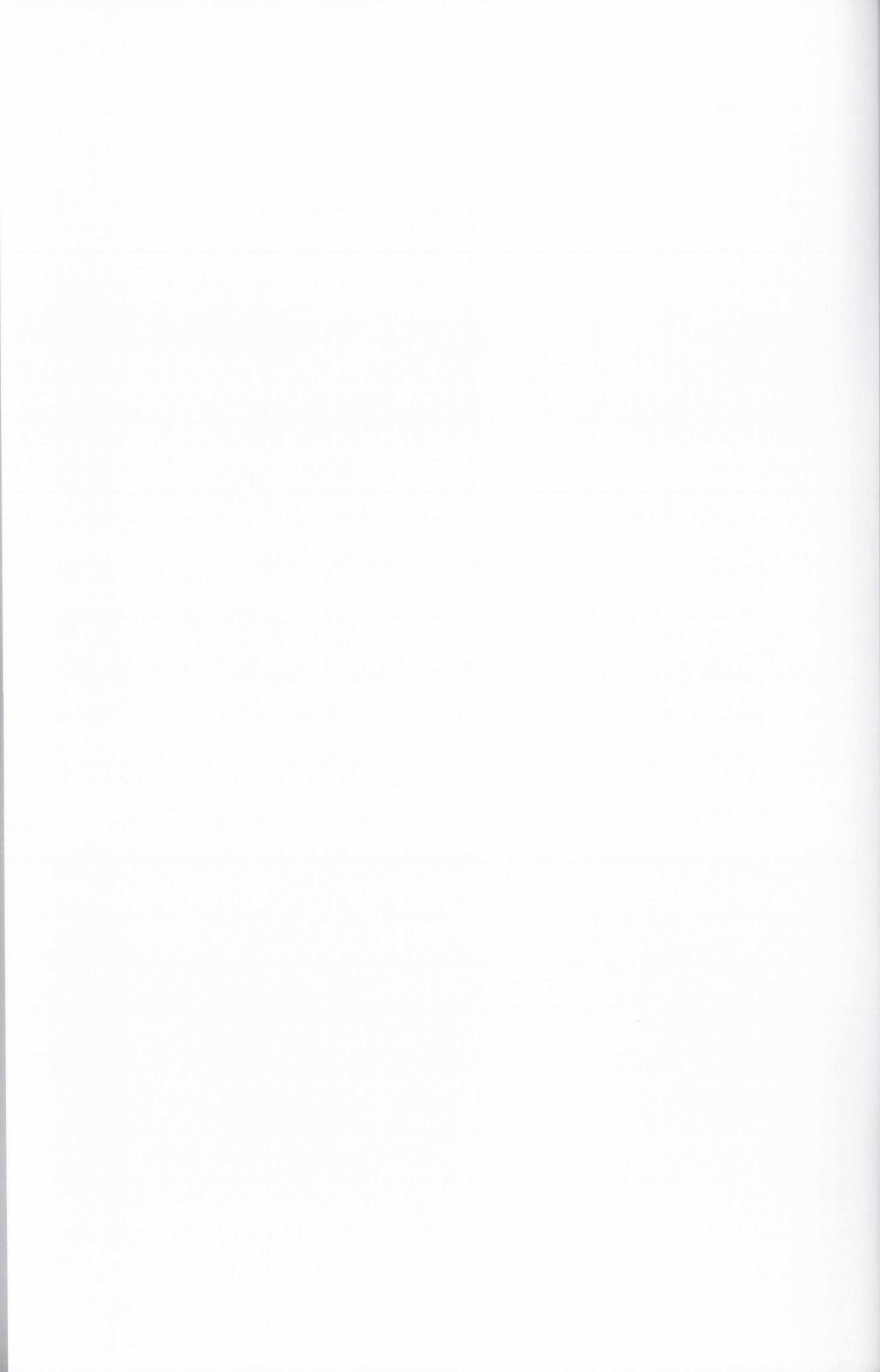
A study of *Pseudomonas aeruginosa* infection in mice has shown that depletion of $CD25^+$ cells has no effect on the outcome of disease in mice, with depleted animals and control animals showing similar cytokine and chemokine production and similar levels of airway neutrophil infiltration and lung histology (Carrigan *et al.*, 2009). Like *B. pertussis*, *P. aeruginosa* is a Gram-negative bacterium which causes acute lung disease. However, the immune response to *P. aeruginosa* is predominantly a Th2 one. Nonetheless, both the present study and that of Carrigan *et al* have collectively demonstrated that an additional regulatory mechanism exists in the lungs of mice and is effective in regulating both Th1 and Th2 immune responses. However, the role of antigen-specific, inducible Treg cells such as Tr1 cells cannot be ruled out.

In summary, this present study has identified a novel population of regulatory T cells in the lungs of mice which are $CD4^+FoxP3^+CD25^-$. They are distinguishable from nTreg cells by their lack of CD25 expression and are the predominant $FoxP3^+$ population in the lungs of naïve mice. Similar populations have been shown to reside in the livers and colons of naïve mice and may represent a Treg cell population specific to mucosal tissues. *In vitro* suppression assays have revealed that these cells can suppress the proliferation of $CD4^+$ responder T cells as well as IFN- γ

production, but only when they have been pre-activated in the presence of IL-2, which results in the upregulation of CD25 on the surface of these cells. CD4⁺FoxP3⁺CD25⁻ Treg cells do not require direct cell-cell contact in order to suppress, and the soluble factor which mediates suppression of responder cells is IL-10.

The *B. pertussis* infection model has demonstrated that the frequencies of CD4⁺FoxP3⁺CD25⁺ Treg cells increase in the lung in response to the infection, but return to pre-infection levels once the pathogen has been eradicated. As it has already been shown that CD4⁺FoxP3⁺CD25⁻ Treg cells upregulate CD25 upon activation *in vitro*, it is possible that this reflects an increase in CD25⁺ Treg cells. CD4⁺FoxP3⁺CD25⁻ Treg cells can produce IL-10 and in response to *B. pertussis* infection, as can CD4⁺FoxP3⁺CD25⁺ Treg cells, although they produce IL-10 later when compared with their CD25⁺ counterparts. Removal or impairment of one population does not affect bacterial burden in *B. pertussis* infection, but mice which lack nTreg cells and have impaired IL-10 production can clear the infection more quickly. CD4⁺FoxP3⁺CD25⁻ Treg cells might therefore represent a distinct subset of Treg cells in the lung, which function in conjunction with CD4⁺FoxP3⁺CD25⁺ Treg cells and play a redundant immunoregulatory role. Further and more detailed investigations into this regulatory subset might aid in the generation of new therapeutic strategies against lung disease in the future.

3.4 Figures



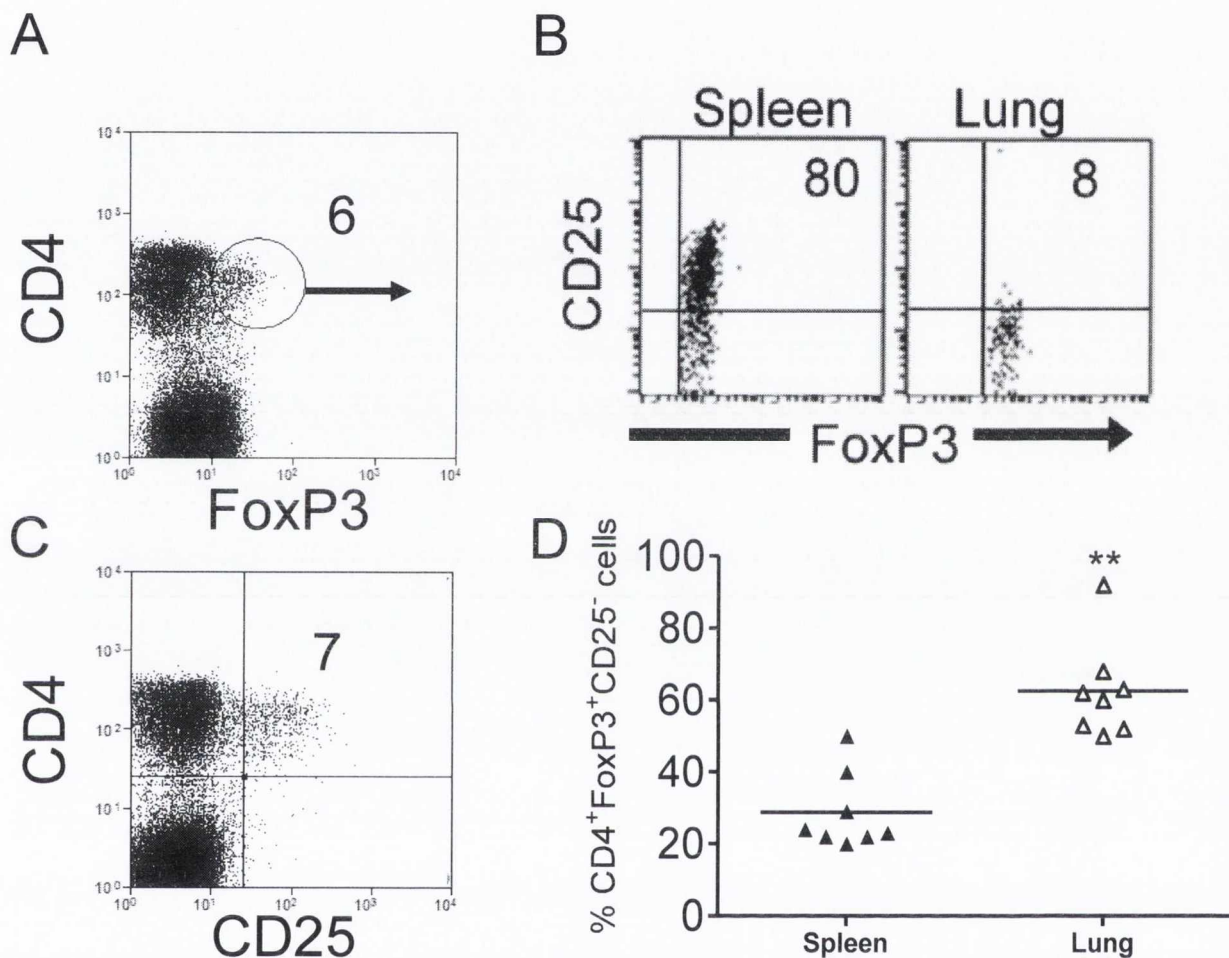


Figure 3.1 The majority of CD4⁺FoxP3⁺ T cells in the lungs of naïve mice do not express the activation marker CD25

Splenocytes from naïve mice were isolated by physical disruption and passed through a 40 µM filter. Lungs from naïve mice were digested with 1 µg/ml collagenase, homogenised and filtered. Isolated cells were stained with rat anti-mouse CD4, CD25 and FoxP3. Representative dot plots show A) the percentage of FoxP3⁺ and CD4⁺ cells (within the total lymphocyte gate) from the lungs of a naïve mouse., B) CD25 expression on viable CD4⁺FoxP3⁺ T cells isolated from naïve spleen or lung, C) Lung CD25 and CD4 expression within the total lymphocyte gate. D) The percentage of viable CD4⁺FoxP3⁺CD25⁻ cells within the CD4⁺FoxP3⁺ gated population from spleens and lungs of naïve mice. Results are pooled from 8 mice. P values were calculated on the difference between CD25 expression on CD4⁺FoxP3⁺ cells isolated from the lungs and spleens. **p<0.01

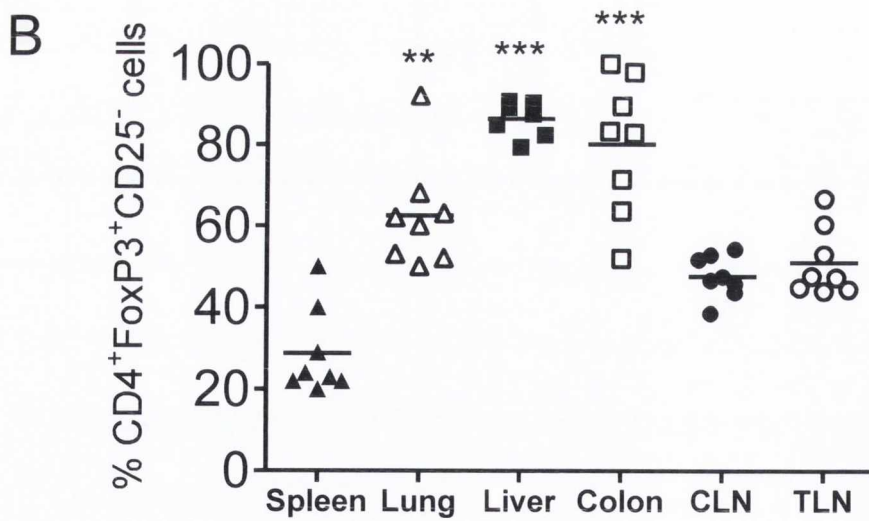
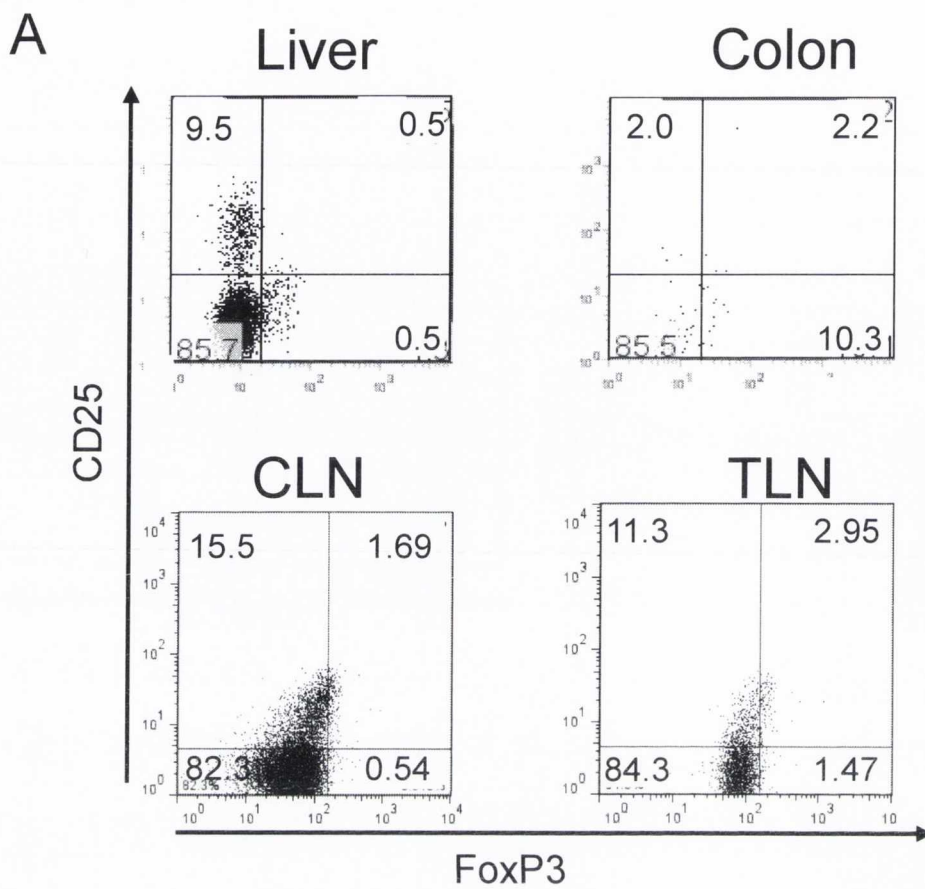
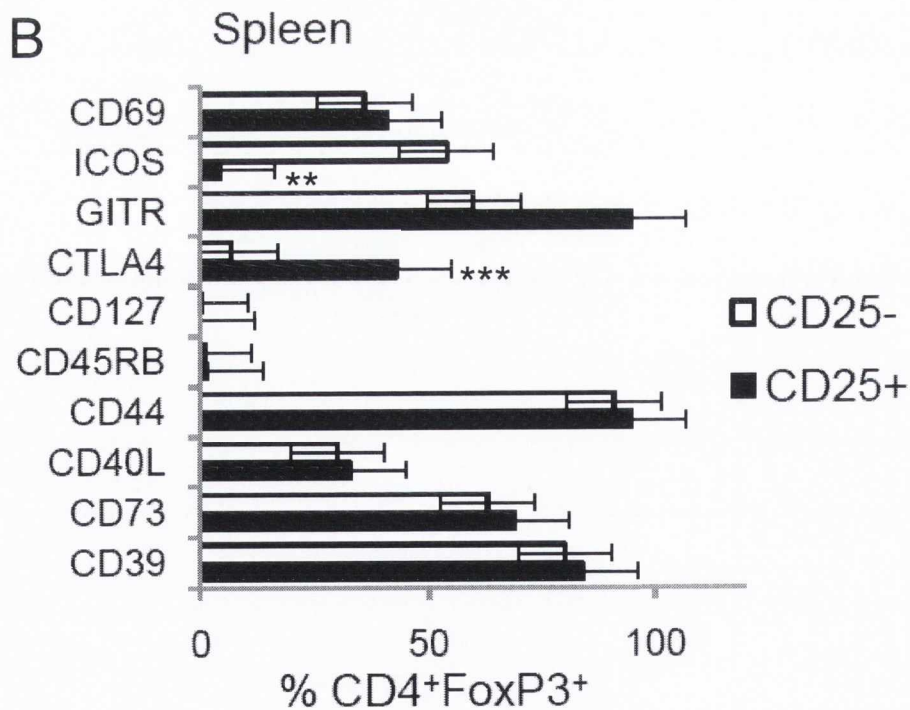
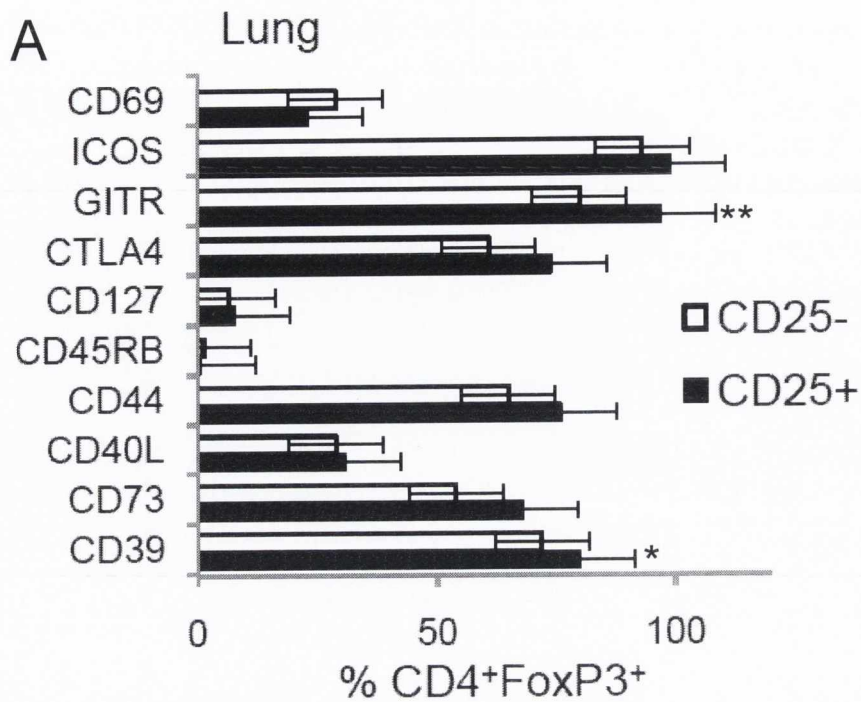


Figure 3.2 The majority of CD4⁺FoxP3⁺ T cells in lungs and other mucosal tissues of naïve mice do not express CD25

Cells were isolated from spleen, cervical (CLN) and thoracic (TLN) lymph nodes and enzymatically digested lung, liver and colon. Cells were stained with rat anti-mouse CD4, CD25 and FoxP3. A) Representative dots plots showing the percentage of FoxP3 and CD25 cells (based on the viable CD4⁺ gate) in liver, colon, CLN and TLN. B) The percentage of viable CD4⁺FoxP3⁺CD25⁻ cells within the CD4⁺FoxP3⁺ gated population from spleens, lungs, livers, colons, CLN or TLN of naïve mice. Results are pooled from 8 mice. P values were calculated on the difference between CD25 expression on CD4⁺FoxP3⁺ cells isolated from the different tissues relative to spleen . **p<0.01, ***p<0.001



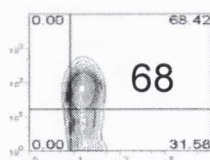
C

Lung CD4⁺FoxP3⁺

CD25⁻

CD25⁺

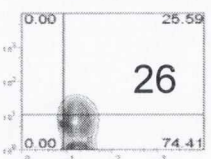
CD73



CD39



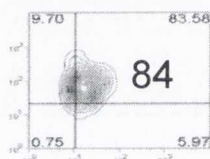
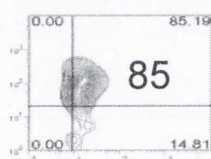
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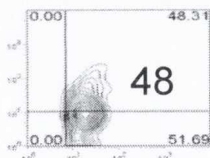
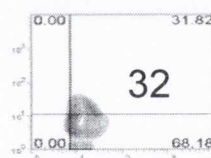
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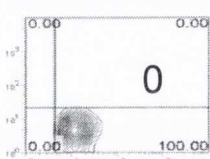
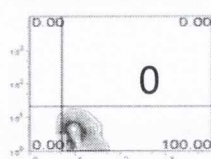
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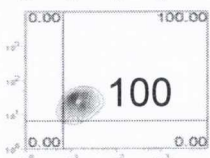
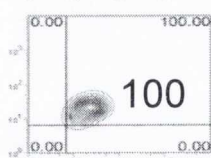
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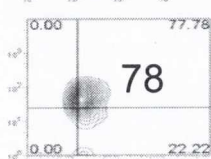
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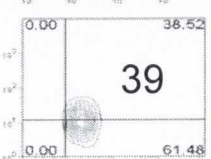
ICOS



GITR



CTLA-4



FoxP3

D

Marker	MFI Lung CD4 ⁺ FoxP3 ⁺ CD25 ⁻ T cells	MFI Lung CD4 ⁺ FoxP3 ⁺ CD25 ⁺ T cells
CD73	997.53 +/- 0.46	107.25 +/- 19.18
CD69	4.5 +/- 9.0	20.2 +/- 4.35
CD45RB	36.03 +/- 6.21	8.18 +/- 9.49
CD44	111.93 +/- 12.15	312.5 +/- 23.27
CD40L	3.18 +/- 6.35	16.88 +/- 1.02
ICOS	60.48 +/- 6.87	157.05 +/- 106.04

Figure 3.3 CD4⁺FoxP3⁺CD25⁻ T cells have a similar phenotype to nTreg cells, but express less GITR and CD73 in the lung, and express more ICOS and less CTLA-4 in the spleen

Cells were isolated from the lungs and spleens of naïve mice and stained with rat anti-mouse antibodies as indicated. The phenotype of CD4⁺FoxP3⁺CD25⁺ or CD25⁻ cells was based on a viable forward by side scatter gate. Results show the mean expression values of surface markers within the CD4⁺FoxP3⁺CD25⁻ and CD4⁺FoxP3⁺CD25⁺ gates in cells isolated from the lungs (A) and spleens (B) of naïve mice. C) Results show representative dot plots. D) Table of average median fluorescence intensities (MFI) for selected surface markers. P values were calculated on the difference in surface marker expression between CD25⁺ and CD25⁻ cells. *p<0.05, **p<0.01, ***p<0.001

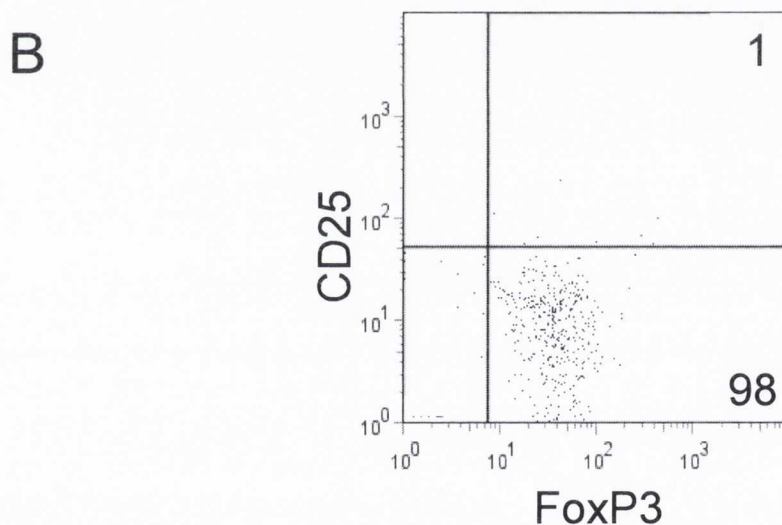
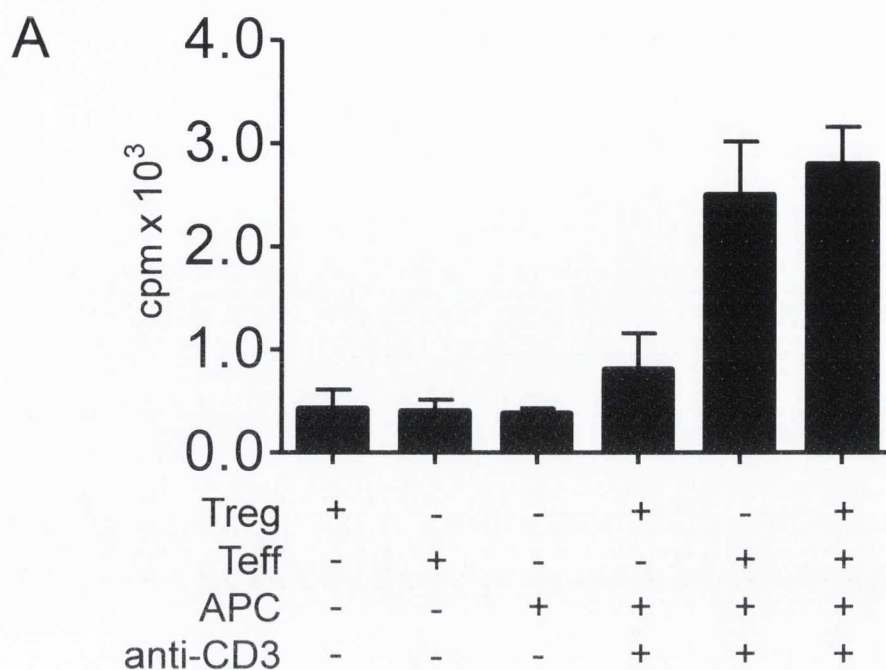


Figure 3.4 Freshly isolated lung CD4⁺FoxP3⁺CD25⁻ T cells do not suppress proliferation of Teff cells *in vitro*

CD4⁺CD25⁻CD45RB^{low} Treg cells were isolated from the digested lungs of naïve mice. CD4⁺CD25⁻CD45RB^{high} Teff cells were isolated from spleens of naïve mice. Teff cells (1 x 10⁴) were cultured with 2 µg/ml immobilised anti-CD3 and irradiated APC with or without Treg cells (1 x 10⁴). A) Proliferation was determined by ³H-thymidine incorporation 72 hours later. B) Representative dot plot showing the expression of FoxP3 in the sorted CD4⁺CD25⁻CD45RB^{low} population. Results are representative of 3 experiments.

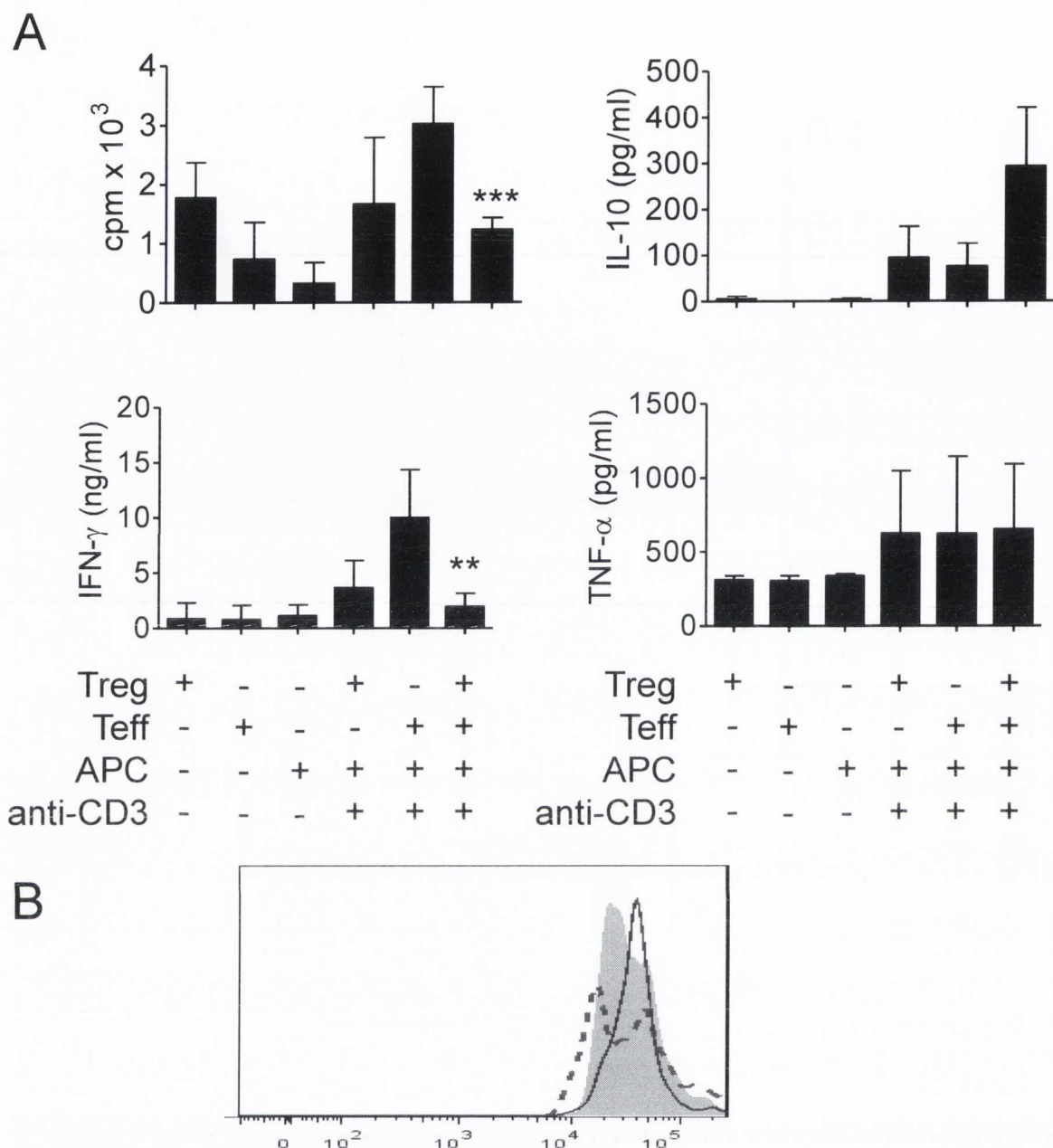


Figure 3.5 Pre-activated lung CD4⁺FoxP3⁺CD25⁻ Treg cells suppress proliferation of Teff cells and IFN-γ production at a 1:1 ratio *in vitro*

CD4⁺CD25⁻CD45RB^{low} Treg cells were isolated from the digested lungs of naïve mice and activated for 5 days with 1 µg/ml immobilised anti-CD3, 20U/ml IL-2 and irradiated APC (5 x 10⁵). CD4⁺CD25⁻CD45RB^{high} Teff cells were isolated from the spleens of naïve mice and were cultured with 10 µg/ml immobilised anti-CD3 and irradiated APC (5 x 10⁵) with or without activated Treg cells. A) After 48 hours the supernatants were removed and concentrations of IL-10, IFN-γ and TNF-α were quantified by ELISA. TGF-β₁ was not detected in supernatants. Proliferation was determined by ³H-thymidine incorporation after 72 hours. B) Proliferation was also determined by labelling freshly isolated CD4⁺CD25⁻CD45RB^{high} Teff cells with 0.5 µM CFSE. Pre-activated CD25⁻ Treg cells were co-cultured with CFSE-labelled effector T cells (1:1) and activated in the presence of 10 µg/ml immobilised anti-CD3 and 5 x 10⁵ irradiated APC for 5 days. Cells were subsequently stained with antibodies directed against surface CD4 and CD25, and CFSE loss was determined by flow cytometry. Results are representative of 3 experiments. P values were calculated based on the difference in proliferation or cytokine production between activated Teff cells and Teff cells activated in the presence of Treg cells. **p<0.01, ***p<0.001

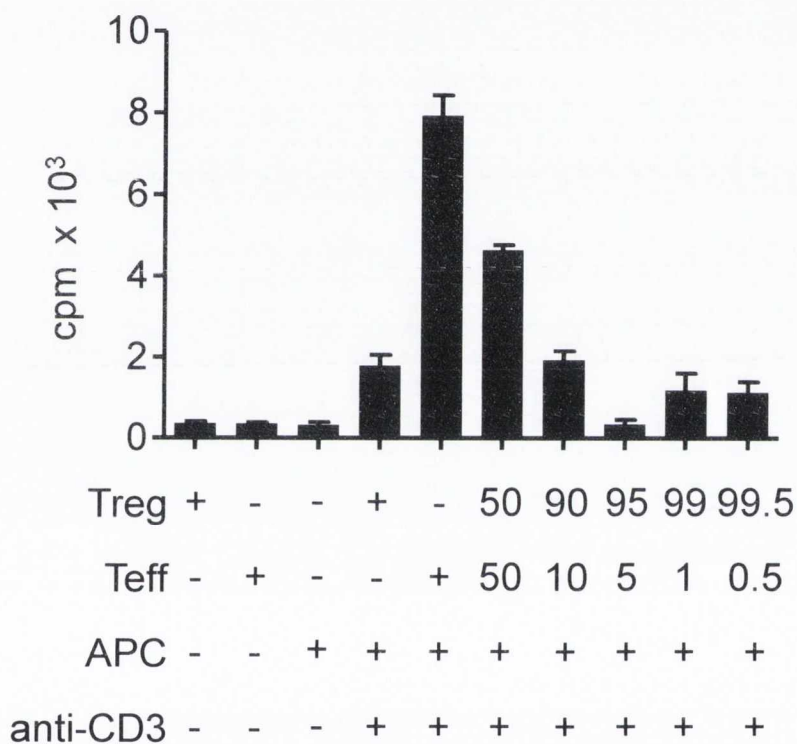


Figure 3.6 Low levels of contaminating Teff cells do not proliferate in the presence of CD25⁻ Treg cells during pre-activation

CD4⁺CD25⁻CD45RB^{low} Treg cells and CD4⁺CD25⁻CD45RB^{high} Teff cells were isolated from the spleens of naïve mice. CD4⁺CD25⁻CD45RB^{low} Treg cells were activated as described in Fig. 3.5, either in the absence of Teff cells or in the presence of different ratios of Teff cells. Proliferation was determined by ³H-thymidine incorporation after 72 hours. Results are representative of 3 experiments.

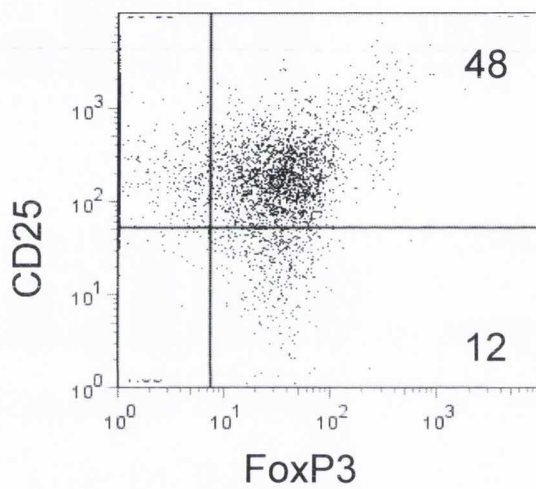


Figure 3.7 Activation of CD4⁺FoxP3⁺CD25⁻ T cells *in vitro* with anti-CD3 and exogenous IL-2 enhances CD25 expression
 CD4⁺CD25⁻CD45RB^{low} Treg cells were isolated from the digested lungs of naïve mice and activated as described in Fig. 3.5. After 24 hours, cells were stained with rat anti-mouse antibodies against CD4, CD25 and FoxP3. Dot plot represents expression of CD25 and FoxP3 in the CD4⁺ population.

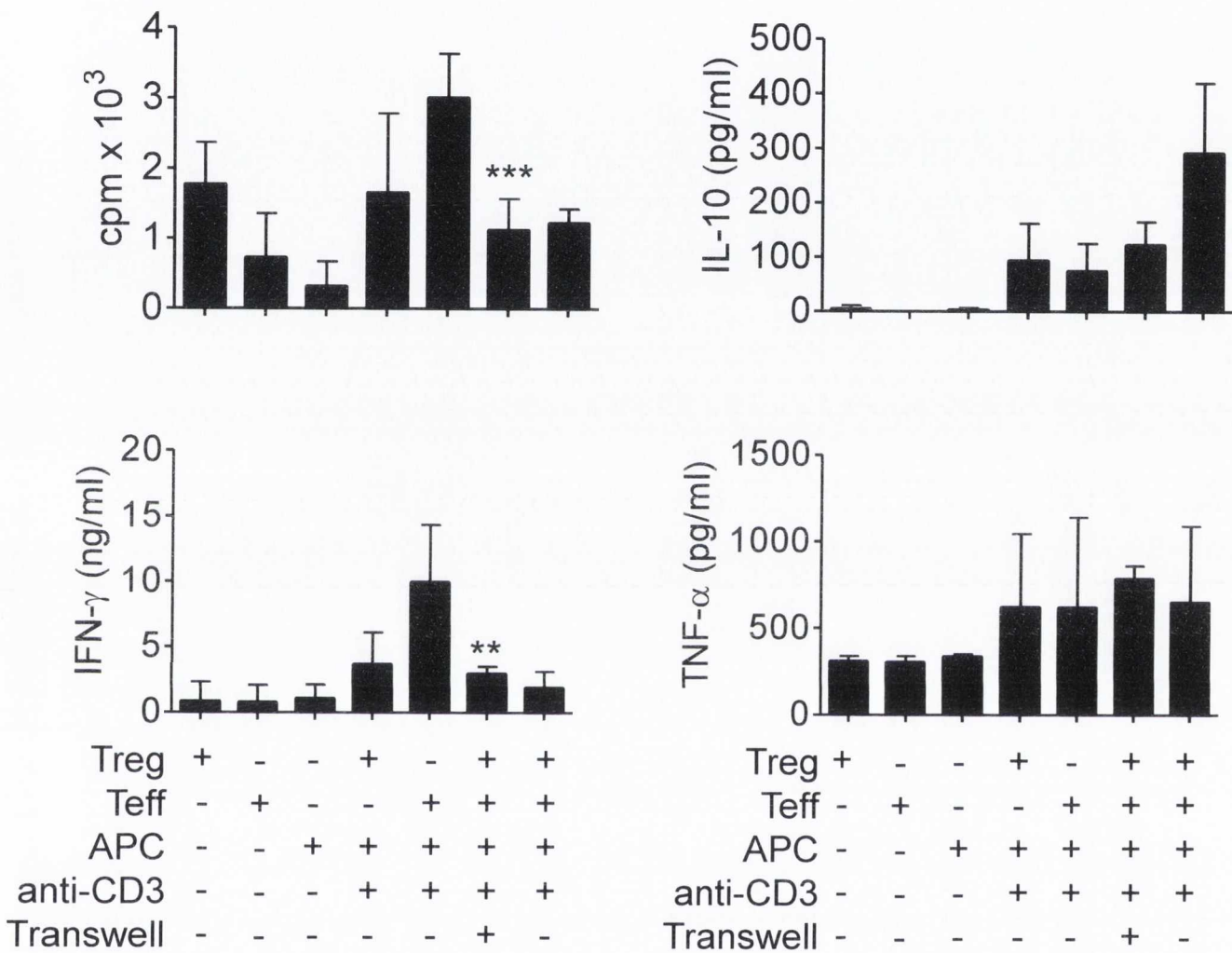


Figure 3.8 CD25⁻ Treg cell suppression of Teff cells is not cell-contact dependent

CD4⁺CD25⁻CD45RB^{low} Treg cells were isolated from the digested lungs of naïve mice and activated as described in Fig. 3.5. After 5 days, CD4⁺CD25⁻CD45RB^{high} Teff cells were isolated from the spleens of naïve mice and were activated as described in Fig. 3.5. Treg cells were cultured under identical conditions, either separated from the Teff cells by a semi-permeable membrane, or in the presence of Teff cells as a positive control. After 48 hours, supernatants were removed and IL-10, IFN-γ and TNF-α concentrations were quantified by ELISA. Proliferation was determined by ³H-thymidine incorporation after 72 hours. Results are representative of 3 experiments. P values were calculated based on the difference in proliferation or cytokine production between activated Teff cells and Teff cells activated but separated from Treg cells by a semi-permeable membrane. **p<0.01, ***p<0.001

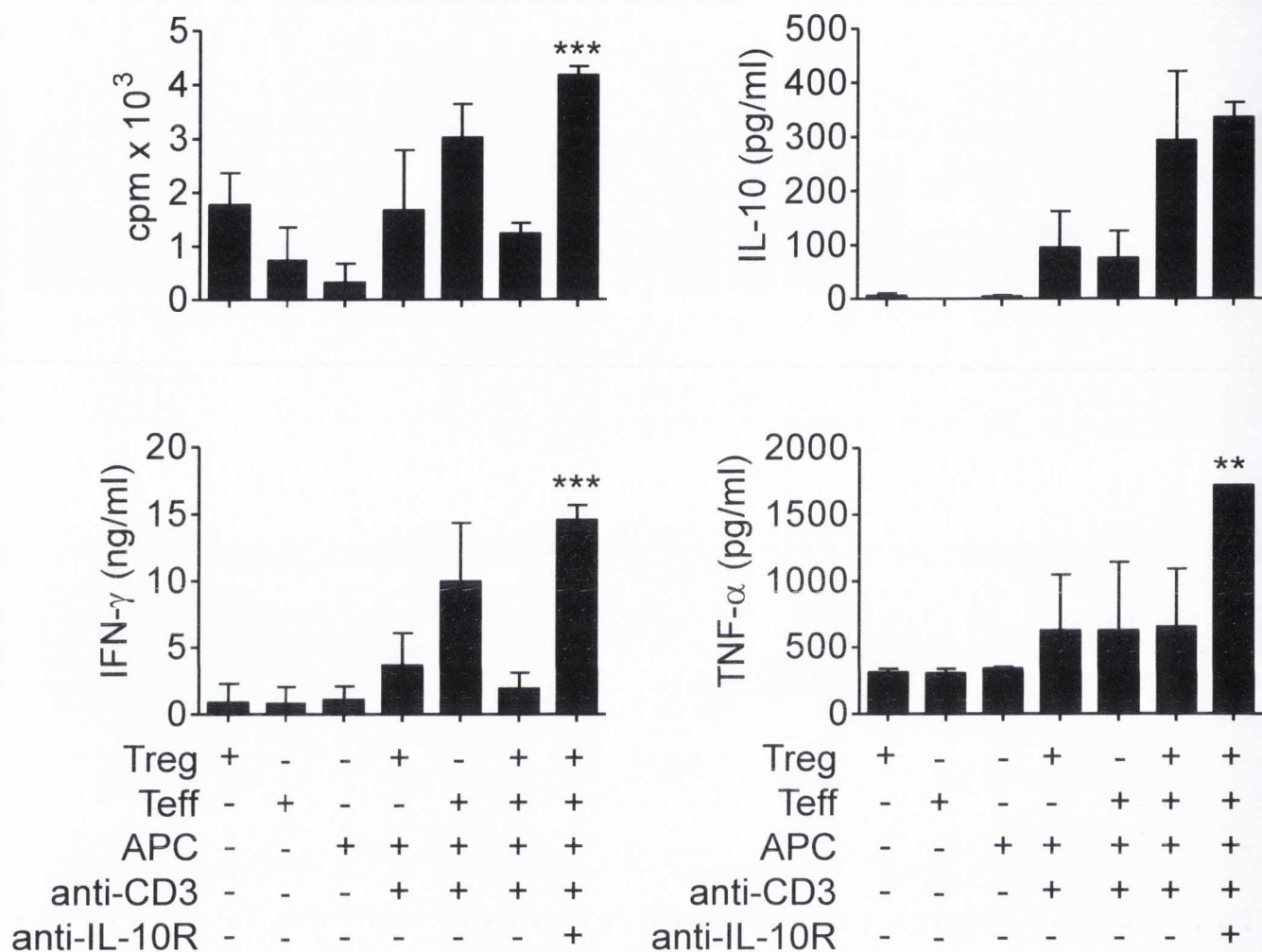


Figure 3.9 Addition of a blocking anti-IL-10 receptor antibody reverses the suppressive effect of CD25⁺ Treg cells on effector T cell proliferation

CD4⁺CD25⁻CD45RB^{low} Treg cells were isolated from the digested lungs of naïve mice and were activated as described in Fig. 3.5. After 5 days, CD4⁺CD25⁺CD45RB^{high} Teff cells were isolated from the spleens of naïve mice and were activated as described in Fig. 3.5, with or without activated Treg cells. Cells were cultured either in the presence or absence of 10 µg/ml anti-IL-10R blocking antibody. After 48 hours, supernatants were removed and IL-10, IFN-γ and TNF-α concentrations were quantified by ELISA. Proliferation was determined by ³H-thymidine incorporation after 72 hours. Results are representative of 3 experiments. P values were calculated based on the differences in proliferation and cytokine production by Teff cells activated in the presence of Treg cells and isotype control antibody, and Teff cells activated in the presence of Treg cells and anti-IL10R. **p<0.01, ***p<0.0001

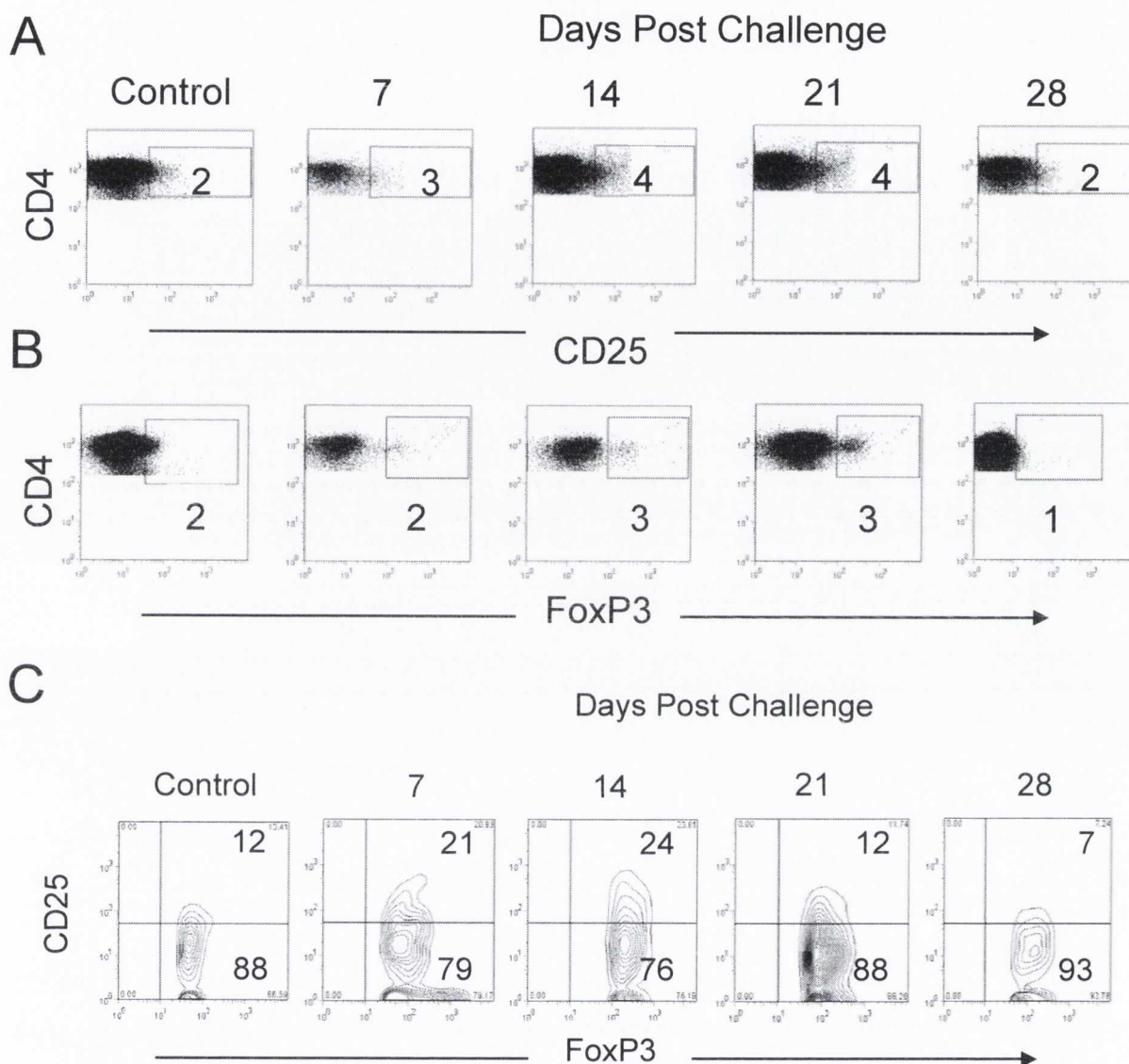


Figure 3.10 Transient expression of CD25 by CD4⁺FoxP3⁺ Treg cells in the lungs during infection with *B. pertussis*

Balb/c mice were challenged with an aerosol of live *B. pertussis*. 7, 14, 21 and 28 days post-challenge, mice were injected with HKBP (4×10^9 bacteria) to induce a recall response. 4 hours after the injection, cells were isolated from the digested lungs of infected mice and incubated overnight in the presence of 2 μ g/ml Brefeldin A. Cells were subsequently stained with rat anti-mouse antibodies against CD4, CD25 and FoxP3. All dot plots shown are based on a viable forward by side scatter gate. A) The percentage of CD4⁺CD25⁺ cells. B) The percentage of CD4⁺FoxP3⁺ cells. C) The percentage of CD25 and FoxP3 gated on viable CD4⁺FoxP3⁺ cells. Results are representative of 6 experiments.

