The Role of TLR2 and GITR agonists in Allergic Airways Disease

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Summary

Asthma is a chronic inflammatory condition characterised by airway hyperresponsiveness, inflammatory infiltrates in the bronchial wall containing eosinophils, and elevated serum IgE levels to common inhaled allergens. Over the last twenty years, the prevalence of asthma and allergies has increased markedly which cannot be explained by changes in genetic predisposition. A greater understanding of the regulation of the inflammatory responses in asthma will assist in identification of potential targets for therapeutic intervention. In this thesis, I have used a murine model of allergic airways disease to investigate (i) the effects of Toll-Like Receptor 2 (TLR2) activation; and (ii) the role of Glucocorticoid-induced tumour necrosis factor receptor (GITR).

Toll-Like Receptors (TLRs) are primary sensors of both innate and adaptive immune systems where they play a pivotal role in the response directed against structurally conserved components of pathogens. Synthetic bacterial lipopeptide Pam3CSK4 (BLP) is a TLR2 agonist capable of modulating Th1 and Th2 responses. I examined the therapeutic effect of Pam3CSK4 on established airways inflammation in a murine model of asthma. In mice previously sensitised and challenged with OVA, Pam3CSK4 given intraperitoneally markedly reduced the total inflammatory cell infiltrate and eosinophilia in bronchoalveolar lavage fluid. Pam3CSK4 therapy was associated with a reduction in OVA-induced IL-4 and IL-5 secretion from thoracic lymph node culture, airway inflammation, bronchial hyperresponsiveness, and serum levels of IgE. Pam3CSK4 therapy was also associated with an increase in OVA-

induced IFNγ, IL-12 and IL-10 production. However, the anti-inflammatory effect of Pam3CSK4 was independent of IL-10 or TGFβ but critically dependent on IL-12, the production of which by dendritic cells was enhanced by Pam3CSK4 *in vitro*. The heightened levels of IL-12 in turn enhanced a specific Th1 response and decreased Th2 activity.

Glucocorticoid-induced tumour necrosis factor receptor (GITR) is expressed at low levels on resting T cells, B cells, and macrophages but at high levels on regulatory T cells. Although GITR expression is up-regulated on $CD4^+$ effector cells upon activation, the role of GITR in Th1 and Th2 cell development is unclear. Here I show that activation of GITR signalling by anti-GITR antibody markedly enhanced the induction of both Th1 (IFNγ) and Th2 (IL-5) cytokine production by naïve $CD4^+CD25^-$ T cells, but had little or no effect on established Th1 or Th2 cell lines or clones. Consistent with this observation, anti-GITR antibody significantly enhanced the expression of the key Th1 (T-bet) and Th2 (GATA3) transcription factors *in vitro*. Administration of anti-GITR mAb also markedly exacerbated airway inflammation of a murine OVA model of asthma with elevated production of OVA-specific IFNγ, IL-2, IL-4, IL-5, and IgE. Furthermore, adoptive transfer of $CD4^+GITR^+$ T cells effectively abolished airway inflammation induced in SCID mice reconstituted with $CD4^+GITR^-$ T cells.

The findings in this thesis provide direct evidence that TLR2 and GITR are important mediators in murine allergic airways disease, suggesting that TLR2 agonists and GITR could represent potential targets for novel therapeutic agents in clinical asthma.

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1. Patel M, Xu D, Kewin P, Choo-Kang B, McSharry C, Thomson NC & Liew FY. TLR2 Agonist Ameliorates Established Allergic Airway Inflammation by Promoting Th1 Response and Not via Regulatory T Cells. *J Immunol* 2005; **174(12)**: 7558-7563.
2. Liew FY, Patel M, Xu D. Toll-like Receptor 2 signalling and inflammation. *Ann Rheum Dis* 2005; **64 Suppl 4**: iv104-5

Presentations

1. McSharry C, Patel M, Kewin P, Kean D, Chaudhuri R, Bordon Y, Harnett MM, Thomson NC & Harnett W. A novel therapeutic approach to airway inflammation using filarial nematode derived ES-62. Oral presentation at European Society Annual Congress 2004.
2. Patel M, Xu D, Kewin P, Choo-Kang B, McSharry C, Thomson NC & Liew FY. TLR2 Agonist Ameliorates Established Allergic Airway Inflammation by Promoting Th1 Response and Not via Regulatory T Cells. Oral presentation at Young Investigator Awards at Winter British Thoracic Society Meeting 2005.

Submissions

1. Patel M, Xu D, Kewin P, Choo-Kang B, McSharry C, Thomson NC & Liew FY. Glucocorticoid-Induced TNFR family related protein (GITR) activation exacerbates murine asthma and collagen-induced arthritis. *Eur J Immunol* 2005. In Press.

Abbreviations

APC	antigen presenting cell
AHR	airway hyperresponsiveness
BM	bone marrow
BAL	bronchoalveolar lavage
CD	clusters of differentiation
cDNA	complementary deoxyribonucleic acid
Con A	Concanavalin A
cpm	counts per minute
CTLA-4	cytotoxic T lymphocyte-associated antigen 4
DC	dendritic cell
EAE	experimental allergic encephalomyelitis
ELISA	enzyme-linked immunosorbent assay
Fab	fragment of antigen binding
FACS	fluorescence activated cell sorter
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
FoxP3	forkhead/winged helix family protein
GITR	Glucocorticoid-induced TNF receptor superfamily member 18
GM-CSF	granulocyte macrophage-colony stimulating factor
GVHD	graft versus host disease
^{3}H	tritium
HPRT	hypoxanthine phosphoribosyltransferase
IBD	inflammatory bowel disease
IDDM	insulin-dependent diabetes mellitus
IFN	interferon
Ig	immunoglobulin
IL-	interleukin
i.n.	intranasal
i.p.	intraperitoneal
IPEX	Immune dysregulation, polyendocrinopathy, entropathy, X-linked syndrome

ko	knockout
LPS	lipopolysaccharide
mAb	monoclonal antibody
MACs	magnetic cell sorting
MALP-2	macrophage activating lipopeptide 2
MHC	major histocompatibility complex
MLN	mesenteric lymph node
MLR	mixed leukocyte reaction
mRNA	messenger ribonucleic acid
NK	natural killer
NOD mice	non-obese diabetic mice
OD	optical density
OVA	ovalbumin
PAMPs	pathogen-associated molecular patterns
PBS	phosphate buffered saline
PBMC	peripheral blood mononuclear cell
PCR	polymerase chain reaction
PE	phycoerythirin
Penh	enhanced pause
PG	prostaglandins
PMA	phorbol 12-myristate 13-acetate
PRR	pathogen recognition receptor
$RAG^{-/-}$	recombinase-activating gene deficient mice
RANTES	regulated on activation, normal T-cell expressed and secreted
RT-PCR	reverse transcriptase PCR
SCID	severe combined immunodeficient mice
SPF	specific pathogen free
STAT	signal tranducer and activator of transcription
TAMRA	6-carboxy-tetramethyl-rhodamine
TCR	T-cell receptor
Tg	transgenic
TGF-β	transforming growth factor-β
Th	T helper

TIR	Toll/IL-1 receptor
TLR	Toll-like receptor
TMB	3,3',5,5'-tetramethylbenzidine peroxidase
TNF	Tumour necrosis factor
Tr1	T regulatory T cell type 1
Treg	regulatory T cell
WT	wild type

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Finally, I would like to dedicate this thesis to my parents and to my uncle Rajni and aunt Raxna. They have always supported me, and given me all the opportunities I could have ever asked for.

Declaration

This study represents original work carried out by the author. This thesis has not been submitted in any form to any other University. Where use has been made of materials provided by others, due acknowledgement has been made.

Manish Patel

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Chapter 1

General Introduction

1.1 Asthma

1.1.1 Introduction

Asthma is a chronic inflammatory condition of the airways characterised by reversible airflow limitation, airway hyperresponsiveness (AHR) and inflammatory infiltrates in the airway walls containing eosinophils, mast cells, and T lymphocytes. Asthma is divided into atopic and non-atopic types, whereby atopic individuals have elevated serum IgE levels directed against particular allergens.

T helper (Th) lymphocyte subsets, defined by the cytokines they secrete are thought to play a key role in the initiation and perpetuation of allergic airway inflammation. Th2 cells, producing interleukin (IL)-4, IL-5 and IL-13, are thought to be of particular importance. (1, 2) The network of cell types and inflammatory mediators involved in asthma is complex and the current understanding is reviewed in references (3-5). This fascinating aspect of applied immunology is rapidly developing, and is a very active area of research.

The aim of the work presented in this thesis is to gain further knowledge into the immunology of asthma, and lead to new therapeutic approaches in the treatment of asthma.

1.1.2 Cellular basis of inflammation in asthma

1.1.2.1 Innate and adaptive immunity

The immune response of mammals can be divided into two parts: innate and adaptive immunity. The innate immune response provides a defence against infection, which is rapid (within a few minutes), but non-specific and has no memory. The innate system utilises many mechanisms to protect us against pathogens. This defence extends from physical barriers (for example skin) to the recruitment of inflammatory cells (such as macrophages, mast cells, eosinophils, neutrophils, and dendritic cells). Thereafter, the immune system produces a more specific response mediated by T and B lymphocytes. These lymphocytes eradicate pathogens more efficiently, and provide immunological memory, which confers increased protection against subsequent re-infections. The innate and adaptive immune systems are not mutually exclusive, with many cells of the innate system also participating in the effector mechanism of the adaptive response. A number of cells of both the innate and adaptive immune system have been implicated in the pathogenesis of asthma. A summary of the interplay of these cells' interaction in the allergic inflammatory response associated in asthma is described below, and is summarised in Figure 1.1.

1.1.2.2 T helper (Th) lymphocytes

An integral cell in adaptive immunity is the $CD4^+$ T cell. Mosmann and Coffmann first described the presence of two functionally distinct $CD4^+$ helper T cell subsets,

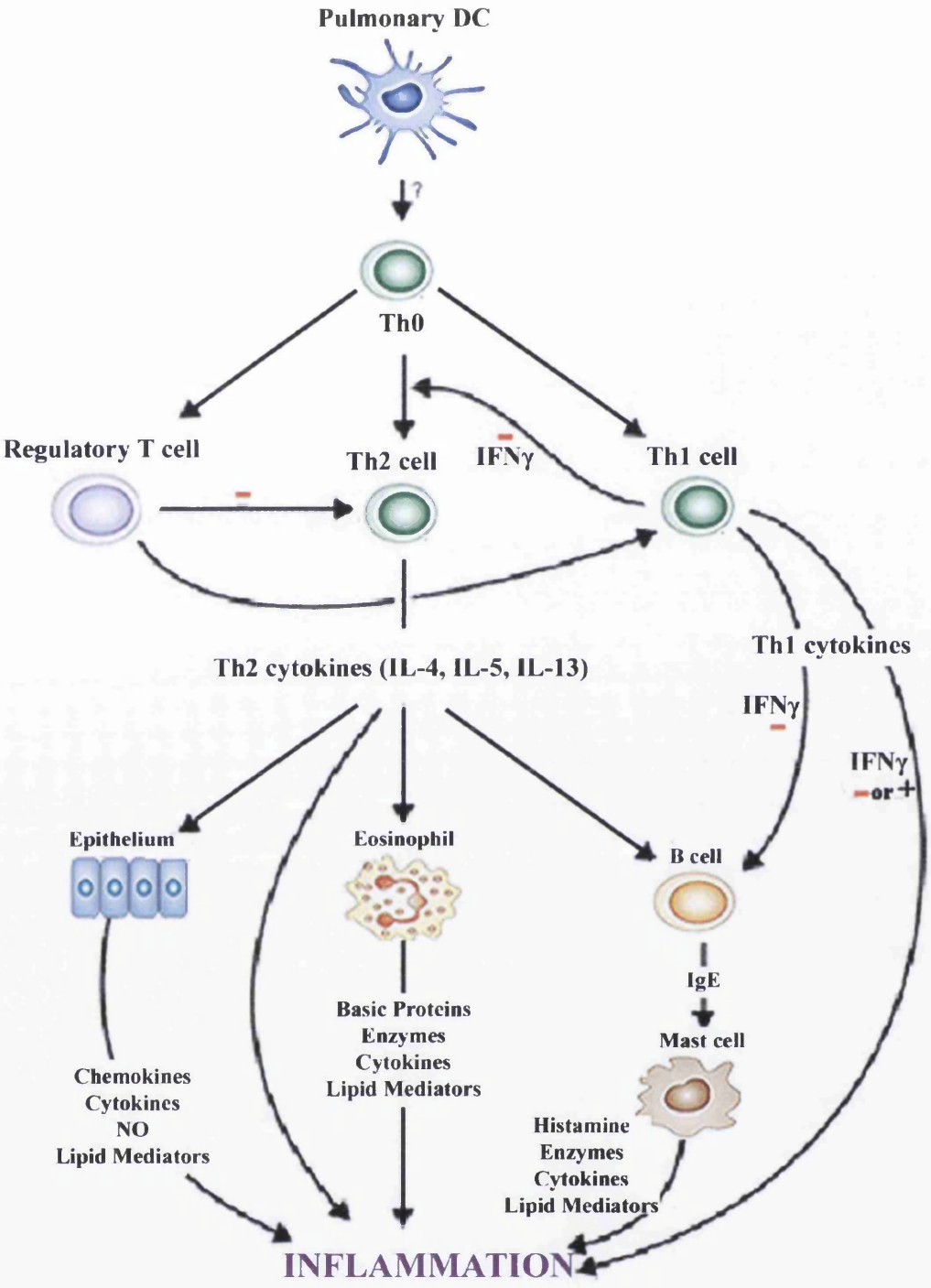

Figure 1.1 Cells thought to be involved in the pathogenesis of asthma

Th2 cytokines have a central role in the pathogenesis of asthma.

Adapted from Lewis et al, 2002; (6).

namely Th1 and Th2, in 1986 (7). Th1 lymphocytes are characterised by their production of interferon (IFN)γ, whereas Th2 lymphocytes secrete interleukin (IL) -4. Th0 lymphocytes produce cytokines characteristic of both Th1 and Th2 clones. Subsequent studies demonstrated that Th1 cells are an integral part of the cell mediated immune response to intracellular organisms such as mycobacteria, leishmania parasites and viruses (8), and involved in the actions of macrophages and cytotoxic T lymphocytes (CTLs). In contrast to Th2 cells, the differentiation of Th1 cells is mainly dependent on IL-12, which signals through STAT-4-dependent pathways (9). Another transcription factor involved in Th1 lineage and IFNγ gene expression is T-bet which induces the transcription of the gene encoding IFNγ (10). Th1 cells are further characterised by the expression of the chemokine receptors CCR1, CCR5 and CXCR3 (11, 12), and the receptors for IL-18 (13) and IL-12 (14).

Th2 cells are characterised by the production of IL-4, IL-5 and IL-13, (8), and expression of the chemokine receptors CCR3, and CCR4 (11, 15, 16), and orphan receptor T1/ST2 (13, 17). Th2 cell differentiation is mainly reliant on IL-4 and STAT-6-dependent signalling pathways which induce the Th2-specific transcription factor, GATA-3 (18, 19). The transcription factor, c-Maf, which trans-activates the IL-4 promoter, has an important role in the transcription of IL-4 (20). Th2 cytokines promote the activation of innate cells, such as mast cells and eosinophils, and can direct B cells to produce murine IgG1 and IgE. These effector mechanisms are important in the elimination of extracellular parasites such as helminths (21). However, Th2-medited responses have also been implicated in pathogenesis of allergic responses, such as asthma.

Asthma is characterised histologically by a predominance of Th2 lymphocytes (1). Bronchial biopsies taken from atopic and non-atopic asthmatics show elevated expression of IL-4, IL-5, and IL-13 (22), and increased expression of GATA-3, c-Maf, and STAT-6 (23). Murine models of asthma also support the importance of Th2 lymphocytes in allergic inflammation. IL-4-deficient mice display reduced airways disease (24), whereas transgenic mice over-expressing IL-5 (25) or IL-13 (26) have more marked disease. In studies designed to demonstrate the importance of IL-4 in asthma, murine adoptive transfer of IL-4-deficient $CD4^+$ cells have shown that IL-4 is not essential for developing allergic airways inflammation. Despite this, it was demonstrated that STAT6 and IL-4 receptor alpha were both necessary for the development of murine airways inflammation (27, 28). It has also been shown that the cytokine IL-13 receptor heterodimer utilises the IL-4 receptor alpha chain, and STAT6 pathway. Further work has shown that although IL-4 is essential for the initiation of Th2 polarisation, subsequent development of allergic airways disease may rely on IL-13. IL-13 can mediate the induction of AHR, pulmonary fibrosis, and goblet cell hyperplasia (29, 30). Another important Th2 cytokine, IL-5, is important in the differentiation, activation and survival of eosinophils (31). IL-5-deficient mice do not develop allergic airways inflammation (32). It is felt that the interplay of Th2 cytokines, such as IL-4, IL-5 and IL-13, are important for the development of allergic airways inflammation.

The balance between Th1 and Th2 cells is thought to be important in the development and propagation of asthma, especially when the two populations are counter-regulatory (7). Cytokines from Th1 lymphocytes were originally thought to be protective of asthma by skewing the immune response from a Th2 towards a Th1

profile (33). Furthermore, the observations that antigen-induced IFNγ production was reduced in peripheral blood lymphocytes of asthmatics (34), and that IFNγ and Tbet-1 was critical for attenuating airways eosinophilia in murine models of asthma (35, 36) gave further support to this hypothesis. However, it has since been demonstrated that IFNγ is produced by bronchoalveolar lavage (BAL) T cells (37) and may enhance airways inflammation (38). In an adoptive murine transfer model of asthma, it was shown that Th1 cells did not reduce airways inflammation, but actually enhanced established disease through the activation and recruitment of neutrophils into the airways (39-41). These observations can be summarised thus; Th2 cells appear to be important in the initiation and maintenance of allergic airways inflammation, and can be regulated by Th1 cytokines. However, once disease has been established Th1 responses may contribute to the perpetuation of disease under certain circumstances.

Some of the T cell-related cytokines and their proposed actions in asthma are summarised in Table 1.1.

1.1.2.3 Dendritic Cells

Dendritic cells are the most effective "professional" antigen presenting cells (APCs), and have been shown to be necessary for disease development in murine models of asthma (42). The interactions between TcRs expressed by naive $CD4^+$ T cells and MHC class II/peptide complexes on APCs can influence T cell differentiation [reviewed in (43)]. This was demonstrated by early work by Bottomly and colleagues, who showed that high density of peptide/MHC complexes promotes strong Th1-mediated immune responses, whereas low density of peptide/MHC complexes induce

Cytokine	*Source*	*Actions*
IL-3	Th1/Th2 cells Eosinophils Basophil Mast cells Macrophages	Differentiation and activation of eosinophils, mast cells, basophils and neutrophils
IL-4	Th2 cells Eosinophils Mast cells Basophils	B-cell switch to IgE synthesis Th2 development Mast cell development Eosinophil and basophil activation Mucous secretion
IL-5	Th2 cells Eosinophils Mast cells Basophils	Eosinophil & Basophil differentiation, maturation, and activation
IL-6	Th1/Th2 cells Macrophages Endothelial cells	T and B-cell growth factor Cofactor for IgE synthesis
IL-9	Th2 cells Eosinophils Mast cells Basophils	Mast cell and Eosinophil development AHR Mucous secretion
IL-10	T cells Macrophages	Suppresses Th1/Th2 function Favours regulatory T cell production B-cell switch to IgG4
IL-13	Th2 cells Eosinophils Mast cells Basophils	Mast cell development B-cell switch to IgE Eosinophilia AHR Mucus secretion
IL-25	Th2 cells	Favors Th2 development and IL-4,IL-5,and IL-13 production
GM-CSF	Th1/Th2 cells Macrophages Eosinophils Mast cells Basophils Fibrobasts & Epithelial cells	Differentiation and activation of eosinophils, neutrophils, and mast cells
IFNγ	Th1 cells	Inhibits IgE synthesis, Inhibits Th2 induction Activates eosinophils and macrophages
IL-12	Macrophages B cells	Favours Th1 expansion Inhibits IgE synthesis
IL-15	Many non-T cells	T cell growth factor
IL-18	Macrophages	Induces IFN-γ production by T cells, NK cells Favours Th1 expansion
IL-23	Various hemopoietic cells	Cofactor for Th1 development, Activates DCs
IL-27	APC	Favours Th1 expansion

Table 1.1 Cytokines involved in asthma

Some of the cytokines important in the pathogenesis of asthma.

Adapted from Larché et al, 2003; (5).

strong Th2-mediated responses (44). In addition, it has been demonstrated that high affinity binding of peptide by TcRs and strong TcR-mediated signalling promotes Th1 differentiation, whereas lower affinity binding and weak TCR signals promote Th2 differentiation (45). Th cell induction by APC is dependent on the nature of antigen, the affinity of interaction of TCR and MHC, the co-stimulatory signals, the cytokine environment and the genetic background of the cells (46).

Potential APCs in the lung are alveolar macrophages, B-cells, epithelial cells, and dendritic cells. The lung macrophage is a poor accessory cell (47), and there is little data showing B cells or epithelial cells process and present antigen in the allergic airway. Hence, the most important APC in the lung is probably the dendritic cell.

Dendritic cells taken from the respiratory mucosal wall preferentially induces Th2 cytokine production (48). Peripheral blood-derived dendritic cells taken from atopic donors secrete IL-13 after stimulation with protein allergens, and enhance Th2 cell development (49). Dendritic cell co-stimulatory signals, such as inducible costimulator (ICOS), may bind T cell surface molecules, such as inducible costimulator ligand ICOS-L, and promote Th2 responses (50, 51). However, ICOS along with IL-10 (produced from DCs) have been implicated as an important factors in tolerance induction to inhaled allergen in murine models of asthma (52, 53).

1.1.2.4 Eosinophils

Antigen presentation and $CD4^+$ T cell responses results in the recruitment and activation of the primary effector cells of the allergic response: the eosinophil, mast cell and basophil.

One of the most striking characteristic in atopic asthma is eosinophilic hyperplasia. Eosinophilic inflammation has always been regarded as a hallmark of both atopic and non-atopic asthma (54, 55). Recently, two different genetically altered ''eosinophil knockout mice'' have been generated. One model was specifically designed to deliver a suicide signal only to eosinophils (56), whereas the other model took advantage of an unexpected observation that a deletion in the GATA-3 promoter site resulted in an eosinophil-free mouse (57). In the eosinophil suicide model, data were presented that eosinophils were an absolute requirement for the asthma-like phenotype in the mouse. Surprisingly, this was different from the other model, in which eosinophils seemed to play little, if any role in the initiation of allergic inflammation. The two models appeared to agree that eosinophil might play a role in airway remodelling, which is in accordance with work showing that anti-IL-5 antibody administration in human subjects reduces eosinophils and the deposition of several tissue matrix proteins (58). Despite this apparently contradictory or incomplete murine data, there is still a great deal of evidence suggesting eosinophils are important in asthma. Eosinophils are integral to the development of bronchoconstriction in the late asthmatic response (59); are increased in number in human atopic (60, 61) and non-atopic (62) lungs; and there is a correlation between BAL eosinophil numbers and severity of asthma (63).

Eosinophils express CD23, low affinity surface receptors for IgE, and this might enhance migration to sites of pulmonary inflammation. Eosinophil degranulation can be induced by binding IgE to surface receptors on the eosinophil. Other factors such as IL-5, GM-CSF and complement component C3b on immune-complexes, along with ligation of IgG receptors can also degranulate eosinophils. This leads to the release of pro-inflammatory substances which include eosinophilic cationic protein (ECP), myelin basic protein (MBP), eosinophil derived neurotoxin (EDN), eosinophil peroxidase, reactive oxygen species, cytokines like IL-3, IL-5, IL-13 and GM-CSF, prostaglandins and leukotrienes. These inflammatory mediators promote the migration and activation of several other cell types into the lung and cause microvascular leakage, increased mucous production, induce bronchoconstriction and epithelial damage [reviewed in (64)]. In summary, eosinophils are important in the late asthmatic response (Figure 1.2) and tissue remodelling.

1.1.2.5 Mast cells and basophils

Eosinophils are important in the late allergic (asthmatic) response, whereas mast cells appear to be pivotal for the immediate response to an inhaled allergen (Fig. 1.2). Both mast cells and basophils are seen in bronchial biopses of asthmatic subjects (65), and express the high affinity IgE (FcεRI) on their cell surface. Allergen-induced cross linking of these receptors by IgE causes the release of pre-formed mediators like histamine, proteoglycans, serine proteases, carboxypeptidase A. These mediators and newly synthetised mediators like prostaglandin D2, leukotriene C4, and platelet

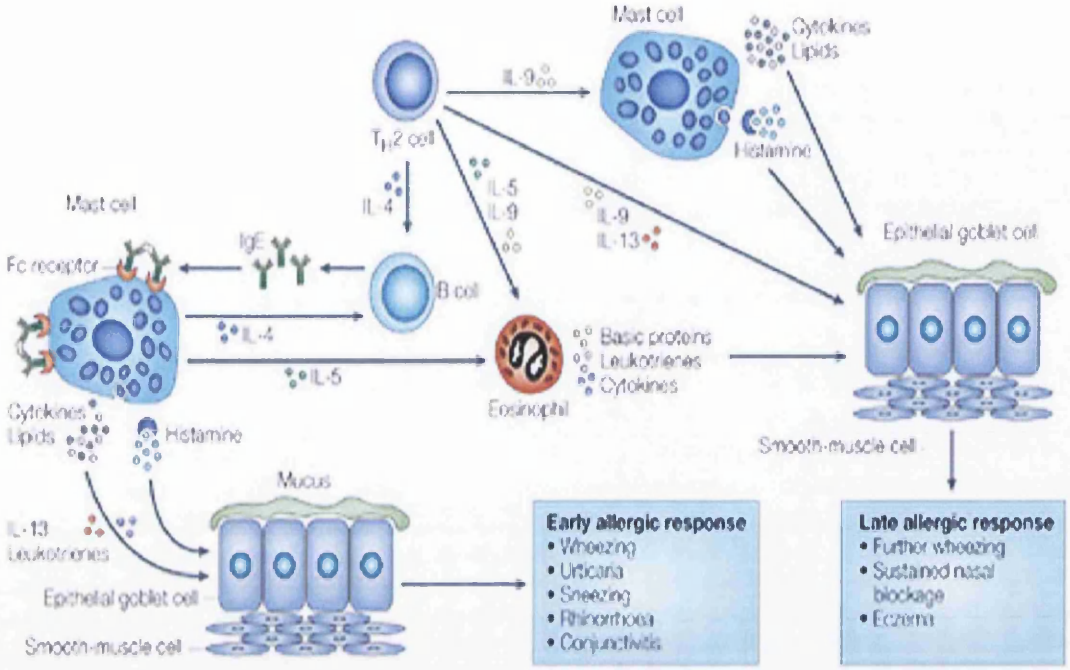

Figure 1.2 Early and Late Allergic Response

Release of pre-formed inflammatory mediators (mainly from mast cells and basophils) leads to the early (acute) allergic response, which occurs within minutes of inhaling allergen. Chemokines released by mast cells and other cells recruit and activate other other inflammatory cells contributing to the late allergic response.

Adapted from Hawrylowicz et al,(66).

activating factor (PAF) cause bronchoconstriction, vasodilatation, and mucosal oedema in the airways. Furthermore, these mediators, and others, will promote the migration and activation of other cells into the lung, which will then further exacerbate the inflammatory response. Mast cells also contribute to the late allergic response, and mediators like histamine, cytokines and proteases are felt to influence airways remodelling. In addition, mast cells produce IL-4 which could promote Th2 cell differentiation and enhance Th2-mediated functions [reviewed in (67)].

1.1.2.6 B cells

IgE is important in the degranulation of mast cells. It has been shown that there is a highly significant relationship between total serum IgE and the prevalence of all asthma in humans (68). IL-4 and IL-13, released by Th2 cells, can induce B cells to class switch their immunoglobulin isotypes IgG1(mice) or IgG4 (humans), and IgE immunoglobulin production (69, 70). It is thought that B cells activated by Th2 cells produce and release IgE which then contributes to the effector mechanisms in asthma described above.

B cells can act as APCs *in vivo* (71), and hence there is a potential that they may have an additional role in allergic inflammation. However, to date there has been no studies specifically examining this in asthma.

1.1.2.7 Other cells involved in asthma pathogenesis

Many other cells types are present in the airways and may have an important role in the pathogenesis of asthma. Airway epithelial cells are thought to be an important source of inflammatory mediators, for example regulated on activation, normal T-cell expressed and secreted (RANTES) and eotaxin. Neutrophils may also have an important role in airways inflammation, with severe and steroid-resistant asthmatics, or asthmatic subjects who smoke having marked airway neutrophilia. Macrophages can also secrete pro-inflammatory cytokines, for example IL-6 and TNFα, and immunoregulatory cytokines, for example IL-12, IL-15 and IL-18, which could potentially also participate in the pathogenesis of asthma. Regulatory T lymphocytes and NKT cells may have an important role in airways inflammation, and I intend to discuss this further in section 1.3.

With so many effector systems interacting in asthma pathogenesis, it would seem sensible that when trying to investigate such a disease one should aim for a cell type or immune system that is integral to all the effector systems. For this reason, I examined and will present data for two surface molecules: Toll-Like Receptor 2 (TLR2) and Glucocorticoid-induced tumour necrosis factor receptor (GITR). TLR2 is found on cells of both the innate and adaptive immune system and been shown to be important in inflammation. GITR is important in the function of regulatory T cells (Tregs), which are integral in controlling immune responses. The next two sections will describe these in turn.

1.2 Toll-Like Receptors

1.2.1 Introduction

Toll was first discovered when *Drosophila* fruitfly embryos deficient in Toll displayed aberrant ventral-dorsal development, and diminished protection against fungal infection (72-74). Subsequent work revealed the presence of a family of mammalian homologues to Toll, termed Toll-like receptors (TLRs) (75, 76), which recognize pathogens, or pathogen-derived products. TLR activation triggers both the early innate and adaptive immune responses. There are now at least 11 TLRs known in mice and 10 in humans (77-79), which recognise distinct structurally conserved components of pathogen-associated molecular patterns (PAMPs).

1.2.2 TLR ligands

TLRs act as primary immune sensors to recognize PAMPs displayed by various microbial components (Table 1.2) including gram-positive and -negative bacteria (78-82); DNA and RNA viruses (83-85); fungi (86) and parasites and protozoa (87-90). TLRs are type 1 transmembrane receptors that possess an extracellular leucine-rich repeat domain and cytoplasmic domain homologous with that of the interleukin 1 receptor (IL-1R) family (75, 91). Upon stimulation, TLRs and IL-1R interact with the adapter molecule, myeloid differentiation factor-88 (MyD88), recruiting the IL-1R-associated kinase (IRAK), ultimately leading to the activation of the transcription

factor nuclear factor-κB and inflammatory gene transcription. All TLRs characterised so far, except TLR3, signal through the adaptor protein MyD88. However some TLRs also appear to signal through a MyD88-independent pathway under specific conditions. An adapter protein named Toll/IL-1R domain-containing adapter inducing IFN-beta (TRIF) is involved in MyD88-independent TLR signalling pathways (92, 93), which specifically activates IFN regulatory factor 3 (IRF-3) and induces the IFNβ production.

TLR2 recognizes a wide variety of PAMPs from bacteria, yeast, fungi, and parasites, and is thought to act as a receptor by forming heterodimers with TLR1 or TLR6 (92, 94). TLR2/6 heterodimer binds gram positive cell wall components, such as peptidoglycan, and mycoplasmal lipopeptides that are diacylated, whereas TLR2/1heterodimers recognises bacterial lipopeptides that are triacylated (92, 94-96).

TLR4 functions as a homodimer or homo-oligomers and detects lipopolysaccharide (LPS), a major cell wall component of gram negative bacteria (75, 82). Recognition of LPS also requires the association of LPS-binding protein (LBP) (97), CD14 (98) and MD2 (99). MyD88-dependent and MyD88-independent pathways both mediate TLR4 signalling (100, 101).

Flagellin, a monomeric constituent of bacterial flagella, binds TLR5 (80), and is important in microbial recognition at mucosal surfaces (102, 103). Virus recognition appears to be mediated by TLR3, 7, 8 or 9. TLR3 recognizes double-stranded RNA by a MyD88-independent pathway to induce IFNβ production (104). In contrast, TLR7 and 8 recognize single-stranded RNA from viruses triggering an immune response

and IFN-γ production (105). TLR9 recognizes unmethylated CpG motifs, which are found abundantly in prokaryotic genomes and DNA viruses (85, 106). There are two types of CpG DNA – A/D type and B/K type- with A/D type CpG being the conventional motif, which induces IL-12 and TNFα. B/K type CpG DNA has a greater ability to induce IFNα, but not IL-12, from dendritic cells (106-108). In contrast to other TLRs, TLR7, TLR8, and TLR9 recognizes its ligand intracellularly, perhaps in acidic compartments like endosomes or lysosomes following bacterial or viral lysis (109). It is still unresolved as to whether TLR3 functions on the cell surface or intracellularly. Bladder epithelial cells expressing TLR11 respond specifically to uropathogenic bacteria, indicating a potentially important role for TLR11 in preventing infection of internal organs of the urogenital system (79). TLR11 has only been identified in mice so far.

1.2.3 TLRs and the immune system

TLRs are a crucial link between the innate and adaptive immunity. Initial recognition of the highly conserved PAMPs on pathogens by the innate immune cells will cause release of chemokines, recruit cells to sites of inflammation, trigger phagocytosis with degradation and presentation of pathogen-derived peptides. TLRs are highly expressed on antigen presenting cells (APCs), which upon activation release inflammatory cytokines and express co-stimulatory molecules. Thus, not only do TLRs activate the innate system, they also initiate the development of an antigen-specific adaptive immune response (Fig. 1.3).

TLR	*Ligands*
TLR1	Tri-acyl lipopeptides (bacteria, mycobacteria) Soluble factors (*Neisseria meningitidis*) Modulin
TLR2	Lipoprotein/lipopeptides Peptidoglycan Lipoteichoic acid Lipoarabinomannan (mycobacteria) Glycoinositolphospholipids (*Trypanosoma cruzi*) Glycolipids (*Treponema maltophilum*) Porins (Neisseria) Zymosan (fungi) *Listeria* (Heat-killed bacteria) LPS (Spirochetae) Modulin
TLR3	Double-stranded RNA (virus) Poly(I:C) (synthetic analogue of double stranded RNA)
TLR4	LPS (Gram-negative bacteria) HSP60 (*Chlamydia pneumoniae*) HSP60 (host) HSP70 (host) Fusion protein (RSV) Taxol (Plant) Envelope proteins (MMTV) ES-62 (filarial nematode)
TLR5	Flagellin (bacteria)
TLR6	Di-acyl lipopeptides (mycoplasma) Modulin, soluble tuberculosis factor STF
TLR7	GU rich Single-strand RNA (ssRNA) Imidazoquinoline (synthetic compounds) Loxoribine (synthetic compounds) Bropirimine (synthetic compounds)
TLR8	GU rich Single-strand RNA (ssRNA)
TLR9	CpG DNA (bacteria) CPG ODN (synthetic oligonucleotides that contain unmethylated CPG dinuleotides)
TLR10	? (unknown)
TLR11 (mice only)	Uropathogenic strains of *E.coli* (UPEC)

Table 1.2 Toll-like receptors and their ligands in humans and mice

Adapted from (75, 76, 79, 91, 104-106, 110-113)

1.2.3.1 TLRs and cells of the innate immune response

Pathogens recognition by TLRs induce the expression of selectins, chemokines and chemokine receptors that regulate cell migration to sites of inflammation (114). Innate cells like neutrophils, NK cells, mast cells, eosinophils, dendritic cells, macrophages, B cells, endothelial and epithelial cells express a variety of TLRs which, upon binding of ligands, will generate pro-inflammatory signals and cytokines that will result in microbial killing (78, 96, 115-119). In the absence of TLRs, particularly TLR2/TLR4 or MyD88, macrophages have shown impaired bacterial killing compared with wild type cells (119). Phagocytosis of bacteria including *Escherichia coli, Salmonella typhimurium* and *Staphylococcus aureus* has also been shown to be impaired due to defective phagosome maturation in these TLR-deficient mice (120). Hence, TLRs are important in the initial recognition, cell migration, phagocytosis, and clearance of pathogens by the innate system.

DCs express a large number of TLRs, however the precise pattern of TLR on different subsets of DCs remains unresolved. Human myeloid DCs express all 10 TLRs except TLR4 and TLR9 and can recognize bacterial, fungal and viral pathogens (121-124). Freshly isolated plasmacytoid DCs (pDCs) express TLR7 and TLR9, whereas $CD11c^+$ human myeloid DCs (mDCs) express TLR1, TLR2, TLR3, TLR5, TLR6, and TLR8 (121-124). Some groups found TLR7 was expressed on both pDCs and mDCs (121, 124), whilst others found TLR7 on pDCs alone (122, 123). Mouse DC TLRs are more heterogeneous than human, with most TLRs expressed on most DC subsets, with the exception of $CD8^+$ DC which lack TLR5 and TLR7 (125, 126).

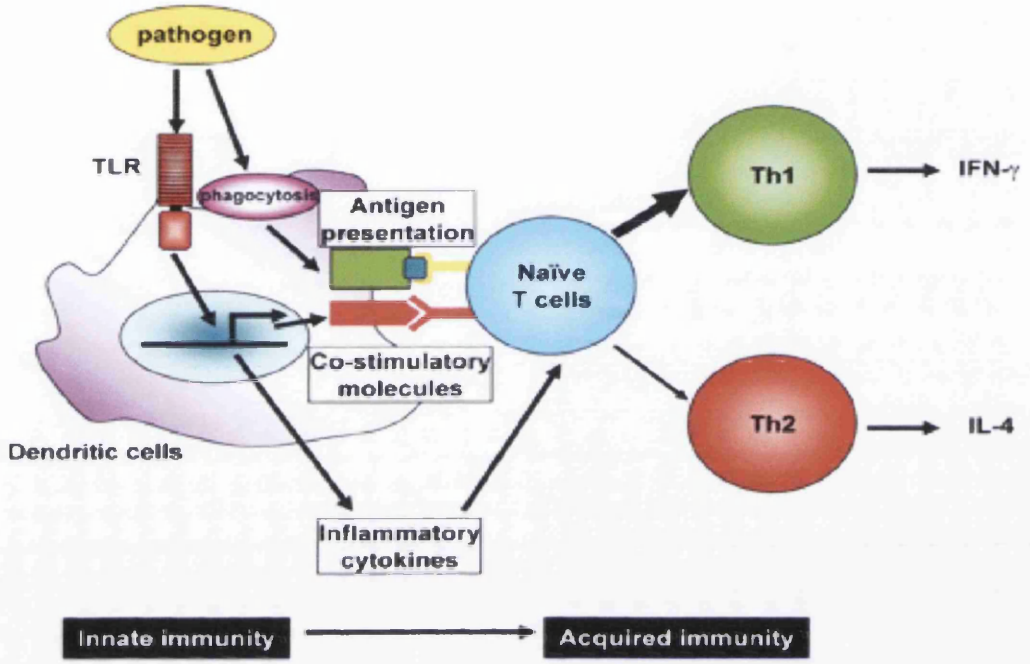

Figure 1.3 TLRs and innate and adaptive immunity

Innate immune cells recognise pathogens via TLRs. TLR activation will induce phagocytosis, pro-inflammatory cytokines production, co-stimulatory molecules expression, and pathogen peptide presentation to T cells. This will in turn lead to antigen-specific immunity (preferentially Th1 responses).

Adapted from Takeda et al (127).

TLRs initiate adaptive immunity mainly through DCs. These cells capture microbial antigens in the peripheral tissues, mature, process the antigen into peptides, and then migrate to draining lymph nodes to present processed peptide to T cells. The activation of TLRs results in downregulation of inflammatory chemokine receptors (like CCR6) and upregulation of receptors for lymphoid chemokines (like CCR7), which encourages DC migration to lymphoid tissue (128, 129). TLR activation also increases the expression of CCR2, CCR5, and CCR7 on DCs; enhances the production of IL-12, tumour necrosis factor α (TNF-α) and IL-6 by DCs; and up-regulates co-stimulatory molecules such as CD40, CD80 (B7-1), CD86 (B7-2) and MHC class II (128-132). These changes allow DCs to mature and present antigenic peptides to T cells more effectively.

Analysis of MyD88-deficient mice has demonstrated the critical role of TLRs in DC maturation and induction of adaptive immune responses. MyD88-deficient DCs stimulated with CpG DNA, but not LPS, failed to mature (101) as demonstrated by the lack of surface co-stimulatory and MHC molecules. Furthermore, these DCs failed to prime antigen-specific naïve T cells *in vitro*. In TLR2 and TLR4-deficient mice, the expression of co-stimulatory molecules and MHC class II molecules, as well as pro-inflammatory cytokines were impaired in DCs upon simulation with PGN, LPS, or Lipid A (133). TLR9-deficient mice also failed to respond to CpG DNA, which resulted in decreased DC maturation, antigen presentation, with subsequent decreased splenocyte proliferation. Hence, TLRs are important in DC maturation and microbial antigen presentation to T cells.

1.2.3.2 TLRs and cells of the adaptive immune response

As well as initiating specific immune responses, TLRs are also critically involved in determining the Th1/Th2 balance (77). The density of antigenic peptide presented, the degree of co-stimulation, and the amount and nature of cytokines and inflammatory mediators produced by DCs at the site of inflammation have been shown to influence T cell differentiation (134).

It has been shown that TLRs recognize PAMPs on pathogens and generate immune responses that are appropriate for their elimination (135). Prokaryotic, fungal, viral, or protozoan pathogens activate DCs through the engagement of TLRs to stimulate IL-12 production and thereby direct the development of Th1 responses leading to their clearance (136-138). LPS from *Escherichia coli*, a TLR4 agonist, promotes the production of IL-12p70 and was associated with Th1 responses (139). CpG-containing bacterial DNA also initiated a strong Th1-biased response by inducing the co-stimulatory molecule CD40 and IL-12, which was dependent on TLR9 (140, 141). Double-stranded RNA (polyI:C, a mimic of viral RNA) is associated with Th1 cell responses (142). It has been shown that MyD88-deficient animals developed a Th2-enhanced response (143). This suggests that the TLR-mediated MyD88 signalling pathway could contribute to Th1 or Th2 cell induction. Thus, in addition to initiating Th1 responses, TLRs are also important in Th2 differentiation by MyD88-dependent and -independent mechanisms. TLR activation of DCs can induce Th2 responses (139, 140, 144-147). It has also been shown that immature dendritic cells can induce both Th1 and Th2 profiles depending on which TLR are activated (148). TLR4 activation of immature DCs promoted the production of the Th1-inducing cytokine

IL-12p70 and the chemokine interferon-gamma inducible protein (IP)-10, which is associated to Th1 responses. In contrast, TLR2 stimulation of these immature DCs failed to induce IL-12p70 or IP-10, but resulted in the release of the IL-12 inhibitory p40 homodimer which acts as an IL-12R antagonist, and favours Th2 development (148). Moreover, it has been shown that TLR2 triggering can promote Th2 differentiation *in vitro* and *in vivo* (139, 146, 149). Flagellin, a TLR5 agonist, can also initiate a MyD88-dependent Th2 response through DC maturation and increased IL-4, IL-13, and IgG1 production (150). Using TLR4-deficient mice, it has been shown that TLR4 is required for the induction of murine allergic airways disease (151). Interestingly, using the TLR4 ligand LPS, it has been shown that the dose of LPS can determine whether a Th1 or Th2 response develops *in vivo*. In a murine model of asthma, LPS stimulation of DCs, at a low dose, enhances Th2 responses. However, when LPS was administered locally into the lungs at high dose a Th1 response predominated (152). Thus, some Th1-inducing pathogens may also induce Th2 development depending on the degree of TLR activation of the DCs.

As well as being found on innate cells, TLRs are also expressed on cells of the adaptive immune system. Recent results have shown a direct role of TLRs on T cell activation and function. Human T cells express cell-surface TLR2 after activation by anti-T cell receptor antibody and IFN-α (153). These activated T cells produced elevated levels of cytokines in direct response to the TLR2 ligand, Pam3CSK4, which is a Gram-positive bacterial lipopeptide. Another study showed that murine $CD4^+$ T cells express TLR3 and TLR9, but not TLR2 or TLR4, and agonists of TLR3 and TLR9 increased T cell survival (154).

Stimulation of TLRs on B lymphocytes directly can lead to T cell-independent activation and production of low-affinity immunoglobulin M (IgM) antibodies (155). Human memory B cells can produce antibodies in response to CpG stimulation independently of antigen-specific T cell help (156). In addition, TLR activation of T cell will result in the generation of signals that will influence B cell maturation and effector function.

In summary, it would appear TLRs can direct the adaptive Th1 and Th2 immune responses by either directly interaction with T cells, or by acting indirectly via APCs. In addition, they can also directly or indirectly influence the humoral immune response.

1.2.3.3 TLRs and inflammatory disease

TLRs and Infectious Disease

TLRs play a critical role in protective immunity *in vivo*. Mice deficient in the TLR-associated adapter molecules MyD88 resulted in increased susceptibility to a wide variety of microorganisms including bacteria, such as *Staphylococcus aureus* (81), *Listeria monocytogenes* (157) and *Mycobacterium avium* (158); parasites, such as *Leishmania major* (159); fungal pathogen, such as *Candida albicans* (160, 161); and the intestinal nematode *Trichuris muris* (162). In addition to its protective role, TLRs also appear to contribute to inflammatory and immune disorders, such as sepsis, atherosclerosis, and asthma. Activation of macrophages and neutrophils by the TLR4 agonist LPS leads to enhanced production of pro-inflammatory cytokines, which are responsible for features of sepsis or septic shock (163). Studies examining a human

TLR4 gene polymorphism, D299G, have shown that the polymorphism increases the risk of gram negative infections (164, 165), and sepsis (166).

TLRs and autoimmune disease

Clearly the TLR responses have evolved for protection, however, the development of some inflammatory diseases is influenced by TLRs include autoimmune diseases such as RA, SLE and EAE. In rheumatoid arthritis (RA), TLR2 and TLR4 are expressed at a higher level in the synovium of RA patients compared to healthy individuals. This increased TLR expression correlates with increased synovial levels of IL-12 and IL-18, which are thought to be important in the RA pathogenesis (167). TLR4 also contributes to the induction of experimental autoimmune encephalomyelitis (EAE), implicating innate immune mechanisms in the development of CNS autoimmune disease (168). Systemic lupus erythematosus (SLE) is a prototype non-organ specific auto-immune disease characterised by inefficient removal of apoptotic cells, and the normally sequestered nucleoproteins exposed on apoptotic cell membranes can trigger auto-reactive B cells to produce IgG auto-antibody. This process appears to involve antigen presentation by dendritic cells using a TLR9-dependent pathway (169). Mammalian DNA has some unmethylated CpG motifs, which are recognised by TLR9. Normally, self DNA is sequestered. However, in SLE the chromatin can be delivered to intracellular compartments that contain TLR9, which results in activation of these B cells which produce antibody against self DNA (170).

TLRs and atherosclerosis

In TLR4 or MyD88-deficient mice, there was a marked reduction in early atherosclerosis due to decreased macrophage recruitment to the arterial intima. In addition, individuals with the D299G polymorphism had reduced carotid artery atherosclerosis (171), and acute coronary events (172). This suggests that TLR4- and MyD88-mediated pathways may be involved in the development of atherosclerosis (173, 174).

TLR and other inflammatory disorders

The MyD88-dependent pathway appears critical in allograft rejection, because minor antigen-mismatched allograft rejection cannot occur in the absence of MyD88 signalling (175). TLRs have been implicated in the initiation and perpetuation of allergic diseases, such as asthma. Exposure to the TLR4 agonist, LPS, has been correlated to the severity of asthma. Indeed, one study actually showed that in people sensitive to house dust mite, asthma severity correlated more closely to LPS levels than with the allergen itself (176). People with allergic asthma are more sensitive to the bronchoconstrictive effects of inhaled LPS (177). In contrast to these findings, exposure to TLR ligands in early childhood may decrease the incidence of asthma in later life (178, 179). It is hypothesised that this protection from asthma may be due to early TLR activation of regulatory T cells which may downregulate the immune response later in life. TLR ligands appear to be able to exacerbate or diminish asthma depending on the timing and nature of exposure. TLRs and asthma will be discussed further Chapters 3 and 4.

