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Peptide Immunotherapy in Models of Allergic Airways Disease

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A thesis submitted for the degree of Doctor of Philosophy
The University of Edinburgh
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Declaration

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

Karen J Mackenzie

June 2011

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Abstract

Allergen-reactive $CD4^+$ T cells are implicated in the pathogenesis of allergic disease. Peptide immunotherapy (PIT) involves therapeutic administration of short immunodominant peptides from within the protein allergen to which $CD4^+$ T cell responses are directed. This approach can induce tolerance of allergen-reactive $CD4^+$ T cells, while negating the risk of severe allergic reactions associated with whole allergen specific immunotherapy. PIT therefore holds promise as a disease-modifying treatment for allergic patients. However, further information regarding the mechanisms of action of PIT are required to aid translation to the allergy clinic.

Chicken ovalbumin (OVA) is a commonly used model allergen in mouse models of allergic airways inflammation (AAI). Trackable, T cell receptor transgenic T cells recognizing the immunodominant 323-339 peptide of OVA (pOVA) allow mechanistic investigation of PIT in response to pOVA. This thesis investigated the hypothesis that strong, systemic T cell responses induced by intravenous administration of soluble pOVA will induce i) tolerance to pOVA and ii) linked suppression to any additional OVA T cell epitopes, hence improving OVA-induced AAI. Contrary to the hypothesis, intravenous pOVA PIT did not improve disease in a C57BL/6 model of OVA-induced AAI. Models of OVA-induced allergic sensitisation and AAI were therefore developed incorporating trackable $CD4^+$ pOVA-reactive T cells (OT-II cells). pOVA PIT induced tolerance of these cells in an allergic sensitisation setting, but had limited impact on the overall OVA response. Yet, in a model of AAI driven solely by Th2 polarised $CD4^+$ OT-II cells, pOVA PIT did improve disease. It was concluded that, in non-transgenic C57BL/6 mice, $CD4^+$ T cells responding to additional epitope(s) within OVA were important in driving disease and that these T cells were not subject to linked suppression following pOVA PIT. Using a panel of overlapping peptides constituting the sequence of OVA, a novel $CD4^+$ epitope within OVA was characterised. The effects of PIT using pOVA in combination with a peptide containing this additional epitope on OVA-induced AAI were then assessed. Findings from this project therefore hold importance for future mechanistic work surrounding PIT in allergic disease.

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List of Abbreviations

AAI	Allergic airways inflammation
AHR	Airway hyperresponsiveness
APC	Antigen presenting cell
APL	Altered peptide ligand
BAL	Bronchoalveolar lavage
BSA	Bovine serum albumin
CCR-	CC Chemokine receptor-
CCL-	CC Chemokine ligand-
Cdyn	Pulmonary dynamic compliance
CFA	Complete Freund's adjuvant
CFSE	Carboxyfluorescein diacetate succinimidyl ester
CLIP	Class II-associated invariant-chain peptide
CTLA-4	Cytotoxic T-lymphocyte antigen 4
D	Day
DC	Dendritic cell
DTR	Diphtheria toxin receptor
EAE	Experimental autoimmune encephalomyelitis
ELISA	Enzyme-linked immunosorbent assay
FoxP3	Forkhead BoxP3 protein
GATA-3	GATA-binding protein 3
GWAS	Genome-wide association studies
H+E	Hemotoxylin and eosin
HDM	House dust mite
HLA	Human leukocyte antigen
ICAM-1	Intracellular adhesion molecule 1
Ig-	Immunoglobulin-
IFN-γ	Interferon – γ
IL-	Interleukin
iLN	Inguinal lymph nodes
i.n	Intranasal
i.p	Intraperitoneal
IPEX	Immune dysregulation polyendocrinopathy enteropathy X-linked syndrome
i.t	Intratracheal
ITAMS	Immunoreceptor tyrosine-based activation motifs
iTreg	Inducible T regulatory cell
i.v	Intravenous

JAK	Janus kinase
Lck	Lymphocyte-specific protein tyrosine kinase
LFA-1	Lymphocyte function-associated antigen 1 integrin
LPS	Bacterial lipopolysaccharide
LN	Lymph nodes
MCh	Methacholine
MD-2	Myeliod differentiation factor 2
MHC II	Major histocompatibility complex II
mLN	Mediastinal lymph nodes
nTreg	Natural T regulatory cell
OVA	Ovalbumin
PALMs	Pollen-associated lipid mediators
PAMPS	Pathogen associated molecular patterns
PAS	Periodic acid-Schiff
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PD-1	Programmed death 1
PIT	Peptide immunotherapy
PLA	Phospholipase A_2
PMA	Phorbol myristate acetate
pOVA	pOVA 323-339
PRR	Pattern recognition receptor
PTM	Post-translational modifications
RAG	Recombination activating gene
Rl	Resistance of the lung
RORαT	Retinoic acid-related orphan receptor αT
RORγT	Retinoic acid-related orphan receptor γT
s.c	Subcutaneous
SIT	Specific immunotherapy
STATs	Signal transducers and activators of transcription
T-bet	T box expressed in T cells
TCR	T cell receptor
TGF-β	Transforming growth factor β
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor α
Treg	T regulatory cell
TSLP	Thymic stromal lymphopoietin
ZAP-70	Zeta-associated protein-70

1 Introduction

1.1 Context

Allergic asthma is a substantial clinical problem and often originates in childhood (Simpson and Sheikh, 2010). It is increasingly evident that allergy can be viewed as a systemic disease, and that allergic asthma may develop in some patients who already have one allergic disease, such as allergic eczema (Punekar and Sheikh, 2009). Current mainstream allergic asthma treatments are directed towards symptom control and generalised suppression of immune responses (Haslett et al., 2002). However, current treatments are not curative and have not reliably been demonstrated to modify disease progression.

$CD4^+$ T cell responses to allergens are strongly implicated in the pathogenesis of allergic asthma [reviewed in (Larché et al., 2003)]. In health, allergen-reactive $CD4^+$ T cells do not mount an allergic response to inhaled aeroallergens, i.e. they are tolerant. Allergen-specific therapies aim to specifically induce tolerance of allergen-reactive T cells. Such targeted therapeutics have the potential to improve/ablate the symptoms of allergic disease such as allergic asthma, but may also be effective in altering the progression of allergic disease, if given early in life (Larché, 2007, Jacobsen et al., 2007). Specific immunotherapy (SIT), which involves administering whole protein allergen to induce tolerance, has been found in some clinical trials to be efficacious in the treatment of allergic disease (Calderon et al., 2007). However, giving allergen as a treatment has the potential to induce severe allergic responses, including anaphylaxis, as a consequence of IgE binding to multiple sites on the allergen and subsequent IgE cross linking on mast cells, leading to mast cell degranulation (Borchers et al., 2004, Peavy and Metcalfe, 2008). Severe allergic reactions induced by SIT are more likely to occur in allergic asthmatics and have been associated with fatalities in rare cases (Abramson et al., 2010, Borchers et al., 2004).

Peptide immunotherapy (PIT) offers a potentially safer approach to allergen-specific immunotherapy. PIT involves administering short allergen-derived peptides to which the T cell response is directed (known as immunodominant peptides), to induce T cell tolerance [reviewed in (Larché, 2007, Wraith, 2009)]. The short nature of these peptides means that they do not facilitate IgE cross linking, therefore abrogating the risk of severe allergic reactions.

PIT has been shown to be capable of inducing T cell tolerance in some animal models (predominantly in the autoimmune setting), and in a small number of clinical trials involving allergic patients (Metzler and Wraith, 1993, Gaur et al., 1992, Campbell et al., 2009, Tarzi et al., 2006). However, the effects of PIT in the clinical setting have varied (Oldfield et al., 2002), and detrimental effects, or lack of therapeutic effect, have been reported in some allergy-based animal studies (Janssen et al., 1999, Barbey et al., 2004). Better understanding of the mechanisms of action of PIT in the allergic setting is therefore required in order to facilitate clinical translation.

In this thesis, the effects of PIT in the context of murine models of chicken ovalbumin (OVA)-induced allergic airways disease are investigated. The focus of this introduction will therefore be:-

- $CD4^+$ T cell biology relevant to this project
- mechanisms thought to be involved in the induction of T cell tolerance
- the immunological aspects of allergic asthma and how these can be modelled *in vivo*
- current knowledge surrounding both specific immunotherapy and peptide immunotherapy

1.2 $CD4^+$ T cell development and selection

1.2.1 $CD4^+$ T cell development in the thymus

T cells develop from haematopoietic stem cells. These migrate from the bone marrow to the thymus, an organ which represents a carefully controlled environment that facilitates T cell development [reviewed in (Zamoyska and Lovatt, 2004)].

Mature $CD4^+$ T cells are generated in the thymus throughout life, but output diminishes with age as a consequence of thymic atrophy [reviewed in (Heng et al., 2010)]. $CD4^+$ and $CD8^+$ T cells develop from the α:β T cell lineage (Zamoyska and Lovatt, 2004). During development, thymocytes must successfully pass through various stages to avoid apoptosis (Zamoyska and Lovatt, 2004). Key phases involve the progression from double negative thymocytes (which do not express CD4 or CD8) to become double positive thymocytes [which express both CD4 and CD8 (Zamoyska and Lovatt, 2004)]. Such progression requires the effective generation of a pre-T cell receptor (-TCR) consisting of a β chain in association with a pre-Tα chain (Zamoyska and Lovatt, 2004, Murphy et al., 2008). Subsequent thymocyte proliferation is followed by expression of the CD4 and CD8 co-receptors (Zamoyska and Lovatt, 2004). Double positive thymocytes then proceed to gene rearrangement of the α chain, leading to the expression of a mature α:β TCR (Zamoyska and Lovatt, 2004). Thymocytes that survive this far then undergo positive and negative selection. Such a developmental pathway ensures diversity in TCR antigen specificity.

1.2.2 Positive $CD4^+$ T cell selection

In order for CD4/CD8 double positive thymocytes to become single positive CD4 or CD8 expressing T cells, requires them to pass through a further checkpoint known as positive selection [reviewed in (Germain, 2002)]. In the context of $CD4^+$ T cell development, the TCR of double positive thymocytes must be capable of binding to self-peptide presented by thymic cortical epithelial cells in the context of major

histocompatibility complex (MHC) II. If this occurs, the T cell escapes apoptosis and down regulates the CD8 co-receptor, becoming a single positive CD4 T cell.

1.2.3 Negative selection

If T cells were only selected based on their ability to recognise self-peptide in the context of MHC, T cells with TCRs with high affinity for self peptides would also be selected, resulting in many high affinity autoreactive T cells in the periphery. T cells developing in the thymus are thus also subject to negative selection [reviewed in (Starr et al., 2003)]. This involves the deletion of T cells with TCRs with high affinity for self peptide/MHC and can be illustrated in a study by Zal et al utilising transgenic mice that possessed $CD4^+$ T cells reactive to an MHC II-restricted peptide from the C5 complement protein (Zal et al., 1994). In that study, $CD4^+$ T cells were present in the periphery of mice that did not express C5, but were deleted in the thymus in mice that did express C5 (Zal et al., 1994). Evidence strongly supports the requirement for negative selection to prevent autoimmunity (Starr et al., 2003, Kishimoto and Sprent, 2001). Hence, negative selection in the thymus is known as central tolerance.

Overall, therefore, the mechanisms involved in T cell development result in a population of $CD4^+$ T cells in the periphery that are restricted to the recognition of antigen in the context of MHC II, and are also tolerant towards self-antigens.

1.3 Innate immune responses

The innate immune response ensures rapid responses to invading pathogens. Innate immune cells such as neutrophils, eosinophils, basophils, mast cells and macrophages are derived from a common myeloid progenitor cell (Murphy et al., 2008). The actions of innate immune cells are vital for efficient responses to pathogens, and can, in some cases, be sufficient to induce pathogen clearance alone, through actions such as phagocytosis and the release of factors which promote the killing of pathogens [reviewed in (Chaplin, 2010)]. However, innate immune

responses alone do not induce immunological memory and do not elicit antigen-specific responses; the key features of adaptive immunity (Murphy et al., 2008).

Triggering of innate immune responses involves the recognition of so-called "danger-signals" by innate immune cells [reviewed in (Matzinger, 2002)]. Innate immune cells possess pattern recognition receptors (PRRs) which can bind to a variety of pathogen associated molecular patterns (PAMPS), [reviewed in (Bianchi, 2007)]. Prime examples of PRRs are the toll-like receptors (TLRs), which can respond to a variety of PAMPs. TLR-4 for example, in association with CD14 and the myeloid differentiation factor-2 (MD-2) protein, can recognise bacterial lipopolysaccharide (LPS), whereas TLR-3 can recognise viral-derived double-stranded DNA (Bianchi, 2007). Other PRRs include mannose-binding lectin and the cytoplasmically located nucleotide-binding oligomerization domain-containing proteins (NOD proteins). Danger can also be signalled through the presence of substances whose presence outside a cell are indicative of cellular damage or distress e.g. uric acid, adenosine-5'-triphosphate (ATP) and heat shock proteins (Gallucci and Matzinger, 2001, Bianchi, 2007). The recognition of danger by innate immune cells can induce the production of an array of inflammatory mediators e.g. IL-12, tumor necrosis factor-α (TNF-α) and IL-1 [reviewed in (Bianchi, 2007)].

1.4 Requirements for the activation of $CD4^+$ T cells

1.4.1 Dendritic cells (DCs) and the importance of Signal 0

The potential for DCs [known to be highly effective antigen presenting cells (APCs)] to respond to PAMPs and other danger-associated signals, means that they are ideally placed to bridge innate and adaptive immunity. Recognition of danger signals (e.g. LPS), by DCs leads to increased antigen sampling, antigen processing and presentation on MHC, and increased expression of costimulatory molecules such as CD80 and CD86 (Bianchi, 2007, Shi et al., 2003, Eisenbarth et al., 2002). Furthermore, the recognition of danger signals by DCs also induces them to migrate

to draining lymph nodes (LNs) where they can present antigen to T cells (Eisenbarth et al., 2002).

The activation of DCs via their detection of danger signals, can be referred to as **Signal 0** in the requirement for activation of $CD4^+$ T cells, since this promotes antigen uptake and processing, and is therefore the first step towards inducing T cell activation. How a DC responds to different danger signals influences the nature of the ensuing T cell effector response (Peters et al., 2010); this is further discussed in later sections.

1.4.2 Antigen processing

In order for a peptide to bind to MHC II and be presented on the surface of an APC, it first has to be generated from an antigen (often a protein). This process is discussed in (Watts et al., 2003, Murphy et al., 2008), and is briefly summarised below.