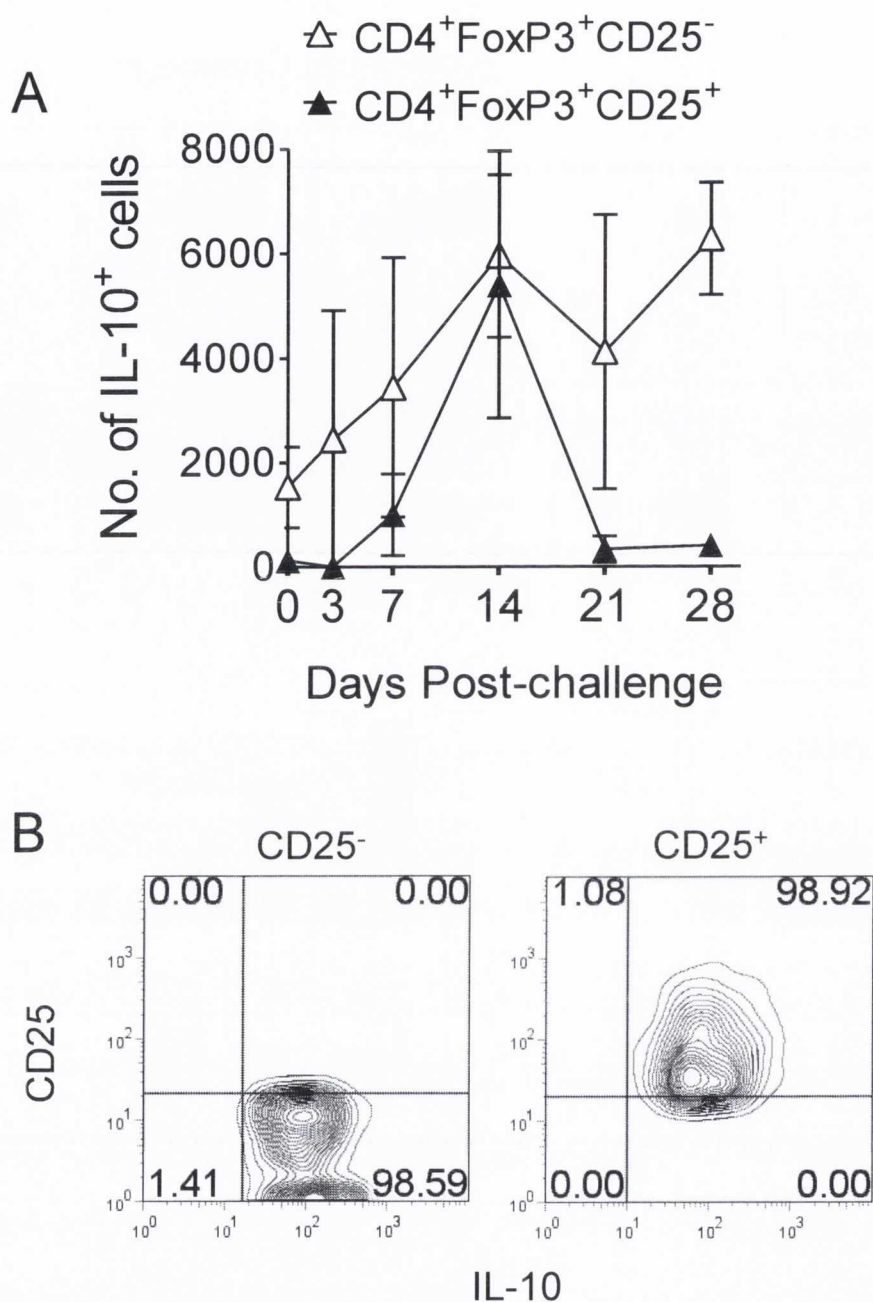


Figure 3.11 Sustained expression of IL-10 by CD4⁺FoxP3⁺CD25⁻ Treg cells during infection with *B. pertussis*

Balb/c mice were challenged with an aerosol of live *B. pertussis*. 7, 14, 21 and 28 days post-challenge, they were then injected with HKBP (4×10^9 bacteria) to induce a recall response. 4 hours after the injection, cells were isolated from the digested lungs of infected mice and incubated overnight in the presence of Brefeldin A. Cells were subsequently stained with rat anti-mouse antibodies against CD4, CD25, FoxP3 and IL-10. Results show A) the percentages of IL-10-producing CD25⁺ and CD25⁻ cells during the course of infection and B) representative dot plots indicating the peak of IL-10 production (14 days post-challenge). Graphs and dot plots are representative of 6 experiments.

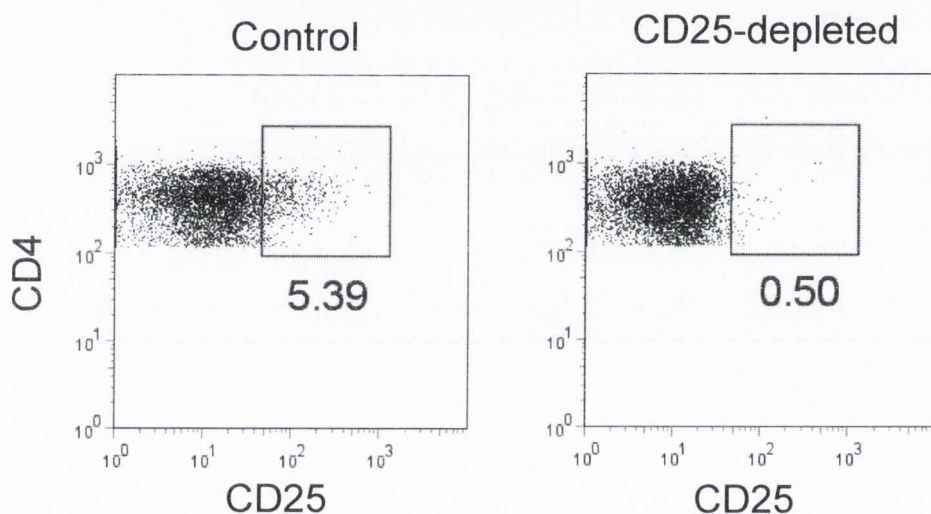


Figure 3.12 Injection of anti-CD25 depleting antibody removes $CD4^+CD25^+$ nTreg cells within 24 hours

C57Bl/6 mice (WT) were injected i.p. with anti-CD25 depleting antibody or with irrelevant isotype-matched IgG1 control antibody. 24 hours after injection, the cells were isolated from the spleens of treated mice and were stained with rat anti-mouse antibodies against CD4 and CD25. CD25 expression was determined by using an alternative conjugated anti-CD25 antibody (7D4) directed against a different epitope to that of the depleting antibody (PC61). Dot plots shown represent the frequencies of $CD4^+CD25^+$ cells in A) mice which received isotype-matched antibody and B) mice which received CD25 depleting antibody based on the viable $CD4^+$ gate. Results are representative of 4 experiments.

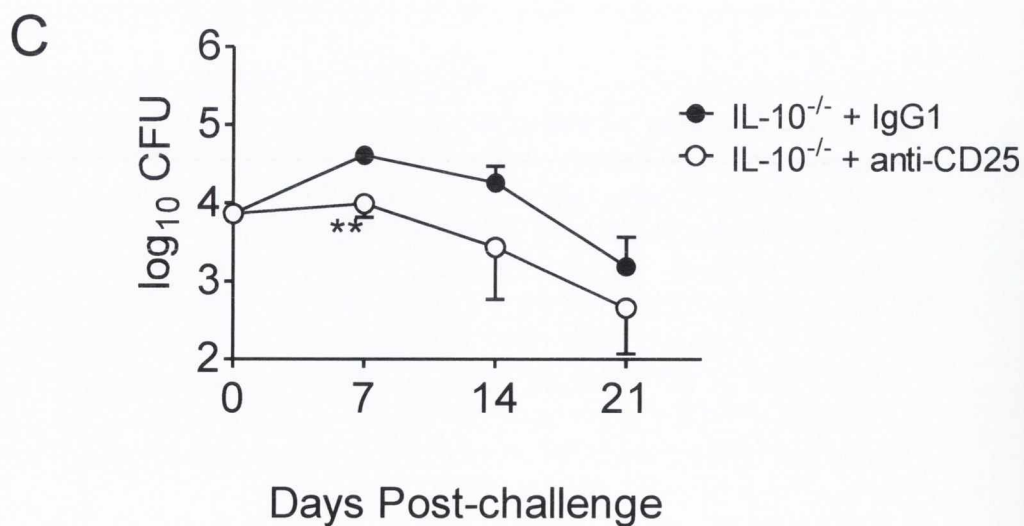
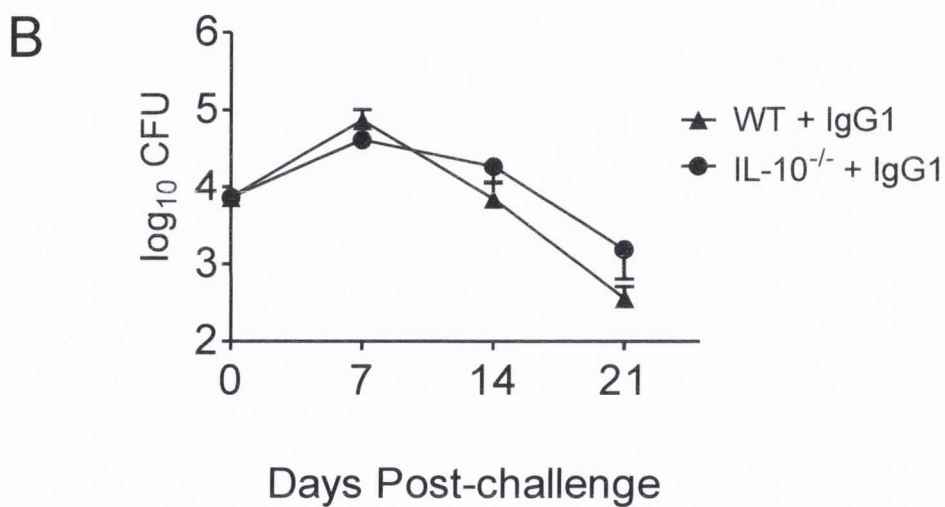
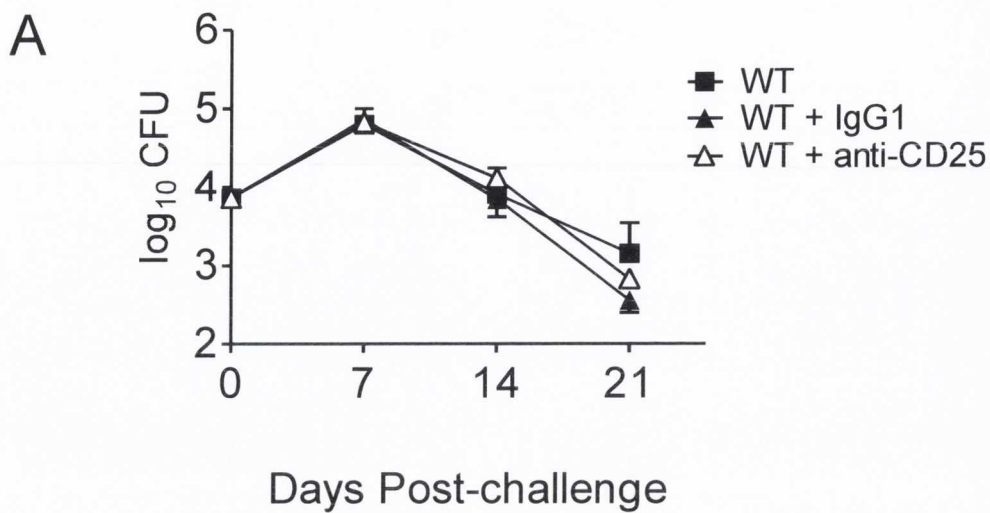


Figure 3.13 Enhanced clearance of *B. pertussis* in IL-10^{-/-} mice depleted of CD25⁺ cells

C57Bl/6 (WT) or IL-10^{-/-} mice were injected (i.p.) with either anti-CD25 depleting antibody or with irrelevant isotype-matched control IgG1 antibody 24 hours prior to aerosol challenge with *B. pertussis*. Control WT mice were infected but did not receive antibody. Each treatment group contained n=4 mice. CFU counts were performed on homogenised lungs 4 hours and 7, 14 and 21 days post challenge. Results are representative of 2 experiments. P values were calculated based on the difference between IL-10^{-/-} mice which received IgG1 isotype control antibody, and IL-10^{-/-} mice which received the depleting antibody. **p<0.01

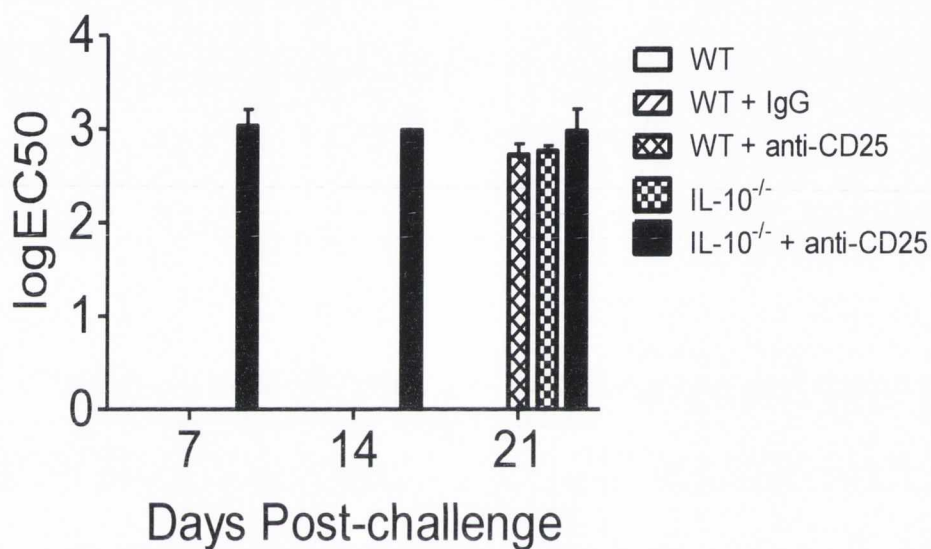


Figure 3.14 Enhanced antibody response to *B. pertussis* in IL-10^{-/-} mice depleted of CD25⁺ cells

C57Bl/6 (WT) and IL-10^{-/-} mice were injected with antibody i.p. prior to *B. pertussis* infection, as described in Fig. 3.13. Serum was collected 7, 14 and 21 days post-challenge and relative levels of *B. pertussis*-specific IgG2a was determined. Results are representative of 2 experiments.

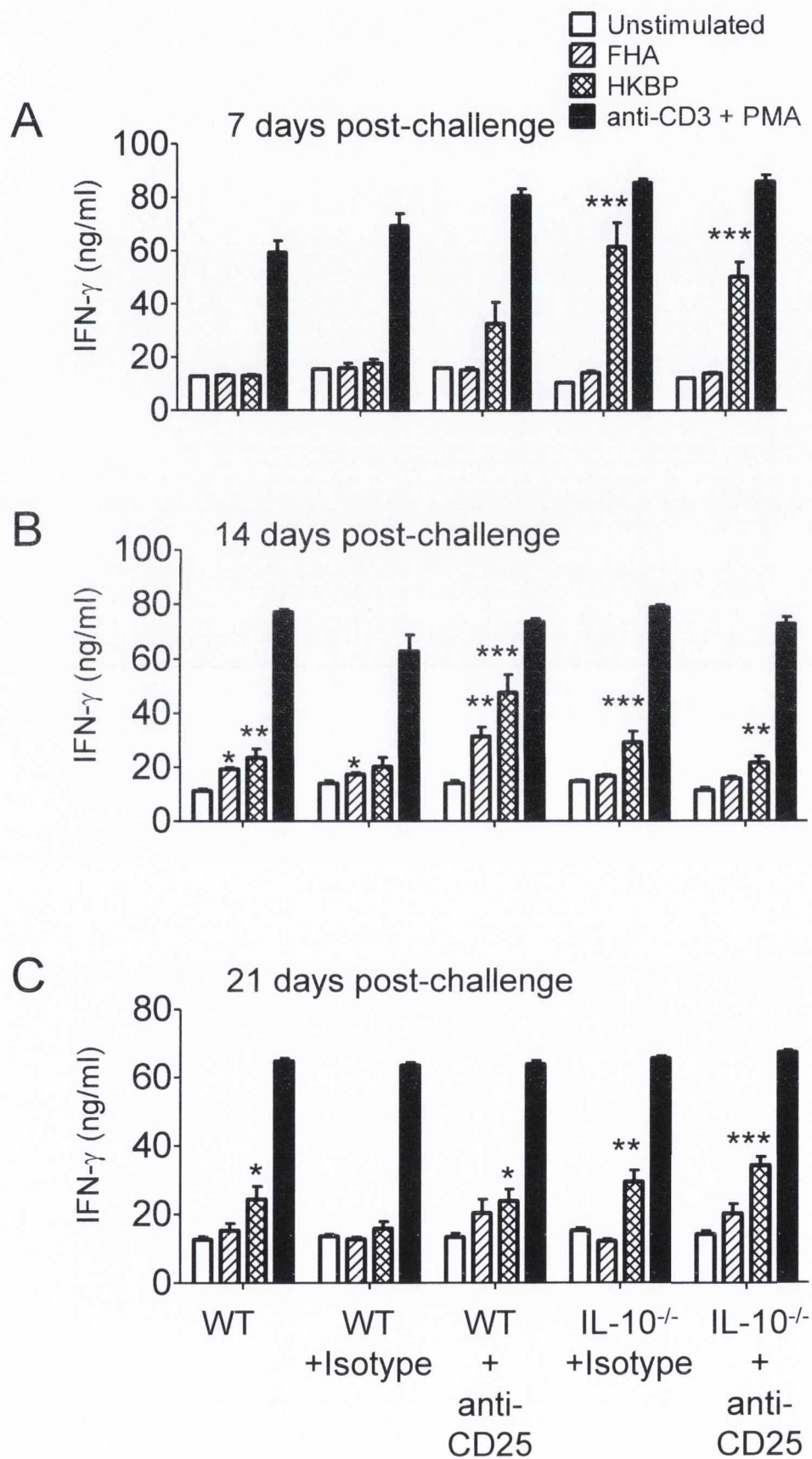


Figure 3.15 Depletion of CD25⁺ cells and/or IL-10-deficiency results in earlier IFN- γ production by *B. pertussis*-specific splenocytes

C57Bl/6 (WT) and IL-10^{-/-} mice were injected with antibody i.p. prior to *B. pertussis* infection, as described in Fig. 3.13. 7, 14 and 21 days post-challenge, freshly isolated splenocytes were cultured for four days with medium alone, FHA, HKBP or anti-CD3 and PMA. IFN- γ concentrations were quantified by ELISA A) 7 days B) 14 days and C) 21 days post-challenge. P values were calculated based on the difference between FHA or HKBP-stimulated cells versus unstimulated control *p<0.05, **p<0.01, ***p<0.001

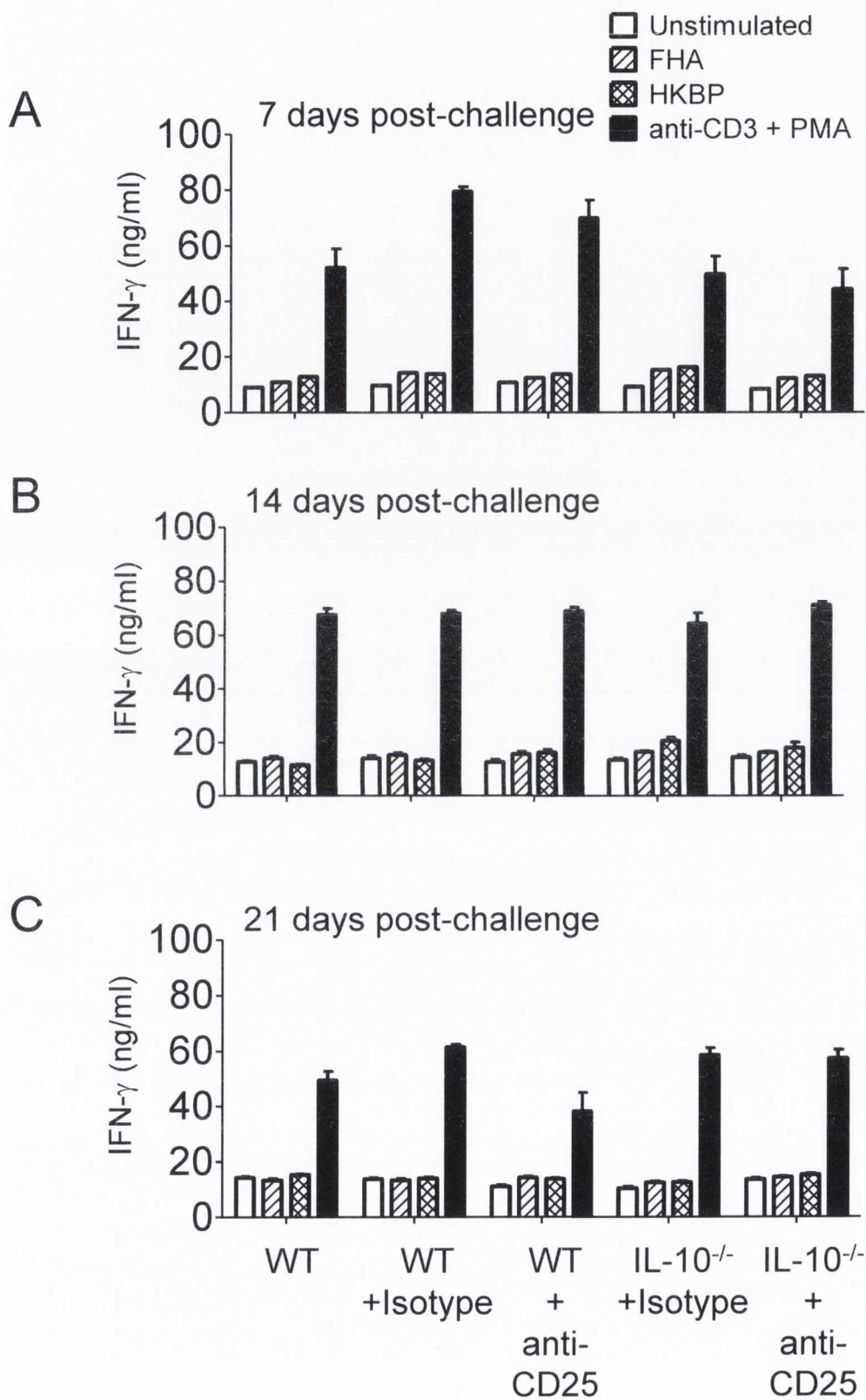


Figure 3.16 Depletion of CD25⁺ cells and/or IL-10 deficiency has no effect on *B. pertussis*-specific IFN- γ production in cervical lymph nodes

C57Bl/6 (WT) and IL-10^{-/-} mice were injected with antibody i.p. prior to *B. pertussis* infection, as described in Fig. 3.13. 7, 14 and 21 days post-challenge, freshly isolated cells from cervical lymph nodes were cultured for four days with medium alone, FHA, HKBP or anti-CD3 and PMA. IFN- γ concentrations were quantified by ELISA A) 7 days B) 14 days and C) 21 days post challenge.

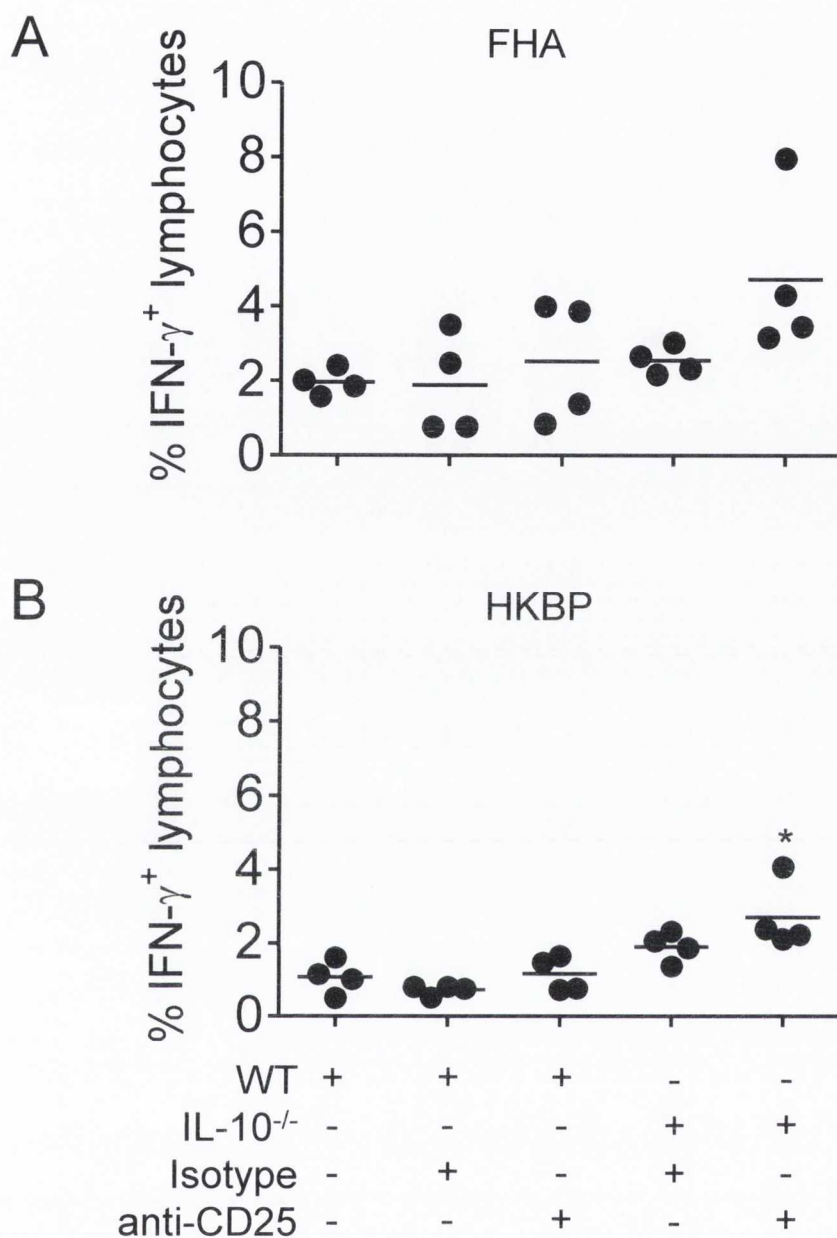


Figure 3.17 Enhanced production of IFN- γ in the spleens of *B. pertussis*-infected IL-10^{-/-} mice depleted of CD25⁺ cells

C57Bl/6 (WT) and IL-10^{-/-} mice were injected with antibody i.p. prior to *B. pertussis* infection, as described in Fig. 3.13. 21 days post-challenge, freshly isolated splenocytes were cultured with (A) FHA or (B) HKBP for four days. Cells were stained with a rat anti-mouse antibody against IFN- γ . Results show the percentage of IFN- γ ⁺ lymphocytes (based on the viable lymphocyte gate) in cells isolated from the spleens of infected mice 21 days after aerosol challenge with live *B. pertussis*. P values were calculated based on the difference between WT mice which received the depleting antibody, and IL-10^{-/-} mice which received the depleting antibody. *p<0.05

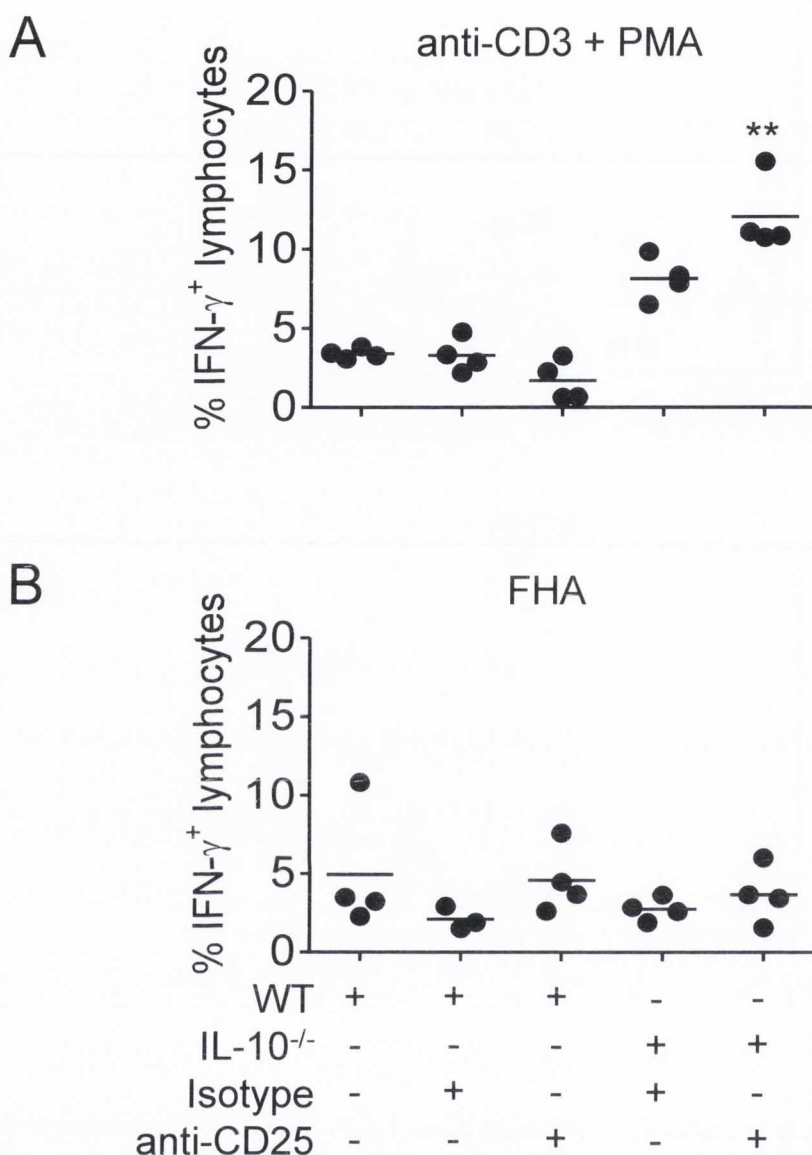


Figure 3.18 Enhanced production of IFN- γ in the CLN of *B. pertussis*-infected IL-10^{-/-} mice depleted of CD25⁺ cells

C57Bl/6 (WT) and IL-10^{-/-} mice were injected with antibody i.p. prior to *B. pertussis* infection, as described in Fig. 3.13. C57Bl/6 (WT) and IL-10^{-/-} mice were injected with antibody i.p. prior to *B. pertussis* infection, as described in Fig. 3.13. 21 days post-challenge, freshly isolated cells from cervical lymph nodes were cultured with (A) anti-CD3 and PMA or (B) FHA for four days. Cells were stained with a rat anti-mouse antibody against IFN- γ . Results show the percentage of IFN- γ^+ lymphocytes (based on the viable lymphocyte gate) examined in cells isolated from the CLN of infected mice 21 days after aerosol challenge with live *B. pertussis*. P values were calculated based on the difference between WT mice which received the depleting antibody, and IL-10^{-/-} mice which received the depleting antibody. **p<0.01

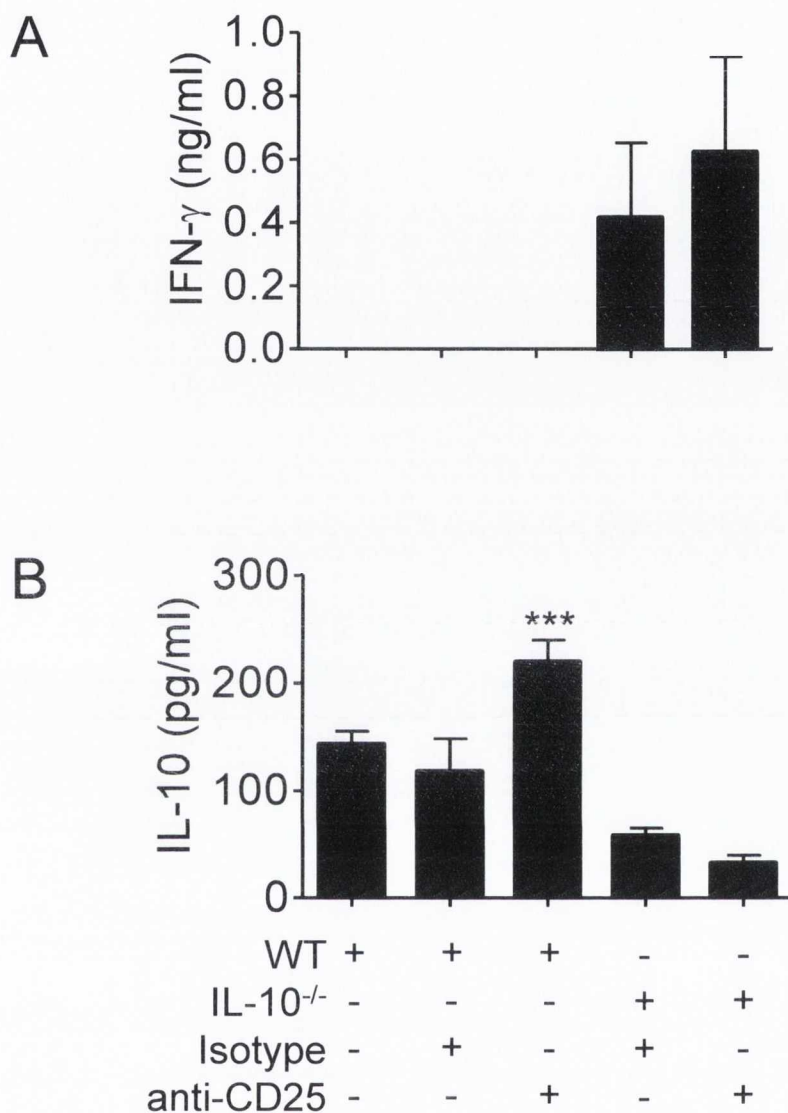


Figure 3.19 IL-10^{-/-} mice produce IFN-γ in response to *B. pertussis* infection, while WT mice depleted of CD25⁺ cells produce significantly more IL-10 than WT controls

C57Bl/6 (WT) and IL-10^{-/-} mice were injected with antibody i.p. prior to *B. pertussis* infection, as described in Fig. 3.13. A) IFN-γ and B) IL-10 concentrations in homogenised lungs were determined by ELISA 7 days post challenge. Results are representative of 2 experiments. P values were calculated based on the difference between WT mice which received the depleting antibody, and WT mice which received isotype control antibody. ***p<0.001

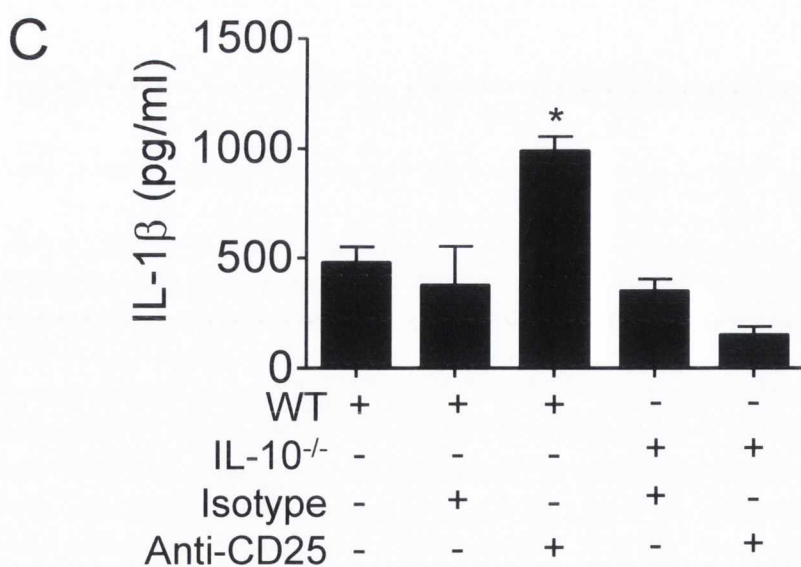
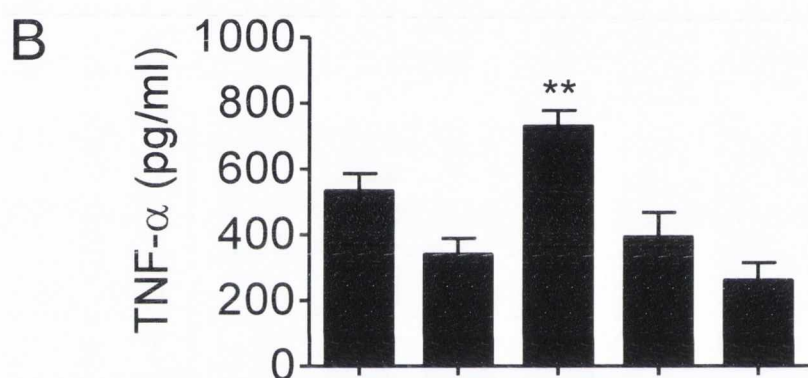
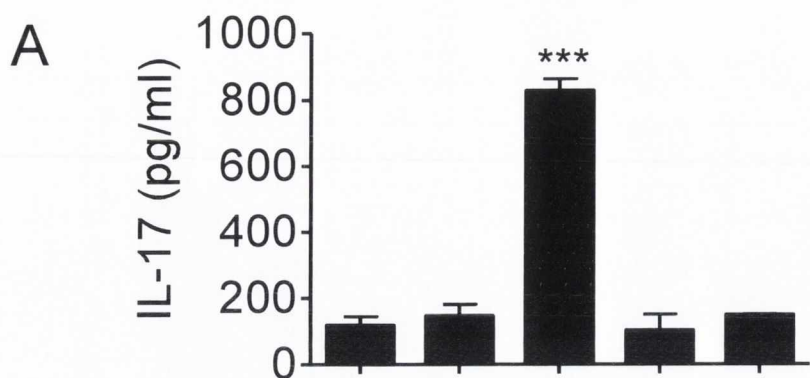


Figure 3.20 Mice depleted of CD25⁺ cells produce significantly higher levels of IL-17, TNF- α and IL-1 β in response to *B. pertussis* infection than controls

C57Bl/6 (WT) and IL-10^{-/-} mice were injected with antibody i.p. prior to *B. pertussis* infection, as described in Fig. 3.13. A) IL-17, B) TNF- α and C) IL-1 β concentrations in homogenised lungs were determined by ELISA 7 days post challenge. Results are representative of 2 experiments. P values were calculated based on the difference between WT mice which received the depleting antibody, and WT mice which received isotype control antibody. *p<0.05, **p<0.01, ***p<0.001

Chapter 4

**Murine alveolar macrophages use retinoic acid to induce
FoxP3⁺ T cells *in vitro* and *in vivo* to promote local
immunosuppression in the lung environment**

CHAPTER 2

THEORY OF THE EARTH AND ITS HISTORY

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4.1 Introduction

A population of CD4⁺FoxP3⁺CD25⁻ Treg cells which are present in the lungs of naïve mice and are dominant over CD4⁺FoxP3⁺CD25⁺ Treg cells in this compartment were described in Chapter 3. Compared with nTreg cells, these CD25⁻ Treg cells have a high activation threshold and can suppress Teff cells *in vitro* via IL-10. These cells appear to comprise a distinct immunoregulatory arm in the lung, as either depleting CD25⁺ or functionally impairing IL-10 has no effect on the course of *B. pertussis* infection. However, removing both populations of Treg cell significantly accelerates clearance of the pathogen, apparently by failing to constrain excessive Th1 immune responses.

It is not clear whether the CD4⁺FoxP3⁺CD25⁻ Treg cells are of thymic origin or whether they are induced in the lung. As they suppress via IL-10, and since the suppressive function of thymically-derived nTreg cells have been shown to be cell contact-dependent *in vitro* (Thornton and Shevach, 1998), it is possible that these cells might be induced in the lung.