Taken together, this evidence implies that TLRs are important in resolving infectious diseases, however the price for this is the tendency to promote autoimmune and chronic inflammatory diseases (180). Thus, the balance of the TLR-mediated pathways is probably involved in many aspects of immune system, from effector mechanisms to regulation.

1.2.3.4 TLRs and immunoregulation

Regulatory T cells (Tregs), including $CD4^+CD25^+$, Tr1 and Th3 cells, are cells that actively suppress immune reactions and play an important role in maintaining self-tolerance (see below). Several studies have indicated that DC activated by specific pathogens or their compounds, such as *Mycobacterium vaccae*, *Plasmodium falciparum*, *Bordetella pertussis* and *Schistosoma mansoni,* can induce regulatory T cells in the peripheral lymphoid organs which is dependent on IL-10 (181-184). Filamentous hemagglutinin (FHA) from *Bordetella pertussis* can stimulate DCs to produce IL-10 which can induce type 1 regulatory T (Tr1) cells. Furthermore, IL-10 production by Ag-specific T cells was significantly reduced, with an associated enhanced inflammation, in TLR4-defective mice. This suggests that TLR4 is important for IL-10-mediated generation of Tr1 cells (184). In another study, activation of TLR2 by schistosome-specific phosphatidylserine on dendritic cells was also essential for the induction of IL-10-producing regulatory T cells (144).

The effect of TLR activation on $CD4^+CD25^+$ regulatory T cell function is receiving intense scrutiny in immunological research. In the setting of *Candida albicans* infection, the yeast can induce host immunosuppression through TLR2 signalling

which was associated with an increased level of IL-10 and enhanced survival of $CD4^+CD25^+$ T cells (160). Furthermore, studies with virus-based vaccines have shown that viruses can trigger TLR signalling in $CD8^+$ T cells in a manner that could bypass regulatory T cell-mediated suppression, and trigger antigen-specific T cell immune responses (185). These investigations therefore indicate that TLR signals may be involved in the suppressive function of $CD4^+CD25^+$ T cells during the course of infection.

The direct effect of TLR triggering on $CD4^+CD25^+$ T cells was demonstrated when LPS, from *Salmonella typhimurium,* enhanced the survival and suppressor function of $CD4^+CD25^+$ regulatory T cells. This phenomenon was independent of APCs (186). In contrast to this, another study has demonstrated that the TLR4 agonist, LPS, can act on $CD4^+CD25^-$ effector cells to make them resistant to Treg suppression. This was independent of co-stimulation and was mediated by IL-6 and other soluble factors produced by DCs in response to TLR activation (187, 188). Furthermore, it has been shown that IL-6 and IL-1 produced by LPS-activated DCs can enhance the proliferative responsiveness of $CD4^+CD25^+$ T cells (188). Hence, TLR signals can manipulate regulatory T cell functions either by modulating DC function or directly acting on $CD4^+CD25^+$ T cells. More importantly, with the evidence to date, it would appear that TLRs can both enhance and abrogate the suppressor action of $CD4^+CD25^+$ regulatory T cells.

1.3 Regulatory T cells

1.3.1 Introduction

The adaptive immune system mounts defensive mechanisms against unanticipated pathogens by creating a diverse repertoire of antigen receptors. With such a system, receptors will be randomly generated that recognise self components, and potentially cause self damage. Various mechanisms including clonal deletion, induction of tolerance, immuno-modulation by suppressive cytokines, and inactivation of autoreactive cells (189-191) have been proposed to prevent this potential problem. However, even in the 1960s it was suggested that a population of T cells could actively "suppress" the immune system. Richard Gershon (192) found a unique subpopulation of $CD8^+$ T lymphocytes from mice which down-regulated the functions of T cells, B cells, and APCs. This finding opened the field of so-called "suppressor T cells". However, this $CD8^+$ I-J restricted suppressor T cells fell into disrepute when the I-J gene was found not to exist by molecular sequencing of the MHC gene by Green & Webb (193).

It has since been demonstrated that the immune system is under T cell-mediated control. Firstly, Nishizuka *et al* showed that neonatal thymectomy of normal mice, resulted in autoimmune-mediated destruction of the ovaries (194). Secondly, Penhale *et al* then demonstrated that thymectomy of adult rats, which were subsequently subjected to sub-lethal X-irradiation, resulted in the development of autoimmune thyroiditis (195). More importantly, it was shown that subsequent inoculation with normal T cells, particularly $CD4^+$ T cells, prevented the development of this disease.

This suggested the existence of a subset of $CD4^+$ T cells within the normal population which possessed regulatory capacities (196-198).

Sakaguchi, *et al* using T cell depletion experiments demonstrated that the ability to suppress autoimmune disease was associated with the actions of $CD4^+CD5^{hi}$ T cells (199). This regulatory subset was further defined by Powrie and colleagues who demonstrated that contained within the population of $CD4^+$ T cells from normal mice, subsets of $CD4^+$ T cells expressing low levels of CD45RB were able to protect against chronic intestinal inflammation (200). Therefore, it would appear that a naturally occurring $CD4^+$ subpopulation exhibited regulatory properties.

1.3.2 Naturally occurring $CD4^+CD25^+$ regulatory T cells

Sakaguchi *et al* showed that the removal of the thymus of mice 3 days after birth resulted in various autoimmune diseases (201, 202). Inoculation of the thymus-free mice with a mixed population of T cells from congenic mice prevented the development of autoimmune diseases if transferred before day 14 (203). It was also shown that a population of cells that naturally developed in the thymus could suppress this T cell-mediated autoimmune disease. Further adoptive transfer studies demonstrated that a subset of peripheral $CD4^+$ T cells in normal adult mice, which expressed the IL-2Rα-chain (CD25), was critical for preventing autoimmune disease induced by $CD4^+CD25^-$ T cells transferred into immune deficient mice (204). These regulatory T cells were also found in the thymus. The transfer of mature $CD4^+CD8^-$ thymocytes (depleted of $CD4^+CD25^+$ T cells) induced a range of autoimmune diseases in syngeneic nude mice, which the co-transfer of $CD25^+CD4^+CD8^-$

thymocytes prevented (205). $CD4^+CD25^+$ T cells, which constitute 5-10% of peripheral $CD4^+$ T cells, are contained within the $CD5^{hi}$ and $CD45RB^{lo}$ fraction of $CD4^+$ T cells, and appear in the periphery 3 days after birth, with their numbers increasing to adult levels by day 14 (203, 204). This agreed with the previous findings of Sakaguchi, Powrie and colleagues - suggesting that peripheral immunological tolerance was maintained through the actions of $CD4^+CD25^+$ T cells.

1.3.2.1 Murine $CD4^+CD25^+$ regulatory T cell suppression *in vitro*

To analyse the suppressive function of $CD4^+CD25^+$ regulatory T cells, *in vitro* studies were devised. Co-culture of freshly-isolated $CD4^+CD25^+$ Treg cells, "effector" $CD4^+CD25^-$ T cells, and APCs under limited TcR stimulation were established. Proliferation and cytokine production were used as end point measures of the suppressor function of Tregs. These *in vitro* studies have shown that $CD4^+CD25^+$ T cells are hypo-responsive, and able to suppress the activation of T cells to polyclonal stimulation (206-210). They have also demonstrated that suppression by $CD4^+CD25^+$ T cells is cell contact dependent. Supernatant collected from activated $CD4^+CD25^+$ T cells or mixture of $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells failed to suppress the proliferation of $CD4^+CD25^-$ T cells *in vitro* (207-209). Furthermore, co-cultures of $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells separated by a semi-permeable membrane failed to demonstrate suppression (211).

$CD4^+CD25^+$ T cells initially require antigen specific TcR activation, however, once activated $CD4^+CD25^+$ T cells can suppress T cell responses in an antigen-independent manner (204, 212). Stimulation with OVA peptides induces a strong suppressive

activity in $CD4^+CD25^+$ T cells from DO11.10 transgenic mice, but fails to do so in the cells from non-transgenic littermates (204). Further studies have shown that in mixed lymphocyte reactions (MLRs), $CD4^+CD25^+$ T cells, activated by allogeneic stimulator cells, suppressed $CD4^+CD25^-$ T cells of the same strain as well as $CD4^+CD25^-$ T cells from a different strain of mouse (212). Therefore, the suppression of $CD4^+CD25^+$ T cells requires TCR stimulation but the activated cells exert their inhibition independent of antigen specificity. Despite this, the effects of $CD4^+CD25^+$ T cells have been shown to be dependent on APCs (207, 213).

1.3.2.2 Human $CD4^+CD25^+$ regulatory T cells

$CD4^+CD25^+$ T cells have also been isolated in human peripheral blood and thymus. They constitute around 5-15% of the peripheral $CD4^+$ population, and are anergic to *in vitro* stimulation by anti-CD3 and anti-CD28 mAb. Human $CD4^+CD25^+$ T cells can inhibit effector T cell responses that have received antigenic or polyclonal stimulation (214-219), as seen in murine studies. It has also been demonstrated that human $CD4^+CD25^+$ T cells can convert effector T cells to regulatory T cells in the peripheral immune system, in a process called infectious tolerance (220-222). This process appears to be dependent on cell-contact, IL-10, and TGFβ (221, 222).

1.3.2.3 Mechanisms of suppression by $CD4^+CD25^+$ regulatory T cells

As well as suppressing $CD4^+CD25^-$ effector T cells, $CD4^+CD25^+$ T cells can also suppress other cell types. Some of these actions are summarised in Table 1.2.

CELL	*ACTION ON CELL TYPE*	*Ref.*
$CD8^+$	1. Proliferation and IFNγ production suppressed 2. Action is APC-independent 3. IL-2 production and CD25 expression reduced 4. High concentrations of IL-2 did not break suppression	(210)
DC	1. Co-stimulatory markers - CD80 and CD86 – downregulated 2. Reduced co-stimulation so that $CD25^-$ cells do not produce IL-2 3. Immature DCs (day 5–6, bone marrow derived) allow $CD25^-$ cells to be suppressed by $CD25^+$ cells *in vitro* 4. Mature DCs (non TLR activated) can reverse $CD25^+$-mediated suppression of $CD25^-$ cells 5. TLR-matured DCs can break anergy & cause proliferation of $CD25^+$ cells	(213) (188, 223, 224)
B cell *	1. Inhibits activation induced proliferation 2. Up-regulates Fas-L expression: inducing cell death	(225) (226)

Table 1.3 Action of $CD4^+CD25^+$ T cells on different cell types

$CD4^+CD25^+$ regulatory T cells can reduce the magnitude of the immune response by many different routes.

* B cells may also act as APCs to recruit T cell help. Activated B ells express CCL4 which induces migration of $CD4^+CD25^+$ T cells.

The precise mechanisms behind the suppressor action of $CD4^+CD25^+$ T cells remain controversial. Several cytokines, cell surface markers and transcription factors have been implicated in the mechanism of $CD4^+CD25^+$ T cells. These are illustrated on Figure 1.4 and discussed below.

IL-2

The population of $CD4^+CD25^+$ T cells in the peripheral circulation is reduced from 5-10% in normal mice to 1-2% in IL-2, IL-2Rα, or IL-2Rβ deficient mice. These knock-out mice died early due to severe lymphoproliferation and autoimmune diseases (207, 227-231). Adoptive transfer of $CD4^+CD25^+$ regulatory T cells from normal mice into IL-2Rβ-/- mice prevented the lethal autoimmune diseases (231, 232). Other work has shown that $CD4^+$ T cells from IL-2-deficient mice protected mice from spontaneous experimental autoimmune encephalomyelitis, while $CD4^+$ T cells from CD25–deficient mice did not. Thus, IL-2 derived from the recipient mouse drove expansion of IL-2-deficient cells, but was unable to drive the expansion of $CD4^+$ cells from CD25-deficient mice (233). Further studies, through use of anti-IL-2 mAb, have shown that IL-2 is necessary for the function of $CD4^+CD25^+$ regulatory T cells i*n vitro* (234). In addition, $CD4^+CD25^+$ T cells proliferate in the presence of higher than normal (physiological) concentrations of IL-2 (207, 232). High concentration of IL-2 also abrogates the suppressive function of $CD4^+CD25^+$ T cells on the proliferation of $CD4^+CD25^-$ T cells *in vitro* (235). Hence, overall IL-2 appears important in $CD4^+CD25^+$ T cell function.

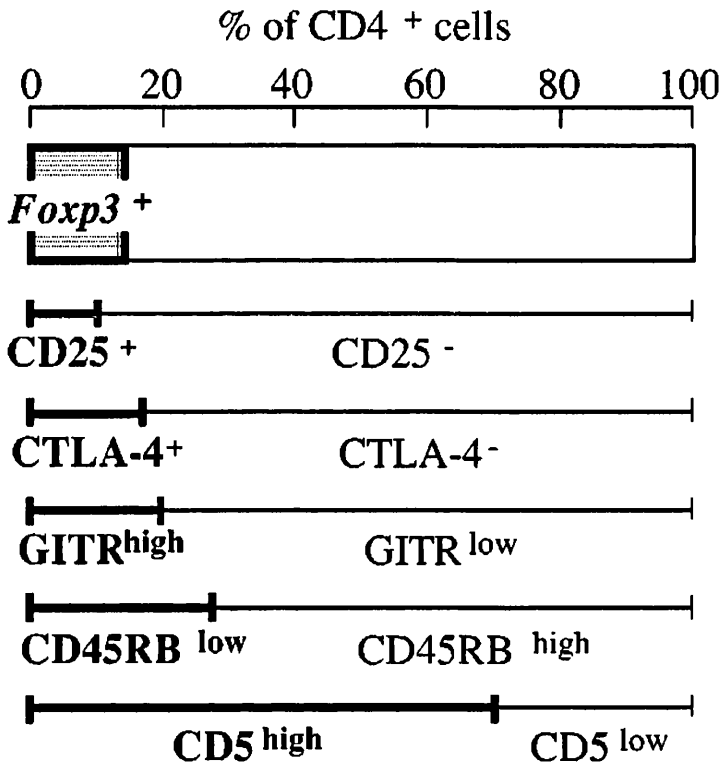

Figure 1.4 Expression of cell surface markers on naturally occurring $CD4^+CD25^+$ Treg cells.

Adapted from Sakaguchi et al, (198).

IL-10 and TGFβ

IL-10 and TGFβ are produced *in vitro* by $CD4^+CD25^+$ T cells after stimulation with plate-bound anti-CD3 antibody and anti-CD28 antibody, or IL-2 (236, 237). Despite this, the suppressor action of $CD4^+CD25^+$ T cells was not abrogated by anti-TGFβ or anti-IL-10 mAb *in vitro* (238, 239). However, administration of monoclonal antibodies against IL-10R or TGFβ completely abrogated the therapeutic ability of $CD4^+CD25^+$ T cells to ameliorate murine colitis *in vivo* (240-242). In addition, TGFβ is important in the $CD4^+CD25^+$ T cell-regulation of $CD8^+$ mediated diseases *in vivo* (243, 244). TGFβ has also been shown to be critical in the generation of $Foxp3^+$ regulatory T cells (245-247). Hence, it is likely that IL-10 and TGFβ are important in the function of $CD4^+CD25^+$ T cells *in vivo*, as well as in the function of other regulatory T cells (see below).

IL-6

IL-6, produced by TLR-activated dendritic cells, can render $CD4^+CD25^-$ T cells resistant to suppression by $CD4^+CD25^+$ T cells (187). Furthermore, IL-6 and IL-1 produced by TLR-activated DCs played an important role in breaking $CD4^+CD25^+$ regulatory T cell's anergy (188). The exact role of IL-6 in $CD4^+CD25^+$ T cell function remains controversial.

CD25

CD25 (α chain of IL-2R) is constitutively expressed at a high level on naive $CD4^+CD25^+$ T cells. As IL-2R is upregulated upon stimulation, all T cells (including $CD25^-$ effector cells) express CD25 upon activation. $CD25^+$ does not confer

regulatory properties, shown by the observation that activated effector cells do not normally act as Tregs (204).

CTLA-4

The Cytotoxic T Lymphocyte-associated Antigen-4 (CD152, CTLA-4) is expressed on T cells, and binds B7-1 and -2 (CD80/CD86) on APCs with higher affinity than CD28. T cell activation, through TcR and CD28 signalling, increases CTLA-4 expression on $CD4^+$ T cells, which will then bind B7. CTLA-4 and B7 ligation cause T cells to de-activate, and cause reduction of the immune response (248, 249). $CD4^+CD25^+$ T cells are the only $CD4^+$ cells that constitutively express CTLA-4 in mice and humans. Blockade of CTLA-4 has been shown to lead to increased T cell–mediated immunity in several experimental models, including tumour immunity (250-253), parasite infection (254-257), and autoimmune diseases (248, 258, 259). In SCID mice colitis models, the protective and therapeutic effects of $CD4^+CD25^+$ T cells were abolished by treatment with anti-CTLA-4 antibody (242, 260). However, $CD4^+CD25^+$ T cells from CTLA-4 deficient mice, exhibited normal development and homeostasis, and also exerted their suppression on $CD4^+CD25^-$ T cells and other cells *in vitro* (261, 262). Studies using anti-CTLA-4 mAb raise the possibility that this antibody could simply block the normal downregulatory signal mediated by CTLA-4-B7 interactions on activated $CD4^+CD25^-$ effector T cells. However, *in vivo* investigation has shown that monoclonal anti-CTLA-4 antibody has no direct effect on the $CD4^+CD25^-$ T cells in the autoimmune colitis model (242). Together, the findings suggest that CTLA-4 may be not the only signal required for the activation and function of $CD4^+CD25^+$ T cells.

GITR

Gene microarray technology has identified more than twenty genes that were preferentially expressed on $CD4^+CD25^+$ over $CD4^+CD25^-$ T cells. One of them is the glucocorticoid-induced tumour necrosis factor receptor (GITR). GITR, also known as TNFRSF18, is a type I transmembrane protein with high homology with other members of the TNFR family, including 4-1BB, CD27, and OX40 (263). It is predominantly expressed on $CD4^+CD25^+$ T cells and on $CD4^+CD8^-CD25^+$ thymocytes. However, like CD25, GITR is also upregulated on naïve T cells after activation, and these stimulated effector cells did not exhibit regulatory function. GITR is expressed and up-regulated on $CD25^+$ Tregs, effector T cells, B cells and macrophages. It binds GITR ligand (GITR-L) which is on DCs, macrophages and B cells but not T cells (264, 265). GITR binding GITR-L or mAb causes activation of the $CD4^+CD25^+$ Tregs which abrogates their suppressive function (264, 265). Other investigators have found that GITR-L binds the effector T cells and makes them resistant to the suppressive action of Tregs (266). GITR activation on effector T cells has also been found to be a co-stimulatory signal on T cells, causing increased proliferation and cytokine production.

So far, no reliable cell surface marker that distinguishes Tregs from activated effector cells has been identified. In mice a transcriptional factor, *Foxp3*, is exclusively expressed at high levels in $CD4^+CD25^+$ regulatory T cell in both the thymus as well as the periphery, but not on activated $CD4^+CD25^-$ effector T cells (213, 267).

Foxp3

The X-linked forkhead/winged helix transcription factor, *Foxp3*, is required for $CD4^+CD25^+$ regulatory T cell development and function (267-271). A mutation in human *FOXP3* is responsible for IPEX (immune dysfunction regulation, polyendocrinopathy, enteropathy, X-linked syndrome), which is associated with autoimmune diseases in multiple organs (272-275). In mice a similar disorder is seen in scurfy mice, which have a mutation in *Foxp3* (274). Retroviral transformation of $CD4^+CD25^-$ T cells with *Foxp3* was sufficient to impart $CD4^+CD25^-$ T cells with suppressive activity (267). These transformed $CD4^+CD25^-$ T cells were anergic and suppressed the proliferation of other T cells *in vitro*, independent of the production of TGF-β or IL-10. Furthermore, the profile of surface markers including GITR, CTLA-4, and CD103 were comparable to those found in naturally occurring $CD4^+CD25^+$ regulatory T cells (276-278). These *Foxp3*-induced $CD4^+CD25^-$ T cells were also able to suppress the development of autoimmune disease like IBD *in vivo* (267, 269, 271). Thus, *Foxp3* (*FOXP3* in humans) appears to be an important gene in the development and function of $CD4^+CD25^+$ regulatory T cells. However, how *Foxp3* is involved in immune suppression is still unknown.

Neuropilin-1 (NrP1)

Since *Foxp3* is a nuclear protein, it has limited value as marker to identify $CD4^+CD25^+$ regulatory T cells *in vitro*. From gene expression profiling, neuropilin-1 was identified as a protein linked to *Foxp3* expression. RT-PCR results reveal that *Foxp3* mRNA correlates with the NrP1 expression on $CD4^+$ T cells and is over-expressed on the surface of $CD4^+CD25^+$ T cells. NrP1 is expressed at a lower level on $CD4^+CD25^-$ T cells and is even further down-regulated upon T cell activation.

Therefore, NrP1 may be an additional surface marker to distinguish $CD4^+CD25^+$ regulatory T cells from recently activated $CD4^+CD25^+$ non-regulatory T cells (279).

Integrin $\alpha_E\beta_7$

The integrin $\alpha_E\beta_7$ was first identified by its expression on more than 90% of $CD8^+$ T cells in the intestinal mucosa, and approximately 40-50% of $CD4^+$ intestinal T cells (280, 281). α_E-integrin is expressed on $CD4^+CD25^+$ T cells, as well as on a small proportion of $CD4^+CD25^-$ murine T cell population. $\alpha_E^+CD4^+CD25^+$ T cells, comprising about 4% of all $CD4^+$ T cells and 25% of $CD25^+CD4^+$ T cells, were shown to have the greatest suppressor function *in vitro* (282). The integrins $\alpha_4\beta_1$ and $\alpha_4\beta_7$ receptors that direct T cell migration to inflamed tissues and to mucosal sites (283, 284). In human studies, peripheral $CD4^+CD25^+$ T cells fall into two categories on the basis of integrin α/β expression. $\alpha_4\beta_7^+$ $CD25^+$ regulatory T cells suppress effector T cells *in vitro* and aid induction of distinct secondary IL-10-producing Tr1 cells, whereas $\alpha_4\beta_1^+$ $CD4^+CD25^+$ T cells induce TGF-β-producing Th3 cells. These induced regulatory T cells cooperated with $CD4^+CD25^+$ regulatory T cells to inhibit activation of other $CD4^+$ T cells (285).

Lymphocyte activation gene-3 (LAG-3, CD223)

The MHC class II ligand, lymphocyte activation gene-3 (LAG-3 or CD223), was found to be selectively expressed on $CD4^+CD25^+$ regulatory T cells and peripherally induced regulatory T cells (286). LAG-3, as a negative regulator, inhibited antigen-driven T cell expansion in mice (287, 288). Furthermore, the suppressive activity of $CD4^+CD25^+$ T cells on $CD4^+CD25^-$ T cell activation can be blocked by anti-LAG-3 antibodies both *in vitro* and *in vivo*. Deficiency of LAG-3 inhibited the regulatory

activity of $CD4^+CD25^+$ T cells, whereas forced expression of LAG-3 induced suppressor activity. LAG-3 can also be released as a soluble form (sLAG-3), which activated antigen-presenting cells through MHC class II signalling, leading to increased antigen-specific T-cell responses *in vivo* (289, 290). Thus, sLAG-3 may be a competitor for ligands binding the membrane form of LAG-3.

Programmed death receptor-1 (PD-1)

Recent gene chip analyses have demonstrated that programmed death receptor-1 (PD-1) mRNA is highly expressed in $CD4^+CD25^+$ regulatory T cells and anergic T cells, suggesting it may be involved in regulating T cell tolerance by two mechanisms (291). PD-1-deficient mice developed lupus-like glomerulonephritis, arthritis and autoimmune-dilated cardiomyopathy, suggesting a regulatory role for PD-1 in controlling lymphocyte responses (292). Although higher ratios of human $CD4^+CD25^+$ T cells were required to suppress proliferation, if the PD-L1 receptor was blocked, regulatory T cell function was still shown to persist in the absence of the PD-1/PD-L1 pathway (215).

CD28

The CD28/B7 costimulation is necessary for the initiation of T cell responses and IL-2 production. Mice deficient in either CD28 or B7 have reduced numbers of $CD4^+CD25^+$ T cells in both the thymus and the periphery (293, 294). CD28 co-stimulation of TcR-signalled, $CD4^+CD8^+$ thymocytes initiated $CD4^+CD25^+$ T cell differentiation by directly inducing *Foxp3* expression, and by up-regulating GITR and CTLA-4 expression, which was independent of IL-2 (295). Furthermore, peripheral $CD4^+CD25^+$ T cells require CD28 co-stimulation of $CD4^+CD25^-$ T cells to maintain

the IL-2 milieu, which is needed for Treg survival (293, 296). Studies have shown that CD28-deficient $CD4^+CD25^+$ T cells were less potent suppressors compared with those from wild-type mice. In addition, $CD4^+CD25^+$ regulatory T cells are anergic in the normal state, but can proliferate *in vitro* with TcR stimulation when anti-CD28 antibody is also present (207). These expanded $CD4^+CD25^+$ T cells exhibited a stronger suppressive ability after resting (297).

Perforin & Granzyme

These are enzymes that are important for the cytolytic activity of CD8+, NK and NKT cells. Human $CD4^+CD25^+$*FOXP3*$^+$ cells can be activated by anti-CD3 and anti-CD46 Ab to express granzyme A, and kill activated $CD4^+$ and $CD8^+$ T cells by a perforin-dependent mechanism (298).

The above findings may suggest $CD4^+CD25^+$ Treg cells are a homogenous population that suppress immune cells by a several mechanisms. The evidence could also indicate that $CD4^+CD25^+$ Treg cells are a heterogeneous population that suppress cells by a number of distinct pathways. The interaction and regulation of these molecular and signalling pathways involved Treg cells suppression is an area of active research and is still being unravelled.

1.3.3 Other $CD4^+$ Regulatory T cells

$CD4^+CD25^+$ Treg cells are thought to "naturally occurring" as they are initially found in the thymus and thought to exit into the periphery around day 3 in mice (203, 205). In addition to these Tregs, there is growing evidence to suggest suppressor cells can

be induced in the periphery from conventional $CD4^+CD25^-$ T cells (184, 299-301). These cells are also anergic under normal stimulatory conditions, and can inhibit immune cell function (Table 1.3).

1.3.3.1 Tr1 cells

The generation of Type-1 $CD4^+$ regulatory (Tr1) cells were first described by Groux *et al* in a murine model of inflammatory bowel disease (299). T cells from naïve OVA TcR-transgenic mice, repeatedly stimulated with OVA and IL-10 *in vitro*, differentiated into a T cell population, which produced high level of IL-10, moderate amounts of IFN-γ and IL-5, and small amounts of IL-2 and IL-4. Tr1 have been shown to produce moderate amounts of TGFβ in some studies, but not in others. These *Foxp3*-negative cells can suppress nïave, and Th1 and/or Th2 responses *in vivo* in an antigen-specific manner (301-303). The suppressive effects of Tr1 cells can be reversed by neutralizing anti-IL-10 mAb or anti-TGFβ antibody, or both (299, 304). This result indicates that the inhibitory capacity of Tr1 cells is mainly mediated through the production of the immunosuppressive cytokine, IL-10. Further work has demonstrated that Tr1 cells can be generated in $CD4^+$ T cells culturesthat have been supplemented with Vitamin D3 and dexamethasone, or have had CD40-CD40L blockade, or been activated by immature dendritic cells (301-303, 305).

	CD4+CD25+	*Tr1*	*Th3*
Origin	Thymus	Periphery	Periphery
Phenotype			
CD25	+++	+/-	+/-
GITR	++	-	-
CTLA-4	+++	+	+
Foxp3	++	- *	+
Cytokine production			
IL-10	Yes	Yes	-
TGFβ	Yes	-	Yes
IL-2	-	+/-	-
Induced by	-	IL-10	TGF-β/IL-10/IL-4
Ag Specificity	self	Tissue/foreign Ag	Tissue/foreign Ag
Mode of action			
in vitro	Cell contact IL-10, TGFβ	IL-10, TGFβ	TGFβ
in vivo	Cell contact	IL-10, TGFβ	TGFβ

Table 1.4 Comparison of $CD4^+$ Regulatory T cells

* Recent description of IL-10 secreting Tbet1-positive *foxp3*-positive Tr cells has been made (278) – see below.

1.3.3.2 Th3 cells

Th3 cells were initially described by Chen *et al.* $CD4^+$ T cells from mesenteric lymph nodes of mice given oral myelin basic protein (MBP), suppress MBP-specific EAE *in vivo,* in a TGFβ-dependent manner (306, 307). Th3 regulatory cells secrete TGFβ, provide help for IgA synthesis, and can suppress both Th1 and Th2 cells (245, 308).

1.3.3.3 Other IL-10 producing T cells

In addition to the cells discussed above, there are a number of other cell groups that also exhibit suppressor function. It has been demonstrated that pulmonary DCs produce IL-10 in response to respiratory allergen. These DCs can induce Tregs in the lung that blocks asthma in a murine model. This interaction is dependent on IL-10 and ICOS- ICOS ligand pathway (52, 53). Further studies have demonstrated that these Tregs are Tbet1-expressing and are *Foxp3* positive. These cells have been shown to effectively block murine asthma *in vivo* (278).

1.3.4 Other regulatory cells

$CD8^+$ Tr cells

$CD8^+$ T cells normally act as effector cells in response to intracellular infections. There is also some evidence suggesting that these cells have regulatory function. $CD8^{-/-}$ mice develop experimental allergic encephalomyelitis (EAE) in a more severe and

chronic manner, than do wild type mice (309). In addition, $CD8^+$ T cells residing in the gut appear to have a regulatory role in intestinal immune responses (310) and antigen-specific $CD8^+$ cells inhibited IgE production and IL-4 production in a murine model of asthma (311).

TCR γδ+ cells

TCR γδ+ cells are important in the induction and regulation of the mucosal immune response (235, 312). TCR γδ+ murine intra-epithelial lymphocytes have a role in tolerance induced by orally administered sheep red blood cells (313). Elimination of TGFβ and IL-10 producing TCR γδ+ T cells from mice with tumours augments CTL and NK (NK) cell activities leading to tumour regression (314).

NKT cells

NKT cells express surface markers characteristic of both T cells and NK cells, and are either $CD4^+$ or $CD4^-CD8^-$. These cells have a limited TcR repertoire (expressing αβ TcR) and recognise glycolipid antigens presented by the non-polymorphic MHC class I-like CD1d. When activated, these NKT cells produce large quantities of IL-4 and IFNγ, and have been shown to exert a suppressive effect in a variety of experimental autoimmune diseases (315-317). LFA-1/ICAM-1 or CD28/B7 blockade, and TGFβ have been proposed as mechanisms for the suppressive function of NKT cells (318, 319), however their mode of action remains unclear. In addition to regulating immune responses, it was demonstrated that NKT cells can produce high levels of IL-4 and IL-13 in response to allergen, and can actually induce airways hyperresponsiveness in a murine model of asthma (320).

In summary, there are a several suppressor $CD4^+$ T cells with common features. It may be that these form a spectrum of $CD4^+$ Tregs, and the environment and nature of immune response that they control may direct the Treg phenotype displayed. Potentially, $CD4^+CD25^+$ Treg, Tr1, Th3 and other regulatory T cells could co-operate *in vivo* to prevent autoimmune reactions and over-active immune responses (321). $CD4^+CD25^+$ regulatory T cell are arguably the best studied regulatory T cells, principally because of the easy with which these cells can be obtained *ex vivo*. For this reason, $CD4^+CD25^+$ regulatory T cells have a great deal of evidence behind their suppressor function *in vitro*; in experimental inflammatory disorders; and in transfer experiments *in vivo*.

1.3.5 $CD4^+CD25^+$ regulatory T cells and disease

Cells that can regulate the immune responses have enormous clinical importance. Controlling the suppression of the immune system could assist the treatment of transplant rejection, autoimmune and allergic disease; whilst decreasing suppression could help in cancer therapy or treatment of persistent infections.

$CD4^+CD25^+$ regulatory T cells and transplantation tolerance

Transplant rejection and tolerance is a T cell-dependent event. Removal of $CD4^+CD25^+$ T cells from donor cell transfers, or *in vivo* $CD25^+$ cell-depletion of the recipients before transplantation resulted in increased graft-versus-host disease in several animal models. Pre-treatment with anti-CD4 antibody plus regulatory $CD4^+CD25^+$ T cells can suppress the rejection of skin grafts mediated by naïve

$CD4^+CD45RB^{high}$ T cells (322, 323). Furthermore, a therapeutic effect has also been obtained by adding regulatory T cells to donor T cells in GVHD (324). A novel human to mouse *trans vivo* model has identified donor reactive immune responses in kidney and liver transplant patients. The results show that the delayed type hypersensitivity reaction was weaker in tolerised patients, and this response suppression was dependent on IL-10 and TGFβ. In addition, kidney transplant patients with no history of acute rejection demonstrated little response to mismatched allopeptides, as compared to patients that had suffered episodes of acute rejection, and this regulation was mediated by $CD4^+CD25^+$ T cells (325, 326).

$CD4^+CD25^+$ regulatory T cells and tumour immunity

In humans, high levels of $CD4^+CD25^+$ regulatory T cells have been found in lung, ovarian, breast and pancreatic tumour specimens (327, 328), which may impede the generation and activation of tumour effector T cell responses. A study addressing the role of $CD4^+CD25^+FOXP3^+$ T cells in ovarian carcinoma, found that human tumour-specific $CD4^+CD25^+$ regulatory T cells suppressed specific anti-tumour immunity *in vitro,* and reversed the inhibition of tumour growth induced by adoptive transfer of tumour-specific effector T cells (329).

$CD4^+CD25^+$ regulatory T cells and inflammatory bowel disease

Evidence shows that $CD4^+CD25^+$ Tregs prevent the development of murine colitis by directly inhibiting effector T-cell proliferation and pro-inflammatory cytokine production (211, 240, 330). Furthermore, IL-10 and TGF-β play an important role in the prevention of intestinal inflammation (240, 331). $CD4^+CD25^+$ T cells can also treat established colitis induced in SCID mice (242). The therapeutic role of

$CD4^+CD25^+$ regulatory T cells was strongly dependent on CTLA-4, IL-10, and TGFβ (242, 332).

$CD4^+CD25^+$ regulatory T cells and rheumatoid arthritis

Rheumatoid arthritis is characterised by synovium infiltrated by activated $CD4^+$ and $CD8^+$ T cells, B cells, plasma cells, neutrophils and macrophages. Depletion of $CD4^+CD25^+$ T cells in susceptible DBA/1 mice resulted in significantly increased severity and incidence of disease (333), and enhanced Ag (type II collagen)-specific proliferation of splenocytes *in vitro* (333). These effects were reversed by adoptively transferring $CD4^+CD25^+$ T cells isolated from syngeneic naïve mice. However, $CD4^+CD25^+$ regulatory T cells are not important in controlling autoimmune arthritis in a proteoglycan-induced murine arthritis model (334). In humans, it was found that $CD4^+CD25^{high}$ T cells isolated from the peripheral blood of patients with active RA were anergic and were unable to regulate pro-inflammatory cytokines released by effector T cells and monocytes *in vitro,* when compared to cells isolated from healthy individuals (335).

$CD4^+CD25^+$ regulatory T cells and diabetes mellitus

Diabetes mellitus is mainly caused by beta islet cell autoreactive $CD4^+$ and $CD8^+$ T cells, particularly those cells producing Th1-type cytokines IFNγ, IL-12 and TNFα. As to whether there is deficiency in $CD4^+CD25^+$ T cells in patients with type 1 diabetes remains controversial. However, the *in vitro* suppressive function of these cells isolated from diabetic patients on T cell proliferation was reduced when compared to healthy control subjects (336, 337). Using the non-obese diabetic (NOD) mice as a model of type 1 diabetes, it has been shown that $CD4^+CD25^+$ regulatory T

cells can inhibit IFNγ production and prevent diabetes (338). In these studies, $CD4^+CD25^+$ regulatory T cells were localised in the pancreas-associated tissues, and it was demonstrated that decreased numbers of $CD4^+CD25^+$ T cells in these locations correlated with acceleration to diabetes. Reconstitution with pancreatic lymph node-derived $CD4^+CD25^+$ T cells prevented diabetes progression. It was also shown that although $CD4^+CD25^+$ Tregs did not inhibit $CD8^+$ T cell recruitment to the islets, they did delay or suppress the anti-islet $CD8^+$ T cell differentiation into effector CTL (339).

$CD4^+CD25^+$ regulatory T cells and infectious disease

The persistence of pathogens in the host is seen in infectious diseases like tuberculosis, leishmaniasis, toxoplasmosis, and many viruses. $CD4^+CD25^+$ regulatory T cells may inhibit the clearance of such pathogens by suppressing effector T or innate cells. $CD4^+CD25^+$ regulatory T cells have been shown to prevent the complete elimination of *Leishmania* parasites in both resistant and susceptible mice (242, 340). Moreover, reactivation of disease was associated with a local increase in $CD4^+CD25^+$ T cell numbers. In another study, it was shown that the transfer of $CD4^+CD25^+$ T cells purified from chronically infected mice into resistant mice was sufficient to trigger disease reactivation (341). $CD4^+CD25^+$ regulatory T cells also have been shown to suppress effector T cell responses, and contribute to pathogen persistence in experimental and clinical infections, including *Pneumocystis carinii* (342), *Candida albicans* (160, 343), *Helicobacter pylori* (344), human cytomegalovirus (345), HIV (345), and Hepatitis C infection (8, 346, 347). Thus, $CD4^+CD25^+$ regulatory T cells have a protective role in infection by limiting pathogen-induced immunopathology, whilst also allowing the development of pathogen-induced immunological memory.

However, the multiple mechanisms underlying pathogen persistence still need further investigation.

$CD4^+CD25^+$ regulatory T cells and Th2-mediated disease

Patients that specifically lack $CD4^+CD25^+$ regulatory T cells develop severe eczema, have elevated IgE levels, display blood eosinophilia, and have food allergies. Therefore, it seems likely that regulatory T cells play a role in controlling the development of allergic disease and asthma (348). Furthermore, $CD4^+CD25^+$ regulatory T cells have been shown to suppress immature dendritic cells, rendering the DCs unable to induce Th immunity (223). Murine asthma work has produced conflicting data. Some investigators demonstrated that $CD4^+CD25^+$ T cells worsened the allergic phenotype in mice, by preferential inhibition of Th1 cells by Tregs (349), whilst others have found that $CD4^+CD25^+$ regulatory T cells can suppress Th2-mediated inflammation, without affecting airways hyperresponsiveness (350, 351). In humans, $CD4^+CD25^+$ cells from the peripheral blood of atopic and non-atopic individuals has demonstrated that the suppressive activity of such cells is reduced in atopic individuals, implying that the disordered activity of Tregs may allow development of allergic disease (352).

In summary, $CD4^+CD25^+$ regulatory T cells have a critical role in the control of immune responses, probably working in close cooperation with other peripheral induced regulatory T cells and/or immunosuppressive cytokines *in vivo*. Dysfunction or deficiency of regulatory T cells could potentially be a cause of autoimmune diseases. However, activation of regulatory T cells is not always beneficial to the host. Regulatory T cells also inhibit show anti-pathogenic and anti-tumour immunity, and

may weaken vaccine induced-immunity. By gaining further knowledge of regulatory T cells, it may be possible to manipulate Tregs to suppress overactive immune responses in autoimmune, allergic and graft-versus-host diseases, whilst not compromising anti-tumour or anti-pathogen immunity. Hence, by carrying out basic scientific research one would hope to translate these works from bench to bedside.

1.4 Hypothesis

As demonstrated above, there is a complex network of cells and factors that interplay in pathogenesis of asthma, and there is a substantial body of evidence supporting the importance of regulatory T cells and Toll-Like Receptors in inflammatory diseases.

As previously discussed, there are numerous ways in which to investigate such a complicated disease like asthma. However, the approach I have taken is twofold. Firstly, I propose to investigate the TLR2 system that is expressed and shown to be important in the function of many of the cells involved in allergic airways inflammation. TLR2 activation will affect effector cells of both the innate and adaptive immune system, as well as potentially affecting Tregs. I postulate that TLR2 activation will ameliorate murine asthma, by either down-regulating or skewing effector Th responses, or by activating regulatory T cells.

Secondly, I propose to look at a surface molecule that is known to be important in the function of regulatory T cells, but also as a co-stimulatory molecule of effector T cells. Hence, I postulate that GITR activation in murine asthma may exacerbate the phenotype by abrogating Treg function, and by enhancing the Th2 effector response.

1.5 Aims of the project

Hence, the aims of this project are:

1. To examine the effects of TLR2 activation in a murine model of allergic airways inflammation.
2. To examine the role of GITR activation in a murine model of allergic airways inflammation.

I will employ a range of *in vitro* and *in vivo* techniques to address these questions. The project will not only provide vigorous training in advanced immunology for a PhD program, but the results obtained should also shed light on the characteristics and function on this crucial area of immune regulation, leading to potential novel therapeutic approaches to a range of inflammatory diseases.

Chapter 2

Materials and Methods

2.1 Mice

BALB/c mice (H-2^d, IgM^a), 129/Sv (H-2^b, IgM^a), and severe combined immunodeficient mice (SCID, C.B-17 of the BALB/c background) were purchased from Harlan Olac (Bicester, Oxon, UK). DO11.10 mice (I-A^d), transgenic for TcR specific to OVA peptide (sequence 323-339), were on a BALB/c background and bred in the Central Research Facilities (CRF), University of Glasgow. IL-12 p40 knockout (ko) mice (on a BALB/c background) and IFNγ ko mice (on a 129/Sv background) were bred in the Central Research Facilities (CRF), University of Glasgow, and kindly supplied by Prof. A. M. Mowat (Division of Immunology, Infection, and Inflammation, University of Glasgow, Glasgow, U.K).

All mice were housed and treated in the Biological Service facilities in the University of Glasgow according to the UK Home Office guidelines under pathogens free condition. SCID mice were kept in micro-isolator cages with filtered air and fed sterilized foods. Mice were used at 6-10 weeks of age. Procedures were carried out under Project License 60/3119, procedure number 9.

2.2 Reagents and Buffers

The reagents either purchased or donated are summarised in Tables 2.1-2.3. Details of buffers are given Table 2.4.

Cytokine	*Supplier*	*Source*	*Concentration*	*Use*
GM-CSF	R&D Systems Oxon, U.K.	Murine	30 ng/ml	BMDC Culture
CSF-1	R&D Systems	Murine	20ng/ml	BMM Culture
IL-2	GlaxoSmithKline Stevenage, U.K.	Human	10 ng/ml	Cell culture maintenance
IL-4	R&D Systems	Mouse	10 ng/ml	Th2 polarisation BMDC culture
IL-12	Genetics Institute, Cambridge, MA	Mouse	5 ng/ml	Th1 polarisation

Table 2.1 Cytokines used for *in vitro* culture

Antibodies (Clone No.)	*Source*	*Isotype*	*Concentration*	*Use*
Anti-CD3 (145-2C11)	BD Bioscience	Hamster IgG1	0.5 - 5 μg/ml	T cell activation
Anti-CD28 (37.51)	BD Bioscience	Syrian Hamster IgG2	1 μg/ml	Co-stimulation of T cells
Anti-CD16/32 (2.4G2)	BD Bioscience	Rat IgG2b	0.5 μg/ 100μl	Fc receptor blocking
FITC or PE- anti-CD4 (RM4-4/5)	BD Bioscience	Rat IgG2a	0.5 μg/100μl	FACS
PE-anti-CD25 (PC61/7D4)	BD Bioscience	Rat IgG2b	3.6 μg /1 x10^8 cells	T cell separation FACS
* Anti-IL-10R (1B1.3A)	DNAX, Palo Alto, CA	Rat IgG1	1 mg/mouse	*in vivo* experiments
Anti-IL-12 (rm IL-12)	R&D Systems	Goat IgG	0.1 μg/ml	Drive Th1 cells
** Anti-IL-12	ATCC Manassas, VA	Rabbit IgG	1mg/mouse	*in vivo* experiments
Anti-IFN-γ (XMG1.2)	BD Bioscience	Rat IgG1	300 ng/ml	Drive Th1 cells
** Anti-TGF-β (1D11.16.8)	ATCC Manassas, VA	Bovine IgG1	1 mg/mouse	*in vivo* experiments
*** Anti-GITR (DTA-1)	Generated in house	Rat IgG1	1 mg/mouse	*in vivo* experiments
FITC-GITR (108619)	R&D Systems	Rat IgG2a	1:10 dilution (Kit)	FACS
FITC-conjugated mouse anti-rat IgG F(ab')	Jackson Immuno-Research Lab	$F(ab')_2$ fragment	1:125 dilution	GITR cell separation FACS

Table 2.2 Antibodies used for *in vivo* and *in vitro* experiments

* Anti-IL-10R was kindly provided by Dr. K. Moore (DNAX, Palo Alto, CA).

** Anti- IL-12 was a polyclonal rabbit anti-mouse IgG antibody prepared from clone Rab74.6 and anti-TGFβ monoclonal antibody was prepared from clone 1D11.16 (both ATCC, Atlanta GA).

*** Anti-mouse GITR mAb was prepared from the DTA-1 hybridoma, kindly donated by Prof. S. Sagakuchi (Department of Experimental Pathology, Kyoto University, Japan).

All control IgG were purchased from Sigma-Aldrich.

Regent	*Supplier*	*Use*
2,2,2-tribromoethanol	Sigma-Aldrich, Poole U.K.	Mouse anaesthesia
2% Alhydrogel	Brentag Biosector, Denmark	Mouse sensitisation
2-Mercaptoethanol	Sigma-Aldrich	T cell culture
Amyl alcohol	Sigma-Aldrich	Mouse anaesthesia
Anti-PE microbeads	Miltenyibiotec, Auburn, CA	$CD25^+$ T cell separation
Anti-rat IgG microbeads	Miltenyibiotec	GITR cell separation
CD4 separation kit	Miltenyibiotec	$CD4^+$ T cell separation
Concanavalin A	Sigma-Aldrich	Cell culture
Extravidin-peroxidase	Sigma-Aldrich	ELISA
Foetal calf serum (FCS)	Harlan, Loughborough, UK	Cell culture
L-glutamine	Invitrogen, Paisley, U.K.	Cell culture
OVA (fraction V)	Sigma-Aldrich	Mouse sensitisation, challenge & cell culture
Synthetic lipopeptide Pam3CSK4/BLP	EMC Microcollections, Germany	*In vitro* & *in vivo* experiments
PBS	Invitrogen, Paisley, U.K.	Cell culture
Penicillin	Invitrogen, Paisley, U.K.	Cell culture
Peptidoglycan (from *S. aureus*)	Sigma-Aldrich	*In vivo* experiments
qPCR core kit	Eurogentec, Hampshire, U.K.	Real time PCR
RNA-Bee	Ams Biotechnology Oxon, U.K.	RNA extraction
Streptomycin	Invitrogen Paisley, U.K.	Cell culture
SuperScript II RNaseH reverse transcriptase	Invitrogen Paisley, U.K.	RT-PCR
TMB	Insight, KPL, Middlesex, U.K.	ELISA
TURBO DNA-free kit	Ambion, Huntingdon, U.K.	Removal of genomic DNA contamination
UNG	Eurogentec	Real time PCR

Table 2.3 Other reagents

Buffer	*Constituents*
Phosphate buffered Saline (PBS)	0.24g KH_2PO_4 0.2g KCL 1.44g NaH_2PO_4 8g NaCL Add to 800ml ddH_2O, stir and allow to dissolve, then pH to 7.0 and make up to a final volume of 1 litre.
FACS buffer	1 x PBS 2% FCS 0.05% sodium azide
MACS buffer	1 x PBS 2% FCS 100 IU/ml penicillin 100 µg/ml streptomycin sterilised with 0.22µM cellulose Acetate filter (VWR International)
Coating buffers for cytokine ELISA	1) 0.1 M $NaHCO_3$: 1.68g in 200ml distilled H_20, pH-adjusted to 8.4 2) 0.05 M carbonate/bicarbonate buffer: 50mM $NaHCO_3$, 50mM Na_2CO_3, pH-adjusted to 9.4 3) 0.1 M carbonate/bicarbonate buffer: 0.1 M $NaHCO_3$, 0.1 M Na_2CO_3, pH-adjusted to9.5
Blocking buffer for cytokines ELISA	PBS & 10% FCS
Washing buffer for ELISA	0.05% Tween-20 in PBS, pH-adjusted to 7.4
IgG purification Buffer A	1:4 (v/v) ratio of 1 M NaH_2PO_4 & 0.5 M Na_2HPO_4, sterilised with 0.22µM cellulose Acetate filter (VWR International)
IgG purification elution buffer	0.1M glycine: 3.75g in 100ml distilled water, pH-adjusted to 2.5
Tris buffer for IgG storage	2M Tris Buffer: 12.11g tris base, pH-adjusted to 9, and sterilised with 0.22µM cellulose Acetate filter (VWR International)

Table 2.4 Buffers

2.3 Cell culture

2.3.1 Cell maintenance

Cells were cultured in complete medium, which consisted of RPMI 1640 medium supplemented with 2 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, 50 mM 2-mercaptoethanol, and 10% heat-inactivated foetal calf serum (FCS). FCS, which had been *Mycoplasma* screened by the manufacturer, was heat-inactivated at 56°C for 30 minutes. Cultures were incubated at 37°C in a humidified incubator supplemented with 5% CO_2.