Proteins that are endocytosed by an APC enter an intracellular processing pathway. During processing, the pH of the endosomes reduces inducing the activation of various proteases. These can then cleave the protein into peptides. Meanwhile, MHC II is generated by the cell which is then transported from the endoplasmic reticulum to endosomes. In the initial stages of transit, peptides are prevented from binding to MHC II by the presence of the invariant chain [which is later cleaved to the class II-associated invariant-chain peptide (CLIP)], in the MHC II binding groove. When endosomes containing newly generated peptides and MHC II fuse, this presents the opportunity for peptide to bind to MHC II [with the aid of the chaperone molecule human leucocyte antigen (HLA) –DM, or the mouse equivalent H-2M]. It is thought likely that long peptides can initially bind to MHC II and these are then shortened by further protease activity (Watts et al., 2003). The end result is a peptide that is typically 10-25 amino acids in length bound to MHC II. This complex is then transported to the cell surface to permit interaction with TCRs on $CD4^+$ T cells.

T cell responses are usually predominantly directed towards one or more epitopes contained within antigen-derived peptides. Such epitopes are thus termed "immunodominant" [discussed in (Sant et al., 2005)]. Many variables influence whether or not a particular antigen-derived peptide is immunodominant. For example, the nature of the peptides generated by APCs will depend upon the actions of proteases (Sant et al., 2005). The peptides that are generated then need to be capable of binding to MHC II, and processes such as peptide editing by HLA-DM ensure that stable peptide-MHC II interactions are favoured (Sant et al., 2005). Hence, some peptides containing particular epitopes may not be displayed on MHC II following antigen processing. This is relevant to the existence of "cryptic epitopes" which only elicit T cell responses when they are contained within exogenously delivered peptides, whereas T cell responses are not directed towards these epitopes during processing of the full antigen (Sweenie et al., 2007). Immunodominance can also be affected by the frequency of $CD4^+$ T cells with TCRs reactive to particular peptide-MHC-II complexes, since this frequency is known to vary (Chu et al., 2010). Overall, the T cell response to an antigen is often predominantly directed towards only a few (or even one) immunodominant epitopes, as a consequence of variations in antigen processing and of the availability, and ensuing response, of peptide-reactive $CD4^+$ T cells.

1.4.3 Signal 1: TCR engagement and resultant signalling

Signal 1 refers to the TCR on a $CD4^+$ T cell recognising peptide expressed on an APC in the context of MHC II [reviewed in (Smith-Garvin et al., 2009)]. The α and β chains together form the site of antigen binding. These chains must also be associated with the CD3 complex (itself formed from 4 chains - one CD3γ chain, one CD3δ chain and two CD3ε chains), and the ζ chain [reviewed in (Qian and Weiss, 1997)]. All these chains possess cytoplasmic tails containing immunoreceptor tyrosine-based activation motifs (ITAMs)], (Qian and Weiss, 1997). Together these associations form the TCR complex. TCR engagement with peptide-MHC II leads to phosphorylation of tyrosines present within the ITAMs. The actions of the tyrosine kinases proto-oncogene tyrosine protein kinase Fyn (Fyn) and lymphocyte-specific

protein tyrosine kinase (Lck) are implicated in this phosphorylation (Qian and Weiss, 1997, Smith-Garvin et al., 2009). In $CD4^+$ T cells, signalling through the TCR is also mediated by the CD4 co-receptor which associates with the TCR, binds to MHC II and is itself associated with Lck, hence promoting ITAM phosphorylation (Janeway, 1992). ITAM phosphorylation enables another tyrosine kinase, known as zeta-associated protein-70 (ZAP-70), to bind to the ζ chain, become phosphorylated itself and then become activated (Qian and Weiss, 1997). This sets into play further down stream signalling events which can cumulate in the activation of the transcription factors nuclear factor κB (NFκB), nuclear factor of activated T cells (NFAT) and activator protein 1 (AP-1), which are required for the $CD4^+$ T cell to proliferate and differentiate (Qian and Weiss, 1997).

1.4.4 Signal 2: Co-stimulation

Signal 2 refers to the additional stimulus provided by co-stimulation between the APC and T cell. TCR ligation with peptide-MHC without signal 2 inhibits the development of an effector T cell response and can induce a state of unresponsiveness, known as anergy [reviewed in (Schwartz, 2003)]. The requirement for Signal 2 to elicit productive effector T cell responses represents a means of regulating against harmful T cell responses to self peptides.

Co-stimulation via CD28, a molecule constitutively expressed on naïve as well as activated T cells, has been well characterised (Acuto and Michel, 2003). CD28 can bind to CD80 and CD86 on the APC, the expression of which are increased upon DC activation (Bianchi, 2007). Costimulation via CD28-CD80/86 facilitates IL-2 production, proliferation and effector cytokine production by the T cell [reviewed in (Alegre et al., 2001)]. In contrast, lack of costimulation via CD28 significantly impairs the T cell response, and often leads to anergy or cell death [discussed in (Alegre et al., 2001, Schwartz, 2003, Croft et al., 1994)]. Co-stimulation also occurs via molecules such as CD40L and OX40 which are expressed by activated T cells (Quezada et al., 2004, Croft et al., 2009). OX40 binds to OX40L on DCs, the expression of which is induced by PRR stimulation or in response to co-stimulation

[reviewed in (Croft et al., 2009)]. OX40-OX40L interactions subsequently promote T cell survival (Rogers et al., 2001).

Negative co-stimulatory molecules also exist and are necessary to prevent excessive immune responses that would be detrimental to the host. These include molecules such as cytotoxic T-lymphocyte antigen 4 (CTLA-4) and programmed death 1 (PD-1). Like CD28, CTLA-4 can also bind to CD80 and CD86, but with greater avidity [reviewed in (Rudd et al., 2009)]. CTLA-4-CD80/86 interactions significantly impair T cell proliferation and IL-2 production (Krummel and Allison, 1995). CTLA-4 is expressed by activated T cells and its importance in limiting immune responses is demonstrated by CTLA-4 deficient mice, which develop extensive lymphoproliferative disease at an early age (Waterhouse et al., 1995).

1.4.5 Signal 3: The effect of cytokines on T cell differentiation

The combined effects that cytokines exert during T cell activation are termed **Signal 3.** The cytokine milieu that is present during $CD4^+$ T cell activation profoundly affects T cell differentiation and, consequently, the nature of the effector response [reviewed in (O'Shea and Paul, 2010)]. Cytokines (such as those produced by innate cells in response to PRR stimulation), can bind to receptors on $CD4^+$ T cells, activating tyrosine kinases from the Janus kinase (JAK) family which in turn activate signal transducers and activators of transcription (STATs), [reviewed in (Zhou et al., 2009, Adamson et al., 2009)]. Different cytokine environments thus promote the activation of different STATs, which, following their translocation to the nucleus, will differentially result in the activation of a variety of T cell genes. The activation of different STATs can also lead to the induction of other transcription factors known as “master-regulators”, the expression of which are associated with particular T cell lineages.

Differentiation of naïve $CD4^+$ T cells into effector T cells therefore requires Signals 0, 1, 2, and 3. Figure 1.1 summarises these requirements for T cell activation.

Figure 1-1: Signals required for the induction of an effector $CD4^+$ T cell response

1.5 $CD4^+$ T cell subsets

Several subsets of $CD4^+$ T cells have been described, which possess different effector properties and can differentiate from naïve $CD4^+$ T cells in response to different cytokines (Signal 3) [reviewed in (Zhou et al., 2009)]. Figure 1.2 provides an overview of $CD4^+$ T cell differentiation, the understanding of which has evolved over time. 4 main lineages have been described [Th1, Th2, Th17 and T regulatory (Treg) cells] which can be identified by their expression of different transcription factors. Considerable plasticity is increasingly being demonstrated between different $CD4^+$ subsets [reviewed in (Zhou et al., 2009)]. The different cytokine signatures of differentiated $CD4^+$ T cells, influences the nature of the immune response (Zhou et al., 2009). The different $CD4^+$ T cells subsets are briefly discussed in the next section. Those subsets thought to be particular relevance in allergic asthma will be further discussed in section 1.7.10.

Figure 1-2: $CD4^+$ T cell subset

1.5.1 Th1 and Th2 cells

Th1 and Th2 cells were the $CD4^+$ T cell subsets first to be described (Mosmann and Coffman, 1989). Th1 cells are key in the defence against pathogens, particularly intracellular pathogens, and are primarily associated with their ability to produce interferon-γ (IFN-γ) [reviewed in (Wan, 2010)]. Th1 differentiation is associated with the activation of STAT1 and STAT4 and of the transcription factor T box expressed in T cells (T-bet), which is crucial for Th1 effector function [reviewed in (Zhu and Paul, 2010, Adamson et al., 2009)]. While vital for anti-pathogenic responses, Th1 responses are also prominent features of inflammatory diseases such as rheumatoid arthritis and inflammatory bowel disease (Wan, 2010).

Th2 cells produce the signature cytokines IL-4, IL-5 and IL-13 [reviewed in (Paul and Zhu, 2010)]. Th2 responses are particularly important for host defence against helminths [reviewed in (Harris and Gause, 2011)], but are also heavily implicated in allergic responses, as further discussed in section 1.7.10. Through the provision of B cell help, Th2 cells enable the B cell class-switching that is required for the generation of IgE [reviewed in (Harris and Gause, 2011)]. GATA-binding protein 3 (GATA-3) is known as the master transcription factor for Th2 cells [reviewed in (Paul and Zhu, 2010)]. Hence, GATA-3 depletion has been found to abrogate Th2 responses, and GATA-3 is required for Th2 differentiated cells to maintain Th2 cytokine production (Pai et al., 2004, Zhu et al., 2004). Th2 cells can differentiate in response to IL-4 which leads to STAT6 activation and the activation of GATA-3 [(Kurata et al., 1999) and reviewed in (Paul and Zhu, 2010, Adamson et al., 2009)]. GATA-3 expression can also be induced via STAT5 activation, for example in response to stimulation of the TCR with low doses of antigen (Yamane et al., 2005). STAT5 activation can also occur via IL-2 signalling (Cote-Sierra et al., 2004) and also via the actions of IL-7 and IL-15 (Liao et al., 2008).

Reciprocal inhibition between the Th1 and Th2 subsets has been described. GATA-3 plays a role in the inhibition of Th1 responses, as evidenced in a study by Zhu et al, who found that conditional depletion of GATA-3 in $CD4^+$ T cells prior to culture in

Th2-promoting conditions, suppressed IL-4 expression and promoted IFN-γ expression (Zhu et al., 2004). IL-4 has also been shown to interfere with the expression of a component of the IL-12 receptor during T cell differentiation, thus further directing the T cell towards a Th2 rather than a Th1 phenotype (Szabo et al., 1997).

It is generally felt that Th2 cells represent a relatively stable cell phenotype, compared to some of the other $CD4^+$ T cell subsets such as Th17 cells (Zhou et al., 2009). However, there is evidence to suggest that culturing Th2 cells in the presence of transforming growth factor-β (TGF-β) can lead to down regulation of Th2 signature cytokines and upregulation of IL-9. The resultant cells have been described as Th9 cells (Veldhoen et al., 2008), and are further discussed below. Hence, Th2 cells may also exhibit a degree of plasticity *in vivo*.

1.5.2 Th17 cells

Th17 cells represent a much more recently identified lineage and have the ability to produce cytokines such as IL-17A, IL-17F, IL-21 and IL-22 [reviewed in (Kolls and Khader, 2010)]. Th17 differentiation is associated with the induction of the transcription factor retinoic acid-related orphan receptor γT (RORγT) and, more recently, with the induction of the retinoic acid-related orphan receptor αT (RORαT) (Yang et al., 2008). Cytokines responsible for Th17 differentiation vary between mice and humans [reviewed in (Oboki et al., 2008)], but in mice IL-6 together with TGF-β are able to promote differentiation of naive $CD4^+$ T cells to Th17 cells. Th17 cells appear to be important for defence against mucosal pathogens, particularly fungi (Kolls and Khader, 2010). Th17 cells have also been implicated in a number of autoimmune diseases such as multiple sclerosis (Durelli et al., 2009) and arthritis (Singh et al., 2010). Increasingly, Th17 cells are being found to exhibit considerable plasticity in terms of their potential to develop different phenotypes, e.g. by developing the ability to produce IFN-γ in response to different cytokine stimuli (Lee et al., 2009).

1.5.3 Th9 cells

Th9 cells have been proposed as an IL-9 producing T cell subset in their own right, and can develop from naïve $CD4^+$ T cells in the presence of TGF-β and IL-4 (Dardalhon et al., 2008), or from Th2 cells in the presence of TGF-β (Veldhoen et al., 2008). Recently a transcription factor PU.1 has been identified as being important for Th9 differentiation (Chang et al., 2010), however a true "master regulator" transcription factor is yet to be defined, meaning that Th9 cells do not as yet represent a fully accepted T cell lineage. The potential role of Th9 cells in allergy is discussed in section 1.7.10.6.

1.5.4 T regulatory cells

1.5.4.1 $FoxP3^+$ T regulatory cells

A variety of subsets of T cells with regulatory properties have been described, these are vital for the inhibition of excessive inflammatory responses [reviewed in (Sakaguchi et al., 2008)]. Natural T regulatory cells (nTregs) develop in the thymus, constitute around 5-10% of the peripheral $CD4^+$ T cell population, possess the transcription factor forkhead box P3 (FoxP3) and express CD25 [(Hori et al., 2003) and reviewed in (Sakaguchi et al., 2008, Hawrylowicz and O'Garra, 2005)]. In humans, mutations in the FOXP3 gene result in the immune dsyregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX), where multiple autoimmune and allergic conditions develop (Wildin et al., 2001). A similar phenotype is seen in scurfy mice, which have mutations in FoxP3 (Bennett et al., 2001). $FoxP3^+$ T regulatory cells can also be induced from naïve $CD4^+$ T cells, and are termed inducible T regulatory cells (iTreg), [reviewed in (Sakaguchi et al., 2010)]. These can be generated *in vitro* by culturing naïve $CD4^+$ $CD25^-$ T cells in the presence of IL-2 and TGF-β (Davidson et al., 2007), although the requirements for their generation *in vivo* may vary [(Coombes et al., 2007) and discussed in (Sakaguchi et al., 2010)]. Both nTregs and iTregs are capable of regulating immune responses as demonstrated by their ability to suppress ongoing inflammatory

responses or prevent the development of autoimmune disease upon adoptive transfer in some animal models (DiPaolo et al., 2005, Aricha et al., 2008).