In humans, lung T cells generally have a memory/ recently activated phenotype (Marathias *et al.*, 1991). Furthermore, human and rat studies on lung interstitium and bronchoalveolar lavage cells have shown that T cells from these environments have reduced cloning efficiency and cytokine production compared with blood T cells (Garlepp *et al.*, 1992; Holt *et al.*, 1988; Nelson *et al.*, 1990). Lung T cells proliferate weakly in response to mitogens in culture, presumably due to the high frequency of memory cells in the lung, which do not respond as readily as naïve cells to such stimuli (Becker *et al.*, 1990).

T cell-mediated immunity is important in the lung and respiratory tract to protect from inhaled pathogens and antigens from the environment. The induction of respiratory T cell immunity must be tightly regulated however, to prevent inflammatory damage to the fragile tissue during an immune response. Additionally, the immune system in the lung continuously encounters a vast amount of antigen, most of which is harmless. It is crucial that the resident immune cells can

distinguish such harmless antigen from pathogen-derived antigens, and effectively “ignore” them, yet respond appropriately and timely when harmful antigen is detected.

The broader lung environment also affects T cell function. It has been suggested that lung T cells are maintained in a state of “partial hyporesponsiveness”, and the lung compartment can employ various immunoregulatory mechanisms to accomplish this, including components of the surfactant lining (Wilsher *et al.*, 1988) and secreted products of alveolar type II epithelial cells (Paine *et al.*, 1992). Alveolar macrophages (AM) have also been implicated (Strickland *et al.*, 1996a). AM are the most numerous (>90%) immune cell in the normal lung (Holt *et al.*, 2008). They are located in the alveolar space, while lung T cells are sequestered in the vascular bed of the lung parenchyma (Holt *et al.*, 2008). In the rat, AM have been shown to induce T cell anergy *in vitro*, which is reversible upon removal of AM from culture (Strickland *et al.*, 1996a). The same group also described the downregulation of the IL-2R (CD25) on T cells isolated from lungs of naïve rats, and that these cells otherwise appear to be “postactivated” and trapped in the G₀/G₁ unproliferative phase of the cell cycle (Strickland *et al.*, 1996b). They conclude that AM are responsible for limiting T cell activation in the lung, presumably to prevent damage to the delicate tissue (Strickland *et al.*, 1996a; Strickland *et al.*, 1996b).

It has long been recognised that the lung is an immunosuppressive organ. The fact that the CD4⁺FoxP3⁺CD25⁻ Treg cell population described in Chapter 3 bears striking similarity to the lung cells described by Strickland *et al* led to the hypothesis that AM might be responsible for the induction of CD4⁺FoxP3⁺CD25⁻ Tregs in the lungs of naïve mice. The importance of FoxP3 as the master regulator of Treg cells was first described in 1995 by Sakaguchi and colleagues (Sakaguchi *et al.*, 1995); therefore many of the AM studies cited thus far pre-date its discovery. While Strickland *et al* reported AM-mediated suppression of T cell proliferation *in vitro*, I expanded on this finding and sought to investigate whether AM could induce FoxP3 expressing T cells, which might in turn suppress T cell proliferation.

The aim of this study was to determine whether AM are involved in inducing FoxP3⁺ T cells in the lungs of naïve mice. I have demonstrated that AM can convert FoxP3⁻ naïve T cells to FoxP3⁺ T cells *in vitro*. The mechanism involves a soluble factor rather than direct cell-cell contact, because *in vitro* culture of naïve T cells with conditioned media from AM (AM-CM) also induces FoxP3⁺ T cells, and leads to increased IL-10 and reduced IFN- γ and IL-4 production by the T cells. The soluble factors responsible for this are retinoic acid (RA) in conjunction with TGF- β_1 , and inhibiting the RA receptor (RAR) and/or neutralisation of TGF- β_1 inhibits the induction of FoxP3⁺ T cells *in vitro* and enhances T cell proliferation in the presence of AM. *In vivo*, intranasal administration of RARi as a treatment does not affect bacterial burden in mice infected with *B. pertussis*. However, RARi is effective at overcoming nasal tolerance when administering vaccines intranasally, as shown by enhanced antigen-specific IgE in the serum of mice which received intranasal antigen, adjuvant and RARi.

4.2 Results

4.2.1 Alveolar macrophages suppress T cell proliferation and can induce FoxP3 expression in naïve CD4⁺FoxP3⁻ T cells

It is well known that AM act to suppress local immune responses in the lung. Depletion of AM *in vivo*, using clodronate liposomes, illustrated the importance of AM-mediated immunosuppression; mice depleted of AM exhibited excessive T cell responses against innocuous antigen (Strickland *et al.*, 1993; Thepen *et al.*, 1991; Thepen *et al.*, 1989). Furthermore, it has been demonstrated *in vitro* that AM from mice, rats and humans inhibited T cell proliferation (Strickland *et al.*, 1996a; Strickland *et al.*, 1993; Upham *et al.*, 1995).

The mechanism by which AM exert control over T cells is unclear, although it is known that they secrete anti-inflammatory mediators such as NO (Hirano, 1998), prostaglandins (De Rose *et al.*, 1997) and the cytokines IL-10 (Chanteux *et al.*, 2007) and TGF- β_1 (Nishimura *et al.*, 2007). TGF- β_1 in particular is associated with the induction of Treg cells (Huter *et al.*, 2008; Selvaraj and Geiger, 2007; Zhou *et al.*, 2008), however, the precise relationship between AM and the induction of FoxP3⁺ T cells has not been reported. Most of the studies cited thus far actually pre-date the discovery of FoxP3 as the most definitive Treg cell marker, and while those *in vitro* studies used purified populations of CD4⁺ T cells, they did not specifically exclude Treg cells from their experiments (Strickland *et al.*, 1996a; Strickland *et al.*, 1993; Upham *et al.*, 1997). It is possible that Treg cells could have been responsible for the reported inhibition of T cell proliferation, so to exclude this possibility it was first investigated whether AM from bronchoalveolar lavage (BAL) could inhibit the proliferation of highly purified naïve effector T cells *in vitro*.

Expression of the naïve T cell marker CD45RB is inversely correlated with FoxP3 expression (Luke *et al.*, 2006). CD4⁺CD45RB^{low} T cells have been shown to be mainly comprised of FoxP3⁺ nTreg cells in naïve mice and can be used to ameliorate colitis in mice, while CD4⁺CD45RB^{high} T cells represent FoxP3⁻ effector cells capable of inducing colitis in immune compromised mice (Luke *et al.*, 2006; Powrie *et al.*, 1996; Powrie *et al.*, 1994a). I therefore

purified splenic CD4⁺CD45RB^{high}CD25⁻ T cells as a source of CD4⁺FoxP3⁻CD25⁻ naïve T effector cells to be co-cultured with AM *in vitro* (Appendix, Fig. 8.2). A proportion of the sorted CD4⁺CD45RB^{high}CD25⁻ cells were tested for FoxP3 expression after each sort; these cells were routinely less than 2% FoxP3⁺.

AM were isolated from the lungs of naïve mice by BAL, while peritoneal macrophages (PM) were isolated from the peritoneal cavity by peritoneal lavage and used as a positive control. Cells were cultured overnight at a concentration of 5×10^5 macrophages/ml in serum-free medium. Freshly isolated CD4⁺CD25⁻CD45RB^{high} splenic T cells and 2 µg/ml soluble anti-CD3 were added and cultured for a further 48 hours. AM comprise approximately 90% of all immune cells in BAL fluid of naïve animals, therefore, AM were co-cultured with naïve T cells in a ratio of 9:1. ³H-thymidine was added to the cells for the final 18 hours of culture. T cells activated in the presence of PM proliferated strongly ($p < 0.05$ compared to controls), whereas very weak proliferation was induced observed in T cells activated in the presence of AM (Fig. 4.1).

Since the purified T cells in the assay were free of contaminating FoxP3⁺ T cells, I next investigated whether AM exerted their suppressive effect by inducing FoxP3 expression in bystander T cells, or whether the inhibition of T cell proliferation is independent of Treg cells. AM were cultured overnight at a concentration of 5×10^5 AM/ml in serum-free medium. Freshly isolated CD4⁺CD25⁻CD45RB^{high} splenic T cells were cultured with AM in the presence or absence of 2 µg/ml soluble anti-CD3 for a further 24 hours. Cells were stained with rat anti-mouse antibodies against CD4, CD25 and FoxP3 and analysed by flow cytometry. T cells cultured with AM expressed FoxP3 compared with unstimulated T cells (Fig. 4.2A-B), while T cells which were activated and co-cultured with AM expressed significant ($p < 0.05$) FoxP3 compared with T cells activated with anti-CD3 in the absence of AM (Fig. 4.2A-B). Culture of T cells with PM also enhanced FoxP3 expression in T cells, regardless of whether anti-CD3 was present or not, but was significant ($p < 0.05$) when the T cells were activated with anti-CD3 (Fig. 4.2A).

While the induction of FoxP3 expression in the presence of PM after 24 hours' culture was notable and interesting, Fig. 4.1 clearly illustrates that the activated T cells were able to proliferate strongly after 48 hours in the presence of PM. It is possible that the FoxP3 expression observed was only transient, and it certainly did not affect T cell proliferation. For this reason, it was decided to focus on AM in subsequent experiments.

4.2.2 AM-mediated FoxP3 induction is independent of T cell activation

Activated $CD4^+CD25^-CD45RB^{high}$ T cells up-regulate CD25 *in vitro* (Fig. 4.3B). This confirms published data which demonstrated that although AM can suppress T cell proliferation *in vitro*, their effect is limited to this final stage in the activation process, and they do not interfere with earlier activation events such as CD25 upregulation (Upham *et al.*, 1995). However, under normal physiological conditions, T cells trafficking through the lung encounter AM in the absence of activating stimuli. I sought to determine whether T cell activation was required for AM to induce FoxP3 expression. Naïve $CD4^+CD25^-CD45RB^{high}$ T cells were cultured with AM for 24 hours *in vitro* in the presence or absence of 2 µg/ml anti-CD3. $CD4^+CD25^-CD45RB^{high}$ T cells cultured with AM without anti-CD3 upregulated FoxP3 to a modest extent (from 1.4 to 3.7%), and these T cells remained predominantly CD25⁻ (Fig. 4.3A). In contrast, T cells cultured with AM and anti-CD3 upregulated CD25 (Fig. 4.3B). Interestingly, for T cells cultured with AM in the absence of anti-CD3 the ratio of induced $CD25^-:CD25^+$ FoxP3⁺ cells was strikingly similar to that observed when FoxP3⁺ cells are isolated from the lungs of naïve mice, as described in Chapter 3. These findings suggest that the resident lung AM can actively induce peripheral tolerance, by converting naïve T cells into FoxP3⁺ T cells that might be regulatory .

4.2.3 AM use a soluble factor to induce FoxP3 expression, and inhibit Th1 immune responses

In order to test whether the induction of FoxP3 was dependent on AM:T cell contact or whether AM produced soluble immunoregulatory molecules, conditioned medium (CM) was harvested from overnight AM culture, diluted and 20 ml of each dilution was added to naïve T cells. $CD4^+CD25^-CD45RB^{high}$ T cells were isolated from the spleens of naïve mice, and these cells

were cultured with 2 µg/ml anti-CD3 and irradiated APC in the presence or absence of decreasing doses of AM-CM for 48 hours. ³H-thymidine was added for the final 18 hours of culture, and proliferation was determined by its uptake. Alternatively, CD4⁺CD25⁻CD45RB^{high} T cells were cultured with 2 µg/ml anti-CD3 and irradiated APC in the presence or absence of decreasing doses of AM-CM for 24 hours, after which FoxP3 expression was determined by flow cytometry. The most concentrated AM-CM (final concentration 1:10) had the greatest anti-proliferative effect on activated effector T cells (Fig. 4.4) and significantly enhanced ($p<0.01$) FoxP3 expression in these cells compared with control cells that received no AM-CM (Fig. 4.5A). Addition of diluted AM-CM (final concentrations 1:20 and 1:40) recapitulated proliferation of naïve T cells activated with anti-CD3 and irradiated APC (Fig. 4.4) and reduced FoxP3 induction (Fig 4.5A and 4.5B). The results reveal that AM might secrete a soluble mediator(s) that could suppress T cell proliferation and induce FoxP3 expression *in vitro*.

Cytokine analysis of the supernatants from these co-cultures demonstrated that AM-CM enhanced IL-10 production, most likely by activated naïve T cells (Fig. 4.6). Very low levels of IL-10 were present in AM-CM (Fig. 4.7), so it is likely that the enhanced IL-10 detected in the co-culture was produced by the induced FoxP3⁺ cells. Interestingly, addition of AM-CM to cultures resulted in a dose-dependent decrease in IL-4 and IFN-γ production (Fig. 4.6). The data implies that the AM-derived soluble mediator inhibits Th1 and Th2 cytokines, presumably due to the induction of suppressive FoxP3⁺ T cells.

Analysis of cytokines present in AM-CM reveals that AM produce TGF-β₁, TNF-α, and IFN-γ, which was greater than that produced following overnight culture of PM (Fig. 4.7). AM and PM produced very low concentrations of IL-4, IL-10 and IL-17 (Fig 4.7) ALDH1A, an aldehyde dehydrogenase involved in the synthesis of retinoic acid (RA), is known to induce FoxP3⁺ Treg cells (Coombes *et al.*, 2007). Analysis of AM revealed that they expressed ALDH1A, which was greater than that expressed by PM (Fig. 4.8).

4.2.4 Induction of FoxP3 by AM is dependent on retinoic acid

In an attempt to identify the soluble factor responsible for FoxP3 induction by AM, T cells were cultured in the presence of AM-CM and inhibitors of RA and/or TGF- β_1 . TGF- β_1 can convert naïve murine CD4⁺FoxP3⁻ T cells to CD4⁺FoxP3⁺CD25⁺ iTreg cells *in vitro* (Chen *et al.*, 2003; Fantini *et al.*, 2004), and this effect has been shown to be reversible (Selvaraj and Geiger, 2007). Studies of DC in the gut have shown that they produce RA, which can induce FoxP3⁺ Treg cells in conjunction with TGF- β_1 (Coombes *et al.*, 2007). Furthermore, it has been reported that gut macrophages express retinal dehydrogenase, an enzyme involved in the conversion of retinal into RA (Manicassamy and Pulendran, 2009). Since TGF- β_1 was present in AM-CM (Fig. 4.7), I examined whether neutralising TGF- β_1 could inhibit FoxP3 induction in activated naïve T cells co-cultured with AM *in vitro*. As AM also express the aldehyde dehydrogenase which converts retinaldehyde to RA (Fig 4.8), the effect of blocking RA signalling was also investigated, using a synthetic RA receptor antagonist (RARi, 5 μ M).

Inhibition of either TGF- β_1 or RA in AM-CM led to a reduction in the percentage of FoxP3⁺ T cells, and a combination of both TGF- β_1 and RARi significantly reduced the percentage of FoxP3⁺ cells ($p < 0.05$), when compared with controls where activated naïve T cells were cultured with AM-CM, implying an additive effect (Fig. 4.9). Inhibition of RA signalling or neutralisation of TGF- β_1 also significantly inhibited ($p < 0.05$) the induction of FoxP3 in activated naïve T cells co-cultured with AM, when compared with controls where T cells were co-cultured with AM in the absence of inhibitors (Fig. 4.10). These data demonstrate that AM suppress effector T cells via TGF- β_1 and RA.

4.2.5 Inhibition of lung T cell proliferation by AM *in vitro* can be overcome by inhibiting RA signalling

Numerous studies have described the anti-proliferative effect that AM have on T cells (Ettensohn *et al.*, 1986; McCombs *et al.*, 1982; Rich *et al.*, 1991; Schauble *et al.*, 1993; Toews *et al.*, 1984; Upham *et al.*, 1995; Upham *et al.*, 1997; Warner *et al.*, 1981). In addition, previous data

from our lab reported the proliferation of macrophage-depleted lung cells or purified lung T cells upon co-culture with splenic APC, but not lung APC, presumably due to the presence of AM (McGuirk *et al.*, 1998). None of these studies address the role of FoxP3 because many of them pre-date the discovery of Treg cells and their associated transcription factor. Based on the data thus far, I hypothesised that the suppression of T cell proliferation by AM might be mediated by the induction of FoxP3⁺ T cells. Since inhibiting RA reduced the frequency of FoxP3⁺ cells *in vitro*, I investigated whether this strategy would enhance T cell proliferation in the presence of AM. I decided to focus on the effect of only inhibiting RA from this point on, as cell cultures were maintained in complete RPMI supplemented with FCS, which contains exogenous TGF- β_1 . AM-CM used in previous experiments did not contain FCS, and was therefore free of contaminating exogenous TGF- β_1 .

Total mononuclear cells from lungs of naïve Balb/c mice (2×10^6 cells/ml) were cultured with 2 μ g/ml anti-CD3 in the presence or absence of 5 μ M RARi for 72 hours. Cells were replenished with either fresh medium or medium containing ³H-thymidine for the final 18 hours of culture and FoxP3 expression and cell proliferation were determined, respectively. RARi significantly enhanced proliferation ($p < 0.05$) of lung cells (Fig. 4.11A). FoxP3 expression was decreased when lung cells were cultured in the presence of RARi (Fig. 4.11B). Lung cells (2×10^6 /ml) which had been depleted of AM were also cultured with anti-CD3 in the presence or absence of RARi. RARi did not affect proliferation of these cells (Fig. 4.11A). Collectively, these results demonstrate that inhibiting the effects of RA enhances proliferation of cells cultured in the presence of AM, presumably due to its inhibition of FoxP3 induction.

4.2.6 Inhibiting RA signalling enhances antigen-specific T cell proliferation

It has been previously demonstrated that lung APC have a suppressive effect on T cell proliferation in response to *B. pertussis* antigens (McGuirk *et al.*, 1998). In these experiments purified T cells from the lungs of mice infected with *B. pertussis* proliferated poorly *in vitro* when cultured with antigen in the presence of lung APC, but not splenic APC. Also, addition of lung APC suppressed proliferation of lung T cells cultured with antigen in the presence of splenic APC,

which strongly indicates a role for AM-mediated suppression. Therefore experiments were designed to examine whether RA and FoxP3⁺ T cells were responsible for the suppressive effects of lung APC on T cell proliferation.

Naïve Balb/c mice were infected with *B. pertussis*. CD3⁺ T cells were purified from lung and spleen 21 days post challenge. CD3⁺ T cells were cultured with FHA (4 µg/ml), anti-CD3 and PMA (5 µg/ml and 25 ng/ml) or medium only, with irradiated lung-derived or splenic APC in the presence or absence of 5 µM RARi. After 48 hours supernatants were removed for cytokine analysis by ELISA and cells were replenished with either fresh medium or medium containing ³H-thymidine, to analyse FoxP3 expression and to determine proliferation, respectively.

Regardless of the stimulus, lung T cells showed reduced proliferation in the presence of lung APC, when compared to lung T cells stimulated in the presence of splenic APC (Fig. 4.12, left panels). Addition of RARi to lung T cells activated with lung APC resulted in slightly reduced FHA-specific proliferation (Fig. 4.12A, left panel), but increased proliferation in response to anti-CD3 and PMA (Fig. 4.12B, left panel). RARi did not affect proliferation of lung T cells cultured in the presence of splenic APC, in response to both stimuli (Fig. 4.12, left panels). In other words, RARi only affected proliferation of T cells cultured in the presence of AM.

Splenic T cells cultured with lung APC showed a dramatic reduction in proliferation in response to both stimuli when compared to splenic T cells stimulated with splenic APC (Fig. 4.12, right panels). RARi slightly enhanced FHA-specific proliferation of splenic T cells cultured with splenic APC, but this was not significant and enhancement was not observed in response to anti-CD3 and PMA stimulation. RARi slightly enhanced FHA-specific proliferation and proliferation in response to anti-CD3 and PMA when splenic T cells were cultured with lung APC (Fig. 4.12, right panels).

RARi only enhanced proliferation when lung APC, and therefore AM, were present in the cultures. However, a simultaneous decrease in FoxP3⁺ cell frequency was not observed in response to antigen (Fig. 4.13A), and was only very slightly reduced when anti-CD3 and PMA were present in culture (Fig. 4.13B). With respect to cytokine production, FHA-specific IFN- γ and IL-10 production by lung T cells was reduced when RARi was added to the co-cultures which contained AM (Fig. 4.14, left panels), while RARi only reduced FHA-specific IL-10 but had no effect on IFN- γ production by lung T cells activated with splenic APC (Fig. 4.14, left panels). RARi decreased FHA-specific IL-10 production by splenic T cells activated in the presence of splenic APC, but increased IFN- γ production by these cells regardless of the source of APC (Fig. 4.14, right panels).

IFN- γ and IL-10 production by lung T cells in response to anti-CD3 and PMA was enhanced when RARi was also present, regardless of the source of APC (Fig. 4.15, left panels). The same effect was observed for splenic T cells (Fig. 4.15, right panels), with the exception of IL-10 production by T cells stimulated with splenic APC. RARi had no effect on IL-10 production in this case (Fig. 4.15A).

4.2.7 Intranasal RARi reduces the frequency of FoxP3⁺ T cells in *B. pertussis*-infected mice, but does not affect bacterial burden

Since RARi can enhance antigen-specific T cell proliferation *in vitro*, I next examined whether inhibition of RA could alter the course of infection in mice with *B. pertussis*. Naïve Balb/c mice were infected with *B. pertussis*. Mice received either 75 μ g or 150 μ g RARi intranasally (i.n.) every second day, from day 1 post-challenge up to day 19 post-challenge. Control mice received vehicle i.n., which was a mix of PBS and ethanol. 7, 14 and 21 days post-challenge lungs were homogenised and CFU counts were performed. The frequencies of CD4⁺FoxP3⁺ T cells were also examined.

Treatment with either dose of RARi had no effect on clearance of *B. pertussis*. CFU values from the lungs of mice which received vehicle were similar to RARi-treated mice (Fig. 4.16).

Nonetheless, 7 days post-challenge the mice which received the higher dose of RARi had significantly reduced ($p < 0.001$) frequency of lung $CD4^+FoxP3^+$ cells compared with vehicle controls (Fig. 4.17A). The lower dose of RARi was seen to have a significant ($p < 0.05$) effect on the frequency of $FoxP3^+$ cells at day 14 post-challenge (Fig. 4.17B), and both doses reduced the frequencies of lung $CD4^+FoxP3^+$ cells when the experiment was terminated on day 21 post-challenge (Fig. 4.17B). These results suggest that nasal administration of RARi is effective at reducing the frequency of induced $FoxP3^+$ T cells in the lungs, but this strategy alone does not affect the clearance of the pathogen. It is possible that the remaining $FoxP3^+$ T cells provide adequate immune regulation. These $FoxP3^+$ T cells may represent thymically-derived nTreg cells.

4.2.8 RARi overcomes nasal tolerance to induce humoral immunity to nasally-administered antigens and may represent a novel strategy for mucosal vaccination

I next examined whether inhibiting RA could break nasal tolerance to vaccination with ovalbumin. Studies have shown that mucosal administration of antigen results in immunological nonresponsiveness to the same antigen (van Halteren *et al.*, 1997). It has also been reported that mucosal administration of antigen prior to systemic sensitisation with the same antigen results in long lasting immunosuppression rather than priming (Garside *et al.*, 1995; Hoyne *et al.*, 1996; McMenamin *et al.*, 1994). Based on the data so far, I investigated whether the induction of $FoxP3^+$ T cells by RA-producing AM was responsible for tolerance in the respiratory mucosa, and whether this tolerance could be overcome using RARi.

Naïve Balb/c mice were immunised twice i.n., 21 days apart with 25 μ g Ovalbumin (Ova) protein, alone or in the presence of 5 μ g CpG as adjuvant. Both types of vaccine were administered in the presence or absence of 100 μ g RARi. Control mice were vaccinated subcutaneously with Ova and CpG or i.n. with vaccine vehicle (a mix of PBS and ethanol).

As expected, control mice which received vehicle i.n. did not have detectable Ova-specific IgE in their serum (Fig. 4.18B). Ova-specific IgE was also not detected at levels above background

in mice which were vaccinated with Ova +/- RAR α or Ova +/- CpG (Fig. 4.18B). These results correlate with other studies showing non-responsiveness to mucosally administered antigen (Garside *et al.*, 1995; Hoyne *et al.*, 1996; McMenamin *et al.*, 1994; van Halteren *et al.*, 1997). However, significant ($p < 0.001$) Ova-specific IgE was detected in the serum of mice which were vaccinated with Ova + CpG in the presence of RAR α , and concentrations were similar to those of mice which were vaccinated subcutaneously with Ova + CpG (Fig. 4.18B). There was no difference in total IgE concentrations in response to the different treatments (Fig. 4.18A). These data suggest that the FoxP3⁺ T cells which are induced by RA-producing AM in the lung might be responsible for the induction of respiratory tolerance, and that inhibiting their induction at the time of antigen administration can overcome tolerance to specific antigen.

Conversely, significant ($p < 0.001$) IgG₁ was detected in mice which received Ova + CpG either i.n. or subcutaneously (Fig. 4.19B). There was no difference in total IgG₁ concentrations between the different groups (Fig. 4.18A). These results confirm the findings of others which have shown that oral tolerance inhibits the production of antigen-specific IgE and IgG₁, but nasal tolerance only blocks IgE production (van Halteren *et al.*, 1997). However, nasal administration of Ova + CpG in the presence of RAR α reduced the serum concentration of Ova-specific IgG₁ compared to vaccine alone (Fig 4.19).

4.3 Discussion

This study has demonstrated for the first time that AM inhibit T cell proliferation by inducing FoxP3 expression in naïve T cells *in vitro*. Importantly, the activation status of the T cells did not affect the induction as AM induced FoxP3 expression in both unstimulated and anti-CD3-stimulated T cells *in vitro*. The phenotype of the Treg cells induced by AM without anti-CD3 stimulation was predominantly $CD4^{+}FoxP3^{+}CD25^{-}$, which resembles the *ex vivo* phenotype of FoxP3⁺ T cells isolated from the lungs of naïve mice, as described in Chapter 3.

The data so far has pointed to a role for soluble mediators in AM-induced FoxP3 expression in naïve $CD4^{+}$ T cells. The induction of FoxP3 and inhibition of T cell proliferation by AM was duplicated using AM-CM. Inhibition studies revealed that AM-derived RA and TGF- β_1 were mostly responsible for the induction of FoxP3. *In vitro*, AM inhibited anti-CD3- or antigen-specific proliferation of T cells, but this inhibition was overcome by blocking RA signalling. Inhibiting RA signalling *in vivo*, by nasal administration of RARi significantly reduced the frequencies of FoxP3⁺ T cells in the lungs of mice infected with *B. pertussis*, but this had no effect on the bacterial burden. However, the addition of RARi to nasally delivered antigen and adjuvant prevented the induction of nasal tolerance and allowed systemic immunity to develop. This might represent a novel vaccination strategy in terms of delivering vaccines to mucosal sites, where antigens are encountered in real life.

The ability of AM to suppress local immune responses has been well documented, both *in vitro* and *in vivo*. AM from mice, rats and humans are able to suppress proliferation of T cells *in vitro* (Strickland *et al.*, 1996a; Strickland *et al.*, 1993; Upham *et al.*, 1995). Of particular importance are *in vivo* studies where AM were completely and specifically eliminated. These studies utilised a cytotoxic drug called dichloromethylene diphosphate (Cl_2MDP), which is specifically targeted to macrophages by encapsulation in liposomes. Intratracheal (i.t.) administration of the drug-loaded liposome into the lung eliminated AM and resulted in overwhelming inflammation to otherwise harmless particulate and soluble antigen (Strickland *et*

al., 1993; Thepen *et al.*, 1992; Thepen *et al.*, 1991; Thepen *et al.*, 1989). The inflammation appeared to be mediated by T cells (Strickland *et al.*, 1993; Thepen *et al.*, 1991).

Clearly AM have a crucial role in regulating T cell responses in the lung, and based on the novel murine lung Treg cell population described in Chapter 3, the possibility that murine AM can influence or induce FoxP3⁺ T cells was examined. It is likely that the CD4⁺FoxP3⁺CD25⁻ Treg cells are induced in the lung rather than derived from the thymus because they suppress via IL-10, unlike thymically-derived Treg cells which suppress in a cell-contact dependent manner (Thornton and Shevach, 1998). It is important to stress that the published studies on AM and T cells did not investigate Treg cells, and in many instances they were published before the relevance of FoxP3 in T cell biology came to light. Therefore, it was first investigated whether *in vitro* culture of highly purified naïve effector T cells with AM resulted in suppression of T cell proliferation, or whether the previously published work merely reflected the presence of contaminating Treg cells in the culture. T cells were purified from the spleen rather than the lung to eliminate the possibility that lung T cells might already be hyporesponsive. Activation of splenic CD4⁺CD25⁻CD45RB^{high} (therefore FoxP3^{low/-}) naïve effector T cells with control peritoneal macrophages resulted in T cell proliferation, whereas culture with AM did not promote proliferation of the same T cell population. Undoubtedly AM exert a profound inhibitory effect on T cell proliferation.

Once it was established that AM suppress the proliferation of highly purified, Treg-free splenic CD4⁺CD25⁻CD45RB^{high} T cells, it made sense to investigate whether or not AM induce FoxP3 expression in these cells. Indeed, culture of stimulated CD4⁺CD25⁻CD45RB^{high} splenic T cells with AM induced significant FoxP3 expression in naïve T cells compared to stimulated controls. This is the first time such a link between AM and FoxP3 induction has been reported; the induction of FoxP3⁺ T cells from naïve effector T cells may account for an intermediary step in the suppression of T cell-mediated immunity by AM in the lung.

In order to examine the effect of AM on T cell proliferation *in vitro*, and to investigate if the suppression of proliferation correlated with FoxP3 induction, the T cells had to be activated. As a result, the T cells upregulated CD25 in response to activation with anti-CD3. Therefore, the ability of AM to enhance FoxP3 expression in unstimulated T cells was examined. The results of this experiment confirm that T cells do not need to be activated in order to express FoxP3 as a consequence of culture with AM. The unstimulated induced Treg cell population bore a striking phenotypic resemblance to resident CD4⁺FoxP3⁺ cells in the naïve mouse lung, in terms of CD25 expression.

This finding has further implications in terms of what actually happens in the lung under normal conditions rather than artificial *in vitro* assays. Obviously most of the T cells which come in contact with AM would not encounter activating stimuli, so would not express CD25. This could explain why CD4⁺FoxP3⁺ T cells isolated from the naïve mouse lung are overwhelmingly CD25⁻. Although these induced Treg cells do not express CD25 at the steady state, they may comprise a “reservoir” of inactive Treg cells which will effectively suppress immune responses, but only when they are sufficiently activated. Furthermore, studies in the rat have also reported that postactivated T cells lose expression of IL-2R (CD25) but not any other markers upon migration to the lung after adoptive transfer *in vivo* (Strickland *et al.*, 1996b). Therefore the induced FoxP3⁺ T cells could be derived from naïve CD25⁻ T cells as well as previously activated T cells which have down-regulated CD25 expression.

With respect to the mechanism by which AM exert suppression over T cells, AM supernatants from several species have been shown to suppress T cell proliferation, indicating a role for a diffusible factor (Pennline *et al.*, 1979; Pennline and Herscowitz, 1981; Strickland *et al.*, 1996a). There is evidence that the diffusible factor differs across species, with rodent AM using NO, but human AM exerting suppression of T cells in an NO-independent manner (Rich *et al.*, 1991; Upham *et al.*, 1995). Nonetheless, the potential contamination by Treg cells in these studies was not addressed, so the ability of conditioned medium from overnight culture of AM (AM-CM) to suppress proliferation and induce FoxP3 expression in highly purified splenic CD4⁺CD25⁻

CD45RB^{high} T cells was investigated. The results demonstrate that AM-CM can promote FoxP3 expression by these cells and effectively inhibit their proliferation in a dose-dependent manner. Clearly, AM-mediated induction of Treg cells is facilitated by the release of a soluble factor.

Furthermore, AM-CM appeared to promote IL-10 production in a dose-dependent manner. AM-CM contained very low concentrations of IL-10, so it is very unlikely that its presence in this assay results from any additive effects in the culture. Rather, it might be produced by the induced FoxP3⁺ T cells, because induced Treg cells use cytokines rather than cell-cell contact to suppress (Thornton and Shevach, 1998). Notably, the conversion of CD4⁺FoxP3⁺CD25⁻ T cells to CD4⁺FoxP3⁺CD25⁻ iTreg cells has also been reported. The existence of antigen-specific CD4⁺FoxP3⁺CD25⁻ iTreg has been shown *in vivo* (Kretschmer *et al.*, 2005), and malaria infection can also drive the conversion of CD4⁺FoxP3⁺CD25⁻ T cells to CD4⁺FoxP3⁺CD25⁻ iTreg cells (Couper *et al.*, 2007). If the AM-induced Treg cells are the source of IL-10, then it represents yet another similarity to the CD4⁺FoxP3⁺CD25⁻ Treg cells described in Chapter 3, which use IL-10 to suppress *in vitro* and *in vivo*.

Importantly, TGF- β_1 was detected in AM-CM, but not PM-CM, suggesting that AM might specifically use TGF- β_1 to induce FoxP3⁺ T cells. AM-CM and PM-CM did not contain foetal calf serum (FCS) and therefore exogenous TGF- β_1 present in FCS was not an issue. *In vitro* conversion of naïve CD4⁺FoxP3⁻ T cells to CD4⁺FoxP3⁺CD25⁺ inducible Treg (iTreg) cells by TGF- β_1 has been reported (Chen *et al.*, 2003; Fantini *et al.*, 2004). DC have been shown to play a role in the TGF- β_1 -dependent induction of functional FoxP3⁺ iTreg cells in a diabetes model (Luo *et al.*, 2007). In the intestine, TGF- β_1 and the vitamin A metabolite RA have been shown to convert naïve CD4⁺ T cells to antigen-specific CD4⁺FoxP3⁺ iTreg cells (Coombes *et al.*, 2007). Therefore, the induction of Treg cells might be an important strategy for maintaining tolerance at mucosal interfaces such as the intestine and the lung.

In vivo studies have demonstrated that blocking TGF- β_1 during feeding of Ova inhibited both the induction of oral tolerance and the peripheral conversion of Ova-specific Treg cells (Mucida *et al.*, 2005). However, TGF- β_1 is a pleiotropic cytokine and can promote Treg cell induction or Th17 cells, depending on the cytokine milieu. When activated CD4⁺ T cells are exposed to TGF- β_1 alone, they can convert to FoxP3⁺ T cells, but if IL-6, IL-1 β and IL-23 or IL-21 are also present then FoxP3 induction is blocked and the induction of Th17 cells is favoured (Bettelli *et al.*, 2006; Manel *et al.*, 2008; Mangan *et al.*, 2006; Veldhoen *et al.*, 2006). The presence of TGF- β_1 in AM-CM is therefore not sufficient to prove it is absolutely responsible for the induction of FoxP3⁺ T cells.

RA has been shown to modulate the pleiotropic effects of TGF- β_1 and promote FoxP3⁺ Treg cell induction (Benson *et al.*, 2007; Coombes *et al.*, 2007; Kang *et al.*, 2007b; Mucida *et al.*, 2007; Sun *et al.*, 2007). When CD4⁺ T cells are activated in the presence of TGF- β_1 and RA, FoxP3⁺ T cell induction is enhanced. Even if IL-6 is also present, Th17 cell differentiation is inhibited because RA blocks the IL-6-dependent expression of ROR γ t, the key Th17-polarising transcription factor (Ivanov *et al.*, 2006; Mucida and Cheroutre, 2007; Mucida *et al.*, 2007; Zhou *et al.*, 2008). It has been reported that gut DCs from GALT and small intestinal lamina propria use RA to induce Treg cells (Coombes *et al.*, 2007; Sun *et al.*, 2007), this is controversial since there is evidence that macrophages from lamina propria induce iTreg cells while DCs induce Th17 cells (Denning *et al.*, 2007).

The production of RA by AM has not previously been reported. To determine whether RA was responsible for enhancing TGF- β_1 -dependent FoxP3 induction, in naïve T cells, the expression of retinal dehydrogenases (RALDH) by AM was examined. RA is derived from dietary vitamin A and it is synthesised when all-trans retinal is irreversibly oxidised by aldehyde dehydrogenase 1A (ALDH1A) (Blomhoff and Blomhoff, 2006; Moise *et al.*, 2007). The expression of ALDH1A is tightly controlled and is restricted to specific cells and tissues. Consequentially RA production is restricted to these specific cells and tissues, such as the gut (Coombes *et al.*, 2007; Germain *et al.*,

2006; Iwata *et al.*, 2004; Lampen *et al.*, 2000). ALDH1A activity is therefore an indirect measure of retinoic acid production. Both AM and PM expressed the enzyme, but AM produced slightly more than PM. This is the first time that RA production by AM has been described.

RA exerts its function by binding to nuclear hormone receptors which belong to the retinoic acid receptor (RAR) family, of which there are three isoforms – α , β and γ (Mora *et al.*, 2008). Unlike RALDH, RAR proteins are ubiquitously expressed (Blomhoff and Blomhoff, 2006; Moise *et al.*, 2007). *In vivo* studies have demonstrated that the injection of RAR antagonists decreased the frequencies of lamina propria FoxP3⁺ cells (Mucida *et al.*, 2007). To determine the soluble factor/s responsible for FoxP3 induction by AM, CD4⁺CD25⁻CD45RB^{high} T cells were activated in the presence of AM-CM and a variety of inhibitors. The effects of TGF- β_1 were blocked by adding a TGF- β_1 -neutralising antibody to the culture. RA signalling was inhibited by a synthetic RAR antagonist (RARi) either alone or in conjunction with the TGF- β_1 blocking antibody. Blocking RA signalling or neutralisation of TGF- β_1 reduced the frequency of FoxP3⁺ T cells. Combining RARi and anti-TGF- β_1 further inhibited the induction of FoxP3⁺ T cells. These experiments identify for the first time a mechanism by which AM induce FoxP3⁺ T cells.

Inhibition of either RA or TGF- β_1 also prevented the induction of FoxP3⁺ T cells when T cells were cultured with AM rather than AM-CM, but combining both inhibitors had a stronger effect. It is important to note that while AM-CM did not contain exogenous FCS-derived TGF- β_1 , co-cultures were maintained in medium supplemented with FCS, so exogenous TGF- β_1 would have been present in these cultures. For this reason, it was decided to focus on the effect of only inhibiting RA in subsequent experiments.

Published data from this lab has demonstrated that unseparated mononuclear cells from the spleens of naïve mice proliferated upon stimulation with the mitogen ConA, whereas lung mononuclear cells were unresponsive (McGuirk *et al.*, 1998). However, macrophage-depleted lung cells were capable of proliferating, and it was concluded that the hyporesponsiveness was due to

the presence of suppressive AM (McGuirk *et al.*, 1998). To test whether these findings were compatible with this published data, the experiment was repeated using anti-CD3 as a T cell-specific stimulus rather than ConA, and the cells were cultured in the presence or absence of RARi. The results revealed that blocking RA signalling significantly enhanced proliferation of total but not AM-depleted lung cells, and this enhanced proliferation was associated with a reduction in the frequency of FoxP3⁺ cells.

This lab has also reported that lung mononuclear cells from mice which had been infected with the respiratory pathogen *B. pertussis* were hyporesponsive to purified *B. pertussis* antigens, but spleen cells from the same mice were responsive (McGuirk *et al.*, 1998). Additionally, purified splenic T cells from infected mice could proliferate strongly against purified *B. pertussis* antigens in the presence of splenic APC and they produced relatively high levels of IFN- γ and IL-2. However, this strong response was dramatically reduced when splenic T cells were cultured in the presence of lung APC, or equal numbers of lung APC and splenic APC (McGuirk *et al.*, 1998). Purified lung T cells proliferated and produced low levels of IL-2 and IFN- γ when stimulated in the presence of splenic APC, but as with the splenic T cells these effects were inhibited if lung APC were present. These studies suggest a role for AM (present in the lung APC population) in the suppression of lung T cell responses, however a mechanism by which they exert this suppressive effect was not proposed.

I hypothesised that AM were inducing FoxP3⁺ T cells which were responsible for inhibiting antigen-specific T cell responses. The *B. pertussis* infection experiment was repeated, and CD3⁺ T cells were cultured in the presence or absence of RARi. Addition of RARi resulted in enhanced proliferation when lung-derived or splenic T cells were stimulated with either FHA or anti-CD3 and PMA in the presence of lung APC – or in other words, in the presence of AM. Increased proliferation was generally associated with a reduction in the frequency of FoxP3⁺ T cells, suggesting that the inhibition of RA signalling prevented FoxP3 induction by AM. However, IFN- γ concentrations were only enhanced in the presence of RARi for cells stimulated with PMA

and anti-CD3. Interestingly, IL-10 was also enhanced in the presence of RARi in response to PMA and anti-CD3. However, FHA-specific IFN- γ and IL-10 production was reduced when RARi was present in culture. It is likely that RARi would not have affected the function of pre-existing nTreg cells or antigen-specific iTreg cells which could have been induced in the mice during *B. pertussis* infection. Rather, RARi would only have prevented AM from inducing FoxP3 expression in FoxP3⁻ T cells *in vitro*, so any pre-existing antigen-specific T cells in the culture would be expected to suppress in response to FHA. This might explain why IFN-g was reduced along with IL-10; while antigen-specific IL-10-producing FoxP3⁺ T cells might be inhibited *in vitro*, nTreg cells might be functioning to compensate, resulting in reduced antigen-specific IFN- γ production.

Since inhibiting RA signalling could enhance T cell proliferation in response to *B. pertussis* antigens, the question arose whether manipulation of the AM/RA/FoxP3 mechanism could be used to accelerate clearance of bacterial infection in the upper respiratory tract. Mice infected with *B. pertussis* received treatment intranasally every second day. Enumeration of CFUs at days 7, 14 and 21 post-challenge demonstrated that neither dose of RARi significantly affected the bacterial burden of mice compared to controls. Perhaps an increased dose of inhibitor would be more successful, but that would be practical because the inhibitor slightly precipitated out of solution at the higher of the two doses used and this made it difficult for the mice to inhale. More frequent administrations of low-dose RARi might circumvent this. However, flow cytometry analysis of cells isolated from the lungs revealed a significant reduction in the frequency of CD4⁺FoxP3⁺ T cells in infected mice which had received RARi compared to infected mice which received vehicle as a control. Evidently, RARi as a treatment was having the effect it was expected to have *in vivo*, but the resultant reduction in FoxP3⁺ T cells did not translate into enhanced protective immunity to *B. pertussis*.

Nonetheless, these results show for the first time successful inhibition of FoxP3⁺ T cell induction *in vivo* as a consequence of nasal administration. It is possible that despite the significant reduction in the frequency of FoxP3⁺ T cells in the lung in response to RARi treatment, those FoxP3⁺ T cells that remained might have been sufficient to regulate the immune response against

B. pertussis. The remaining FoxP3⁺ T cells might represent thymically-derived nTreg cells. It is therefore possible that the findings from this experiment reflect the observation in Chapter 3 that two populations of Treg cells are present in the lungs of mice, and that both must be removed or functionally impaired in order to eradicate the pathogen more rapidly.

Alternatively, it was investigated whether RARi could be applied to enhance the efficacy of nasal vaccination. It is well known that the administration of antigen via the mucosal surfaces of the body (i.e. nasal, oral or urogenital) results in systemic immunological nonresponsiveness to the same antigen (Garside *et al.*, 1995; Hoyne *et al.*, 1996; McMenamin *et al.*, 1994; van Halteren *et al.*, 1997). The profound immunological nonresponsiveness induced upon mucosal administration of antigen is characterised by reduced production of Th1 and Th2 cytokines, and suppressed immunoglobulin production (van Halteren *et al.*, 1997). Specifically, tolerance induced by the nasal or oral route is mediated by CD4⁺ T cells and results in decreased T cell proliferation, which in turn inhibits isotype switching to IgE (Garside *et al.*, 1995; Hoyne *et al.*, 1996). Importantly, oral tolerance has been shown to inhibit IgE and IgG₁ responses, while nasal tolerance inhibits IgE only (van Halteren *et al.*, 1997). It was hypothesised that the reason for this nonresponsiveness was due to the induction of mucosal FoxP3⁺ T cells in response to the antigen. Since it has been demonstrated that RARi can be successfully administered intranasally to inhibit the induction of FoxP3⁺ T cells by AM *in vivo*, it was possible that this might represent a novel strategy for developing nasal vaccines.

It was proposed that inhibiting AM-mediated FoxP3⁺ T cell induction by administering RARi at the same time as Ova and CpG would prevent the induction of nasal tolerance and result in systemic immunity to Ova. Analysis of antibodies in the serum of experimental mice revealed no difference in total IgE in response to the different treatments. However, only mice that were immunised subcutaneously and those which received intranasal Ova, CpG and RARi had detectable Ova-specific IgE. All mice that were immunised with Ova and CpG had Ova-specific serum IgG₁, regardless of the route of immunisation or whether RARi was present. However, those

which received antigen and adjuvant in the presence of RAR α had reduced IgG $_1$ in serum. It is possible that the enhanced production of IgE in these mice comes at the expense of IgG $_1$.