Viable cells were counted with a Neubauer haemocytometer (Weber Scientific International Ltd, UK) on a Nikon Labphot microscope, staining with 0.1% (w/v) trypan blue. During cell purification and washing, cells were centrifuged at 1400 rpm at 4°C unless otherwise stated.

2.3.2 Lymphocyte function assay – cell proliferation

Proliferation was assessed by culturing cells in triplicate in U-bottom 96 well plates (Nunc, Roskilde, Denmark) for 72 h. The final volume per well was 200 μl. Plates were pulsed with [^{3}H]thymidine (1 μCi/well, Amersham Life Science, Bucks, UK) for the last 8 h of culture. Plates were harvested onto a glass fibre filter (Wallac, Milton Keynes, UK) using a 295-0054 Betaplate^{TM} 96 well harvester (Wallac). [^{3}H]-

thymidine incorporation was measured using a Matrix 96 Direct Beta Counter (Wallac).

2.3.3 Cultures for cytokine assessment

Cytokine production was assessed by analysing supernatants from 24 well flat bottom plates or from 96 well plates (either U-shaped or flat bottom) after 72-96 hours of culture. Supernatants were stored at -20°C until cytokine analysis by ELISA was performed.

2.4 T Cell isolation

T cells were purified from murine lymph nodes by using magnetic beads. In brief, single cell suspension was prepared by forcing LN cells through 100 μm Nytex membrane (Cadisch & Sons, London, UK) twice using a 10 ml disposable syringe in the presence of RPMI-1640 medium. Cells were counted and then incubated with either non-conjugated rat, biotinylated, or PE-conjugated monoclonal antibodies. MACs buffer (Table 2.4) was added to make the reaction volume 40 μl per 10^7 total cells. After 20 minutes on ice, anti-rat, anti-biotin or anti-PE microbeads were added and incubated for another 20 minutes on ice. T cells were then washed twice and centrifuged at 200 g for 10 minutes. Thereafter T cells were separated by either negative or positive selection using an AutoMACS machine (Miltenyi Biotech) (353).

2.4.1 Negative selection of $CD4^+$ T cells

$CD4^+$ T cells were purified from BALB/c lymph nodes by negative selection using magnetic beads. Cells were incubated with a cocktail of biotin-conjugated monoclonal antibodies [against CD8a (Ly-2, Rat IgG2a), CD11b (Mac-1, Rat IgG2b), CD45R (B220, Rat IgG2a), DX5 (Rat IgM) and Ter-119 (Rat IgG2b)], at a concentration of 5 µl antibody per 10^7 total cells. Anti-biotin microbeads (10 µl per 10^7 cells) were added and $CD4^+$ T cells were then separated by negative selection.

2.4.2 Positive selection of $CD4^+CD25^+$ T cells

$CD4^+$ cells were then further separated into $CD25^+$ and $CD25^-$ populations by *auto*MACS using PE-labelled anti-CD25 Ab and anti-PE Ab conjugated with microbeads. Briefly, 1 x 10^8 $CD4^+$ T cells were incubated with PE-anti-CD25 antibody (3 µg) for 15 minutes at 4°C in the dark, and then washed twice with 20 x volume of MACS buffer (Table 2.4). The cells were then incubated with 35 µl anti-PE microbeads for 15 minutes at 4°C in the dark. The cells were re-suspended in 2 ml MACS buffer, and then separated by positive selection using the *auto*MACS machine. The purity of each final cell preparations was determined by staining cells with murine anti-CD4-FITC and anti-CD25-PE Ab and analysed by FACSCalibur using CellQuest software (BD). The purity of $CD4^+CD25^-$ T cells was routinely >97%, and $CD4^+CD25^+$ T cells >94%.

2.4.3 Positive selection of $CD4^+GITR^+$ cells

$CD4^+$ T cells were also further separated into $CD4^+GITR^+$ and $CD4^+GITR^-$ populations by positive selection using rat anti-GITR mAb (DTA-1 hybridoma) and anti-rat IgG Ab conjugated with microbeads. The purity of cell preparations was determined by FACS analysis using rat anti-GITR mAb and FITC-conjugated mouse anti-rat IgG $F(ab')_2$ fragment, and was routinely >90%.

2.5 Specific *in vitro* culture protocols

2.5.1 Anti-GITR mAb production

A hybridoma cell line producing rat IgG2a against mouse GITR was generously gifted by Professor Shimon Sakaguchi (Department of Experimental Pathology, Kyoto University, Japan). Antibody was generated as previously described (264). Briefly, hybridoma cells were cultured for 3 days in normal complete medium supplemented with 10% FCS. The cells were then washed in PBS, and cultured for a further 3 days in RPMI medium supplemented with immunoglobulin (Ig) -deplete FCS (Harlan, Loughborough, UK). The supernatant was then collected and frozen at -20°C, until antibody purification was performed. A protein G Sepharose 4 Fast Flow column (Pharmacia Biotech) was used for IgG purification. Prior to use the protein G column was washed with 20% ethanol (v/v), and balanced with 400ml of Buffer A (Table 2.4). Two and a half litres of supernatant was loaded at 4°C onto the protein G column. After removing low affinity proteins bound to the column with 200ml of

Buffer A, the antibody was washed off the column with an elution buffer. 2 ml aliquots were collected and mixed with 20 µl 2M tris HCl (Table 2.4). The protein concentration of each elute was estimated by measuring optical density (O.D.) at 280 nm. The aliquots with the highest O.D. values were then dialysed overnight in PBS (pH 7.4) at 4°C. The samples of antibodies were then stored at -20°C until used. The protein concentration for each aliquot was using Coomassie Protein Assay (Pierce, USA). Aliquots of antibody were routinely tested for LPS using an endotoxin kit (Sigma-Aldrich).

2.5.2 Regulatory T cell suppressive function assays *in vitro*

$CD4^+CD25^+$ and $CD4^+CD25^-$ T cells were separated from naïve BALB/c mice using an *Auto*MACS machine (as described above). Suppressor function assays were set up in 96-well U-bottom plates, with "effector" $CD4^+CD25^-$ T cells (1 x 10^4 cells/well) being cultured with "regulatory" $CD4^+CD25^+$ in a 1:1 ratio. Soluble anti-CD3 antibody (1 µg/ml), and mitomycin C-treated (50 µg/ml) $CD4^-$ spleen or lymph nodes cells (5 x 10^4 cells/well) were used as antigen presenting cells (APC). In some experiments, either 10 µg/ml control rat IgG Ab or anti-GITR mAb were added to cultures. Cells were cultured at 37°C in 5% CO_2 for 3 days, and proliferation and cytokine production was assessed.

2.5.3 Polarisation of T helper cells

$CD4^+$ T cells or $CD4^+CD25^-$ T cells were separated as above. Cells (1 x 10^6 cells) were then cultured in conditions to generate Th0 (pluripotent), Th1, or Th2 cells. For Th0 cell induction, cells were cultured with complete medium alone. Th1 cells were generated by culturing with murine IL-12 (10 ng/ml) and anti-IL-4 antibody (1 μg/ml). Th2 cells were produced by culturing with murine IL-4 (10 ng/ml), anti-IFNγ (500ng/ml), and anti-IL-12 antibody (1 μg/ml). At the end of a 3 day culture, the supernatants were collected and stored at -20 °C for cytokine analysis. The cells were rested for 3-4 days in medium containing IL-2 (10 ng/ml; BD PharMingen), and re-cultured in the conditions described above. After 2 cycles of culture the supernatants were again collected and stored at -20°C for cytokine analysis. The cells were phenotyped for Th1 or Th2 by cytokine secretion and CD44 and CD62L expression. More than 95% of the cells were $CD44^+CD62L^-$ and produced the expected type 1 or type 2 cytokines.

Where indicated, 10 – 100 μg/ml control rat IgG Ab or anti-GITR mAb was added to cell culture at the same time as the Th polarising cytokines and antibody. In some experiments, Pam3CSK4 (10 or 100 ng/ml) was added to the Th polarisation culture conditions. Cells were stimulated with graded doses of soluble or plate bound anti-CD3 mAb (0.5-5 μg/ml), as indicated in the text.

For antigen-specific stimulation, T cells were purified from DO11.10 mice - transgenic for TcR specific to OVA peptide (sequence 323-339). The OVA-specific

$CD4^+$ T cell or $CD4^+CD25^-$ T cells were cultured in the conditions described above, except that 0.3 μM $OVA_{323-339}$ peptide was used instead of anti-CD3 mAb.

2.5.4 Bone marrow derived dendritic cell (BMDC) and bone marrow derived macrophage (M) cell generation

BMMs and BMDCs were generated as described previously (354, 355). Briefly, femurs and tibiae of mice were removed and separated from the surrounding muscle in sterile conditions. Bone marrow was flushed out with RPMI-1640 medium using a 5 ml syringe with $26^{1/2}$G "brown" needle, and cell clusters were washed and passed through Nitex twice. For dendritic cell generation, cells (2 x 10^6 /ml) were seeded into 6-well plate with complete medium in the presence of GM-CSF (30 ng/ml) and IL-4 (10 ng/ml), and incubated for 3 days at 37°C in 5% CO_2. Half of the culture medium was then replaced with fresh complete medium supplemented with GM-CSF and IL-4, and cells were cultured for another 3 days. Non-adherent cells (DCs) were then removed and washed by cold RPMI. For macrophage cell generation, cells (5 x 10^6 /ml) were cultured in a total volume of 10 mls complete medium supplemented with CSF-1 (20 ng/ml) in petri dishes. After 3 days half the medium was replaced with fresh medium supplemented with CSF-1, and adherent cells were scraped off the bottom of ice-cooled petri dishes after a further 3 days culture.

The macrophage and dendritic cells were cultured at 1 x 10^6 cell/ml with 0.3 μM $OVA_{323-339}$ peptide and Pam3CSK4 (10 or 100 μg/ml) for 12 h. Supernatants were collected and stored at -20°C for cytokine analysis. The residual cells were then washed and cultured with 1 x10^6 $CD4^+$ T cells (purified from DO11.10 transgenic

mice), 0.3 μM $OVA_{323-339}$ peptide and Pam3CSK4 (10 or 100 μg/ml) for a further 12 h. Supernatants were collected and stored at -20 °C for cytokine analysis.

2.6 Murine model of allergic asthma

Murine models of allergic asthma are widely used (reviewed in (356)). Mice are sensitised to protein allergens with adjuvant and subsequently re-challenged repeatedly with allergen into the airways. This leads to the development of airway eosinophilia and airway hyperresponsiveness. Female BALB/c mice (6 - 8 weeks old) were used except in studies involving IL-12 and IFNγ gene knockout (ko) mice. Female 129/Sv mice were used as control mice for IFNγ ko experiments.

Avertin (357) was used as an anaesthetic during all experiments. A stock solution was made by dissolving 2,2,2 tri-bromoethanol in a 1:1 ratio (w/v) with amyl alcohol. This was stored at 4°C until immediately before use when a working solution was made by diluting the stock 1:40 in LPS-free sterile PBS.

2.6.1 Induction of airway eosinophilia in mice

Mice were sensitised to ovalbumin (OVA), as described in Figure 2.1 by modification of a previously published protocol (358). All OVA solutions were filter sterilised prior to use (Millex-GV 0.22 μm, Millipore, Livingston, UK), and aliquots were routinely tested for LPS using an endotoxin kit (Sigma-Aldrich). An alum suspension (Alhydrogel 2%) was used as an adjuvant. To adsorb the OVA to the alum, a 1 mg/ml

solution of OVA in PBS was added to an equal volume of the alum suspension and mixed using a vortex mixer. Mice were immunised intraperitoneally (i.p.) on day 0 and 14 with 100 μg OVA in an alum suspension made up to a volume of 200 μl. On day 14, mice were anaesthetised with 250 μl of avertin, and 100 μg of OVA in 40 μl of PBS was administered intranasally (i.n.). Mice were again anaesthetised before being challenged i.n. on each of the days 25, 26, and 27 with 10 μg OVA in 40 μl of PBS. Control mice were given PBS in place of OVA in both the sensitisation and the challenge stages.

2.6.2 Physiological measure of airways hyperresponsiveness – Enhanced Pause

Whole Body Plethysmography is a technique that has been used to monitor lower airways resistance in unrestrained conscious mice. Enhanced Pause (Penh) is a function of the small pressure differences observed in the chambers housing mice compared to a reference chamber. Penh was measured at 12.5 to 50 mg/ml methacholine on day 28, as previously described (359). Briefly, mice were placed in a 4 chamber plethysmography unit (EMMS, England, U.K.), and left undisturbed for a 30 minute acclimatisation period. Mice were then challenged for 2 minute periods with saline (control) or graded doses of methacholine. Airways hyperresponsiveness was then estimated as Penh, using EMMS EDAQ® software.

2.6.3 Immunological measures of allergic airways disease

Mice were sacrificed on day 29 by administration of a fatal dose of avertin. Blood, bronchooalveolar lavage, lymph nodes and spleen were harvested and kept on ice

until processed (see below).

In some experiments, disease-modifying agents were administered. The amounts, timings and routes of delivery of these compounds are outlined in the relevant results chapter.

2.6.4 Bronchoalveolar lavage (BAL)

The thorax was opened carefully and blood was taken by cardiac puncture. The trachea was then exposed and a small transverse incision made in it immediately below the level of the larynx. Polythene tubing (0.58mm ID, 0.78mm OD; VWR International) threaded over a 23 gauge 'blue' needle was inserted into the trachea and held in place using 12 cm blunt forceps. The lungs were then lavaged by sequentially inserting and withdrawing two volumes of 0.8ml of PBS, ensuring that both lungs were seen to inflate during the lavage process and that there was no leakage of lavage fluid from the trachea. The two lavage samples from each mouse were pooled in a 1.5 ml microcentrifuge eppendorf tube (Thistle Scientific, Uddingston, UK) and kept on ice until processing. The volume of each sample was estimated by weighing each sample and subtracting the weight of an empty eppendorf. The BAL fluid was centrifuged at 1400 rpm for 5 minutes to pellet the cells and the supernatant removed. Supernatants were frozen at –20°C until analysis. The cells were re-suspended in 1ml of PBS, and the cell numbers were counted. The absolute cell count per lavage was divided by the lavage volume to standardise the cell count per ml of lavage fluid.

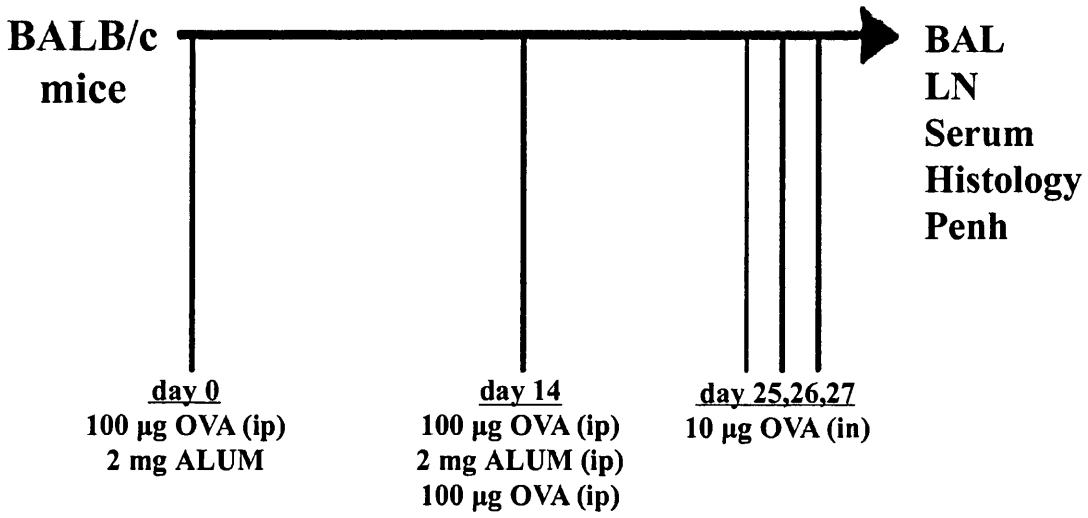

Figure 2.1 Experimental protocol used to induce allergic airways inflammation.

Mice were injected i.p. with 100 µg OVA and 100 µl 2 % Alum on day 0 and 14. The mice were boosted i.n. with 100 µg OVA on day 14. All the mice were then challenged i.n. on 3 consecutive days beginning on day 25. Penh was determined on day 28 and mice were sacrificed on day 29. Serum, BAL and lymphoid cells were collected and lung histology studied.

2.6.4.1 BAL differential cellularity

The BAL cellularity was investigated by preparing and staining slide cyto-centrifuge preparations using a Shandon Cytospin. Approximately 1 x 10^5 cells were centrifuged at 400 rpm for 6 minutes then fixed in methanol for 10 minutes. The cell preparations (cytopreps) were stained with Diff-Quik (Triangle Biomedical Sciences, Skelmersdale, UK), a staining kit for rapid Romanowsky staining. Differential cell counting was done using standard morphological criteria (Fig. 2.2). Four hundred cells were counted per slide. Differential cell counts were expressed as a percentage of the number of cells then multiplied by the total cell count per ml to give an absolute cell number.

2.6.5 Lung histology

After BAL sampling, the lungs were removed and the lungs inflated with 1ml of 10% neutral buffered formalin (10% (v/v) of 37% aqueous solution of formaldehyde (Aldrich), in 30mM $NaH_2PO_4.H_2O$, 45 mM Na_2HPO_4, pH 7). The trachea was tied with 2-0 silk suture (Ethicon, Edinburgh, UK) and the lungs immediately immersed in 10% neutral buffered formalin for at least 72 hours. After fixation, the left lung was then dissected free, embedded in paraffin and 6 μm sections cut (kindly performed by Mr. Roderick Ferrier, Department of Pathology, Western Infirmary, Glasgow). Sections were stained with haematoxylin and eosin (H&E). Sections were examined at x 20 – 100 magnification and peri-bronchial and peri-vascular inflammation assessed.

2.6.6 Lymphoid cell culture

Thoracic lymph nodes and spleens were obtained by careful dissection, and stored in complete RPMI on ice until processing. A single cell suspension was obtained by gently pressing lymph nodes and spleens through sterile Nytex using a 5 ml syringe plunger. The cell suspension was washed three times in complete RPMI, passed again through Nytex to remove cellular aggregates and debris. After re-suspended in complete medium, cells were count using a haemocytometer.

Proliferation assays using thoracic lymph node cells and spleens were performed in triplicate in U-bottom 96 well culture plates at 2 x 10^5 cells/well in 100 μl complete medium. Additional cell stimulating agents or medium were added in volumes of 100 μl to give a final culture volume of 200 μl per well. Cells were stimulated with a final concentration of 100 – 1000 μg/ml OVA or 2-5 μg/ml Concanavalin A. Cell culture was allowed to proceed for 72 hours.

Cytokine production by lymphoid cells was measured using supernatants derived from parallel cultures set up in 24 well flat-bottom plates (Nunc). These culture supernatants were harvested after 96 hours. Supernatants were frozen at –20°C until cytokine levels were measured by ELISA.

Mice sensitised and challenged with:

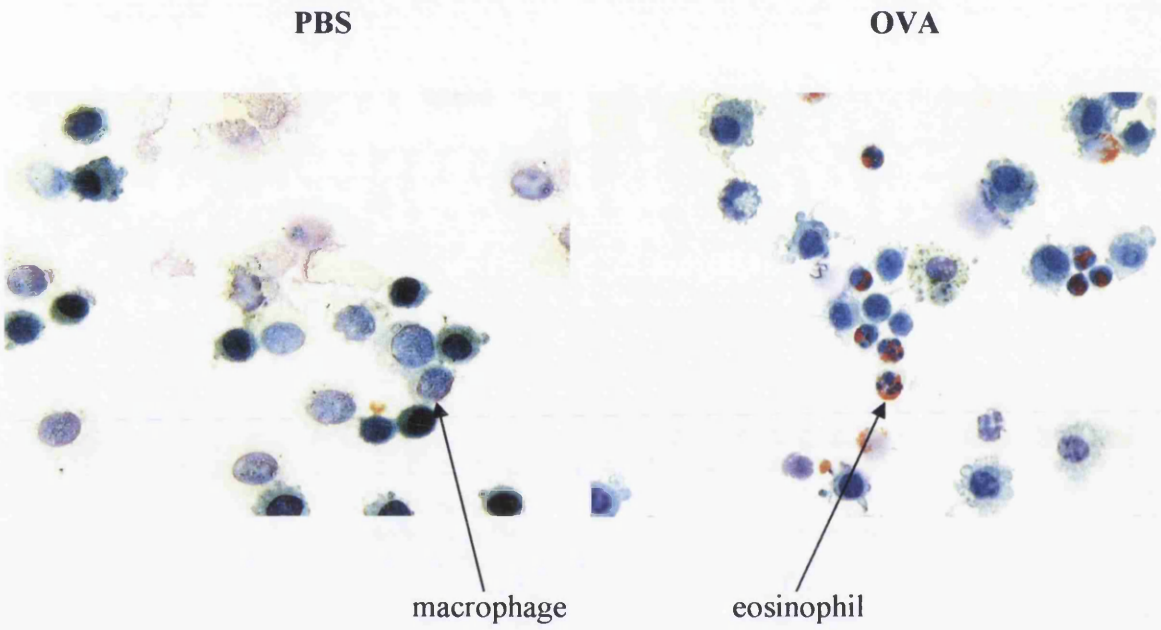

Figure 2.2 Typical bronchoalveolar cytospin preparations.

PBS-sensitised and –challenged mice demonstrated that macrophages were the predominant cell type in BAL. OVA-sensitised and –challenged mice displayed BAL eosinophilia and macrophages.

2.7 Peritoneal lavage of mice

In some experiments, peritoneal lavage was performed to determine the differential cellularity. Mice were terminated by dislocation of the neck, and their peritoneal cavities exposed by careful dissection. 5 mls of sterile PBS was instilled into the peritoneum using a 20 G "yellow" needle. After 15 seconds, the fluid was slowly removed and stored on ice until processing. The volume of each sample was estimated, and the peritoneal fluid was centrifuged at 1400 rpm for 5 minutes to pellet the cells. The supernatant was removed, and the cells were re-suspended in 1ml of PBS. The cell numbers were counted, and the absolute cell count per lavage was divided by the lavage volume to standardise the cell count per ml of lavage fluid. Peritoneal fluid cellularity was investigated by preparing and staining slide cyto-centrifuge preparations using a Shandon Cytospin (as described above in Section 2.6.4.1, p96). The cell preparations (cytopreps) were stained with Diff-Quik. Differential cell counting was done using standard morphological criteria. Four hundred cells were counted per slide. Differential cell counts were expressed as a percentage of the number of cells then multiplied by the total cell count per ml to give an absolute cell number.

2.8 Flow cytometry

2.8.1 Cell surface markers

Freshly purified or activated cells (5 x 10^5 cells) were re-suspended in 100 µl FACS buffer (PBS with 2% FCS and 0.05% NaN_3) in 12 x 75 mm polystyrene tubes (Falcon BD, Oxford, UK), and incubated with anti-CD16/32 antibody (FcR block, 0.5 µg/ 100 µl) for 15 minutes at 4°C in order to block non-specific Fc bindings.

Samples were stained with directly conjugated antibodies: αCD4 (PE), αCD4 (FITC), αCD25 (PE), αGITR (FITC), αCD69 (FITC), and αCD44 (FITC), together with appropriate isotype controls were prepared, and incubated in the dark for 30 minutes at 4°C in FACS buffer. After one wash, cells were re-suspended in 500 µl FACSFlow and analysed with a FACSCalibur flow cytometer (Becton Dickinson) equipped with a 488 nm Argon laser and a 635 nm red diode laser, data were analyzed by Cellquest software (BD).

In initial experiments with GITR staining, a directly conjugated antibody was not available. Hence, indirect staining was performed with rat anti-GITR mAb (1µg/ 5 x 10^5 cells) binding the cells and FITC-conjugated mouse anti-rat IgG F(ab') (1:125, (v/v)) used as a secondary label.

2.9 Enzyme linked immunosorbance assay (ELISA)

2.9.1 Cytokine assays

The concentration of cytokines in cell culture supernatants, lung lavage fluid, and serum were determined by ELISA with paired antibodies (BD. PharMingen). Details of the concentrations of antibodies used and the lower limit of detection of each assay are given in Table 2.4. Immulon 4 plates (Thermo Labsystems, Franklin, USA) were incubated overnight at 4°C with the appropriate concentration of capture antibody in 0.1 M $NaHCO_3$ coating buffer. Plates were then washed 3 times with ELISA washing buffer (Table 2.4). For each wash, wells were filled with washing buffer and allowed to stand for at least 1 minute prior to removal of the wash buffer. Plates were washed 4-6 times between each of the subsequent steps. Non-specific binding was reduced by incubating 1-2 h with ELISA blocking buffer (200 µl/ well) at room temperature. Culture supernatant (50 µl/well) were then added to the plate in triplicate. Serial two-fold dilutions of recombinant cytokines were used as standard in duplicate. The plates were incubated for 2 h followed by a matched biotinylated antibody (detection antibody) for 1 h at room temperature. Extravidin Peroxidase (1:1000 dilution) was added, and the plates were incubated for 30-45 minutes at 37°C. 100 µl TMB (3',3',5'5'- tetra -methylbenzidine) Microwell Peroxidase Substrate was then added. Plates were incubated in the dark for 5-30 minutes depending on the rate of development of colour. The plates were then read at 630 nm on a MRX II microplate reader (Dynex Technologies, U.K.), always within 1 hour of the TMB being added. In some experiments, IL-10, IL-13 and TGFβ detections were performed by Cytoset Kit (Biosource, UK) according to the manufacturer's instructions.

2.9.2 Murine serum immunoglobulin measurements

2.9.2.1 Serum OVA-specific IgG1, IgG2a and IgE levels

Blood collected from mice coagulated at room temperature for 6 hours. Therafter, samples were centrifuged at 14,000 rpm for 30 minutes and sera aliquoted off. Murine serum OVA-specific IgG1, IgG2a and IgE levels were determined using a modification of the ELISA protocol described above, and of a previously published method (360). At least five washes with ELISA wash buffer (Table 2.4) were done between each step of the protocol. Immunolon 4 micro-ELISA plates were coated at 4°C overnight with 50 µl of a 10 µg/ml solution of OVA in a coating buffer of 0.05 M carbonate/bicarbonate buffer. The plates were then blocked with 200 µl of ELISA blocking buffer (Table 2.4) for 1 hour at room temperature. Serum was diluted appropriately in wash buffer, then 100 µl of the diluted serum added across the top row of the plate, leaving column 12 with buffer alone. The starting dilutions were 1 in 250 for serum IgG1 assays, 1 in 50 for Ig2a assays and 1 in 20 for OVA-specific IgE assays. The samples were then serially diluted with wash buffer through rows two to eight of the plate. IgG1 samples were diluted 1:3 each time and IgG2a and IgE assays. The samples were then serially diluted with wash buffer through rows two to eight of the plate. IgG1 samples were diluted 1:3 each time and IgG2a and IgE samples diluted 1:2. Samples were then incubated at room temperature for 1½ hours. Biotinylated anti-mouse IgG1, IgG2a and IgE (BD Biosciences) antibodies were added to the appropriate plates at a concentration of 0.5 µg/ml in 50 µl wash buffer. The plates were incubated for 1 hour at room temperature. Thereafter, 50 µl of a 1:1000 dilution of ExtrAvadin in wash buffer was added to each well and incubated

Cytokine	Capture Antibody (μg/ml)	Detection Antibody (μg/ml)	Lower Limit of Detection of Assay (pg/ml)
IL-2	2	1	40
IL-4	2	2	30
IL-5	2	2	30
IL-12	2	0.5	40
IFN-γ	1	0.5	40

Table 2.5 Antibodies used in murine cytokine ELISAs.

The antibodies used for the quantification of murine cytokines by ELISA in cell culture supernatants.

All antibodies were purchased from BD Biosciences, Cowley, UK.

for 30 minutes at room temperature. The IgG plates were developed using 100 µl of TMB substrate and then read at 630 nm, as described in section 2.8.1 above. In the case of OVA-specific IgE, the reaction was stopped at 30 minutes by the addition of 50 µl 1 M H_3PO_4. Plates were then read at 450 nm with 570 nm correction. The mean optical density for a designated serum dilution for each of OVA-specific IgG1, IgG2a and IgE was recorded to give a comparison of the serum levels of these antibodies among different treatment groups.

2.9.2.2 Measurement of serum total IgE levels

Total murine serum IgE levels were measured using commercial ELISA kit (OptEIA, BD Biosciences) following the manufacturer's guidelines. The working volume was 100 µl. At least 4 washes were performed between each step. Immunolon 4 micro-ELISA plates were coated with a 1:250 dilution of capture antibody in 0.1 M carbonate/bicarbonate buffer (0.1 M $NaHCO_3$, 0.1 M Na_2CO_3, pH 9.5) and incubated at 4°C overnight. Plates were blocked by incubating with 200 µl of ELISA blocking buffer for 1 hour at room temperature. Mouse serum samples diluted 1:200 in ELISA wash buffer and then incubated for 2 hours at room temperature. Samples and the supplied IgE standard were tested in duplicate. The standard curve ranged form 1.6 ng/ml to 100 ng/ml. Detection antibody and detection agent, both at a dilution of 1:250 in ELISA buffer, were then added and samples incubated for 1 hour at room temperature. TMB substrate was used to develop the ELISA. This reaction was stopped at 30 minutes by the addition of 50 µl 1 M H_3PO_4. Plates were then read at 450 nm with 570 nm correction.

2.10 Reverse transcription – polymerase chain reaction (RT-PCR)

Real time PCR was used when investigating the mechanism behind the effects of anti-GITR mAb on $CD25^-$ effector T cells *in vitro*. By quantifying the messenger RNA signals, we examined the stage of protein synthesis at which anti-GITR mAb was having its effect.

2.10.1 RNA purification

Total RNA was extracted from freshly purified or activated T cells. Cells ($0.5\text{-}1 \times 10^6$) were re-suspended in 400 µl RNA-Bee (Table 2.3) by pipetting up and down. RNA-Bee is a reagent that isolates and preserves RNA. Samples were treated immediately or stored at -70°C before processing. Frozen samples were thawed on ice and 50 µl of Choloroform (Sigma-Aldrich) added. The mixtures were then incubated on ice for 15 minutes. RNA, DNA and protein were separated by centrifugation at 14,000 rpm at 4°C for 15 minutes. The upper aqueous phase was removed into a clean RNAase-free eppendorf tube (Ambion) and an equal volume of cold RNAase-free 2-isopropanol added. Samples were incubated at -20°C for 1 h before centrifuging as above. The RNA containing pellet was washed in 100 µl ice cold RNAase-free 70% ethanol and re-suspended in 20-50 µl cold RNAase-free water depending on the original cell numbers.

2.10.2 DNase treatment

The RNA preparations may have genomic DNA contamination that could potentially be amplified in later steps. Therefore, TURBO DNA-free^{TM} kit (Table 2.3) was used to remove genomic DNA. Briefly, 1-2 units TURBO DNase were added to 20 µl of RNA in the presence of the TURBO DNAse buffer, and incubated in a 37°C water bath for 1 h. DNAse inactivation regent (2 µl) was then added for 2 minutes at room temperature, and removed by centrifuging at 14,000 rpm for 1 minute. RNA samples were transferred into RNAse- free tube to perform RT-PCR.

2.10.3 Reverse Transcription PCR

Cloned (c) DNA was synthesized using superscript II RNase H-reverse transcriptase and OligodT_{12-18} (Invitrogen) was used as primers. Briefly, 1-5 µg RNA was incubated with 1 µl Oligo (500 µg/ml) and 1 µl dNTP mix (10 mM) in a total volume of 12 µl. The mixture was heated at 65°C for 5 minutes and quickly chilled on ice. Samples were reverse-transcribed using 1 µl (200 units) SuperScript II reverse transcriptase with 4 µl 5 x first-strand buffer, 1 µl 0.1 M DTT, 1 µl distilled H_2O at 42°C for 50 minutes and then inactivated by heating samples at 70°C for 15 minutes. Negative control samples (without first-strand synthesis) were prepared in the absence of reverse transcriptase.

2.10.4 Primers and probe design

Oligonucleotide primers and fluorescent probes were designed to the interest genes using the PrimerExpress™ 1.5 program. Sequence of interest gene was obtained from genebank. For primers, optimal primer Tm is 59-60°C, less than 2 degree between the forward and reverse primers and including 20-80% GC; for probe, optimal Tm is 69°C or 10°C higher than the Tm of primers, no G on the 5' end and with less than 4 contiguous Gs, and have more Gs than Cs. The fluorescent probes contained a reporter dye (FAM) covalently attached at the 5' end and a quencher dye (TAMRA) attached at the 3' end. Either one of the primers or probe was designed to span two intron-exon boundaries, allowing discrimination between products resulting from amplification of cDNA and contamination of genomic DNA. Primers specificity was checked against the BLAST® database, found within the PubMed World Wide Web site. This was to ensure that there is no significant homology with other genes from the same species.

2.10.5 Real-time PCR

Real-Time PCR was performed using an ABI prism 7700 sequence detector (PerkinElmer Applied Biosystems, Foster City, CA) according to the manufacturer's instruction, primers and probes are shown in Table 2.6. The principle of the method is outlined in Figure 2.3. Each reaction contained 12.5 µl Master mix (10 mM dNTP mix, 5 mM $MgCl_2$, 0.625 U AmpliTaqGold™, 0.25 U AmpErase Uracil N-Glycosylase, 10x qPCR™ buffer), 1 µl probe, 0.75 µl of each of the primers, 9 µl

Gene	*Forward Primer*	*Reverse Primer*	*Probe (5'-FAM&3'-TAMRA)*
T-bet	5'-GCCAGGGAACCGCTTATATG -3'	5'-AACTTCCTGGCGCATCCA -3'	5'-CCCAGACTCCCCCAACACCGGA-3'
GATA3	5'-TCCTCCTCTACGCTCCTTGCTA -3'	5'-ACACTGATTCCTTGGCGCTC -3'	5'-TCGTGATCGGAAGAGCAACCGTCTC -3'
HPRT	5'-GCAGTACAGCCCCAAAATGG-3'	5'-AACAAAGTCTGGCCTGTATCCAA -3'	5'-TAAGTTGCAAGCTTGCTGGTGAAAAGGA-3'

Table 2.6 Taqman (real time) PCR primer and probes

H_2O, 1 µl cDNA template in a total volume of 25 µl. Amplifications were performed in triplicates in a Thermo-Fast 96 Semi-skirt plate (ABgene). The cycling was initiated at 50°C for 2 minutes and followed at 95°C for 10 minutes for 1 cycle; 95°C for 15 seconds and 60°C for 1 minute for 45 cycles. Data analysis was performed using Sequence Detector software (PerkinElmer Applied Biosytems). This represents the PCR cycle at which an increase in fluorescence above a set threshold level can first be detected (Figure 2.4). Expression of the gene of interest in a sample was normalised by comparison to the expression of a reference reporter gene, HPRT. Firstly, the ΔCt was obtained by subtracting the Ct value of HPRT from the Ct value of the gene of interest. The formula $2^{-\Delta\Delta Ct}$ was then used to calculate a value for the fold increase in gene expression relative to HPRT. Multiplication of this value by 100 gives expression of the gene of interest as a percentage of HPRT. The positive error is the standard deviation of the difference, $s = \sqrt{(s_1^2 + s_2^2)}$ where s_1 and s_2 are the standard deviations of the Cts of the HPRT and the gene of interest.

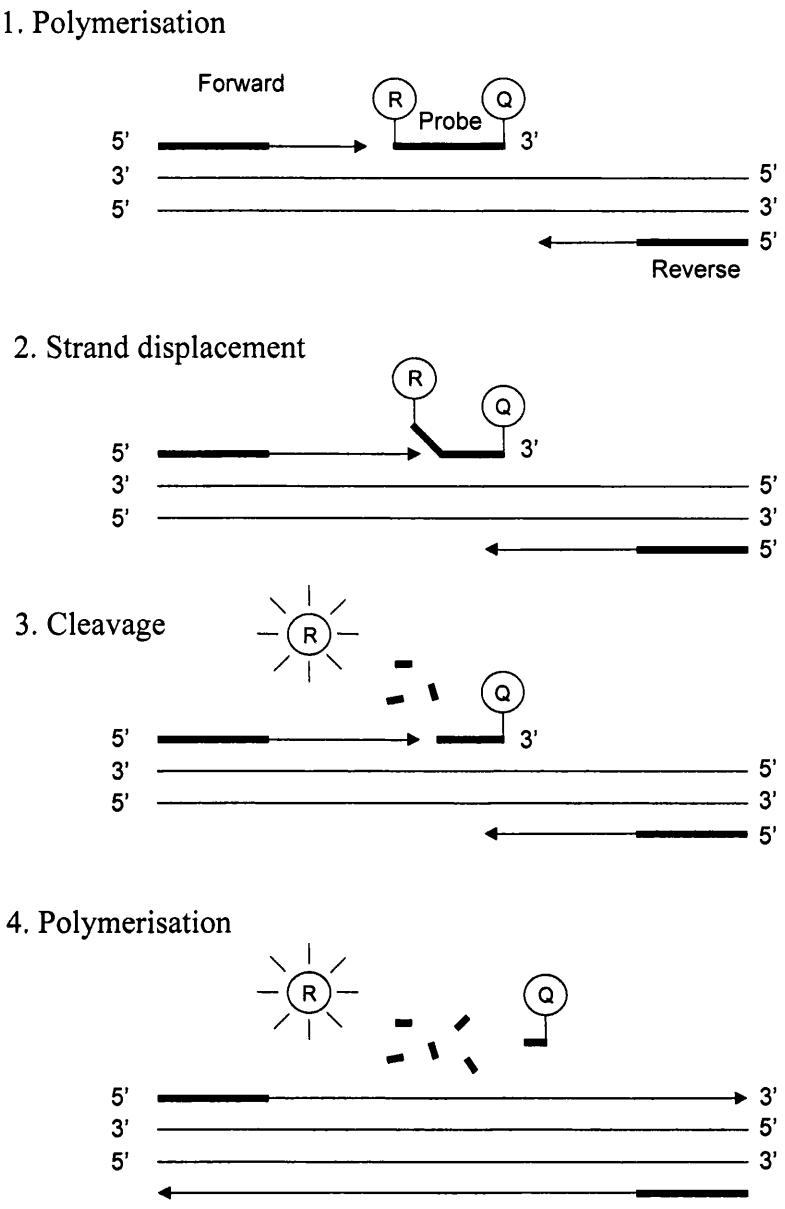

Figure 2.3 Taqman real-time RT-PCR.

The Taqman probe is an oligonucleotide with a 5' reporter dye (R) and a 3' quencher (Q). In an intact probe, the proximity of the quencher to the reporter causes suppression of reporter fluorescence. During PCR, the probe is cleaved by the 5' → 3' exonuclease activity of Taq DNA polymerase separating the reporter from the quencher. Reporter fluorescence increases and can be used to determine the quantity of specific product generated. The 3' end of the probe is blocked to prevent extension of the probe during PCR.

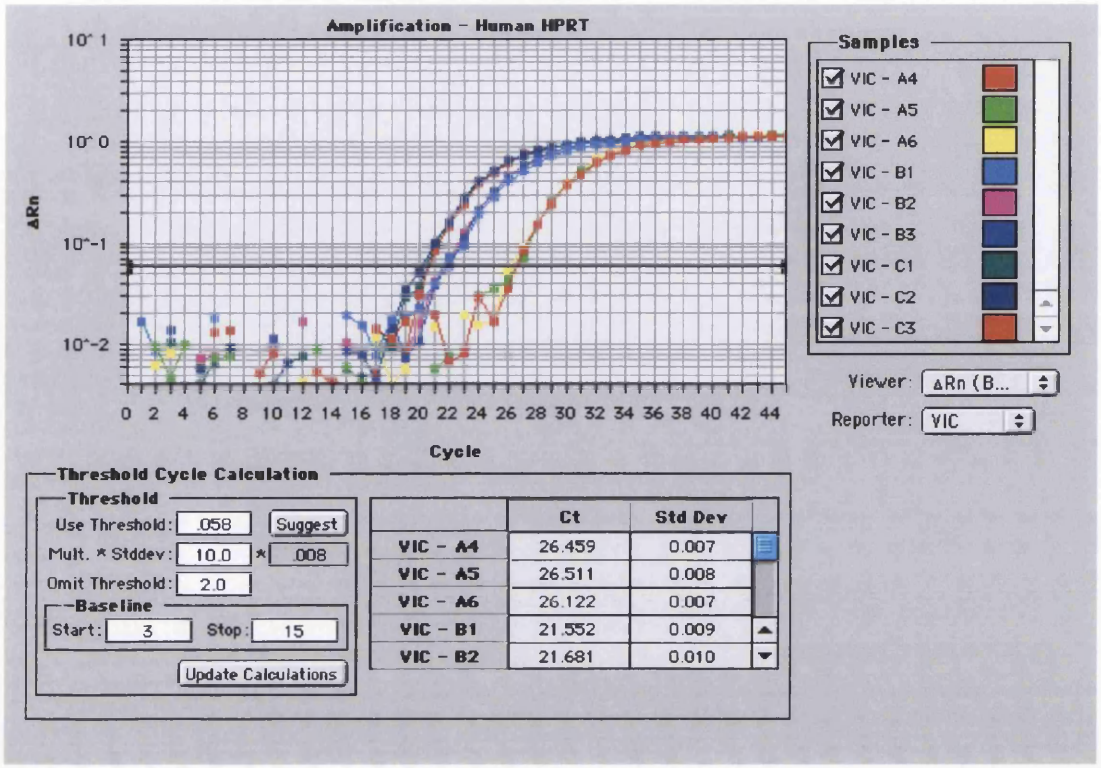

Figure 2.4 Typical Taqman amplification plots.

The amplification plot shows how the fluorescence emission (normalised Reporter, Rn) varies with the PCR cycle number. Initially the fluorescent signal is below the detection limit of the Sequence Detector. As the PCR reaction proceeds the signal can be detected as it continues to increase in direct proportion to the amount of specific amplified product. The Rn reaches a plateau when the ratio of polymerase enzyme to PCR product decreases preventing the amount of PCR product from increasing exponentially. The Ct (threshold cycle) is determined during the exponential phase (threshold indicated by the black line). The panel above displays plots for three triplicate samples (A4-A6, B1-B3 and C1-C3).

2.11 Statistical analysis

Data were collated and statistical analyses performed using a statistical software package (Minitab Statistical Software, Minitab Inc., State College, PA, USA). Power calculation to estimate sample size to examine for statistical differences in BAL eosinophilia were performed using MINITAB (Fig. 2.5). The Student's *t*-test and analysis of variance (ANOVA) were used to compare data sets with normal distribution. Non-parametric statistics were used (Mann-Whitney *U* test) for comparison between data sets with skewed distribution. *$p<0.05$ was considered significant, and **$p<0.01$ was deemed highly significant.

Eosinophil percentage difference we felt was significant is: 10%
Standard deviation of eosinophilia is: 5%
Significance level is: 0.05
Power value is: 0.95

The calculation below shows that each group in an experiment requires at least 8 mice (copied from MINITAB worksheet).

Power and Sample Size

```
2-Sample t Test

Testing mean 1 = mean 2 (versus not =)
Calculating power for mean 1 = mean 2 + 10
Alpha = 0.05  Sigma = 5

Sample   Target  Actual
  Size    Power   Power
     8   0.9500  0.9602
```

Figure 2.5 Power calculation to estimate sample size required to demonstrate differences in bronchoalveolar lavage eosinophilia

Chapter 3

The immunomodulatory effect of Pam3CSK4 in a murine model of allergic airways disease

Data in this chapter has been published in

Patel *et al* (2005), *J Immunol*; **174(12)**:7558-7563.

3.1 Introduction

The prevalence of asthma in developed countries has increased approximately threefold over the last twenty years, which cannot be explained by changes in genetic predisposition. Environmental factors, especially in industrialised countries, are now thought to be responsible for this rapid increase (361, 362).

Respiratory infections have been linked to asthma in either a preventative or exacerbating role (361, 362). Hence, investigating the components of the immune system that initially recognise the respiratory tract pathogens seems imperative to the further understanding of this inflammatory disease. Toll-Like receptors (TLRs) are pattern recognition receptors, which act as primary sensors of microbial products. There are now 10 TLRs identified in humans, in both the innate and adaptive immune systems, where they play a pivotal role in the response directed against distinct structurally conserved components of pathogens (153, 363). Bacterial endotoxin (LPS) is recognised by TLR4, and administration of low dose intranasal LPS in a murine model of asthma resulted in an increase in the Th2-mediated allergic inflammation (152). However, in the same murine model administration of high dose LPS ameliorated airways inflammation. Immunomodulatory oligodeoxynucleotides (ISS-ODN) and CpG DNA can reduce allergic inflammation in experimental models of asthma, via the activation of TLR9 (364). Thus far, CpG DNA is the only TLR agonist that has the ability to reverse established airway inflammation in both acute and chronic murine models of asthma (364, 365).

Recently, it has been demonstrated that TLR2 polymorphism is a major determinant of the susceptibility to asthma and allergies in children of farmers in Germany (366). TLR2 recognises microbial components by forming a heterodimer with TLR1 or 6 (78), and studies have examined the effect of the TLR2 agonists synthetic lipopeptide (Pam3CSK4) and peptidoglycan (PGN) on murine models of asthma. The results were contradictory. When administered during the sensitisation period, early TLR2 agonist treatment was reported to worsen asthma (149, 367). In contrast, TLR2 activation immediately prior to the intranasal challenge was found to reduce allergic airways inflammation (368, 369). It is important to note, however, that although PGN was thought to activate TLR2/6 heterodimer, recent work suggests that lipoprotein- and lipotechoic acid-contamination of PGN may be responsible for its TLR2 activation (370). All studies to date have examined TLR2 activation prior to the airway challenge in these models (149, 367, 368), and none have examined the therapeutic potential of TLR2 agonists in established airways inflammation.

We investigated the therapeutic effect of the synthetic TLR2 agonist Pam3CSK4 in a murine model of airways inflammation. TLR2 agonists were administered both immediately before (termed "prophylactic" administration) and after intranasal allergen challenge (termed "established inflammation" administration). We report here that intraperitoneal Pam3CSK4 profoundly attenuated OVA-specific asthma in mice. Our data therefore provide direct evidence that Pam3CSK4 or other TLR2 agonists could have potential as a new therapeutic approach in the treatment of clinical asthma.

3.2 Prophylactic intraperitoneal (i.p.) TLR2 agonist ameliorates airways inflammation in a murine model of asthma

Initial experiments were performed to determine if the TLR2 agonists - Pam3CSK4 or PGN - could influence the development of allergic airways inflammation. Bacterial lipopetides are characterized by unique NH_3-terminal lipo-amino acid, *N*-acyl-*S*-diacylglycerol cysteine, and are the found in the outer membrane of gram-positive and gram-negative bacteria. Pam3CSK4 (BLP or $Pam_3CysSerLys_4$) is a synthetic analogue of a bacterial lipopeptide, and is recognised by TLR2/1 heterodimer (371). Peptidoglycan (PGN) consists of a glycan backbone with alternating β 1-4 linked residues of N-acetyl-D-glucosamine and muramic acid. PGN is a major component of gram-positive bacteria, and activates the immune system via TLR2.

BALB/c mice were sensitised and challenged with OVA, as outlined in Figure 3.1, and Pam3CSK4 or PGN was given by intraperitoneal injection (i.p.) 2 hours prior to the antigen challenge on day 25. OVA control mice were treated with i.p. PBS. PBS control mice were sensitised and challenged with PBS. The dose of PGN or Pam3CSK4 used was determined by *in vivo* titration, with the optimal dose being 100 μg/mouse.