1.5.4.2 FoxP3$^-$ T regulatory cells

FoxP3$^-$ T regulatory cells also exist. Th3 cells, which produce TGF-β and exhibit regulatory properties, appear to be particularly relevant to the regulation of immune responses in the gut [reviewed in (Weiner, 2001)]. IL-10 is known to have potent immunoregulatory activities as illustrated in animal studies where IL-10 deficiency or blockade leads to immunopathology (Berg et al., 1996, Asseman et al., 2003). Tr1 cells have been defined by some as CD4$^+$ T cells with regulatory properties that produce IL-10, yet also require IL-10 for their differentiation [reviewed in (Grazia Roncarolo et al., 2006)], as depicted in Figure 1.2. Tr1 cells encompassed by this definition consist of a heterogeneous population for, although they may only produce IL-10, they may also produce other cytokines such as IFN-γ and IL-5 in different situations [(Barrat et al., 2002, Buer et al., 1998, Groux et al., 1997) and reviewed in (Grazia Roncarolo et al., 2006, Hawrylowicz and O'Garra, 2005)]. A variety of protocols can lead to the generation of Tr1 cells *in vitro,* such as culturing naïve CD4$^+$ T cells together with antigen and IL-10 (Groux et al., 1997), or with vitamin D3 and the glucocorticoid dexamethasone (Barrat et al., 2002), or by pre-treating of DC (e.g. with TGF-β) prior to culture with naïve CD4$^+$ T cells (Sato et al., 2003). However, others would argue that Tr1 cells have little relevance *in vivo* and that emphasis should put on the production of IL-10 by effector cells, such as Th1 cells and other subsets, which can be promoted in settings of chronic or strong antigenic stimulation, thus modifying the effector response (Jankovic et al., 2007, Gabrysova et al., 2009). Overall, the induction of IL-10 secreting T cells is highly therapeutically desirable [as discussed in (Sabatos-Peyton et al., 2010)], and will be further discussed in later sections.

In summary, a variety of T cell responses can be elicited through the combined effects of Signals 0-3; these responses can be inflammatory or regulatory in nature.

1.6 Peripheral Tolerance

1.6.1 Requirement for peripheral tolerance

Despite the robust mechanisms that constitute central tolerance, T cells capable of reacting to self-antigens still exist within the periphery (Liu et al., 1995). Furthermore, T cells in the periphery will have the capacity to recognise harmless antigens (Jenkins et al., 2010). It is paramount that mechanisms exist to maintain peripheral T cell tolerance in order to guard against autoimmunity and inflammatory responses against harmless antigens [reviewed in (Ryan et al., 2007)]. Peripheral tolerance holds particular importance for the lung, which is continually exposed to environmental antigens and where continually mounting an inflammatory response would be extremely detrimental to the host [discussed in (Lloyd and Hawrylowicz, 2009)]. So how is peripheral tolerance effected?

1.6.2 Mechanisms of tolerance induction

Several mechanisms have been demonstrated to be integral in maintaining peripheral tolerance [reviewed in (Ryan et al., 2007)]. The way in which DCs present antigen to T cells is key in orchestrating whether a tolerogenic or inflammatory response is elicited (Hochweller and Anderton, 2005). Hence a DC in a steady state, e.g. one which has not become activated by the detection of PAMPs, will not undergo upregulation of costimulatory molecules such as CD80 and CD86 (Hochweller and Anderton, 2005). Insufficient costimulation of T cells by DCs promotes a tolerogenic T cell response, rather than an inflammatory T cell response (Hochweller and Anderton, 2005, Sotomayor et al., 1999). Tolerogenic responses may occur in a variety of ways. 3 pillars of peripheral tolerance have been proposed, encompassing deletion, adaptation/anergy and regulation.

1.6.2.1 Deletion

Deletion represents a crucial mechanism whereby central tolerance is effected, but also remains an important way in which T cell tolerance is maintained in the periphery [reviewed in (Mueller, 2010, Ryan et al., 2007)]. For example, abnormalities in apoptotic pathways can lead to loss of peripheral tolerance and autoimmunity (Hutcheson et al., 2008). Deletion of antigen-reactive T cells is also implicated in some models of therapeutic tolerance induction. For example, administration of high doses of soluble peptide has been found to lead to deletion of peptide-reactive $CD4^+$ T cells (Hochweller and Anderton, 2005, Kearney et al., 1994). Analysis of the kinetics of such tolerogenic responses have shown that high dose, soluble peptide induces a T cell proliferative phase followed by a deletional phase (Hochweller and Anderton, 2005, Kearney et al., 1994). OX40-OX40L interactions have been shown to be important in such situations (Bansal-Pakala et al., 2001). OX40 is expressed following T cell activation. DCs are induced to express OX40L when they become activated, for example in response to PRR stimulation, and/or in response to CD40 ligation [reviewed in (Croft et al., 2009)]. OX40L-OX40 interactions have been found to increase T cell expression of anti-apoptotic proteins such as Bcl-2 and Bcl-xL (Rogers et al., 2001). Using agonistic antibodies against OX40 has been shown to prevent T cell tolerance induced by high dose peptide (Bansal-Pakala et al., 2001). The timing of OX40L expression on DCs together with the kinetics of T cell responses to high-dose soluble peptide, are thus consistent with T cells entering into a proliferative phase, but later dying on account of deficient OX40-OX40L mediated survival signals (Bansal-Pakala et al., 2001, Hochweller and Anderton, 2005).

1.6.2.2 Adaptation/anergy

The term anergy is very broad and can be taken to mean a state of unresponsiveness of a T cell in response to antigenic stimulation [reviewed in (Mueller, 2010, Schwartz, 2003)]. Different forms of anergy have been described. Clonal anergy tends to refer to an anergic state that develops *in vitro* in effector T cells (or T cell

lines) that receive insufficient costimulation [(Jenkins et al., 1987, Mueller et al., 1991) and discussed in (Schwartz, 2003)]. This results in (reversible) inhibition of proliferation and IL-2 production, but usually does not prevent effector cytokine production *in vitro* (Schwartz, 2003, DeSilva et al., 1991, Mueller et al., 1991, Jenkins et al., 1987). However, the relevance of clonal anergy in maintaining peripheral tolerance *in vivo* is uncertain [discussed in (Schwartz, 2003, Mueller, 2010)].

Another form of anergy known as adaptive tolerance has been proposed to be most applicable *in vivo* [discussed in (Schwartz, 2003)]. Adaptive tolerance is thought to be particularly relevant to naïve T cells and usually results in widespread inhibition of proliferation and cytokine production (Schwartz, 2003, Pape et al., 1998). Evidence suggests that such effects can be mediated by a block in the translation of mRNA that encodes effector cytokines (Villarino et al., 2011), and that persistence of antigen is required for the maintenance of adaptive tolerance (Rocha et al., 1993). The reversible nature of adaptive tolerance has implications for therapies designed to induce tolerance, since T cells undergoing adaptive tolerance are likely to retain the potential to become pathogenic in the future (Villarino et al., 2011, Rocha et al., 1993, Schwartz, 2003).

1.6.2.3 Regulation

The vital role that Tregs play in maintaining peripheral tolerance is evidenced by the autoimmune and allergic phenotypes displayed by patients with IPEX syndrome (described in section 1.5.4.1), and by multiple studies demonstrating that targeted deletion of Tregs leads to autoimmune manifestations [reviewed in (Sakaguchi et al., 2008)]. Regulatory cell subsets such as iTregs and Tr1 cells, have also been shown to be important in the induction of peripheral tolerance (Thompson et al., 2011, Meiler et al., 2008), and will be discussed later in the context of immunotherapy.

Mechanisms such as bystander suppression and linked suppression are particularly applicable to the regulation pillar of tolerance, and have been demonstrated after the therapeutic induction of tolerance [discussed in (Larche and Wraith, 2005, Wraith, 2009, Hochweller et al., 2006b)]. Bystander suppression refers to a situation where tolerance induced towards an epitope within one molecule (for example a protein), also results in tolerance towards epitope(s) within another protein. For example, Anderton et al found that inducing tolerance using a peptide contained within proteolipid protein (a constituent of myelin), followed by immunisation with whole myelin, induced tolerance to proteolipid protein and also to peptides derived from other myelin proteins (Anderton and Wraith, 1998). Bystander suppression has also been demonstrated in other studies (Backstrom and Dahlgren, 2004). Linked suppression refers to the scenario when tolerance induced towards one epitope within a protein leads to tolerance towards other epitopes within the same protein, and is thought to require both epitopes to be presented by the same APC [discussed in (Cobbold et al., 2010, Hochweller et al., 2006b)]. Linked suppression has been demonstrated in animal models of therapeutic tolerance induction (Bauer et al., 1997, Anderton and Wraith, 1998, Briner et al., 1993), and also recently in humans (Campbell et al., 2009).

Another mechanism related to regulation is that of infectious tolerance where $CD4^+$ T cells are induced to become Tregs through the effects of other Tregs [reviewed in (Cobbold et al., 2010)]. Shevach et al have recently proposed that this can occur by nTregs inducing FoxP3 expression in $FoxP3^-$ T cells via the actions of surface-bound TGF-β (Shevach et al., 2008).

In summary, peripheral tolerance harnesses mechanisms that comprise deletion, adaptation and regulation. Mechansims such as linked suppression hold particular interest for the field of therapeutic tolerance induction, and will therefore be further discussed in this thesis.

1.7 Allergic asthma

1.7.1 Clinical aspects

Asthma is a chronic inflammatory disease of the airways characterised by reversible airways obstruction and airway hyperresponsiveness (AHR), [reviewed in (Holgate, 2008)]. In allergic asthma, bronchoconstriction occurs in response to aeroallergens, leading to symptoms such as wheeze, cough and shortness of breath (Haslett et al., 2002). Allergic asthma is also associated with increases in the size and frequency of goblet cells within the airways; these produce mucous, further compromising airway patency (Holgate, 2008). Chronic inflammation leads to airway remodelling over time, which can lead to a non-reversible component of the airways obstruction (Holgate, 2008, Haslett et al., 2002).

1.7.2 Epidemiology

Asthma is a heterogeneous disease, however the majority of asthma is thought to be allergic in nature [discussed in (Kim et al., 2010)]. Asthma is a particularly apparent problem in the paediatric population since around 10-20% of children have a diagnosis of asthma, although this figure can vary between studies, often on account of variations in study design (Anderson et al., 2007, Punekar and Sheikh, 2009). Allergic asthma, together with other allergic diseases such as allergic rhinitis and allergic eczema, are a significant burden on the healthcare system (Anandan et al., 2009, Hoskins et al., 2000). Furthermore, the emergence of one allergic condition is often followed by additional allergic conditions (Punekar and Sheikh, 2009). Allergic eczema is the most common allergic condition to be initially diagnosed and, in many children, is later followed by the diagnosis of allergic asthma and/or allergic rhinitis (Punekar and Sheikh, 2009). Recently, Cadot et al have demonstrated that sensitisation to one aeroallergen can promote sensitisation to others (Cadot et al., 2010).

1.7.3 The atopic march

The observations described above have given rise to the concept of the "atopic march" whereby allergic sensitisation via the skin leads to allergic eczema, which in turn can progress to allergic rhinitis and/or asthma [reviewed in (Zheng et al., 2011)]. This is supported by studies demonstrating that mutations in the gene encoding filagrin, a protein important for the barrier function of the skin, are not only associated with the risk of atopic dermatitis (Palmer et al., 2006), but are also associated with the combination of asthma and atopic dermatitis (Rodríguez et al., 2009). Recently, an association between filagrin deficiency, atopic dermatitis and peanut allergy has also been demonstrated (Brown et al., 2011).

1.7.4 The hygiene hypothesis

In 1989 Strachan hypothesised that the increased prevalence of allergic disease was as a result of a reduction in exposure to pathogens in childhood, as a consequence of the improved hygiene standards associated with modern living (Strachan, 1989). Strachan found that children from large families (particularly children born further down the birth order), were less likely to have allergic rhinitis or allergic eczema (Strachan, 1989). Subsequent studies have often supported this initial hypothesis. For example, children who live on a farm or attend day-care early in life, both of which are associated with an increased exposure to potential pathogens, have been shown to have a reduced risk of developing allergic disease (von Mutius and Vercelli, 2010, Nicolaou et al., 2008). Ever since, the "hygiene hypothesis" has continually been revisited, adapted and modulated. Nowadays, the hygiene hypothesis is no longer taken to be simply an imbalance between infection-induced Th1 responses and allergy-promoting Th2 responses, since this would not account for recent increases in Th1-mediated diseases such diabetes mellitus, nor their reported association with allergic disease (Simpson et al., 2002). Such an interpretation of the hygiene hypothesis would also not support the protective role of Th2-inducing helminth infections against allergic disease (Yazdanbakhsh et al., 2002). More recently, there has been more emphasis on a dsyregulation facet to the hygiene hypothesis. Hence the influence of factors such as exposure to pathogens and microbial products in

early life (and even *in utero*), is now thought to be important in the development of regulatory responses, which together with Th1 responses, may act to counteract allergic responses [discussed in (Warner, 2004)].

1.7.5 What constitutes an allergen?

Many hundreds of allergens have been identified (which can be derived from sources such as house dust mite (HDM) faeces, pollen, animal dander, foods and insect-venom) and, while no general consensus has been reached regards what constituents an allergen, certain features are often present [reviewed in (Traidl-Hoffmann et al., 2009)]. Most allergens are proteins but, although structural similarities have been described for some allergens, the allergenicity of a protein cannot yet be predicted from structure alone (Traidl-Hoffmann et al., 2009). Particular biological activities of some allergens seem important for their allergenic potential. Some of the most well-characterised (and common) allergens are derived from HDM faeces. HDM allergen contains proteins such as Der p1 which has protease activity and has been shown capable of inducing epithelial tight junction breakdown (Wan et al., 1999). This may lead to increased allergen uptake and presentation by APC beneath the epithelium and facilitate allergen sensitisation. Other allergens exhibit other biological characteristics that may aid their allergenicity. For example some allergens have lipid-binding properties. These can facilitate TLR activation and may activate innate immune cells (Karp, 2010). Indeed, pollen-associated lipid mediators (PALMS) have been shown to be capable of directly activating eosinophils, and the actions of PALMS on DCs can favour the induction of Th2 rather than Th1 responses (Gilles et al., 2009).

Overall, allergens often possess properties that aid their presentation to the immune system and facilitate the development of Th2 responses. Despite possessing these properties, most potential allergens remain innocuous for the vast majority of people. So why do some people become allergic and others do not?