It is well known that Th2 cytokines, in particular IL-4, promote IgG $_1$ and IgE production (van Halteren *et al.*, 1997). This can be inhibited by IFN- γ (Snapper and Paul, 1987), and it has been shown that tolerised mice have increased IFN- γ production in the spleen along with reduced levels of serum IgG $_1$ (van Halteren *et al.*, 1997). It appears that CD4 $^+$ are responsible because the transfer of CD4 $^+$ T cells from nasally tolerised mice suppressed IgE but not IgG $_1$ production in primed recipients (Garside *et al.*, 1995; van Halteren *et al.*, 1997) and nasal tolerance can be induced in CD8 $^{-/-}$ mice (Hoyne *et al.*, 1996). Furthermore, blocking IFN- γ prior to adoptive transfer abrogated the suppression of IgE production (van Halteren *et al.*, 1997). It was hypothesised that the “regulatory” CD4 $^+$ T cells transferred from nasally tolerised mice suppressed primary and ongoing IgE responses in Th2-skewed recipients, via IFN- γ (van Halteren *et al.*, 1997). It has also been suggested that while low levels of IL-4 might be sufficient for IgG $_1$ class switching, higher levels might be necessary for IgE class switching and would explain why nasal tolerance only suppresses IgE and not IgG $_1$ (van Halteren *et al.*, 1997).

A specific role for AM in regulating this system is supported by a study in which the elimination of AM via intratracheal injection of clodronate liposomes resulted in increased IgE production in the lungs of pre-sensitised rats upon subsequently exposed to antigen aerosols (Thepen *et al.*, 1992). Considerable infiltration of CD4 $^+$ T cells into the lungs and airways was also observed (Thepen *et al.*, 1992). My data confirms that nasal tolerance inhibits IgE and not IgG $_1$ immune responses. It also provides evidence that AM and the FoxP3 $^+$ T cells they induce are responsible for promoting nasal tolerance because this tolerogenic mechanism can be overcome to promote protective systemic immunity to soluble, nasally-administered antigens.

In conclusion, the results from this study have shown for the first time that AM actively induce FoxP3 in naïve T cells that might have regulatory function but do not appear to be anergic..

This gives further insight into how AM maintain an immunosuppressive environment in the lungs. Interestingly, AM-mediated Treg cell conversion is independent of T cell activation, which might explain why the majority of CD4⁺FoxP3⁺ T cells isolated from the lungs of naïve mice do not express CD25. AM produce both RA and TGF- β_1 , which can synergise to mediate FoxP3⁺ T cell induction and promote suppression of effector T cell responses. The induced Treg cells appear to function in an IL-10-dependent manner to inhibit Th1 and Th2 responses, which is also similar to how lung CD4⁺FoxP3⁺CD25⁻ Treg cells suppress *in vitro* and *in vivo*.

Intranasal administration of an RAR antagonist alone does not represent an effective treatment for respiratory infection, because in the *B. pertussis* model the prevention of FoxP3⁺ T cell induction did not affect the bacterial burden. Nonetheless, this further highlights the multifaceted ways in which protective immunity is stringently regulated in the lungs; therefore targeting both nTreg cells and induced FoxP3⁺ T cells might be required for treating respiratory infections. However, the manipulation of this novel regulatory network reflects a more promising strategy in terms of mucosal vaccination, as inhibition of RA signalling at the same time as i.n. immunisation with soluble antigen can overcome the overwhelming immune non-responsiveness that is associated with nasal vaccines.

4.4 Figures

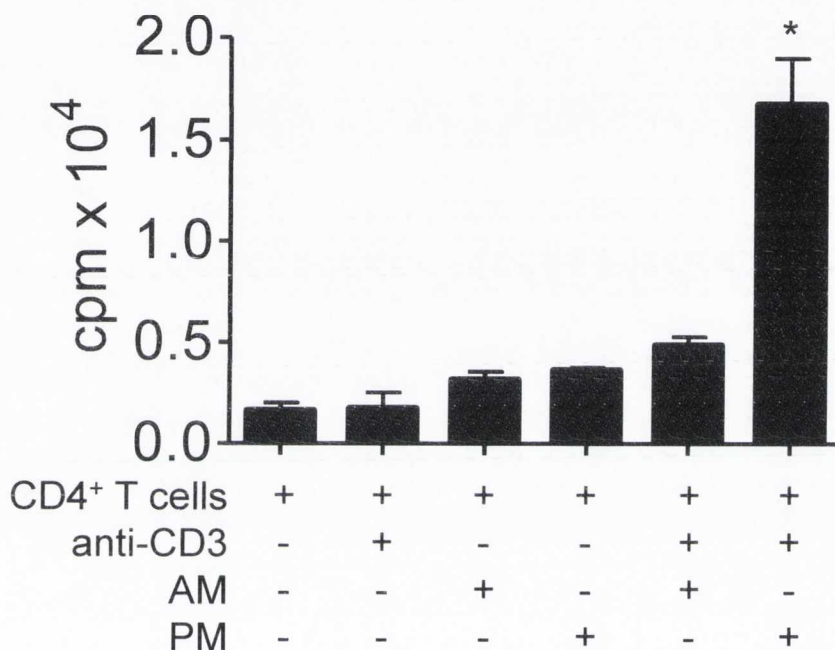
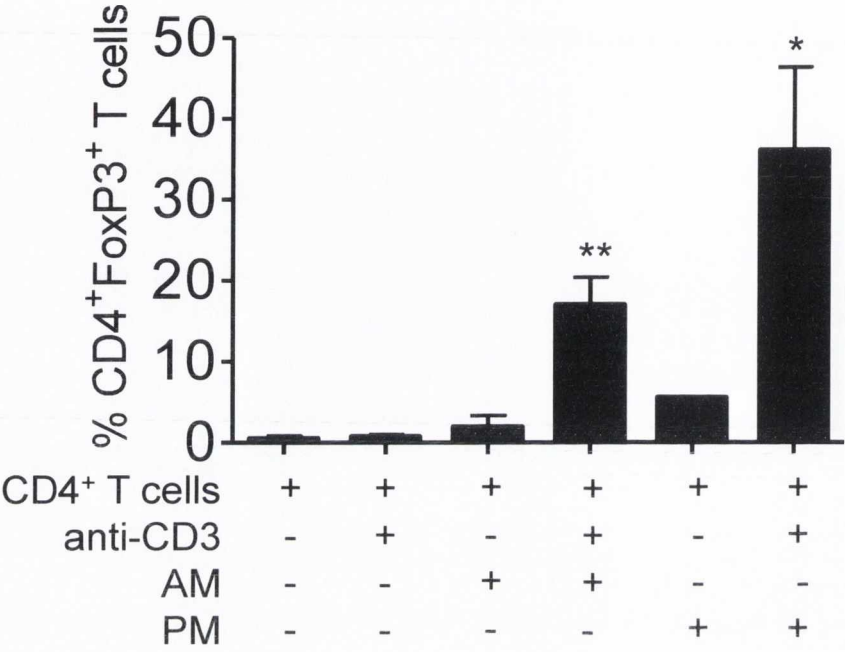


Figure 4.1 AM do not support proliferation of naïve T cells

AM were isolated from the lungs of naïve female Balb/c mice by bronchoalveolar lavage and PM were isolated from the peritoneal cavity by peritoneal lavage. Macrophages were cultured at $5 \times 10^5/\text{ml}$ *in vitro* and allowed to adhere overnight. CD4⁺CD25⁻CD45RB^{high} naïve effector T cells were purified from the spleens of naïve mice and were added to macrophages with 2 $\mu\text{g}/\text{ml}$ soluble anti-CD3 48 hours at a ratio of 9:1 macrophages:T cells. Proliferation was determined by ³H-thymidine incorporation after 48 hours. P values were calculated based on the difference in proliferation between T cells activated with anti-CD3 and CD4⁺ T cells activated with anti-CD3 in the presence of PM. * $p < 0.05$

A



B

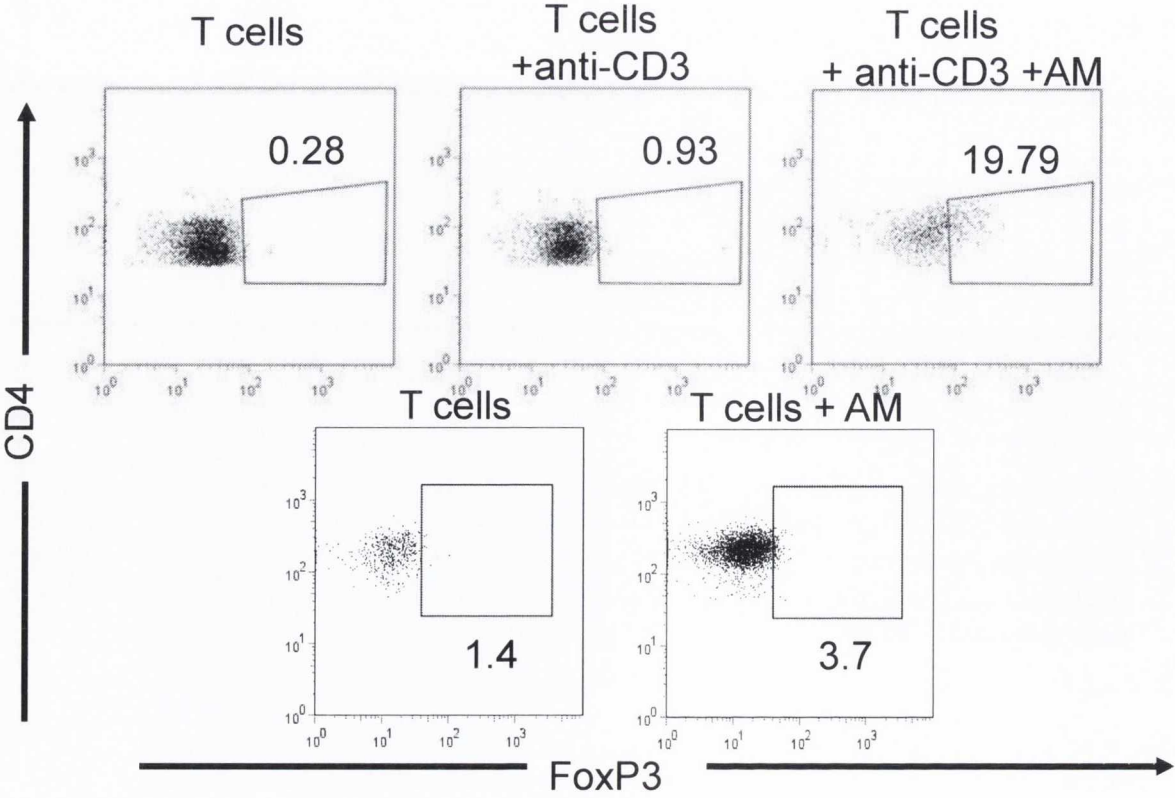


Figure 4.2 AM and PM can induce FoxP3 expression in FoxP3⁻ naïve T cells

AM and PM were obtained and cultured as described in Fig 4.1 CD4⁺CD25⁻CD45RB^{high} naïve effector T cells were purified from the spleens of naïve mice, activated with 2 µg/ml soluble anti-CD3 and cultured with AM or PM at a ratio of 9:1 macrophages:T cells. After 24 hours, cells were stained with rat anti-mouse CD4, CD25 and FoxP3 and the frequency of CD4⁺FoxP3⁺ T cells was determined following gating on the viable CD4⁺ lymphocytes. A) Results show mean FoxP3 expression. B) Representative dot plots showing the percentage of induced FoxP3⁺ CD4⁺ T cells by AM. P values were calculated based on the difference in FoxP3 expression on CD4⁺ T cells activated with anti-CD3 alone versus CD4⁺ T cells activated in the presence of AM or PM. *p<0.05, p<0.01. Results are representative of 6 experiments.

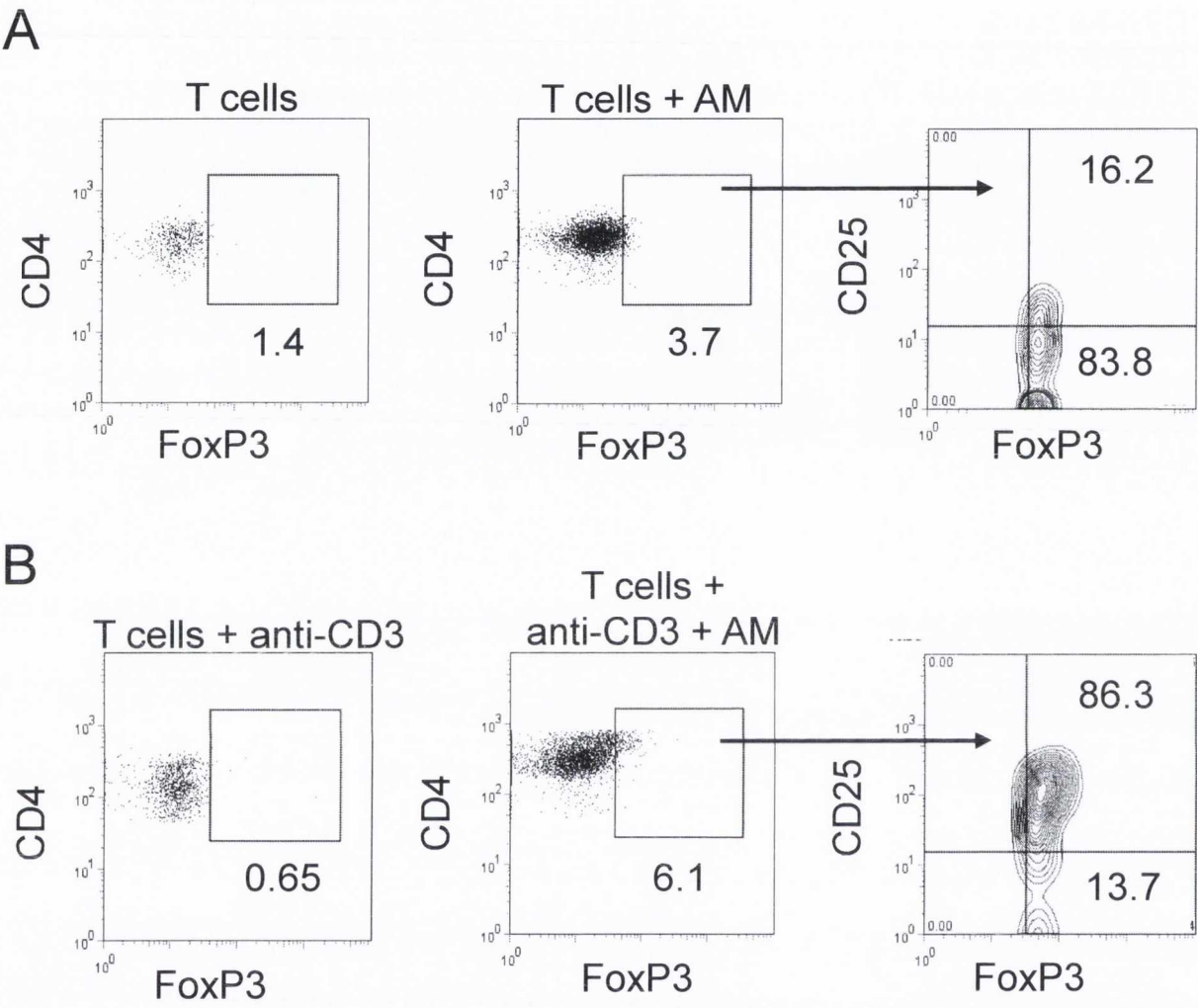


Figure 4.3 Induction of FoxP3 expression in T cells by AM does not require T cell activation *in vitro*

AM were obtained and cultured as described in Fig 4.1. CD4⁺CD25⁻CD45RB^{high} naïve effector T cells were purified from the spleens of naïve mice and cultured with AM at a ratio of 9:1 AM:T cells, in the absence (A) or presence (B) of 2 µg/ml soluble anti-CD3. After 24 hours, cells were stained with rat anti-mouse CD4, CD25 and FoxP3 and the frequency of CD4⁺FoxP3⁺CD25⁺ cells was determined following gating on the viable CD4⁺ lymphocytes. Dot plots are representative of 3 experiments.

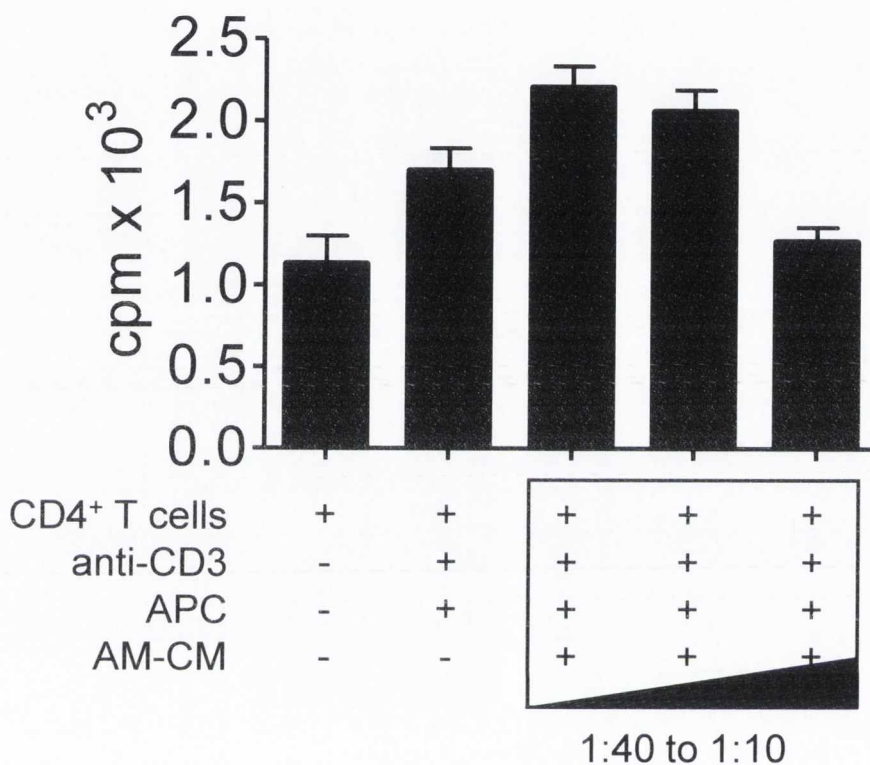


Figure 4.4 Conditioned media from AM culture can suppress the proliferation of naïve T cells

AM were obtained as described in Fig 4.1. AM-conditioned medium (AM-CM) was harvested after 24 hours and twofold dilutions were prepared. CD4⁺CD25⁻CD45RB^{high} naïve effector T cells were purified from the spleens of naïve mice, activated with 2 µg/ml soluble anti-CD3 and 5 x 10⁵ irradiated APC and cultured with decreasing dilutions of AM-CM for 48 hours. 20 µl of AM-CM was added to 180 µl of cells, so the final dilution factors were 1:10, 1:20 and 1:40. Proliferation was determined by ³H-thymidine incorporation after 48 hours. Results are representative of 3 experiments.

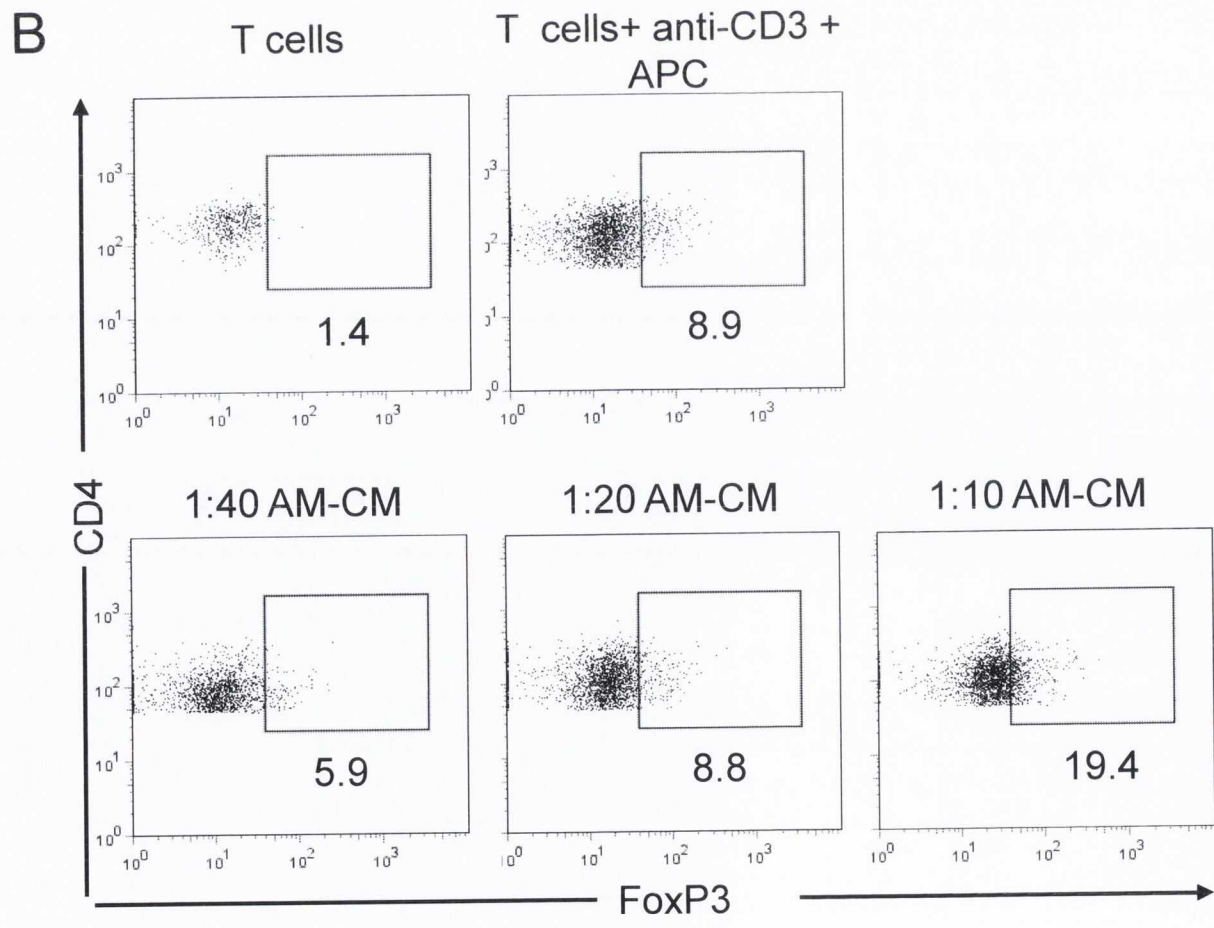
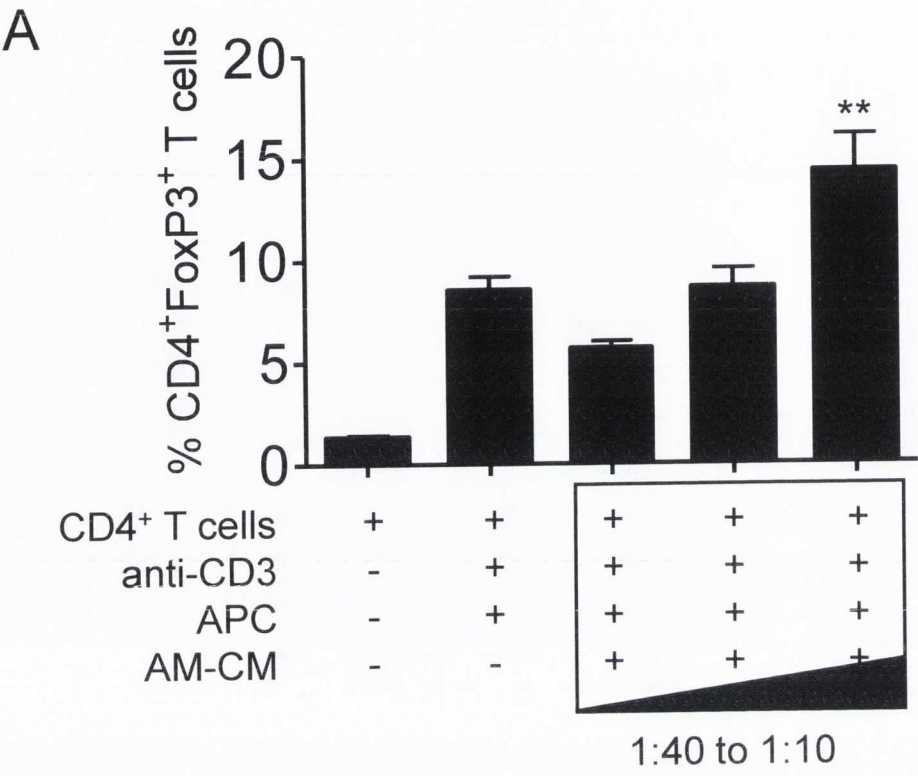
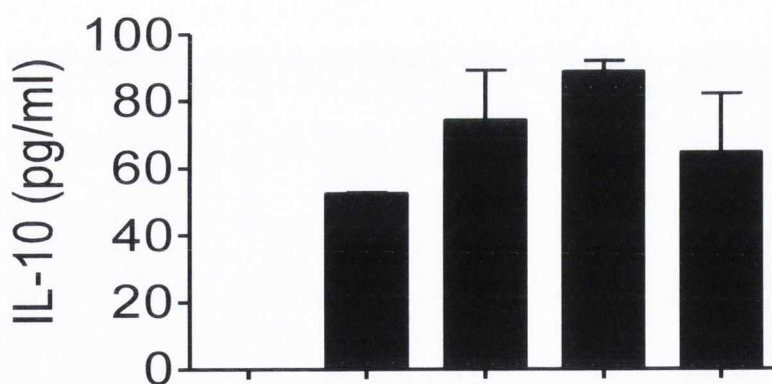
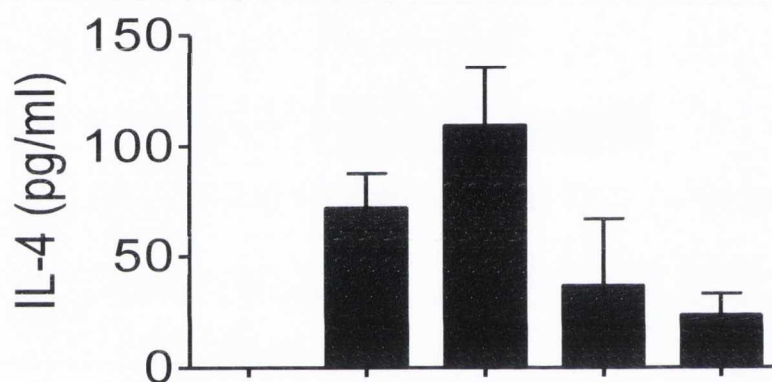
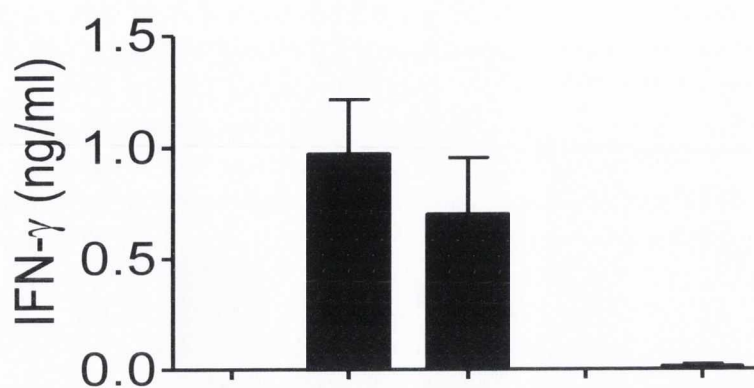
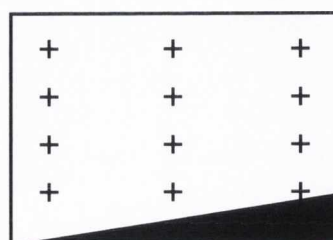


Figure 4.5 AM-CM can induce FoxP3 expression in FoxP3⁻ naïve T cells

AM-CM was produced as described in Fig 4.4. CD4⁺CD25⁻CD45RB^{high} naïve effector T cells were purified from the spleens of naïve mice, activated with 2 µg/ml soluble anti-CD3, 5 x 10⁵ irradiated APC and decreasing dilutions of AM-CM, as described in Fig 4.4. After 24 hours, cells were stained with rat anti-mouse CD4, CD25 and FoxP3 and the frequency of CD4⁺FoxP3⁺ T cells was determined following gating on the viable CD4⁺ lymphocytes. A) Results show the mean of FoxP3 expression. B) Representative dot plots showing the percentage of induced FoxP3⁺ CD4⁺ T cells. P values were calculated on the difference in FoxP3 expression on CD4⁺ cells activated with anti-CD3 and irradiated APC versus CD4⁺ T cells activated in the presence of AM-CM. **p<0.01. Results are representative of 6 experiments.



CD4⁺ T cells + +
 anti-CD3 - +
 APC - +
 AM-CM - -



1:40 to 1:10

Figure 4.6 AM-CM promotes IL-10 and inhibits IFN- γ and IL-4 production by T cells in a dose-dependent manner

AM-CM was produced as described in Fig 4.4. CD4⁺CD25⁻CD45RB^{high} naïve effector T cells were purified from the spleens of naïve mice, activated with 2 μ g/ml soluble anti-CD3 and 5×10^5 irradiated APC and cultured with decreasing dilutions of AM-CM, as described in Fig 4.4. After 24 hours, supernatants were removed and the concentrations of IFN- γ , IL-4 and IL-10 were quantified by ELISA. Results are representative of 4 experiments.

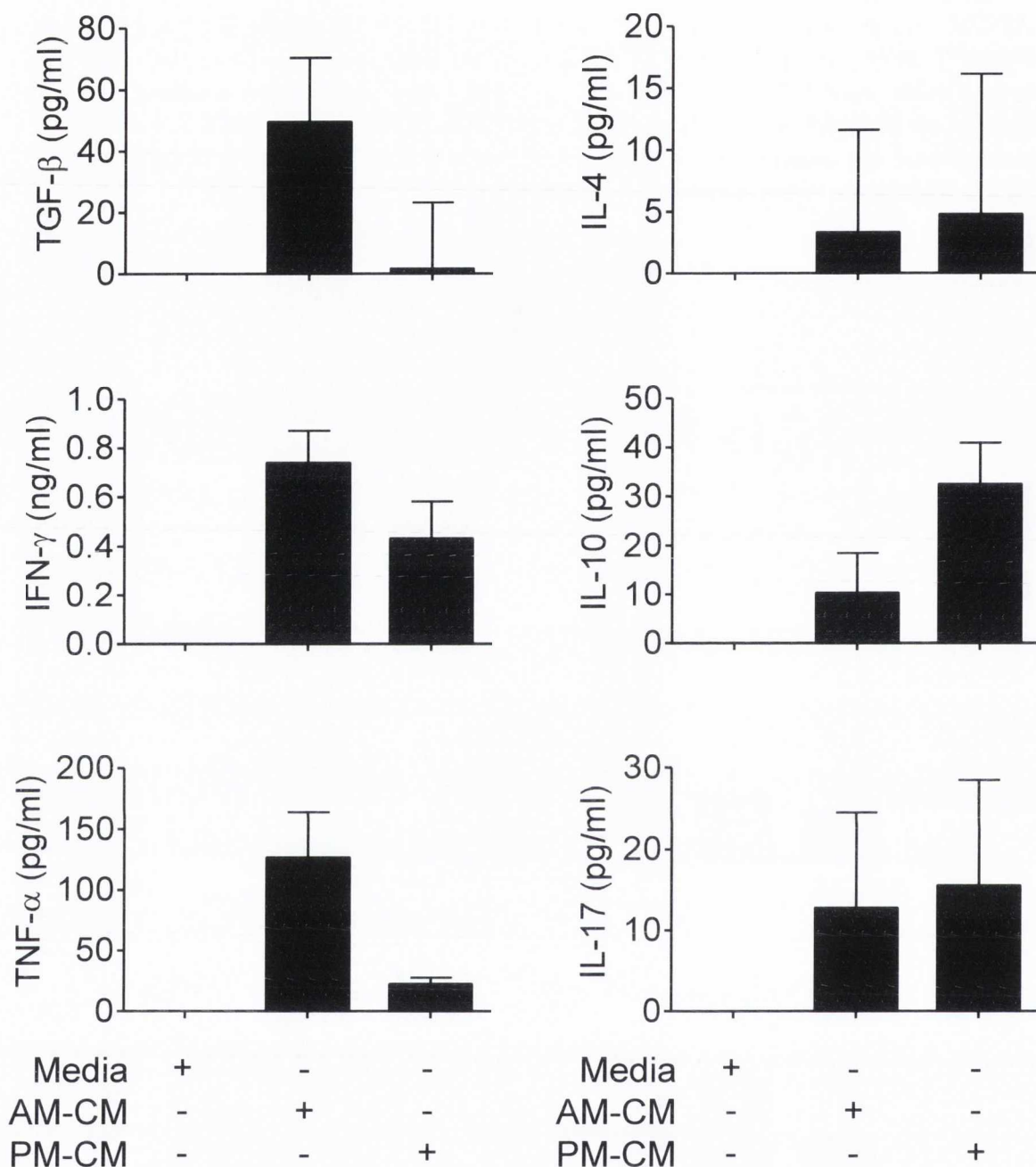


Figure 4.7 AM produce TGF- β_1 , IFN- γ , and TNF- α but not IL-4 IL-10 or IL-17

AM and PM were obtained and cultured as described in Fig 4.1. Culture media was then harvested and the concentrations of TGF- β_1 , IFN- γ , TNF- α , IL-4, IL-10 and IL-17 were quantified by ELISA.

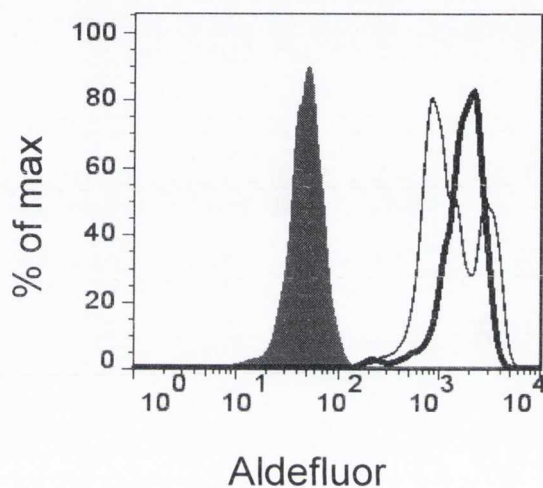
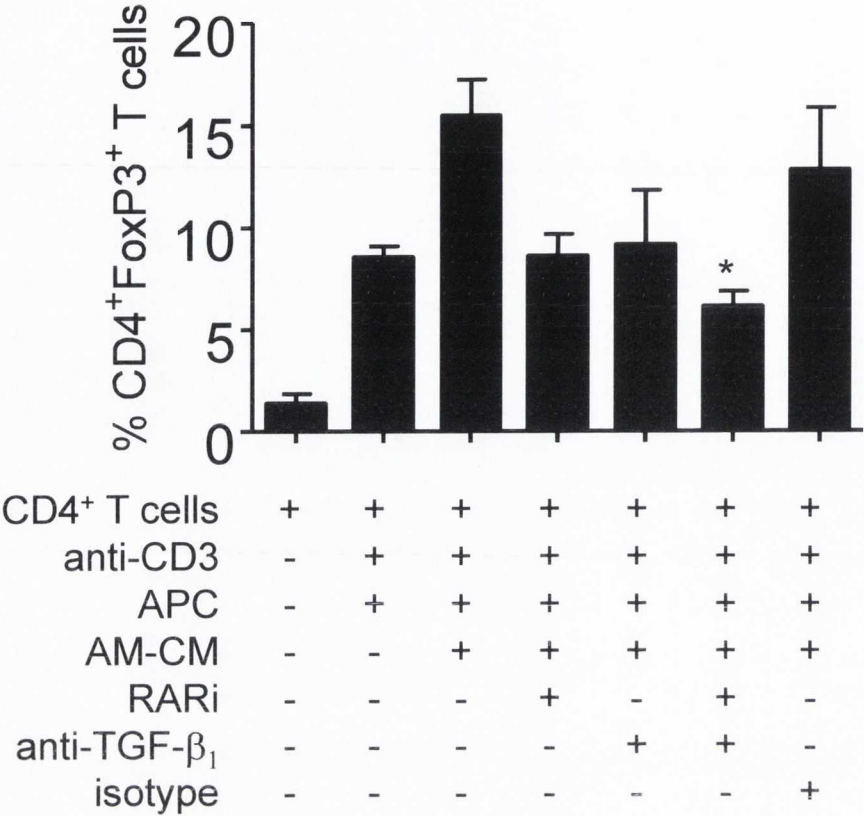


Figure 4.8 AM express aldehyde dehydrogenase A1 at a higher intensity than PM

AM and PM were obtained as described in Fig 4.1. Cells were resuspended at $1 \times 10^6/\text{ml}$ and aldehyde dehydrogenase 1A (ALDH1A) activity was estimated using the Aldefluor® flow cytometry staining assay. Representative histogram depicts ALDH1A (Aldefluor®) expression as an indirect measurement of RA production on viable unstained control cells (filled grey histogram), peritoneal exudate cells (thin line) and BAL cells (thick line) isolated from a naïve mouse.

A



B

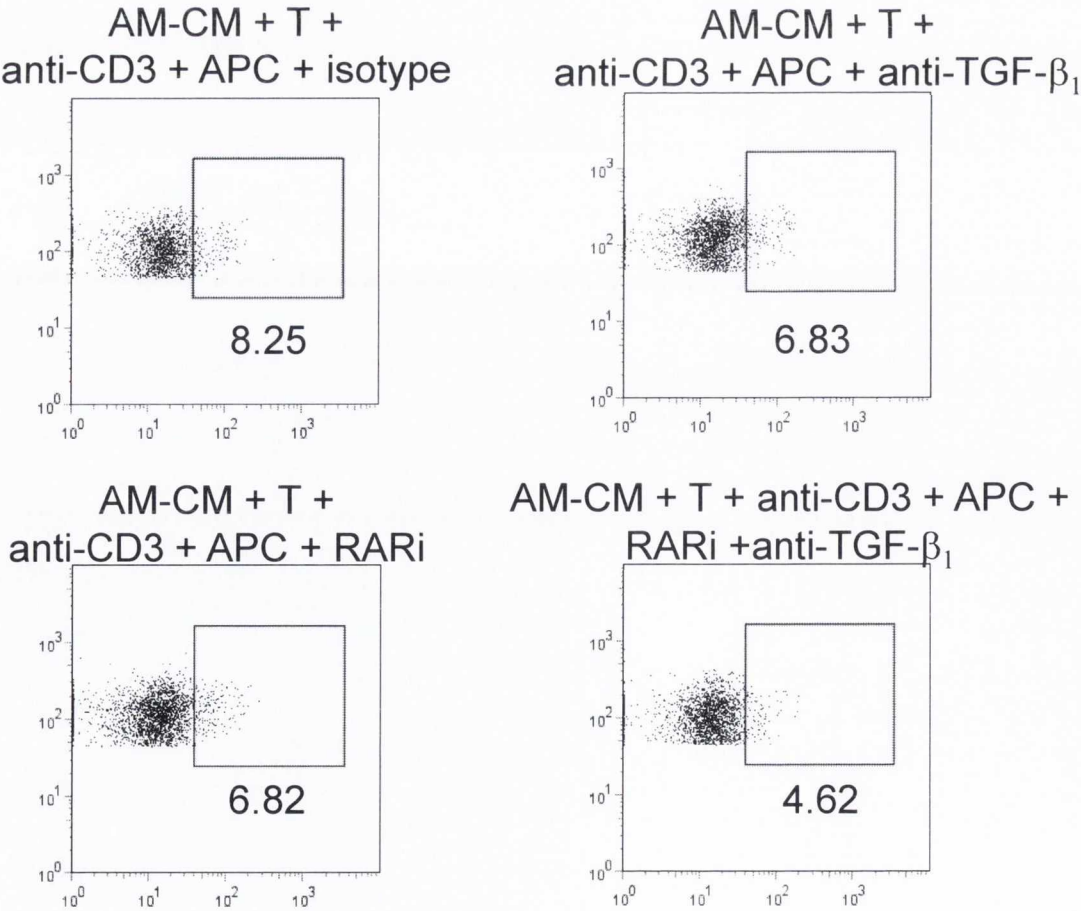
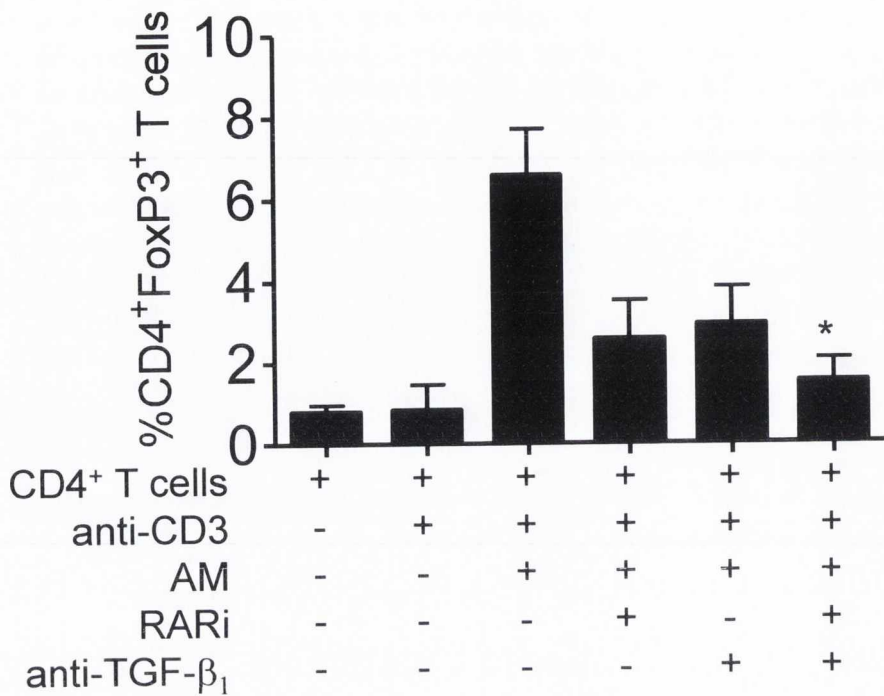


Figure 4.9 Inhibiting RA and/or TGF- β_1 signalling reduces the induction of FoxP3⁺ T cells by AM-CM

AM-CM was produced as described in Fig. 4.4 CD4⁺CD25⁻CD45RB^{high} naïve effector T cells were purified from the spleens of naïve mice. The cells were activated with 2 μ g/ml soluble anti-CD3 and AM-CM, in the presence or absence of a blocking antibody against TGF- β_1 , isotype control antibody, and/or an antagonist of the retinoic acid receptor (RARi). After 24 hours, cells were stained with rat anti-mouse CD4, CD25 and FoxP3 and the frequency of CD4⁺FoxP3⁺ T cells was determined following gating on the viable CD4⁺ lymphocytes. A) Results show the mean FoxP3 expression. B) Representative dot plots showing the inhibition of FoxP3 induction by RARi. P values were calculated on the difference in FoxP3 expression on CD4⁺ cells activated with anti-CD3 and irradiated APC in the presence of AM-CM versus CD4⁺ T cells activated in the presence of AM-CM, RARi and anti-TGF- β_1 . * $p < 0.05$. Results are representative of 5 experiments.