The results for prophylactic i.p. Pam3CSK4 and PGN administration in murine asthma are very similar. Hence, to avoid duplication the data for Pam3CSK4 are shown and discussed below. Any differences seen between Pam3CSK4 and PGN are highlighted in the text.

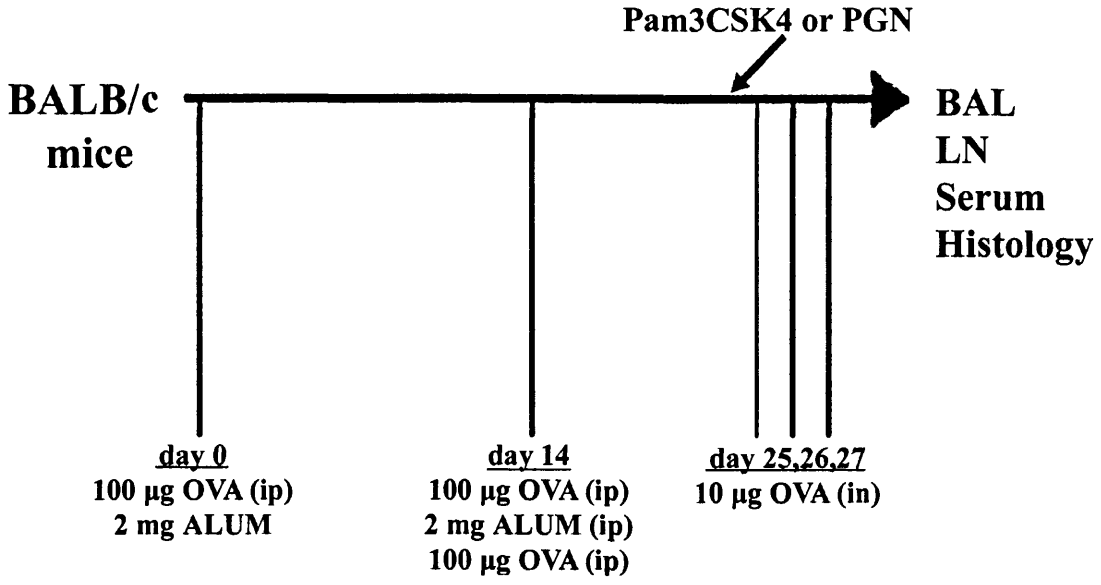

Figure 3.1 Experimental protocol to examine the role of prophylactic i.p. TLR2 agonists on allergic airways disease.

BALB/c mice were injected i.p. with 100 µg OVA and 100 µl 2 % Alum on day 0 and 14. The mice were boosted i.n. with 100 µg OVA on day 14. All the mice were then challenged intranasally (i.n). on 3 consecutive days beginning on day 25. Pam3CSK4 or PGN (25-100 µg per mouse) was administered i.p only once on day 25. TLR2 agonist was given 2 h prior to i.n. challenge. Serum, BAL and lymphoid cells were collected and lung histology studied on day 28.

3.2.1 Prophylactic intraperitoneal Pam3CSK4 treatment reduces total bronchoalveolar lavage cellularity and eosinophilia

Pam3CSK4 administration prior to i.n. allergen challenge resulted in a marked reduction in BAL eosinophil numbers, as compared to untreated OVA-sensitised and -challenged mice (Fig. 3.2). BAL cellularity was not significantly affected by TLR2 agonist administration. BAL cytokines were not detected.

3.2.2 The effect of prophylactic intraperitoneal Pam3CSK4 treatment on OVA-induced thoracic lymph node responses *in vitro*

To investigate the immunological mechanism involved in the Pam3CSK4 treatment, thoracic lymphoid node cells were harvested from the Pam3CSK4-treated and untreated control mice and cultured with OVA *in vitro*. Pam3CSK4 therapy did not affect the T cell proliferation against OVA *in vitro* (data not shown). However, the lymphoid cells from Pam3CSK4-treated OVA sensitised/challenged mice produced significantly more IFNγ compared to untreated OVA-sensitised/challenged mice. In contrast, Pam3CSK4 treatment led to a marked reduction in IL-4 and IL-5 synthesis by the lymphoid cells from OVA-sensitised/challenged mice (Fig. 3.3). Con A-induced responses from thoracic cell node cells from mice treated with Pam3CSK4 showed a similar cytokine profile to that of OVA stimulation. Culture of LN cells with medium alone resulted in no cytokines being detected (data not shown).

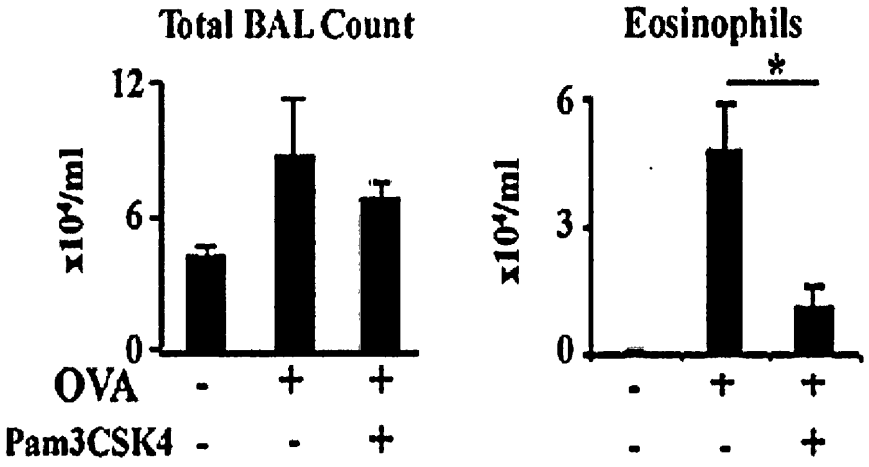

Figure 3.2 BAL eosinophilia fell after prophylactic i.p. Pam3CSK4 therapy.

BAL total cell concentration and eosinophil proportion fell markedly after Pam3CSK4 therapy. Mice given i.p. Pam3CSK4 (100 μg/mouse) on day 25 demonstrated a highly significant fall in their BAL eosinophilia (**$p<0.01$ compared with mice given OVA alone). Data are mean +/- SEM, n=5 and are representative of 3 experiments.

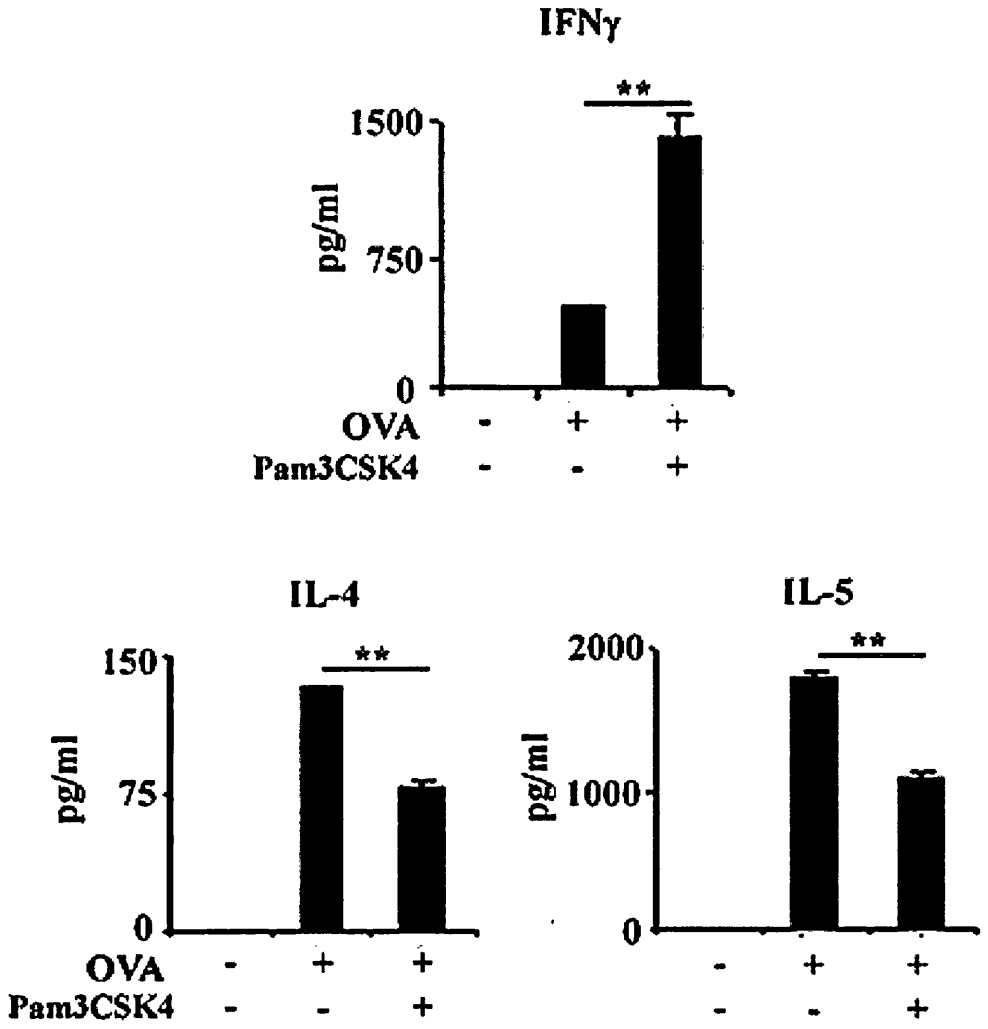

Figure 3.3 ***In vitro*** **OVA-induced IFNγ, IL-4, and IL-5 responses after prophylactic i.p. Pam3CSK4 therapy.**

Lymph node cells were harvested on day 28 and cultured with 1 mg/ml OVA *in vitro* (as described in Materials and Methods, Section 2.6.6, p97). Culture supernatant was collected at 96 h and cytokine concentrations assayed by ELISA. IFNγ production was significantly increased (**$p<0.01$), and IL-4, and IL-5 production was significantly reduced (**$p<0.01$ compared with mice given OVA alone). Data are mean +/- SEM, n=5 and are representative of 3 experiments.

3.2.3 Histological evidence that prophylactic intraperitoneal Pam3CSK4 treatment attenuates airways inflammation

Histology demonstrated that mice that had received i.p. Pam3CSK4 therapy prior to their i.n. OVA challenges had reduced peri-bronchial and peri-vascular inflammation (Fig. 3.4). The degree of airways inflammation seen in TLR2 agonist-treated mice appeared to be equivalent to the minimal amount of inflammation associated with PBS control mice lungs.

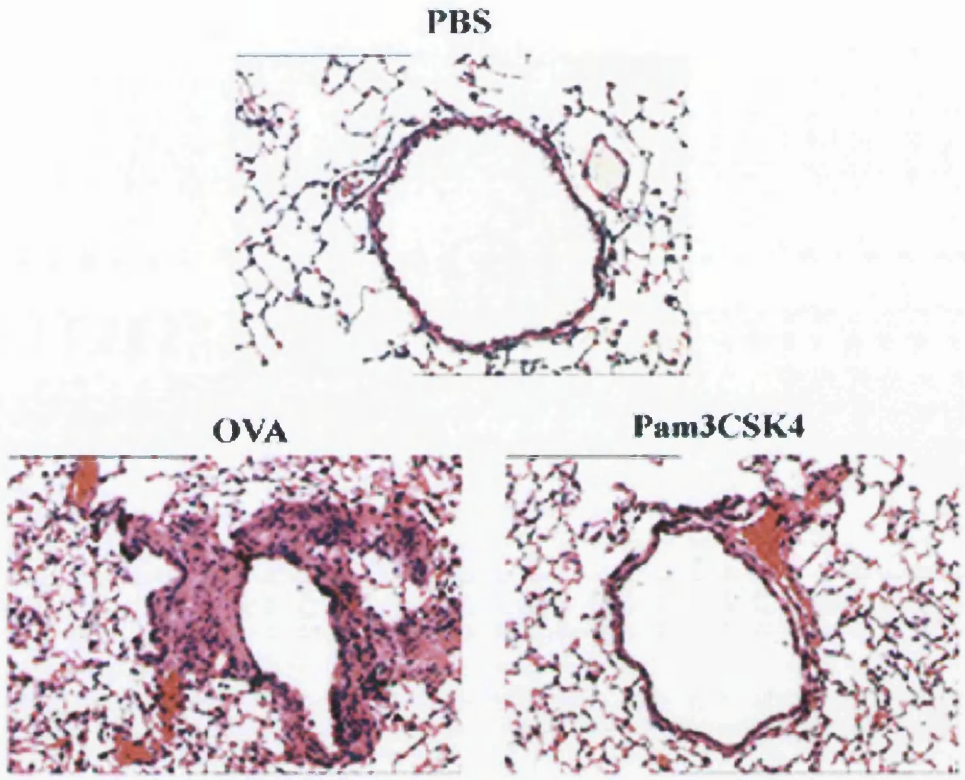

Figure 3.4 Histological evidence that airways inflammation is decreased after prophylactic i.p. Pam3CSK4 therapy.

H&E sections of lungs (x20 magnification) demonstrate that airways inflammation was ameliorated by Pam3CSK4 therapy. Pam3CSK4-treated mice demonstrated reduced peri-vascular and peri-bronchial inflammation. Lungs were collected on day 28 and are representative of 5 mice per group.

3.3 Intranasal (i.n.) TLR2 agonist exacerbates allergic airways inflammation in a murine model of asthma

Having found that prophylactic i.p. TLR2 agonists attenuated the development of allergic airways inflammation, further investigations were performed to examine if TLR2 agonists administered by another route could affect allergic inflammation. BALB/c mice were sensitised and challenged with OVA, as outlined in Figure 3.5. One dose of intranasal (i.n.) Pam3CSK4 or PGN was given at the same time as the allergen challenges. Thus i.n. TLR2 agonist was administered on one occassion on day 25, 26 or 26. The mice still received all three i.n. OVA challenges. The dose of i.n. TLR2 agonist administered was either 25, 50 or 100 μg per mouse. OVA control mice were challenged with i.n. OVA alone and PBS control mice were sensitised and challenged with OVA. Initial experiments showed that all doses of i.n. TLR2 agonist had a similar detrimental effect on airways inflammation. However, TLR2 agonist administration in a dose greater than 25 μg resulted in an unacceptably high mortality rate, which appeared to be representative of excessive airways disease, which was seen in lungs of the surviving mice treated with higher dose TLR agonists. Hence, 25 μg was selected for all subsequent experiments.

The data for i.n. Pam3CSK4 and PGN effect on murine asthma are very similar. Hence, to avoid duplication the data for Pam3CSK4 are discussed below and any differences seen between Pam3CSK4 and PGN are highlighted.

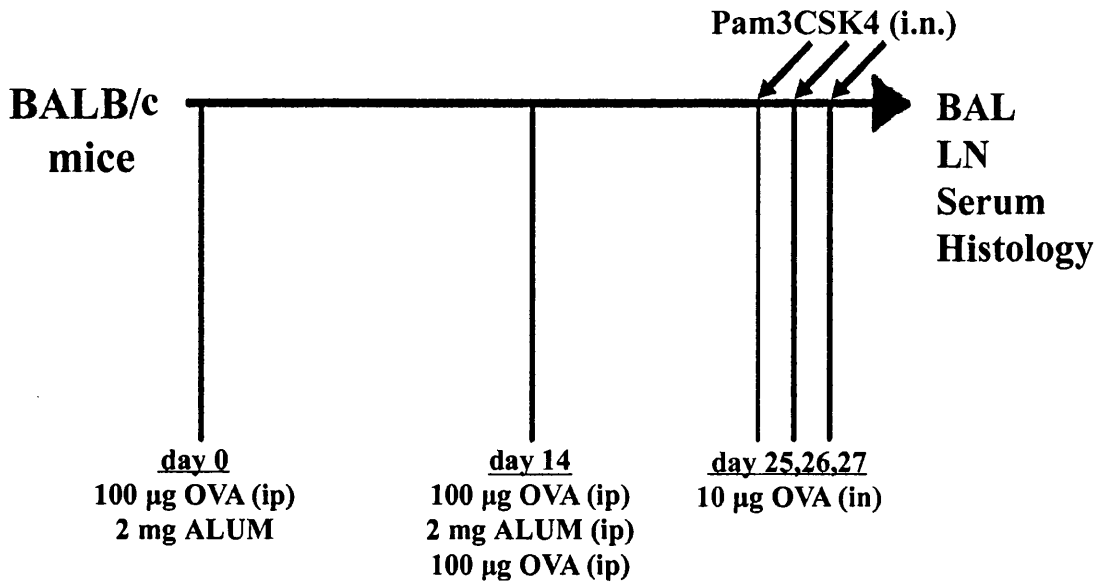

Figure 3.5 Experimental protocol to investigate the effect of intranasal TLR2 agonist on allergic airways disease.

BALB/c mice were injected i.p. with 100 µg OVA and 100 µl 2 % Alum on day 0 and 14. The mice were boosted i.n. with 100 µg OVA on day 14. All the mice were then challenged i.n. on 3 consecutive days beginning on day 25. Pam3CSK4 or PGN (25-100 µg per mouse) was administered i.n. at the same time as the OVA challenges. Serum, BAL and lymphoid cells were collected and lung histology studied on day 28

3.3.1 Intranasal Pam3CSK4 administration increases bronchoalveolar total cellularity and eosinophilia

Intranasal Pam3CSK4 administration resulted in a marked increase in total BAL cellularity and eosinophil numbers, as compared to untreated OVA sensitised and challenged mice (Fig. 3.6). It was noted that mice treated with i.n. PGN displayed the same increase in total BAL cell number and eosinophilia, however the proportion of BAL neutrophilia also increased, as compared to i.n. Pam3CSK4 treatment. It was also noted that higher doses of i.n. PGN resulted in bloody BAL, and macroscopic evidence of pulmonary haemorrhage. BAL cytokines were not detected.

3.3.2 Intranasal Pam3CSK4 treatment enhances OVA-induced cytokine responses by thoracic lymph node cells *in vitro*

Pam3CSK4 administration did not affect the T cell proliferation against OVA *in vitro* (data not shown). However, the lymphoid cells from i.n. Pam3CSK4-treated OVA sensitised/challenged mice produced significantly more IFNγ, IL-4 and IL-5 compared to untreated OVA-sensitised/challenged mice (Fig. 3.7). Con A-induced responses from thoracic cell node cells from mice treated with Pam3CSK4 showed a similar cytokine profile to that of OVA stimulation. Culture of LN cells with medium alone resulted in no cytokines being detected.

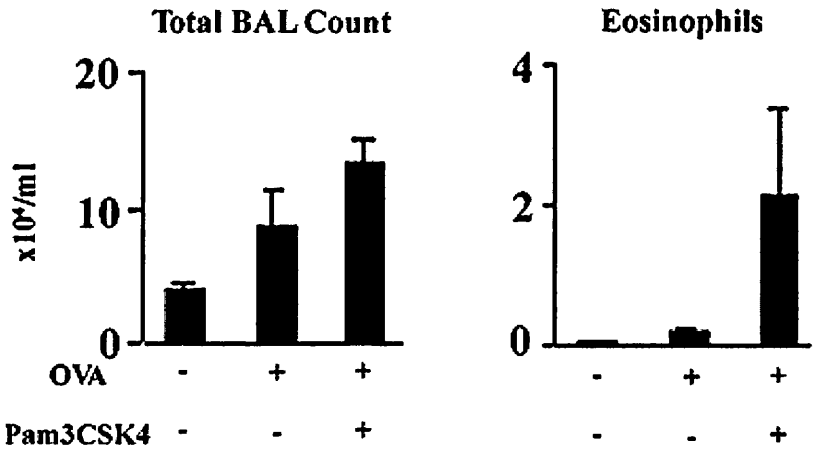

Figure 3.6 Total bronchoalveolar cell count and eosinophil proportion are increased after intranasal (i.n.) Pam3CSK4 administration.

BAL total cell concentration and eosinophil proportion were enhanced after i.n. Pam3CSK4 administration. The increase in BAL indices was statistically not significant as compared to mice given OVA alone. Data shown are for mice given i.n. Pam3CSK4 on day 25 alone, however they are also representative of mice treated with Pam3CSK4 on day 26 or day 27. Data are mean +/- SEM, n=5 and are representative of 3 experiments.

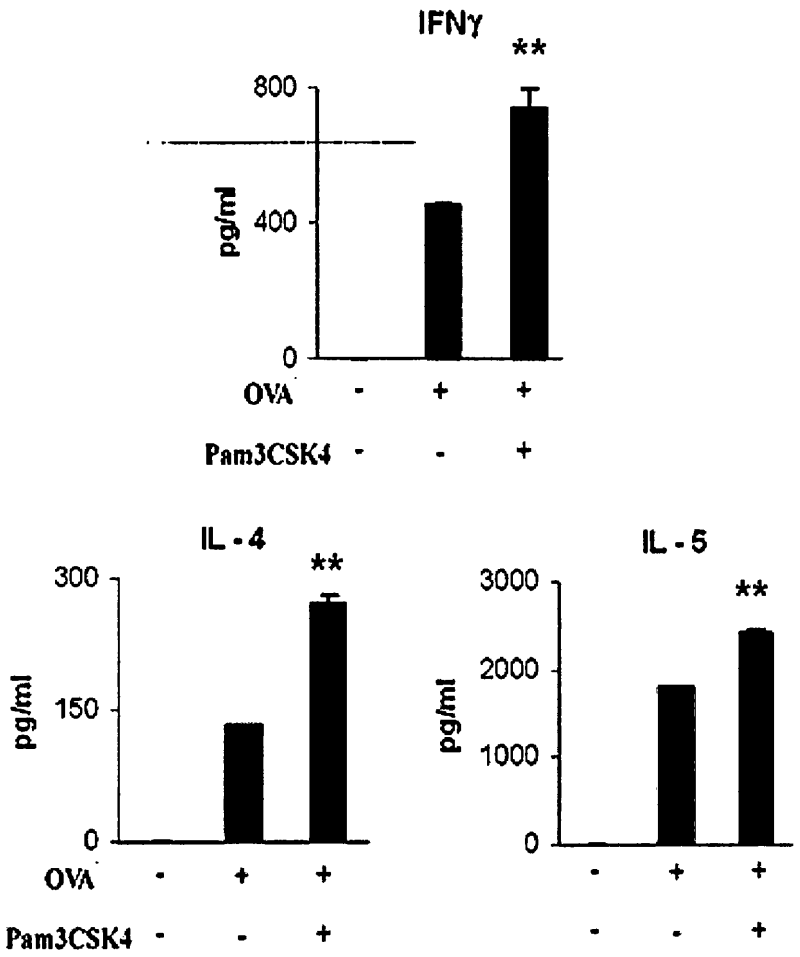

Figure 3.7 OVA-induced IFNγ, IL-4, and IL-5 *in vitro* production were enhanced after i.n. Pam3CSK4 administration.

Thoracic lymph node cells were harvested on day 28 and cultured with 1 mg/ml OVA *in vitro* (as described in Materials and Methods, Section 2.6.6, p97). Culture supernatant was collected at 96 h and cytokine concentrations assayed by ELISA. IFNγ, IL-4, and IL-5 production was significantly increased (**$p<0.01$ compared with mice given OVA alone). Data shown are for mice given i.n. Pam3CSK4 on day 25 alone, however are also representative of mice treated with Pam3CSK4 on day 26 or day 27. Data are mean +/- SEM, n=5 and are representative of 3 experiments.

3.3.1 Histological evidence that intranasal Pam3CSK4 administration worsens airways inflammation

Histological analysis showed that mice treated with TLR2 agonist had increased peri-bronchial and peri-vascular inflammation (Fig 3.8). In addition, it was noted that peptidoglycan administration resulted in worsened inflammatory infiltrates, with intra-pulmonary haemorrhages being evident.

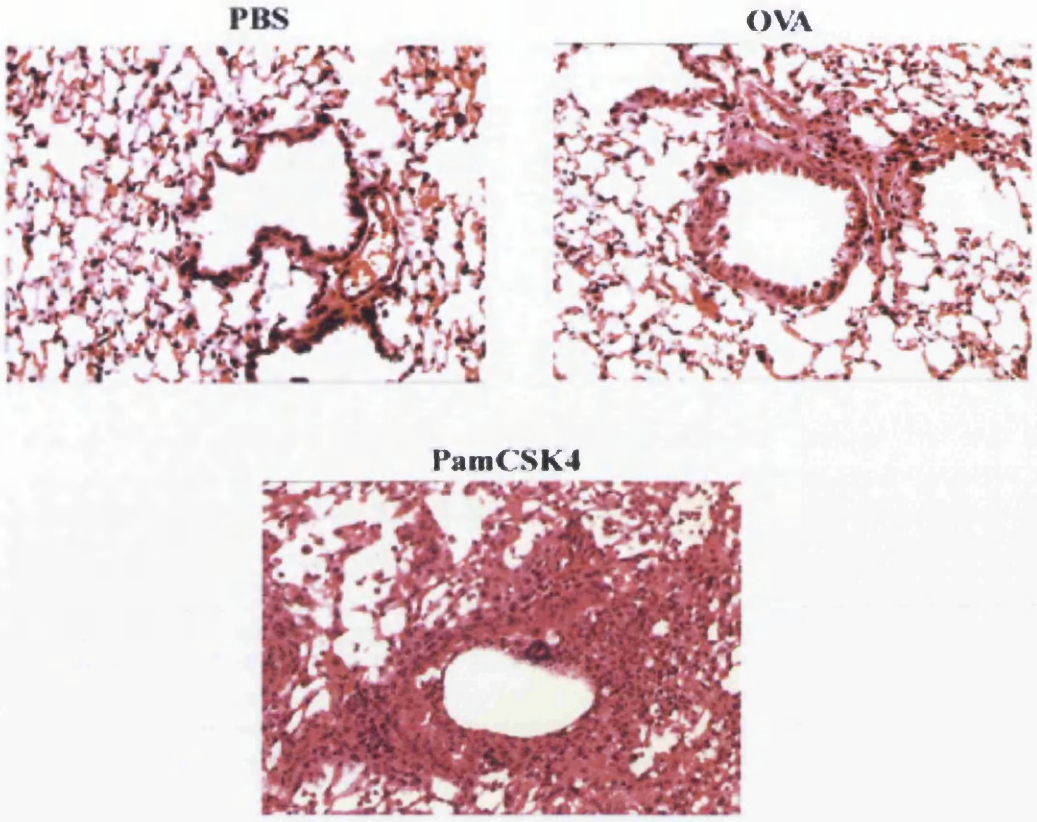

Figure 3.8 Histological changes demonstrate that i.n. Pam3CSK4 administration worsened airways inflammation.

H&E sections of lungs (x20 magnification) demonstrate that peri-bronchial and peri-vascular inflammation was enhanced by i.n. Pam3CSK4 therapy. Lungs were collected on day 28. Histology shown are for mice given i.n. Pam3CSK4 on day 25 alone, however are representative of mice treated with Pam3CSK4 on day 26 or day 27. Sections are representative of 5 mice per group.

3.4 Intraperitoneal Pam3CSK4 therapy reverses established OVA-induced airways inflammation

Having found that prophylactic i.p. TLR2 agonist administration resulted in amelioration of murine allergic airways inflammation, the next aim was to establish whether i.p. Pam3CSK4 therapy could reverse inflammation that was already established? To investigate the potential of TLR2 agonist in reversing inflammation in allergic asthma, BALB/c mice were primed and boosted with OVA (as described in Materials and Methods, Section 2.6.1 p92). All the mice were challenged i.n. on 3 consecutive days beginning on day 25. Pam3CSK4 (100 μg per mouse) was administered i.p only once on either day 25, 26 or 27. Pam3CSK4 was given 2 h after challenge (Fig. 3.9). Thus, mice treated with Pam3CSK4 on day 27 alone would have been challenged on the previous 3 days with OVA. In these sets of experiments, PGN was not used.

3.4.1 Intraperitoneal Pam3CSK4 treatment reduces BAL total cell count, and eosinophilia in established airways inflammation

Pam3CSK4 treatment produced a significant reduction in the BAL total cell count and eosinophilia (Fig. 3.10). This decrease in BAL cellularity was observed on all of the days that BLP was administered. Even mice that had received all three i.n. OVA challenges showed a decrease in their BAL indices, when BLP was administered 2 hours after their last i.n. allergen dose (see day 27 data). BAL cytokines were not detected.

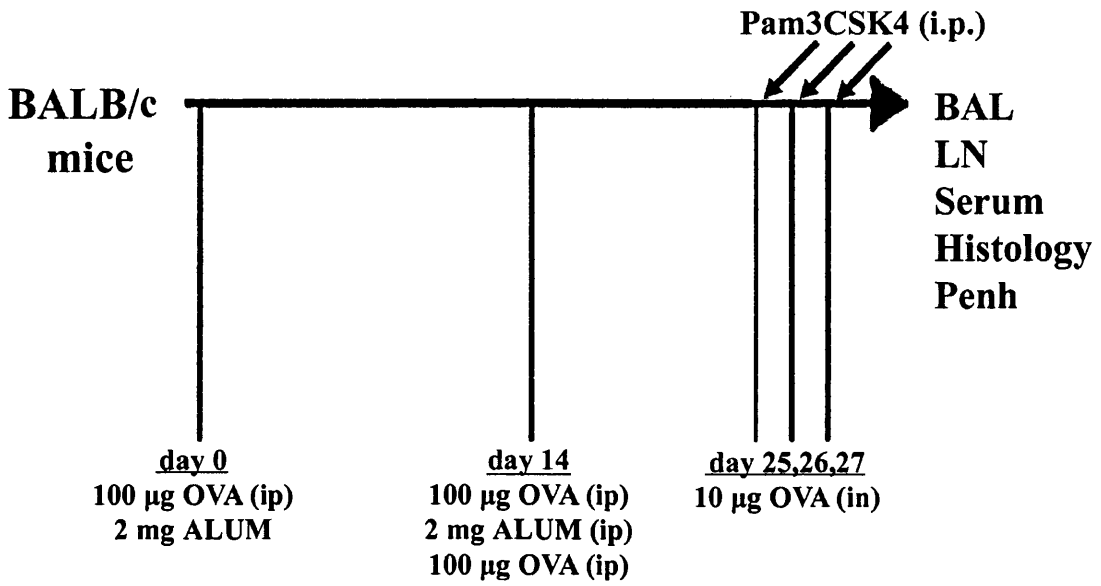

Figure 3.9 Experimental protocol to examine the role of intraperitoneal Pam3CSK4 in established allergic airways disease.

BALB/c mice were injected i.p. with 100 µg OVA and 100 µl 2 % Alum on day 0 and 14. The mice were boosted i.n. with 100 µg OVA on day 14. All the mice were then challenged i.n. on 3 consecutive days beginning on day 25. Pam3CSK4 (100 µg per mouse) was administered i.p only once on either day 25, 26 or 27. Pam3CSK4 was given 2 h after challenge. Penh was determined on day 28 and mice were sacrificed on day 29. Serum, BAL and lymphoid cells were collected and lung histology studied.

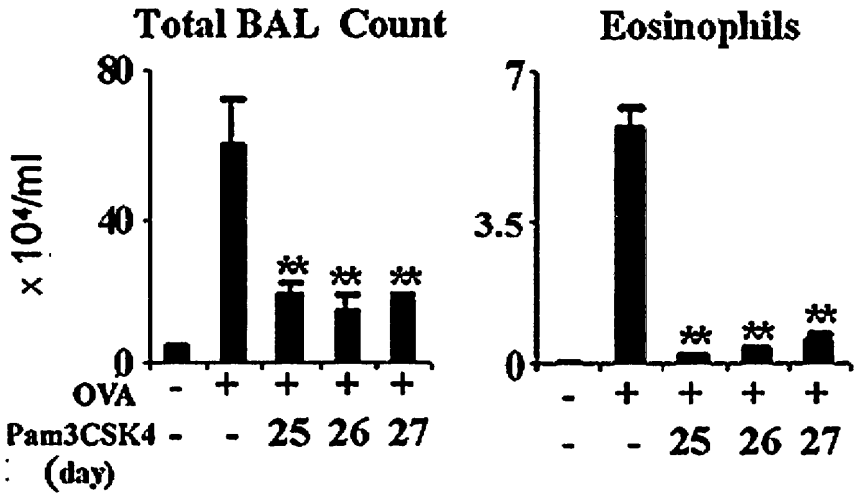

Figure 3.10 BAL total cell concentration and eosinophil proportion fell markedly after Pam3CSK4 therapy.

BALB/c mice were challenged i.n. on 3 consecutive days beginning on day 25. Pam3CSK4 (100 μg per mouse) was administered i.p only once on either day 25, 26 or 27, 2 h after i.n. challenge. Mice given Pam3CSK4 after i.n. challenge demonstrated a highly significant fall in their BAL indices (**$p<0.01$ compared with mice given OVA alone). Data are mean +/- SEM of individual mice, n= 8 and are representative of 3 experiments.

3.4.2 The effect of intraperitoneal Pam3CSK4 treatment on OVA-induced thoracic lymph node responses *in vitro*

Thoracic lymph node cells were harvested from the Pam3CSK4-treated and untreated control mice and cultured with OVA *in vitro*. Pam3CSK4 did not affect the T cell proliferation against OVA *in vitro*. However, the lymphoid cells from Pam3CSK4-treated OVA sensitised/challenged mice produced significantly more IFNγ compared to untreated OVA-sensitised/challenged mice (Fig. 3.11). Pam3CSK4 treatment led to a marked reduction in IL-4 and IL-5 synthesis by the lymphoid cells from OVA-sensitised/challenged mice (Fig 3.12). In addition, the lymphoid cells from Pam3CSK4-treated OVA sensitised/challenged mice produced significantly more IL-10 compared to untreated OVA-sensitised/challenged mice (Fig. 3.13), however as expected IL-12 production was not altered after Pam3CSK4 treatment. Con A-induced proliferative and cytokine responses from thoracic cell node cells from mice treated with Pam3CSK4 were similar to that of OVA stimulation. Culture of LN cells with medium alone resulted in no cytokines being detected.

3.4.3 Serum IL-12 levels are increased in mice with established inflammation receiving Pam3CSK4 treatment

To further investigate whether the enhanced IFNγ production by LN cells was due to IL-12 induction by Pam3CSK4 *in vivo*, serum IL-12 levels were measured. After Pam3CSK4 administration the level of serum IL-12 was greatly increased (Fig. 3.14), as compared to mice receiving i.p. PBS alone. This elevated IL-12 level peaked at 2 h

after Pam3CSK4 therapy and steadily declined until it was not detectable at 24 h (data not shown). Serum levels of IFNγ, IL-4 and IL-5 were not detected.

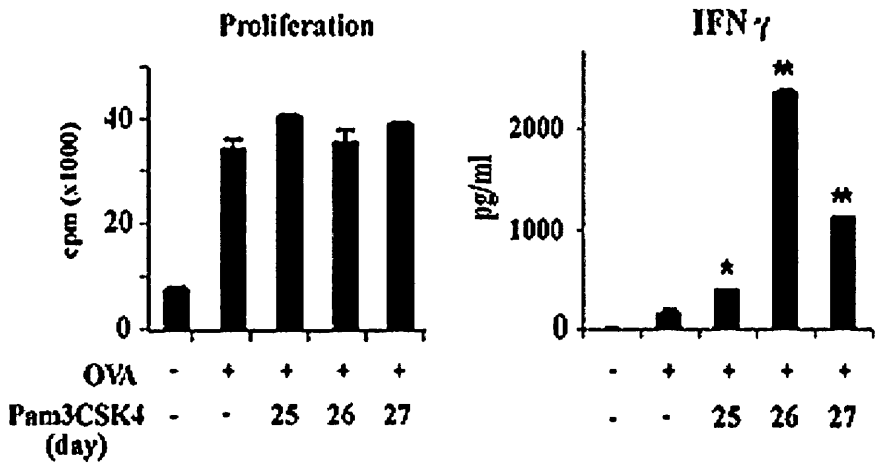

Figure 3.11 OVA-induced proliferation and IFNγ production *in vitro* by thoracic lymph node cells taken from mice with established airways inflammation and given i.p. Pam3CSK4.

Lymph node cells were harvested on day 29 and cultured with 1 mg/ml OVA *in vitro* (as described in Materials and Methods, Section 2.6.6, p97). Cellular proliferation was determined after 8 hours culture with [^{3}H] thymidine. Culture supernatant was collected at 96 h and cytokine concentrations assayed by ELISA. Cells from OVA-sensitised/challenged mice treated with Pam3CSK4 did not show any change in their proliferative response, however IFNγ production was signicantly increased (*$p<0.05$, and **$p<0.01$ compared with mice given OVA alone). Data are mean +/- SEM, n=8 and are representative of 3 experiments

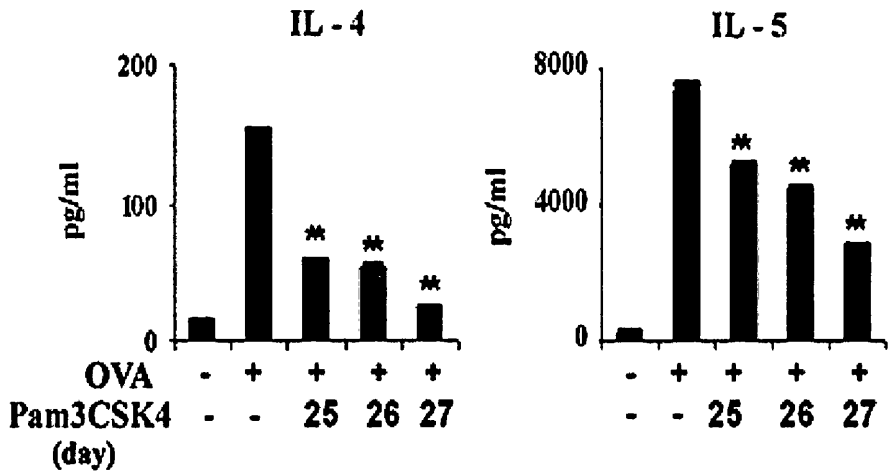

Figure 3.12 ***In vitro*** **OVA-induced IL-4 and IL-5 production by thoracic lymph node cells taken from mice with established airways inflammation that had been given i.p. Pam3CSK4 is reduced.**

Lymph node cells were harvested on day 29 and cultured with 1 mg/ml OVA *in vitro* (as described in Materials and Methods, Section 2.6.6, p97). Culture supernatant was collected at 96 h and cytokine concentrations assayed by ELISA. Cells from mice treated with Pam3CSK4 showed a significant reduction in their IL-4 and IL-5 production (**$p<0.01$ compared with mice given OVA alone). Data are mean +/- SEM, n=8 and are representative of 3 experiments

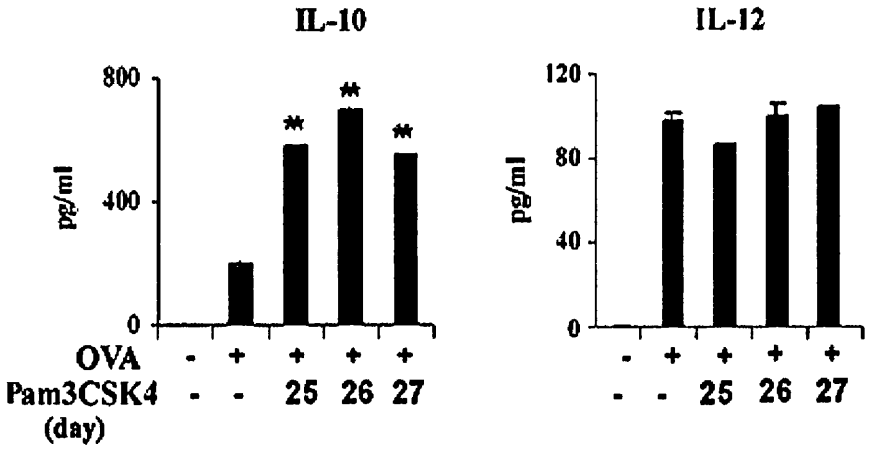

Figure 3.13 ***In vitro*** **OVA-induced IL-10 and IL-12 production** ***in vitro*** **by thoracic lymph node cells taken from mice with established airways inflammation and given i.p. Pam3CSK4.**

Lymph node cells were harvested on day 29 and cultured with 1 mg/ml OVA *in vitro* (as described in Materials and Methods, Section 2.6.6, p97). Culture supernatant was collected at 96 h and cytokine concentrations assayed by ELISA. Cells from OVA-sensitised/challenged mice treated with Pam3CSK4 did not show any change in their IL-12 response, however IL-10 production was markedly increased (**$p<0.01$ compared with mice given OVA alone). Data are mean +/- SEM, n=8 and are representative of 3 experiments.

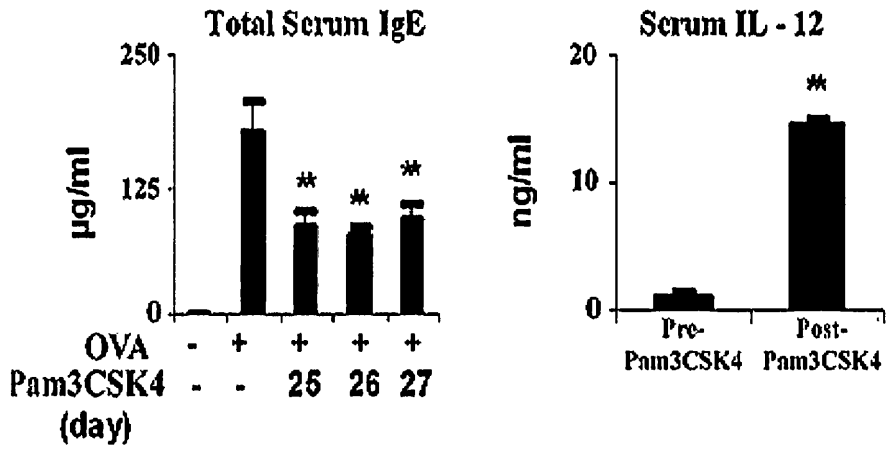

Figure 3.14 Serum total IgE is reduced after i.p. Pam3CSK4 therapy, however serum IL-12 levels are elevated.

Sera for IL-12 estimation were collected 2 h after Pam3CSK4 treatment on day 27. Pam3CSK4 treatment led to highly enhanced IL-12 synthesis (up to 15 ng/ml). Sera collected on day 29 showed a marked reduction in total IgE (**$p<0.01$ compared with mice given OVA alone). OVA-specific IgE showed a similar reduction after Pam3CSK4 treatment (data not shown). Data are mean +/- SEM of individual mice, n= 8 and are representative of 3 experiments.

3.4.4 Serum immunoglobulin levels from mice with established inflammation receiving Pam3CSK4 treatment

Serum obtained from OVA-sensitised/challenged mice after Pam3CSK4 treatment showed a marked reduction in the concentration of total IgE antibody (Fig 3.14). OVA-specific IgE also demonstrated a similar after Pam3CSK4 therapy (data not shown). Serum IgG1 levels were not affected by Pam3CSK4 therapy. The levels of serum IgG2a were low and again not significantly affected by i.p. Pam3CSK4 treatment (Fig. 3.15).

3.4.5 Histological evidence that Pam3CSK4 therapy can reverse established airways inflammation

Histological analysis demonstrated a reduction in the inflammatory infiltrates seen in the peri-bronchial and peri-vascular areas of the murine lungs treated with Pam3CSK4 (Fig. 3.16). This decrease in airway inflammation was observed on all of the days that Pam3CSK4 was administered. Even mice that had received all three i.n. OVA challenges showed a profound decrease in their airways inflammation, when Pam3CSK4 was administered 2 hours after their last i.n. allergen dose (see day 27 data, Fig. 3.16).

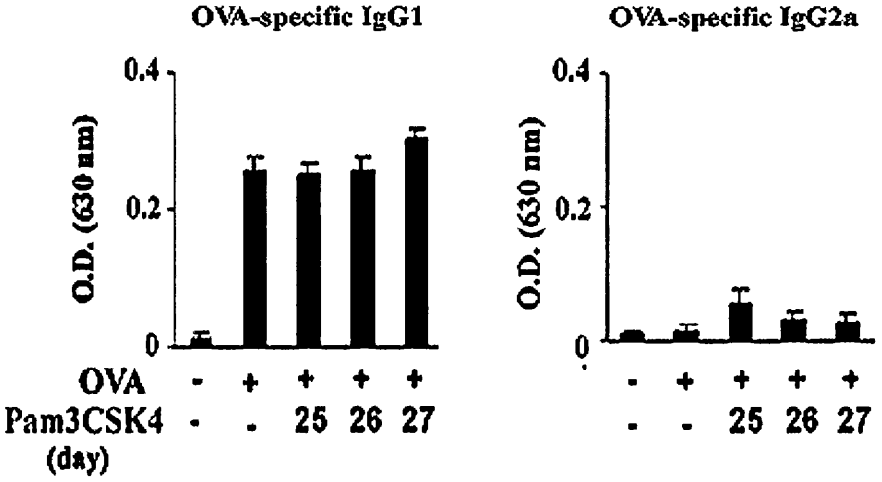

Figure 3.15 Serum OVA-specific IgG1 and IgG2a levels are not affected by Pam3CSK4 therapy.

Sera collected on day 29 showed little difference in their IgG1 and IgG2a levels. Data are mean +/- SEM of individual mice, n= 8 and are representative of 3 experiments.

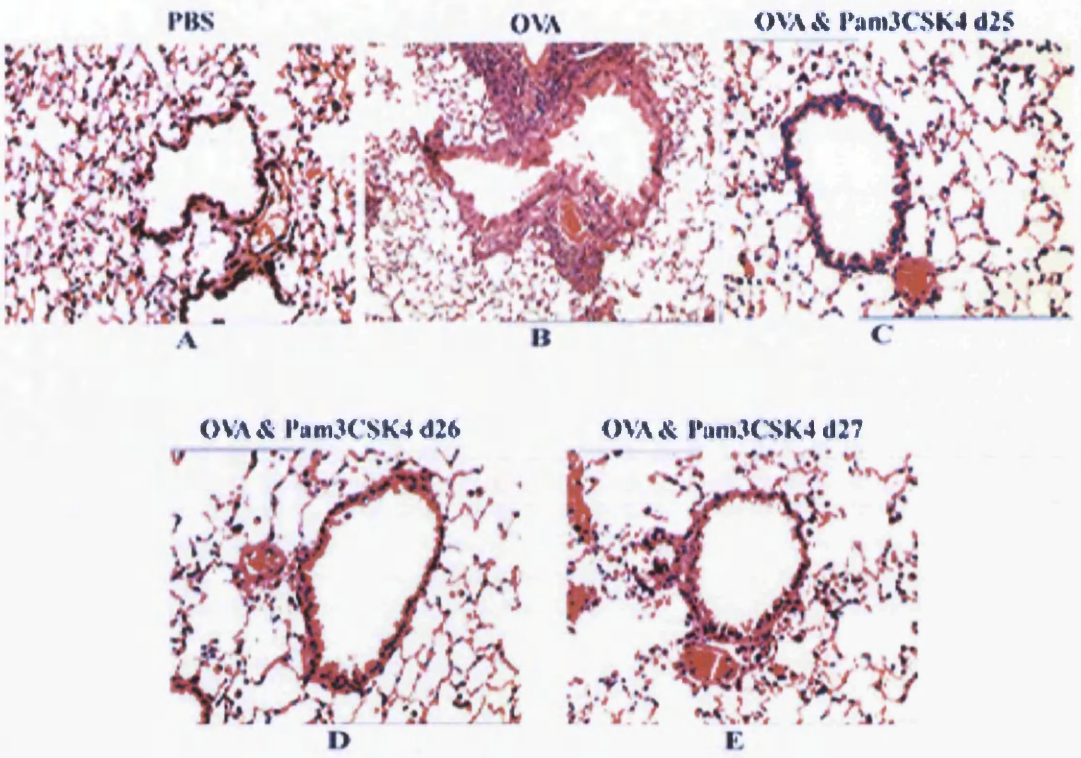

Figure 3.16 Histological evidence that Pam3CSK4 therapy ameliorated established airways inflammation.

H&E sections of lungs (x20 magnification) demonstrate that airways inflammation is reduced in mice treated with i.p. Pam3CSK4 between days 25 and 27 (panels C-E), compared with the inflammation seen in OVA-sensitized and -challenged mice (panel B). Pictures are representative of 8 mice per group.

3.4.6 Pam3CSK4 therapy reduces airways hyperresponsiveness to methacholine in mice with established airways inflammation

Whole Body Plethysmography is a technique that has been used to monitor lower airways resistance in unrestrained conscious mice. Enhanced Pause (Penh) was used as physiological measure of airways hyperresponsiveness. Penh was measured after nebulisation for 2 minute with methacholine on day 28. Mice were challenged with 0, 12.5, 25, and 50 mg of nebulised methacholine (as described in Materials and Methods, Section 2.6.2, p93). Mice that had received Pam3CSK4 treatment showed a significant reduction in the airway hyperresponsiveness, as compared to untreated OVA-sensitised and –challenged mice (Fig. 3.17).