1.7.6 Genetic factors

It is generally accepted that a complex interplay of genetic and environmental factors are implicated in the susceptibility to, and pathogenesis of, allergic asthma [reviewed in (Holloway et al., 2010)]. Although some HLA-associations do exist for asthma and for other allergic diseases, these are not as strong nor as consistent as, for example, is the case for some autoimmune diseases [as discussed in (Shiina et al., 2004)]. The heterogeneous nature of asthma can also make it difficult to accurately assess genetic associations, since genetic studies have often investigated asthma as a whole, rather than studying asthma in respect to different phenotypic categories. That said, the majority of twin studies support a strong genetic component to asthma [discussed in (Koppelman et al., 1999)]. It is likely that a multitude of genetic factors are associated with asthma and that reciprocal interactions between these genetic factors and the environment are key (Holloway et al., 2010). For example, as discussed in section 1.7.3, mutations in the gene encoding filagrin, a protein important for epithelial integrity, have been associated with asthma (Rodríguez et al., 2009). Genes implicated in the way in which the innate immune system responds to its environment, such as in TLRs, have also been associated with asthma (Tesse et al., 2011). As would be expected, genome-wide association studies (GWAS) studies also highlight a multitude of genes associated with Th2 immune responses (Holloway et al., 2010). Genes expressed by cells such as smooth muscle cells and fibroblasts, such as the disintegrin and metalloproteinase domain-containing protein 33 (ADAM 33), have also been associated with asthma (Van Eerdewegh et al., 2002). Mutations in such genes may be implicated in the airways remodelling seen in some asthmatic patients (Jie et al., 2009).

It is thus likely that a combination of genetic factors and how these modify the body's response to its environment, together with variations in environmental factors themselves, contribute to the pathogenesis of allergic asthma.

1.7.7 Early life influences

There is evidence that *in utero* influences and early life events may alter the risk of a child developing allergy, including allergic asthma [discussed in (Warner, 2004)]. Initial priming to a vast number of environmental proteins appears to occur *in utero* (Prescott et al., 1998). Exposure of the fetus to environmental proteins and potential allergens has been proposed to occur via the placenta and the amniotic fluid (Warner, 2004).

Evidence suggests that, at birth, an infant's immune response is skewed towards Th2 responses (Prescott et al., 1998), and that the extent of these Th2 responses, and the degree to which they are modulated by Th1 and regulatory components of the immune response, may be shaped early in life (Warner, 2004). For example, mononuclear cells from cord blood of infants whose mothers were exposed to a farming environment, have reduced Th2 responses *in vitro* and increased regulatory T cells, when compared to control infants (Schaub et al., 2009).

The potential for a pregnant mother's diet to influence a child's risk of allergic disease is also evident. Pregnant mothers have previously been advised to avoid highly allergenic foods such as peanuts, with the aim of reducing the risk of their child developing peanut allergy (McLean and Sheikh, 2010). However, recent evidence suggests that the opposite may in fact be true and has led to changes in advice for pregnant mothers (McLean and Sheikh, 2010). Either way, there is increasing interest in the effects of maternal nutrition upon a child's risk of developing allergic disease. Influences such as maternal diet and other environmental factors have been proposed to lead to epigenetic changes in the fetus (Kabesch et al., 2010). These are phenotypic changes that are not due to changes in DNA sequence (Kabesch et al., 2010). In this respect, alterations in DNA methylation have been found to be associated with allergic asthma in some animal models (Hollingsworth et al., 2008), and epigenetics in relation to allergy is likely to be an important area for future study (Kabesch et al., 2010).

1.7.8 Airway epithelium

Abnormalities in, and responses of, airway epithelium are increasingly being attributed in the pathogenesis of allergic asthma [discussed in (Holgate et al., 2010)]. Epithelial damage has been found in bronchial biopsies from asthmatic children (Barbato et al., 2006). Such a reduction in epithelial integrity has been proposed to lead to increased allergen uptake (Holgate et al., 2010). Furthermore, primary epithelial cells derived from asthmatic patients appear to more readily undergo epithelial-mesenchymal transition than primary epithelial cells derived from non-asthmatic patients (Hackett et al., 2009). Such cells were found to lose markers associated with epithelial cells and gain expression of molecules associated with mesenchymal cells (Hackett et al., 2009). This has been hypothesised to play a role in asthma-associated airway remodelling (Hackett et al., 2009, Holgate et al., 2010).

The way in which epithelial cells respond to their environment is also likely to be implicated in the pathogenesis of allergic asthma. For example, thymic stromal lymphopoietin (TSLP) has been shown to be released by epithelial cells in response to TLR stimulation (Kato et al., 2007), and also in response to proteases present within allergens (Kouzaki et al., 2009). TSLP is known to promote Th2 responses through its actions on DCs (Ito et al., 2005). Such data point to an important role for airway epithelial cells in bridging the innate and adaptive immune responses.

1.7.9 Experimental models of allergic airways inflammation

There continues to be debate as to how best to model allergic asthma *in vivo*. The majority of models of allergic airways inflammation (AAI) utilise the mouse, which does not naturally develop asthma [these models are reviewed in (Finkelman and Wills-Karp, 2008)]. There are key differences too in the anatomy of the mouse lung compared to the human lung. These include major differences in the gross anatomy such as the number of lung lobes and airway branches (Finkelman and Wills-Karp, 2008). Importantly, some therapeutic approaches such as the blocking of Th2 cytokines which have shown therapeutic effects in mice, have not shown widespread

efficacy when translated to asthmatic patients [discussed in (Holgate, 2010)]. It is therefore important to remember the limitations of mouse models of AAI. That said, mouse models of AAI do represent an important tool for the study of allergic asthma and for the initial development of therapeutic approaches.

Acute models of AAI in mice often involve a sensitisation phase to a model allergen, followed by an airway inhalation phase [reviewed in (Nials and Uddin, 2008)]. Many allergens have been used, including antigens from HDM- and animal dander- derived allergens (Nials and Uddin, 2008). Chicken ovalbumin (OVA), is also often used as a model allergen mainly because of the availability of tools such as OVA-reactive transgenic mice (Barnden et al., 1998), which can aid mechanistic studies. Protocols for the induction of acute AAI vary widely in terms of exact time points, doses and routes of allergen exposure (Nials and Uddin, 2008). AAI can also be induced following adoptive transfer of allergen-reactive Th2 cells (as well as other $CD4^+$ T cell subsets), and subsequent allergen challenge via the airways (McKinley et al., 2008). Models also exist whereby allergen-pulsed DCs can induce AAI (van Rijt et al., 2005). Such approaches can induce disease with important similarities to human asthma such as eosinophil infiltration into the lungs, the development of allergen-specific IgE, the presence of goblet cells in the airways and AHR (Finkelman and Wills-Karp, 2008).

Many AAI protocols utilise BALB/c rather than C57BL/6 mice (Janssen et al., 1999, Takeda et al., 2001). This is because of the natural tendency of the BALB/c response to be towards a Th2 phenotype compared to the Th1 phenotype associated with C57BL/6 mice (Heinzel et al., 1989). As a result of this, AAI protocols involving BALB/c mice tend to require lower antigen doses than required for C57BL/6 mice (Janssen et al., 1999, de Vries et al., 2009). That said, C57BL/6 can be induced to develop all the features of AAI (although notably lung function changes are more difficult to detect) (Morokata et al., 1999, Takeda et al., 2001). Furthermore, the wealth of transgenic mice on the C57BL/6 background means that AAI models developed in C57BL/6 mice are likely to have an advantage in terms of rapid

mechanistic assessment. Thus when using AAI models decisions need to be made as to whether to use BALB/c mice (in which allergic response is easier to induce, lung function changes are usually measurable but future work requiring transgenics will be delayed), or C57BL/6 mice (where allergy is still inducible, transgenic tools can be readily employed, but with the caveat that lung function changes may be more difficult to detect). After weighing up the options the C57BL/6 background was chosen for this project.

At present, AAI models are usually acute in nature and are likely to resolve over time (Leech et al., 2007a). There are continued efforts to develop and modify acute models of AAI which could offer new ways in which to model human asthma. Chronic AAI models are also being developed. These are particularly interesting with respect to their ability to induce airway remodelling, often a feature in chronic asthma (McMillan and Lloyd, 2004). There are also continued calls for the development of more epithelial-focused AAI models, in view of the apparent contribution of epithelial responses to the development of allergic responses in the airways (Holgate, 2010).

1.7.9.1 The role of alum

The alum preparation used to induce allergic sensitisation in many animal models (and used in this project), consists of a preparation of aluminium hydroxide and magnesium hydroxide [further discussed in (Flach et al., 2011)]. Similar preparations are also often used as adjuvants in vaccines for humans (Flach et al., 2011). Originally, alum was thought to primarily exert its adjuvant properties by acting as a depot, enabling antigen to remain accessible for long periods of time, but this explanation is no longer widely accepted (Marrack et al., 2009). A recent study by Flach et al provides insight into how alum may exert its effects. They demonstrated that alum crystals strongly bind to lipid molecules in the cell membrane of DCs, but are not phagocytosed (Flach et al., 2011). These finding led to the hypothesis that soluble antigen is released from alum crystals and is then endocytosed into the DC,

by an undefined, non-phagocytic process (Flach et al., 2011). In response to alum crystal binding, DCs were shown to upregulate intercellular adhesion molecule 1 (ICAM-1) and co-stimulatory molecules such as CD86. ICAM-1 facilitated strong binding of DCs to $CD4^+$ T cells via the lymphocyte function-associated antigen 1 integrin [LFA-1, (Flach et al., 2011)]. Alum has previously been shown to increase the recruitment of other immune cells such as neutrophils, macrophages and eosinophils to the site of injection (Kool et al., 2008), yet Flach et al only found alum to be strongly bound to DCs and not to other cell types (Flach et al., 2011). Furthermore, exposing DCs *in vitro* to alum, then transferring them to recipient mice together with OVA, was sufficient to induce OVA-specific antibody responses (Flach et al., 2011). These data argue against an obligatory role for uric acid in the adjuvant properties of alum, as had been previously described (Kool et al., 2008), and strongly support a role for DCs in immune responses induced using alum adjuvants.

1.7.10 Cells implicated in allergic responses in the lung

1.7.10.1 Dendritic Cells

DCs play a vital role in orchestrating $CD4^+$ T cell responses in models of AAI [reviewed in (Lambrecht and Hammad, 2009)]. DCs are known to line the airways and are often found positioned beneath the epithelium where they are able to extend their processes between epithelial cells to facilitate sampling of antigens within the airway lumen (Lambrecht and Hammad, 2010). Conventional DCs highly express the molecule CD11c (Lambrecht and Hammad, 2009). In mouse models of AAI, depletion of CD11c positive cells using a CD11c-diptheria toxin receptor (DTR) system has been found to inhibit Th2 cells responses during allergen challenge, thereby reducing AAI (van Rijt et al., 2005). Furthermore, some of the therapeutic effects of inhaled corticosteroids are thought to be related to a reduction in DCs in the lung (Moller et al., 1996).

The work of Lambrecht et al, has greatly aided understanding of DC biology in the lung, particularly in relation to mice (Lambrecht and Hammad, 2009). Variations in the phenotype of DCs present in the non-inflamed versus the inflamed lung have been identified (Lambrecht and Hammad, 2009). The majority of DCs resident in the non-inflamed lung are thought to be $CD11c^{hi}$ $CD11b^{-}$ MHC II^{hi} (Sung et al., 2006, Lambrecht and Hammad, 2009). During pulmonary inflammation, there is an influx of so-called "inflammatory DCs" into the lung, thought to mainly be derived from circulating blood monocytes (Hammad et al., 2009, Lambrecht and Hammad, 2009). But, not all DCs promote inflammation. Plasmacytoid DCs (pDCs), (typically $CD11c^{+}$, $Cd11b^{-}$ PDCA-1^{+}), have been implicated in the induction of tolerance towards inhaled proteins (de Heer et al., 2004).

Factors produced by epithelial cells in response to the airway environment can influence DCs, and consequently affect the nature of the resultant T cell response. In mice, stimulation of TLR4 on epithelial cells has been shown to increase the production of cytokines such as IL-33 (Hammad et al., 2009). IL-33 can act on DCs via the receptor ST2 (Rank et al., 2009) and IL-33 stimulated bone-marrow derived DCs have been found to induce Th2 cytokines from naïve $CD4^{+}$ T cells (Rank et al., 2009). TLR4 stimulation of epithelial cells can also induce TSLP (Hammad et al., 2009).

DCs are thus instrumental in the development of Th2 responses to allergen in the airways, representing a vital link between the innate and adaptive immune responses. Increasing evidence indicates that epithelial-DC interactions can alter the nature of the developing T cell response.

1.7.10.2 Basophils

Basophils have previously been proposed to play a key role as APCs during the initiation of Th2 $CD4^{+}$ responses (Perrigoue et al., 2009). However, more recent studies have reaffirmed the importance of DCs as the predominant APC in models of

allergic disease and helminth infection (Hammad et al., 2010, Phythian-Adams et al., 2010). In a model of HDM-induced AAI, Hammad et al found that, although basophils were recruited to mediastinal lymph nodes (mLN) following HDM inhalation, specific depletion of basophils caused only a limited reduction in the number of eosinophils in BAL, and did not reduce Th2 responses of mLN cells *in vitro* (Hammad et al., 2010). Furthermore, antigen presentation by basophils, in contrast to DCs, did not induce proliferation of naïve $CD4^+$ T cells in that study (Hammad et al., 2010). The FCεR1 antibody used previously in other studies to deplete basophils, was found by Hammad et al to also result in depletion of FCεR1 expressing DCs (Hammad et al., 2010). This led them to hypothesise that DC depletion, and not basophil depletion, was responsible for the reduction in Th2 responses seen by others (Perrigoue et al., 2009). In support of this hypothesis, Phythian-Adams et al have found that depleting DCs by targeted depletion of $CD11c^+$ cells, severely impaired the Th2-mediated protective response to a helminth infection, whereas depletion of basophils had no significant impact (Phythian-Adams et al., 2010). Taken together these findings suggest that while in certain circumstances basophils may be involved in augmenting Th2 responses, the predominate APC implicated in inducing Th2 responses remains the DC.

1.7.10.3 T cell responses in allergic asthma

T cell responses are strongly implicated in the development of allergic sensitisation to allergens and in the pathogenesis of allergic asthma [(Corrigan et al., 1988, Robinson et al., 1993b, Walker et al., 1991) and reviewed in (Larché et al., 2003)]. Roles for many of the $CD4^+$ T cell subsets in the pathogenesis of different asthma phenotypes have previously been proposed [discussed in (Lloyd and Hessel, 2010)], however Th2 cells are the subset most strongly associated with the development of allergy and allergic asthma (Larché et al., 2003). The focus of this section will therefore primarily be on Th2 cells; however roles for other T cells subsets such as Th17 and Th9 cells in allergic asthma will also be discussed.