A



B

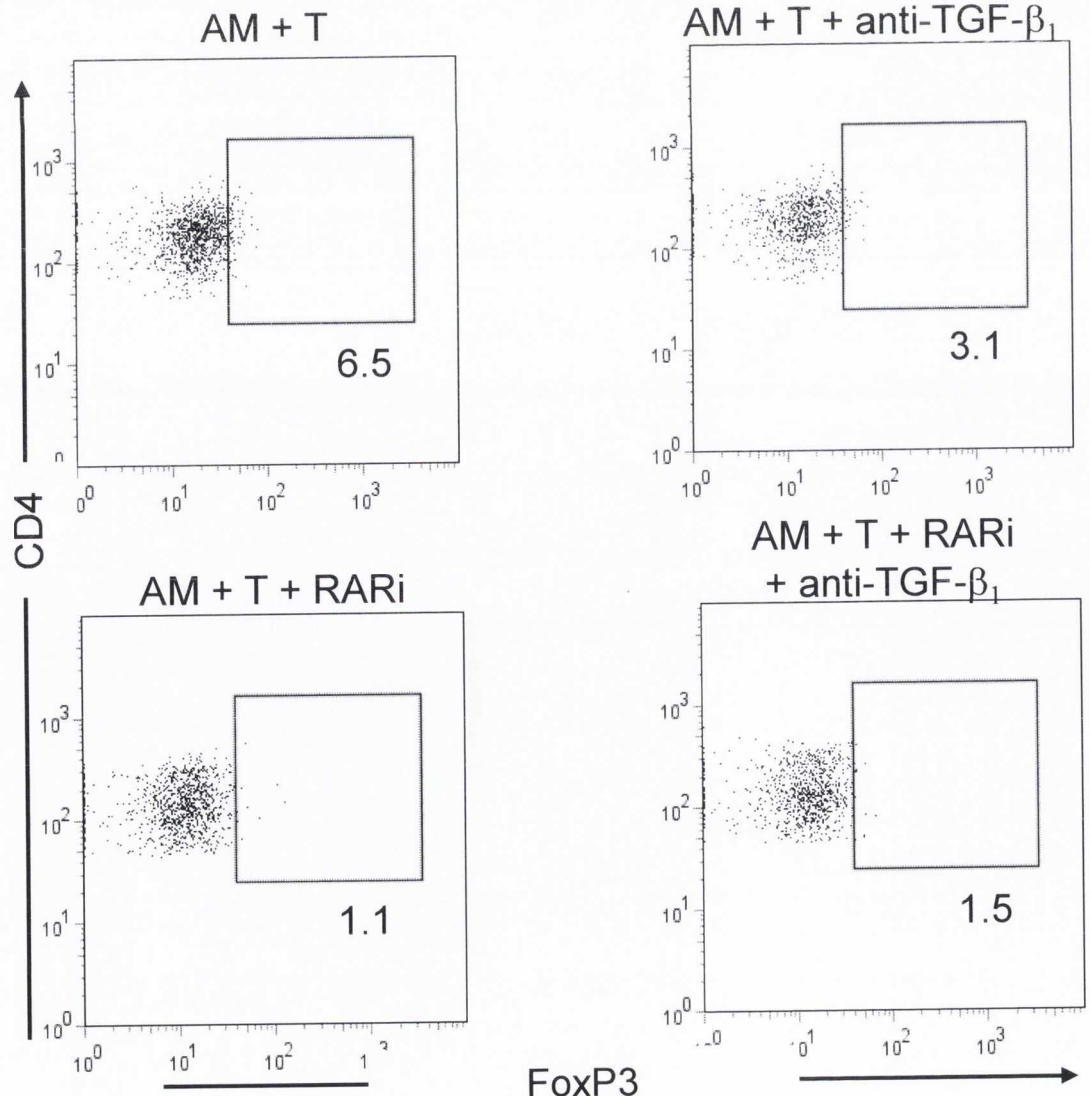


Figure 4.10 Inhibiting RA and/or TGF- β_1 signalling reduces the induction of FoxP3⁺ T cells by AM

AM were obtained as described in Fig 4.1. CD4⁺CD25⁻CD45RB^{high} naïve effector T cells were purified from the spleens of naïve mice, activated with 2 μ g/ml soluble anti-CD3 and cultured with AM at a ratio of 9:1 AM:T cells, in the presence or absence of a blocking antibody against TGF- β_1 , and/or an antagonist of the retinoic acid receptor (RARi). After 24 hours, cells were stained with rat anti-mouse CD4, CD25 and FoxP3, and the frequency of CD4⁺FoxP3⁺ T cells was determined following gating on the viable CD4⁺ lymphocytes. A) Results show the mean FoxP3 expression B) Representative dot plots showing the inhibition of FoxP3 induction by RARi and/or TGF- β_1 . P values were calculated on the difference in FoxP3 expression on CD4⁺ cells activated with anti-CD3 in the presence of AM versus those activated in the presence of AM, RARi and anti-TGF- β_1 . * $p < 0.05$. Results are representative of 3 experiments.

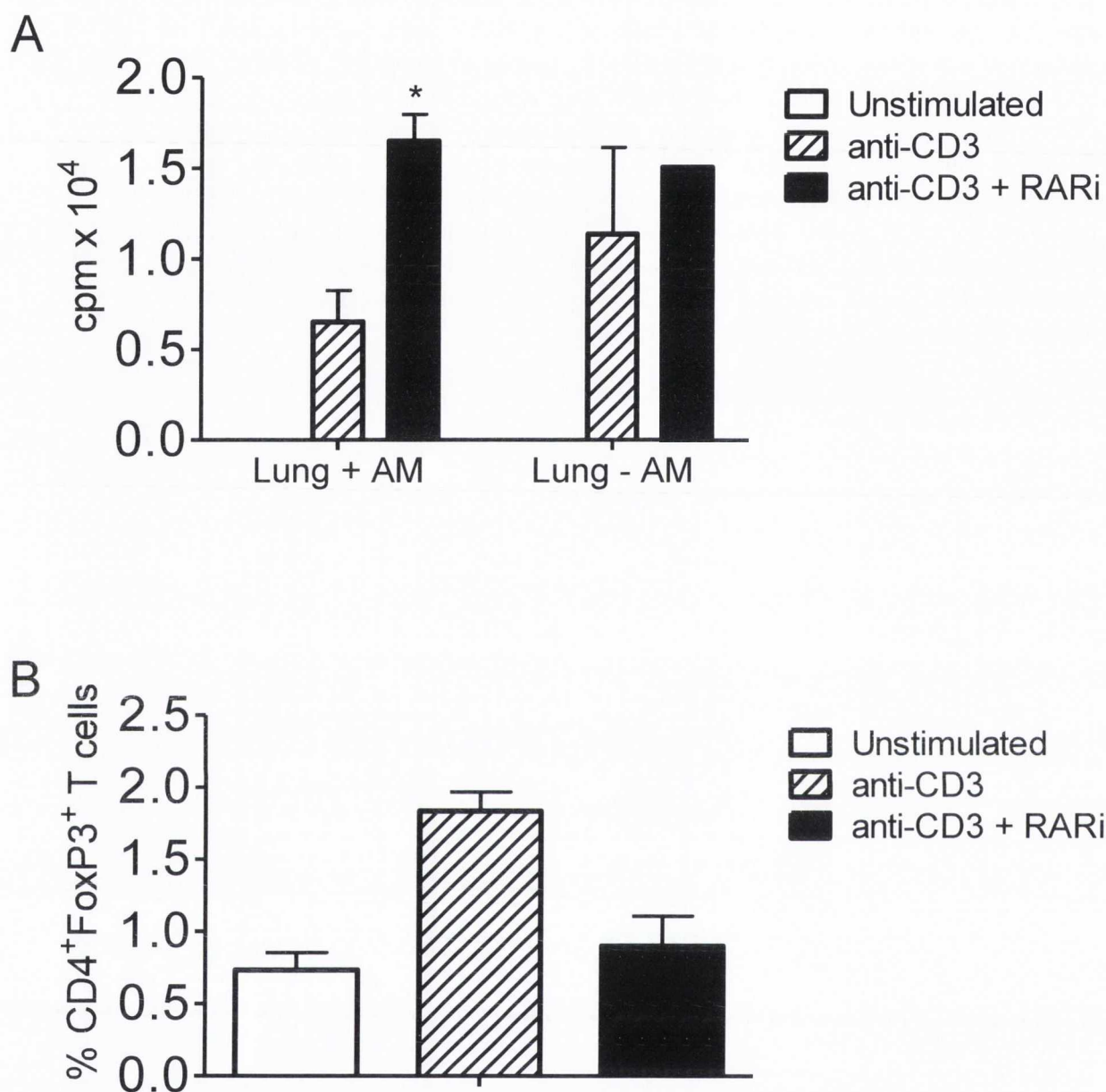


Figure 4.11 AM-mediated suppression of naïve T cells is a consequence of FoxP3⁺ T cell induction, which is mediated by RA

Total lung mononuclear cells from naïve Balb/c mice were activated with anti-CD3 in the presence or absence of RARi. Separately, AM were depleted from total lung homogenate by allowing them to adhere to plastic and removing the non-adherent cells after 3 hours incubation at 37°C. 2 x 10⁶/ml non-adherent lung cells were also activated with anti-CD3 in the presence or absence of RARi. (A) Proliferation was determined by ³H-thymidine incorporation after 72 hours. (B) Lung mononuclear cells were stained with rat anti-mouse CD4, CD25 and FoxP3 and the frequency of CD4⁺FoxP3⁺ T cells was determined following gating on the viable CD4⁺ lymphocytes. Results are representative of 2 experiments. P values were calculated on the difference in proliferation between cells activated with anti-CD3 in the presence of RARi versus cells activated without RARi. *p<0.05

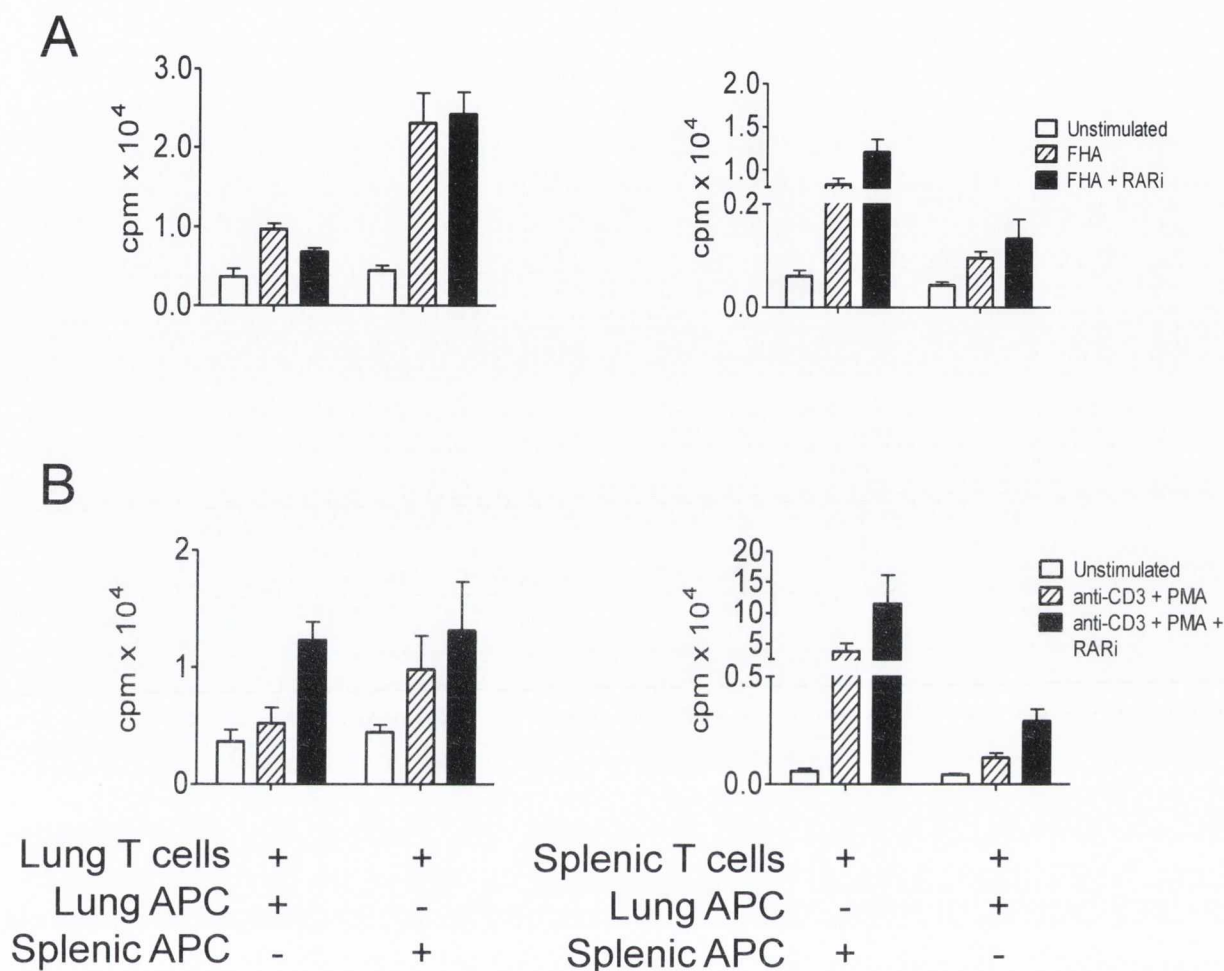


Figure 4.12 Inhibiting RA signalling overcomes AM-mediated suppression of T cell proliferation *in vitro*

Balb/c mice were infected with *B. pertussis*. 21 days post-challenge CD3⁺ cells were purified from lungs and spleens of infected mice and were activated with A) FHA from *B. pertussis* or B) anti-CD3 and PMA, with irradiated APC from infected lungs or spleens, in the presence or absence of RARi. ³H-thymidine was added for the final 18 hours of culture and proliferation was determined by uptake of ³H-thymidine.

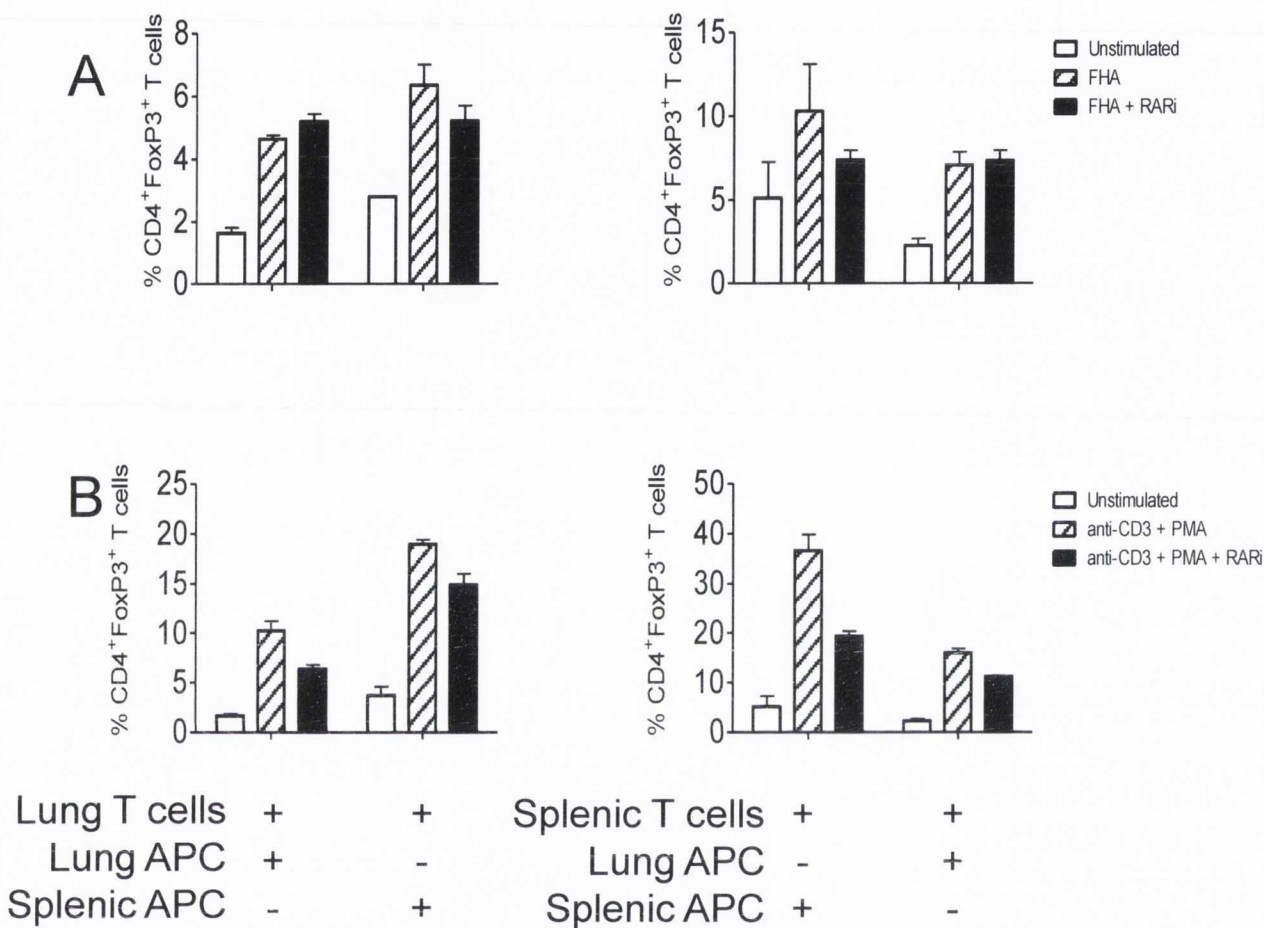


Figure 4.13 Inhibiting RA signalling inhibits AM-mediated induction of FoxP3⁺ T cells *in vitro*

Balb/c mice were infected with *B. pertussis*. 21 days post-challenge CD3⁺ cells were isolated from lungs and spleen of infected mice and activated with A) FHA from *B. pertussis* or B) anti-CD3 and PMA, with irradiated APC from infected lungs or spleens, in the presence or absence of RARi. After 48 hours cells were stained with rat anti-mouse CD4, CD25 and FoxP3.

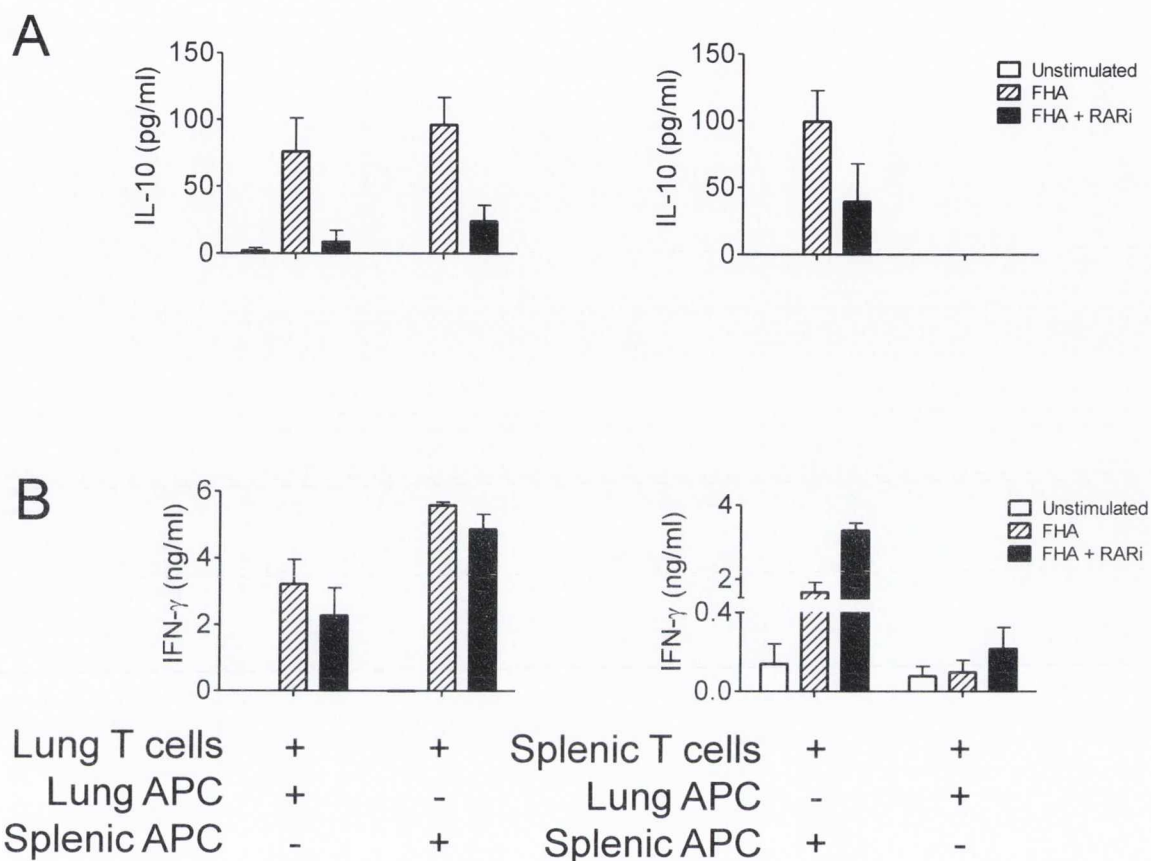


Figure 4.14 Inhibiting RA signalling reduces antigen-specific IL-10 and increases splenic antigen-specific IFN- γ production

Balb/c mice were infected with *B. pertussis*. 21 days post-challenge CD3⁺ cells were isolated from lungs and spleen of infected mice and activated with FHA from *B. pertussis*, with irradiated APC from infected lungs or spleens, in the presence or absence of RARi. After 48 hours, supernatants were removed and IL-10 and IFN- γ concentrations were quantified by ELISA.

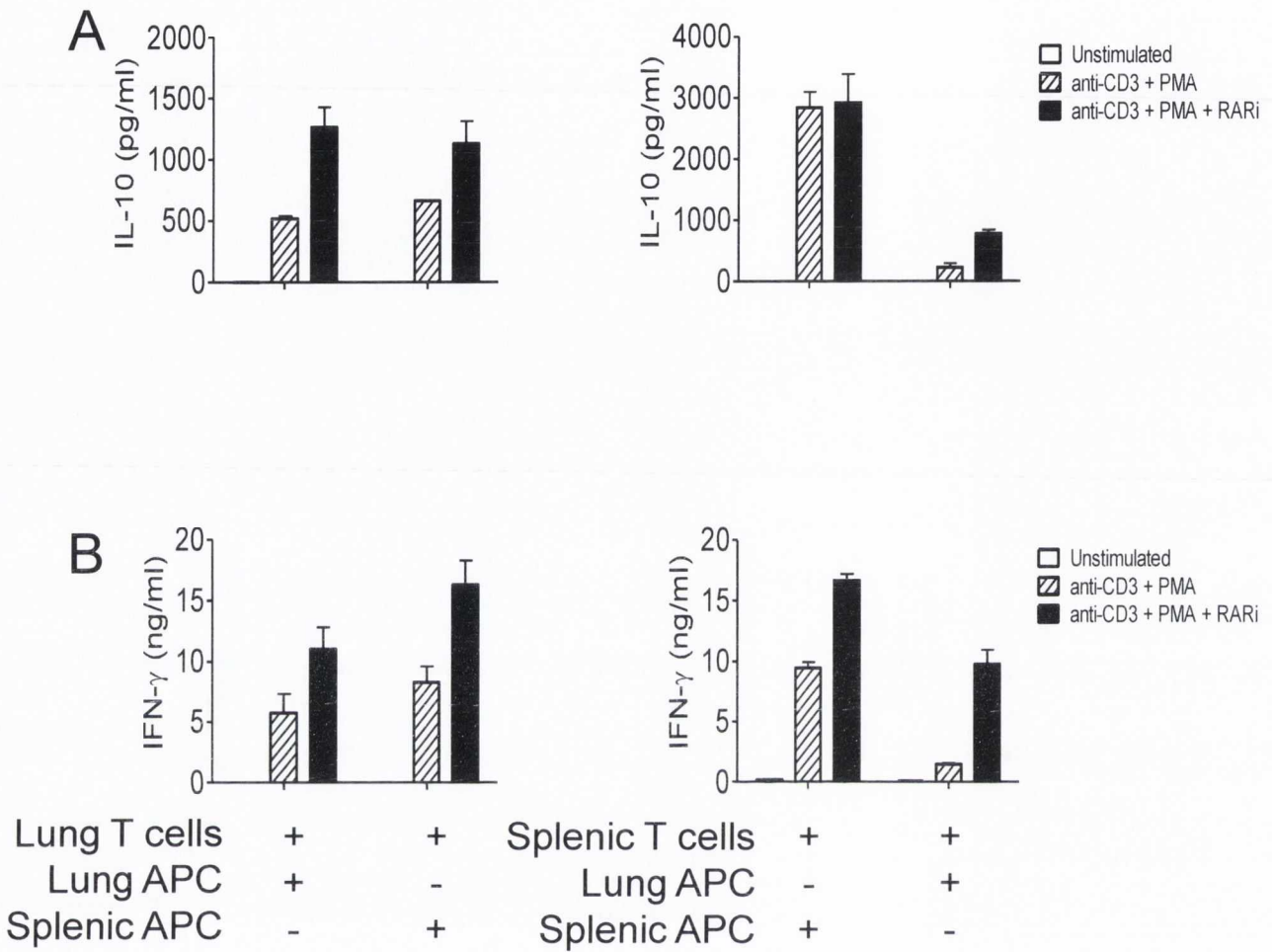


Figure 4.15 Inhibiting RA signalling enhances IL-10 and IFN- γ production in response to anti-CD3 and PMA

Balb/c mice were infected with *B. pertussis*. 21 days post-challenge CD3⁺ cells were isolated from lungs and spleen of infected mice and activated with anti-CD3 and PMA, with irradiated APC from infected lungs or spleens, in the presence or absence of RARi. After 48 hours, supernatants were removed and IL-10 and IFN- γ concentrations were quantified by ELISA.

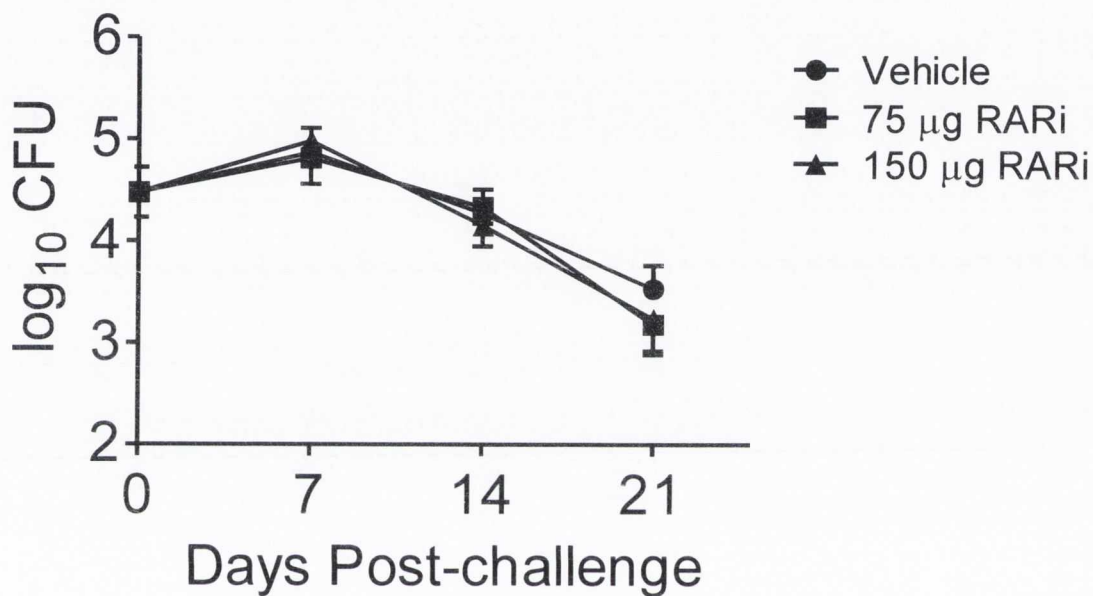


Figure 4.16 RARi treatment does not affect the burden of *B. pertussis* infection

Naïve Balb/c mice were infected with *B. pertussis*. Mice were treated every second day from day 1 post-challenge by intranasal administration with 75 μ g or 150 μ g RARi or vehicle, containing a PBS/ethanol mix. 7, 14 and 21 days post-challenge, lungs were homogenised and CFU counts were performed.

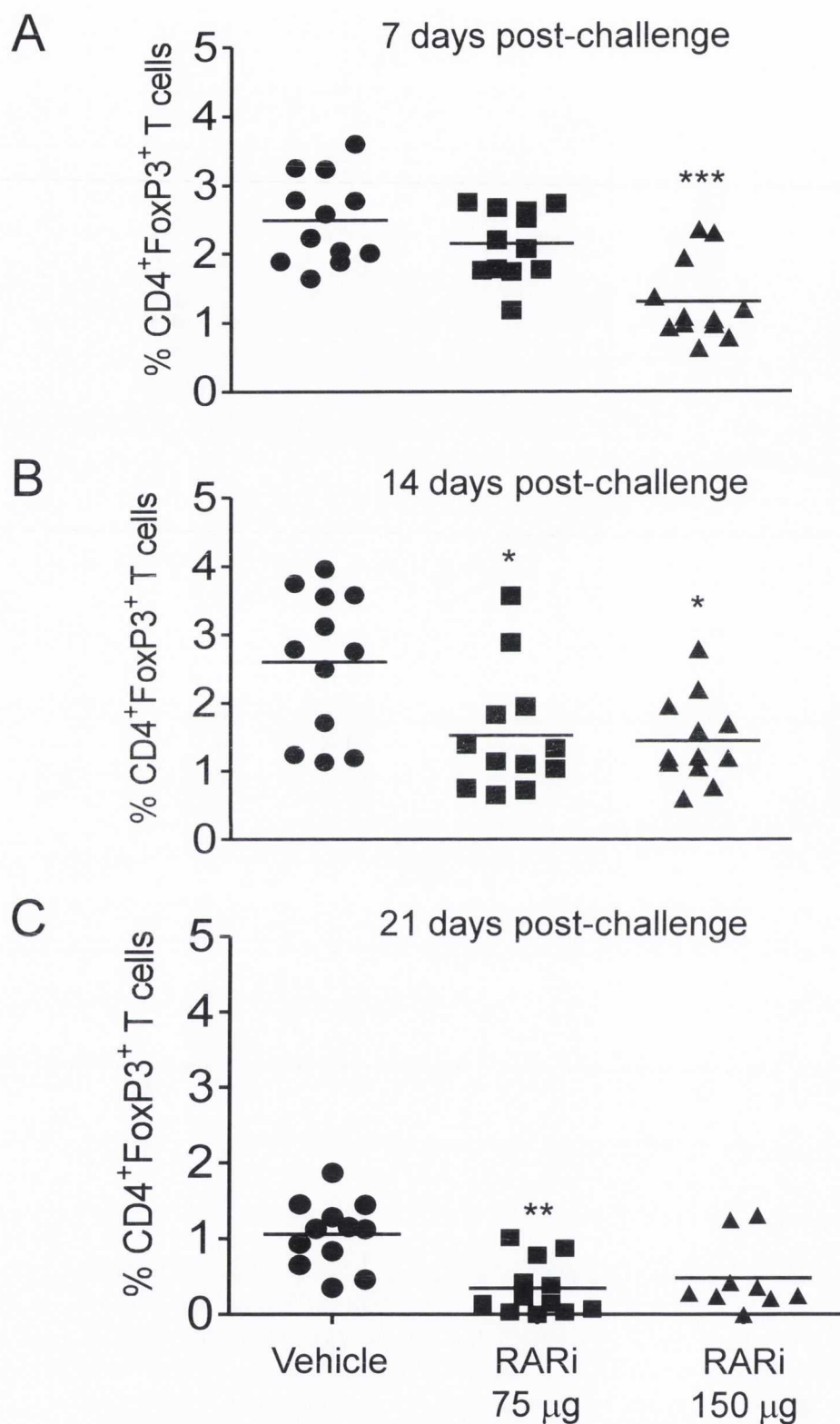


Figure 4.17 RARi treatment significantly decreases the amount of CD4⁺FoxP3⁺ cells in the lungs of infected mice

Naïve Balb/c mice were infected with *B. pertussis* and immunised as described in Fig 4.16. 7, 14 and 21 days post-challenge, lungs were homogenised and cells were stained with rat anti-mouse CD4, CD25 and FoxP3. P values were calculated on the difference in CD4⁺FoxP3⁺ cell frequencies between mice which received RARi and mice which received vehicle. *p<0.05, **p<0.01, ***p<0.001

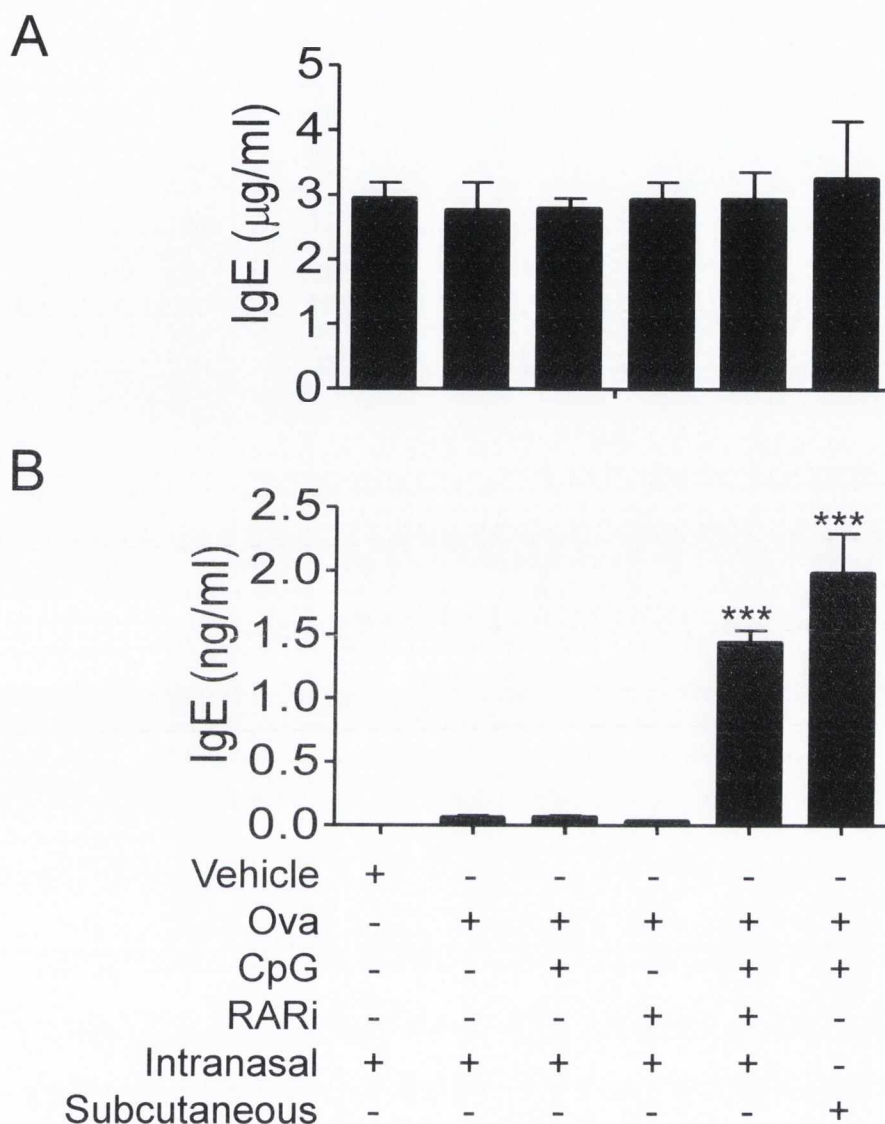


Figure 4.18 RARi breaks tolerance to nasally delivered antigen with a TLR agonist as adjuvant

Naïve Balb/c mice were immunised twice (0, 21 days) with vehicle, Ova, Ova + CpG, Ova + RARi or Ova + CpG + RAR i.n. or Ova + CpG subcutaneously. One week after the second vaccine, mice were sacrificed, and serum was analysed for the presence of (A) total IgE and (B) Ova-specific IgE. P values were calculated on the difference in IgE concentrations between mice which received Ova versus mice which received vehicle. *** $p < 0.001$

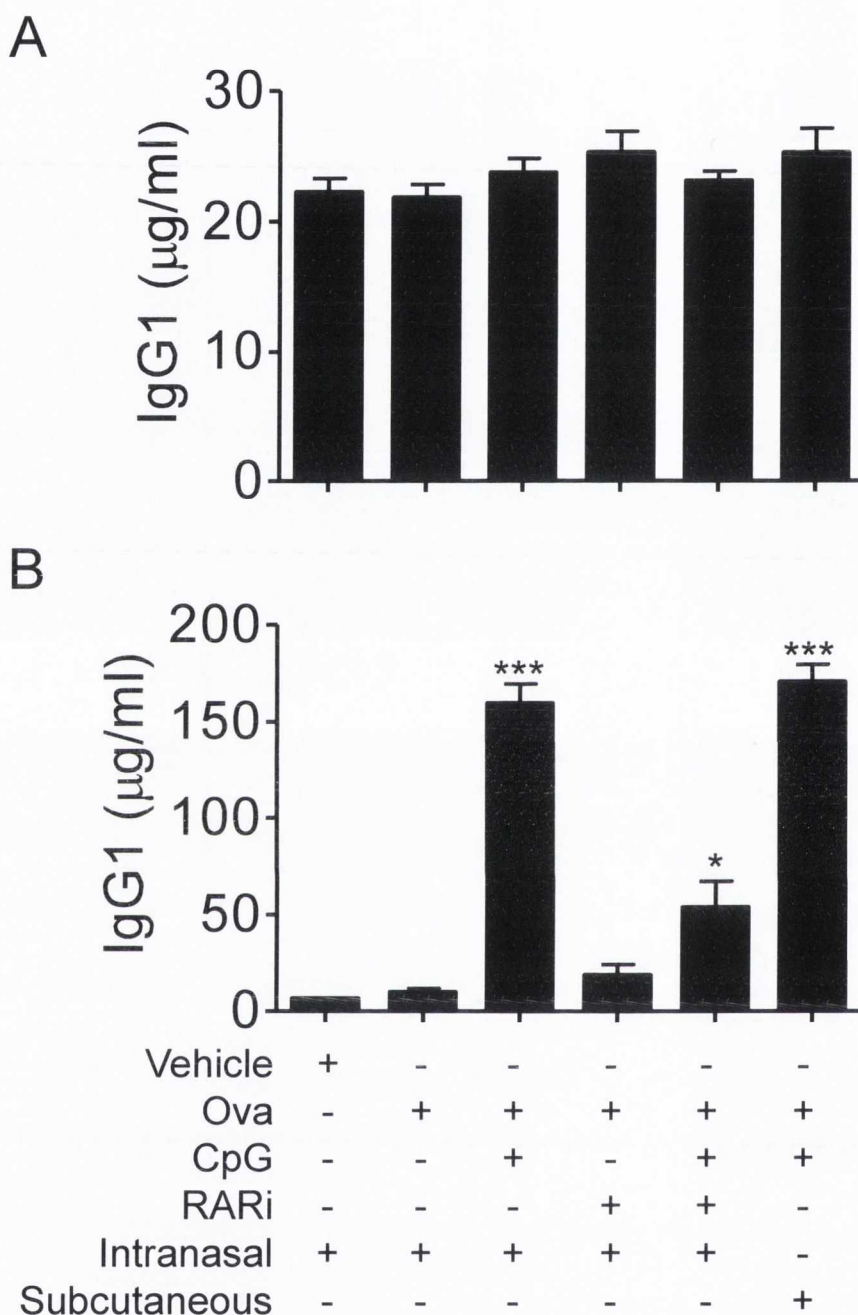


Figure 4.19 RARi breaks tolerance to nasally delivered antigen with a TLR agonist as adjuvant

Naïve Balb/c mice were immunised as described in Fig 4.18. One week after the second immunisation, mice were sacrificed, and serum was analysed for the presence of (A) total IgG1 and (B) Ova-specific IgG1. P values were calculated on the difference in IgG1 concentrations between mice which received Ova versus mice which received vehicle. * $p < 0.05$, *** $p < 0.001$

Chapter 5

**Human alveolar macrophages use retinoic acid to induce
FoxP3⁺ T cells – implications for cigarette smoking as a risk
factor for reactivation tuberculosis**

5.1 Introduction

The results from Chapter 4 illustrate the existence of a novel mechanism of tolerance in the lungs of mice, where AM promote the induction of FoxP3⁺ T cells via RA secretion and perhaps to a lesser extent, TGF- β ₁. Similar to mouse AM, human AM also suppress T cell proliferation *in vitro*, however the mechanism by which they achieve this is not clear; both cell-cell contact mechanisms and the secretion of soluble mediators have been proposed (Rich *et al.*, 1991; Upham *et al.*, 1995; Upham *et al.*, 1997). Additionally, T cells isolated from lung tissue or obtained from bronchoalveolar lavage of normal subjects display reduced proliferative ability relative to T cells isolated from peripheral blood (Garlepp *et al.*, 1992; Holt *et al.*, 1988; Lecossier *et al.*, 1988). Despite reports that human and murine AM differ in the mechanism by which they inhibit T cell proliferation, the potential role of Treg cells was not investigated in those studies. Therefore, I sought to determine whether human AM exert their suppressive function by inducing FoxP3⁺ T cells *in vitro* and whether RA is involved in human respiratory tolerance.

Respiratory immune tolerance is important in the context of infection with microorganisms that target the airways. An adequate immune response is desirable in order to eradicate pathogens, but this needs to be strictly regulated to minimise inflammatory damage to the lungs and airways and to prevent overactive responses to innocuous environmental antigen. In pulmonary TB infection, AM represent an intracellular niche required for *M. tuberculosis* to establish infection in the host (Lee *et al.*, 2009).

Ultimately, the outcome of TB infection is determined by the type and the magnitude of the host's Th1 response (Flynn and Chan, 2001; Kaufmann, 2001; North and Jung, 2004). Inadequate TB-specific Th1 responses are believed to result from Treg cell-mediated immunosuppression (Kaufmann, 2001). Indeed, Treg cells are directly associated with poorer outcomes of TB infection. There are increased frequencies of nTreg cells in PBMC of patients with active TB compared with uninfected controls (Guyot-Revol *et al.*, 2006). Moreover, TB patients have further enrichment of nTreg cells at the site of infection compared with peripheral blood (Chen *et al.*, 2007; Guyot-Revol

et al., 2006; Ribeiro-Rodrigues *et al.*, 2006; Scott-Browne *et al.*, 2007). Expression of FoxP3 mRNA in combination with IL-8 and IL-12 mRNA can be used to differentiate LTBI from active disease, as patients with active disease have higher expression of these three genes when compared with latently infected individuals (Wu *et al.*, 2007). Additionally, FoxP3 expression in whole blood correlates directly with active disease (Burl *et al.*, 2007; Roberts *et al.*, 2007).

It is also thought that increased numbers of Treg cells at the site of infection facilitates the dissemination of *M. tuberculosis* bacilli and the development of extrapulmonary TB. Indeed, higher frequencies of Treg cells have been found in the airways of patients with disseminated TB compared with patients with localised pulmonary TB; these cells appear to suppress immune responses in part via IL-10 secretion (Sharma *et al.*, 2009).

Depletion of AM in mice after challenge with virulent *M. tuberculosis* enhances protective immunity against the pathogen, with evidence of increased survival, reduced CFUs and increased production of Th1 cytokines in lung and spleen (Leemans *et al.*, 2001). It has been demonstrated that depletion of Treg cells *in vitro* enhances the Th1 response against mycobacterial antigens as measured by increased IFN- γ production (Chen *et al.*, 2007; Guyot-Revol *et al.*, 2006; Ribeiro-Rodrigues *et al.*, 2006), and is corroborated by the findings that depletion of Treg cells prior to *M. tuberculosis* infection reduced bacterial growth in mice (Kursar *et al.*, 2007; Scott-Browne *et al.*, 2007).

Interestingly, the number of AM obtained from BAL of smokers is approximately 4-6 times greater than from non-smokers (Brody, 1998; Brody and Craighead, 1975; Harris *et al.*, 1970). Furthermore, there is significant epidemiological evidence that exposure to cigarette smoke is an independent risk factor for tuberculosis infection, with greater progression of primary tuberculosis, development of reactivation tuberculosis, more severe cavitory disease and death from tuberculosis in smokers (Alcaide *et al.*, 1996; Altet-Gomez *et al.*, 2005; Altet *et al.*, 1996; Bates *et al.*, 2007; Davies *et al.*, 2006; den Boon *et al.*, 2005; Gajalakshmi *et al.*, 2003; Jha *et al.*, 2008; Kolappan and Gopi, 2002; Leung *et al.*, 2010; Leung *et al.*, 2004; Lin *et al.*, 2009; Lin *et al.*, 2007;

Lin *et al.*, 2008; Moran-Mendoza *et al.*, 2008; Pai *et al.*, 2007; Slama *et al.*, 2007; Zellweger, 2008). Recently it has also been reported that smoking prolongs the infectivity of treated TB patients (Siddiqui *et al.*, 2010). Despite this, the biological mechanisms have not been elucidated (Shang *et al.*, 2011).

Since increased frequencies of Treg cells and cigarette smoking are independently associated with poorer outcomes of TB infection, I investigated whether there was a connection, particularly because higher frequencies of AM are found in the lungs of smokers. I hypothesised that the increased numbers of AM in the lungs of smokers might induce more FoxP3⁺ T cells compared with non-smokers, and that this might explain why cigarette smoking is associated with progression of primary tuberculosis and development of reactivation tuberculosis. I also investigated whether the capacity to induce FoxP3 expression in T cells was different in AM from smokers and non-smokers, and whether infection of AM with *M. tuberculosis* H37Ra affected FoxP3 expression in T cells.

The results demonstrate that human AM can induce FoxP3 expression in FoxP3⁺ T cells *in vitro*, and this is achieved at least in part by RA. AM from smokers and non-smokers expressed similar levels of ALDH1A and induced FoxP3⁺ T cells to the same extent *in vitro*. However, the fact that more AM are present in the lungs of smokers *in vivo* means that more FoxP3⁺ T cells might be induced in these individuals. Indeed, *in vitro* culture of T cells with increasing numbers of AM results in a dose-dependent increase in the frequency of induced FoxP3⁺ T cells. This might explain why smokers have a higher risk for TB infection, progression of primary TB, development of reactivation TB, more severe cavitary disease and death from TB. Finally, I demonstrate that *in vitro* infection of AM with *M. tuberculosis* H37Ra does not affect their ability to induce FoxP3⁺ T cells.