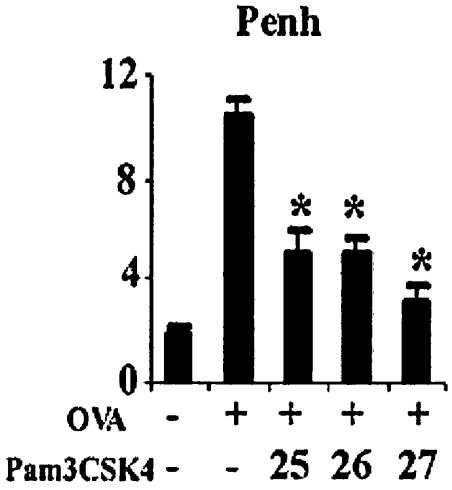

Figure 3.17 Pam3CSK4 therapy reduced Penh values in mice with established allergic airways inflammation.

Enhanced Pause (Penh), after a 2 min nebulisation with 50 mg/ml methacholine was performed on day 28. Mice treated with Pam3CSK4 showed a significant reduction in their Penh measurement (*$p<0.05$ compared with mice given OVA alone). Data are mean +/- SEM, n=8 and are representative of 3 experiments.

3.5 Chapter Discussion

The main findings from the work in this chapter are that:

i) Intraperitoneal Pam3CSK4 or PGN administration immediately prior to the i.n. OVA challenges significantly reduces airways inflammation.

ii) Intranasal Pam3CSK4 or PGN given simultaneously with the i.n. OVA challenges exacerbates murine asthma, with PGN administration being associated with worsened airways inflammation

iii) Intraperitoneal Pam3CSK4 administration after the i.n. OVA challenges ameliorates established allergic airways inflammation.

The results of these investigations demonstrate an impressive therapeutic role of intraperitoneal Pam3CSK4 in murine asthma. The beneficial effect was immediate and sustained, with the effect of i.p. Pam3CSK4 being sustained for at least 5 days after treatment (data not shown). The amelioration of allergic airways disease seen with i.p. TLR2 agonist treatment was evident in developing and, more importantly, established experimental asthma.

Interestingly, i.n. TLR2 agonist administration worsened the allergic phenotype of mice in this model. It was felt that i.n. TLR2 agonists may be having a local effect on inflammatory cells such as macrophages, neutrophils, mast, and NK cells, whereas i.p. TLR2 agonists was having a more systemic effect. Certainly, other investigators have found that 15-150 μg i.n. PGN itself can actually induce acute lung injury in mice. Peptidoglycan enhanced BAL neutrophilia, and increased the production of pro-inflammatory cytokines TNFα, IL-6 and MIP-1α from inflammatory cells in the lung

(372). Work with human mast cells found that TLR2 agonists caused degranulation of mast cells which results in a local inflammatory response. Furthermore, it was demonstrated that PGN and yeast zymosan (working through TLR 2/6 heterodimer) caused greater mast cell degranulation with greater release of inflammatory mediators than Pam3CSK4 (working through TLR 2/1 heterodimer) (373). In our studies, we observed that i.n. PGN caused a greater degree of airways inflammation, with a greater degree of BAL neutrophilia, than i.n. Pam3CSK4. It may be that when TLR2 agonists are given i.n., there is activation and degranulation of airways inflammatory cells, such as mast cells, neutrophils and eosinophils, which leads to greater allergic airways inflammation. Furthermore, the observation of increased airways inflammation seen with i.n. PGN as compared to Pam3CSK4 could be explained by worsened local mast cell degranulation through TLR 2/6 signalling. Further studies are needed to examine the mechanism(s) of i.n. TLR2 agonist's aggravation of allergic airways inflammation, and possibly also look at effects of TLR2 administration by different routes in experimental models of asthma.

Systemic administration of TLR2 agonists by i.p. route significantly attenuates allergic airways inflammation. It was interesting to note that although the serum IgG levels remained unchanged, there was a rapid decrease in both the serum OVA-specific and total IgE levels. This has also been reported by other investigators (11). The mechanism of this rapid IgE reduction is unclear. However, it should be noted that the half life of rodent serum IgE can be as short as 12 h (374, 375). The half life of rodent IgG is around 4-5 days (376, 377). Despite this, the IgG profile 5 days after the last i.n. challenge showed that TLR2 agonists still had little effect on IgG levels.

Our data demonstrating different outcomes depending on the timing and route of administration of agonist is supported by previous studies looking at TLR2 agonists and experimental asthma. These papers have produced apparently contradictory results. When administered during the sensitisation period (14-21 days prior to i.n. OVA challenge), early TLR2 agonist treatment was reported to worsen a Th2-mediated asthma (149, 367). In contrast, TLR2 activation immediately prior to the intranasal challenge was found to reduce allergic airways inflammation (368, 369). Recently, Velasco *et al* (369) have demonstrated that early administration of PGN (21 days prior to intratracheal OVA challenge) can also reduce airways inflammation. Having observed, in our own studies, that the route of administration is critical to the TLR2 agonist effect on airways disease, it is may be that these divergent results are a consequence of different routes of administration. However, reviewing the studies reveals administration of TLR2 agonist directly into the lungs, by either an intranasal or intratracheal route, can both exacerbates and ameliorates airways inflammation (367, 369). The action of TLR2 agonists in different murine models of asthma is complex and unresolved. The next chapter presents data on potential mechanisms involved, and further discussions are made regarding the possible reasons for the disparate observations made in Chapters 4 and 6.

Our observation that TLR2 agonist treatment results in a reduced OVA-induced Th2 cytokine response, with enhanced Th1 cytokine production by draining lymph nodes suggests that TLR2 agonists are modulating the asthma phenotype by skewing the Th2-mediated immune response to a Th1 response. As previously discussed, there is evidence that Th1 lymphocytes are protective in asthma by skewing the immune response towards a Th1 profile (33). However, in adoptive murine transfer models of

asthma, Th1 cells can actually enhance airways disease once it has been established (39-41) It has also been shown that IFNγ and Tbet-1 are critical for the attenuation of airways eosinophilia in murine models of asthma (35, 36). Hence, there is some debate as to the contribution of Th1 cells in the pathogenesis of asthma. In our work, it was noted that the serum IL-12 levels were significantly increased shortly after Pam3CSK4 treatment, which gives further support to the idea that, in our model, TLR2 agonists are influencing the Th1/Th2 balance in allergic airways disease.

The enhancing effect of Pam3CSK4 on IL-10 production also raised the possibility that regulatory T cells may play a role in the beneficial outcome of Pam3CSK4 treatment. Many investigators have observed that regulatory T cells' ($CD4^+CD25^+$, Tr1 and Th3) function is dependent on IL-10 and TGFβ *in vivo* (204, 242, 299, 306). It is also known that Tregs express TLRs, and their suppressive function can be enhanced by TLR activation (186, 378).

Hence, our observations of the therapeutic effect of i.p. TLR2 agonist in experimental asthma may be attributed to enhanced Treg function, or to a skewing of the immune response towards a Th1 response. The next chapter will further examine the possible mechanisms involved in the therapeutic effect of i.p. Pam3CSK4.

Chapter 4

The mechanisms involved in Pam3CSK4 modulation of inflammation in a murine model of allergic airways disease

Data in this chapter has been published in

Patel *et al* (2005), *J Immunol*; **174(12)**:7558-7563.

4.1 Introduction

In Chapter 3, we reported that the synthetic TLR2 agonist Pam3CSK4 profoundly attenuated allergic airways inflammation in OVA-specific asthma in mice, when administered intraperitoneally. This chapter investigates the immunological mechanisms involved in i.p. Pam3CSK4 treatment.

Based on our observations of increased IL-10 production by OVA-stimulated LN cultures seen in Pam3CSK4-treated mice, we proposed that regulatory T cells (Tregs) were one of the cell types important in the beneficial outcome of Pam3CSK4 treatment. IL-10 and TGFβ have been implicated in the effector mechanism of several subclasses of regulatory T cells *in vivo* (204, 242, 299, 306). As such one of the primary aims of this chapter is to further examine the role of IL-10 and TGFβ in Pam3CSK4 therapy, and to provide evidence as to whether Tregs are important in Pam3CSK4 therapy.

Based on our observations of increased serum levels of IL-12, and the enhanced IFNγ and reduced IL-4 and IL-5 production by OVA-stimulated LN cultures observed in Pam3CSK4-treated mice, we propose that a skewing of the Th2 to a Th1 response contributes to TLR2 agonist treatment. We postulate that Pam3CSK4 may induce IL-12 production from APCs (mainly DCs) which can in turn enhance the development of Th1 cells. The secondary aims of this chapter are to determine if IL-12 and IFNγ are required for TLR2 agonist therapy in allergic airways disease; and if they are, to try and identify the cellular source of these cytokines.

The data presented below demonstrate that the therapeutic effect of Pam3CSK4 is critically dependent on IL-12 and IFNγ, but not on IL-10 or TGFβ. Our results further demonstrate that Pam3CSK4 is capable of inducing DCs to produce IL-12, which in turn enhances the development of Th1 cells and production of IFNγ. This provides evidence that Pam3CSK4 therapeutic effect in allergic airways disease is through Th1 skewing, and is unlikely to involve regulatory T cells.

4.2 The effect of intraperitoneal Pam3CSK4 therapy on peritoneal inflammation

The anti-inflammatory effects of Pam3CSK4, described in the previous chapter, demonstrated that TLR2 agonist therapy was dependent on the route of administration. Pam3CSK4 therapy profoundly attenuated Th2-mediated inflammation when given i.p., whereas i.n. TLR2 agonist administration exacerbated the allergic phenotype. Hence, we felt it important to ensure that i.p. Pam3CSK4 therapy was not a result of sequestration of inflammatory cells from the lungs into the peritoneal cavity.

To investigate this, the murine model of allergic airways inflammation was used as described in Fig. 4.1. Mice were sensitised with OVA, and then all the mice were challenged i.n. on 3 consecutive days beginning on day 25. Pam3CSK4 (100 μg per mouse) or PBS was administered i.p only once on day 25, 2 h after i.n. challenge. Mice were sacrificed on day 28, and peritoneal lavage was performed with 5 mls of ice cold PBS (as described in Materials and Methods section 2.7, p99). Similar results were obtained for mice receiving Pam3CSK4 therapy on day 26 and 27.

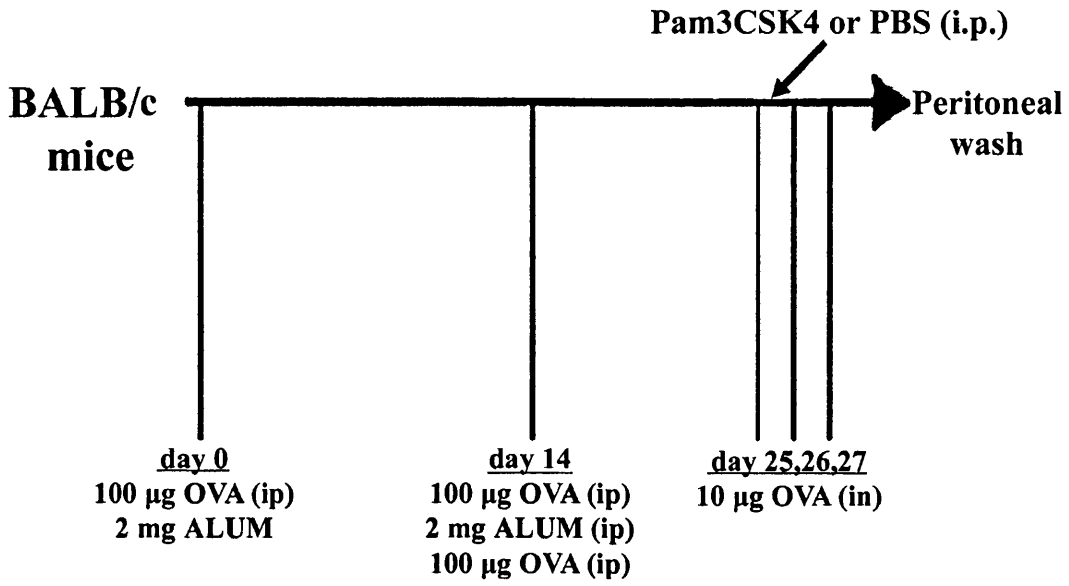

Figure 4.1 Experimental protocol to investigate if i.p. Pam3CSK4 therapy sequesters inflammatory cells into the peritoneal cavity.

BALB/c mice were injected i.p. with 100 µg OVA and 100 µl 2 % Alum on day 0 and 14. The mice were boosted i.n. with 100 µg OVA on day 14. All the mice were then challenged i.n. on 3 consecutive days beginning on day 25. Pam3CSK4 (100 µg per mouse) or PBS was administered i.p only once on day 25, 2 h after i.n. challenge. Mice were sacrificed on day 28, and peritoneal lavage was performed with 5 mls of ice cold PBS (as described in Materials and Methods section 2.7, p99).

4.2.1 Intraperitoneal Pam3CSK4 therapy did not sequester inflammatory cells into the peritoneal cavity.

Pam3CSK4 treatment of mice significantly reduced the eosinophil numbers in the peritoneal cavity. The number of peritoneal macrophages, neutrophils and lymphocytes were not significantly altered after Pam3CSK4 treatment (Fig. 4.2). The total number of inflammatory cells in the peritoneum was not affected (Pam3CSK4-treated mice total peritoneal cell count 10.75 +/- 2.2 x 10^5 vs PBS-treated mice total peritoneal count 7.05 +/- 5.7 x 10^5, p=0.38. Data are mean +/- SEM, n=6).

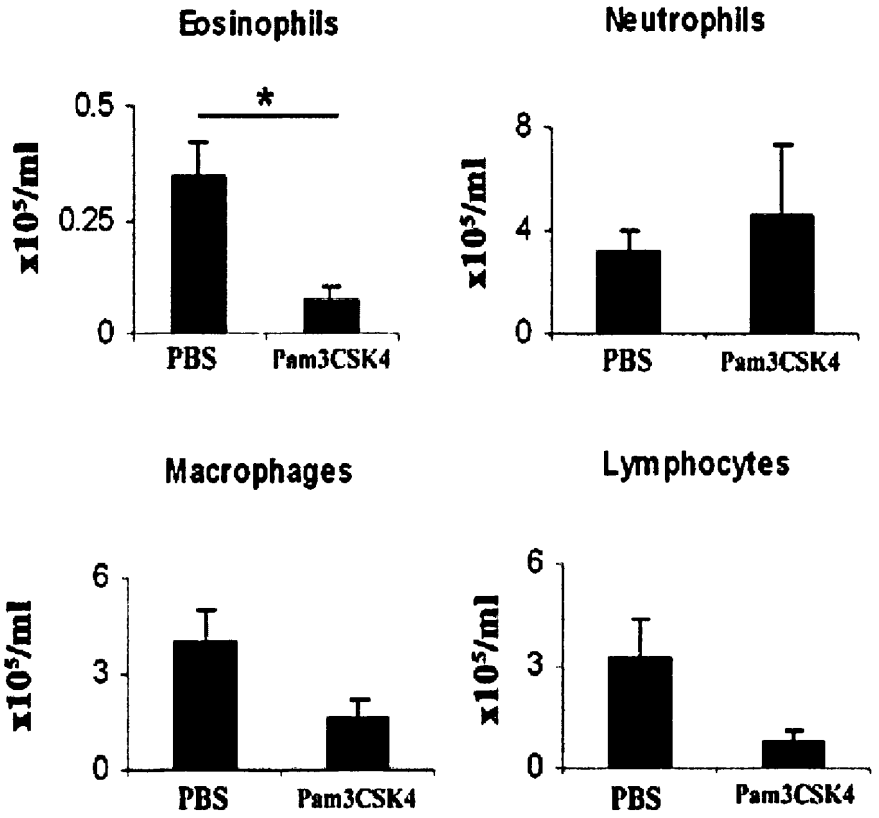

Figure 4.2 Intraperitoneal Pam3CSK4 therapy is not due to sequestration of inflammatory cells into the peritoneal cavity.

Mice were sensitised with OVA, and then all the mice were challenged i.n. on 3 consecutive days beginning on day 25. Pam3CSK4 (100 μg per mouse) or PBS was administered i.p only once on day 25, 2 h after i.n. challenge. Mice were sacrificed on day 28, and peritoneal lavage was performed with 3 mls of ice cold PBS. Pam3CSK4 treatment of mice significantly reduced the eosinophil numbers in the peritoneal cavities (*$p<0.05$ as compared to PBS vehicle control). The number of macrophages, lymphocytes, neutrophils was not significantly different. The total numbers of inflammatory cells in the peritoneum were not affected. Data are mean +/- SEM, n=6.

4.3 Pam3CSK4 therapy is not dependent on IL-10

To investigate whether IL-10 is important in Pam3CSK4 therapy, mice were sensitised and challenged with OVA using the standard protocol. The animals were injected i.p with a blocking anti-mouse IL-10 receptor (R) antibody or control normal IgG, 2 h before the i.n. OVA challenge of day 27. Pam3CSK4 therapy was administered 2 h after the last i.n. challenge on day 27 (Fig. 4.3). This antibody and the amounts administered are routinely found to be effective in abrogating $CD4^+CD25^+$ regulatory T cells in this laboratory (242).

4.3.1 Anti-IL-10R antibody does not reverse the therapeutic effect of Pam3CSK4 on BAL indices

The total BAL cellularity, eosinophil proportion, and absolute eosinophil numbers were consistently reduced in OVA-sensitised and –challenged mice after Pam3CSK4 therapy. Administration of anti-IL-10R antibody did not affect the beneficial effect of Pam3CSK4 therapy (Fig. 4.4). BAL cytokines were not detected.

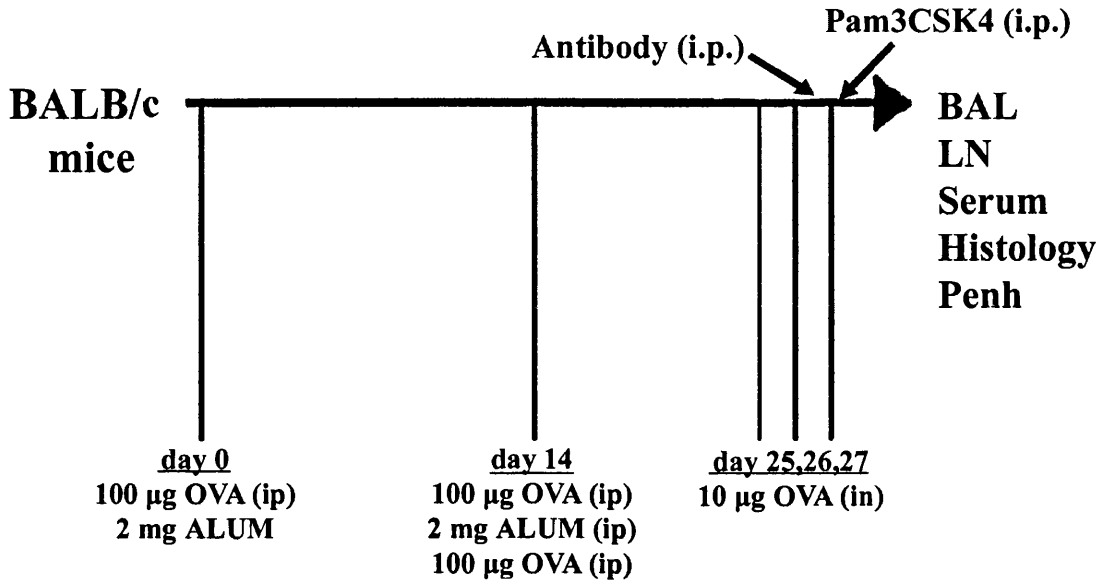

Figure 4.3 Experimental protocol to examine the role of IL-10, TGFβ, and IL-12 in Pam3CSK4 therapy of established airways inflammation.

Mice were sensitized and challenged as described in Fig. 3.1. Some mice were injected i.p. with 1 mg of anti-IL-10R antibody, anti-TGFβ antibody, anti-IL-12 antibody, or isotype-matched normal control IgG as indicated. The antibodies were given 2 h before the last i.n. OVA challenge on day 27. Pam3CSK4 (100 µg/mouse) was injected i.p. 2 h after the day 27 i.n. challenge. Penh was determined on day 28 and mice were sacrificed on day 29. Serum, BAL and lymphoid cells were collected and lung histology studied.

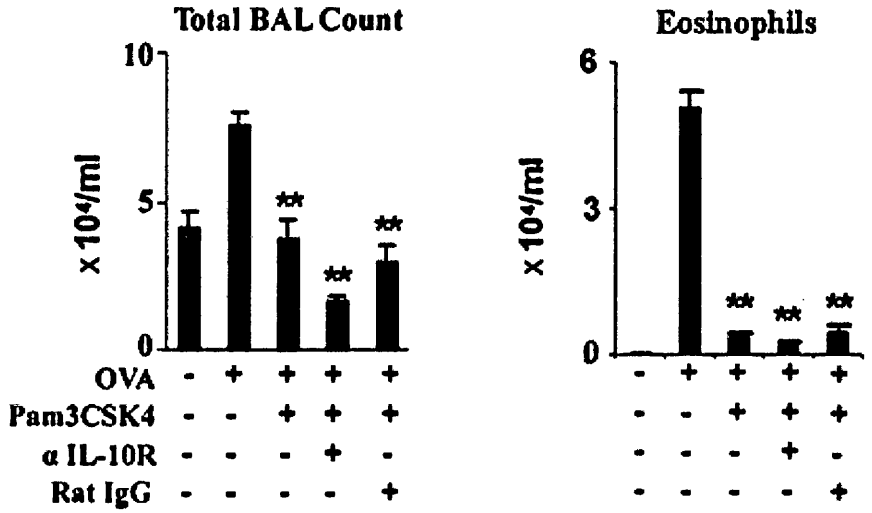

Figure 4.4 The therapeutic benefit of Pam3CSK4 on BAL indices was not affected by anti-IL-10R antibody administration.

Mice were sensitised and challenged with OVA and injected i.p with anti-mouse IL-10R antibody or control normal IgG, 2 h before the i.n. OVA challenge of day 27. Pam3CSK4 therapy was administered 2 h after the last i.n. challenge on day 27. All mice that received Pam3CSK4 therapy demonstrated a highly significant fall in their BAL indices (**$p<0.01$ compared with mice given OVA alone). Administration of anti-IL-10R antibody did not affect total BAL cell count or eosinophil numbers. Data are mean +/- SEM, n=8.

4.3.2 The effect of anti-IL-10R antibody on Pam3CSK4 therapy of OVA-induced thoracic lymph node responses and enhanced pause

Thoracic lymph node cells were harvested from the Pam3CSK4-treated and untreated control mice and cultured with OVA *in vitro*. Pam3CSK4 did not affect the T cell proliferation against OVA *in vitro* from LN cells from mice treated with anti-IL-10R or control IgG. Anti-IL-10R antibody did not affect the cytokine production from thoracic lymph node cells from mice treated with Pam3CSK4 and stimulated *in vitro* with OVA. The IFNγ production was still enhanced, with attenuation of IL-4 and IL-5 (Fig. 4.5). However, the reversal of airway hyperresponsiveness measured by Penh, which was associated with Pam3CSK4 therapy, was reversed with anti-IL-10R administration (Fig. 4.5).

4.3.3 Anti-IL-10R antibody does not reverse histological improvement in inflammation seen with Pam3CSK4 therapy

Pam3CSK4 treatment of mice resulted in an attenuation of the histological evidence of airways inflammation, as compared to untreated mice. Anti-IL-10R antibody administration did not alter the reduction in inflammatory cell infiltrate, peri-bronchial inflammation, or peri-vascular inflammation demonstrated in the lungs after Pam3CSK4 therapy (Fig. 4.6).

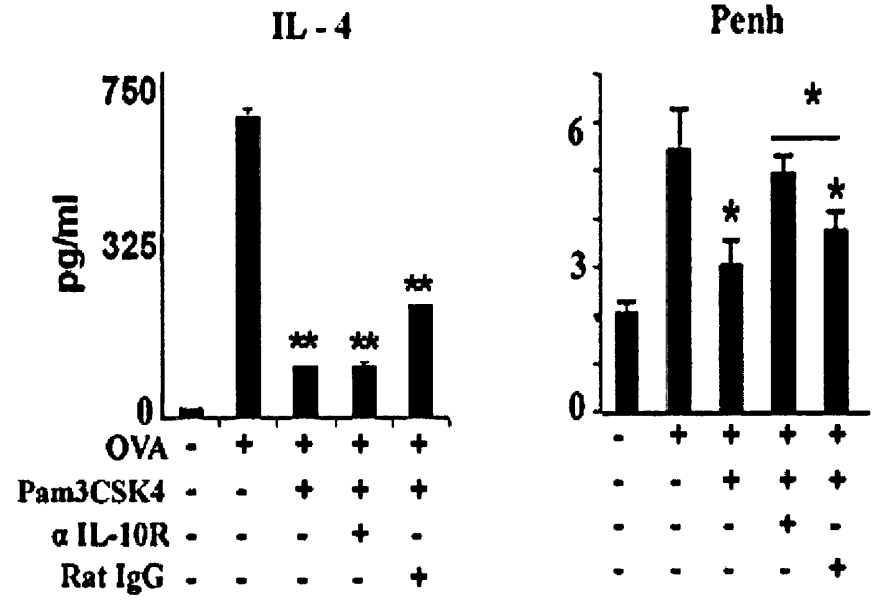

Figure 4.5 The effect of anti-IL-10R antibody on the OVA-induced IL-4 responses in thoracic lymph node cells *in vitro*, and Penh values from mice treated with Pam3CSK4.

Mice were sensitised and challenged with OVA and injected i.p with anti-mouse IL-10R antibody or control normal IgG, 2 h before the i.n. OVA challenge of day 27. Pam3CSK4 therapy was administered 2 h after the last i.n. challenge on day 27. Mice that received Pam3CSK4 therapy had a significant reduction in IL-4 production by draining thoracic lymph node (LN) cells (**$p<0.01$ compared with mice given OVA alone). Administration of anti-IL-10R antibody did not affect the reduction of IL-4 production by thoracic lymph node cells treated with Pam3CSK4 therapy (all mice that received Pam3CSK4 had a significant reduction, **$p<0.01$ compared with mice given OVA alone). However, anti-IL-10R antibody did reverse the beneficial effects in Penh values seen after Pam3CSK4 therapy. Data are mean +/- SEM, n=8.

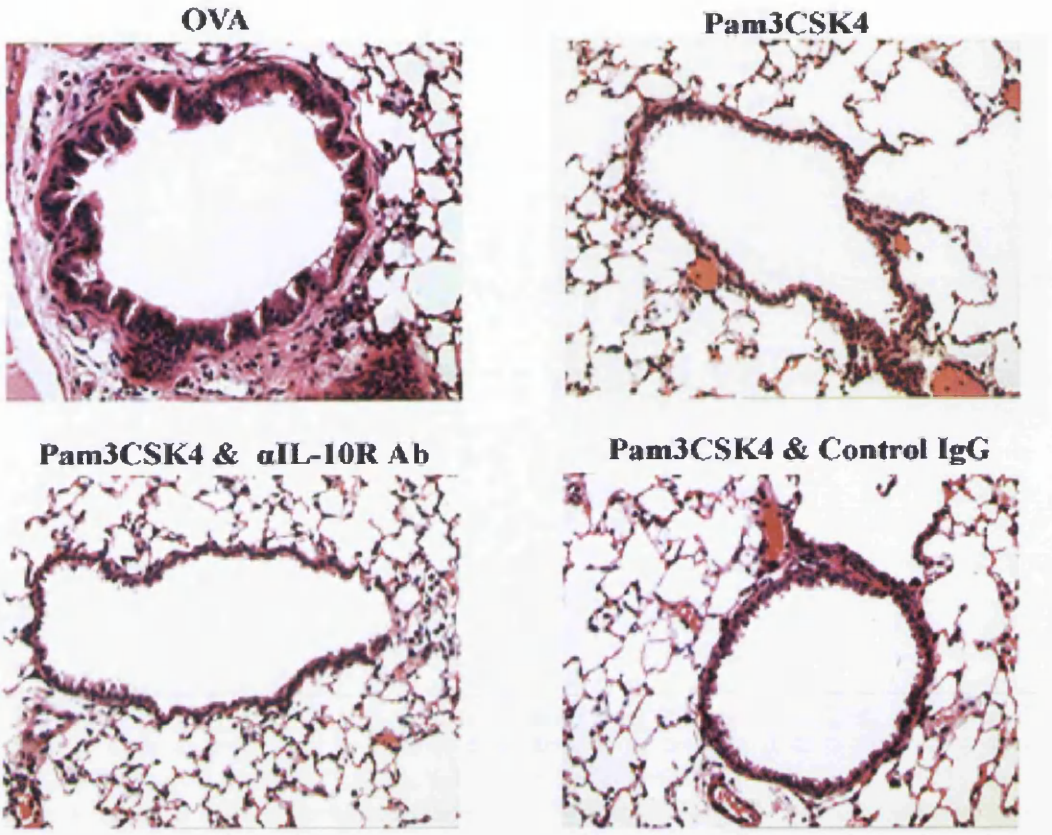

Figure 4.6 Anti-IL-10R antibody did not affect the histological evidence of reduced airways inflammation induced by Pam3CSK4.

Mice were sensitised and challenged with OVA and injected i.p with anti-mouse IL-10R antibody or control normal IgG, 2 h before the i.n. OVA challenge of day 27. Pam3CSK4 therapy was administered 2 h after the last i.n. challenge on day 27. Histology showed that administration of anti-IL-10R antibody did not affect the attenuation of airways inflammation seen after Pam3CSK4 therapy. H&E sections at x20 magnification. Data are representative of 8 mice per group.

4.4 Pam3CSK4 therapy is not dependent on TGFβ

To investigate the possibility that TGFβ is important in Pam3CSK4 therapy, mice were sensitised and challenged with OVA using the standard protocol. The animals were injected i.p with blocking anti-mouse TGFβ antibody or control normal IgG, 2 h before i.n. OVA challenge of day 27. Pam3CSK4 therapy (day 27) was administered 2 h after the last i.n. challenge (Fig. 4.3). This antibody and the amounts administered are also routinely found to be effective in abrogating $CD4^+CD25^+$ regulatory T cells in this laboratory (242).

4.4.1 Anti-TGFβ antibody does not reverse the therapeutic effect of Pam3CSK4 on BAL indices

The total BAL cellularity, and eosinophil proportion and number were consistently reduced after Pam3CSK4 therapy. Administration of anti-TGFβ antibody did not alter this effect of Pam3CSK4 therapy (Fig. 4.7). BAL cytokines were not detected.

4.4.2 The effect of TGFβ antibody on Pam3CSK4 therapy of OVA-induced thoracic lymph node responses and enhanced pause

Thoracic lymph node cells were harvested from the Pam3CSK4-treated and untreated control mice and cultured with OVA *in vitro*. Pam3CSK4 did not affect the T cell proliferation against OVA *in vitro*, from LN cells from mice treated with anti-TGFβ

or control IgG. Anti-TGFβ antibody did not affect the cytokine production from thoracic lymph node cells from mice treated with Pam3CSK4 and stimulated *in vitro* with OVA. The IFNγ production was still enhanced, with attenuation of IL-4 and IL-5 (Fig. 4.8). However, the reversal of airway hyperresponsiveness measured by Penh which was associated with Pam3CSK4 therapy was reversed with anti-TGFβ Ab administration (Fig. 4.8).

4.4.3 Anti-TGFβ antibody does not reverse histological evidence of anti-inflammatory action of Pam3CSK4 therapy

Pam3CSK4 treatment of mice resulted in an attenuation of the histological evidence of airways inflammation, as compared to untreated mice. Anti-TGFβ antibody administration did not alter the reduction in inflammatory cell infiltrate demonstrated in the lungs after Pam3CSK4 therapy (Fig. 4.9).

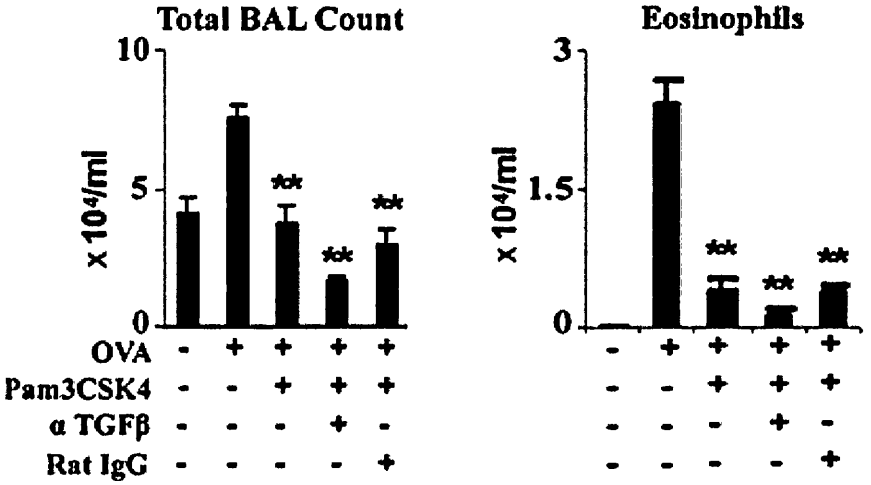

Figure 4.7 The therapeutic benefit of Pam3CSK4 on BAL indices was not affected by anti-TGFβ antibody administration.

Mice were sensitised and challenged with OVA and injected i.p with anti-mouse TGFβ antibody or control normal IgG, 2 h before the i.n. OVA challenge of day 27. Pam3CSK4 therapy was administered 2 h after the last i.n. challenge on day 27. All mice that received Pam3CSK4 therapy demonstrated a highly significant fall in their BAL indices (**$p<0.01$ compared with mice given OVA alone) Administration of anti-TGFβ antibody did not affect the reduction in total BAL count or eosinophil numbers seen after Pam3CSK4 therapy. Data are mean +/- SEM, n=8.

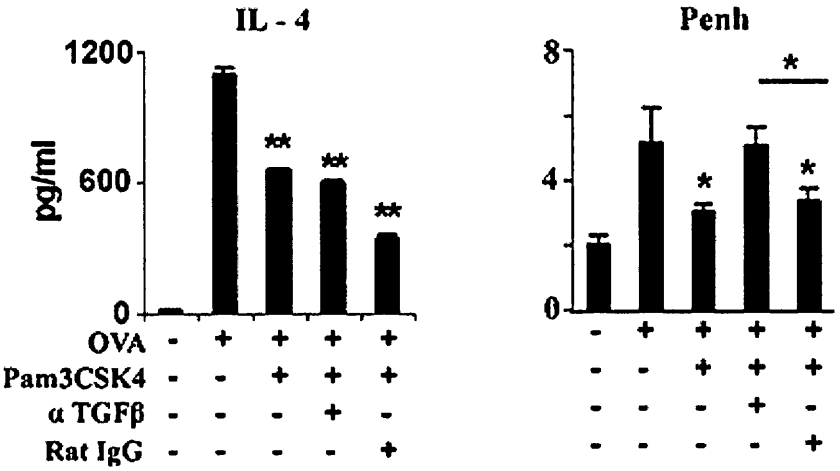

Figure 4.8 The effect of anti-TGFβ antibody on OVA-induced IL-4 responses in thoracic lymph node cells *in vitro*, and Penh values from mice treated with Pam3CSK4.

Mice were sensitised and challenged with OVA and injected i.p with anti-mouse TGFβ antibody or control normal IgG, 2 h before the i.n. OVA challenge of day 27. Pam3CSK4 therapy was administered 2 h after the last i.n. challenge on day 27. Mice that received Pam3CSK4 therapy had a significant reduction in IL-4 production by draining thoracic lymph node (LN) cells (**$p<0.01$ compared with mice given OVA alone). Administration of anti-TGFβ antibody did not affect the reduction of IL-4 production by thoracic lymph node cells treated with Pam3CSK4 therapy (all mice that received Pam3CSK4 had a significant reduction, **$p<0.01$ compared with mice given OVA alone). However, anti-TGFβ antibody did reverse the reduction in airway hyperreactivity measured by Penh following Pam3CSK4 therapy. Data are mean +/- SEM, n=8.

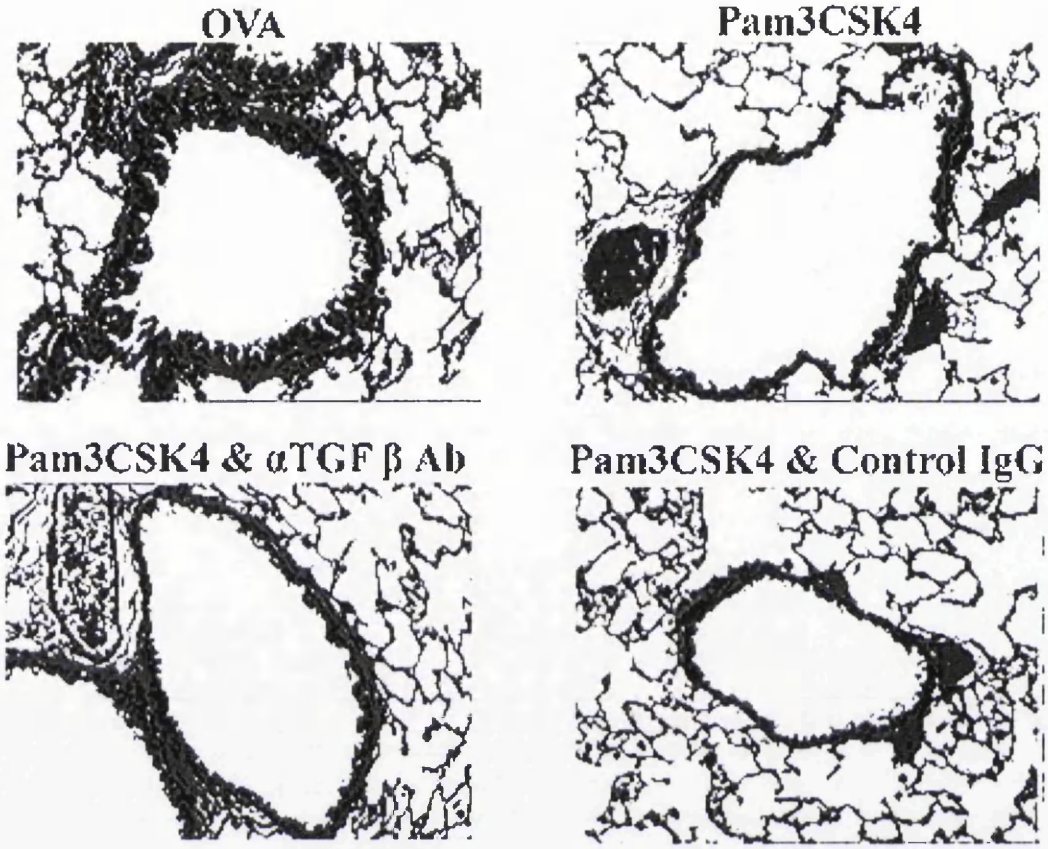

Figure 4.9 Anti-TGFβ antibody did not affect the histological evidence of reduced airways inflammation induced by Pam3CSK4.

Mice were sensitised and challenged with OVA and injected i.p with anti-mouse TGFβ antibody or control normal IgG, 2 h before the i.n. OVA challenge of day 27. Pam3CSK4 therapy was administered 2 h after the last i.n. challenge on day 27. Histology showed that administration of anti-TGFβ antibody did not affect the attenuation of airways inflammation seen after Pam3CSK4 therapy. H&E sections at x20 magnification. Data are representative of 8 mice per group.

4.5 The therapeutic effect of Pam3CSK4 is IL-12 dependent

We then investigated the role of IL-12 in the therapeutic effect of Pam3CSK4. Mice were sensitised and challenged with OVA, as described above, and injected i.p. with blocking anti-mouse IL-12 Ab or control rabbit IgG 2 h prior to the i.n. challenge of day 27. Pam3CSK4 was than administered 2 h after the allergen challenge on day 27 (Fig 4.3). The function of this antibody was confirmed *in vitro*, and is routinely used in Th2 cell polarisation.

4.5.1 Anti-IL-12 antibody reverses the therapeutic effect of Pam3CSK4 on BAL indices

Fig. 4.10 shows that the therapeutic effect of Pam3CSK4 was completely abrogated by blocking IL-12. This was clearly demonstrated by the reversal of the Pam3CSK4 beneficial effect on BAL cellularity and eosinophila, seen after anti-IL-12 antibody administration.

4.5.2 Anti-IL-12 antibody reverses OVA-induced lymph node responses induced by Pam3CSK4 therapy

Thoracic lymph node cells were taken from mice treated with Pam3CSK4, and stimulated *in vitro*. The OVA-induced LN proliferative response was not affected by Pam3CSK4 treatment. However, the Th2 cytokine (IL-4 and IL-5) production was

reduced with enhancement of the Th1 cytokine (IFNγ) production by LN cells taken from Pam3CSK4-treated mice. Administration of anti-IL12 antibody completely abolished these changes induced by Pam3CSK4 therapy (Fig. 4.11).

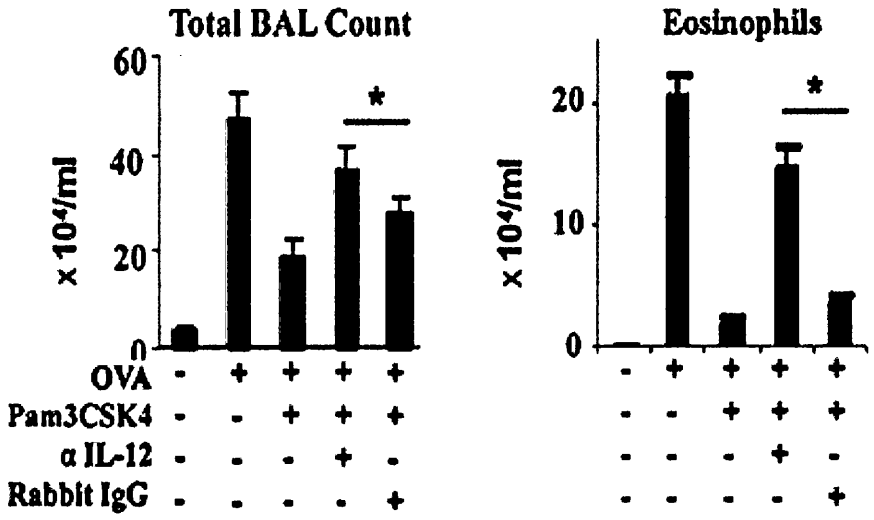

Figure 4.10 The therapeutic benefit of Pam3CSK4 on BAL indices was abolished by anti-IL-12 antibody administration.

Mice were sensitised and challenged with OVA and injected i.p with anti-mouse IL-12 antibody or control normal IgG, 2 h before the i.n. OVA challenge of day 27. Pam3CSK4 therapy was administered 2 h after the last i.n. challenge on day 27. Administration of anti-IL-12 antibody significantly reversed the reduction in total BAL count or eosinophil numbers seen after Pam3CSK4 therapy (*$p<0.05$ compared with mice given control Ab). Data are mean +/- SEM, n=8.

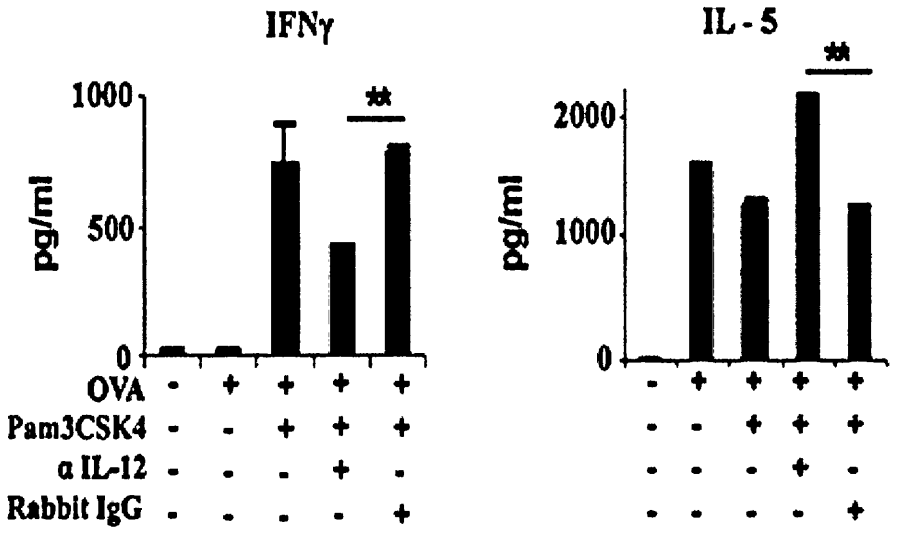

Figure 4.11 The effect of anti-IL-12 antibody on OVA-induced IFNγ and IL-5 responses in thoracic lymph node cells *in vitro*.

Mice were sensitised and challenged with OVA and injected i.p with anti-mouse IL-12 antibody or control normal IgG, 2 h before the i.n. OVA challenge of day 27. Pam3CSK4 therapy was administered 2 h after the last i.n. challenge on day 27. Administration of anti-IL-12 antibody significantly reversed the reduction in IL-5 production from thoracic lymph node cells by mice given Pam3CSK4 therapy. Antibody administration also abrogated the enhanced production of IFNγ by cells from Pam3CSK4-treated mice (**$p<0.01$ compared with mice given control Ab). Data are mean +/- SEM, n=8.

4.5.3 Anti-IL-12 antibody reverses the beneficial effect of Pam3CSK4 on airway hyperresponsiveness

Anti-IL-12 antibody administration resulted in a significant reversal of the amelioration in Penh values seen in Pam3CSK4-treated mice. Blocking IL-12 resulted in Pam3CSK4-treated mice continuing to have increased Penh levels, as seen in untreated OVA-sensitised and -challenged mice (Fig. 4.12).

4.5.4 Anti-IL-12 antibody effect on serum immunoglobulin levels of Pam3CSK4-treated mice

The beneficial effect of Pam3CSK4 treatment seen in serum total and OVA-specific IgE levels was completely abolished after anti-IL-12 antibody administration (Fig. 4.12). Serum levels of IgG1 and IgG2a were unaffected by Pam3CSK4 therapy and anti-IL-12 administration (data not shown).

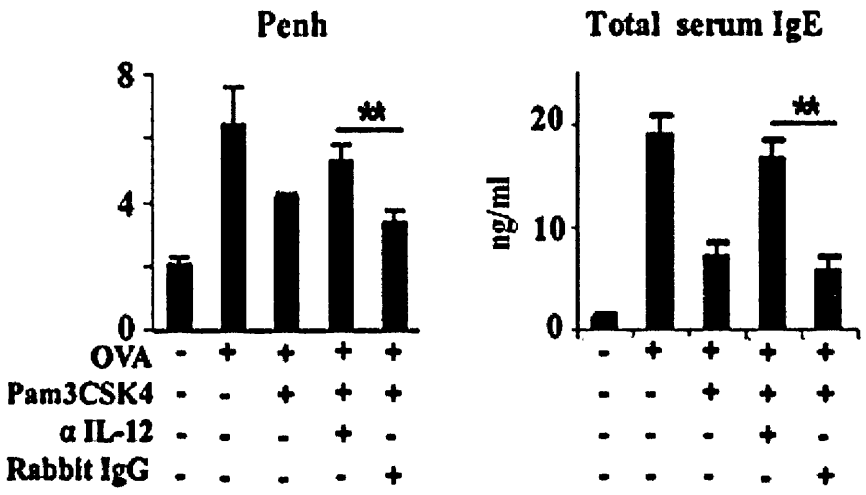

Figure 4.12 The effect of anti-IL-12 antibody on airway hyperresponsiveness measured by Penh and total serum IgE levels from mice treated with Pam3CSK4.

Mice were sensitised and challenged with OVA and injected i.p with anti-mouse IL-12 antibody or control normal IgG, 2 h before the i.n. OVA challenge of day 27. Pam3CSK4 therapy was administered 2 h after the last i.n. challenge on day 27. Administration of anti-IL-12 antibody significantly reversed the beneficial effects on Penh values and total serum IgE seen in mice given Pam3CSK4 therapy (**$p<0.01$ compared with mice given control Ab). Data are mean +/- SEM, n=8.