1.7.10.4 Th2 cells

In general, studies of allergic and allergic asthmatic patients have found that Th2 responses play a vital role in the pathogenesis of allergy and allergic asthma [(Robinson et al., 1993a, Del Prete et al., 1993) and reviewed in (Larché et al., 2003)]. Stimulation of peripheral blood mononuclear cells (PBMCs) from allergic patients usually elicits a strongly Th2 polarised response, compared to that elicited from PBMCs from non-allergic patients (Movérare et al., 2000). Th2 cytokines also tend to predominate in the lung in allergic asthmatics. For example, Pilette et al found high levels of Th2 cytokines in bronchoalveolar lavage (BAL) from patients following allergen exposure, and increased levels of the chemokines CCL22 and CCL17 (known to be ligands for CCR4, expressed on Th2 cells), compared to non-allergic controls (Pilette et al., 2004). Levels of these chemokines correlated both with the number of lymphocytes and levels of IL-5 and IL-13 in BAL (Pilette et al., 2004).

Animal models of AAI have consistently demonstrated that a Th2 response towards allergens generates features that often correspond with those seen in allergic asthma. These include the development of allergen-specific IgE, eosinophil infiltration into the lungs, goblet cell metaplasia and AHR, which can be all induced via allergen-induced Th2 responses [reviewed in (Nials and Uddin, 2008)]. Furthermore, Th2 polarised allergen-specific T cells can induce AAI upon adoptive transfer and subsequent allergen airway challenge of naïve recipients (Hansen et al., 1999). If Th1 polarised cells are transferred, although features of pulmonary inflammation ensue, these are not consistent with the features of AAI (Hansen et al., 1999).

The signature Th2 cytokines IL-4, IL-5 and IL-13, combine to promote AAI. Studies using mice deficient in specific Th2 cytokines, or using blocking antibodies, have aided understanding of the contribution of different Th2 cytokines to AAI [reviewed in (Larché et al., 2003)]. IL-13 is predominantly associated with goblet cell development and mucous production and AHR (Yang et al., 2001). IL-5 is mainly associated with eosinophilic responses (Hamelmann et al., 2000) and IL-4 probably

exerts a variety of effects including facilitation of class-switching to produce IgE and effects on mast cells [discussed in (Larché et al., 2003)]. Targeted depletion of GATA-3 in T cells has been found to significantly inhibit the development of AAI (Zhang et al., 1999). In some mouse models, Th1 cytokines such as IL-12 delivered into the lung, inhibit features of AAI, such as AHR and pulmonary eosinophilia (Schwarze et al., 1998). Furthermore, T-bet deficient mice have been found to spontaneously develop some features of AAI (Finotto et al., 2002, Finotto et al., 2005). These studies imply that the antagonist effects of Th1 responses can be important for the inhibition of allergic Th2 responses.

1.7.10.5 Th17 cells

The role of Th17 cells in the pathogenesis of allergic asthma remains unclear [reviewed in (Lloyd and Hessel, 2010)]. Th17 responses have been identified as being prominent in particular groups of asthmatics such as those with severe, often neutrophilic phenotypes. Al-Ramli et al found that the frequency of IL-17 expressing cells was significantly higher in airway biopsies from severe asthmatics compared to asthmatics with mild or moderate asthma (Al-Ramli et al., 2009). IL-17 mRNA has also been found to be increased in induced sputum from some asthmatics and correlates with the presence of neutrophils in sputum (Bullens et al., 2006). In animal models, adoptive transfer of allergen-reactive Th17 cells followed by allergen challenge can induce some features of AAI – for example goblet cells and pulmonary cellular infiltration (McKinley et al., 2008). However, a neutrophilic rather than eosinophilic picture usually develops (McKinley et al., 2008).

The consensus appears to be that Th17 cells may be implicated in some asthma phenotypes, particularly those that are severe and have a more neutrophilic, rather than eosinophilic, phenotype.

1.7.10.6 Th9 Cells

A role for Th9 cells in allergic asthma has also been postulated [reviewed in (Lloyd and Hessel, 2010)]. Some studies have found an increased frequency of IL-9 expressing cells in the allergic asthmatic lung (Shimbara et al., 2000). Temann et al found that selective over expression of IL-9 in the lungs of mice led spontaneously to features of AAI (Temann et al., 1998). However, more recently McMillan et al found that the severity of OVA-induced AAI was not significantly different between IL-9 deficient mice and wild type mice (McMillan et al., 2002). Therefore, although Th9 producing T cells may be involved in promoting the features of AAI, IL-9 does not appear to be required for AAI to develop.

1.7.10.7 T regulatory cells

Tregs are likely to be involved in protecting against the development of allergic asthma [reviewed in (Lloyd and Hawrylowicz, 2009)]. Treg production of cytokines such as IL-10 (such as that produced by Tr1 cells), has been postulated to suppress allergic responses to aeroallergens and be involved in the therapeutic development of tolerance [(Francis et al., 2003, Campbell et al., 2009) and reviewed in (Hawrylowicz and O'Garra, 2005, Larché, 2005)].

Several findings particularly highlight the importance of regulatory cells in maintaining health in the airways. Patients with IPEX syndrome (resulting in loss of nTreg frequency or function), often have allergic manifestations, which can include asthma (Wildin et al., 2001). In mice, depletion of Tregs (using depleting antibodies against CD25), has been found to increase the severity of AAI, upon exposure to HDM (Lewkowich et al., 2005). In that study, this appeared to be as a result of increased pulmonary DC activation that occurred following Treg depletion (Lewkowich et al., 2005). Adoptive transfer of (polyclonal) iTregs in a model of HDM-induced AAI, has also been shown to reduce disease (Chen et al., 2003), as has adoptive transfer of allergen-reactive $CD4^+$ $CD25^+$ Tregs after allergic sensitisation and prior to allergen challenge (Kearley et al., 2005). In the latter study,

inhibition of AAI was found to be dependent on IL-10, although interestingly the source of this IL-10 was not derived from the transferred T cell population (Kearley et al., 2005). FoxP3$^+$ Tregs have also been implicated in the natural resolution of AAI in mouse models (Leech et al., 2007a).

The induction of Tregs is thus an attractive therapeutic aim for the treatment of allergic asthma, and is further discussed below.

1.7.10.8 Eosinophils

Eosinophils are particularly important in the immune response to helminths [reviewed in (Shamri et al., 2010)] but are also strongly associated with allergy. Eosinophils are often found in BAL from asthmatics, and are usually present in mouse models of AAI (Schwarze et al., 1998, Robinson et al., 1993a, Tanizaki et al., 1993, Janssen et al., 1999). Eosinophils can become activated in response to a range of danger signals such as TLR stimulation, uric acid, and in response to allergen-associated molecules such as pollen-associated lipid mediators (PALMS) (Shamri et al., 2010, Kobayashi et al., 2010, Plotz et al., 2004). Eosinophils contain granules which can release a multitude of cytotoxic products, cytokines and growth factors which are capable of influencing the allergic inflammatory response [reviewed in (Shamri et al., 2010)]. Such a cocktail of eosinophil-derived products can damage epithelium and can influence airway remodelling [e.g. via the actions of growth factors such as vascular endothelial growth factor (VEGF) and cytokines such as TGF-β] (Bochner and Gleich, 2010). Cysteinyl leukotrienes released from eosinophils can induce bronchoconstriction and increase mucus production (Shamri et al., 2010, Sampson et al., 2000). In certain circumstances, eosinophils have also been shown to act as APCs and induce CD4$^+$ T cell responses (Wang et al., 2007).

IL-5 is known to be key for eosinophil maturation and recruitment from the bone marrow [discussed in (Bochner and Gleich, 2010)], and attempts have been made to utilise IL-5 blockade therapeutically. However, although murine AAI studies have

found therapeutic benefits of blocking IL-5 (Hamelmann et al., 1999a, Bochner and Gleich, 2010), initial clinical trials using a humanised IL-5 blocking antibody did not result in therapeutic effects in asthmatics, despite significantly reducing the frequency of eosinophils in blood (Leckie et al., 2000). More recently, this approach has been shown to reduce the frequency of severe asthma exacerbations in carefully selected patients with high levels of eosinophilia in BAL (Haldar et al., 2009).

1.7.10.9 Mast cells

Mast cells are implicated in allergic responses because of their ability to release and synthesise an abundance of inflammation-promoting mediators and cytokines such as histamine, proteases, leukotrienes and factors such as TSLP [(Okayama et al., 2009) and reviewed in (Metz and Maurer, 2007)]. Known to be important for innate responses to helminths, and in wound healing, mast cells often reside in the tissues and can be activated, for example through activation of TLRs or complement components [(Nigo et al., 2006) and reviewed in (Stone et al., 2010)]. Mast cells also express the high affinity IgE receptor FcεR1. When IgE binds to multiple sites on an allergen and then binds to FcεR1 receptors on a mast cell's surface, cross-linking of FcεR1 receptors induces mast cell degranulation (Stone et al., 2010, Segal et al., 1977). Mast cells have been found to be particularly increased in the lung parenchyma of patients with uncontrolled allergic asthma (Andersson et al., 2011). The effects of mast cell derived mediators are diverse but include; direct effects upon smooth muscle contractility (driving bronchoconstriction), increased vascular permeability, increased mucous production and effects on other cell types such as eosinophils (Metz and Maurer, 2007, Alkhouri et al., 2011, Vliagoftis et al., 2004).

1.7.11 Allergen-specific IgE

In health, IgE is found in low concentrations in serum [discussed in (Stone et al., 2010)]. Allergic individuals develop allergen-specific IgE, which can then bind to IgE receptors, such as FcεR1, present on mast cells. FcεR1 is also present on

basophils and DCs (Holgate et al., 2009) and binding of allergen-IgE complexes to DCs facilities allergen uptake and presentation (Maurer et al., 1998).

The presence of allergen-specific IgE indicates that an individual has become sensitised to that allergen, however this does necessarily translate to clinical manifestations of allergy (Celik-Bilgili et al., 2005). As a general rule, the higher the level of allergen-specific IgE in a patient's serum, the more likely it is that they will display clinical allergy to that allergen (Celik-Bilgili et al., 2005). Such associations led to the development of the humanised monoclonal IgE blocking antibody Omalizumab, which can bind to circulating IgE resulting in a reduction of IgE in serum (Holgate et al., 2009). This also reduces the expression of FcεR1 receptors on cells such as mast cells, reducing the likelihood of allergen-induced mast cell degranulation (Holgate et al., 2009). Omalizumab is not effective in all patients and is an expensive treatment modality (Holgate, 2010). This means that the use of Omalizumab is currently restricted to patients with severe asthma. This targeted approach has been found to be effective in improving asthma control in some patients (Rodrigo et al., 2011).

In summary, allergic asthma results from a complex interplay of immunological responses directed towards allergen(s). Evidence suggests that allergen-reactive Th2 cells, whose effector responses are important for the recruitment of eosinophils, the development of allergen-specific IgE and features such as goblet cell hyperplasia, play a key role in the pathogenesis of allergic asthma, as summarised in Figure 1.3.

Figure 1-3: Th2 cells can induce many of the features of allergic asthma through their responses to inhaled allergen

1.7.12 Current treatments for allergic asthma

Reducing a patient's exposure to an allergen is vital to improve the symptoms of allergic asthma (van den Bemt et al., 2004). However, in practice this can often be difficult as is the case for allergens such as HDM or pollen, which are found ubiquitously in the environment. The mainstay of pharmacological treatment for allergic asthma involves treating symptoms as they arise using β2-agonist inhalers, which induce bronchodilatation (Haslett et al., 2002). In addition, corticosteroids (either delivered locally via inhalation, or in more severe cases, given systemically) are used to non-specifically dampen allergic responses. Add-on treatments can include drugs such as leukotriene receptor antagonists and rarely, in severe steroid-resistant asthma, the IgE blocking antibody Omalizumab (Haslett et al., 2002). Adequate treatment of other manifestations of allergic disease such as allergic rhinitis, can also improve asthma (Fuhlbrigge and Adams, 2003). Current pharmacological treatments are both vital and efficacious for the management of allergic asthma, however none of these seem to modify disease progression (Guilbert et al., 2006).

The increasing awareness of allergy as a systemic disease and the potential for an "atopic march" means that treatments that could be given early in life, and that reduce disease progression, must remain a key goal. This is a primary aim of allergen-specific immunotherapy.

1.8 Allergen-specific immunotherapy

1.8.1 Specific immunotherapy (SIT) for allergy and allergic asthma

In health, tolerance exists towards aeroallergens. Specific immunotherapy (SIT) is built upon the concept of therapeutically inducing tolerance to an allergen. It has long been known that administering a protein such as an allergen in the absence of danger signals i.e. in the absence of adjuvant, can tolerise T cells reactive towards epitopes within that protein. The first clinical report of specific immunotherapy was

from Noon, who found that injecting hayfever patients subcutaneously with grass pollen could improve their symptoms (Noon, 1911).

Animal models have consistently demonstrated that administration of whole protein allergen in the absence of adjuvant can induce tolerance to that allergen and improve allergic sensitisation and AAI. Many routes of administration have proven effective including oral, subcutaneous, and intranasal approaches (Nakao et al., 1998, Ostroukhova et al., 2004, Janssen et al., 1999). Furthermore, whole protein SIT in animal models has been found to be effective if given prior to initial sensitisation (Keller et al., 2006), following sensitisation but prior to airway challenge (Vissers et al., 2004), and also after airway challenge and before re-challenge (Boudousquié et al., 2009). SIT is used on a relatively small scale in some allergy clinics in the UK and is currently predominantly used in patients with insect venom allergies, allergic asthma (usually mild or moderate forms), and severe allergic rhinitis [reviewed in (Calderón et al., 2011)]. Different regimes for SIT administration exist, however treatment most often involves subcutaneous administration of protein allergen with escalating doses given weekly and a maintenance dose (usually given around once every 6 weeks) given thereafter for up to 5 years [reviewed in (Frew, 2010)]. Individual clinical trials have reported clinical improvements with SIT in various allergic settings, but variations in patient criteria, disease phenotypes, SIT regimes and outcome measures, present a challenge to assess the overall efficacy of SIT in allergic disease [discussed in (Calderón et al., 2011)]. Recent Cochrane systematic reviews assessing outcomes from randomised-controlled SIT trials have provided valuable evidence regards the effectiveness of SIT in patients with respiratory allergy. These findings can be summarised as follows:-

i) SIT can improve allergic rhinitis (Calderon et al., 2007).

ii) A particular form of SIT known as sublingual immunotherapy (SLIT) is also efficacious in the treatment of allergic rhinitis (Radulovic et al., 2010). However, others have noted that SLIT appears to be less effective

than subcutaneously delivered SIT [further discussed in (Akdis and Akdis, 2009)].

iii) There is some evidence that SIT can be effective at improving allergic asthma (Abramson et al., 2010).

Evidence also suggests that if maintenance SIT treatments are continued for 3-4 years, improvements can be maintained in the longer-term (Durham et al., 2010).