5.2 Results

5.2.1 Human AM induce FoxP3 expression in naïve CD4⁺FoxP3⁻ T cells

It has been known for some time that human AM, like murine AM, actively suppress T cell proliferation (Ettensohn *et al.*, 1986; McCombs *et al.*, 1982; Rich *et al.*, 1991; Schauble *et al.*, 1993; Toews *et al.*, 1984; Upham *et al.*, 1995; Upham *et al.*, 1997). Furthermore, T cells isolated from human lungs produce less IL-2 than blood-derived T cells from the same donors (Holt *et al.*, 1988). The results from Chapter 4 demonstrated that murine AM achieve their suppressive effects in part by secreting RA, which induces FoxP3⁺ T cells. It has been reported that while human and rodent AM suppress T cell proliferation *in vitro*, they differ in the mechanisms of suppression (Rich *et al.*, 1991; Upham *et al.*, 1995). While it has been demonstrated that murine AM use RNI to inhibit T cell proliferation *in vitro*, this does not appear to be the case for human AM (Upham *et al.*, 1995). It has been proposed that human AM suppress T cell proliferation via cell-cell contact mechanisms (Rich *et al.*, 1991). I therefore investigated whether human AM are able to induce FoxP3 expression in human T cells and whether they differ from their murine counterparts in this regard.

The *foxp3* gene and FoxP3 transcription factor is primarily found in quiescent human CD4⁺CD25⁺CD45RO⁺ memory T cell populations (Yagi *et al.*, 2004). Therefore, CD4⁺CD25⁻CD45RO⁻ T cells were purified from peripheral blood as a source of CD4⁺FoxP3⁻CD25⁻ naïve T effector cells to be co-cultured with AM *in vitro*. Approximately 5% of CD4⁺ T cells in peripheral blood express FoxP3, as demonstrated in Fig. 5.1A. A proportion of the sorted CD4⁺CD25⁻CD45RO⁻ T cells were tested for FoxP3 expression after each sort; these cells were routinely less than 1% FoxP3⁺ (Fig. 5.1B). The sorting protocol also efficiently depletes CD45RO⁺ memory T cells; as shown in Fig. 5.1, frequencies decrease from ~14% to 0.04% post-sort.

Human alveolar macrophages were obtained at bronchoscopy, after written consent, under a protocol approved by the St. James's Hospital/ AMNCH ethics board. AM were cultured overnight at a concentration of 5×10^5 /ml. Alternatively, MDM were generated from PBMC of

healthy donors and cultured *in vitro* for 7-10 days prior to co-culture with T cells. Freshly isolated CD4⁺CD25⁻CD45RO⁻ T cells (6×10^3) were activated with 2 µg/ml soluble anti-CD3 in the presence or absence of AM or MDM for a further 24 hours. AM comprise approximately 90% of all immune cells in BAL fluid of healthy, non-smoking donors, so to replicate this “normal” cell ratio, macrophages were cultured with effector T cells in a ratio of 9:1. Cells were stained with anti-human antibodies against CD4 and FoxP3 and analysed by flow cytometry. T cells, which were stimulated and co-cultured with AM expressed significantly more FoxP3 ($p < 0.01$) compared with anti-CD3 stimulated T cells alone, or T cells activated with anti-CD3 in the presence of MDM (Fig 5.2). It has been reported that MDM can suppress T cell proliferation in response to mitogen (Schauble *et al.*, 1993). However, they did not induce FoxP3 expression in T cells stimulated with anti-CD3 (Fig 5.2).

This mechanism by which AM induce FoxP3 in effector T cells, appears to be conserved between mice and humans. This previously uncharacterised mechanism adds a new dimension to the body of evidence which describes how the lungs actively suppress immune responses. These findings might represent an important therapeutic target in terms of treating allergy and respiratory diseases.

5.2.2 Human AM-mediated FoxP3 induction is independent of T cell activation

Human CD4⁺CD25⁻ T cells upregulate CD25 in response to activation *in vitro*, in a similar manner to murine CD4⁺ CD25⁻ T cells. The results in Chapter 4 showed that the induction of FoxP3 expression by culture with murine AM was independent of T cell activation; therefore I investigated whether this was also the case for human AM and T cells.

Naïve CD4⁺CD25⁻CD45RO⁻ T cells were cultured with AM at a 1:9 ratio for 24 hours in the absence of stimulation with anti-CD3. Unstimulated CD4⁺CD25⁻CD45RO⁻ T cells cultured with AM significantly ($p < 0.0001$) upregulated FoxP3 compared to those cultured in the absence of AM (Fig 5.3). These findings might explain why T cells isolated from human lungs are

hypoproliferative and show reduced IL-2 production and enhanced IFN- γ production compared with blood T cells (Becker *et al.*, 1990; Holt *et al.*, 1988).

5.2.3 Human AM use a soluble factor to suppress T cell proliferation and simultaneously induce FoxP3 expression *in vitro*

In mice, the induction of FoxP3 expression by T cells co-cultured with AM is in part dependent on RA. The mechanism by which human AM exert their suppressive effect remains unclear. An absolute requirement for cell-cell contact has been proposed by Rich *et al* (Rich *et al.*, 1991), while others have reported that suppression of effector T cells by AM cells requires a combination of soluble factors and cell-cell contact (Upham *et al.*, 1997). Since other mechanistic differences between human and murine AM have been reported (Upham *et al.*, 1995), I investigated whether conditioned medium (CM; containing any potential soluble factors) from human AM culture could induce FoxP3 expression in naïve CD4⁺CD25⁻CD45RO⁻ T cells.

CM was harvested from overnight AM culture and twofold dilutions were prepared. CD4⁺CD25⁻CD45RO⁻ T cells (6×10^3) were activated with 2 μ g/ml anti-CD3 and irradiated APC in the presence or absence of 50 μ l of decreasing doses of AM-CM for 48 hours. Proliferation was determined by uptake of ³H-thymidine. Alternatively, CD4⁺CD25⁻CD45RO⁻ T cells were activated with 2 μ g/ml anti-CD3 and irradiated APC in the presence or absence of 50 μ l decreasing doses of AM-CM for 24 hours, after which FoxP3 expression was determined by flow cytometry. High concentrations of AM-CM (final dilutions 1:4 or 1:8) had an anti-proliferative effect on activated naïve CD4⁺ T cells (Fig. 5.4) and enhanced FoxP3 expression in these cells compared to naïve CD4⁺ T cells activated in the presence of irradiated APC (Figs. 5.5 and 5.6). These findings suggest that a soluble factor(s) produced by human AM is responsible for the induction of FoxP3 expression in CD4⁺ T cells *in vitro* that may have regulatory function.

5.2.4 Human AM can synthesise RA

The results to date have shown that human AM function in a similar manner to murine AM by utilising a soluble mediator to induce FoxP3 expression in naïve T cells. The default mechanism

in the normal lung is a non-inflammatory Th2 immune response (Stumbles *et al.*, 1998). RA is the biologically active isoform of vitamin A. Deficiency of this vitamin promotes a Th1 phenotype in humans, while RA supplementation restores Th2 and inhibits Th1 cytokine production by PBMCs (Dawson *et al.*, 2006). Furthermore, RA treatment effectively prevents radiation-induced lung fibrosis (Tabata *et al.*, 2006), and cytosolic ALDH1 activity was detected in the rat lung but the ALDH1⁺-cell type/s were not determined (Yoon *et al.*, 2006). Therefore, I next investigated whether human AM expressed the ALDH1A enzyme. ALDH1A is an aldehyde dehydrogenase which irreversibly synthesises RA from dietary vitamin A (Coombes *et al.*, 2007; Hsu *et al.*, 1999). The results from flow cytometric (Fig. 5.7) and fluorescence microscopic analysis (Fig. 5.8) showed that human AM strongly express this ALDH1A.

Fig. 5.7 shows a slight difference in ALDH1A expression between the two donors depicted. It would appear that the non-smoker expressed more ALDH1A than the smoker, as fluorescence intensity directly correlated with the enzyme's expression. Further studies would need to be undertaken to determine whether this is a reproducible result. Unfortunately, donor cell numbers were not always sufficient to perform this experiment. RT-PCR and/or western blotting could be used to determine relative levels of ALDH1A expression, but this too would require greater cell numbers, which was not always possible to obtain. Nonetheless, these data provide indirect evidence that human AM can produce the immunomodulatory molecule RA and is the first time this activity has been reported in these cells.

5.2.5 Induction of FoxP3 and suppression of T cell proliferation by AM is dependent on RA

A synthetic retinoic acid receptor antagonist (RARi) was used to determine the role of RA in FoxP3 induction in effector T cells. CD4⁺CD25⁻CD45RO⁺ T cells (6×10^3) were activated with 2 µg/ml anti-CD3 and irradiated APC in the presence of AM-CM and a synthetic RARi (5 µM or 20 µM).

Addition of RARi at a concentration of 20 μ M to T cell/AM-CM culture significantly ($p<0.05$) reversed the effect of AM-CM on FoxP3 expression on T cells (Fig. 5.9). Furthermore, RARi reversed the suppressive effect of AM-CM on T cell proliferation, suggesting that the RA-induced FoxP3⁺ T cells suppress effector T cell proliferation (Fig. 5.10). These results suggest that both human and murine AM can actively promote respiratory tolerance via the induction of FoxP3 in T cells. In these experiments, I chose to focus on the effect of inhibiting RA alone on the induction of human FoxP3⁺ T cells rather than TGF- β_1 , or RA and TGF- β_1 synergy. The reason for this was AM were maintained in complete medium supplemented with FCS, which can contain exogenous TGF- β_1 . Therefore inhibiting this cytokine might not reflect any AM-specific effects.

5.2.6 AM from smokers induce FoxP3 expression in T cells to the same extent as AM from non-smokers *in vitro*

Light microscopy analysis has shown that AM obtained from smokers appear morphologically distinct from those obtained from non-smokers. AM from smokers display pigmented cytoplasmic granules (Fig. 5.11). Others have reported that AM from smokers contain more Golgi vesicles, endoplasmic reticulum and residual bodies when compared with AM from non-smokers (Harris *et al.*, 1970). Therefore it seemed possible that cigarette smoking might affect the ability of AM to convert naïve T cells to FoxP3⁺ T cells, so I examined the effect of AM from smokers and non-smokers on naïve T cells. AM and AM-CM from cigarette smokers and non-smokers were able to induce FoxP3 expression on naïve T cells to the same extent *in vitro* (Figs. 5.12 and 5.13). Despite AM from both cohorts also expressing similar but not identical levels of ALDH1A (Fig. 5.7), this data suggests that there is no qualitative difference in the ability of AM to induce FoxP3 in T cells in response to cigarette smoking. However, consistent with previous studies (Brody, 1998; Brody and Craighead, 1975; Harris *et al.*, 1970), there was a significantly higher number of AM in BAL of smokers compared with non-smokers (Fig. 5.14). It is important to note that the length of time that patients smoked for did not seem to have an impact on AM numbers within the smoking cohort. For example a patient who smoked for 60 years did not have more AM in their lungs when compared with someone who smoked for 30 years. An equal number of AM from smokers and non-smokers were used for *in vitro* experiments.

whether increasing the number of AM could induce greater frequencies of FoxP3⁺ T cells *in vitro*. CD4⁺CD25⁻CD45RO⁻ T cells (6×10^3) were activated with 2 µg/ml anti-CD3 with increasing numbers of AM. A dose-dependent increase in FoxP3 expression was observed when T cells were cultured with increasing numbers of AM (Fig. 5.15). Therefore it is possible that increased numbers of AM in the airways of smokers might result in a more tolerogenic environment when compared with the airways of non-smokers.

5.2.7 Infection of AM with *M. tuberculosis* H37Ra does not affect their ability to induce FoxP3 in T cells.

It is well established that smoking is an independent risk factor for progression of primary tuberculosis, development of reactivation tuberculosis, more severe cavitary disease and death from tuberculosis. (Alcaide *et al.*, 1996; Altet-Gomez *et al.*, 2005; Altet *et al.*, 1996; Bates *et al.*, 2007; Davies *et al.*, 2006; den Boon *et al.*, 2005; Gajalakshmi *et al.*, 2003; Jha *et al.*, 2008; Kolappan and Gopi, 2002; Leung *et al.*, 2010; Leung *et al.*, 2004; Lin *et al.*, 2009; Lin *et al.*, 2007; Lin *et al.*, 2008; Moran-Mendoza *et al.*, 2008; Pai *et al.*, 2007; Slama *et al.*, 2007; Zellweger, 2008). It has also been reported that smoking prolongs the infectivity of treated TB patients (Siddiqui *et al.*, 2010). Impaired Th1 responses against TB lead to poorer outcomes of infection, and this is attributed to the dampening of protective immunity by Treg cells (Ribeiro-Rodrigues *et al.*, 2006). These studies may explain why cigarette smokers fare worse than non-smokers in terms of the outcome of infection.

Since AM provide a niche for *M. tuberculosis* to replicate, I next investigated whether infection of AM with an avirulent *M. tuberculosis* strain H37Ra affected their ability to induce FoxP3 expression in naïve T cells. AM were infected with a high multiplicity of infection (MOI), so that most of the cells would be infected with a high bacterial burden (>10 bacilli/cell). The results of staining AM with Auramine and Hoechst showed that most AM were highly infected with *M. tuberculosis* (Fig. 5.16). AM were infected for 3 hours, after which extracellular bacteria were removed. After 24 hours, AM-CM was harvested from infected and uninfected AM cultures

and incubated with CD4⁺CD25⁻CD45RO⁻ naïve T cells (6×10^3), purified from the blood of healthy donors and activated with 2 µg/ml anti-CD3.

Infection of AM with *M. tuberculosis* H37Ra had no effect on the ability of AM to induce FoxP3 expression in naïve T cells. CM from infected and uninfected AM induced FoxP3 to the same extent (Fig. 5.17). AM-CM from infected and uninfected cultures both inhibited proliferation of T cells (Fig. 5.18). These results indicate that the H37Ra strain of *M. tuberculosis* does not affect the ability of AM to induce FoxP3 in T cells.

5.3 Discussion

The results from this chapter demonstrate that AM from normal human lung can induce FoxP3 expression in naïve human T cells. This is consistent with the findings in mice and suggests the existence of a previously unreported mechanism of peripheral tolerance in the human lung. This indicates that a highly conserved strategy exists for maintaining immune tolerance in the lung and it has implications for the treatment of respiratory conditions such as TB infection.

The results revealed that human AM can induce significant FoxP3 expression in activated or unactivated FoxP3⁻ T cells from allogeneic donors *in vitro*. As in the murine studies, a soluble factor, identified as RA, was found to be at least partly responsible. Interestingly, cigarette smoking does not affect the ability of AM or AM-CM to induce FoxP3⁺ T cells. Furthermore, infection of AM with *M. tuberculosis* H37Ra does not influence the induction of FoxP3⁺ T cells either. However, both cigarette smoking and increased frequencies of Treg cells are independently associated with poorer outcomes of TB. An explanation for this and a potential link between these two observations comes from evidence that the lungs of smokers contain more AM than those of non-smokers (Brody, 1998; Brody and Craighead, 1975; Harris *et al.*, 1970). Activation of T cells in the presence of increasing numbers of AM results in a dose-dependent increase in the frequencies of induced FoxP3⁺ T cells. Therefore it is possible that smokers' lungs contain more Treg cells than non-smokers' lungs by virtue of having greater numbers of AM, and this could make them more susceptible to TB infection. Additionally, the increased frequencies of AM-induced Treg cells in these individuals might be responsible for the increased morbidity and mortality reported in TB-infected smokers compared with TB-infected non-smokers, possibly as a result of inadequate Th1-mediated anti-TB immunity (Altet-Gomez *et al.*, 2005; Bates *et al.*, 2007; Davies *et al.*, 2006; den Boon *et al.*, 2005; Gajalakshmi *et al.*, 2003).

Like murine AM, it has long been known that human AM are immunosuppressive. They inhibit proliferation but not effector function of T cells *in vitro* (Upham *et al.*, 1995; Upham *et al.*, 1997). This selective inhibition is thought to prevent excessive local T cell responses but permits

some functional activity. Indeed, when compared with T cells in peripheral blood, those obtained from lung tissue or airways of healthy individuals proliferate much more weakly (Garlepp *et al.*, 1992; Holt *et al.*, 1988; Lecossier *et al.*, 1988), and are phenotypically distinct (Davidson *et al.*, 1985). However, the mechanism by which human AM mediate suppression of T cell proliferation has not been defined, and there is uncertainty as to whether they secrete soluble factors to achieve this or whether they suppress via cell-cell contact (Rich *et al.*, 1991; Upham *et al.*, 1995; Upham *et al.*, 1997). While it has been reported that rodent AM suppress via NO, human AM are able to suppress T cells in an NO-independent manner (Rich *et al.*, 1991; Upham *et al.*, 1995).

Despite the fact that AM from humans and rodents exert the same anti-proliferative effect on T cells *in vitro*, it has been suggested that they differ in how they achieve this. Studies have shown that rodent AM use RNI, but human AM might suppress proliferation of T cells via direct cell-cell contact rather than a soluble factor (Upham *et al.*, 1995). Nonetheless, these studies did not investigate whether or not FoxP3⁺ T cells were involved in suppressing T cell proliferation. Furthermore, the T cell populations used were not very highly purified. Consistent with the murine studies described in Chapter 4, the data in this chapter has revealed a role for soluble RA in the induction of FoxP3⁺ T cells by human AM.

I did not investigate the effect of inhibiting TGF- β_1 , as AM were cultured in medium containing exogenous TGF- β_1 and any observed effects could have been attributed to this, rather than endogenous TGF- β_1 . Furthermore, TGF- β_1 is a pleiotropic cytokine and RA is the key cofactor which modulates the effect of TGF- β_1 to promote FoxP3⁺ T cells induction and directly inhibit ROR γ t⁺ Th17 cell induction (Benson *et al.*, 2007; Coombes *et al.*, 2007; Ivanov *et al.*, 2006; Kang *et al.*, 2007b; Mucida and Cheroutre, 2007; Mucida *et al.*, 2007; Sun *et al.*, 2007; Zhou *et al.*, 2008). Additionally, it is already known that TGF- β_1 is found in the lungs and that normal AM can secrete this cytokine (Pons *et al.*, 2005). Therefore the presence of RA in an environment also containing pleiotropic TGF- β_1 strongly suggests that FoxP3⁺ T cells can be induced, *in vitro* and *in vivo*. I have not shown directly that FoxP3⁺ cells induced by AM or AM-CM in mice and humans

can suppress effector T cells *in vitro*, but it is now firmly established in the literature that FoxP3 expression is inextricably linked with both inducible and natural Treg cells. Further work will be required to demonstrate, using *in vitro* suppression assays, that the FoxP3⁺ T cells induced by AM are in fact Treg cells, inducible or otherwise.

Culture of highly purified CD4⁺CD25⁻CD45RO⁻ naïve effector T cells from human blood with allogeneic AM significantly enhanced the frequency of FoxP3⁺ T cells after 24 hours. This result was not merely due to combining T cells and AM from different individuals, or an inherent effect of macrophages on T cells, because activation with allogeneic MDM did not induce FoxP3 expression. Indeed, other studies have reported that blood monocytes readily promote antigen-specific proliferation by autologous T cells *in vitro*, but AM are much less potent APC (Toews *et al.*, 1984). It is possible that this is due to the presence of AM-induced Treg cells.

The results suggest that human AM can specifically convert naïve CD4⁺FoxP3⁻ T cells to CD4⁺FoxP3⁺ T cells *in vitro*. These assays involved activation of the T cells with anti-CD3, however I have also shown in subsequent studies that CD4⁺FoxP3⁺ T cells can be induced by AM in the absence of anti-CD3. These findings are consistent with those observed with murine AM. The CD4⁺FoxP3⁺ T cells induced by murine AM in the absence of anti-CD3 activation were predominantly CD25⁻. However, the AM and T cells in the human assays were from allogeneic donors, and in this instance the T cells might become activated in response to HLA mismatch, and it is likely they could upregulate CD25 as a result. Unfortunately it proved impossible to obtain both BAL samples and blood from the same individuals to investigate whether CD25 expression remained low in AM-converted autologous Treg cells *in vitro*.

Further evidence that the observed FoxP3 induction was probably not due to any donor-mismatch effects comes from the studies in which AM-CM was capable of inducing FoxP3 expression and suppressing T cell proliferation in a dose-dependent manner *in vitro*. These results suggest that a soluble factor secreted by AM induces FoxP3⁺ T cells. The findings are consistent

with the murine data described in Chapter 4, and with reports from others which demonstrated inhibition of T cell proliferation by AM-derived regulatory molecule(s) (Holt, 1979).

Flow cytometric and fluorescence microscopic analysis of human AM revealed that they express high levels of ALDH1A, and this provides strong evidence that these cells can produce RA. While ALDH1 expression in rat and human lungs has been documented (Stewart *et al.*, 1996; Yoon *et al.*, 2006), the specific cell types which express this enzyme in the lung have not been determined. Collectively, the data from human and murine cells in the previous study confirm the expression of this enzyme by AM. Clearly, AM from both species have the ability to produce RA and, by extension, promote the induction of FoxP3⁺ T cells. It is notable that AM from smokers and non-smokers might express different levels of ALDH1A, as a comparison of a smoker and a non-smoker revealed greater fluorescence intensity (approx half a log) for the non-smoker. The amount of fluorescence in the Aldefluor assay should correlate with the amount of enzyme present. This could be confirmed via RT-PCR or western blotting experiments. Unfortunately it is difficult to obtain sufficient cells from a non-smoker to perform several different experiments, so it limited the ability to perform the Aldefluor assay and quantify mRNA and/or protein levels. Furthermore, for this particular experiment, the smoker sample had an unusually low number of cells. The non-smoker's sample contained approximately 10% more cells than the smoker's. Both sample yields were barely within the minimum detection range for the assay, so it is possible that this small difference in cell numbers might be reflected by the fluorescence intensity.

Evidence that RA was involved in FoxP3 induction by human AM was provided by blocking RA signalling with a specific inhibitor. RARi prevented the induction of FoxP3⁺ T cells by AM-CM, and this was significant at the higher dose of RARi (20 μ M). Furthermore, inhibiting RA signalling also promoted T cell proliferation in a dose-dependent manner. When the higher dose of inhibitor was present, T cells were able to proliferate to the same extent as control T cells. These data provide strong evidence for a conserved mechanism employed by human and murine AM to promote peripheral tolerance in the lung. This mechanism might represent a novel way to target therapies for respiratory infections or allergies.

Cigarette smoking results in increased numbers of AM in the human lung, which are larger and more metabolically active than AM from non-smokers (Bowden, 1984; Harris *et al.*, 1970), and are more debris-laden (Brody, 1998; Brody and Craighead, 1975). It is likely that the enhanced frequencies of AM result from a requirement to “clean up” the airways in response to cigarette smoke exposure. Since AM obtained from the lungs of smokers are more metabolically active and smoking induces a local inflammatory response, this might suggest that these AM might have a greater ability to induce local Treg cells. However, this was not the case, as AM or AM-CM from smokers and non-smokers induced FoxP3⁺ T cells to the same extent *in vitro*. This is consistent with the flow cytometric analysis, which showed that almost 100% of AM from both smokers and non-smokers expressed ALDH1A; there did not appear to be any qualitative differences in the ability of these cells to produce RA. These findings also suggest that any difference in ALDH1A fluorescence intensity between smokers and non-smokers might just reflect differences in cell numbers between samples, and might not indicate any qualitative differences in ALDH1A expression. However, the relative expression of the enzyme could be confirmed by RT-PCR and/or western blotting if the sample yield facilitated additional studies.

Due to practical limitations, an equal number of AM from smokers and non-smokers were used for *in vitro* experiments. In general, however, greater numbers of AM were routinely present in BAL samples acquired from smokers. This was to be expected as the lungs of smokers contain far more AM than non-smokers (Brody and Craighead, 1975). Therefore it seemed probable that the *in vitro* assays did not accurately reflect the ratio of AM:T cells *in vivo* in the lungs of smokers. It is also possible that the increased numbers of AM in their lungs might induce more FoxP3⁺ T cells. Furthermore, there was an increase in FoxP3⁺ T cells when T cells were cultured with increasing numbers of AM. Therefore it is likely that smokers' lungs contain more FoxP3⁺ T cells as a direct consequence of the enhanced numbers of RA-producing resident AM. High frequencies of FoxP3⁺ T cells have been reported in the large airways of smokers when compared with non-smokers, and this correlates positively with the length of time as a smoker (Isajevs *et al.*, 2009). The data in the present study may explain why this increase in FoxP3⁺ T cells is found in smokers when compared with non-smokers.

Thus far, the data suggests a tolerogenic role for AM in the human lung which can be augmented in individuals who smoke cigarettes. It also suggests that a delicate balance must be struck to ensure optimal immune responses can occur with minimal pathology. The induction of Treg cells is certainly beneficial in terms of limiting overactive immune responses to inhaled antigens and preventing tissue damage. However, excessive immune regulation in the lung can promote the development of tumours and can impair the eradication of microorganisms (Mills, 2004). The increased frequencies of AM-induced Treg cells in the lungs of smokers might facilitate this. Unfortunately, it was difficult to obtain sufficient T cell numbers from BAL to investigate whether increased frequencies of FoxP3⁺ T cells were present in the lungs of smokers. It is well known that exposure to cigarette smoke is the main epidemiologically proven cause of lung cancer (CDC, 2006; Kuper *et al.*, 2002; Stewart *et al.*, 2008). Smoking also increases the risk of microbial infection and can enhance pathogen load (Drannik *et al.*, 2004; Stampfli and Anderson, 2009).

Certainly, individuals exposed to cigarette smoke are more likely to become infected with *M. tuberculosis* (Bates *et al.*, 2007), and smoking is associated with worse outcomes of infection such as the development of reactivation TB from LTBI (Davies *et al.*, 2006; Zellweger, 2008), disseminated disease (Chiang *et al.*, 2007), and increased mortality (Gajalakshmi *et al.*, 2003; Jha *et al.*, 2008). Treated TB patients who smoke have a prolonged infectivity period when compared with non-smokers (Siddiqui *et al.*, 2010). However, it is not known why this is the case.

Treg cells are also associated with negative outcomes of TB infection. Th1 responses have a major role in immunity to TB, but this can be impaired by Treg cells and allows the persistence of the pathogen (Joosten and Ottenhoff, 2008; Kaufmann, 2001; Scott-Browne *et al.*, 2007). In fact, enhanced expression of FoxP3 mRNA in combination with IL-8 and IL-12 mRNA can differentiate patients with active disease and those with LTBI (Wu *et al.*, 2007), and there is a positive correlation between FoxP3 expression in whole blood and active disease (Burl *et al.*, 2007; Roberts *et al.*, 2007). Patients with active TB have higher frequencies of CD4⁺CD25⁺ T cells in blood when compared with uninfected individuals (Guyot-Revol *et al.*, 2006; Hougardy *et al.*, 2007) and LTBI patients (Hougardy *et al.*, 2007). Depletion of CD4⁺CD25⁺ T cells from PBMC

prior to stimulation with *M. tuberculosis* antigen *in vitro* results in enhanced antigen-specific IFN- γ production (Guyot-Revol *et al.*, 2006). Patients with active TB have greater numbers of Treg cells at the disease site compared with peripheral blood (Chen *et al.*, 2007; Guyot-Revol *et al.*, 2006; Ribeiro-Rodrigues *et al.*, 2006).

It is also thought that increased numbers of Treg cells at the site of infection facilitates the dissemination of *M. tuberculosis* bacilli and the development of extrapulmonary TB. Indeed, enhanced frequencies of Treg cells have been found in the airways of patients with disseminated TB compared to patients with localised pulmonary TB (Guyot-Revol *et al.*, 2006; Sharma *et al.*, 2009). AM provide an intracellular niche required for *M. tuberculosis* to establish infection in the host (Lee *et al.*, 2009). The results of this study have demonstrated that infection of AM with a high MOI of *M. tuberculosis* does not affect their ability to induce FoxP3⁺ T cells *in vitro*, and that AM-CM from infected cells inhibits T cell proliferation to the same extent as that from uninfected AM. A high MOI was chosen to make sure that most if not all AM were infected, thus ensuring that any observed effects could not be attributed to uninfected AM present in the co-culture. These results suggest that neither *M. tuberculosis* infection nor the innate bactericidal mechanisms of the AM influence ALDH1A activity, and therefore *M. tuberculosis*-infected AM can induce FoxP3⁺ T cells to the same extent as uninfected AM *in vitro*.

Since cigarette smoking and enhanced frequencies of Treg cells are independently associated with reactivation TB and the development of extrapulmonary TB, this suggests a potential link between these two risk factors. In view of the fact that in general, the lungs of smokers have greater numbers of AM than non-smokers, and since increasing the number of AM induces more FoxP3⁺ T cells *in vitro*, it is likely that there is greater induction of FoxP3⁺ T cells in the lungs of smokers that might have regulatory function. If a smoker becomes infected with *M. tuberculosis*, then lung-infiltrating T cells stand a greater chance of being converted to a regulatory phenotype, and the already enhanced numbers of pre-existing induced Treg cells could severely impair local protective Th1 immunity against the pathogen. Ultimately, inadequate anti-TB immunity could prevent effective granuloma formation, or if the bacilli are sufficiently contained,

then the Treg cells and their anti-inflammatory products might upset the dynamic of the granuloma and facilitate reactivation TB. Further failure to eliminate *M. tuberculosis* could permit dissemination of the bacteria and the development of extrapulmonary TB. This hypothesis is supported by a study which demonstrates that the elimination of AM from the lungs of mice has a protective effect in pulmonary TB. Despite effective granuloma formation as a result of AM depletion, the mice had reduced bacterial growth and dissemination, 100% survival, enhanced cellular recruitment to the lungs and augmented Th1 responses (Leemans *et al.*, 2001).

In conclusion, human AM can induce CD4⁺FoxP3⁺ T cells at least in part by secreted RA. It is possible that CD4⁺FoxP3⁺ T cells induced by RA in the lung lack CD25 expression in the absence of an activated immune response. As a result these CD4⁺FoxP3⁺CD25⁻ T cells might represent an under-activated “reservoir” of cells that must be activated by IL-2 in order to optimally function *in vivo*. However, if the numbers of AM increase, then they enhance the induction of FoxP3⁺ T cells with potential regulatory function. AM numbers increase *in vivo* as a consequence of cigarette smoke exposure, and this can skew the delicate immune equilibrium in the lung to become excessively immunosuppressive, which could favour the development of tumours. It could also increase susceptibility to respiratory infection and impair clearance of microorganisms. The induced Treg cells possibly contribute to this excessive local immunosuppression.

Smokers are more likely to become infected with *M. tuberculosis* than non-smokers and they have greater morbidity and mortality than infected non-smokers. Infection of AM with *M. tuberculosis* does not impair the ability of AM to induce FoxP3 in T cells, which means that smokers infected with this pathogen have greater frequencies of Treg cells in their lungs, when compared with *M. tuberculosis*-infected non-smokers. These Treg cells may well be responsible for the morbidity and mortality that is associated with smoking and TB. Aside from the obvious solution of giving up smoking, perhaps RAR antagonists could be localised to the airways as part of treatment, or immunisation against respiratory infections such as TB in the future.

5.4 Figures

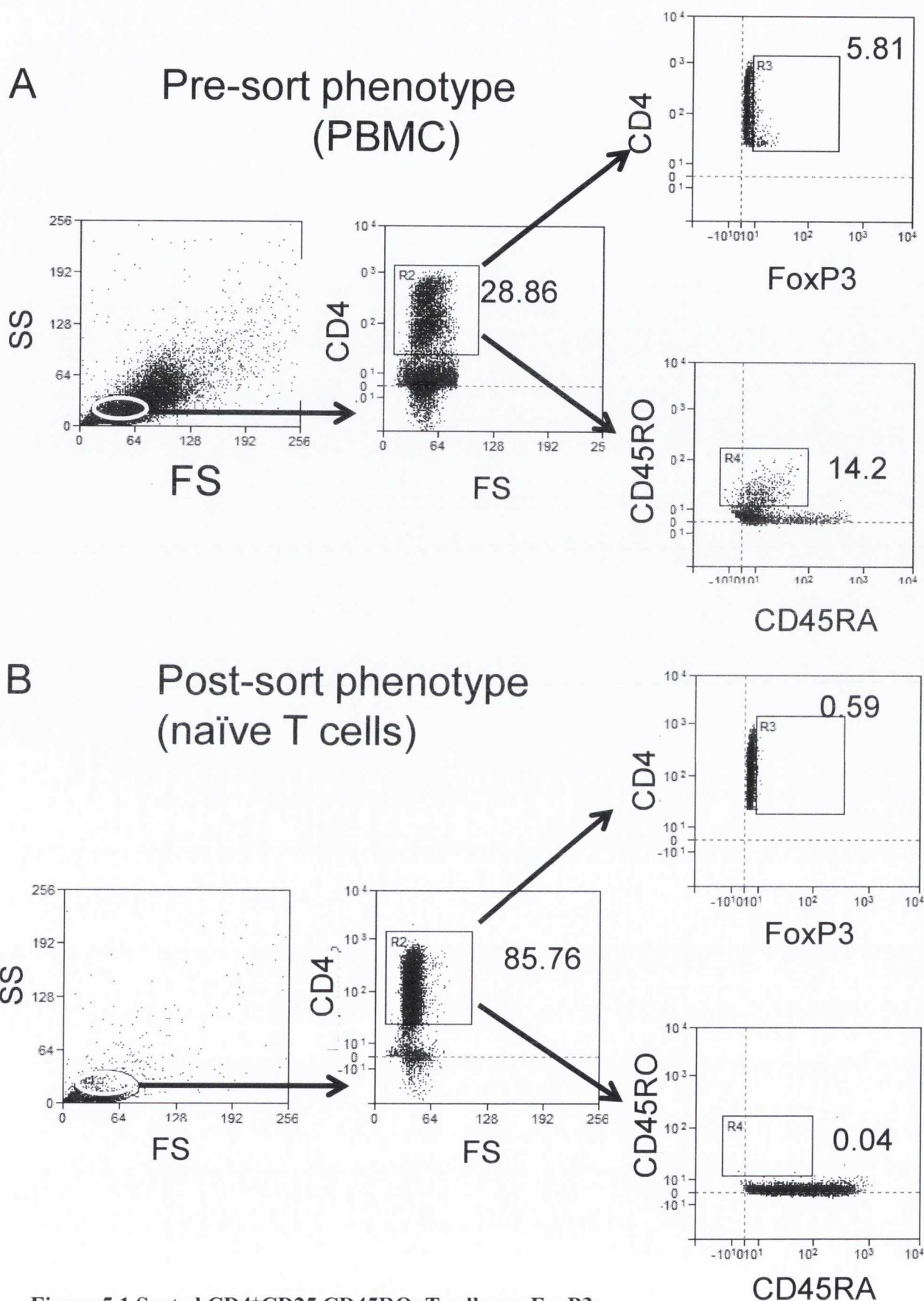


Figure 5.1 Sorted $CD4^+CD25^-CD45RO^-$ T cells are FoxP3 $^-$

$CD4^+CD25^-CD45RO^-$ naïve effector T cells were purified from PBMC isolated from the buffy coats of healthy blood donors (obtained, with permission, from the Irish Blood Transfusion Service). To test the purity, sorted cells were stained with anti-human CD4, FoxP3, CD45RA and CD45RO, and the frequency of $CD4^+FoxP3^+$ cells was determined following gating on the viable $CD4^+$ lymphocytes. A) Phenotype of cells prior to sorting. B) Phenotype of cells post-sort.

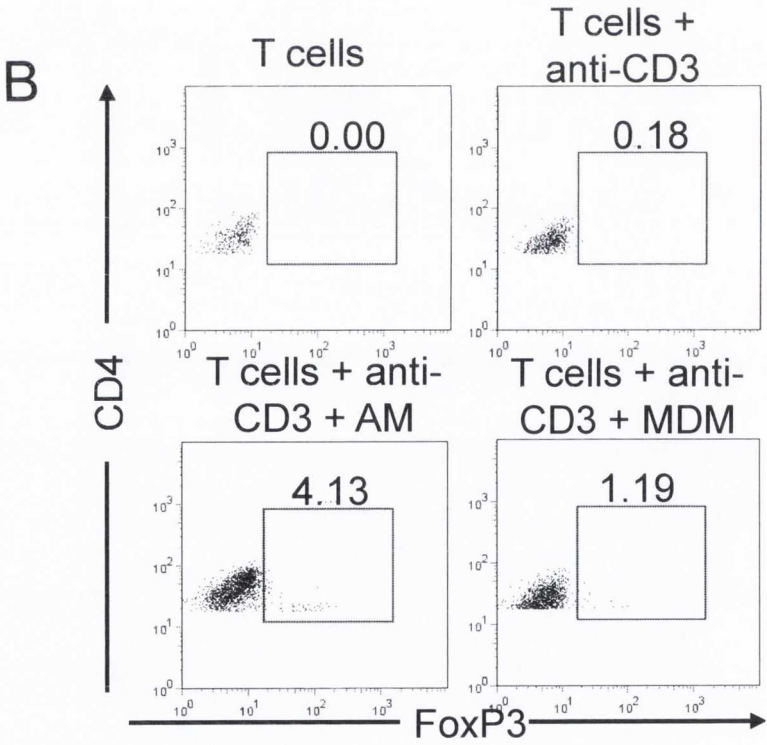
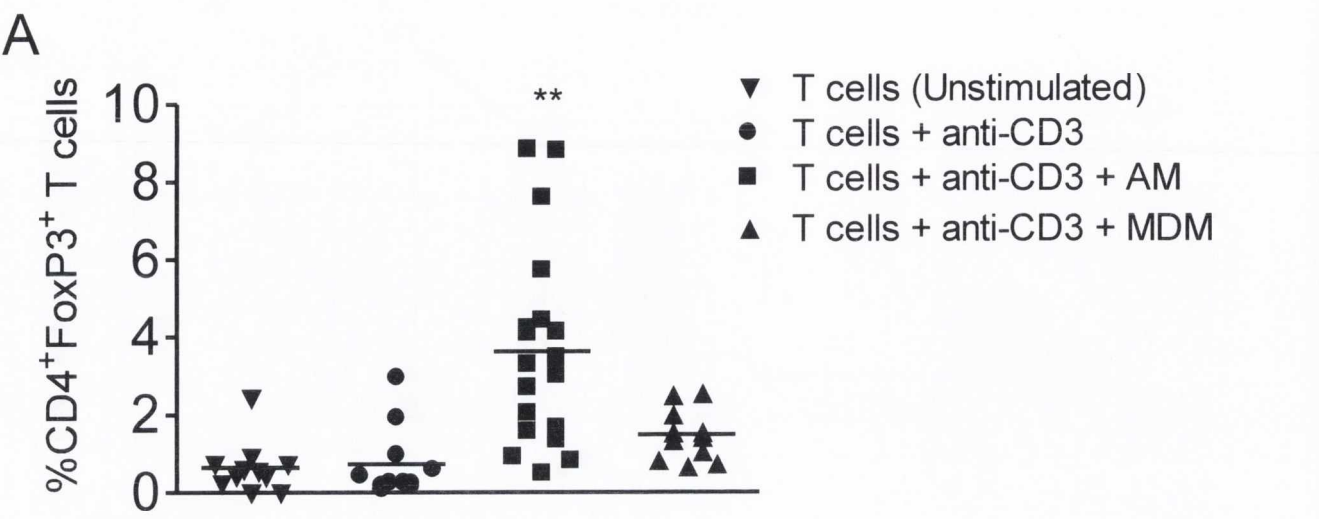


Figure 5.2 Human AM induce FoxP3 expression in allogeneic naïve CD4⁺FoxP3⁻CD25⁻ T cells cultured with anti-CD3

Human AM were obtained by bronchoalveolar lavage (BAL). BAL was performed on patients undergoing bronchoscopy. Human MDM were differentiated from PBMC isolated from the buffy coat of healthy blood donors. Macrophages were cultured at $5 \times 10^5/\text{ml}$ and incubated for at least 24 hours prior to co-incubation with T cells. CD4⁺CD25⁻CD45RO⁻ naïve effector T cells were purified from PBMC as described in Fig. 5.1. T cells were activated with 2 µg/ml soluble anti-CD3 and cultured in the presence or absence of AM or MDM at a ratio of 9:1 macrophages:T cells. After 24 hours, cells were stained with anti-human CD4 and FoxP3 and the frequency of CD4⁺FoxP3⁺ cells was determined following gating on the viable CD4⁺ lymphocytes. A) Results are pooled from 20 patients. B) Representative dot plots showing the percentage of induced FoxP3⁺ CD4⁺ T cells. P values were calculated on the difference in FoxP3 expression between CD4⁺ T cells cultured with anti-CD3 and AM versus CD4⁺ T cells cultured in the absence of AM.