4.5.5 Anti-IL-12 antibody administration abolishes the histological improvement in inflammation seen after Pam3CSK4 therapy

Histological examination of the murine lungs demonstrated that anti-IL-12 antibody therapy reverses the improvement in lung inflammation seen in mice after Pam3CSK4 administration (Fig 4.13).

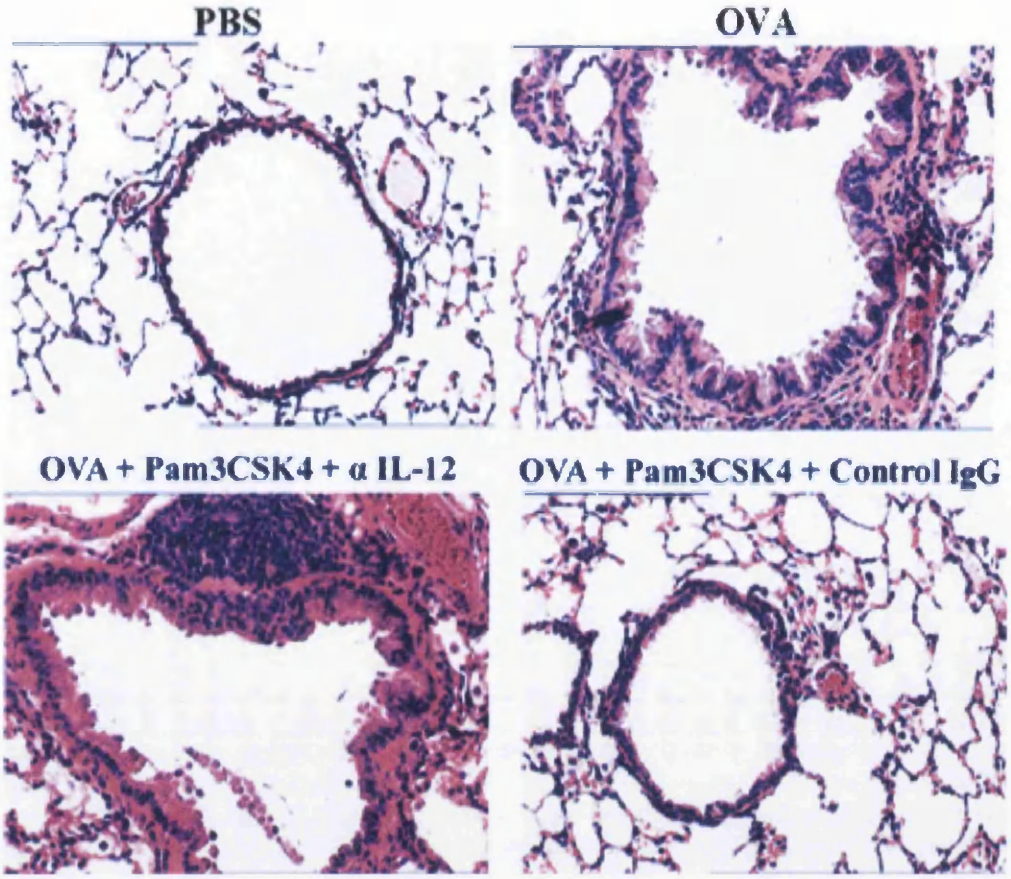

Figure 4.13 Anti-IL-12 antibody abolished the histological evidence of reduced airways inflammation induced by Pam3CSK4.

Mice were sensitised and challenged with OVA and injected i.p with anti-mouse IL-12 antibody or control normal IgG, 2 h before the i.n. OVA challenge of day 27. Pam3CSK4 therapy was administered 2 h after the last i.n. challenge on day 27. Histology showed that administration of anti-IL-12 antibody completely reversed the reduction of airways inflammation seen after Pam3CSK4 therapy. H&E sections at x20 magnification. Data are representative of 8 mice per group.

4.5.6 Pam3CSK4 therapy has no effect on BAL indices in IL-12 deficient mice

To confirm the role of IL-12 in this model, we sensitised and challenged IL-12 knockout (ko) mice with OVA, and Pam3CSK4 was then administered 2 h after the allergen challenge on day 27. While the wild type (BALB/c) control mice showed the expected beneficial effect of Pam3CSK4 treatment, IL-12 ko mice did not. IL-12 ko mice exhibited the same degree of eosinophilia (Fig. 4.14) and total BAL cellularity (data not shown) as the untreated IL-12 ko or wild-type mice.

4.5.7 Pam3CSK4 therapy has no effect on OVA-induced thoracic lymph node cells response taken from IL-12 deficient mice

The wild type mice demonstrated that although the proliferative response to OVA *in vitro* is unaltered after Pam3CSK4 therapy, the Th2 response (IL-4 and IL-5 production) is attenuated whilst the Th1 response (IFNγ production) is enhanced. In IL-12 ko mice there was no change in the cytokines produced by thoracic lymph nodes after Pam3CSK4 therapy (Fig 4.14).

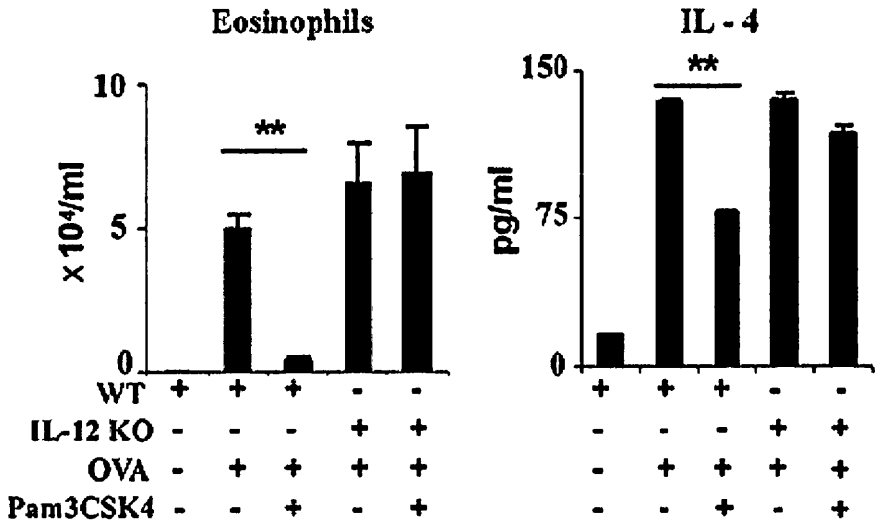

Figure 4.14 Pam3CSK4 had no effect on the eosinophilia or IL-4 synthesis by the lymphoid cells of IL-12 deficient mice.

Wild-type and IL-12 ko mice (on a BALB/c background) were sensitized and challenged as in Fig. 3.1. Pam3CSK4 was administered i.p. on day 27, 2 h after the last i.n. OVA challenge. Pam3CSK4 had no effect on the eosinophilia or IL-4 synthesis by the lymph node cells of IL-12 ko mice. IFNγ was not detectable in the culture of cells from the IL-12 ko mice. Data are mean +/- SEM of 5 mice.

4.5.8 Pam3CSK4 therapy has no effect on histological inflammation in IL-12 deficient mice

Lung sections stained with H&E demonstrated that IL-12 ko mice showed little change in their peri-bronchial and peri-vascular inflammation after Pam3CSK4 therapy. Wild type mice demonstrated the expected reduction in pulmonary inflammation after Pam3CSK4 therapy (Fig. 4.15).

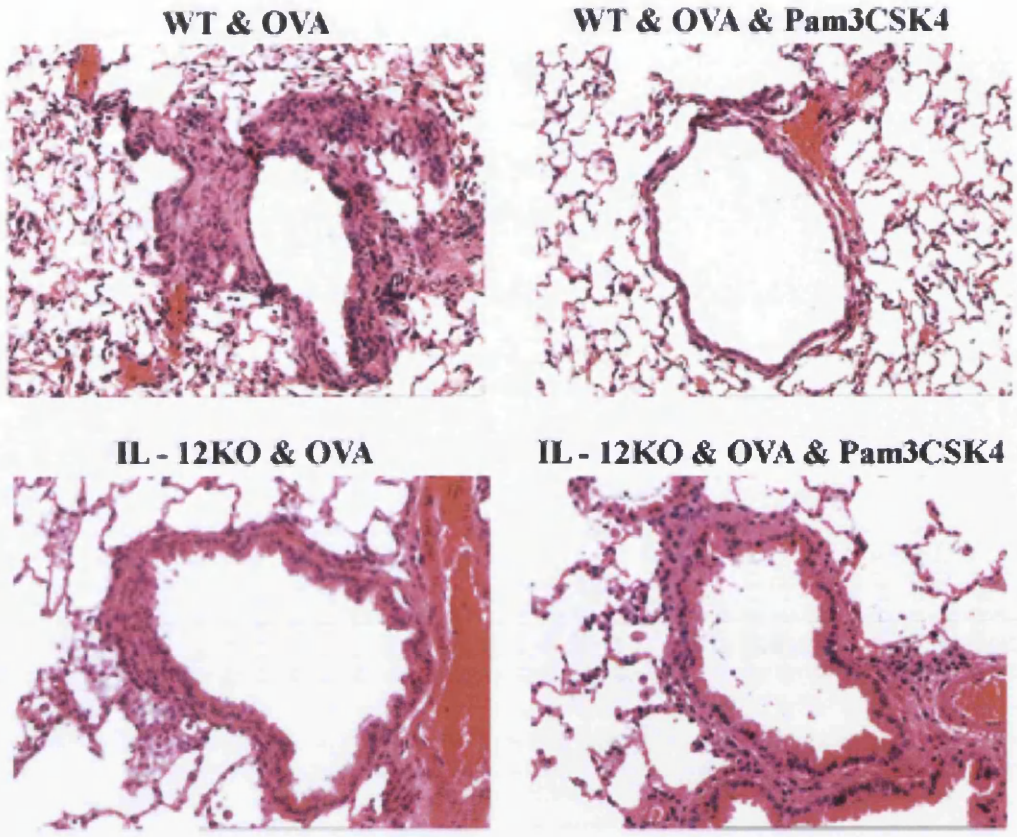

Figure 4.15 Histological evidence that Pam3CSK4 therapy did not reduce airways inflammation in IL-12 deficient mice.

Wild-type and IL-12 ko mice (on a BALB/c background) were sensitized and challenged as in Fig. 3.1. Pam3CSK4 was administered i.p. on day 27, 2 h after the last i.n. OVA challenge. Histology examination of the lungs clearly demonstrated that Pam3CSK4 did not prevent inflammatory cell infiltrations in the IL-12 ko mice. Data are representative of 5 mice per group.

4.6 Pam3CSK4 therapy is Interferon γ dependent

The enhanced IFNγ production by draining thoracic LN cells from mice treated with Pam3CSK4 would suggest that TLR2 agonist therapy may induce Th1 cell skewing. This hypothesis was further supported by our finding that Pam3CSK4 therapy was dependent on IL-12. To confirm the role of IFNγ in this model, we sensitised and challenged IFNγ ko mice (on a 129SV background) with OVA and treated them with Pam3CSK4 as above.

4.6.1 Pam3CSK4 therapy has no effect on BAL indices in IFNγ deficient mice

While the 129SV wild type mice sensitised and challenged with OVA showed typical airway eosinophilia which was reversed by Pam3CSK4 treatment, the IFNγ ko mice did not demonstrate this reversal. IFNγ ko mice exhibited the same degree of eosinophilia (Fig. 4.14) and total BAL cellularity (data not shown) as the untreated IFNγ ko or wild-type mice.

4.6.2 Pam3CSK4 therapy has no effect on OVA-induced lymph node cells response taken from IFNγ deficient mice

The IFNγ ko mice demonstrated no change in the cytokines produced by thoracic lymph nodes taken from mice given Pam3CSK4 therapy. This is in contrast to the wild type mice that demonstrated increased Th1 cytokine (IFNγ) production and a

reduced Th2 response (IL-4 and IL-5 production) by LNs taken from mice given Pam3CSK4 therapy (Fig 4.14). IFNγ was not detectable in the culture of cells from the IFNγ ko mice.

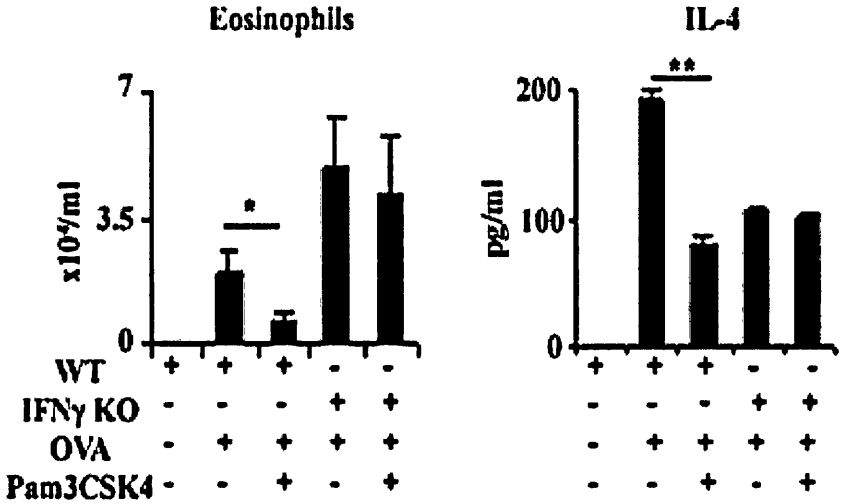

Figure 4.16 Pam3CSK4 had no effect on the eosinophilia or IL-4 synthesis by the lymphoid cells of IFNγ deficient mice.

Wild-type and IFNγ ko mice were sensitized and challenged as in Fig. 3.1. Pam3CSK4 was administered i.p. on day 27, 2 h after the last i.n. OVA challenge. Pam3CSK4 had no effect on the BAL eosinophilia, or on IL-4 synthesis by the lymphoid cells of IFNγ ko mice. Data are mean +/- SEM of 6 mice.

4.6.3 Pam3CSK4 therapy has no effect on histological inflammation in IFNγ deficient mice

Histological examination demonstrated that IFNγ ko mice showed little change in their peri-bronchial and peri-vascular inflammation after Pam3CSK4 therapy. Wild type mice demonstrated the expected reduction in pulmonary inflammation after Pam3CSK4 therapy (Fig.4.17).

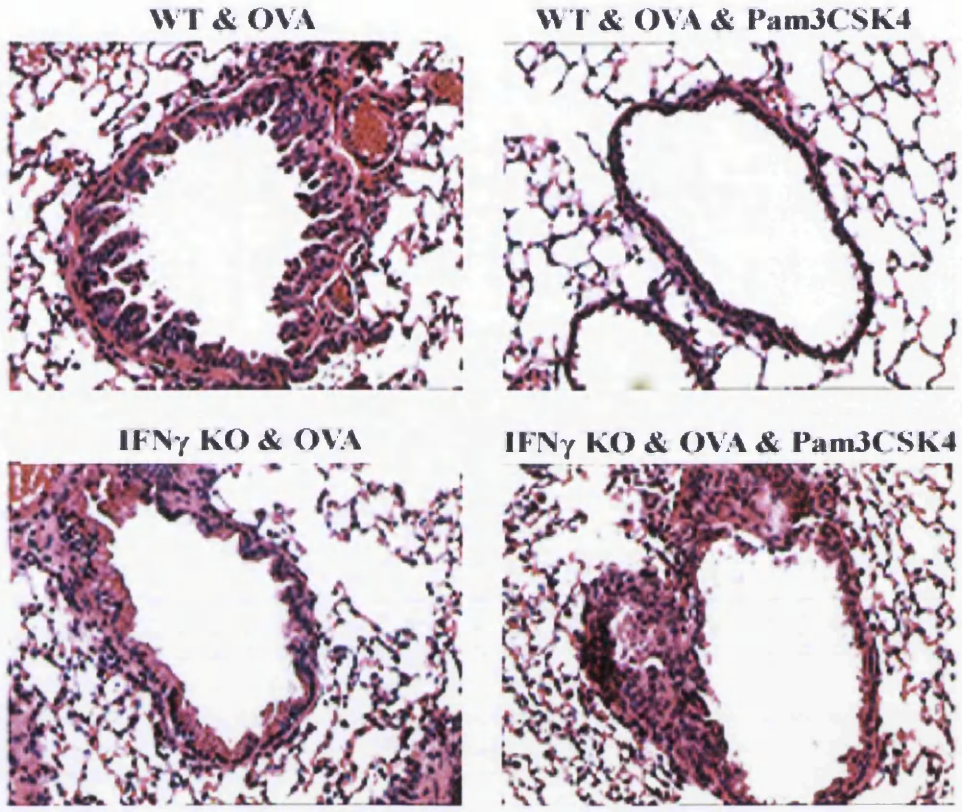

Figure 4.17 Histological evidence that Pam3CSK4 therapy did not reduce airways inflammation in IFNγ deficient mice.

Wild-type and IFNγ ko mice were sensitized and challenged as in Fig. 3.1. Pam3CSK4 was administered i.p. on day 27, 2 h after the last i.n. OVA challenge. Histology examination of the lungs clearly demonstrated that Pam3CSK4 did not prevent inflammatory cell infiltrations in the IFNγ ko mice. Data are representative of 6 mice per group.

4.7 Pam3CSK4 enhances IL-12 and IFNγ synthesis *in vitro*

We have demonstrated above that IL-12 and IFNγ are critical for the ability of Pam3CSK4 to downregulate airways inflammation associated with reduced eosinophilia, IL-4, IL-5, and IgE production. To investigate the cell source and mechanism by which Pam3CSK4 induced IL-12 production and Th1 cell development, we cultured bone marrow derived dendritic cells (BMDCs) and bone marrow derived macrophages (BMMs) with graded doses of Pam3CSK4 *in vitro*.

4.7.1 Pam3CSK4 induces IL-12 production from dendritic cells and macrophages *in vitro*

In response to Pam3CSK4, BMDCs produced significant levels of IL-12, and low but detectable levels of IL-10 (Fig. 4.18). IFNγ, IL-4 and IL-5 were not detected (data not shown). Pam3CSK4 induced BMMs to produce IL-12 and IL-10 (Fig. 4.19), but IFNγ, IL-4 and IL-5 were not detected (data not shown).

4.7.2 Pam3CSK4-stimulated dendritic cells and macrophages induce polarisation of naïve $CD4^+$ T cells to Th1 cells

Pam3CSK4 could enhance IL-12 production from BMDCs and BMMs and IL-10 production from BMMs. To further investigate the effects on these TLR2 agonist-activated APCs on T cell differentiation, Pam3CSK4-stimulated BMDCs and BMMs

were washed and cultured with highly purified $CD4^+$ T cells from OVA-TcR transgenic mice (DO10.11), in the presence of OVA peptide and Pam3CSK4. $CD4^+$ T cells cultured in the presence of Pam3CSK4-stimulated BMDCs produced significant amounts of IFNγ, but little or no detectable IL-4 (Fig. 4.20). $CD4^+$ T cells cultured with Pam3CSK4-stimulated BMMs produced a small amount of IFNγ but no IL-4 (Fig. 4.21). These results demonstrate that Pam3CSK4 is capable of inducing APCs (mainly DCs) to produce IL-12, which in turn enhanced the development of Th1 cells and the production of IFNγ.

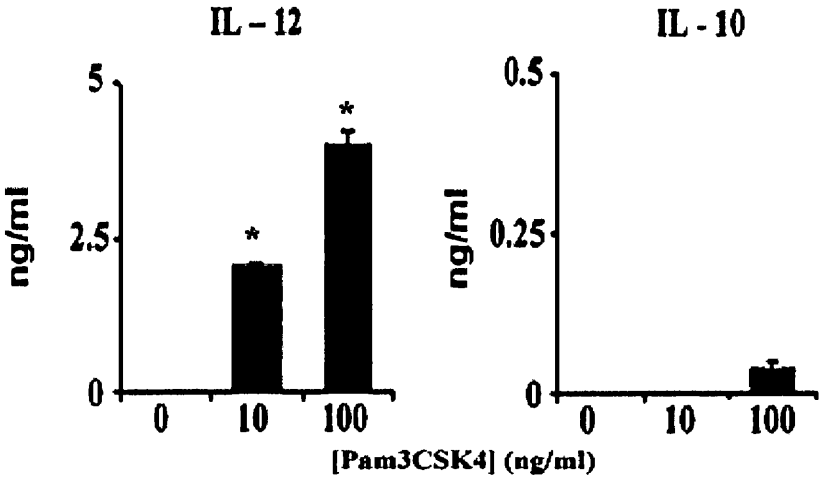

Figure 4.18 Pam3CSK4 enhanced IL-12 production by DCs *in vitro*.

BMDC were cultured with 10 or 100 ng/ml Pam3CSK4 for 12 h. Cytokine concentrations in the culture supernatant were assayed by ELISA. No cytokine was detected in the cultures without Pam3CSK4. In the presence of Pam3CSK4, BMDC produced IL-12 (*$p<0.05$ compared to cultures without Pam3CSK4). IL-10, IFNγ, IL-4 and IL-5 were not detected in significant amounts. Data are mean +/- SEM, n=3 and are representative of 3 experiments.

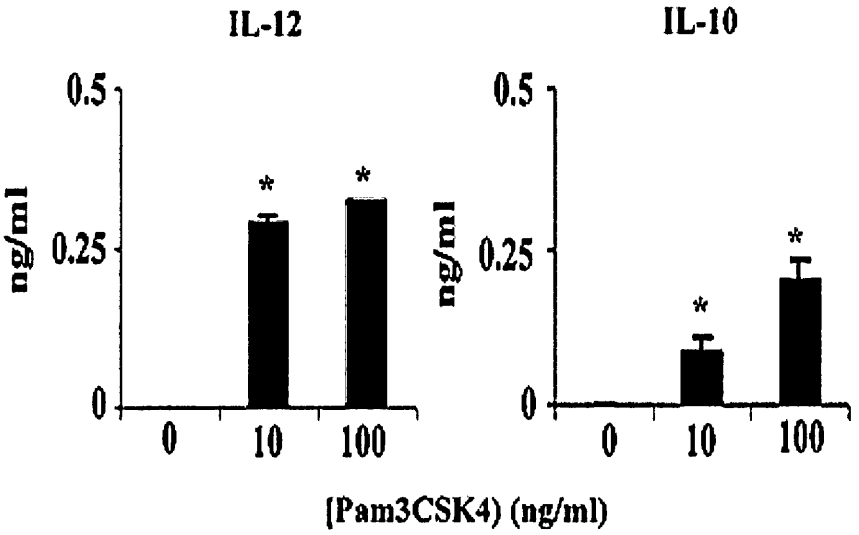

Figure 4.19 Pam3CSK4 enhanced IL-12 and IL-10 production by macrophages *in vitro*.

BMMs were cultured with 10 or 100 ng/ml Pam3CSK4 for 12 h. Cytokine concentrations in the culture supernatant were assayed by ELISA. No cytokine was detected in the cultures without Pam3CSK4. In the presence of Pam3CSK4, BMMs produced low levels of IL-12 and IL-10 (*$p<0.05$ compared to cultures without Pam3CSK4). IFNγ, IL-4 and IL-5 were not detected. Data are mean +/- SEM, n=3 and are representative of 3 experiments.

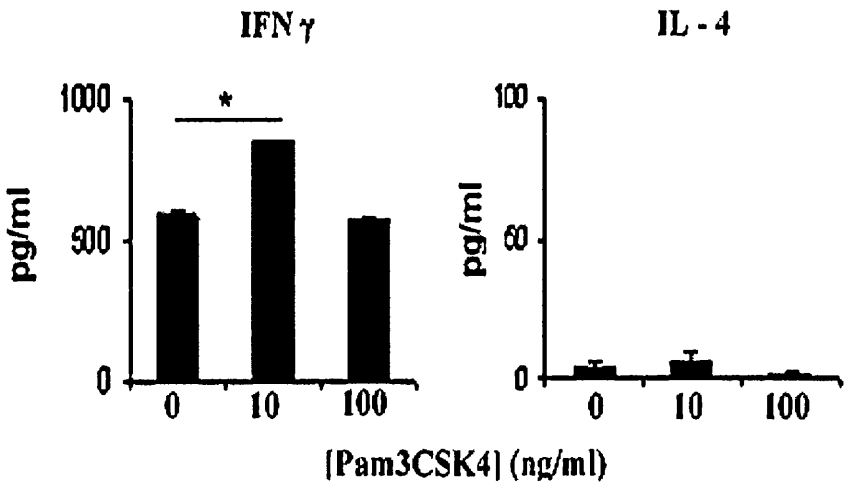

Figure 4.20 Pam3CSK4-stimulated DCs enhanced IFNγ synthesis by T cells *in vitro*.

$CD4^+$ T cells were purified from DO11.10 mice and cultured in a 5:1 ratio with BMDCs, in the presence of OVA peptide and Pam3CSK4 for 72 h. Cytokine concentrations in the culture supernatant were assayed by ELISA. Pam3CSK4 significantly enhanced IFNγ (*$p<0.05$ compared to cultures without Pam3CSK4) but not IL-4 synthesis. Data are mean +/- SEM, n=3 and are representative of 3 experiments.

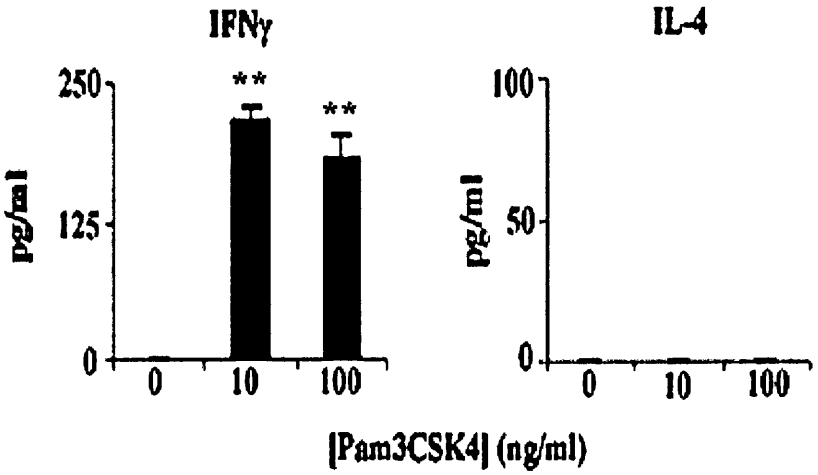

Figure 4.21 Pam3CSK4-stimulated macrophages enhanced IFNγ synthesis by T cells *in vitro*.

$CD4^+$ T cells were purified from DO11.10 mice and cultured in a 5:1 ratio with BMMs, in the presence of OVA peptide and Pam3CSK4 for 72 h. Cytokine concentrations in the culture supernatant were assayed by ELISA. Pam3CSK4 significantly increased IFNγ production (**$p<0.01$ compared to cultures without Pam3CSK4) but had no detectable effect on IL-4 synthesis. Data are mean +/- SEM, n=3 and are representative of 3 experiments.

4.7.3 Pam3CSK4 directly enhances Th1 and Th2 differentiation of $CD4^+$ cells

Having found Pam3CSK4 could influence Th1 differentiation via APCs, we were interested to investigate the direct effect of Pam3CSK4 on Th differentiation. $CD4^+$ T cells were purified from OVA-TcR transgenic mice (DO10.11) and stimulated twice with OVA peptide under neutral (Th0), Th1 or Th2 polarising conditions in the presence of Pam3CSK4 (as described in Materials and Methods, Section 2.5.3, p90). $CD4^+$ cells cultured under all three conditions showed enhanced IFNγ and IL-5 production in the presence of Pam3CSK4 treatment after 1 round of polarisation (Fig. 4.22). This enhanced Th1 and Th2 cytokine production was not evident after the 2^{nd} round of stimulation.

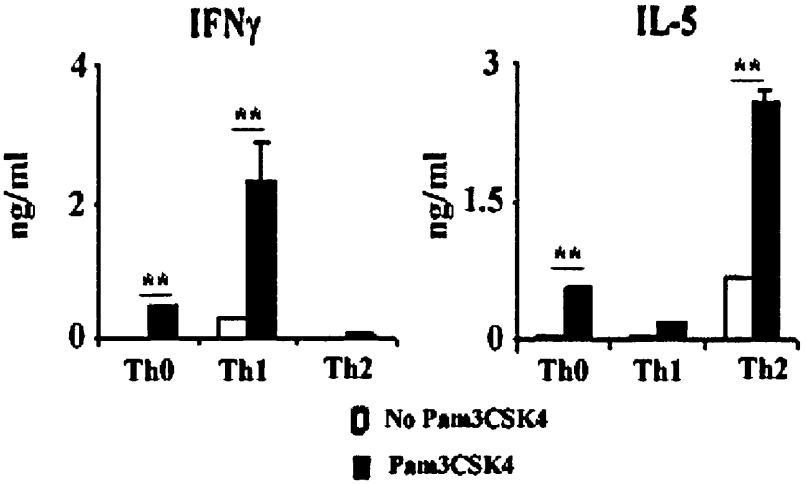

Figure 4.22 Pam3CSK4 enhanced *in vitro* production of IFNγ and Il-5 by $CD4^+$ T cells directly.

$CD4^+$ T cells were purified from DO11.10 mice and cultured with OVA peptide under neutral (Th0), Th1 or Th2 polarising conditions in the presence of 10 ng/ml Pam3CSK4. Cytokine concentrations in the culture supernatant were assayed by ELISA. Pam3CSK4 increased IFNγ and IL-5 production from Th0 cells (**$p<0.01$ compared to cultures without Pam3CSK4). Pam3CSK4 also enhanced IFNγ and IL-5 synthesis from Th1 and Th2 cells respectively (**$p<0.01$ compared to cultures without Pam3CSK4). Pam3CSK4 also increased both IFNγ and IL-5 production from Th0 cells. Data are mean +/- SEM, n=3 and are representative of 2 experiments.

4.8 Chapter Discussion

The main findings from the work in this chapter are that:

iv) Intraperitoneal Pam3CSK4 therapy does not sequester eosinophils into the peritoneal cavity

v) Pam3CSK4 therapy is dependent on IL-12 and IFNγ *in vivo*

vi) Pam3CSK4 therapy is independent of IL-10 or TGFβ *in vivo*

vii) Pam3CSK4 can induce IL-12 synthesis from APCs, which in turn can enhance Th1 differentiation of $CD4^+$ cells *in vitro*

viii) Pam3CSK4 can directly enhance early Th1 and Th2 differentiation of $CD4^+$ cell *in vitro.*

The immunomodulatory effect of Pam3CSK4 therapy in allergic airways disease appears to be based, at least in part, on its ability to induce IL-12 synthesis by DCs. The heightened levels of IL-12 in turn enhance a specific Th1 response and decrease Th2 activity. The therapeutic effect is independent of IL-10 and TGFβ and, by extension, unlikely to involve regulatory T cells.

In an equivalent study in asthma patients, it has been shown that recombinant IL-12 administration could reduce blood and sputum eosinophilia, but did not significantly change airway hyperresponsiveness (379). The reasons for these observations are difficult to explain. It may be that the short term administration of IL-12 was sufficient to attenuate acute indices of allergic inflammation, however therapy that alters the Th1/2 profile in a more prolonged fashion is required to alter parameters like airway hyperresponsiveness. It is known that IFNγ or IL-12 can reverse Th2-

mediated airways hyperresponsiveness and inflammation in murine models of allergic airways inflammation (380). However, once disease has been established it has also been reported that high levels of IFNγ and IL-12 can promote Th2-induced eosinophilic inflammation, and that a strong Th1 response (independent of Th2 involvement) can induce airway hyperresponsiveness (381-383). This is perhaps not surprising since at high concentrations, IFNγ is a well-recognised pro-inflammatory cytokine that appears to have a role in airways inflammation. In as much as Th2 cytokines are a dominant detrimental feature in airways diseases, the key therapeutic approach is perhaps to achieve a balance between the Th2 and Th1 cytokines, where there is a decrease in the Th2 cytokines without a large increase in the Th1 response. The role of TLR2 signalling in the induction of Th1 and Th2 cell development from naïve T cells remains unresolved. TLR2 activation has been reported to lead to the initiation of both Th1 and Th2 differentiation (149, 384, 385). It is likely that whether the TLR signalling predominantly activates the Th1 or the Th2 pathway depends on the timing (relative to specific antigenic stimulation), dose and nature of TLR2 agonists, and the genetic background of the responding hosts. In our model, although there is a mild increase in the Th1 cytokines after TLR2 agonist therapy, the levels do not appear to be high enough to exacerbate airway hyperresponsiveness, or increase the Th1-dominant IgG2a antibody subtype. Our data is supported by recent reports investigating the effect of a TLR2 agonist in the sensitisation phase (369), and the effect of a TLR 2/4 agonist in the challenge phase (386) of murine models of asthma which have shown that TLR activation could lead to a beneficial decrease in airways disease by down-regulating the Th2 response, with a mild non-detrimental increase in Th1 response.

Several contradictory reports have been made on the effect of TLR2 agonist administration in the sensitisation phase of airways disease induction. Investigators (149, 367) have shown that early TLR2 agonist administration worsens the development of murine asthma, whilst others (369) have demonstrated early TLR2 agonist therapy can ameliorate allergic airways disease. One possible explanation for these observations is that different strains of mice were used by different investigators, and therefore the nature of the T-helper response that predominates in different strains of mouse may influence the effect of TLR2 activation. The majority of work to date has been performed using BALB/c mice, which have a dominant Th2 immune system. However, administration of TLR2 agonist therapy to BALB/c mice has resulted in both amelioration (368, 369) and aggravation (367) of allergic airways disease. Furthermore, Redecke *et al* (149) used 129SV mice which have a dominant Th1 immune system, and showed that Pam3CSK4 administration in the sensitisation phase of airways disease induction resulted in a worsening of experimental asthma. Interestingly, our own work utilised 129SV mice as wild type controls for the IFNγ ko studies, and demonstrated that Pam3CSK4 administration had a therapeutic role in allergic airways disease in this strain of mouse. Hence, it is unlikely that strain differences alone will fully explain the different results reported for TLR2 agonist treatment in murine models of asthma. The amount and route of administration of TLR2 agonist have been proposed as alternative explanations for the differences observed. With 129SV mice, Redecke *et al* administered 500 μg of Pam3CSK4 per mouse by a subcutaneous route, whereas I administered only 100 μg of Pam3CSK4 per mouse by an intraperitoneal route. Although this might partly explain the different observations observed with TLR2 agonist administration in 129SV mice, it does not account for all the observations made in BALB/c mice (367, 369). These differences

and the mechanisms behind TLR2 agonist administration in different experimental asthma models remain unresolved and require further investigation. Possible explanations for the different action of TLR2 agonists observed in murine models of asthma are further discussed in Chapter 6.

There are other possible roles for TLR agonist in the lung. TLR activation of inflammatory cells and respiratory epithelial cells increase their adhesion molecule expression and migration into sites of inflammation (387, 388). In Chapter 3, we demonstrated that Pam3CSK4 administration by i.p. injection ameliorates airways disease, whilst TLR2 agonists given by the i.n. route exacerbate inflammation. One mechanism that may account for this difference is that i.p. Pam3CSK4 therapy could be creating a local inflammatory response in the peritoneal cavity, and sequestering inflammatory cells (such as eosinophils) away from the lung to the peritoneum. However, in this chapter we have demonstrated that this is not the case. Mice that had received i.p. Pam3CSK4 displayed a reduction in the eosinophil numbers in the peritoneum. In addition, mice that had received i.p. Pam3CSK4 therapy demonstrated little difference in their peritoneal lymphocyte, neutrophil and macrophage numbers; did not look unwell; and macroscopically their intestines and peritoneal cavities looked normal. It may be that the timing of the peritoneal washes at 24h, 48h and 72h after Pam3CSK4 therapy did not detect an acute peritonitis. However, any detrimental effects that may have occurred in the peritoneal cavity out with these times are certainly not long-lived, whereas the beneficial effects of TLR2 agonist treatment on airways inflammation is seen up to 5 days after the last i.n. challenge.

Another possible explanation for the attenuation airways eosinophilia observed after TLR2 agonist i.p. administration may relate to increased cell death through increased apoptosis and/or necrosis. Necrosis seems unlikely, as increased cell death would result in increased release in inflammatory mediators which would in turn enhance inflammation. We are currently examining the migration and activation status, including markers of apoptosis, in immune cells in the mouse after TLR2 agonist therapy. This will further add to our knowledge of how TLR2 agonists are functioning in this murine model of allergic airways disease.

The enhancing effect of Pam3CSK4 on IL-10 production *in vivo* and *in vitro* raised the possibility that regulatory T cells may play a role in Pam3CSK4 treatment. Many investigators have observed that regulatory T cells ($CD4^+CD25^+$, Tr1 and Th3) function is dependent on IL-10 and TGFβ (204, 242, 299, 306). It is also known that Tregs express TLRs, and their suppressive function can be enhanced by TLR activation (186, 378). Using blocking antibodies, we demonstrate here that the anti-inflammatory effect of Pam3CSK4 appears to be independent of IL-10 or TGFβ, and hence Treg cells are unlikely to play an important role in Pam3CSK4 therapeutic effect.

The beneficial effect of Pam3CSK4 in murine allergic airways disease and its mechanism of action appear to be akin to the TLR9 agonist, CpG oligonucleotides. TLR9 activation also led to an increase in IL-12 production from DCs and an enhancement of IFNγ synthesis by T cells, resulting in decreased Th2 cell differentiation and attenuated eosinophilic airways inflammation (365). However, it has been reported that CpG could enhance the risk of aggravation of autoimmune

disorder in immunocompetent hosts (170). Thus TLR2 agonists, such as synthetic Pam3CSK4, may represent additional and alternative potential reagents for controlling allergic diseases.

Chapter 5

The role of Glucocorticoid-induced tumour necrosis factor receptor (GITR) in a murine model of allergic airways disease

Data in this chapter has been submitted for publication.

5.1 Introduction

Glucocorticoid-induced tumour necrosis factor receptor (GITR, TNFRSF 18), is a receptor belonging to the TNFR superfamily (TNFRSF), which was cloned in 1997 in a hybridoma T cell line treated with dexamethasone (389). GITR is expressed at low levels on naive T cells (264, 265, 291), and is abundant on $CD4^+CD25^+$ T regulatory (Treg) cells (264, 265). GITR was proposed as a cytological marker for $CD4^+CD25^+$ Tregs, however, GITR expression is up-regulated after activation of $CD4^+CD25^-$ effector T cells (264, 265).

Its ligand, GITRL, is a 173 amino acid transmembrane protein and is expressed on macrophages, DCs, and B cells, but not on T cells (390-392). The interaction of GITR with its ligand provides an early co-stimulatory signal for $CD4^+$ T cells, enhancing proliferation and cytokine production of both $CD4^+CD25^+$ and $CD4^+CD25^-$ populations (390, 393, 394). $CD4^+CD25^+$ Tregs have a direct inhibitory effect on the proliferation of $CD4^+CD25^-$ effector T cells, and this suppressive activity is reversed by an agonistic anti-GITR mAb. Thus, GITR is an important molecule that modulates both regulatory and effector T cell functions. However, the role of GITR in disease, especially Th2- mediated disorders, has not yet been investigated and is still poorly understood. We have investigated the role of GITR in the function of Th1 and Th2 cells *in vitro* and in a murine model of asthma *in vivo*.

The aims of the work in this chapter are to investigate the role of GITR in the polarisation of Th1 and Th2 cells *in vitro* by activating cells with anti-CD3 and anti-GITR mAb, and then examine cytokine production and expression of the key

transcription factors T-bet (Th1) and GATA3 (Th2). We then aim to subsequently examine the role of GITR in murine asthma *in vivo*.

We report here that anti-GITR antibody enhanced both Type 1 (IFNγ) and Type 2 (IL-5) cytokine production by anti-CD3-activated $CD4^+$, and $CD4^+CD25^-$ T cells cultured under neutral, Th1 or Th2 polarising conditions *in vitro*. Consistent with this, anti-GITR antibody treatment also significantly increased the expression of the key transcription factors T-bet (Th1) and GATA3 (Th2) by $CD4^+CD25^-$ T cells cultured with anti-CD3 antibody *in vitro*. *In vivo*, GITR activation led to disease exacerbation of asthma with concomitant elevation of Type1 and Type 2 cytokine synthesis. Thus, our study suggests that GITR is a potential therapeutic target for both Th1- and Th2-mediated inflammatory diseases.

5.2 The effects of anti-GITR antibody on Th polarisation *in vitro*

Our experiments to examine the role of GITR in lymphocyte function started with *in vitro* studies to investigate the effect of anti-GITR mAb treatment on Th1 and Th2 differentiation from naïve $CD4^+$ T cell precursors. Previous work has demonstrated that anti-GITR mAb can abrogate $CD4^+CD25^+$ Treg suppressor function (264, 265), and that GITR is an early co-stimulatory signal for $CD4^+$ T cells - enhancing proliferation and cytokine production of both $CD4^+CD25^+$ and $CD4^+CD25^-$ populations (390, 393, 394). Given the importance of Th1 and Th2 cells in infectious and inflammatory diseases, we therefore investigated the role of GITR in the development of these two key subsets of T cells.

5.2.1 Anti-GITR mAb partially abrogates the suppressor function of $CD4^+CD25^+$ T regulatory cells

In vitro experiments were performed to re-confirm that anti-GITR mAb abrogated the suppressor function of regulatory T cells. These experiments were performed as originally described by Shimuzu *et al* (264), and then performed with graded doses of anti-CD3 and anti-GITR antibody (as described in Materials and Methods, Section 2.5.2, p89). $CD4^+CD25^-$ and $CD4^+CD25^+$ cells from naïve BALB/c mice were purified by MACS. $CD4^+CD25^+$ cells were pre-incubated for 30 minutes with either anti-GITR or control Ab. $CD4^+CD25^-$ and $CD4^+CD25^+$ cells (1:1 ratio) were then cultured with soluble anti-CD3 Ab, and mitomycin C-treated $CD4^-$ cells (acting as APC) for 72 hours. Cellular proliferation was determined for the last 8 hours of

culture by [^{3}H] thymidine incorporation. $CD4^+CD25^-$ T cells showed typical proliferation under these conditions, but $CD4^+CD25^+$ T cells did not proliferate above background. The regulatory function of the $CD4^+CD25^+$ T cells was demonstrated by their suppression of proliferation of the $CD4^+CD25^-$ cells in co-culture experiments. Anti-GITR mAb partially abrogated the suppressor function of $CD4^+CD25^+$ cells, as demonstrated by the recovery of proliferation of $CD4^+CD25^-/CD4^+CD25^+$ T cell co-cultures to the proliferation level seen by $CD4^+CD25^-$ T cells alone (Fig. 5.1). APC cells did not proliferate in the presence of anti-CD3 mAb. Anti-GITR mAb did not significantly affect the proliferation of $CD4^+CD25^+$ T cells (data not shown).

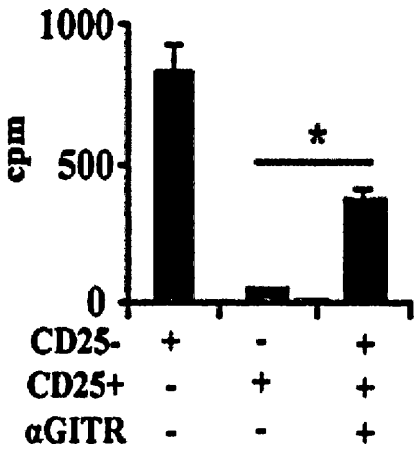

Figure 5.1 Anti-GITR mAb partially abrogated the suppressor function of CD25$^+$ T regs *in vitro*.

$CD4^+CD25^-$ and $CD4^+CD25^+$ cells from BALB/c mice were purified by MACS. $CD4^+CD25^+$ cells were pre-incubated for 30 minutes with either 10 μg/ml anti-GITR or control Ab *in vitro*. $CD4^+CD25^-$ and $CD4^+CD25^+$ cells (1:1 ratio) were then cultured with soluble anti-CD3 Ab (1 μg/ml), and mitomycin C-treated $CD4^-$ cells (as APC) for 72 hours. Cellular proliferation was determined for the last 8 hours of culture by [^{3}H] thymidine incorporation. Anti-GITR mAb partially abrogated the suppressor function of $CD4^+CD25^+$ T cells (*$p<0.05$). Data are representative of 7 independent experiments, and are shown as mean +/- SEM.

5.2.2 Anti-GITR mAb enhances proliferation and IFNγ and IL-5 production from naïve $CD4^+$ T cells

To examine the effect of GITR activation of $CD4^+$ cells as a whole, $CD4^+$ cells were purified from the lymph nodes of naïve BALB/c mice. These $CD4^+$ cells, which have a physiological ratio of $CD4^+CD25^+$ and $CD4^+CD25^-$ cells of approximately 1:20, were stimulated with anti-CD3 mAb and either anti-GITR or control Ab, in the presence of mitomycin C-treated $CD4^-$ cells (acting as APC) for 72 h (as described in Materials and Methods, Section 2.5.3, p90). Proliferation of $CD4^+$ T cells was enhanced in the presence of anti-GITR mAb. Both IFNγ and IL-5 production from $CD4^+$ cells was significantly increased after anti-GITR mAb treatment (Fig. 5.2). APCs did not proliferate or produce cytokines.

5.2.3 Anti-GITR mAb enhances IFNγ and IL-5 production from purified $CD4^+CD25^-$ T cells

To examine the effect of GITR activation on $CD4^+CD25^-$ effector cells alone, $CD4^+$ cells were separated in to $CD4^+CD25^+$ and $CD4^+CD25^-$ populations. $CD4^+CD25^-$ cells were stimulated with anti-CD3 Ab under neutral, Th1 or Th2 polarising conditions in the presence of anti-GITR or control IgG for 72 h (as described in Materials and Methods, Section 2.5.3, p90). Under neutral conditions, $CD4^+CD25^-$ cultured with anti-GITR mAb showed significantly enhanced Th1 and Th2 cytokine production compared to control Ab, after the 1^{st} and 2^{nd} round of stimulation (Fig. 5.3). Proliferation of $CD4^+CD25^-$ cells was enhanced by culture with anti-GITR mAb. Anti-GITR mAb treatment of $CD4^+CD25^+$ cells did not significantly alter the

proliferative response or cytokine production, as compared to control antibody (data not shown).

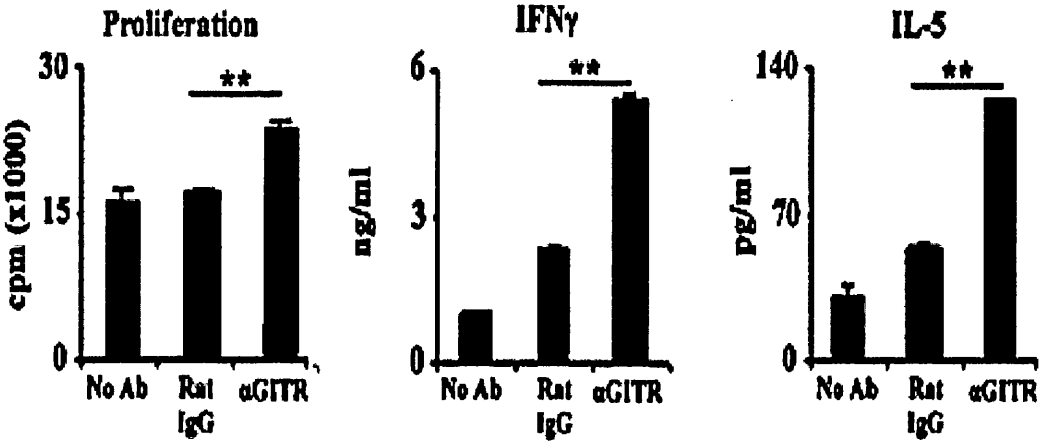

Figure 5.2 Anti-GITR mAb enhanced proliferation, and IFNγ and IL-5 production by $CD4^+$ cells *in vitro*.

Naïve $CD4^+$ cells from BALB/c mice were cultured with plate-bound anti-GITR or rat IgG Ab (10 μg/ml), soluble anti-CD3 Ab (1 μg/ml), and mitomycin C-treated $CD4^-$ cells (as APC) for 72 h. Culture supernatant was collected and cytokine concentrations assayed by ELISA. Cellular proliferation was determined for the last 8 hours of culture by [^{3}H] thymidine incorporation. Anti-GITR mAb significantly increased proliferation and IFNγ and IL-5 production from $CD4^+$ T cells (**$p<0.01$ as compared to control IgG). Data are representative of 3 independent experiments, and are shown as mean +/- SEM.