1.8.1.1 Proposed mechanisms involved in SIT

An array of mechanisms have been proposed to be involved in successful induction of tolerance using SIT [reviewed in (Akdis and Akdis, 2009)]. Janssen et al examined the effects of SIT using OVA given subcutaneously or intranasally in a mouse model of OVA-induced AAI (Janssen et al., 2000a). Using trackable, OVA-reactive T cells, they found that subcutaneous SIT resulted in an initial rapid and widespread T cell response which led to tolerance (Janssen et al., 2000a). In contrast, intranasal SIT did not induce tolerance and was associated with a slower, more localised T cell response. This led them to hypothesise that a "strong, synchronised and systemic T cell response" promoted tolerance induction (Janssen et al., 2000a).

Other SIT studies in mice have suggested a role for the induction of $FoxP3^+$ Tregs, and associated TGF-β production, in SIT-mediated tolerance (Ostroukhova et al., 2004). Interestingly, a study by Boudousquié et al suggested an initial role for $FoxP3^+$ Tregs following SIT, but demonstrated that TGF-β expressing $CD4^+$ $CD25^-$ cells, which also developed later in that model, were able to transfer tolerance to recipient mice (Boudousquié et al., 2009).

The induction of IL-10 has also been implicated in tolerance induced by SIT in animal models. Vissers et al found that SIT using subcutaneous OVA after sensitisation reduced the severity of AAI (Vissers et al., 2004). Although the IL-10

concentration in BAL did not increase, a fall in BAL IL-5 levels occurred, resulting in an increased IL-10:IL-5 ratio. Blocking IL-10 in that study significantly inhibited the tolerogenic effects of SIT (Vissers et al., 2004). IL-10 has similarly been identified as being important for tolerogenic outcomes in several human SIT studies. IL-10 has been found to be important for the natural acquisition of tolerance to the bee venom allergen, phospholipase A_2 (PLA), in beekeepers (Meiler et al., 2008). In keeping with these findings, Akdis et al found that bee-venom SIT given to bee-venom allergic patients reduced the production of Th2 signature cytokines (and IFN-γ) in response to PLA, but increased IL-10 production (Akdis et al., 1998). In that study, the frequency of IL-10 positive $CD4^+CD25^+$ T cells was increased shortly after SIT (Akdis et al., 1998). IL-10 has also been attributed to tolerance induced following grass pollen SIT (Francis et al., 2003). In that study, although SIT did not alter the *in vitro* Th2 (or IFN-γ) recall responses of PBMCs to grass pollen, significantly more IL-10 was produced after grass pollen stimulation of PBMCs from SIT-treated patients, compared PBMCs from non-SIT controls (Francis et al., 2003).

The mechanisms of action of SIT therefore still remain unclear and may vary with different modes of application. However, the induction of IL-10 has often been attributed to tolerance induced by SIT.

1.8.1.2 Impact of SIT on multiple allergen sensitisations

One of the most attractive aspects of allergen-specific therapy is the potential for tolerance induction to modify the course of disease. This is perhaps most easily envisaged if the allergen-specific therapy induces regulatory mechanisms, since these may have the potential to regulate against additional allergic responses, e.g. via bystander or linked suppression. There is some evidence that SIT given to induce tolerance to one allergen, may reduce the likelihood of future sensitisation to additional allergens. Des Roches et al compared the development of new allergen sensitisations in children who were mono-sensitised to HDM, and who were either receiving SIT against HDM or not receiving SIT (Des Roches et al., 1997).

Interestingly, while no children in the SIT group developed sensitisation to additional allergens, 10 out of 22 children in the non-SIT group did, during the 3 year follow-up period (Des Roches et al., 1997). A more recent, larger study compared the percentage of mono-sensitised HDM allergic children who developed sensitisation to additional allergens over a total period of 6 years. For the first 3 years, children either received SIT against HDM or no SIT (Pajno et al., 2001). Only 33% of the non-SIT group did not develop sensitisation to additional allergens, compared to 75% of the SIT treated group (Pajno et al., 2001). There is also some evidence that SIT for allergic rhinitis can reduce the risk of a child going on to develop asthma, even up to 7 years after cessation of SIT (Jacobsen et al., 2007).

Viewed together, these findings therefore enable the tentative proposal shown in Figure 1.4, that early treatment of allergic children with allergen-specific therapy could reduce the progression of disease, and possibly even prevent allergic asthma from developing.

Figure 1-4: Proposed impact that early treatment with allergen-specific immunotherapy may have upon allergic disease progression

1.8.1.3 Is SIT safe?

SIT comes with risks. The administration of whole protein allergen to allergic patients incurs the risk of inducing severe allergic reactions, including anaphylaxis, by facilitating cross linking of IgE on mast cells and inducing degranulation [reviewed in (Peavy and Metcalfe, 2008)]. SIT is associated with the induction of allergic responses and, although rare, fatalities have been reported (Borchers et al., 2004). Caubet et al found that systemic allergic reactions (though not necessarily anaphylaxis) occurred after around 4% of SIT injections (Caubet and Eigenmann, 2008). In that study, systemic reactions were more likely to occur in asthmatics. A systematic review of SIT in asthmatics concluded that overall, the risk of a systemic allergic reaction occurring was around 1 for every 9 allergic asthmatic patients treated with SIT (Abramson et al., 2010).

Hence, although SIT can improve allergic disease, it is associated with the risk of severe allergic reactions, particularly in allergic asthmatics. This has meant that SIT is not currently recommended in the UK for the treatment of severe allergic asthmatics [discussed in (Calderón et al., 2011)]. Furthermore, for allergen-specific immunotherapy to have most impact upon the course of disease (as proposed in Figure 1.4), treatment may need to be targeted to young children. The risks associated with SIT may be too great for such an approach to be widely implemented. The development of safer approaches to allergen-specific immunotherapy is therefore highly desirable. Peptide immunotherapy (PIT) offers such an approach.

1.9 Peptide Immunotherapy (PIT)

Peptide immunotherapy (PIT) refers to the administration of peptides containing immunodominant T cell epitopes, with the aim of inducing tolerance towards the full antigen [reviewed in (Larche and Wraith, 2005)]. This approach has been shown in a range of animal models to be capable of inducing T cell tolerance [reviewed in

(Anderton, 2001, Larche and Wraith, 2005)]. The main advantage of PIT over whole protein SIT is that the peptides administrated are short in length meaning they do not facilitate cross linking of IgE on mast cells, thus are generally thought to reduce the risk of allergic reactions such as anaphylaxis. Furthermore if IgE is generated towards a peptide this is likely to recognise a different conformation than found on whole protein antigens [discussed in (Larché, 2007, Larche and Wraith, 2005)]. Hence, although Pedotti et al reported (IgG1-mediated) anaphylaxis in a model of experimental autoimmune encephalomyelitis (EAE), a mouse model of multiple sclerosis (Pedotti et al., 2001), this occurred in the context of disease induction using immunisation with peptide, rather than protein (Pedotti et al., 2001).

1.9.1 Evidence for therapeutic effects of PIT in animal models

1.9.1.1 Autoimmune disease

Initial studies of PIT were carried out in animal models of EAE. Inflammatory T cell responses directed towards components of myelin are important for the pathogenesis of EAE (Libbey et al., 2010). It has consistently been shown that administration of soluble myelin-derived peptides in the absence of adjuvant can reduce/abrogate EAE, when disease is induced via immunisation with the same peptide in complete Freund's adjuvant (CFA) (Liu and Wraith, 1995, Metzler and Wraith, 1993, Gaur et al., 1992). PIT has also been shown to be effective therapeutically in other models of autoimmune disease (Daniel and Wegmann, 1996, Bayrak and Mitchison, 1998). Similarly to SIT, multiple routes and regimes of peptide administration are capable of inducing tolerance [discussed in (Larche and Wraith, 2005)].

1.9.1.2 Using PIT for the treatment of allergic disease

In mice, PIT has been found to induce tolerance in some allergy-relevant models. Various models have been employed in which PIT can be give prophylactically (i.e. prior to sensitisation) (Janssen et al., 1999), after sensitisation and prior to challenge (Bauer et al., 1997), or after challenge and prior to rechallenge (Campbell et al.,

2009). Each approach has its own merits. A prophylactic approach has often been favoured for PIT in various allergic and autoimmune models (Janssen et al., 1999, Konkel et al., 2010). It has generally been felt that this approach may be the most likely to induce tolerance and hence is valuable when trying to assess whether or not tolerance can be induced using PIT in a particular model. Clinically, however, it is difficult to envisage a situation where PIT is given to a patient prior to sensitisation, although this could perhaps occur if an approach was eventually developed which involved giving PIT against a variety of common allergens to children at high risk of atopic disease. Campbell et al have recently demonstrated therapeutic effectiveness in a model where PIT was administered between challenge and re-challenge (Campbell et al., 2009). That type of approach has advantages in terms of clinical translation as it could mirror the situation of using PIT to treat a patient who has allergic asthma.

Studies investigating the effect of PIT on allergic disease can be divided in those investigating the effects of PIT on the response to allergen/CFA induced responses (hence modelling Th1 rather than Th2 responses), and those examining the effects of PIT in allergic disease.

1.9.1.2.1 PIT in allergen/CFA immunisation models

Hoyne et al investigated PIT in a model where mice were immunised with Der p 1 in CFA (Hoyne et al., 1993). They found that PIT using a peptide containing an immunodominant T cell epitope from Der p 1 induced tolerance, resulting in reduced *in vitro* recall responses of LN cells to both the peptide and to Der p 1 (Hoyne et al., 1993). Fel d 1 is a common cat allergen which is composed of two polypeptide chains. Briner et al found that PIT using two immunodominant peptides from polypeptide chain 1 of Fel d 1, prior to immunisation with chain 1 in CFA, induced tolerance both to the peptides themselves and also reduced *in vitro* recall responses to chain 1 (Briner et al., 1993). There was also evidence suggestive of linked suppression in that study (Briner et al., 1993).

1.9.1.2.2 PIT in models of allergic sensitisation and AAI

PIT in models of allergic sensitisation and AAI are limited in number. In an allergic sensitisation model where mice were sensitised to the birch pollen allergen bet v 1 using alum as an adjuvant, Bauer et al found that PIT using one immunodominant peptide abrogated *in vitro* proliferative responses of LN cells to the peptide (Bauer et al., 1997). Significant reductions in proliferation of LN cells in response to Bet v 1 were only found in mice given PIT after sensitisation and not if PIT was given prior to sensitisation, although the reasons for this were unclear (Bauer et al., 1997). In a recent study, Campbell et al examined the effects of PIT using an immunodominant peptide from Fel d 1, in a model of Fel d 1-induced AAI (Campbell et al., 2009). PIT using one Fel d 1-derived peptide was given after sensitisation and challenge, but before re-challenge (Campbell et al., 2009). This was found to significantly improve AAI, and again there was evidence of linked suppression (Campbell et al., 2009).

Importantly, not all models of PIT in allergy have resulted in improvements. Janssen et al found that in a model of OVA-induced AAI, PIT using the reported immunodominant epitope, pOVA 323-339 (pOVA), given after OVA sensitisation and prior to OVA challenge, increased the severity of AAI (Janssen et al., 1999). Furthermore, in a model of OVA sensitisation (and not AAI), Barbey et al found that intranasal administration of pOVA prior to OVA/alum sensitisation, did not induce tolerance to OVA, whereas intranasal application of OVA did (Barbey et al., 2004).

1.9.2 Evidence of therapeutic effects of PIT from human studies

1.9.2.1 Autoimmunity

PIT has been used in some patients with autoimmune disease. Clinical improvements have been seen in some studies in patients with rheumatoid arthritis (Koffeman et al., 2009) and multiple sclerosis (Warren et al., 2006). However, early clinical trials of PIT in multiple sclerosis patients had to be halted because of hypersensitivity

reactions (later found to be IgG-mediated) and possible disease exacerbations (Bielekova et al., 2000, Kappos et al., 2000). The peptides used in those studies contained alterations in their amino acid sequences, designed to improve their therapeutic effects. Concerns have been raised over such altered peptide ligands (APLs), for although they can be designed to promote certain responses from responding T cells (Janssen et al., 2000b), or reduce side effects such as IgG1 binding (Leech et al., 2007b), in practice their effects can be unpredictable [reviewed in (Anderton, 2001)].

1.9.2.2 Allergic disease

The PIT studies that have taken place in the context of allergy mainly relate to cat allergy and bee venom allergy [reviewed in (Moldaver and Larche, 2011)]. Initially, PIT using two 27 amino acid long, immunodominant peptides from Fel d 1 were used clinically. Several clinical trials demonstrated some clinical improvements in some cat allergic patients following PIT, however some patients developed early allergic reactions (Norman et al., 1996, Maguire et al., 1999, Pene et al., 1998). It appeared that the peptides used in those trials could, in some instances, enable IgE cross linking on mast cells. This led to the use of multiple shorter 15-17 amino acid peptides which overlapped and encompassed the majority of the sequence of the Fel d 1 protein allergen. Again, improvements in readouts such as rhinitis symptoms and late-phase cutaneous responses to allergen were demonstrated in some patients (Alexander et al., 2005, Oldfield et al., 2002). PIT using immunodominant epitopes from the bee venom allergen PLA, have also been demonstrated to significantly improve late phase skin reactions induced in response to bee venom (Tarzi et al., 2006).

To summarise, although PIT holds promise as potentially safer approach to allergen-specific immunotherapy, outcomes are variable. If PIT is to be effectively translated to the allergy clinic, then further understanding of the mechanisms of action of PIT, and of potential factors influencing therapeutic outcomes, is required.

1.9.3 Mechanisms involved in PIT

Mouse models have enhanced our understanding of the mechanisms involved in tolerance induction using PIT. Some of these have already been discussed in section 1.6.2 and the mechanistic effects of PIT will also be discussed in detail in subsequent chapters. High dose PIT is often associated with deletion of peptide-reactive T cells (Hochweller and Anderton, 2005, Konkel et al., 2010). Such protocols also often induce a population of anergic cells (Konkel et al., 2010, Pape et al., 1998). The induction of $FoxP3^+$ T regulatory cells following PIT has not often been reported. However, Kretschmer et al found that $FoxP3^+$ cells were induced following administration of a peptide that was specifically targeted to the DC (Kretschmer et al., 2005), and very low dose PIT given subcutaneously and in multiple doses has also been postulated to have led to iTreg induction in another study (Kang et al., 2005). The induction of IL-10 following PIT has been cited in clinical (Campbell et al., 2009, Tarzi et al., 2006) and animal (Gabryšová and Wraith, 2010) studies. Furthermore, IL-10 dependent mechanisms have also been attributed to linked suppression seen following PIT in cat allergic patients (Campbell et al., 2009). Interestingly, Gabrysova et al found that peptides with high affinity for MHC, that thus provide a strong signal to responding T cells, promoted the production of IL-10 from peptide-reactive T cells in a model using repeated intranasal peptide administrations (Gabryšová and Wraith, 2010). These effects are discussed further in Chapter 6.