**p<0.01

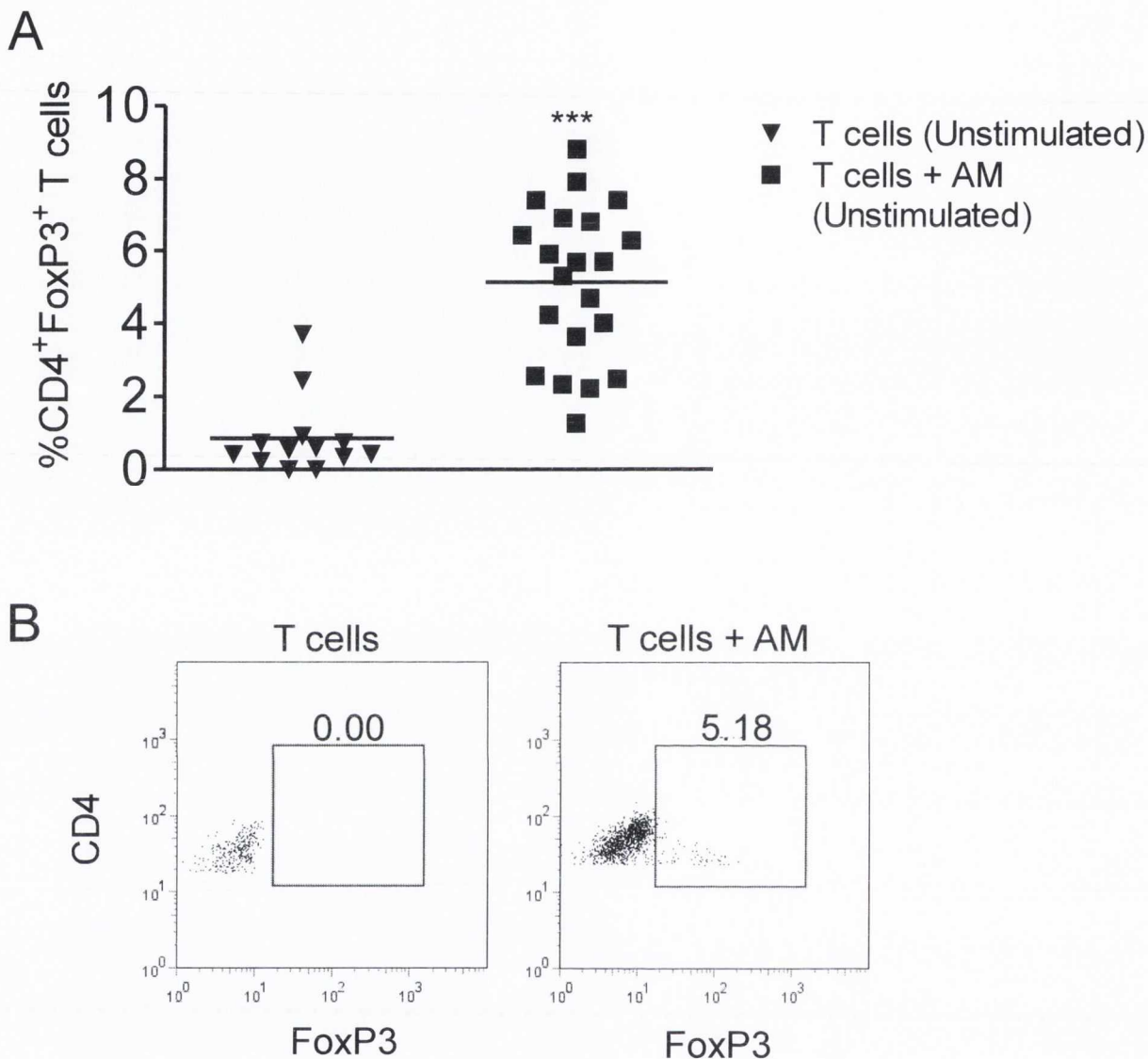


Figure 5.3 Induction of FoxP3 expression by AM does not require T cell stimulation

Human AM were obtained and cultured as described in Fig. 5.2. CD4⁺CD25⁻CD45RO⁻ naïve effector T cells were obtained as described in Fig. 5.1. T cells were cultured in the presence or absence of AM at a ratio of 9:1 AM:T cells. After 24 hours, cells were stained with anti-human CD4 and FoxP3 and the frequency of CD4⁺FoxP3⁺ cells was determined following gating on the viable CD4⁺ lymphocytes. A) Results are pooled from 21 patients. B) Representative dot plots showing the percentage of induced FoxP3⁺ CD4⁺ T cells. P values were calculated on the difference in FoxP3 expression between CD4⁺ cells cultured with AM versus CD4⁺ T cells cultured without AM ***p<0.0001

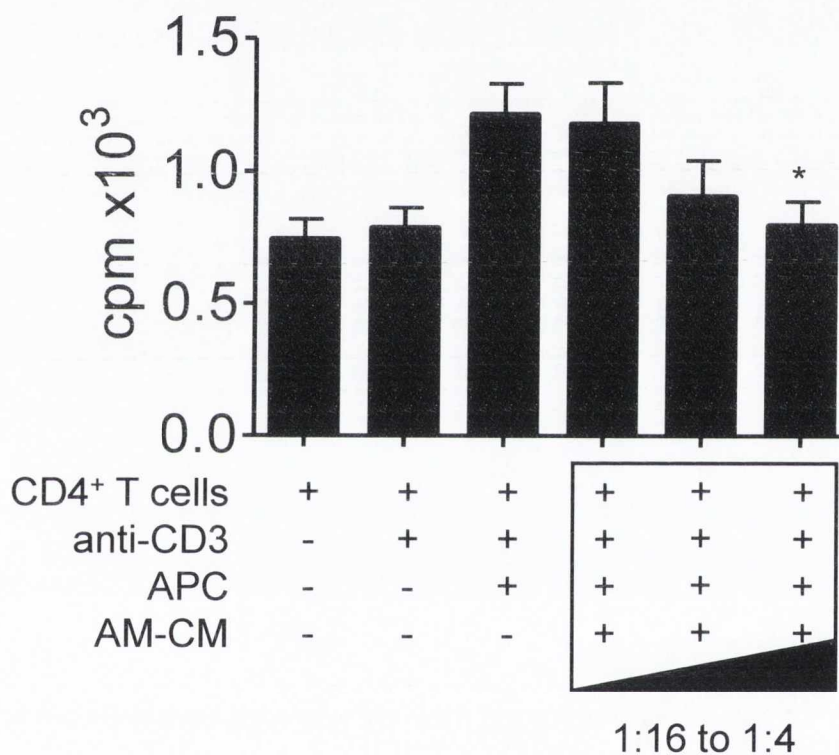
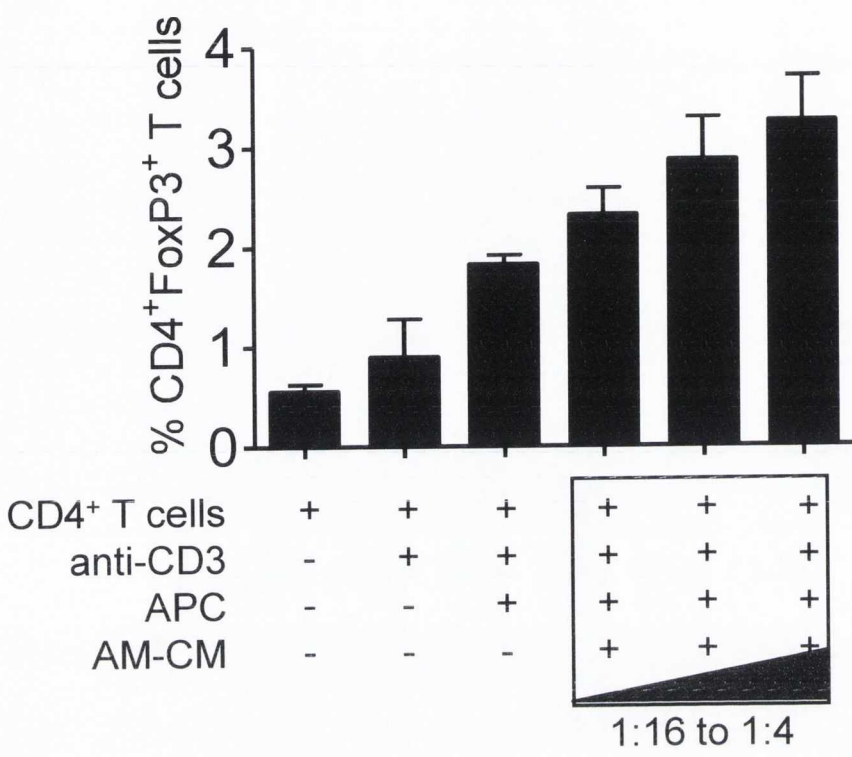


Figure 5.4 Conditioned Media from alveolar macrophage culture can suppress the proliferation of naïve T cells

Human AM were obtained and cultured as described in Fig. 5.2. AM conditioned media (AM-CM) was harvested after 24 hours, and twofold dilutions were prepared. CD4⁺CD25⁻CD45RO⁻ naïve effector T cells were purified from PBMC as described in Fig. 5.1. T cells were activated with 2 µg/ml soluble anti-CD3 and 5 x 10⁵ irradiated APC and cultured with of decreasing dilutions of AM-CM. 50 µl of AM-CM was added to 150 µl of cells, so the final dilution factors were 1:4, 1:8 and 1:16. Proliferation was determined by ³H-thymidine incorporation after 48 hours. Results are the mean values for 3 blood donors, each tested with 6 AM-CM samples. P values were calculated on the difference in proliferation between CD4⁺ cells activated with AM-CM versus CD4⁺ T cells activated without AM-CM *p<0.05

A



B

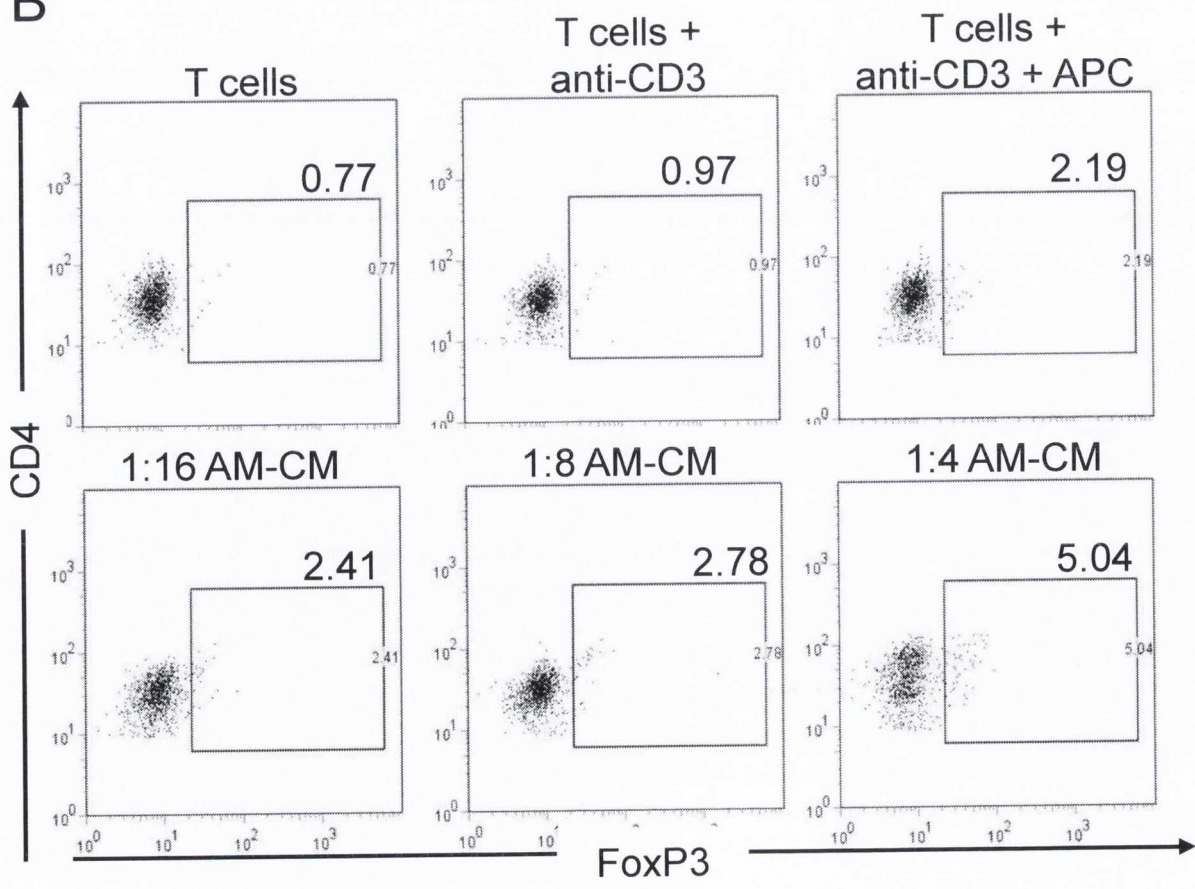
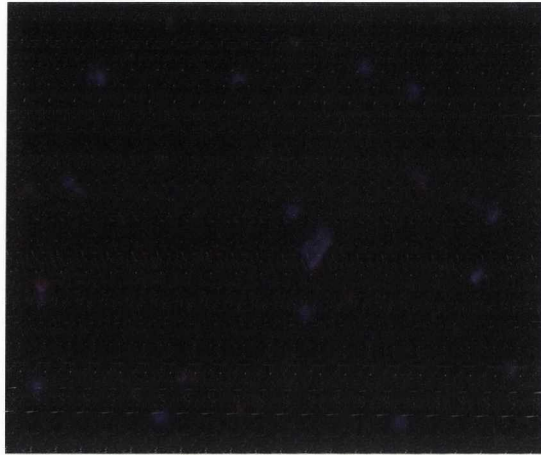


Figure 5.5 AM-CM can induce FoxP3 expression in FoxP3⁻ naïve T cells

AM-CM was produced as described in Fig. 5.4. CD4⁺CD25⁻CD45RO⁻ naïve effector T cells were purified from PBMC as described in Fig. 5.1. T cells were activated with 2 µg/ml soluble anti-CD3 and 5 x 10⁵ irradiated APC and cultured with decreasing dilutions of AM-CM. After 24 hours, cells were stained with anti-human CD4 and FoxP3 and the frequency of CD4⁺FoxP3⁺ cells was determined following gating on the viable CD4⁺ lymphocytes. A) Results are the mean values for 3 blood donors, each tested with 6 AM-CM samples. B) Representative dot plots showing the percentage of induced FoxP3⁺ CD4⁺ T cells.

A) T cells + anti-CD3



B) T cells + anti-CD3 + AM

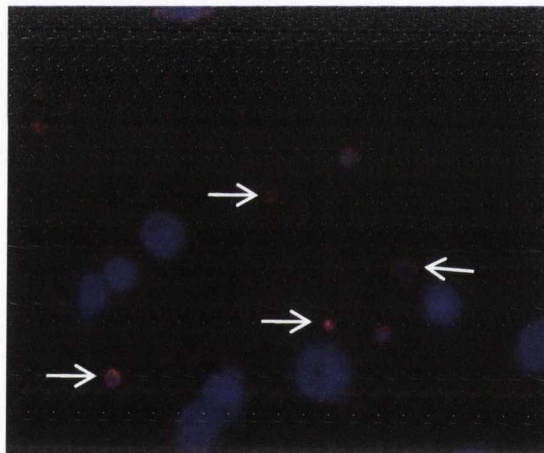


Figure 5.6 AM-CM can induce FoxP3 expression in FoxP3⁻ naïve T cells

AM were obtained as described in Fig. 5.2. CD4⁺CD25⁻CD45RO⁻ naïve effector T cells were obtained as described in Fig. 5.1. T cells were activated with 2 µg/ml soluble anti-CD3 in the presence or absence of AM at a ratio of 9:1 AM:T cells. After 24 hours, cells were stained with anti-human FoxP3-PE (red) and Hoechst 3358 nuclear stain (blue), and the extent of FoxP3⁺ T cell induction was examined by fluorescent microscopy using a Leica photomicroscope. Representative images show A) T cells + anti-CD3 and B) T cells + anti-CD3 + AM. All images are 100x magnification. White arrows indicate FoxP3⁺ T cells.

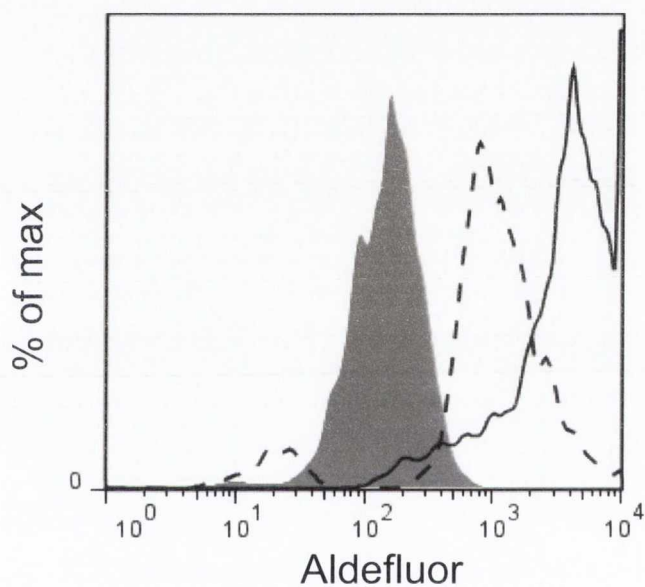
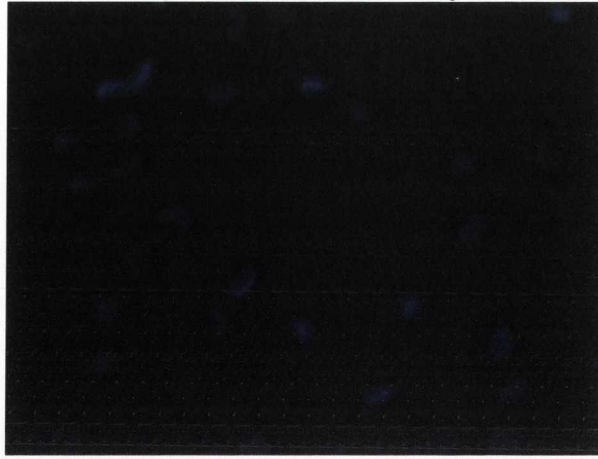


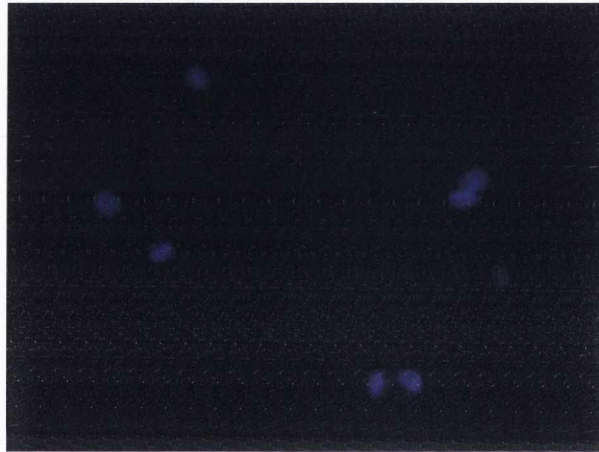
Figure 5.7 Human AM express the enzyme which produces retinoic acid

AM were obtained as described in Fig. 5.2. AM were resuspended at $1 \times 10^6/\text{ml}$ and aldehyde dehydrogenase activity was estimated using the Aldefluor® staining assay. Representative histogram depicts ALDHA1 (Aldefluor®) expression as an indirect measurement of RA production on viable unstained control cells (filled grey histogram), AM from a smoker (dashed line) and AM from a non-smoker (solid line).

A) Hoechst only



B) Inhibitor + Aldefluor + Hoechst



C) Aldefluor + Hoechst

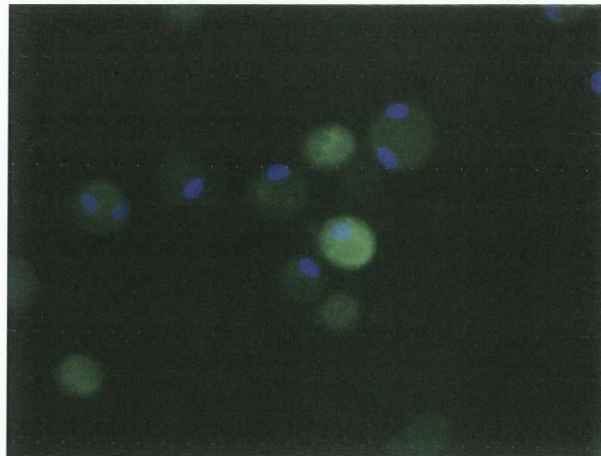


Figure 5.8 Human AM express the enzyme which produces retinoic acid

AM were obtained as described in Fig. 5.2. AM were cultured at $1 \times 10^6/\text{ml}$ and aldehyde dehydrogenase activity was estimated using the Aldefluor® staining assay (green) and Hoechst 3358 nuclear stain (blue) by fluorescent microscopy using a Leica photomicroscope. Representative images depict A) unstained AM, B) AM stained with Aldefluor® reagent in the presence of the ALDH1A inhibitor DEAB and C) AM stained with Aldefluor® reagent. All images are 100x magnification.

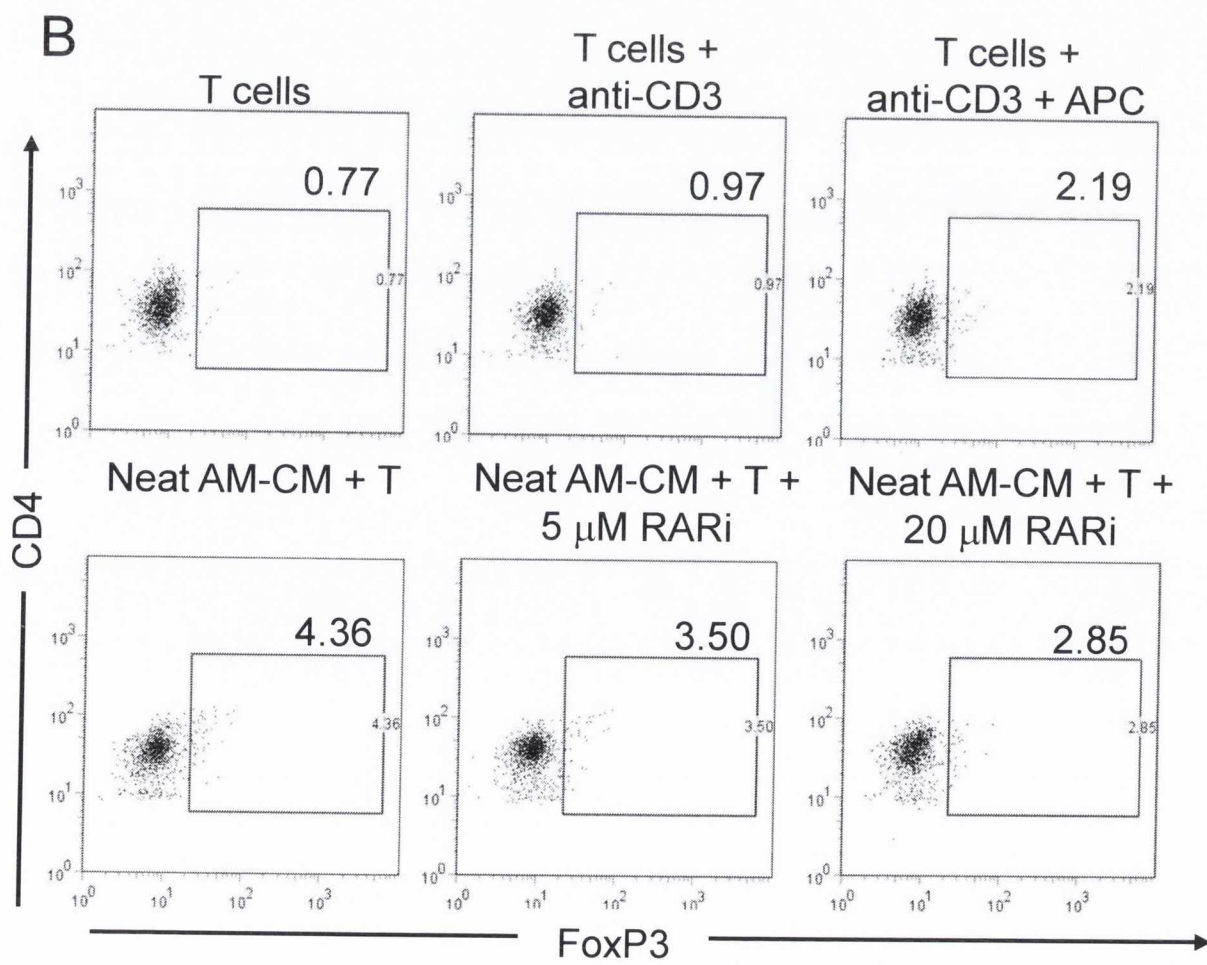
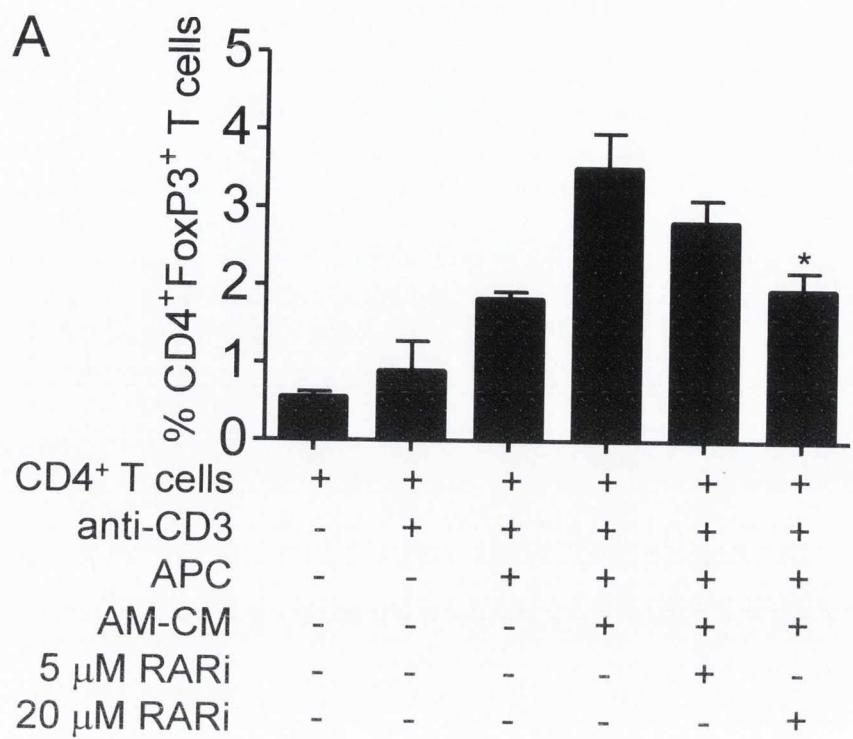


Figure 5.9 Inhibiting retinoic acid signalling significantly reduces the induction of FoxP3⁺ T cells by AM-CM

AM-CM was produced as described in Fig. 5.4. CD4⁺CD25⁻CD45RO⁻ naïve effector T cells were purified from PBMC as described in Fig. 5.1. T cells were activated with 2 µg/ml soluble anti-CD3 and 5 x 10⁵ irradiated APC and cultured with AM-CM in the presence or absence of an antagonist of the retinoic acid receptor (RARi). After 24 hours, cells were stained with anti-human CD4 and FoxP3 and the frequency of CD4⁺FoxP3⁺ cells was determined following gating on the viable CD4⁺ lymphocytes. A) Results are the mean values for 3 blood donors, each tested with 6 AM-CM samples. B) Representative dot plots showing the percentage of induced FoxP3⁺ CD4⁺ T cells. P values were calculated on the difference in FoxP3 expression between CD4⁺ T cells activated in the presence of AM-CM and RARi versus CD4⁺ T cells activated with AM-CM. *p<0.05

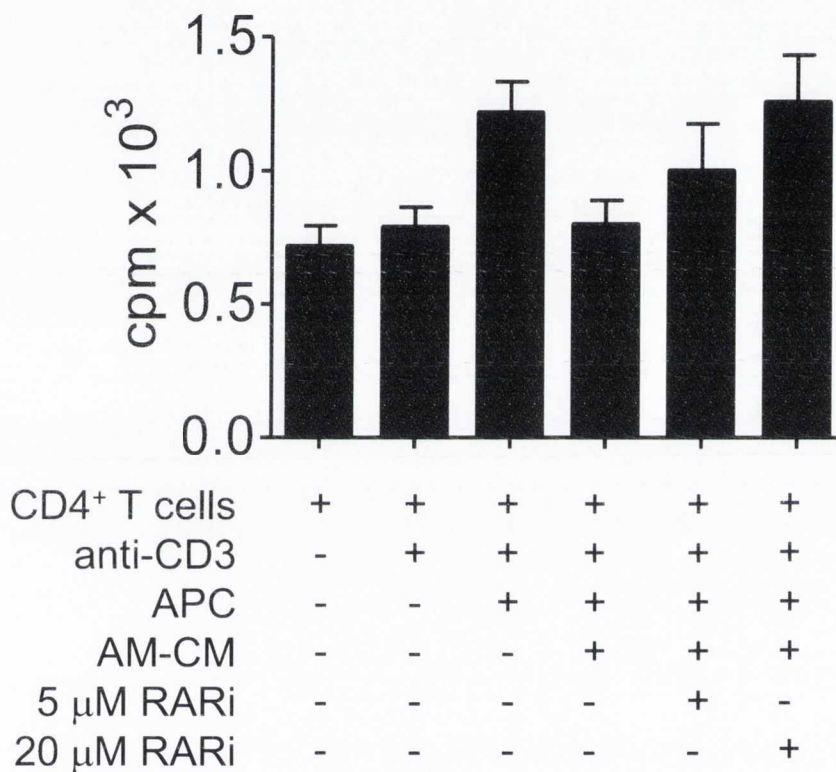
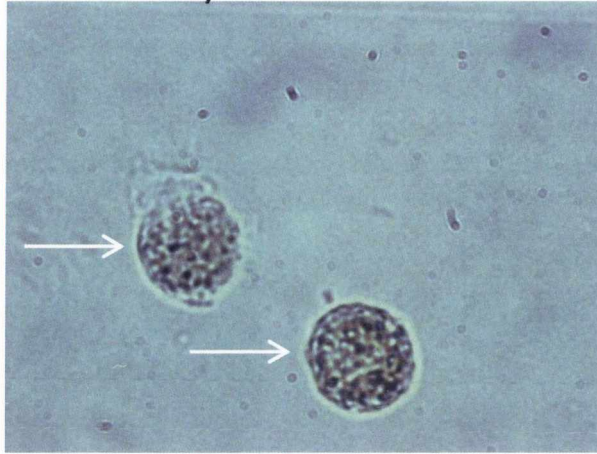


Figure 5.10 Inhibiting retinoic acid signalling overcomes the anti-proliferative effect of AM-CM on T cells

AM-CM was produced as described in Fig. 5.4. CD4⁺CD25⁻CD45RO⁻ naïve effector T cells were purified from PBMC as described in Fig. 5.1. T cells were activated with 2 μg/ml soluble anti-CD3 and 5 x 10⁵ irradiated APC and cultured with AM-CM in the presence or absence of RARi. Proliferation was determined by ³H-thymidine incorporation after 48 hours. Results are the mean values for 3 blood donors, each tested with 6 AM-CM samples

A) Smoker



B) Non-smoker

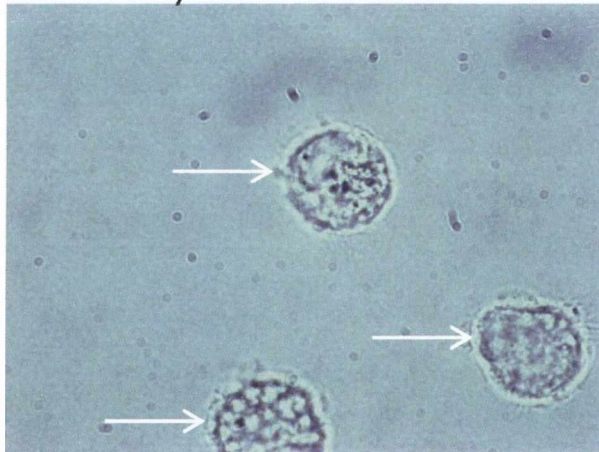


Figure 5.11 AM from smokers are morphologically distinct from AM from non-smokers

AM were obtained as described in Fig. 5.2. AM from each donor were observed using a light microscope (100x magnification). Arrows indicate AMs.

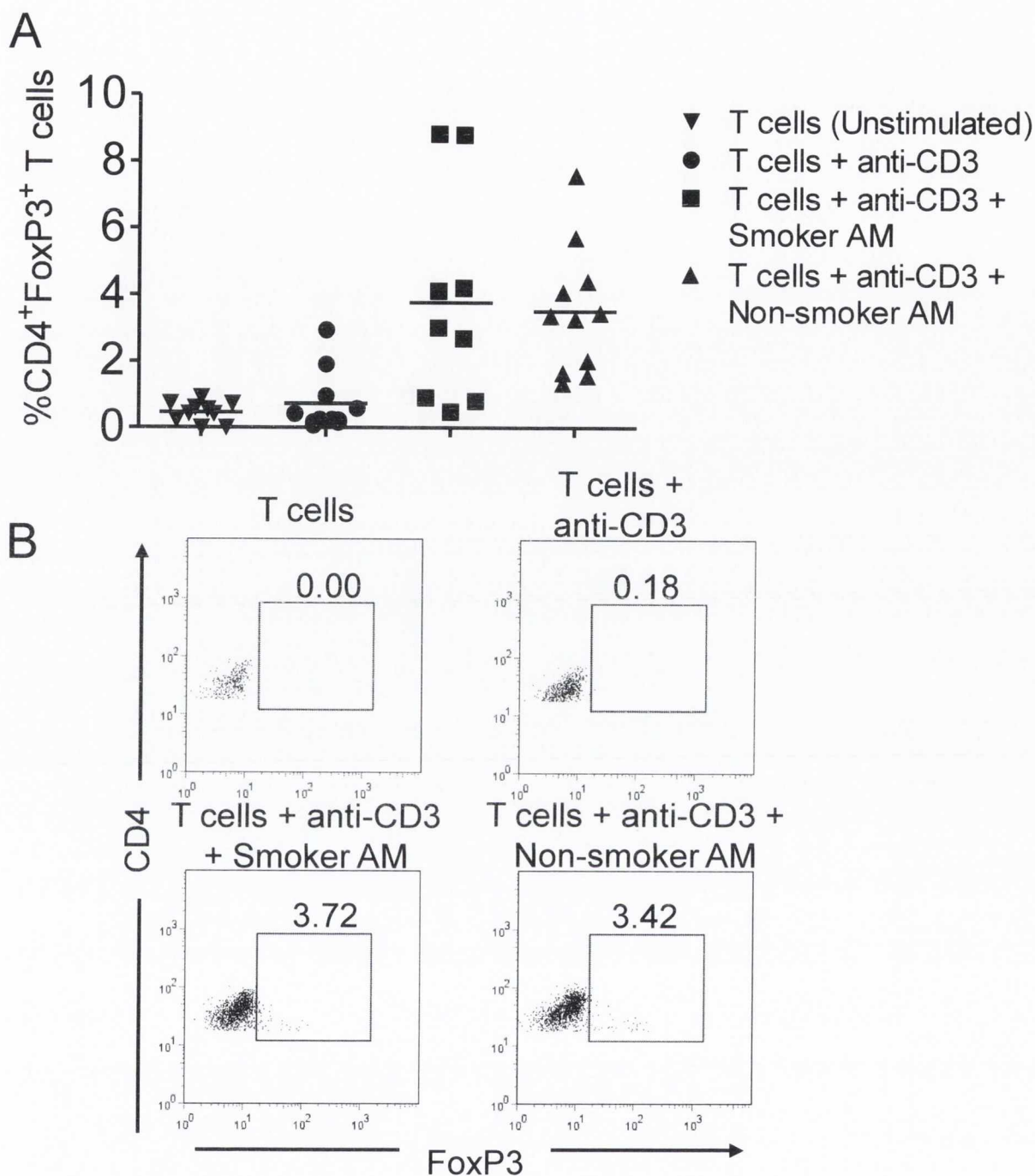
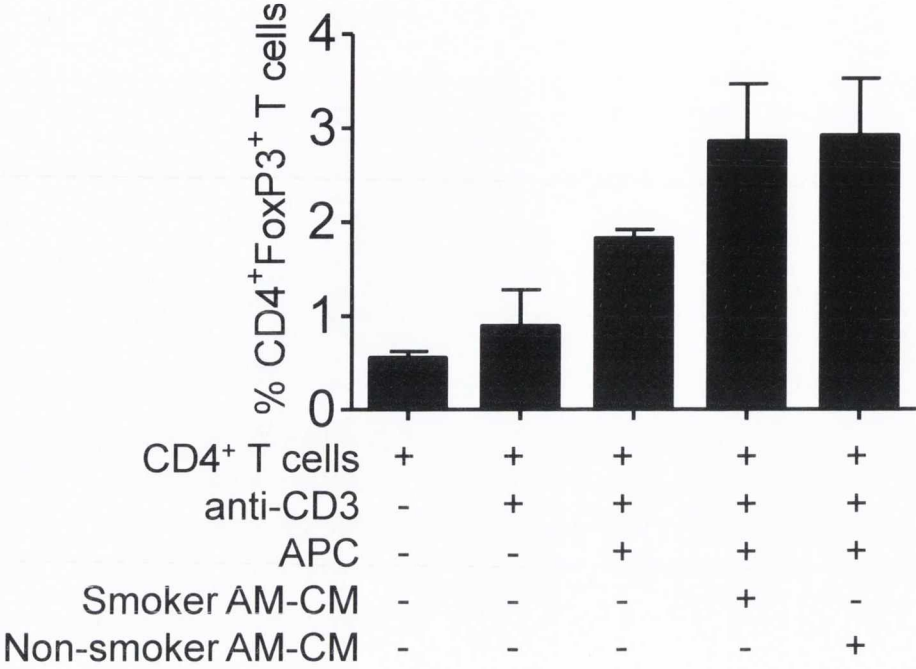


Figure 5.12 AM from smokers induce FoxP3⁺ T cells to the same extent as AM from non-smokers

AM were obtained as described in Fig. 5.2. CD4⁺CD25⁻CD45RO⁻ naïve effector T cells were purified from PBMC as described in Fig. 5.1. T cells were activated with 2 µg/ml soluble anti-CD3 in the presence or absence of AM from smokers or non-smokers at a ratio of 9:1 AM:T cells. After 24 hours, cells were stained with anti-human CD4 and FoxP3 and the frequency of CD4⁺FoxP3⁺ cells was determined following gating on the viable CD4⁺ lymphocytes. A) Results are pooled from 21 patients. B) Representative dot plots showing the percentage of induced FoxP3⁺ CD4⁺ T cells.

A



B

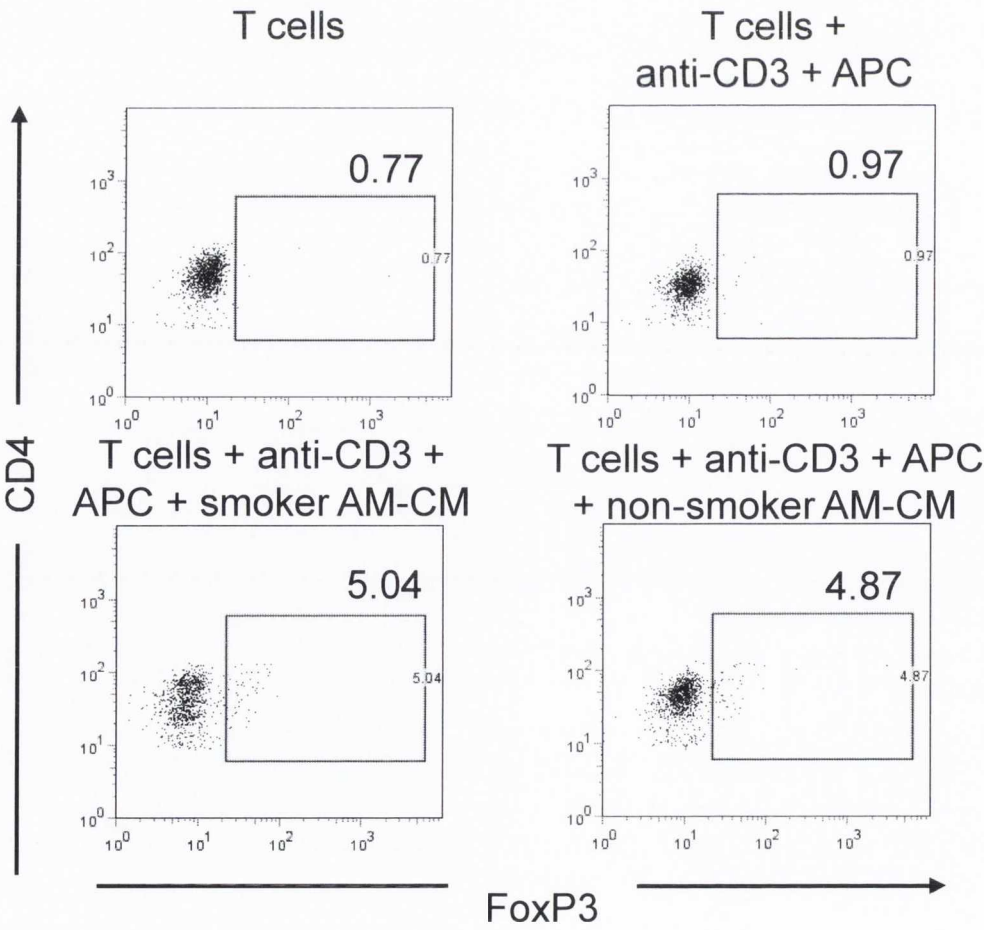


Figure 5.13 AM-CM from smokers induces FoxP3⁺ T cells to the same extent as AM-CM from non-smokers

AM-CM was produced as described in Fig. 5.4. CD4⁺CD25⁻CD45RO⁻ naïve effector T cells were purified from PBMC as described in Fig. 5.1. T cells were activated with 2 µg/ml soluble anti-CD3 and 5 x 10⁵ irradiated APC in the presence or absence of AM-CM from smokers or non-smokers. After 24 hours, cells were stained with anti-human CD4 and FoxP3 and the frequency of CD4⁺FoxP3⁺ cells was determined following gating on the viable CD4⁺ lymphocytes. A) Results are the mean values for 3 blood donors, each tested with 6 AM-CM samples (3 x smokers, 3 x non-smokers) B) Representative dot plots showing the percentage of induced FoxP3⁺ CD4⁺ T cells.

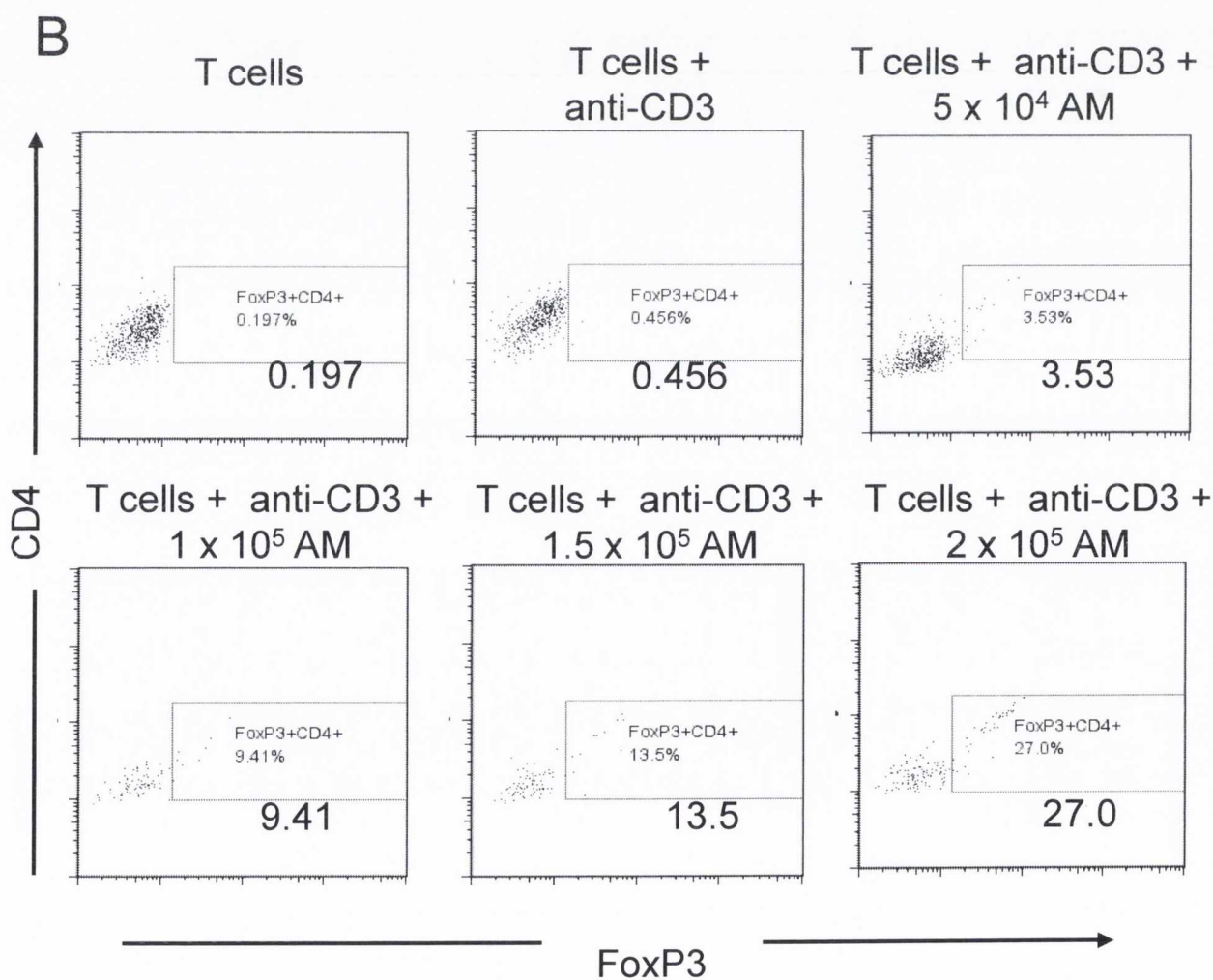
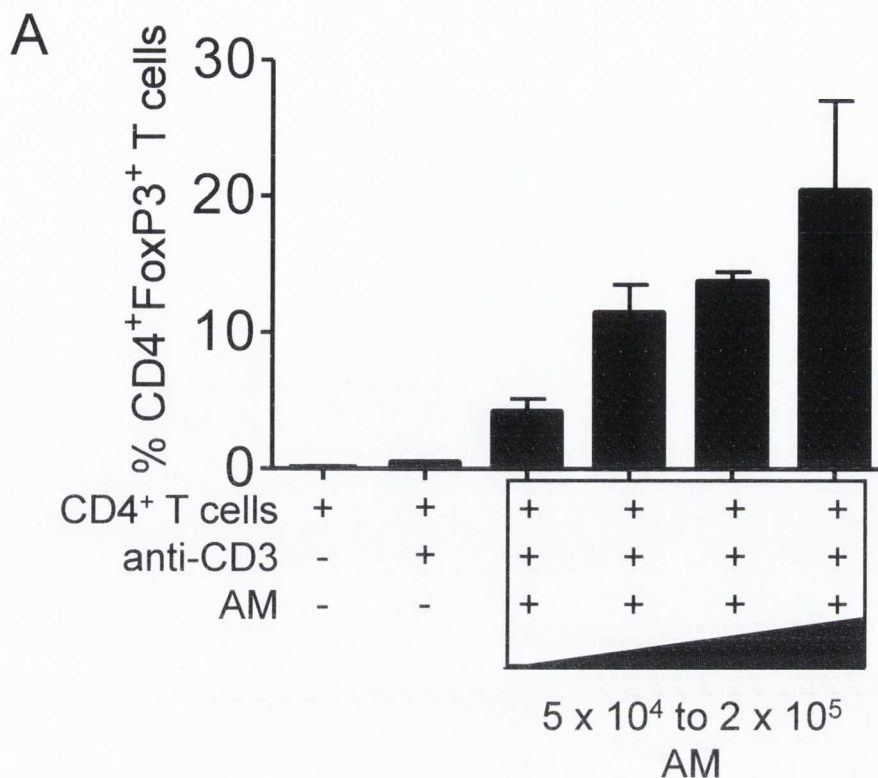


Figure 5.15 AM induce FoxP3⁺ T cells in a dose-dependent manner

AM were obtained as described in Fig. 5.2 and were cultured at different concentrations. CD4⁺CD25⁻CD45RO⁻ naïve effector T cells were purified from PBMC as described in Fig. 5.1. T cells were activated with 2 µg/ml soluble anti-CD3 in the presence or absence of AM. After 24 hours, cells were stained with anti-human CD4 and FoxP3 and the frequency of CD4⁺FoxP3⁺ cells was determined following gating on the viable CD4⁺ lymphocytes. A) Results are pooled from 2 AM donors. B) Representative dot plots showing the percentage of induced FoxP3⁺ CD4⁺ T cells.