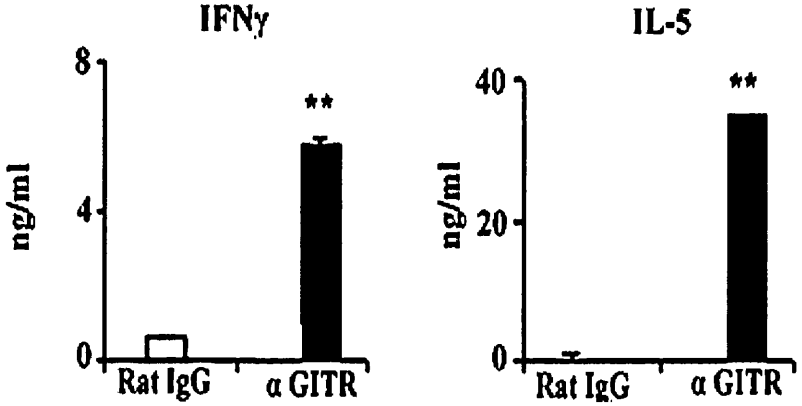

Figure 5.3 IFNγ and IL-5 production by $CD4^+CD25^-$ cells *in vitro* is enhanced by anti-GITR mAb.

Naïve $CD4^+CD25^-$ cells were stimulated by anti-CD3 antibody (plate bound 1 μg/ml) and anti-GITR or control antibody (soluble 10 μg/ml), without Th skewing agents. Culture supernatant was collected at 72 h and cytokine concentrations assayed by ELISA. Naive $CD4^+CD25^-$ showed enhanced Th1 and Th2 cytokine production in the presence of anti-GITR mAb (**$p<0.01$ as compared to control antibody). Data are representative of 3 independent experiments, and are shown as mean +/- SEM.

5.2.4 Anti-GITR mAb enhances early Th1 and Th2 polarisation by purified $CD4^+CD25^-$ T cells *in vitro*

Having found that anti-GITR mAb can enhance both Th1 and Th2 cytokine production from $CD4^+$ and $CD4^+CD25^-$ effectors T cells under neutral conditions, we then further investigated the effect of GITR activation on $CD4^+CD25^-$ effector cells under Th1 and Th2 polarising conditions. $CD4^+CD25^-$ cells purified from BALB/c mice were stimulated with plate bound or soluble anti-CD3 Ab (0.5-5 μg/ml), and anti-GITR or control Ab (10 μg/ml), under Th1 or Th2 skewing conditions. After the 1^{st} round of polarisation, IFNγ and IL-5 production was significantly enhanced by Th1 and Th2 cells respectively, cultured in the presence of anti-GITR mAb (Fig. 5.4). Proliferation of Th1 and Th2 cells was not affected by culture with anti-GITR mAb (data not shown). Flow cytomeric analysis was performed to check the GITR expression on $CD4^+CD25^-$ T cells. Consistent with earlier reports (264, 265, 394), GITR expression on naïve $CD4^+CD25^-$ T cells was up-regulated within 24 h of CD3 engagement.

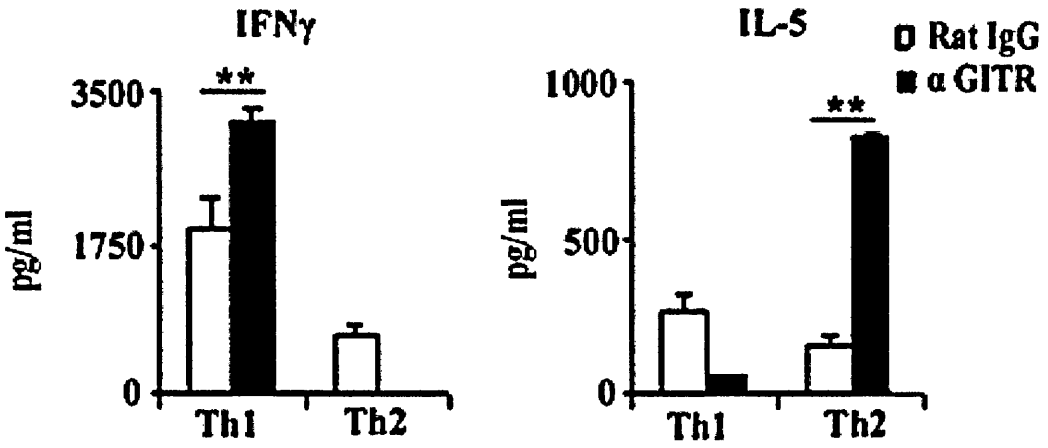

Figure 5.4 IFNγ and IL-5 production *in vitro* by $CD4^+CD25^-$ cells cultured in Th polarising conditions is enhanced by anti-GITR mAb.

$CD4^+CD25^-$ cells from BALB/c mice were purified by MACS, and stimulated with soluble anti-CD3 Ab (1 μg/ml), and anti-GITR or control Ab (10 μg/ml), under Th1 or Th2 skewing conditions for 72 h. Culture supernatant was collected and cytokine concentrations assayed by ELISA. After the 1st round of polarisation, IFNγ and IL-5 production was significantly enhanced by Th1 and Th2 cells respectively in the presence of anti-GITR mAb (**$p<0.01$ as compared to control IgG). Data are representative of 3 independent experiments, and are shown as mean +/- SEM.

5.2.5 Anti-GITR mAb has no effect on Th polarisation of mature effector cells *in vitro*

We then investigated the effect of anti-GITR antibody on established T cell lines. Th1 and Th2 cell lines polarised above were then subjected to a second round of polarisation in the presence of anti-GITR antibody or normal IgG. Anti-GITR antibody had no significant effect on the proliferation or cytokine production of these cell lines (data not shown). An earlier report showed that anti-GITR antibody enhanced the cytokine production of cloned Th1 and Th2 cells (390). We therefore investigated the effect of anti-GITR antibody on a more established cell line, cloned Th1 (X12) and cloned Th2 (X9) cells. Again, anti-GITR antibody had no effect on the proliferation or cytokine production by these cells following CD3 activation (Fig. 5.5). Flow cytometric analysis was performed to check the GITR expression of the cloned Th cells. GITR expression on cloned Th cells was up-regulated within 24 h of CD3 engagement. Anti-GITR mAb treatment did not significantly alter the proliferative response or cytokine production by $CD4^+CD25^+$ T cells (data not shown).

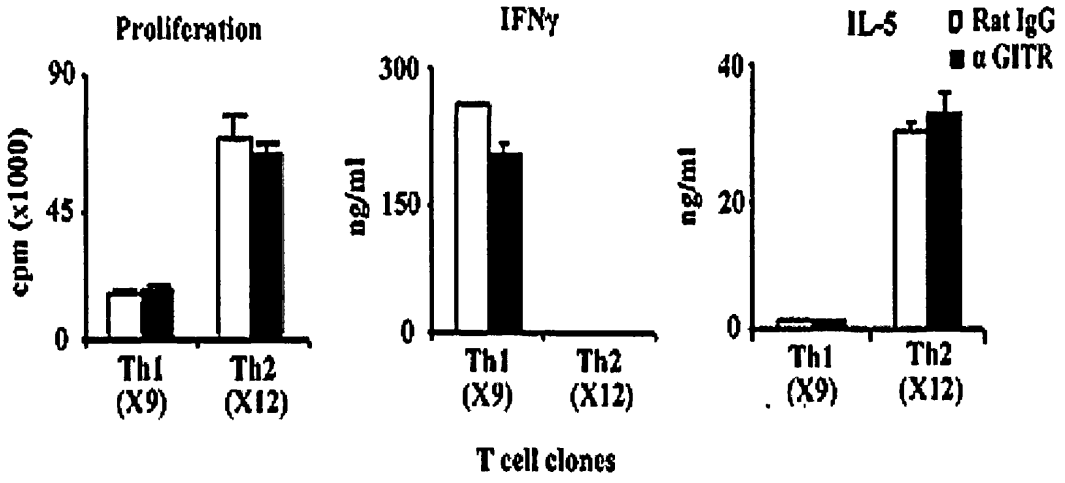

Figure 5.5 IFNγ and IL-5 production *in vitro* by Th clones is not altered by anti-GITR mAb.

Th1 (X9) and Th2 (X12) clones were stimulated with anti-CD3 antibody (plate bound 1 μg/ml) and anti-GITR or control antibody (soluble 10 μg/ml) for 72 h. Culture supernatant was collected and cytokine concentrations assayed by ELISA. The presence of anti-GITR mAb did not result in significant differences in proliferation, or IFNγ or IL-5 produced by cloned T cells. Data are mean +/- SEM, and are representative of 3 independent experiments.

5.2.6 Anti-GITR mAb enhances T-bet and GATA3 mRNA expression in early Th polarisation

We then explored the mechanism for the anti-GITR antibody mediated enhancement of Type 1 and Type 2 cytokine synthesis in the naïve or early polarised cells. Since T-bet and GATA3 are the master switches of Th1 and Th2 development respectively (10, 23), we investigated the effect of anti-GITR antibody on the expression of these two transcription factors.

5.2.6.1 Anti-GITR mAb enhances early T-bet and GATA3 mRNA expression of purified $CD4^+CD25^-$ T cells

Naïve $CD4^+CD25^-$ cells from BALB/c mice were purified and stimulated by anti-CD3 antibody and anti-GITR or control antibody, without Th skewing agents for 48 h. Real time PCR analysis shows that both T-bet and GATA3 messages were markedly enhanced in naïve $CD4^+CD25^-$ T cells cultured with anti-CD3 antibody under neutral condition in the presence of anti-GITR antibody compared with normal IgG control (Fig. 5.6).

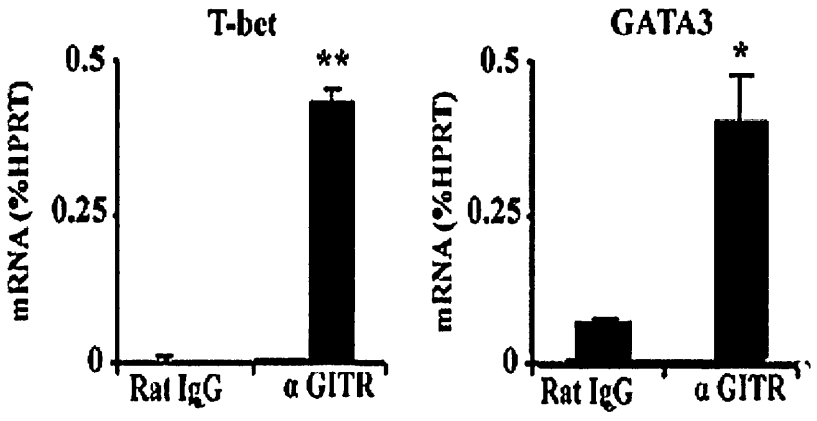

Figure 5.6 Anti-GITR mAb enhanced the expression of T-bet and GATA3 mRNA by Th0 cells.

Naïve $CD4^+CD25^-$ cells from BALB/c mice were purified by MACS and stimulated by anti-CD3 antibody (plate bound 1 μg/ml) and anti-GITR or control antibody (soluble 10 μg/ml), without Th skewing agents for 48 h. The expression of T-bet and GATA3 were determined by quantitative PCR. $CD4^+CD25^-$ cells showed a significant increase in their T-bet and GATA3 expression, when stimulated in the presence of anti-GITR mAb (**$p<0.01$ and *$p<0.05$ compared with control Ab). Data are representative of 2 independent experiments, and are shown as mean +/- SEM.

5.2.6.2 Anti-GITR mAb enhances early T-bet and GATA3 mRNA expression in polarised Th1 and Th2 cells

$CD4^+CD25^-$ cells from BALB/c mice were purified and stimulated by anti-CD3 antibody, and anti-GITR or control antibody. Cells had one round of polarisation toward Th1 and Th2 phenotypes for 48 h (as described in Material and Method, Section 2.5.3, p90). Anti-GITR mAb enhanced T-bet expression in $CD4^+CD25^-$ T cells cultured under Th1 polarising conditions (Figs. 5.7), and GATA3 expression in $CD4^+CD25^-$ T cells stimulated under Th2 polarising conditions (Fig. 5.8).

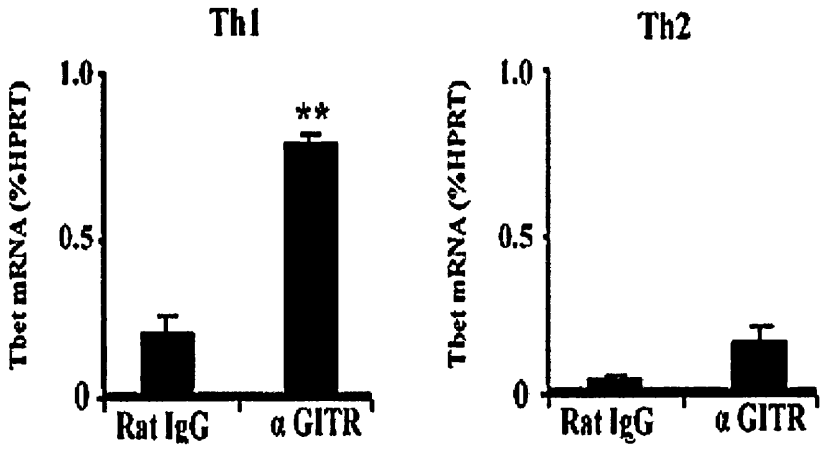

Figure 5.7 T-bet mRNA expression is enhanced by anti-GITR mAb treatment of Th1 cells.

Naïve $CD4^+CD25^-$ cells from BALB/c mice were purified by MACS and stimulated by anti-CD3 antibody (plate bound 1 μg/ml) and anti-GITR or control antibody (soluble 10 μg/ml). Cells were polarised toward Th1 and Th2 phenotypes for 48 h. The expression of T-bet was determined by quantitative PCR. Anti-GITR mAb increased the T-bet mRNA expression of $CD4^+CD25^-$ cells polarised under Th1 skewing conditions (**$p<0.01$ compared with control Ab). Data are representative of 2 independent experiments, and are shown as mean +/- SEM.

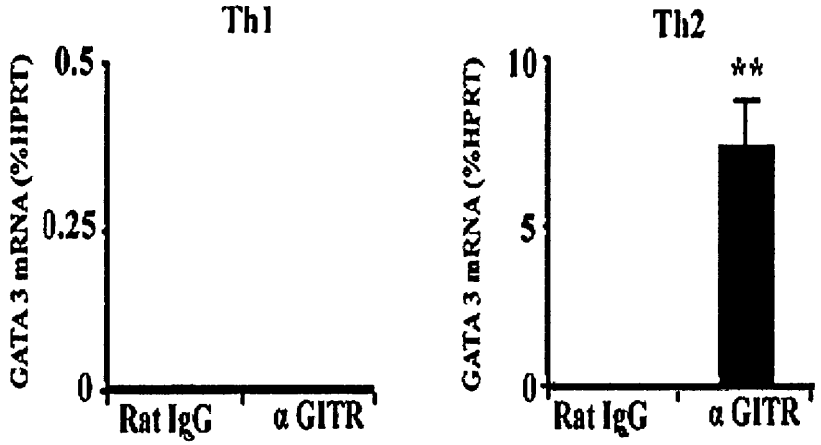

Figure 5.8 GATA3 mRNA expression is enhanced by anti-GITR mAb treatment of Th2 cells.

Naïve $CD4^+CD25^-$ cells from BALB/c mice were purified by MACS and stimulated by anti-CD3 antibody (plate bound 1 μg/ml) and anti-GITR or control antibody (soluble 10 μg/ml). Cells were polarised toward Th1 and Th2 phenotypes for 48 h. The expression of GATA3 was determined by quantitative PCR. Anti-GITR mAb increased the GATA3 mRNA expression of $CD4^+CD25^-$ cells polarised under Th2 skewing conditions (**$p<0.01$ compared with control Ab). Data are representative of 2 independent experiments, and are shown as mean +/- SEM.

5.3 Anti-GITR monoclonal antibody administration exacerbates allergic airways inflammation in a murine model of asthma

We have established that anti-GITR mAb can augment the early Th1 and Th2 responses of $CD4^+$ T cells and $CD4^+CD25^-$ T cells. This enhancing effect is likely to involve the transcription factors T-bet and GATA3. Previous work has demonstrated that anti-GITR mAb can abrogate $CD4^+CD25^+$ Treg suppressor function (264, 265) We then proceeded to investigate the relevance of the anti-GITR antibody on a Th2 (asthma) models of inflammatory disease *in vivo*.

To investigate the potential role of an activating anti-GITR mAb in murine allergic airways inflammation, we used an OVA-induced murine asthma model. BALB/c mice were primed and boosted with OVA (as described in Materials and Methods, Section 2.6.1, p92). All the mice were challenged i.n. on 3 consecutive days beginning on day 25. Anti-GITR mAb or control IgG (1 mg per mouse) was administered i.p on day 24 and 26 (Fig. 5.9). The dose of anti-GITR mAb used was determined by preliminary *in vivo* titration.

Several control groups were used in these experiments. The group labelled below as "PBS" represent mice that were sensitised and challenged with PBS (to control for OVA), and also given i.p. PBS was on days 24 and 26 in place of anti-GITR mAb. The group labelled "Rat IgG" represent mice that were sensitised and challenged with OVA, and received control rat IgG i.p. on day 24 and 26.

One further control group was examined to ensure the effect of anti-GITR mAb was not antigen-independent. Here, mice were sensitised with OVA, received anti-GITR mAb i.p. on days 24 and 26, and were challenged with i.n. PBS on days 25, 26, and 27. This last group of control mice did not develop any features of allergic airways disease (data not shown).

Initial experiments were carried out with varying doses of i.n. OVA challenge. The amount of i.n. OVA administered was between 1 to 50 micrograms per mouse. The i.n. OVA challenge dose was titrated at 2 μg per mouse, which induced a moderate amount of airways inflammation that still had the potential to be enhanced.

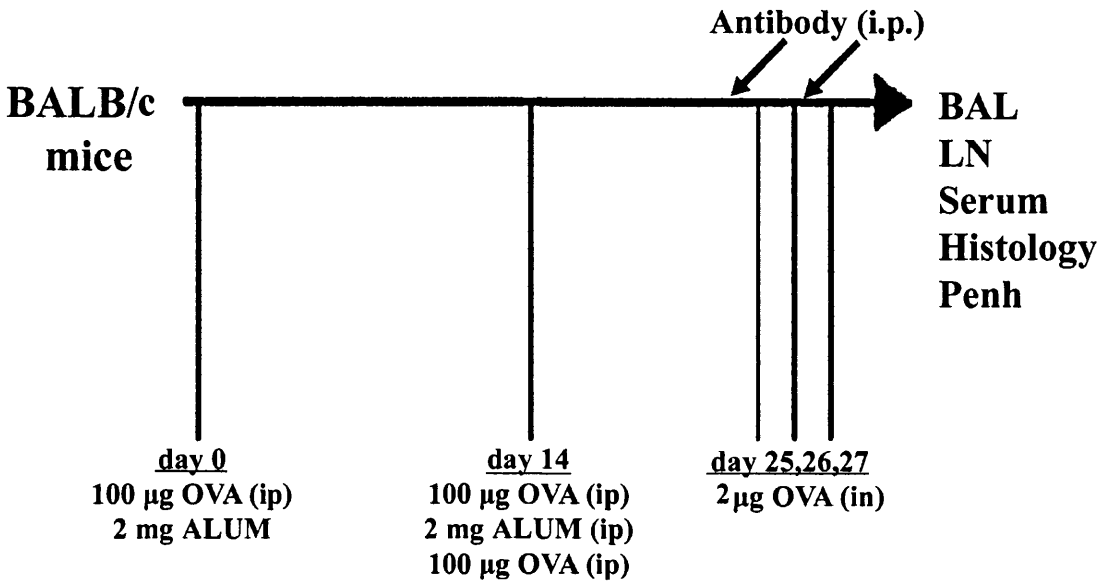

Figure 5.9 Experimental protocol to investigate the role of anti-GITR mAb in a murine model of allergic airways disease.

BALB/c mice were injected i.p. with 100 µg OVA and 100 µl 2 % Alum on day 0 and 14. The mice were boosted i.n. with 100 µg OVA on day 14. All mice were then challenged i.n. on 3 consecutive days beginning on day 25. Anti-GITR mAb or control Rat IgG (1 mg per mouse) was administered i.p on day 24, and 26. Antibody was given 1 h prior to i.n. challenge. Penh was determined on day 28 and mice were sacrificed on day 29. Serum, BAL and lymphoid cells were collected and lung tissue was harvested for histology.

5.3.1 Anti-GITR antibody increases BAL eosinophilia

Mice treated with anti-GITR mAb but not control rat IgG showed a profound increase in their BAL eosinophilia, without affecting the BAL total cell count (Fig. 5.10).

5.3.2 Anti-GITR antibody administration enhances OVA-induced thoracic lymph node responses *in vitro*

To investigate the immunological mechanism involved in anti-GITR mAb treatment which resulted in the increased airway eosinophilia, thoracic lymph node cells were harvested from the anti-GITR-treated and rat IgG-treated mice, and cultured with OVA *in vitro*. Lymphocytes from mice treated with anti-GITR mAb had augmented OVA-specific T cell proliferation compared to those treated with control rat IgG (Fig. 5.11). The lymphoid cells from anti-GITR mAb-treated OVA sensitised/challenged mice produced significantly more IL-2, IL-4, IL-5, IL-10, and IFNγ compared to cells from mice treated with control antibody (Fig. 5.11 – Fig. 5.13).

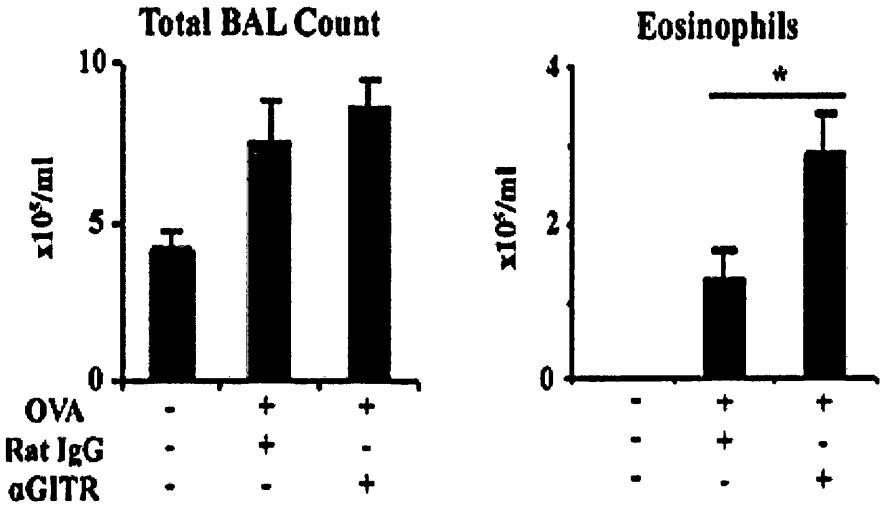

Figure 5.10 Anti-GITR mAb exacerbated BAL eosinophilia in murine allergic airways disease.

BAL total cell concentration remained unchanged, however the total eosinophil numbers and proportion increased markedly after anti-GITR mAb administration (*$p<0.05$ compared with control Ab). Data are mean +/- SEM, n=10 and are representative of 3 independent experiments.

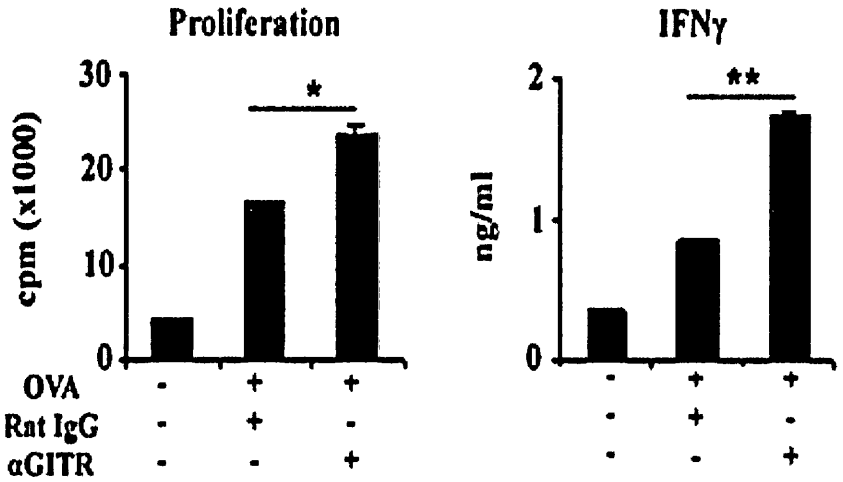

Figure 5.11 Anti-GITR mAb enhanced the proliferative and Th1 cytokine response in murine asthma.

Thoracic lymph node cells were harvested on day 29 and cultured with 1 mg/ml OVA *in vitro*. Culture supernatant was collected at 96 h and cytokine concentrations assayed by ELISA. Cellular proliferation was determined after 8 hours culture with [^{3}H] thymidine. Anti-GITR mAb administration resulted in enhanced cellular proliferation, and IFNγ production (*$p<0.05$ and **$p<0.01$ compared with control Ab). Data are mean +/- SEM, n=10 and are representative of 3 experiments.

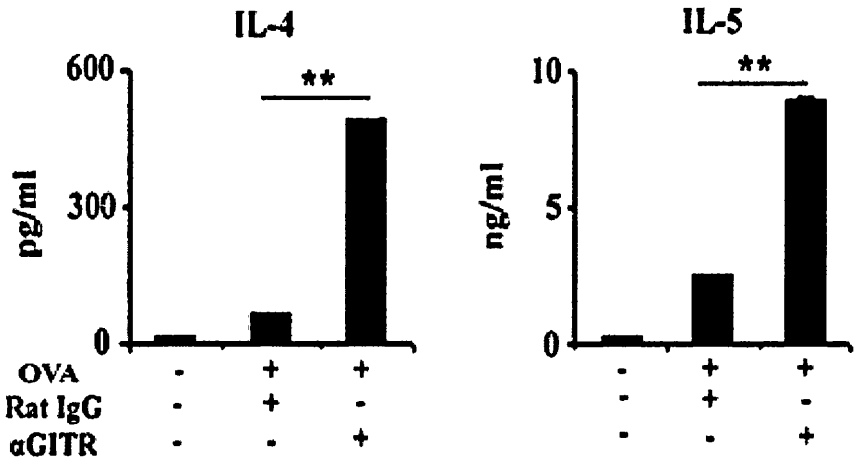

Figure 5.12 Anti-GITR mAb enhanced the Th2 cytokine response in murine asthma.

Thoracic lymph node cells were harvested on day 29 and cultured with 1 mg/ml OVA *in vitro*. Culture supernatant was collected at 96 h and cytokine concentrations assayed by ELISA. Anti-GITR mAb administration resulted in significantly increased production of IL-4 and IL-5 (**$p<0.01$ compared with control Ab). Data are mean +/- SEM, n=10 and are representative of 3 experiments.

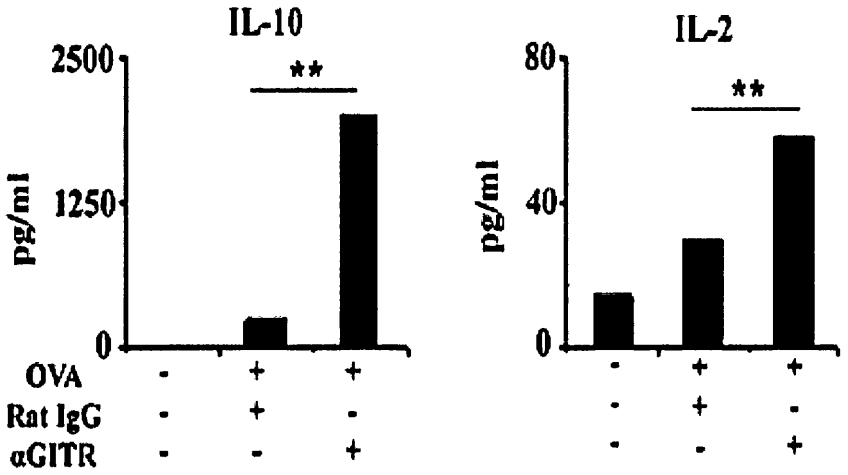

Figure 5.13 Anti-GITR mAb enhanced IL-10 and IL-2 production in murine asthma.

Thoracic lymph node cells were harvested on day 29 and cultured with 1 mg/ml OVA *in vitro*. Culture supernatant was collected at 96 h and cytokine concentrations assayed by ELISA. Anti-GITR mAb administration resulted in enhanced IL-10 and IL-2 production (**$p<0.01$ compared with control Ab). Data are mean +/- SEM, n=10 and are representative of 3 experiments.

5.3.3 Anti-GITR antibody administration increases serum IgE levels

Serum obtained from mice after anti-GITR mAb treatment showed significantly raised titres of OVA-specific IgE antibody (Fig. 5.14), and total IgE (data not shown). The level of serum OVA-specific IgG1 was not significantly altered by anti-GITR mAb treatment (Fig. 5.14). Serum IgG2a levels were low in both groups and unaffected by anti-GITR administration (data not shown), which reflects the strong Th2 bias in this model.

5.3.4 Anti-GITR antibody increases airway hyperresponsiveness measured by enhanced pause

On day 28, the airway hyper-reactivity (a physiological index measure of asthma) was assessed by whole body plethysmography. Enhanced pause (Penh) was measured after a 2 min nebulisation with 50 mg/ml methacholine (as described in Materials and Methods Section 2.6.2, p93). Anti-GITR mAb-treated mice demonstrated increased Penh, however this difference was not statistically significant when compared to control IgG-treated mice (Fig. 5.15). The p value of 0.055 suggests that the difference may have reached statistical significance if the number of mice in each group was increased.

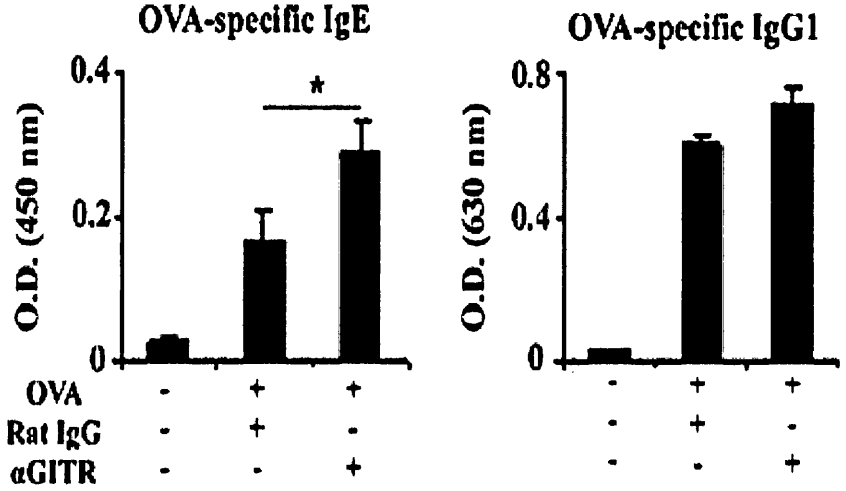

Figure 5.14 Anti-GITR mAb enhanced the OVA-specific IgE levels in murine asthma.

Sera were collected on day 29. Anti-GITR mAb treatment led to a significant increase in OVA-specific IgE (*$p<0.05$ compared to control rat IgG) but no significant difference in OVA-specific IgG1 production. Data are mean +/- SEM of individual mice, n= 10 and are representative of 3 experiments.

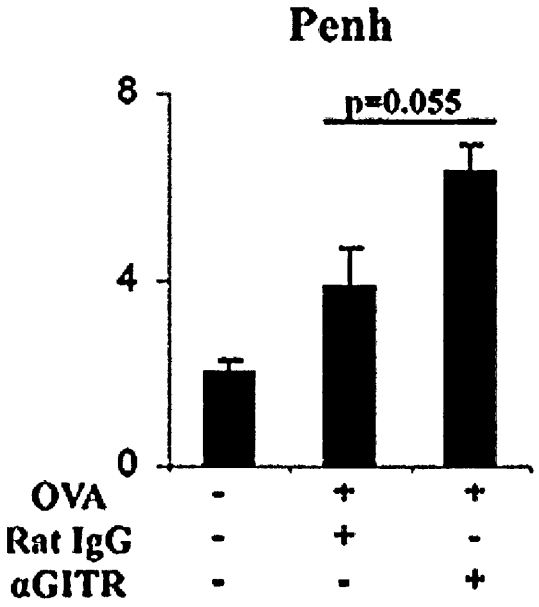

Figure 5.15 Anti-GITR mAb modulates airway hyperresponsiveness in murine asthma.

Enhanced Pause (Penh) measurement after a 2 min nebulisation with 50 mg/ml methacholine was performed on day 28. A non-significant increase in airways hyper-reactivity was seen in the anti-GITR mAb-treated mice. Data are mean +/- SEM of individual mice, n= 10 and are representative of 2 experiments.

5.3.5 Anti-GITR antibody administration exacerbates histological evidence of inflammation in murine lungs

Histological analysis demonstrated an amplification of the inflammatory infiltrates seen in the peri-bronchial and peri-vascular areas of the lungs treated with anti-GITR mAb (Fig. 5.16).

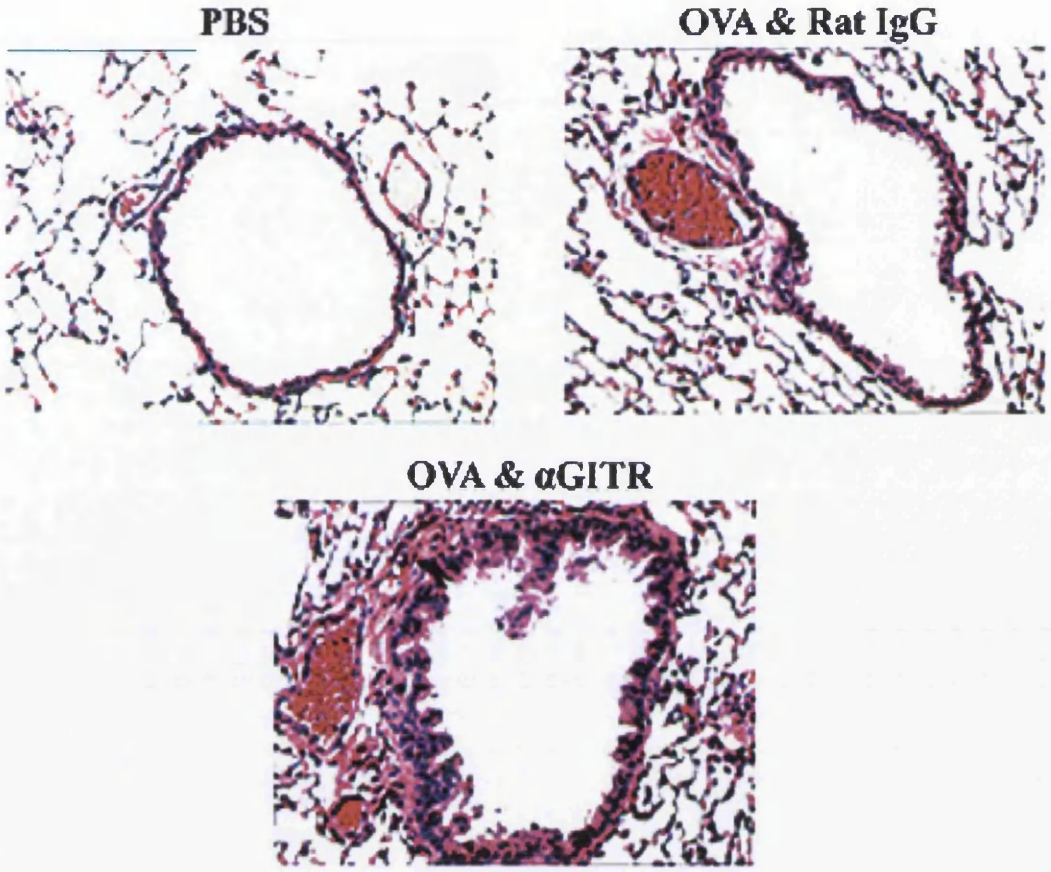

Figure 5.16 Histological evidence that anti-GITR mAb exacerbated murine allergic airways disease.

H&E sections of lungs (x20 magnification) demonstrate that peri-bronchial and peri-vascular inflammation was increased after anti-GITR mAb administration. Pictures (day 29) are representative of 10 mice per group.

5.4 $CD4^+GITR^+$ cells attenuate allergic airways inflammation in SCID mice

Previous studies have demonstrated that $CD4^+GITR^+$ cells which consist mainly of $CD4^+CD25^+$ Tregs but also contain $CD4^+CD25^-$ Tregs, suppress Th1-mediated inflammatory bowel disease (IBD) in the mouse (395). To investigate whether $CD4^+GITR^+$ cells could exert a regulatory effect in a Th2 driven allergic airways disease, we adoptively transferred $CD4^+GITR^+$, $CD4^+GITR^-$, or a combination of $CD4^+GITR^+$ and $CD4^+GITR^-$ cells into SCID mice, which then had airways inflammation induced by the standard protocol (Fig. 5.17).

5.4.1 $CD4^+GITR^+$ cells attenuates BAL eosinophilia induced in SCID mice

Mice transferred with $CD4^+GITR^-$ cells developed marked eosinophilia after airway challenge with OVA, whereas mice transferred with $CD4^+GITR^+$ cells failed to show eosinophilia. Furthermore co-transfer of both cell types demonstrated that the $CD4^+GITR^+$ T cells completely suppressed $CD4^+GITR^-$-induced eosinophilia (Fig. 5.18). There was no significant difference in the total BAL cell count among the three groups of mice.

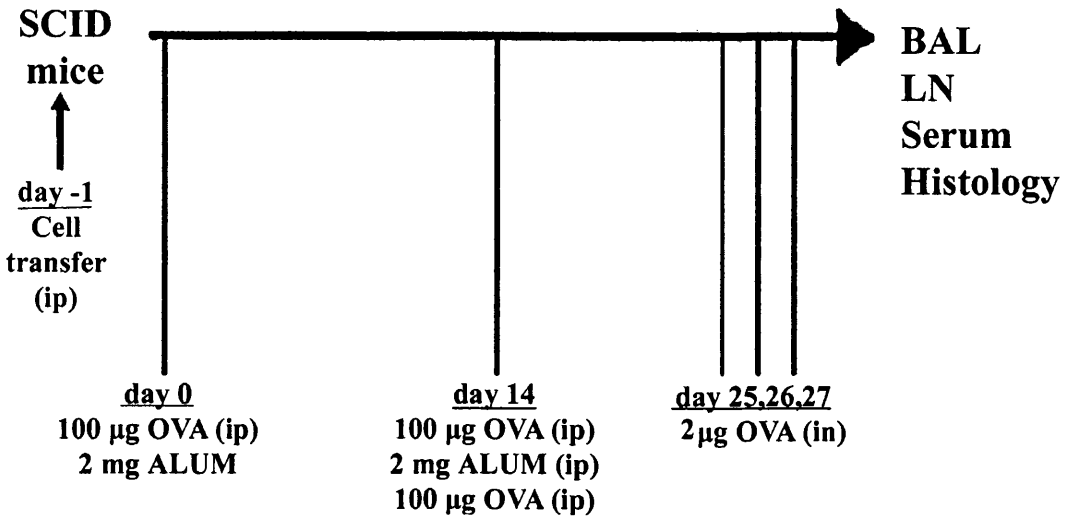

Figure 5.17 Experimental protocol for the adoptive transfer of $CD4^+$ $GITR^+$ and $CD4^-GITR^+$ cells into SCID mice with subsequent induction of allergic airways disease.

$CD4^+GITR^+$, $CD4^+GITR^-$, or equal numbers of $CD4^+GITR^+$ and $CD4^+GITR^-$ cells were transferred i.p. into SCID mice, and allergic airways inflammation was induced. SCID mice were injected i.p. with 100 µg OVA and 100 µl 2 % Alum on day 0 and 14. The mice were boosted i.n. with 100 µg OVA on day 14. All mice were then challenged i.n. on 3 consecutive days beginning on day 25. Mice were sacrificed on day 28. Serum, BAL and lymphoid cells were collected and lung histology studied.

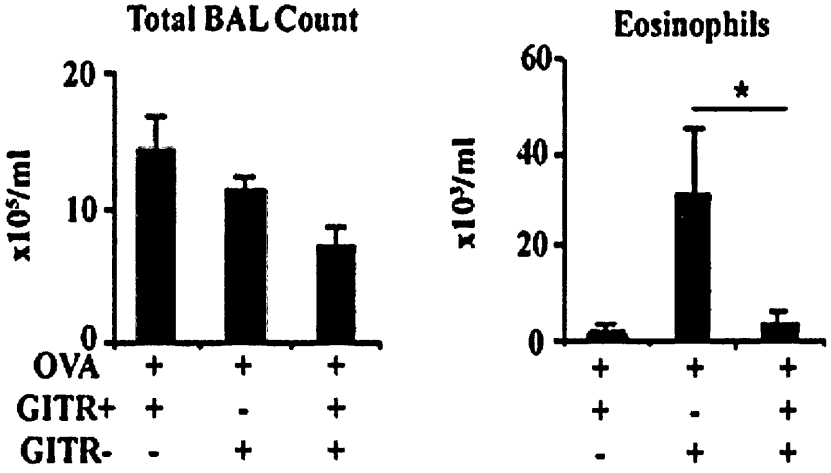

Figure 5.18 $CD4^+GITR^+$ cells suppress BAL eosinophilia in SCID mice.

$CD4^+GITR^+$, $CD4^+GITR^-$, or equal numbers of $CD4^+GITR^+$ and $CD4^+GITR^-$ cells were transferred i.p. into SCID mice, and allergic airways inflammation was induced. There was no significant difference in the total BAL cell count among the three groups of mice. However, co-transfer of both cell types demonstrated that the $CD4^+GITR^+$ T cells completely suppressed $CD4^+GITR^-$-induced eosinophilia (*$p<0.05$ compared to $CD4^+GITR^-$ cell transfer alone). Data are mean +/-SEM, n=5.

5.4.2 The effect of $CD4^+GITR^+$ adoptive cell transfer on OVA-induced lymph node cell responses *in vitro*

Thoracic lymph node cells were harvested and cultured with OVA *in vitro*. The proliferative response of $CD4^+GITR^-$ cells was unaffected by $CD4^+GITR^+$ cells. $CD4^+GITR^+$ cells alone did not proliferate (data not shown). Cells from $CD4^+GITR^-$-transferred mice produced significant levels of IL-5 and IFNγ, whilst cells from $CD4^+GITR^+$ transferred mice did not. Interestingly $CD4^+GITR^+$ cells significantly suppressed the production of IL-5 but enhanced the synthesis of IFNγ by $CD4^+GITR^-$ T cells *ex vivo* (Fig. 5.19). IL-10 was not detected in appreciable amounts in culture supernatants.

5.4.3 $CD4^+GITR^+$ cell transfer attenuates histological evidence of inflammation

Histological analysis demonstrated that SCID mice reconstituted with $CD4^+GITR^-$ but not $CD4^+GITR^+$ cells developed airways inflammation. The airway inflammation in mice reconstituted $CD4^+GITR^-$ cells was completely abolished by co-transfer of $CD4^+GITR^+$ cells (Fig. 5.20).

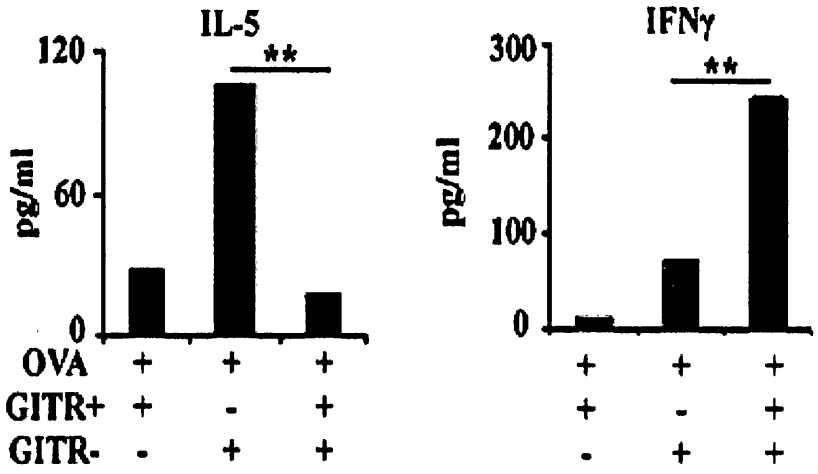

Figure 5.19 OVA-induced LN culture responses in SCID mice adoptive transfer experiments.

Lymph node cell culture supernatant, collected at 96 h, shows that the IL-5 produced by $CD4^+GITR^-$ recipients was significantly suppressed in the presence of $CD4^+GITR^+$ cells. However, IFNγ production by LN cultures from $CD4^+GITR^-$-treated mice was markedly enhanced when $CD4^+GITR^+$ cells were co-transferred. Data are mean +/- SEM, n=5.

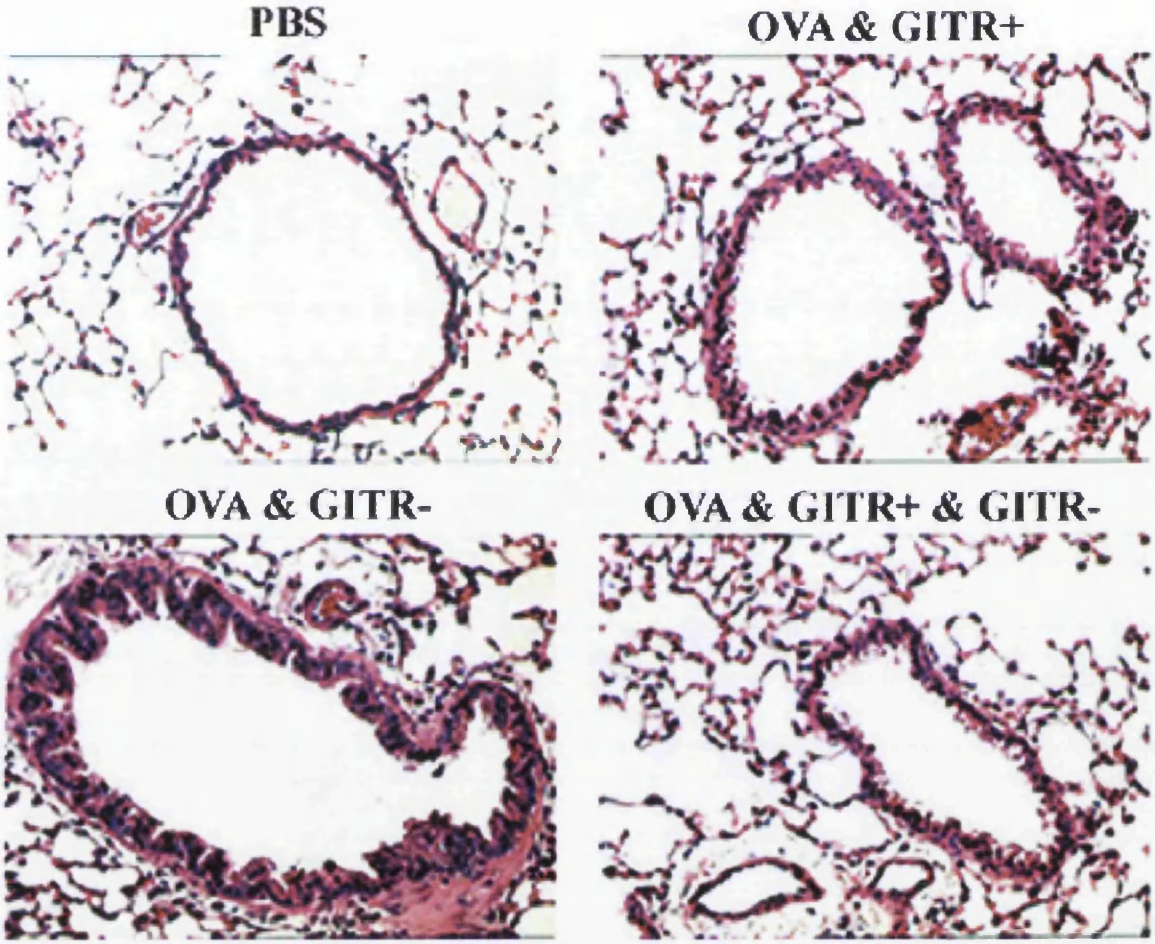

Figure 5.20 Histological evidence that $CD4^+GITR^+$ cells suppressed allergic airways inflammation in SCID mice.

H&E sections of lungs (x20 magnification) demonstrate that airways inflammation was increased after $CD4^+GITR^-$ cell transfer. This allergic airway inflammation was ameliorated if $CD4^+GITR^+$ cells were co-transferred with the $CD4^+GITR^-$ cells. Pictures (day 29) are representative of 5 mice per group.