Thus, the mechanisms of tolerance effected by PIT appear to vary in different models. This has important implications for how PIT can best be translated to the clinic in order to maximise clinical efficacy.

1.9.4 Issues relevant to the clinical application of PIT

1.9.4.1 Route, regime and dose

It is likely that the nature of the peptide, the route of delivery and the PIT regime employed all have the potential to affect the mechanism(s) of tolerance induction and/or the efficacy of PIT. The induction of regulatory responses using PIT is highly desirable because of the potential to generate long-term tolerance to an allergen, and the possibility that this may regulate immune responses to other allergens. In contrast, induction of an adaptive tolerance form of anergy presents the problem that, upon removal of the peptide, anergic cells may return to pathogenicity (Schwartz, 2003). If PIT is to be given to young children, then PIT regimes that start with a low dose and then escalate, are likely to represent the safest approach. This could also bring advantages by enhancing the likelihood of inducing IL-10 producing regulatory cells [discussed in (Sabatos-Peyton et al., 2010)]. Furthermore, if particular delivery regimes were found to induce bystander and/or linked suppression, this would also be likely to be advantageous to the overall goal of modifying progression of allergic disease.

1.9.4.2 HLA variation

The issue of HLA diversity represents a particular challenge regarding which peptides to use in a diverse population, since the expression of MHC II molecules varies widely between individuals. MHC diversity means that for any given allergen, immunodominant T cell epitopes can vary between individuals [discussed in (Larché, 2008)]. One approach might be to characterise the T cell immunodominant epitopes within an allergen on a personalised basis, but this is unlikely to be feasible in terms of practicality and cost. Importantly however, peptides are often promiscuous - meaning they can bind to many different MHC II molecules (Friedl et al., 1999, Kobayashi et al., 2000). This is partly on account of the non-polymorphic nature of the HLA-DR α chain which results in fundamental similarities between different HLA-DR molecules. Thus one or several peptides might be capable of inducing tolerance in HLA diverse individuals. Furthermore, using multiple overlapping

peptides as adopted in some recent studies (Campbell et al., 2009), should also promote the induction of T cell tolerance in HLA-mismatched patients.

1.9.4.3 Could PIT induce immunity and not tolerance?

The induction of tolerance is associated with peptide presentation by non-activated DCs (Hochweller and Anderton, 2005). Therapeutic administration of peptides theoretically risks inducing an inflammatory response, rather than a tolerogenic response, if DCs are activated. One particular situation where this could be envisaged is during illness such as bacterial or viral infection, when PAMPs could activate DCs. Currently SIT is contraindicated during acute systemic illness (Zuberbier et al, 2010), as is also the case for routine vaccinations, and it would probably also be advisable to adopt this approach for PIT. The effects of mild viral infection upon PIT are, however, unknown. Exposure to allergen at the time of respiratory viral infection can lead to allergic sensitisation, (O'Donnell and Openshaw, 1998) and intranasal SIT at the time of influenza virus was found to prevent tolerance and exacerbate AAI (Tsitoura et al., 2000). It is, therefore, possible that PIT given via an intranasal route at this time, could interfere with tolerance induction or induce an inflammatory response. In order to thoroughly address such issues, initially requires the development and optimisation of *in vivo* models of PIT in allergic respiratory disease.

1.10 Concluding remarks

PIT offers promise as an allergen-targeted therapy with the potential not just to improve the symptoms of allergic disease, but also to modify disease progression. PIT represents a potentially safer alternative to SIT, which is particularly pertinent to allergic asthmatics since they have an increased risk of SIT-induced adverse reactions. Several clinical trials have shown that PIT can have therapeutic effects in allergic respiratory disease. However, there remains uncertainty about the mechanisms involved in successful PIT, how these may vary with different PIT

regimes and the effects of potential confounding factors such as concurrent viral infection. Improved animal models of PIT in allergic disease and AAI should aid our understanding of these issues, thus facilitating progression of PIT into the allergy clinic.

1.11 Hypothesis and aims of this project

The following points influenced the way in which this project was constructed:-

- Further development of models of PIT in allergic sensitisation and allergic airways disease are required in order to facilitate further mechanistic assessment of the effects of PIT in allergic disease.
- The model allergen chicken ovalbumin (OVA) is often used in allergic models. OVA can induce AAI and transgenic mice are available with $CD4^+$ T cells which have OVA-reactive TCRs (Barnden et al., 1998). Congenic marking of such cells enables OVA-reactive T cells to be tracked *in vivo.*
- The induction of tolerance using SIT has previously been associated with the generation of "strong, synchronous and systemic T cell responses" (Janssen et al., 2000a)
- High dose, intravenous PIT using the reported immunodominant epitope of OVA (pOVA 323-339) has previously been demonstrated to be capable of inducing tolerance of pOVA-reactive T cells via the initial induction of a vigorous and widespread T cell response (Hochweller and Anderton, 2005, Konkel et al., 2010).
- The induction of linked suppression is therapeutically desirable.

1.11.1 Hypothesis

This thesis investigated the hypothesis that strong, systemic T cell responses induced by intravenous administration of soluble pOVA 323-339 (pOVA) will induce:-

i) tolerance to pOVA
ii) linked suppression to any additional OVA T cell epitopes, hence improving OVA-induced AAI.

1.11.2 Aims

The aims of this project were to:-

i) Establish a model of OVA-induced AAI in C57BL/6 mice and characterise the immune parameters within it.

ii) Assess the effects of pOVA PIT, given intravenously and in high dose, on the severity of AAI.

iii) Assess the effects of pOVA PIT on known, trackable pOVA-reactive $CD4^+$ T cells in models of OVA-induced allergic sensitisation and AAI.

iv) Assess whether pOVA PIT induces linked suppression to any additional T cell epitope(s) within OVA.

2 Materials and Methods

2.1 Mice

Female C57BL/6J and BALB/c mice were purchased from Charles River (Kent, UK). All mice were maintained in specific pathogen-free conditions at the University of Edinburgh, UK and fed on OVA-free diets. Age-matched, 6-12 week old female mice were used for all experiments. The experimental unit used for statistical analysis was individual mice. Each cage consisted of one treatment group, decided at random. A colony of OT-II transgenic mice expressing an I-A^b-restricted TCR reactive to OVA peptide 323-339 (Barnden et al., 1998), congenically labelled with CD45.1 was maintained at the University of Edinburgh, UK. All experiments were conducted under UK home office regulations and had local ethical approval.

2.1.1 Phenotyping OT-II mice

The OT-II mice used were recombination activating gene (RAG) sufficient thus they retain the potential for α and β chain rearrangement. However, all OT-II mice were screened using flow cytometric analysis of blood taken after weaning to ensure the presence of the Vβ5.1 and Vα2 TCR chains on >90% $CD4^+$ cells. The presence of the congenic marker CD45.1 and the absence of the C57BL/6 CD45 isoform CD45.2 was also confirmed (Appendix 1). Splenocytes from rederived OT-II mice were confirmed to proliferate *in vitro* to OVA and to pOVA (data not shown).

2.2 General reagents

2.2.1 Wash buffer

RPMI 1640 (Gibco, Paisley, UK)

2.2.2 Tissue culture medium

RPMI 1640 (Gibco) supplemented with 2mM L-glutamine, 100U/ml penicillin, 100µg/ml streptomycin (all from PAA, Austria), 50µM 2-β-mercaptoethanol and 5% heat-inactivated fetal calf serum [HI-FCS (Gibco)].

2.2.3 Flow cytometry buffer (FACS buffer)

Phosphate-buffered saline [PBS (Gibco)] supplemented with 1% bovine serum albumin (BSA) and 0.05% sodium azide (both from Sigma-Aldrich, Dorset, UK).

2.2.4 MACS buffer

Hanks Balanced Salt Solution (Gibco) supplemented with 2% HI-FCS (Gibco) and 2mM EDTA (Sigma-Aldrich).

2.3 Antigens

Grade V chicken Ovalbumin (OVA) was used for sensitisation (Sigma-Aldrich). Chromatographically purified chicken OVA (Worthington Biochemical Corporation, Lakewood, USA) was used *in vitro* and for sensitisation and airway challenges. OVA peptide 323-339 (referred to throughout as pOVA) was synthesised by PepLogic, Essex, UK. The lipopolysaccharide (LPS) level was quantified using a limulus amebocyte lysate (LAL) test (Sigma-Aldrich). 0.6-3.6ng/ml LPS was detected in OVA (Worthington Biochemical Corporation) at the concentration used for intranasal administration (1mg/ml). The LPS concentration for pOVA (PepLogic) was undetectable (<0.06EU/ml), however the positive control for this assay did not clot completely meaning there is the possibility that an inhibitor of the LAL assay may exist within the reconsitituted pOVA. For epitope mapping experiments, a panel of 15mer peptides were synthesised by Peptide 2.0, Chantilly, USA covering the amino acid sequence of OVA. Each peptide shifted by 5 amino acids to generate the panel shown in Table 1. OVA peptide 263-278 (referred to throughout as p263-278, sequence KLTEWTSSNVMEERKI) was synthesised by Cambridge Research

Biochemicals, Cleveland, UK. The peptides in Table 1 were reconstituted in 100µl dimethyl sulfoxide [DMSO (Sigma-Aldrich)] and diluted with RPMI 1640 (Gibco) to 3mg/ml and stored at -80°C. pOVA and p263-278 were reconstituted using sterile water only (Sigma-Aldrich) and stored at -80°C.

2.4 $CD4^+$ T cell isolation, polarisation and adoptive transfer

2.4.1 Positive selection of naïve $CD4^+$ OT-II cells

Spleens and peripheral lymph nodes from female OT-II mice were harvested and disaggregated through gauze to form single cell suspensions. Spleen cell suspensions underwent red cell lysis using 2ml per spleen of red cell lysis buffer (Sigma-Aldrich) for 2 minutes, followed by the addition of 20ml wash medium and centrifugation (300xg, 5 minutes). Following red cell lysis, spleen cells were combined with lymph node cell suspensions. CD4 (L3T4) MACS Microbeads (Miltenyi Biotec, Surrey, UK) were used to positively select for $CD4^+$ cells according to manufacturer's instructions. Briefly, 45µl of MACS buffer and 5 µl CD4 MACS beads were added for every $1x10^7$ cells, followed by incubation at 4°C for 15 minutes. Cells were washed in MACS buffer, and run through MACS LS columns (Miltenyi Biotec) as per manufacturer's instructions. After washing columns three times with MACS buffer, positively selected cells were collected by removing the column from the magnet, applying a further 5ml MACS buffer to columns and rapidly flushing with a plunger. Cells were washed in PBS, counted and resuspended in PBS prior to adoptive transfer. The purity of positively selected cells was consistently >90%, as determined by flow cytometry.

2.4.2 Carboxyfluorescein diacetate succinimidyl ester (CFSE) staining of naïve $CD4^+$ cells

In experiments where OT-II cells were CFSE labelled prior to adoptive transfer, 5µM CFSE (eBioscience, Hatfield, UK) was added to every $5x10^7$ cells (in a concentration of $5x10^7$ cells/ml), mixed well and incubated at 37°C for 5 minutes. Cold RPMI (Gibco) containing 10% HI-FCS (Gibco) was added to cells, prior to

centrifugation at 300xg for 5 minutes followed by two additional washes in RPMI (Gibco) containing 10% HI-FCS and two washes in PBS (Gibco) prior to counting.

2.4.3 Th2 polarisation of OT-II cells

Spleen and peripheral lymph node single cell suspensions from female OT-II mice were prepared as described in section 2.4.1 and cultured at $4x10^6$ cells/ml in 6 well plates (Costar UK Ltd, Buckinghamshire, UK) in tissue culture medium supplemented with 40U/ml rIL-2 (Peprotech EC Ltd, London, UK), 4ng/ml rIL-4 (Peprotech EC Ltd), 5µg/ml anti-IL-12 (BioXcell, New Hampshire, USA) and 5µg/ml anti-IFNγ (BioXcell) and 10µg/ml pOVA (PepLogic). At 72 hours, cells were split using tissue culture medium containing the same cytokine and antibody combination but without further addition of pOVA. Cells were cultured for a total of 96 hours after which they were harvested, washed and $CD4^+$ cells positively selected using CD4 MACS Microbeads (Miltentyi Biotec) as described for naïve $CD4^+$ cell selection in section 2.4.1. Supernatants from polarised cells were checked for the presence of Th2 cytokines by enzyme-linked immunosorbent assay (ELISA).

2.4.4 Polarising conditions for Th0 and Th1 polarisation of OT-II cells

In some experiments intracellular cytokine staining was conducted following different polarisation regimes. For Th0 polarisation, tissue culture medium was supplemented with 20U/ml rIL-2 (Peprotech EC Ltd) and 10µg/ml pOVA (PepLogic) only. For Th1 conditions 20U/ml rIL-2 (Peprotech EC Ltd), 25ng/ml rIL-12 (Peprotech EC Ltd) and 25ng/ml rIL-18 (Peprotech EC Ltd) together with 10µg/ml pOVA (PepLogic), were used. Th2 cells were cultured in the conditions described above. In these smaller experiments OT-II single cell suspensions were cultured at 3×10^6 /ml in 48-well plates (Costar UK Ltd) for 6 days. Cells were split after 3 days using antibody/cytokine supplemented tissue culture medium but without further addition of pOVA.

2.4.5 Adoptive transfer of $CD4^+$ OT-II cells

Purified $CD4^+$ OT-II cells (either naïve or following Th2 polarisation) were injected intravenously into recipient C57BL/6 mice via the tail vein (2-4.5 x 10^6 cells per mouse depending on the experiment).

2.5 Administration of soluble peptides

500µg pOVA or 500µg p263-278 or 500µg of both peptides in combination, diluted in sterile PBS (Gibco), were given intravenously via the tail vein in a volume of 200µl. Soluble peptide was given 7 days prior to immunisation with OVA/alum or, in the Th2 polarised $CD4^+$ OT-II AAI model, soluble peptide was given 2 days after cell transfer and 4 days prior to first intratracheal challenge.

2.6 Allergic airways inflammation (AAI) and sensitisation models

2.6.1 Induction of AAI and allergic sensitisation without cell transfer

2.6.1.1 Sensitisation

Mice were sensitised with two intraperitoneal (i.p) injections of 100µg OVA (grade V, Sigma-Aldrich) adsorbed to 2mg aluminium hydroxide [alum, (Alum Imject, Pierce, USA)] on days 0 and 14. Controls instead received two i.p injections of sterile PBS (Gibco) plus 2mg alum (Pierce). In some experiments only the sensitisation injections (either one or two injections) were given and spleen and mediastinal lymph nodes harvested 7 days after the last sensitisation.