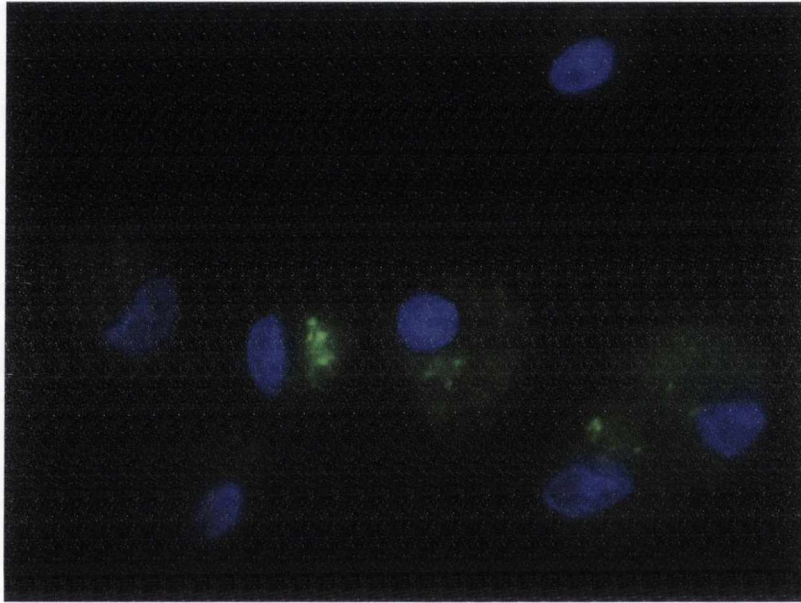
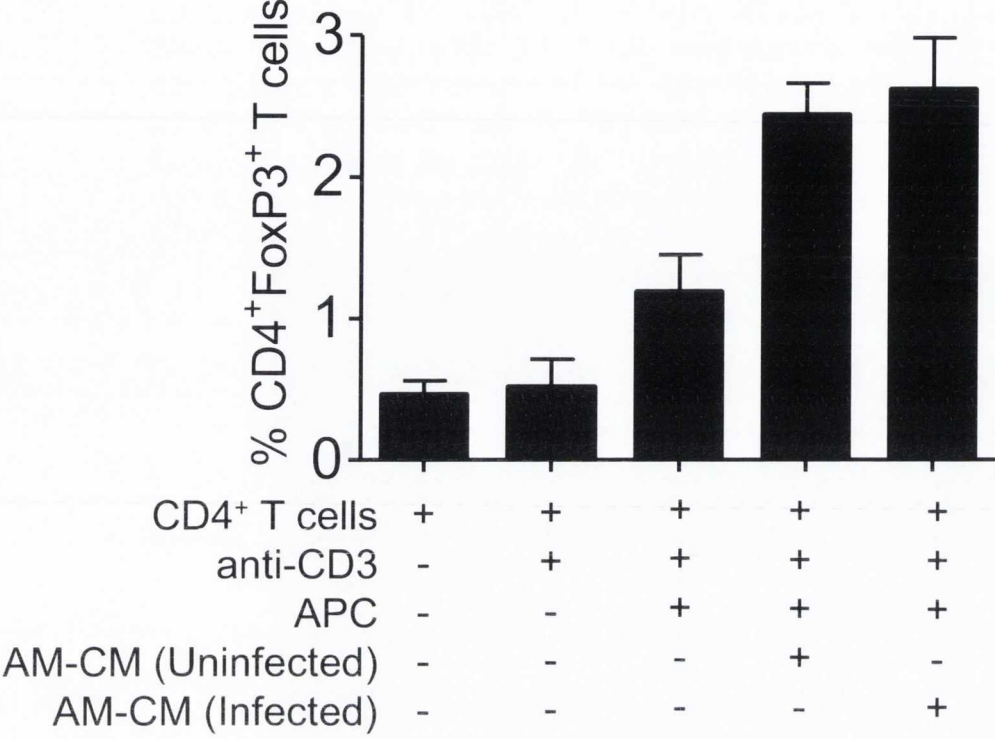


Figure 5.16 Infection of AM with high MOI of *M. tuberculosis* H37Ra

Dilutions of prepared bacilli were added to AM for 3 hours, after which extracellular bacteria were removed and cells were fixed. Infected AM were stained with auramine TB stain (green) and Hoechst 3358 nuclear stain (blue). The MOI was determined by fluorescent microscopy using a Leica photomicroscope. A high MOI was desired for these experiments – i.e. most AM should be infected with a high burden of bacteria (>10 bacilli/cell). Once the ideal MOI was determined, AM were infected with the appropriate volume of bacilli for 3 hours and extracellular bacteria were washed away. 24 hours later, AM were cultured with T cells

A



B

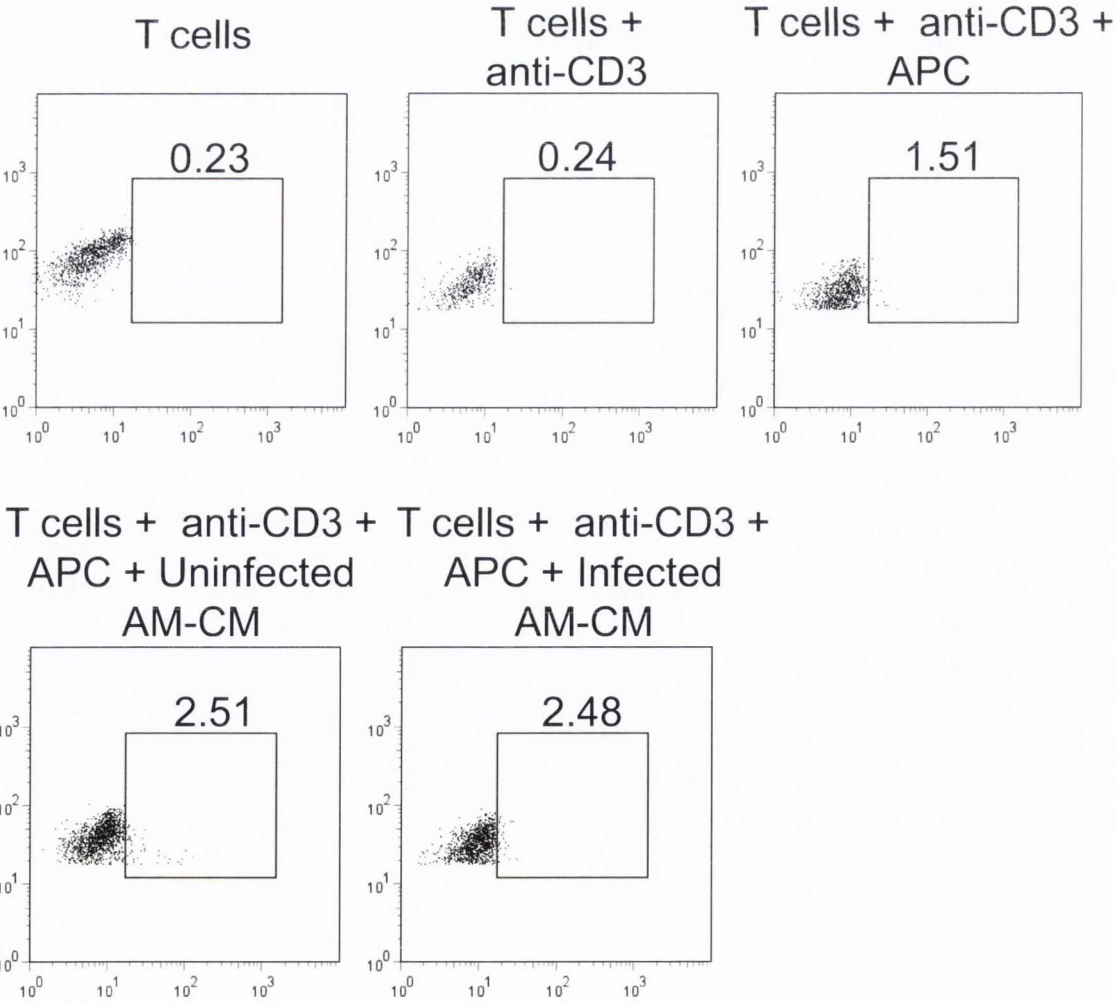


Figure 5.17 *M. tuberculosis* infection does not affect the induction of FoxP3⁺ T cells by human AM

AM were obtained as described in Fig. 5.2. AM were either uninfected, or were infected with *M. tuberculosis* H37Ra at a high MOI . After 3 hours extracellular bacteria were removed and AM were replenished with fresh medium. AM-CM was harvested 24 hours later. CD4⁺CD25⁻CD45RO⁻ naïve effector T cells were purified from PBMC as described in Fig. 5.1. T cells were activated with 2 µg/ml soluble anti-CD3 and 5 x 10⁵ irradiated APC in the presence or absence of AM-CM from infected or uninfected AM. After 24 hours, cells were stained with anti-human CD4 and FoxP3 and the frequency of CD4⁺FoxP3⁺ cells was determined following gating on the viable CD4⁺ lymphocytes. A) Results are the mean values for 3 blood donors, each tested with 6 AM-CM samples. B) Representative dot plots showing the percentage of induced FoxP3⁺ CD4⁺ T cells.

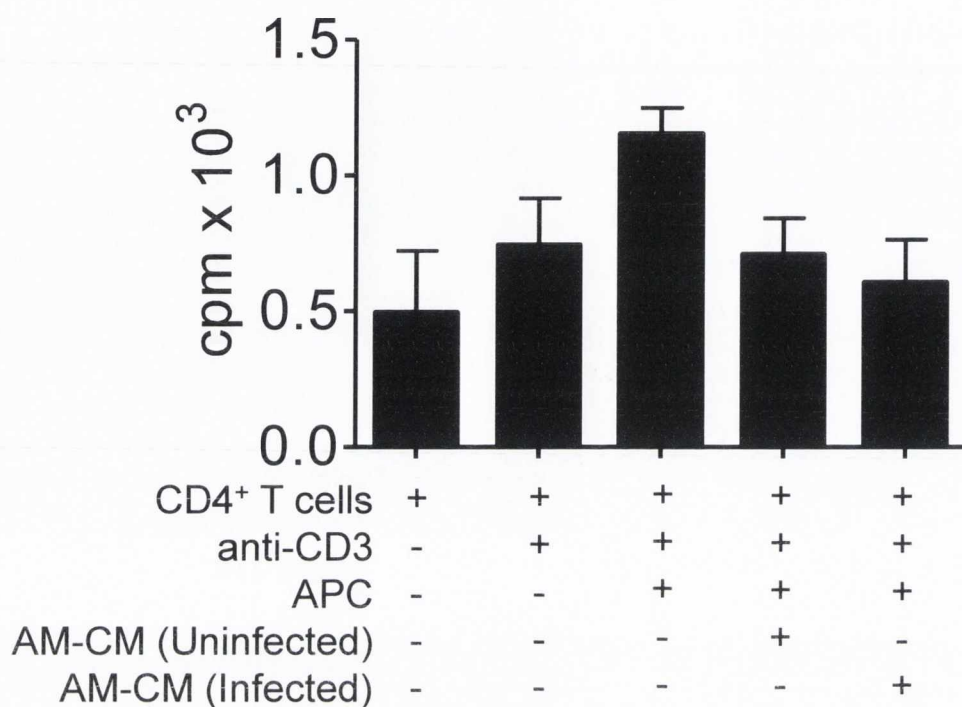


Figure 5.18 The induction of FoxP3⁺ T cells by CM from uninfected and *M. tuberculosis*-infected AM is associated with a reduction in T cell proliferation

AM were obtained as described in Fig. 5.2. AM were either uninfected, or infected with *M. tuberculosis* H37Ra at a high MOI . After 3 hours extracellular bacteria were removed and AM were replenished with fresh medium. AM-CM was harvested 24 hours later. CD4⁺CD25⁻CD45RO⁻ naïve effector T cells were purified from PBMC as described in Fig. 5.1. T cells were activated with 2 µg/ml soluble anti-CD3 and 5 x 10⁵ irradiated APC in the presence or absence of AM-CM from infected or uninfected AM. Proliferation was determined by ³H-thymidine incorporation after 48 hours. Results are the mean values for 3 blood donors, each tested with 6 AM-CM samples.

Chapter 6

General Discussion

The airways are constantly exposed to inhaled antigen, yet the lung microenvironment is predominantly non-inflammatory. Most of the antigens encountered in the lung are not from infectious agents. Therefore the lung's immune system need to "ignore" inhaled innocuous antigen, because excessive inflammation could damage the lung tissue and cause thickening of the alveolar wall, both of which could impair gas exchange and allow inhaled pathogens or toxins to gain access to the bloodstream and spread to other sites of the body. This explains why immune responses in this compartment need to be so highly regulated. Nonetheless, the airways are a common entry point for microorganisms, so when pathogens are encountered, the local immune system of the lung must be capable of initiating a timely, appropriate response.

Evidently, an intricate regulatory network exists in the lung that controls the magnitude of immune responses to inhaled antigen. However, the particular organ-specific mechanisms that are responsible for this immune regulation are unclear. This study examined the role of Treg cells in the lung – specifically $CD4^+FoxP3^+CD25^-$ Treg cells. The results demonstrated how these cells function *in vitro*, and *in vivo* in response to infection with *B. pertussis*. They have also revealed that $FoxP3^+$ T cells can be induced by AM-derived RA in both mice and humans. This mechanism might represent a target for enhancing the efficacy of mucosal vaccines, or as a treatment for respiratory infections. Furthermore, the results have identified a potential link between enhanced frequencies of Treg cells in the lungs as a consequence of cigarette smoking, which might explain why smokers who are infected with *M. tuberculosis* tend to have more severe disease when compared with non-smoking patients.

The discovery that the majority of $CD4^+FoxP3^+$ T cells in the lungs of naïve mice were overwhelmingly $CD25^-$, or inactive, seemed paradoxical at first. It seemed counterintuitive that most of the Treg cells in the lung would be "switched off", particularly given the activated/memory phenotype of these cells. Nevertheless, $CD25$ is a T cell activation marker rather than a specific Treg cell marker, and $FoxP3$ is the archetypal marker of Treg cells. It has been proposed that $CD4^+FoxP3^+CD25^-$ Treg cells represent a "reservoir" of inactive Treg cells

which can become activated when required (Zelenay *et al.*, 2005). In the context of lung immunology, this hypothesis seems plausible - if an adaptive immune response is initiated in the lungs, then these pre-existing local Treg cells may become activated and exert their regulatory function, presumably to minimise tissue damage.

It appears that lung $CD4^{+}FoxP3^{+}CD25^{-}$ Treg cells have a high threshold of activation. They are unable to suppress proliferation of effector T cells *in vitro* when they are freshly isolated from the lungs of naïve mice. However, if they are activated in the presence of IL-2, CD25 expression is enhanced and they subsequently suppress effector T cell proliferation *in vitro*, most likely via IL-10. Nevertheless, $CD4^{+}FoxP3^{+}CD25^{-}$ T cells are not as suppressive as their nTreg cell counterparts, at least *in vitro*. I have shown by phenotypic analysis that lung $CD4^{+}FoxP3^{+}CD25^{-}$ Treg cells express ICOS, a surface marker that is strongly associated with IL-10 production, and is crucial for the induction of peripheral tolerance, in particular mucosal tolerance (Akbari *et al.*, 2002; Bonhagen *et al.*, 2003; Miyamoto *et al.*, 2005; Tuettenberg *et al.*, 2009).

A role for IL-10 in the suppression mediated by these cells is supported by the *in vivo* studies where lung $CD4^{+}FoxP3^{+}CD25^{-}$ Treg cells were found to produce IL-10 during infection with *B. pertussis*. Interestingly, while the frequencies of $CD4^{+}FoxP3^{+}CD25^{+}$ Treg cells increase around the peak of infection, they are still outnumbered by $CD4^{+}FoxP3^{+}CD25^{-}$ Treg cells. Ultimately, the ratios of $CD25^{-}:CD25^{+}$ $CD4^{+}FoxP3^{+}$ return to pre-infection levels once the pathogen has been cleared. It might have been expected that some activated Treg cells would remain, but it is possible that they underwent AICD or simply CD25 expression was downregulated, which has been shown in adoptive transfer experiments in rats (Strickland *et al.*, 1996b). Therefore, under normal conditions, the lung environment might actively keep resident Treg cells in a quiescent state.

The induction of CD4⁺FoxP3⁺CD25⁻ Treg cells may represent an additional population of Treg cells that exist in conjunction with thymically-derived CD4⁺FoxP3⁺CD25⁺ nTreg cells in the lungs of naïve mice. Certainly, depletion of CD25⁺ cells prior to infection with *B. pertussis*, which removed CD4⁺FoxP3⁺CD25⁺ nTreg cells did not enhance bacterial eradication, suggesting that the residual CD4⁺FoxP3⁺CD25⁻ Treg cells function sufficiently in the absence of nTreg cells. Indeed, enhanced concentrations of IL-10 were detected in the lungs of these mice compared with controls. Depleting CD4⁺FoxP3⁺CD25⁺ nTreg cells in IL-10^{-/-} mice accelerated *B. pertussis* clearance, in association with early/enhanced Th1 immune responses. These results suggest that the suppressive function of CD4⁺FoxP3⁺CD25⁻ Treg cells is mediated by IL-10.

It appears that two populations of Treg cell are present in the lungs of mice, and it seems likely that the previously unreported CD4⁺FoxP3⁺CD25⁻ Treg cells are an induced population, unlike the thymically-derived nTreg cells. It has previously been reported that T cells which are activated in the presence of AM are hypoproliferative, and have an activated phenotype with downmodulated CD25 expression (Strickland *et al.*, 1996b). These properties are similar to those of CD4⁺FoxP3⁺CD25⁻ Treg cells described in the present study. AM are the most numerous immune cells in the lung, and many studies have shown that they promote immunosuppression. Indeed, the removal of AM using liposomes containing Cl₂MDP results in massive inflammation in response to otherwise harmless antigen (Strickland *et al.*, 1993; Thepen *et al.*, 1992; Thepen *et al.*, 1991; Thepen *et al.*, 1989). The inflammation appeared to be mediated by T cells (Strickland *et al.*, 1993; Thepen *et al.*, 1991), suggesting that AM have a specific inhibitory effect on T cells *in vivo*.

Previous studies on the effect of AM on T cells did not examine whether Treg cells were involved, with many of these studies published before the discovery of FoxP3 and its importance in Treg cell biology. The experiments in the present study were based on the hypothesis that FoxP3 expression in T cells might be induced by AM, and that these T cells might be responsible for the suppression of effector T cell responses. The experiments employed highly purified Treg-

free populations of naïve T cells - $CD4^+CD25^-CD45RB^{high}$ murine T cells and $CD4^+CD25^-CD45RO^-$ human T cells.

The results of this study demonstrated that AM from humans and mice can inhibit T cell proliferation by inducing FoxP3 expression in naïve T cells *in vitro*. Importantly, the activation status of the T cells does not affect the induction as AM induced FoxP3 expression in both unstimulated and anti-CD3-stimulated T cells *in vitro*. In mice, the phenotype of the Treg cells induced by AM without anti-CD3 stimulation was predominantly $CD4^+FoxP3^+CD25^-$, which strongly resembles the *ex vivo* phenotype of $FoxP3^+$ T cells isolated from the lungs of naïve mice. Unfortunately, it was not possible to culture human AM with T cells from the same donor, and establish the expression of CD25 by the induced $FoxP3^+$ T cells. The induced $FoxP3^+$ T cells could have become activated as a consequence of MHC mismatches in an MLR-type reaction. Nonetheless, these results have suggested for the first time that AM contribute to immune tolerance in the lungs of mice and humans by promoting the induction of $FoxP3^+$ T cells.

$CD4^+$ effector T cells are constantly recruited to the lung and its draining lymph nodes. In the lymph nodes, they encounter inhaled antigen via APC. The vast majority of inhaled antigen is innocuous. In order to prevent unnecessary inflammation it is possible that AM induce $FoxP3^+$ T cells from these infiltrating effector T cells, which are $CD25^-$. However, if pathogenic microorganisms are present then the local immunosuppression must be inhibited. The stimulation of AM via TLRs, in particular TLR2, TLR4 and TLR9, can apparently overcome their inhibitory function and is permissive to the development of an appropriate immune response to the specific pathogen (Fernandez *et al.*, 2004; Lambrecht, 2006; Takabayshi *et al.*, 2006).

AM could induce FoxP3 expression in unactivated T cells as well as activated T cells *in vitro*. Certainly in SPF mice *in vivo*, most of the T cells which encounter AM would not be activated, so would not express CD25. This could explain why $CD4^+FoxP3^+$ T cells isolated from the naïve mouse lung have a predominantly $CD25^-$ phenotype, and it gives further credence to the

Treg cell “reservoir” theory. In other words, inactive FoxP3⁺ T cells are present in the lung and will effectively suppress immune responses, but only when they are sufficiently activated.

The results have shown that human and mouse AM induce FoxP3 expression in T cells using a common mechanism involving RA, suggesting that it is highly conserved. The study showed for the first time that AM have the ability to produce RA. This is consistent with studies on the gut, where DC and macrophages have been reported to produce RA (Coombes *et al.*, 2007; Denning *et al.*, 2007), so this strategy might be common to APC at mucosal surfaces. The studies in mice have shown that RA synergises with TGF- β_1 to augment induction of FoxP3 expression on naïve T cells, and that blocking RA signalling can enhance antigen-specific and non-specific T cell proliferation in the presence of AM *in vitro*. These studies also indicate that the AM-induced murine FoxP3⁺ T cells produce IL-10, which represents another similarity with the CD4⁺FoxP3⁺CD25⁻ Treg cells found in the lungs at high frequencies.

This study also examined the possible role of RA-induced Treg cells in controlling immunity to infection in the lungs, using a model of respiratory infection with *B. pertussis*. The results revealed that nasal administration of RARi significantly reduced the frequency of FoxP3⁺ T cells in the lungs of mice, but this had no effect on the pathogen load. Although RARi appears to be effective in preventing Treg cell conversion by RA, it does not of course remove nTreg cells which may be sufficient at suppressing the immune response against *B. pertussis*. The results from this experiment are consistent with the experiments in which IL-10^{-/-} mice were depleted of nTreg cells, and provide additional evidence for two complementary and possibly redundant immunoregulatory mechanisms in the lung. These results point to future experiments that may shed better light on the mechanism of nasal tolerance. For example, depletion of CD25⁺ cells in conjunction with RARi-mediated inhibition of FoxP3⁺ T cell induction during *B. pertussis* infection. It is likely that disabling both populations of lung Treg cells would eradicate the pathogen more quickly.

Manipulation of RA-induced Treg cells may be a more promising strategy for enhancing the efficacy of mucosal vaccines. It is known that nasal and oral administration of soluble antigens results in extreme immunological non-responsiveness which is mediated by CD4⁺ T cells and is characterised by decreased T cell proliferation, reduced production of Th1 and Th2 cytokines, and suppressed immunoglobulin production (van Halteren *et al.*, 1997). The results of the present study suggest that AM-induced FoxP3⁺ T cells contribute to the immune non-responsiveness to nasally delivered antigens, and that inhibiting RA signalling might overcome it. Experiments to address this hypothesis found some evidence to support it. Nasal delivery of antigen and adjuvant in conjunction with RAR α successfully prevent the induction of nasal tolerance to the antigen and allows systemic immunity to develop, as determined by antigen-specific serum IgE. These findings support the conclusion that the FoxP3⁺ T cells which are induced by RA from AM are at least partly responsible for promoting nasal tolerance. This has implications for the development of vaccines targeted to mucosal sites such as the lung, where so many pathogens and antigens gain entry to the host.

Cigarette smoking is the causative agent of serious respiratory diseases such as lung cancer and chronic obstructive pulmonary disease (COPD) (Domagala-Kulawik, 2008). The pathogenesis of these smoking-related disorders is associated with complex inflammatory processes and changes in the immune system. The respiratory tract is the system most affected by cigarette smoke, and immune consequences include metaplastic and dysplastic changes in the bronchial epithelium, and enhanced secretion of inflammatory cytokines and adhesion molecules (Domagala-Kulawik, 2008). In particular, chronic cigarette smoke exposure results in increased production of matrix metalloproteinases (MMP) by macrophages, which contribute to the destruction of the alveolar wall (Domagala-Kulawik, 2008; Tetley, 2002).

Others have reported that AM in the lungs of smokers are more metabolically active than AM from non-smokers (Harris *et al.*, 1970), so it was hypothesised that they might also differ in the production of RA. However, cigarette smoking had no effect on the expression of ALDH1A

or the ability of human AM to induce FoxP3⁺ T cells, when compared with similar numbers of AM from non-smokers. Furthermore, a comparison of uninfected AM with AM infected with *M. tuberculosis* H37Ra demonstrated that they can induce FoxP3⁺ T cells to the same extent. However, the lungs of smokers contain significantly greater numbers of AM and Treg cells than non-smokers, and culture of T cells with increasing numbers of AM *in vitro* resulted in the enhancement of FoxP3⁺ T cell induction, presumably due to increased production of RA. It is therefore likely that there is greater induction of FoxP3⁺ T cells with regulatory potential *in vivo* in the lungs of smokers compared with non-smokers. This might explain why smokers are more susceptible to respiratory infections and are more likely to develop lung cancers.

Not only are smokers more susceptible to infection with *M. tuberculosis*, but they are also more likely to develop reactivation TB, extrapulmonary TB and even die from the disease in comparison with infected non-smokers. Even with effective chemotherapy, smokers have a longer period of infectivity relative to non-smokers. Independently of this, in *M. tuberculosis*-infected patients increased frequencies of Treg cells are associated with worse outcomes of disease (Chen *et al.*, 2007; Qin *et al.*, 2008; Sharma *et al.*, 2009). This suggests that there is a relationship between cigarette smoking and the induction of Treg cells during *M. tuberculosis* infection. If it was possible to get blood and BAL samples from the same patients, then it would also be interesting to examine whether FoxP3⁺ T cells induced by AM in the absence of activating stimuli lack CD25 expression. It would also be worthwhile to examine whether the induced FoxP3⁺ T cells can inhibit killing of *M. tuberculosis* by infected AM, and whether inhibiting RA signalling can promote killing.

If smokers have enhanced frequencies of AM in their lungs, then elevated levels of AM-induced FoxP3⁺ T cells could impair anti-TB immunity and prevent the eradication or containment of the pathogen. If *M. tuberculosis* cannot be eliminated or contained, active disease ensues with the possibility that it can spread elsewhere in the body. Smokers with contained LTBI are also more likely to progress to reactivation TB, but the mechanism is not known. If the AM-

induced FoxP3⁺ T cells in humans also function via IL-10, then perhaps IL-10 can antagonise TNF- α , which plays a key role in anti-TB immunity. The importance of TNF- α in tuberculosis is highlighted by the reactivation of LTBI in individuals prescribed TNF-blocking drugs, such as infliximab (Keane *et al.*, 2001).

The *in vivo* studies in mice involving nasal delivery of an RAR antagonist have established that this is an effective strategy to inhibit the induction of FoxP3⁺ T cells in the lungs. As a result, this approach can be incorporated into a useful immunisation strategy, and I have demonstrated how nasal administration of antigen and adjuvant in combination with RARi prevents tolerance to the antigen. It is possible that this approach could be applied to humans where the RARi could be delivered via an inhaler to enhance immune responses to *M. tuberculosis* in infected patients, especially smokers. Inhalers would eliminate risk associated with improper and safe needle use as well as disposal, and it might be more cost-effective, as trained medical personnel would not be required to inject patients. From a patient perspective, this approach is more accessible and less invasive than injections. It also makes sense to direct vaccines against respiratory infections to the lungs. In the case of TB, which is endemic in developing countries, this simplified and potentially cheaper vaccination technique might make it accessible to more individuals. Inhalation therapy of RA antagonists might also complement anti-TB chemotherapy, especially as smokers have a prolonged period of infectivity after commencing treatment compared with non-smokers, possibly due to the presence of excessive numbers of FoxP3⁺ T cells.

In conclusion, this study has shown that AM in the lung have the ability to induce FoxP3 expression in naïve, FoxP3⁻ T cells, in part via RA secretion (Fig. 6.1). This is independent of the activation status of the T cell, and this may explain why the majority of CD4⁺FoxP3⁺ T cells in the lungs of naïve mice do not express CD25. These CD4⁺FoxP3⁺CD25⁻ Treg cells comprise a population distinct from CD4⁺FoxP3⁺CD25⁺ nTreg cells because unlike nTreg cells, they suppress via cytokine production, namely IL-10. They also have weaker suppressive capacity than

nTreg cells. These features suggest that the $CD4^{+}FoxP3^{+}CD25^{-}$ Treg cells are an induced population rather than thymically-derived Treg cells. Furthermore, *in vivo* studies using the *B. pertussis* infection model have suggested that two complementary Treg cell populations exist in the lung: one expressing CD25 and the other secreting IL-10.

It is possible that the induced $FoxP3^{+}$ T cells exist in this under-activated state to prevent excessive immunoregulation in the lung. However, when they are appropriately stimulated in the presence of IL-2 *in vitro*, they become sufficiently activated and function to limit T cell responses. *In vivo*, Teff cells may be the source of IL-2, so this represents a neat feedback loop, in which the Treg cells are only fully activated when inflammatory effector T cells are present, and which must be suppressed to prevent tissue damage (Fig. 6.2). This mechanism of peripheral tolerance could be manipulated by inhibiting RA signalling and it might represent a potential therapeutic approach for promoting immune responses to pathogens in the lungs. Additionally, an imbalance of AM, such as the increase in number as a result of cigarette smoking, may skew the immune dynamic in the lung, due to greater induction of $FoxP3^{+}$ T cells (Fig. 6.3). Excessive frequencies of Treg cells can impair protective immune responses such as the Th1 response to *M. tuberculosis* infection. Indeed, smokers infected with *M. tuberculosis* have increased morbidity and mortality compared with infected non-smokers.

Therefore, the results identify a complex system for maintaining tolerance in the lung and preventing tissue damage, but they also illustrate how an imbalance can negatively impact on protective immunity to microorganisms.

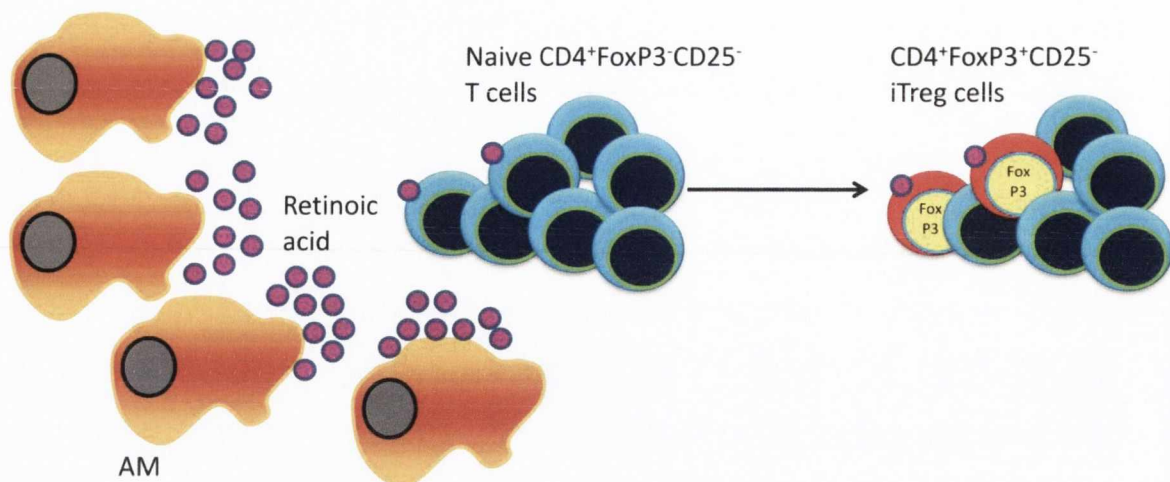


Figure 6.1 Induction of peripheral tolerance in the lung by AM

AM produce RA, which can induce expression of FoxP3 in naïve CD4⁺FoxP3⁻CD25⁻ T cells. The induced CD4⁺FoxP3⁺CD25⁻ T cells might comprise an under-active “reservoir” of Treg cells which must be fully activated before they can exert a suppressive effect *in vivo*.

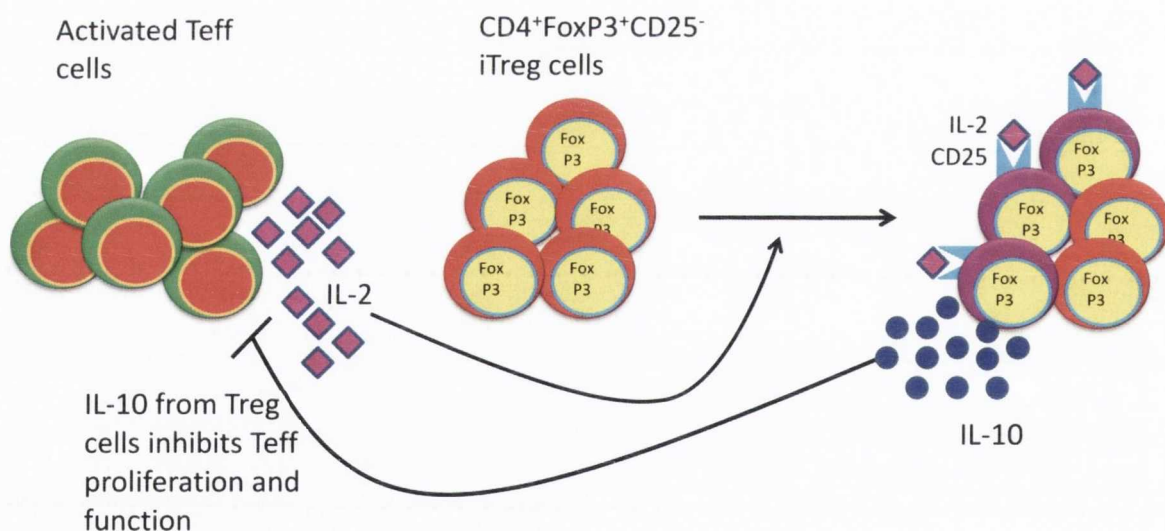


Figure 6.2 CD4⁺FoxP3⁺CD25⁻ Treg cell activation and function

CD4⁺FoxP3⁺CD25⁻ induced T cells become fully activated in the presence of IL-2, which is secreted by Teff cells during an immune response. Activated FoxP3⁺ T cells subsequently produce IL-10, which suppresses proliferation of Teff cells and prevents excessive immunopathology as a result of an overactive immune response.

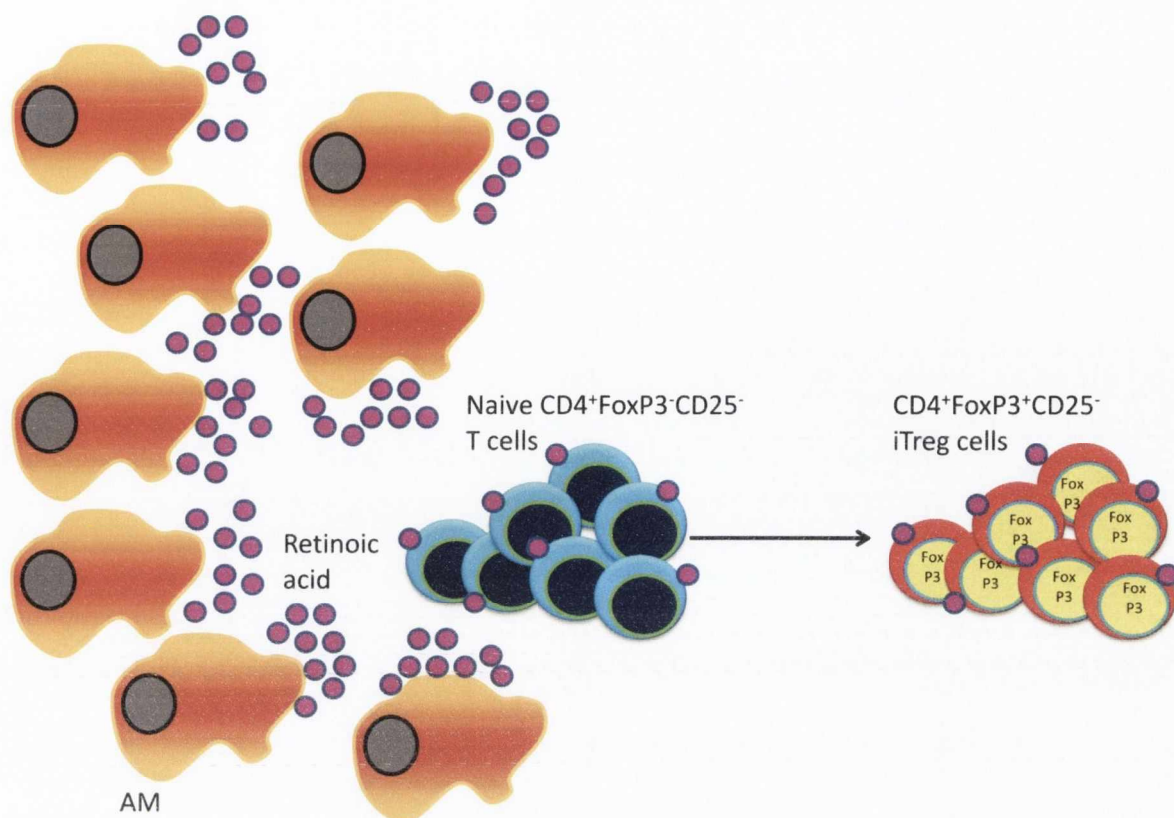


Figure 6.3 Cigarette smoking can enhance the induction of peripheral tolerance, which can skew the immune dynamics in the lung and promote excessive immunoregulation

Cigarette smoking results in increased numbers of AM in the lungs, which results in enhanced conversion of FoxP3⁺ T cells, which might have regulatory function. In general immune cell dynamics need to be delicately balanced – if there are too few Treg cells, then autoimmune diseases can develop; on the other hand, too many Treg cells can impair protective immune responses against pathogens and promote tumor development. In the lung in particular, excessive immunoregulation means that pathogens may persist. Smokers infected with *M. tuberculosis* have poorer outcomes of infection than non-smokers, and Treg cells are independently associated with poorer outcomes as well. These risk factors may be linked, i.e. the augmented numbers of AM in the lungs of smokers may induce increased frequencies of FoxP3⁺ T cells which might impair protective anti-TB immunity.

Chapter 7

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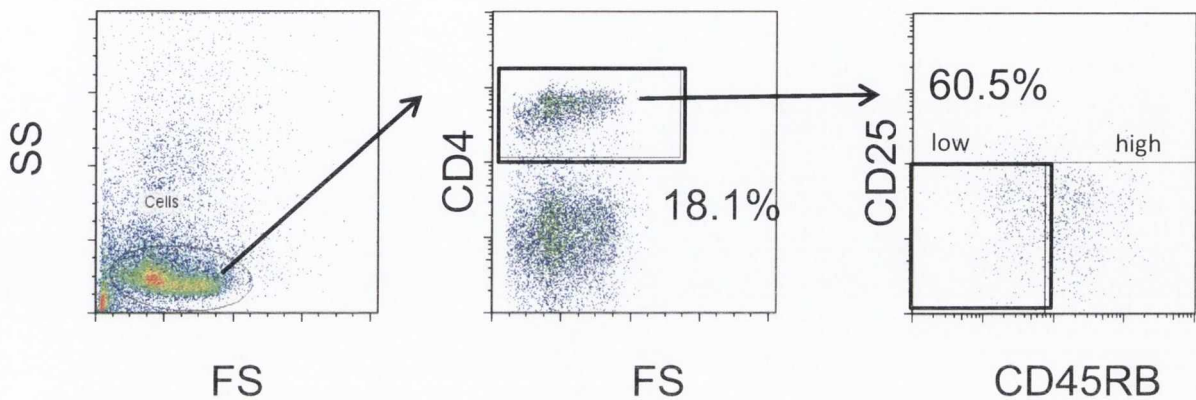
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Chapter 8

Appendix

A) Pre-sort



B) Post-sort

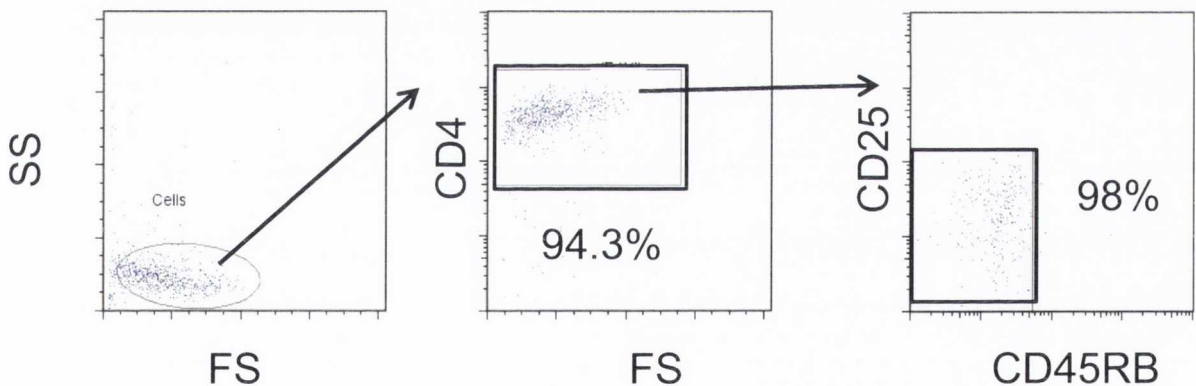
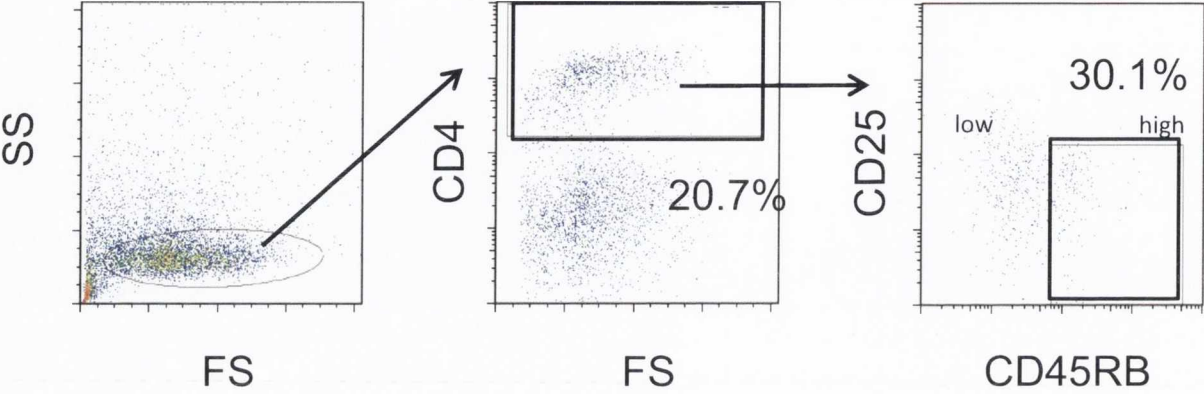


Figure 8.1 Purification of $CD4^+CD25^-CD45RB^{low}$ Treg cells from the lungs of naïve mice

Lungs from naïve mice were digested with 1 $\mu\text{g}/\text{ml}$ collagenase, homogenised and filtered. Isolated cells were stained with rat anti-mouse CD4, CD25 and FoxP3. $CD4^+CD25^-CD45RB^{low}$ Treg cells were purified using a MoFlow cell sorter. A) Representative dot plots showing the gating strategy and percentages of $CD4^+$ and $CD4^+CD25^-CD45RB^{low}$ cells prior to purification. B) Representative dot plots showing the percentages of $CD4^+$ and $CD4^+CD25^-CD45RB^{low}$ cells after purification.

A) Pre-sort



B) Post-sort

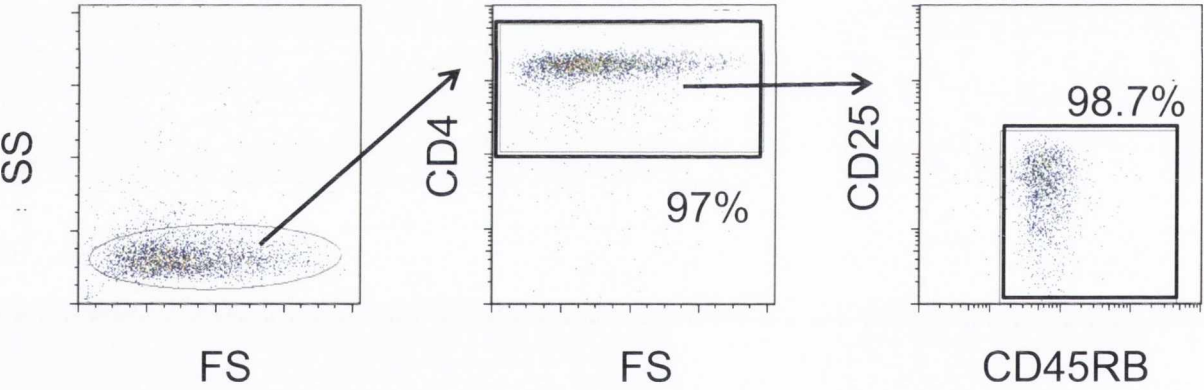


Figure 8.2 Purification of $CD4^+CD25^-CD45RB^{high}$ Teff cells from the spleens of naïve mice
Spleens from naïve mice were homogenised and filtered Isolated cells were stained with rat anti-mouse CD4, CD25 and FoxP3. $CD4^+CD25^-CD45RB^{high}$ Teff cells were purified using a MoFlow cell sorter. A) Representative dot plots showing the gating strategy and percentages of $CD4^+$ and $CD4^+CD25^-CD45RB^{high}$ cells prior to purification. B) Representative dot plots showing the percentages of $CD4^+$ and $CD4^+CD25^-CD45RB^{high}$ cells after purification.