5.5 Chapter Discussion

The work in this chapter demonstrates that:

(i) Activating anti-GITR mAb enhances Th1 and Th2 differentiation of naïve $CD4^+$ and $CD4^+CD25^-$ T cells *in vitro*

(ii) Anti-GITR mAb elevates the expression of the transcription factors, T-bet in Th1 cells and GATA3 in Th2 cell

(iii) Anti-GITR mAb exacerbates murine asthma, associated with an increase of both the Th1 and Th2 response

(iv) Passively transferred $CD4^+GITR^+$ cells can attenuate murine asthma induced in SCID mice reconstituted with $CD4^+GITR^-$ cells.

These results therefore demonstrate that activation of GITR not only enhances Th1 and Th2 development *in vitro*, but has profound effect on Th2-mediated disease *in vivo*.

Regulatory T cells have a pivotal role in asthma pathogenesis (350-352), and GITR is an important factor in controlling Treg function. GITR is expressed at high levels in murine and human $CD4^+CD25^+$ Treg cells, and to a lesser degree on naïve $CD4^+CD25^-$ cells (238, 264, 265, 394-396). Extensive work has demonstrated that the addition of anti-GITR mAb to co-cultures of murine responder ($CD4^+CD25^-$) and suppressor ($CD4^+CD25^+$) cells completely abrogates the suppressive function of these regulatory cells (264, 265). Our study demonstrates that apart from abrogating the suppressive effect of Treg cells reported previously, the reversal of suppression by

anti-GITR antibody in this culture system could also be due to a direct enhancing effect on the $CD4^+CD25^-$ effector cells.

Anti-GITR mAb administration in experimental murine asthma made it worse, and was associated with an increase of both Th1 and Th2 cytokines production. In addition, separate *in vitro* data demonstrates that anti-GITR mAb markedly increased both Th1 and Th2 cytokine production from antigen-naïve $CD4^+CD25^-$ cells. The mechanism of this enhancing effect on $CD4^+CD25^-$ cells is likely due to the ability of the antibody to elevate T-bet and GATA3, the master switch of Th1 and Th2 cells respectively. The effect of anti-GITR antibody in this Th1 and Th2 driven system is likely secondary to the TcR activation and the polarisation cytokines. Thus, anti-GITR antibody elevates the expression of these transcription factors following their initial induction by the polarising conditions, suggesting that there is a common pathway by which GITR signalling enhances the expression of these two opposing transcription factors. Given the importance of T-bet and GATA3 in Th cell differentiation, the mechanism by which GITR signalling enhances the expression of these transcription factors merits further investigation.

It is intriguing that GITR signalling had little or no effect on established Th1 and Th2 cell lines or clones. This is in apparent contrast to a previous report showing that a murine recombinant GITR ligand (rmGITR) was able to activate Th1 and Th2 cell clone (390). The difference may be due to the relative strength of GITR signalling delivered by a cross-linking antibody versus GITR ligand. Naïve $CD4^+CD25^-$ cells express a low level of GITR whereas polarised Th1 and Th2 cell have high density of GITR. It may be that the cross-linking antibody had no further effect on the already

densely packed GITR on highly activated T cell lines or clones. Similar reasoning may also apply to the lack of an enhancing effect of the anti-GITR antibody on $CD4^+CD25^+$ Treg cells which express constitutively high density of GITR and are regarded as memory T cells (264, 265). To ensure that this lack of enhancement in primed cells was not due to differential cell surface expression, the GITR was quantified by flow cytometry. The GITR surface expression was found to be upregulated on T cell clones and on established Th cell lines after TcR stimulation, as previously described (264, 265). Thus, our results show that cross-linking GITR with an agonist anti-GITR antibody enhances the differentiation of naïve T cells into Th1 and Th2 cells, but has little or no effect on the proliferation or cytokine production of established Th1 or Th2 cells or $CD4^+CD25^+$ Treg cells.

In order to further examine the role of GITR activation on established Th2 cells and Tregs, *in vivo* studies were carried out in a murine model of asthma. The administration of anti-GITR mAb in our experiments was relatively late (day 24 and day 26). In this setting, anti-GITR mAb is likely to engage cells that are antigen primed, and are likely to be Th2 committed as a consequence of alum adjuvant administration. Our *in vitro* data, showing the lack of enhancement of proliferation or cytokine production from primed $CD4^+CD25^-$ cells, would suggest that anti-GITR mAb would not augment the function on these Ag-primed effector T cells *in vivo*. It has been demonstrated that anti-GITR mAb abrogates the suppressive function of Tregs *in vitro* and *in vivo* (264, 265, 395). Therefore, it may be that the exacerbation of allergic airways disease seen in our murine model is mainly a consequence of abrogation of Treg function. This explanation may be an over-simplification of the T cell-mediated processes that will be involved in our murine model, and it is more than

likely that antigen-naïve $CD4^{+}CD25^{-}$ cells as well as other cell types are involved in the worsened Th2-mediated phenotype observed in anti-GITR mAb-treated mice. The contribution made by individual T cell populations in the worsened experimental asthma observed in anti-GITR mAb-treated mice could be further clarified by carrying out adoptive transfer experiments. Here, one could transfer effector and regulatory T cells from wild type and GITR-deficient mice into SCID mice, and thereafter induce allergic airways disease. Any difference observed between transfer groups may elucidate the cells responsible for the GITR-mediated worsening of murine asthma.

As previously discussed, many inflammatory cells and mediators are involved in asthma pathogenesis. The aims of this study were to examine key mechanisms that were involved in many of these complex effector systems. For this reason, the investigations outlined in this chapter have primarily concentrated on T cells, which are integral to the initiation, propagation and regulation of asthma. As well as T lymphocytes, GITR is also expressed on other immune cells, for example macrophages and B cells, and it is possible that GITR activation of these cells may contribute to aggravation of Th2-mediated inflammation observed *in vivo*. It would be important to investigate the role of GITR activation of these non-T cells in murine models of asthma. One experimental approach would be to use distinct cell populations from GITR-deficient or GITR-over-expressing mice and transfer them into SCID mice, with subsequent induction of allergic airways disease. This technique and further *in vitro* studies could further define the role of GITR on different cell populations and their modulation of asthma and other inflammatory disorders.

As well as being a marker for regulatory T cells in the $CD4^+CD25^+$ population, several investigators have found that GITR is also expressed on naïve $CD4^+CD25^-$ T cells. Furthermore, it has also been demonstrated that GITR may be a surface marker of $CD4^+CD25^-$ Treg cells (395, 397). To investigate the role of $CD4^+GITR^+$ cells in murine asthma, we used an adoptive transfer asthma model. The results demonstrate that $CD4^+GITR^+$ cells suppressed allergic airways inflammation induced in SCID mice reconstituted by $CD4^+GITR^-$ T cells. The disease-suppressing effect was associated with a decrease in the Th2 cytokine and an increase in the Th1 cytokine production. We have not used anti-GITR mAb treatment in this model, as it would not be possible to differentiate the effect of the antibody on $CD4^+$ Treg cells and the $CD4^+CD25^-$ effector cells *in vivo*. Previously it has been reported that $CD4^+GITR^+$ cells could adoptively suppress inflammatory bowel disease (IBD), induced in SCID mice reconstituted with $CD4^+CD45RB^{high}$ cells (395). In this IBD system, $CD4^+GITR^+$ cells reduced IFNγ synthesis, and had no effect on the minimal level of IL-4. Thus, although $CD4^+GITR^+$ T cells have a general beneficial effect on a range of inflammatory diseases, the immunological parameters affected by these cells could differ, depending perhaps on the prevailing pro-inflammatory cytokine presence in the disease milieu. In our study, it is not completely clear why the OVA-induced LN production of IFNγ is increased when $CD4^+GITR^+$ cells are co-transferred into SCID mice. It may be that $CD4^+GITR^+$ are mainly inhibiting Th2 cells *in vivo* and the balance between the Th2 and Th1 cytokines has been disrupted, leading to an enhanced Th1 profile. It is known that IFNγ can reverse Th2-mediated airways inflammation if given early during the induction phase of disease (380), however, in some models, IFNγ have been reported to promote eosinophilic inflammation if given late in established disease (381). The low level of IFNγ, produced by LN cells after

$CD4^+GITR^+$ cells transfer, does not appear to affect the degree of airways inflammation in our model. Thus, $CD4^+GITR^+$ cells appear to effectively suppress the predominant Th2-mediated response in murine asthma without significantly increasing the Th1 profile, and would appear to be a promising candidate for a new therapy for inflammatory diseases.

In summary, we demonstrate here that GITR activation results in an exacerbation of Th2-mediated inflammatory diseases through the activation of Th1 as well as Th2 cytokines. This is due to the activation of a pathway common to T-bet and GATA3. Thus, our results suggest that GITR may be a potential novel target for a range of Th1 and Th2 mediated inflammatory diseases. Therapeutic potential of reagents such as soluble recombinant GITR receptor is currently being explored in our laboratory.

Chapter 6

General Discussion

6.0 General Discussion

6.1 Extrapolating information from murine models of allergic airways inflammation to human asthma

In this thesis, a murine model of allergic airways inflammation has been used to investigate the immunological processes involved in asthma. This *in vivo* system displays many of the key features seen in acute atopic asthma clinically, such as airway eosinophila, elevated serum IgE, allergen-induced production of Th2-mediated cytokines by lymph node cells, and histological evidence of allergic inflammation. This murine model has allowed the mechanisms of allergic airways inflammation to be examined, and more importantly has permitted initial investigation of immunomodulatory agents, such as TLR2 agonists and anti-GITR mAb, to be carried out with no risk to human subjects. Although this experimental model has been used extensively by many investigators and is robust and reproducible I had minor concerns over two of the many endpoint parameters used to assess inflammation in this model: whole body plethysmography, and the objective interpretation of lung histology.

Enhanced pause (Penh) was used as a physiological marker of airways hyperresponsiveness. The advantage of using whole body plethysmography is that the mice are alive, unrestrained and the measurement of Penh is non-invasive. There is substantial evidence showing Penh is a valid measure of airway hyperresponsiveness, which also correlates strongly with BAL eosinophilia, and serum IgE levels (359, 398, 399). However, there have also been criticisms of the validity of Penh as a measure of

airways hyperreactivity (400, 401). These investigators have demonstrated that Penh does not always relate to invasive measures of airways resistance and lung compliance. Furthermore, it has been suggested that Penh might be a measure of nasal passage smooth muscle contraction in response to methacholine (400, 401). Additional studies suggest that association between Penh and airways resistance in mice may be strain related. It was shown that Penh correlates well with invasive measures of airways hyperreactivity in BALB/c mice, however this correlation was not as strong in C57BL/6 (402, 403). Enhanced pause is generally, but not universally, accepted as an accurate measure of airways hyperresponsiveness. The main concern regarding Penh is that it is a derived ratio of expiratory to inspiratory pressures, and not an absolute physiological measure. Nevetheless, with this limitation in mind we have used a whole body plethysmography in our studies as one of the multiple outcomes of allergic airways disease.

In this thesis, representative histological pictures have been used to demonstrate changes in allergic airways inflammation. Although many histological scoring systems for inflammatory changes are used, there is no universally agreed technique in murine allergic airways disease. With the help of a veterinary experimental pathologist, Dr Hal Thompson, we have made initial investigations into the inflammatory changes that occur at different stages in our murine model of allergic airways disease. This work is preliminary however we propose to develop this as a validated histology scoring system for the allergic murine lung.

Although there is considerable homology between the human and murine genome, with only about 300 genes unique to one or the other species (404), there are

significant differences between their immune systems (405). As such, murine models can only approximate to the complex mechanisms of spontaneous and variable airflow obstruction, disease exacerbations, or naïve airway hyperresponsiveness associated with the spectrum of clinical asthma [reviewed in (406, 407)]. Although experimental models have their limitations, the information gained by their use is invaluable in unravelling the intricacies involved in a disease as complicated as asthma. In particular, the manipulations possible with murine models are far greater than is possible in humans. Therefore, murine models of asthma are important as one of preliminary *in vivo* methods for gaining further knowledge into the pathogenesis of diseases, such as asthma. However, extrapolation of experimental asthma work to the pathophysiology of human disease needs caution, for example murine models generally use inbred strains and this potential bias may not translate to outbred human populations. For this reason, additional experimental animal and human studies will be required to support the main findings of this thesis which suggest that the manipulation of TLR2 and GITR results in therapeutic modulation of the inflammatory response in asthma.

6.2 TLRs, Regulatory T cells and allergic airways inflammation

The prevalence of asthma and allergies has increased dramatically over the last twenty years in developed countries, which cannot be explained by changes in genetic predisposition (361, 362, 408). Environmental factors are now thought to be responsible for this rapid increase in asthma. The "Hygiene Hypothesis" was proposed as a possible explanation for the decreasing prevalence of asthma with increasing numbers of siblings. This hypothesis was extended to suggest that with

improved public health measures and through the use of vaccinations and antibiotics, there has been a reduction in childhood exposure of viral and bacterial infections. This has lead to reduced development of Th1 immunity, leading to a predisposition to Th2-mediated conditions, such as asthma (361). However, there is some debate over the validity of this thesis. It has been shown that childhood Th2-dominated immune responses against helminths are also associated with protection against atopic diseases in adulthood [reviewed in (409)]. Furthermore, it has been shown that the increase in prevalence of Th1-mediated autoimmune diseases, such as Type I diabetes mellitus, has also risen in parallel with atopic diseases (410). This would suggest that a lack of Th1-stimulating immune responses is not the full explanation as to why the prevalence of asthma, and other inflammatory disorders, is increasing.

With both Th1- and Th2-mediated inflammatory diseases increasing, it was proposed that an immuno-regulatory system that controls both these effector immune responses may be affected by environmental changes. Regulatory T cells (Treg) have been suggested as a susceptible immunoregulatory system. It has been shown that microbial pathogens can influence Treg development and function (411-413). Studies have demonstrated that microbial pathogens may induce regulatory T cell development through Toll-like receptors (TLRs). Tregs express TLRs, and their suppressive function can be enhanced by TLR activation (186, 378). This effect itself may be further controlled because TLR signalling can induce DCs to produce soluble mediators, such as IL-6, that can make effector T cells resistant to Treg suppressor action (187). The importance of TLRs in Treg development *in vivo* was suggested in animal model studies using *Mycobacterium vaccae*. It was shown that mice treated with heat-killed *M. vaccae* during allergen sensitisation resulted in reduced airway

hyperresponsiveness, eosinophila, IgE and Th2 cytokine production. The attenuation of this Th2-mediated disease was dependent on the production of IL-10 and TGFβ by regulatory T cells (183). It is known that mycobacteria are recognised by T cells via TLR2 and TLR4.

6.3 The role of TLRs in allergic airways inflammation

In our study, the down-regulatory effect of the synthetic TLR2 ligand (Pam3CSK4) on airways inflammation was associated with enhanced production of IL-10. This raised the possibility that regulatory T cells may play a role TLR2 agonist therapy. Many investigators have observed that regulatory T cells' ($CD4^+CD25^+$, Tr1 and Th3) function is dependent on IL-10 and TGFβ *in vivo* (204, 242, 299, 306). Using blocking antibodies, we demonstrate in Chapter 4 that the anti-inflammatory effects of Pam3CSK4 are mainly independent of IL-10 or TGFβ. The only endpoint measure that suggested IL-10 and TGFβ had a role in the beneficial effect of Pam3CSK4 therapy was Penh. This apparent disassociation of airways hyperresponsiveness with other immunological measures of acute inflammation has been demonstrated before (414-416), and may imply that bronchial hyperreactivity may reflect more established airways changes. Overall, it would appear that regulatory T cells are unlikely to be involved in the beneficial effects of Pam3CSK4 therapy in acute allergic airways inflammation. Although in this model, it would appear Treg cells do not play an important role in the therapeutic effect of TLR2 agonists, this does not necessarily infer that TLR2 or other TLRs will not influence Treg function in other inflammatory disorders. Observations in our laboratory suggest that Pam3CSK4 treatment of

$CD4^+CD25^+$ Tregs can transiently abrogate their suppressor action in a murine model of inflammatory bowel disease (unpublished data).

It is important to remember that, in addition to Tregs, many other cells of both the innate and adaptive immune system express TLRs. It has been demonstrated that several APCs express multiple TLRs. In Chapter 4, it was demonstrated that *in vitro* stimulation of BMDCs and BMMs with Pam3CSK4 could modulate the cytokines produced, which can influence the polarisation of naïve $CD4^+$ T cells. In addition, previous studies have demonstrated that TLR activation up regulates the expression of surface co-stimulatory molecules on DCs (128-132). Thus, it is likely that TLR activation of cells of the innate immune system can influence their APC function by changing their cytokine production, and their surface co-stimulatory signals. In the context of asthma, APCs other than DCs that express multiple TLRs include epithelial cells, fibroblasts, eosinophils, and B cells. In addition, the effector cells of asthma, such as NK cells, smooth muscle cells, and mast cells express TLRs, and may also be influenced through TLR activation. In our laboratory, investigations into the effect of TLR activation on these cells are in progress and will provide important insights into how TLR activation may affect inflammatory diseases such as asthma.

The effect of TLR2 activation in asthma is under intense investigation. Chisholm *et al* and Redecke *et al* (149, 367) have shown that early TLR2 agonist administration worsens the development of murine asthma, whilst Velasco *et al* (369) have demonstrated early TLR2 agonist therapy can ameliorate allergic airways disease. These apparently contradictory findings cannot be attributed to single factors such as the amount or route of administration of TLR2 agonist, or the strain of mouse used –

as discussed in Chapter 4. However, we propose that a combination of factors may determine whether TLR2 agonists attenuates or aggravates allergic airways inflammation. Work in our laboratory has demonstrated that TLR2 can act as co-stimulatory molecule for T cell activation (153). Furthermore, data presented in Chapter 4 showed that direct TLR2 activation of Th cells resulted in enhanced Th1 and Th2 cytokine production *in vitro*. This TLR2-dependent augmentation of Th polarisation is only seen when naïve $CD4^+$ are stimulated. Thus, whether TLR signalling predominantly activates the Th1 or the Th2 pathway depends on a combination of factors such as timing (relative to specific antigenic stimulation), dose and nature of TLR2 agonists; dose and nature of antigen and adjuvant used; and the genetic background of the responding hosts. These factors will then determine if TLR2 agonists predominantly activate T cells, or DCs. Although it has been shown that TLR activation of $CD4^+$ T cells can enhance Th1 responses (153), we postulate that under certain *in vivo* condition, for example in the presence of Alum adjuvant, TLR2 agonists may act on T cells directly and enhance Th2 cytokine production by $CD4^+$ T cells. This may then explain why some investigators found TLR2 agonist administration aggravated allergic murine asthma (149, 367). However, if TLR2 agonists are used in a manner so that DCs are mainly activated, then as shown by our *in vitro* work IL-12 production by the APCs will be increased. This will favour Th1 cell differentiation *de novo*, and will not only increase the number of Th1 cells but will also increase the amount of Th1 cytokines being produced, which in turn will antagonise naïve and antigen-primed Th2 cells. Therefore, as long as the levels of IFNγ induced are not excessive, allergic airways inflammation may then be ameliorated by disrupting the Th1/2 balance. This hypothesis is illustrated in Figure 6.1.

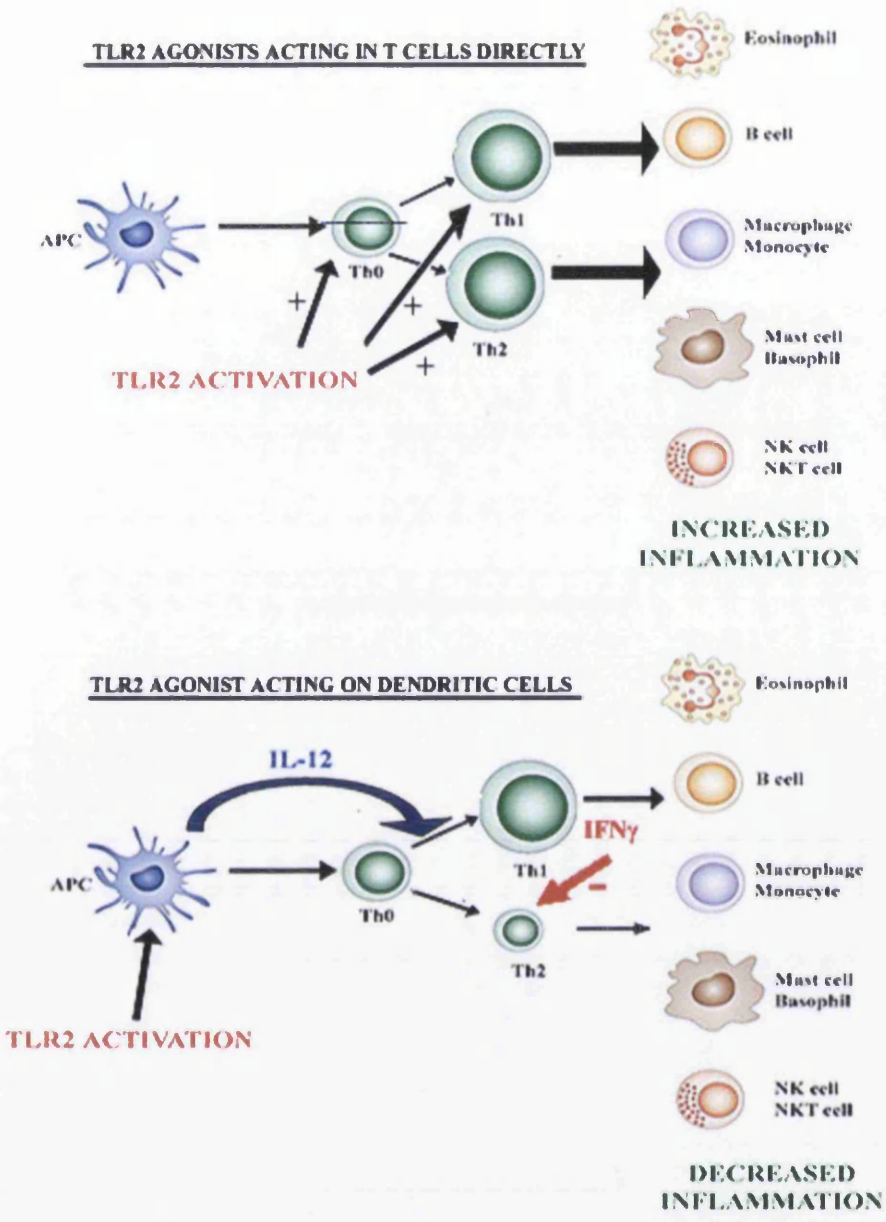

Figure 6.1 Possible explanation for the different action of TLR2 agonists in murine models of allergic airways disease

Depending on factors such as timing (relative to specific antigenic stimulation), dose and nature of TLR2 agonists; dose and nature of antigen and adjuvant used; and the genetic background of the responding hosts, TLR2 activation may directly enhance Th1 and Th2 cytokine production from naïve $CD4^+$ T cells. This will result aggravating allergic murine asthma. However, under different circumstances TLR2 agonists may influence DC function to enhance their production of IL-12, which in turn will favour Th1 cell differentiation. As long as the Th1 response is not excessive, airways inflammation will then be attenuated.

The hypothesis suggested above and the conclusions of the data presented in Chapters 3 and 4 suggest that TLR2 agonists can ameliorate airways inflammation by skewing the immune response from a Th2 profile to a Th1 profile. In addition to the murine work discussed in Chapter 4, studies in humans have shown that Th1 cytokines cannot only ameliorate allergic disease, but may also exacerbate it. For example, human asthma may be characterized by increased expression of both Th2 and Th1 cytokines by T cells (417), and the airway of human asthmatics is characterised by constitutively increased activation of the Th1-dependent transcription factor STAT1 (418), and enhanced expression of IL-12 p40 (419) compared to tissue from non-atopic individuals. However, studies examining patients who had received effective allergen-desensitisation immunotherapy have demonstrated a shift from a Th2 cytokine profile into Th1 cytokine profiles, which correlated with symptomatic benefit in atopic patients (420-422). Thus, although the central requirement for Th2 cytokines in the pathogenesis of allergic disease remains clear, Th1 and Th2 responses are not necessarily mutually exclusive or antagonistic in the context of chronic inflammatory responses to allergens. This might partly account for the disappointing results from administration of subcutaneous IL-12 in asthma patients who exhibited no improvement in airway hyperresponsiveness to histamine, no significant effect on the late asthmatic reaction after inhaled allergen challenge, and experienced a high frequency of serious side effects (379).

Although direct administration of Th1 cytokines or Th1-inducing cytokines (such as IL-12) have had poor results in human studies, preliminary results using CpG-containing DNA vaccines for the treatment of allergic asthma in humans have been reported as being promising. CpG-containing vaccines are composed of DNA with

immunostimulatory sequences (ISS) containing unmethylated cytosine residues adjacent to guanine residues [CpG motif; (423)]. These sequences activate DCs to induce robust Th1 responses, which is dependent on TLR9 (106). Oligodeoxynucleotides that incorporate these ISS (known as CpG-ODNs) are highly effective at preventing or ameliorating allergen-induced pulmonary disease and Th2 responses in mice (423, 424), and these studies have demonstrated that TLR9 signalling can attenuate allergic airways inflammation by skewing a Th2-dominant immune response to a Th1-dominant response. In addition, studies using CpG-containing DNA vaccines in human allergic diseases have reported very few side effects (425). The reasons why TLR9 activation induces a Th1 response in allergic disease with few side effects, whilst Th1 cytokine administration causes systemic upset remains unclear. Hence, targeting TLR9 and other TLRs that induce Th1 responses would appear to be a novel therapy for Th2-mediated diseases.

Although the clinical trials have initially shown CpG-containing DNA vaccines to have few side effects in asthmatic patients, concerns have been raised over the use of CpG-containing vaccines in patients with a predisposition to autoimmune diseases, such as systemic lupus erythematosus (SLE). SLE is a non-organ specific disease characterised by inefficient removal of apoptotic cells, and the normally sequestered nucleoproteins exposed on apoptotic cell membranes that can trigger auto-reactive B cells to produce IgG auto-antibody. This process appears to involve antigen presentation by dendritic cells using a TLR9-dependent pathway (169). It has been suggested that CpG-containing vaccines could enhance the risk of aggravating autoimmune disorder in a small number of immunocompetent hosts (169, 170). In our model, the total IgG level in Pam3CSK4-treated and -untreated mice, and found to be

similar. Although this does not exclude the development of autoreactive antibodies, it does show that the total level of immunoglobulin does not increase after TLR2 agonist therapy. Thus TLR2 agonists, such as synthetic Pam3CSK4, may represent additional and alternative potential reagents for controlling allergic diseases.

Together the results obtained here using TLR agonists suggest that established Th2-mediated inflammation could be ameliorated by TLR agonists, probably by skewing towards a Th1 response. Further studies are needed to elucidate the mechanism of TLR agonist therapy in inflammation, and several suggestions will be discussed in the prospective studies paragraph below. However, synthetic lipopeptide, or other TLR2 agonists have minimal toxicity and may have potential as therapeutic agents in human asthma.

6.4 The role of GITR and allergic airways inflammation

Glucocorticoid-induced tumour necrosis factor receptor (GITR) was described as a surface molecule involved in regulatory T cell function, and our initial experiments were designed to investigate this in a murine model of asthma. Subsequent work has demonstrated that GITR activation not only abrogate $CD4^+CD25^+$ Treg function, but also act as a co-stimulatory signal of $CD4^+CD25^-$ effector T cells, and thus broadened our experimental approach to studying this molecule in asthma. In addition, it has since been shown that GITR expression may represent a marker for all naive $CD4^+$ regulatory T cells. Our preliminary work initially focused on regulatory T cells in asthma, however, as our knowledge of GITR increased the direction of our efforts moved towards how GITR activation affected effector cells, in particular Th polarisation. Furthermore, we investigated whether isolated $CD4^+GITR^+$ cells could suppress allergic airways disease by passive transfer *in vivo*.

Our results demonstrate that an activating anti-GITR mAb enhances both Th1 and Th2 differentiation of naïve $CD4^+CD25^-$ effector T cells *in vitro*. The work also demonstrates that anti-GITR mAb causes an impressive exacerbation of murine asthma, which is associated with an increase of both the Th1 and Th2 response *in vivo*.

There is conflicting published data regarding the role of $CD4^+CD25^+$ regulatory T cells in experimental allergic disease in mice. Some investigators have demonstrated that passive transfer of $CD4^+CD25^+$ T cells worsened the allergic phenotype in mice, and suggested that this was due to the preferential inhibition of Th1 cells by the Tregs

(349). Others have found that $CD4^+CD25^+$ regulatory T cells could suppress Th2-mediated inflammation, without affecting airways hyperresponsiveness (350, 351). The explanation for these different findings is not completely clear, however these were just three studies and a consensus will probably emerge in time with more experimental work. It may also reflect the complex interplay of different inflammatory mechanisms that are involved in airway hyperresponsiveness, only some of which may be under the control of Tregs.

In human studies, $CD4^+CD25^+$ cells isolated from peripheral blood of atopic patients are less effective than the same cells from non-atopic control subjects in their suppressive function and in their ability to regulate Th2 responses (352, 426, 427). Proposed explanations for this finding include reduced numbers of Tregs, dysfunctional Tregs, a higher proportion of $CD4^+CD25^-$ effector T cells, or refractoriness of effector cells to regulation of activated $CD4^+CD25^+$ T cells. Ling *et al* (352) showed that the peripheral blood $CD4^+CD25^+$ T cell count was normal in atopic patients, but the function of these Tregs seemed reduced. This was shown when purified $CD4^+CD25^+$ Tregs from healthy non-atopic individuals had a greater ability to suppress allergen-specific Th2 responses *in vitro* than observed from $CD4^+CD25^+$ Tregs from atopic individuals. Furthermore, suppression was further diminished when $CD4^+CD25^+$ T cells were isolated from grass pollen allergic patients during hayfever season. This reduced Treg function offers a plausible alternative explanation to the hygiene hypothesis for the increased prevalence of autoimmune and allergic disease observed over the last 20-30 years. If Tregs, that normally can suppress Th1- and Th2-mediated responses, became dysfunctional as a consequence of some

environmental change, this would then account for the increase in prevalence of both Th1- and Th2-mediated inflammatory diseases.

There is a condition in nature that illustrates this hypothesis. A mutation in the gene coding the transcription factor *FOXP3* results in the immunodysregulation, polyendocrinopathy, and enteropathy, X-linked (IPEX) syndrome. This is also associated with allergic symptoms including severe eczema, increased serum IgE levels, eosinophilia, and food allergies. This implies that dysfunction of the Treg population results in multiple diseases, including Th2-mediated allergy (272) Furthermore, it has been demonstrated that an imbalance between IL-10-secreting Tregs and Th2 cells are responsible for the occurrence of allergic symptoms (428). Consisitent with this, Akdis *et al* demonstrated an increased frequency of IL-4 secreting, allergen-specific T cells in atopic patients and an increased frequency of IL-10-secreting, allergen-specific T cells in non-atopic individuals (428).

Tregs have also been found to be important in the induction of airways tolerance to inhaled allergen. Murine work has indicated a role for *foxp3* and membrane bound TGFβ-expressing $CD4^+$ Tregs in induction of airways tolerance elicited by low dose inhaled antigen (429). Studies by Umetsu and colleagues (52, 53) have shown that, in a murine model of allergic airways disease, tolerance can be induced by intranasal administration of high dose allergen. This tolerance is dependent on IL-10-producing DCs, which in turn promote the induction of IL-10-secreting regulatory T cells. These Tr cells also produce IL-4 early in their development, and are dependent on IL-10 production by DCs and the inducible T-cell co-stimulator (ICOS) molecule expressed

on DCs for their suppressive function. Subsequent studies have shown these Tr cells to be T-bet and *foxp3*-positive (278).

Hence, Tregs have been shown to inhibit allergic inflammation in animal models, and *ex vivo* experiments suggest that human Tregs may be dysfunctional in atopic individuals. To further support the notion that Tregs are important in the pathogenesis of allergic disease, there is evidence showing that treatments commonly used for asthma and allergy may promote Treg numbers and/or function. Allergen-desensitisation immunotherapy is used for patients with IgE-mediated diseases, such as allergic rhinitis, asthma and bee venom anaphylaxis. Effective allergen immunotherapy is associated with immune deviation from a Th2 profile to a Th1 response (422, 430). However, not all reports agree that immune deviation is necessary for allergen-desensitisation immunotherapy to be successful (431). In this case, it was shown that immunotherapy to insect venom is associated with a decrease in allergen-specific Th2 response, and the induction of IL-10-secreting T regulatory cells (432, 433). Another line of evidence is that an important therapy for asthma and allergic disease is glucocorticoids. It has been shown that glucocorticoid-induced synthesis of IL-10 by human T cells is associated with a suppressive effect on $CD4^+$ T cells (434). More importantly, steroid-resistant asthmatics have $CD4^+$ T cells with a reduced capacity to synthesise IL-10 in response to glucocorticoids *in vitro* (435). Glucocorticoids and Vitamin D3 can induce IL-10 producing Tregs (301, 436) which can reduce Th2 cytokine production by human T cells *in vitro*. Furthermore, pre-incubating $CD4^+CD25^+$ regulatory T cells from the peripheral blood of atopic patients with the steroid fluticasone propionate resulted in enhancement of Treg function (437). Thus, Tregs appear to have an important regulatory role in the pathogenesis of

asthma and allergy, and current treatments may work by inducing Treg numbers and function.

$CD4^+CD25^+$ regulatory T cells constitutively express GITR at high levels, and moreover the suppressive function of these Tregs is abrogated by the activating anti-GITR antibody. If GITR signalling could be blocked, this would lead to an enhancement of the regulatory function of $CD4^+CD25^+$ T cells and potentially new therapies for inflammatory diseases could be developed. In Chapter 5, it was demonstrated the OVA-induced IL-10 production *in vitro* from thoracic LN cells was enhanced in mice that had been treated with anti-GITR mAb. In this situation, one would expect anti-GITR mAb treatment to abrogate $CD4^+CD25^+$ Treg function, with an associated reduction of IL-10 production. If anti-GITR mAb were activating other Tregs, for example $CD4^+CD25^-$ IL-10-producing Tr1 cells, again an increase in IL-10 production would not correlate with the observed exacerbation of murine asthma. However, it has been demonstrated that GITR is also expressed on $CD4^+CD25^-$ effector T cells and macrophages, and we suggest that anti-GITR mAb may be inducing IL-10 production from these cells. Thus, macrophages or $CD4^+CD25^-$ effector T cells may be producing IL-10 in response to anti-GITR mAb treatment *in vivo*, and this may even be enhanced if Treg function is abrogated by GITR activation. It would be important to further investigate this by examining the effects of GITR activation on these cells *in vitro*, as well as examining possibly isolating the role of GITR on other cells such as Tr1 cells which are known to produce large quantities of IL-10.

Regulatory T cells have been shown to have suppressive effects on $CD4^+$, $CD8^+$, B cells and APCs. However, there have been no reports of Tregs directly influencing other cells of the innate system, for example mast cells, NK(T) cells or eosinophils. It would be interesting to investigate the effects of Tregs (and GITR) on the effector function of cells of the innate system, as well as other cells important in airway remodelling of chronic asthma such as fibroblasts and smooth muscle cells. One murine study has demonstrated the importance of $CD4^+CD25^+$ Tregs in suppressing Th2-mediated immune responses, but not bronchial hyperresponsiveness, in a model of chronic asthma (351). Having demonstrated that GITR has an important role in a mouse model of acute asthma, it would be of interest to examine the role of GITR in a chronic model of murine asthma, which may resemble more closely the human disease. It would then be important to determine whether $CD4^+CD25^+$ T cells have a role in regulating chronic airways inflammation in humans.

It is important to remember that GITR is also expressed at low levels on $CD4^+CD25^-$ effector T cells. Upon TcR engagement, GITR expression is upregulated on effector T cells, and has been shown to act as a co-stimulatory molecule (390, 394). Our study demonstrates that activating anti-GITR mAb enhances Th1 and Th2 differentiation of naïve $CD4^+$ and $CD4^+CD25^-$ T cells *in vitro*. The mechanism of the enhancing effect on $CD4^+CD25^-$ cells is likely due to its ability to elevated T-bet and GATA3, the two master switches of Th1 and Th2 cells. Given the importance of T-bet and GATA3 in Th cell differentiation, the mechanism by which GITR signalling enhances the expression of these transcription factors merits further investigation. The mechanism by which GITR activation inhibits Treg function, but co-stimulates effector T cells also requires further elucidation. Shimuzu *et al* showed that NF-κB expression was

increased in $CD4^+CD25^+$ T cells after GITR cross-linking. Ronchetti *et al*, using GITR-deficient mice, found that $GITR^{-/-}$ effector T cells proliferated more than wild type control T cell in response to anti-CD3 stimulation (438). The same investigators also found that GITR provided a signal to T cells that protected them from activation-induced cell death (AICD) (389, 438). However, other investigators suggest that GITR ligation can induce both AICD and apoptosis (396, 439). Therefore the role for GITR as a cell survival signal is unresolved, and recently, it has been shown that GITR cross-linking on naïve and activated T cells resulted in activation of MAPKs and NF-κB. However these signals were not sufficient to directly inhibit AICD of T cells (440). Recently, it has been demonstrated that TNFR-associated factor 2 (TRAF2) plays an inhibitory role in GITR-triggered NF-κB activation, which is not observed in other TNFRs, and may represent a target for GITR manipulation (441). Hence, GITR signalling is incompletely understood, and further studies are required to elucidate the mechanisms of GITR activation of immune cells, which may lead to novel targets for GITR modulation.

Recent studies carried out in our laboratory have shown that GITR activation can also exacerbate a Th1-mediated murine model of arthritis *in vivo* (unpublished data). Hence, GITR activation by monoclonal antibody increases both Th1- and Th2-mediated diseases *in vivo*. Although this would not be beneficial in inflammatory disease, it could potentially have a clinical use when enhancement of the immune system was required, for example in tumour cell or microbial pathogen clearance (Fig. 6.2, Ib). Developing soluble recombinant GITR receptor or increasing TRAF2 signals are potential targets for inhibiting the GITR signal, which could be used in many inflammatory disorders (Fig. 6.2, Ia). The balancing of an over-active immune system

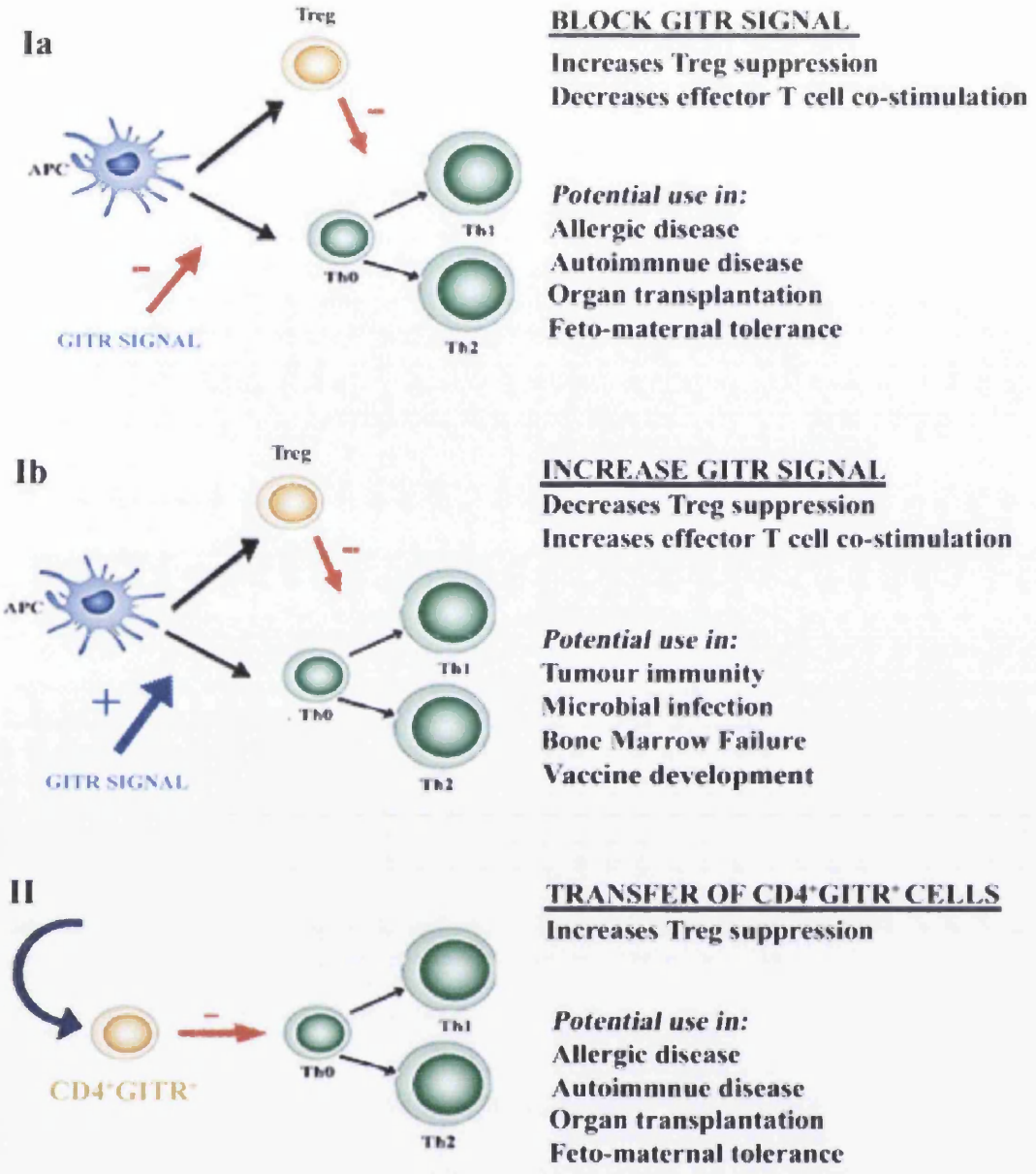

Figure 6. 2 Potential therapeutic interventions with GITR modulation

Manipulation of GITR function could possibly lead to new therapeutic approaches. We hypothesise that blocking GITR signal could increase Treg function and reduce the co-stimulatory signal to effector T cells. If the GITR signal could be blocked sufficiently to dampen the immune system without inducing immunosuppression, then the clinical application in inflammatory and related disorders would be of value (panel Ia). The opposite would then apply if the GITR signal was enhanced (panel Ib). Furthermore, the transfer (panel II) or depletion of $GITR^+$ cells could potentially induce suppression or activation of the immune response.

without over-suppressing it represents a considerable future challenge. In addition to suppressing or enhancing GITR function, GITR expression may represent a marker for all naive $CD4^+$ regulatory T cells. Our study demonstrated that passively transferred $CD4^+GITR^+$ cells could attenuate murine asthma induced in SCID mice reconstituted with $CD4^+GITR^-$ cells. The transfer or induction of $CD4^+GITR^+$ cells could possibly dampen an overactive immune response (Fig. 6.2, II), or the removal of these cells could "boost" the immune system when required. The potential difficulty of using GITR as marker of Tregs is that, like CD25, it is also upregulated upon activation of other cells. Therefore in the peripheral blood of humans, who are continuously exposed to antigen, $GITR^+$ cells will be a heterogeneous mix of Tregs and activated effector cells.

Although these potential problems exist, GITR is an exciting new molecule that is important in the function of both regulatory and effector T cells, and through its modulation may develop into new therapies for many inflammatory disorders.

6.5 Future work

Based on the results of the work presented in this thesis I would like to suggest that our initial hypothesis can be advanced and that several new experimental lines of investigation have been opened.

1. One possible target is GITR, which has been shown to be a molecule that is important in the function of $CD4^+CD25^+$ Tregs and effector T cells. The potential exists for manipulating GITR function that may lead to therapies in human diseases. Again, before translating findings from our murine model of allergic inflammation into clinical diseases such as asthma, extensive *in vitro* and *in vivo* work will be required to determine if GITR activation can be controlled so as not to induce unacceptable adverse effects, and to also examine the effect of GITR activation on other cells of the immune system. These studies could also be designed to examine the role of GITR activation on cells of the innate and adaptive immune system, such as macrophages and B cells. Furthermore, the role of GITR activation on other Treg populations, for example Tr1 and Th3 cells, could be performed. These investigations would hopefully not only provide further information into the cellular and molecular mechanisms behind GITR in acute models of murine asthma, but also address hypotheses regarding the role of GITR and Tregs in chronic asthma pathogenesis as well. Further knowledge into the mechanisms of GITR activation of immune cells would hopefully direct future work into modulating GITR function, which would hopefully lead to safe manipulation of the immune response, as summarised in Figure 6.2.

2a. Our work shows that TLR2 agonist therapy in a BALB/c model of asthma was effective in attenuating Th2 inflammation by reducing a Th2 response and inducing a mild Th1 response. Further work is required to investigate the detailed mechanisms involved in TLR2 activation of APCs and T cells in Th2-mediated inflammation. In particular, examining the molecular basis of TLR2 agonist action on these cells would be of interest. As well as investigating the effect of TLR2 activation on APCs and T cells, it would be important to carry out experiments designed to address whether TLR2 activation of other cells, such as eosinophils, mast cells, epithelial cells, fibroblasts, smooth muscle cells, B cells and NK cells, influenced their function and as to whether this had a bearing on asthma pathogenesis. This experimental approach would also be of value in investigating why different routes and different timing of TLR2 agonist administration resulted in apparently contradictory results. A secondary aim of this work could also address whether these TLR2 agonists had a role in airway remodelling, seen in patients with chronic irreversible asthma, and may also be extended to investigate the role of TLR2 activation in non-allergic airways inflammatory disease such as non-atopic asthma, COPD or Acute Lung Injury.

2b. As TLR2 activation induces a mild Th1 response in our model, it would be important to ensure that side effects from such a therapy are not evident. In our preliminary studies, we showed that TLR2 agonist therapy did not appear to increase IgG levels, did not induce peritonitis, and the mice did not look sick. The primary aim of this work was to examine the potential benefits of TLR2 agonist administration, and was not designed to investigate potential adverse effects. Having demonstrated a potential use for TLR2 agonists in experimental asthma, it would be important to address whether TLR2 activation induces symptoms such as fever, peritonitis over a

longer time scale, or even induced Th1-mediated inflammatory disease. Such work has been performed for TLR9-mediated CpG DNA vaccine therapy in Th2-mediated disease, and appears to have minimal side effects.

3. Further work is required to examine the effects of TLR2 agonists and GITR manipulation in our murine model of acute asthma, and in models of chronic asthma. Airway hyperreactivity was assessed in our work using enhanced pause. Some investigators have found that in some strains of mouse, Penh does not always correlate with invasive measures of airway hyperresponsiveness. It would be important to ensure that our assessment of airway hyperreactivity using whole body plethysmography was validated. This could be performed by running an experiment where non-invasive whole body plethysmography was performed on the same mice that had invasive physiological measures taken. It would also be useful to devise a lung histology score that could be used as validated measure of allergic airways inflammation.

4. If further experimental animal work indicates that TLR2 agonists and GITR have important roles in asthma pathogenesis, and more importantly are safe, then the ultimate aim of this work would be conduct clinical studies in human asthmatics. These studies would initially be aimed at examining the expression of GITR and TLR2 on different cell groups obtained from peripheral blood, sputum, bronchoalveolar lavage (BAL), and lung biopsy specimens from asthmatic and normal healthy individuals. This work could then be taken forward by examining the effect of these immunomodulatory agents on the cells isolated from the peripheral blood and lung samples *ex vivo*. It is noted that the number of cells obtained, especially T cells,

is poor from sputum and BAL samples, and hence lung biopsies may be necessary when examining the role of TLR2 and GITR on pulmonary cells. If TLR2 and GITR were found to have a role in either the protection from asthma in healthy individuals, or in the development of disease in asthmatics then the potential for conducting clinical trials investigating the safety and the therapeutic benefits of these novel immunomodulatory agents should be considered.

6.6 Conclusions

TLR2 agonists appear to represent a novel approach to treat established Th2-mediated inflammation by skewing the immune response towards a Th1 response. GITR activation activates the immune system by abrogating regulatory T cell function, and enhancing effector T cell response. GITR manipulation may lead to new therapies, especially if the benefits of activating or suppressing the immune system are maximised, with minimal induction of side effects. Both TLR2 agonists and GITR merit further investigation, and may result in novel therapies not only for asthma, but a range of many inflammatory disorders.

7.0 References

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