2.6.1.2 Intranasal airway challenge

Mice were intranasally challenged under brief isoflurane anaesthesia (Merial Animal Health Ltd, Essex, UK) with 30-50µg OVA (Worthington Biochemical Corporation)

or sterile PBS (Gibco) as a control on days 28, 29 and 30. Mice were culled on day 32 for assessment of disease parameters.

2.6.1.3 Intratracheal airway ahallenge

In later experiments airway challenges were given via intratracheal instillation on days 28, 31 and 34. Mice were deeply anaesthetised using i.p medetomidine hydrochloride (Pfizer Ltd, Surrey, UK) and ketamine (Fort Dodge Animal Health Ltd, Hampshire, UK), using the protocol in Appendix 2. Mice were supported in a frame facing the researcher, the mouth held open and the glottis and vocal cords visualized using a cold lamp applied to the neck. A blunt needle attached to a syringe was then used to introduce 50µg of OVA directly into the trachea. Anaesthesia was reversed using atipamezole hydrochloride (Pfizer Ltd, Surrey, UK) as detailed in Appendix 2. Mice were culled two days after the last intratracheal instillation for assessment of disease parameters.

2.6.2 Induction of allergic airways disease using transfer of Th2 polarised OT-II $CD4^+$ T cells

4-4.5x10^6 $CD4^+$ Th2 polarised OT-II cells were injected intravenously in a total volume of 200µl via the tail vein into C57BL/6 recipients. 1 day later mice received the first of three 50µg intratracheal OVA challenges, given every 3 days. Mice were culled 2 days after the last airway challenge to assess the extent of disease.

2.6.3 Assessment of AAI

2.6.3.1 Invasive plethysmography

Lung function was assessed 24 hours after the last airway challenge as described previously (Glaab et al., 2007) under deep anaesthesia using a FinePointe resistance and compliance system (Buxco Research Systems, Winchester, UK). Mice were anaesthetised using a combination of ketamine (Fort Dodge Animal Health Ltd) and xylazine hydrochloride (Bayer Healthcare, Uxbridge, UK) as described in Appendix

2. The trachea was surgically exposed and cannulated and the cannula tightly secured using elastic. Mice were mechanically ventilated using 120 breaths/minute with a maximum tidal volume of 0.25ml. Flow and pressure data in response to nebulised PBS alone (Gibco) and to increasing doses of nebulised methacholine [MCh (Sigma-Aldrich)] were automatically processed using biosystem XA software (Buxco Research Systems) to generate values for resistance of the lung (Rl) and dynamic compliance (Cdyn) as previously described (Glaab et al., 2007).

2.6.3.2 Bronchoalveolar lavage (BAL)

The trachea was exposed, canulated and lungs were lavaged with 1ml sterile PBS. BAL cells were counted and cytospins prepared using a Shandon Cytospin 3 centrifuge at 300 rpm for 3 minutes. Slides were air dried for 1 hour, fixed in 100% methanol for 20 minutes and stained with Quick-Diff red stain for 1.5 minutes followed by Quick-Diff blue stain for 15 seconds (both Gamidor Technical Services, UK). Slides were rinsed in water and allowed to air dry. Differential cell counts were performed by light microscopy with the researcher blinded to experimental conditions. A total of 300 cells were counted per cytospin.

2.6.3.3 Histological scoring

Lungs were perfused via the heart with sterile PBS (Gibco), inflated and fixed in methacarn [60% methanol, 30% chloroform, 10% glacial acetic acid, (all Fisher Scientific, Leicestershire, UK)] and embedded in paraffin. Sections were stained with hematoxylin and eosin (H+E) or Periodic acid-Schiff (PAS) stains (staining provided by on-site histology service). Inflammatory scoring of H+E stained slides (1 slide per mouse) was done manually at x200 magnification as previously described (Leech et al., 2007a). 10 consecutive fields were scored. To qualify for scoring, fields had to contain at minimum one complete bronchiole and a blood vessel. Only bronchioles less than half the width of a field (equivalent to less than 300µm in diameter) were scored. Inflammation scores of 1-4 were applied to the

peri-vascular compartment (1 no cells, 2 <20 cells, 3 <100 cells, 4 >100 cells) and the peri-bronchiolar compartment (1 no cells, 2 <20 cells, 3 <100 cells, 4 >100 cells). The percentage of goblet cells in small airways (less than half the width of a field - equivalent to less than 300µm in diameter) was determined by calculating the average percentage of goblet cells within 10 consecutive small airways at x200 magnification. These percentages were then averaged to give the average percentage of goblet cells in small airways for each mouse. The percentage of goblet cells in large airways (more than half a field width in diameter i.e. > 300µm) for each mouse was calculated in the same way, but since these often numbered less than 10, as many large airways as possible (up to a maximum of 10) were scored. All histological scoring was carried out blinded to experimental conditions.

2.6.3.4 Detection of OVA-specific IgE, IgG1 and IgG2a

Blood was taken via an axillary approach, serum collected and frozen at -80°C prior to detection of OVA specific IgE. Serum was diluted 1:4 using PBS (Gibco) and incubated with fast flow protein G sepharose beads (Sigma-Aldrich) overnight at 4°C on a rotator, in order to deplete serum IgG as described previously (Leech et al., 2007a). High binding EIA/RIA 96-well plates (Costar UK Ltd) were coated overnight with 5µg/ml OVA in 0.05M carbonate buffer [0.795g Na_2CO_3, 1.465g $NaHCO_3$ plus 500ml dH_2O, pH 9.6, (all Sigma-Aldrich)], 100µl/well. The following day plates were blocked with 200µl/well PBS/1% BSA (Gibco/Sigma-Aldrich) for 1 hour at 37°C. Serum was centrifuged at 14000xg for 5 minutes to remove beads. Serum was transferred to a clean eppendorf and centrifuged again to ensure bead removal. Recovered serum was diluted further in PBS/1% BSA (Gibco/Sigma-Aldrich) to a final 1:10 dilution. Serial dilutions of serum were prepared in a flexiplate using doubling dilutions in PBS/1% BSA (Gibco/Sigma-Aldrich). OVA-coated plates were washed 5 times in PBS containing 0.05% Tween [PBS-T, (Sigma-Aldrich)] and 50µl of pre-diluted serum added in duplicate to wells. Plates were incubated at 4°C overnight. The following day plates were washed 5 times with PBS-T. Biotin conjugated rat anti-mouse IgE antibody (Clone R35-118, BD bioscience) was diluted to 2 µg/ml in PBS+1%BSA and 100µl of detection antibody solution

added to each well. Plates were incubated for 1 hour at 37°C and washed 5 times in PBS-T (Sigma-Aldrich) prior to the addition of 100µl horseradish peroxidase labelled streptavidin [(streptavidin-HRP, R+D Systems Ltd, Oxon, UK) diluted 1:200 in PBS/1% BSA)] per well. Plates were incubated for 1 hour at room temperature, washed 6 times in PBS-T (Sigma-Aldrich) and the ELISA was developed using 100µl/well 3,3',5,5'-tetramethylbenzidine [(TMB), Invitrogen Ltd, Paisley, UK]. The reaction was stopped using 100µl/well 2M H_2SO_4 (Sigma-Aldrich). As no standard was used, each plate was developed for exactly the same time prior to stopping the reaction. Plates were read at a wavelength of 450nm (with a wavelength correction set at 630nm) using a Biotek Synergy HT plate reader (Biotek, Bedfordshire, UK) and Gen5 software (Biotek).

OVA-specific IgG1 and IgG2a were detected in serum using the same protocol as for IgE, without the IgG depletion step. The IgG1 antibody used was biotinylated rat anti-mouse IgG1 (clone LO-MG1-2, AbD serotec, Oxfordshire, UK), used at 0.125µg/ml and the IgG2a antibody used was biotinylated rat anti-mouse IgG2a (clone 5.7, BD biosciences) used at 1µg/ml.

2.6.3.5 Isolation of cells from lung tissue

In some experiments following perfusion and prior to fixation, the left lobe was tied off, removed and placed into PBS (Gibco). Lung tissue was finely chopped and incubated in collagenase (type I-AS, Sigma-Aldrich) solution (final concentration 0.23mg/ml collagenase) for 45-60 minutes at 37°C. Tissue was disaggregated using rapid flushing through a 20G needle. Cell suspensions were washed twice in PBS (all centrifugation steps were carried out at 300xg for 5 minutes) and subjected to red cell lysis (2ml/lung for 2 minutes) followed by a further wash in PBS (Gibco). Following the final wash, cells were resuspended in PBS (Gibco) and pushed through a 40µM cell strainer prior to counting.

2.7 Immunisations with OVA/ Complete Freund's Adjuvant (CFA)

Mice were immunised with a total of 100-200µg OVA emulsified in CFA which contained 4mg/ml of heat-killed *Mycobacterium tuberculosis* H37a (Sigma-Aldrich). 50µl of OVA/CFA was injected subcutaneously into each hind leg.

2.8 Assessing T cell function *in vitro*

2.8.1 *Ex-vivo* recall proliferation assays

Lymph nodes [mediastinal LN (mLN), or inguinal (iLN) and para-aortic LN, dependent upon the experiment] and/or spleens were processed into single cell suspensions as described in 2.4.1. Cells were cultured in 96-well flat bottomed plates (Costar UK Ltd) at concentrations of $8x10^5$/well for spleen and $6x10^5$/well for LN. Cells were cultured in tissue culture medium in the presence of OVA (0-11µM) or pOVA (0-100µM) or p263-278 (0-30µM) or in equimolar combination (0-30µM pOVA plus 0-30µM p263-278). Proliferation was assessed by pulsing cells at 48 hours with [^{3}H]-thymidine (0.5µCi/well, Amersham Biosciences, Buckinghamshire, UK). Incorporation of [^{3}H]-thymidine was assessed using a liquid scintillation β–counter (Wallac, Turku, Finland) 16 hours later. Cytokines were measured in culture supernatants using ELISA. IL-2 was assessed after 48 hours and other cytokines after 72 hours of culture.

2.8.2 Enzyme-linked Immunosorbent Assay (ELISA) for cytokines

Cytokines were detected in culture supernatants and BAL using ELISA. The concentrations of antibodies used are shown in Table 2. Capture antibodies were diluted in 1 x bicarbonate buffer (10 x bicarbonate buffer comprising 0.15M Na_2CO_3, 0.35M $NaHCO_3$ (all Sigma-Aldrich) plus 400ml dH_2O, pH 9.6 was made initially and subsequently diluted to working concentration in dH_2O). High binding EIA/RIA 96-well plates (Costar UK Ltd) were coated overnight with 50µl/well of capture antibody solution. The following day plates were washed twice with PBS-T (Sigma-Aldrich) and blocked with 200µl/well PBS/1% BSA (Gibco/Sigma-Aldrich) for 1 hour at 37°C. Plates were washed twice in PBS-T (Sigma-Aldrich) prior to addition

of 100µl/well of samples in triplicate. In experiments where samples were diluted prior to ELISA, dilutions were carried out in 1%BSA/PBS. A 10 point standard curve comprising serial doubling dilutions of standard (diluted in 1%BSA/PBS) in duplicate, and wells containing 1%BSA/PBS (Gibco/Sigma-Aldrich) alone, were included on each plate. All standards were obtained from BD Biosciences. Plates were incubated at room temperature for 2 hours and washed 4 times with PBS-T (Sigma-Aldrich)**.** Biotinylated antibodies were diluted to the concentrations shown in Table 2 in PBS/1% BSA (Gibco/Sigma-Aldrich) and 100µl detection antibody solution added to each well. Plates were incubated for 1 hour at room temperature and washed 6 times in PBS-T prior to the addition of 100µl streptavidin–HRP (R+D Systems Ltd) diluted 1:200 in PBS/1% BSA (Gibco/Sigma-Aldrich) per well. Plates were incubated for 30 minutes at room temperature, washed 6 times in PBS-T (Sigma-Aldrich) and the ELISA developed using 100µl/well TMB (Invitrogen Ltd). The reaction was stopped using 100µl/well 2M H_2SO_4 (Sigma-Aldrich). Plates were read at a wavelength of 450nm (with a wavelength correction set at 630nm) using a Biotek Synergy HT plate reader and Gen5 software (Biotek, Bedfordshire, UK).

2.9 Generating short term T cell lines

Mice were immunised with 200µg OVA emulsified in CFA containing 4mg/ml of heat-killed *Mycobacterium tuberculosis* H37a (Sigma-Aldrich) as described in section 2.7. 7-10 days after immunisation para-aortic LN and iLN were harvested, and a protocol derived from one previously been shown to generate T cell lines (Anderton et al., 1998), was used. Briefly, LN cells were cultured at $5x10^6$/well in tissue culture medium together with 7µM OVA in 24 well tissue culture plates (Costar UK Ltd). Cells were harvested after 72 hours and 2ml of Ficoll (Ficoll-Pague Plus, GE Healthcare, Buckinghamshire, UK) laid under 4.5ml of cell suspension in a 15ml Falcon tube (Beckton Dickinson, UK). Cells were centrifuged at 650xg for 15 minutes without brake. Cells were removed from the interface, wash buffer was added followed by further centrifugation at 650xg for 5 minutes, without brake. Cells were washed twice in wash buffer, counted and resuspended at $4\text{-}5x10^6$ cells per well in tissue culture medium supplemented with 2.5% rat Con A supernatant as a source

of IL-2 [previously generated in the host laboratory by stimulating rat splenocytes with concanavalin A (Sigma-Aldrich)]. Cells were incubated at 37°C, 5% CO_2 for a total of 4 days, splitting as necessary using tissue culture medium supplemented with 2.5% rat Con A supernatant.

2.10 *In vitro* recall assays involving short-term T cell lines

T cells were cultured at $2x10^4$/well with $5x10^5$/well of irradiated splenocytes [exposed to 30Gy of gamma-irradiation (caesium isotope)] and peptide or OVA (Worthington Biochemical Corporation) in 96 well flat bottom plates (Costar UK Ltd). Proliferation was assessed by pulsing cells at 48 hours with [^{3}H]-thymidine (0.5µCi/well, Amersham Biosciences). Incorporation of [^{3}H]-thymidine was assessed using a liquid scintillation β–counter (Wallac) 16 hours later. Cytokines were measured in culture supernatants after 72 hours of culture, by ELISA.

2.11 Flow cytometry

2.11.1 Antibodies

Table 3 lists all antibodies used for flow cytometry.

2.11.2 Staining of surface markers

Cells to be stained were centrifuged at 300xg for 5 minutes and the supernatant removed. Antibodies were diluted in FACS buffer and 50µl added to cell pellets which were then mixed well prior to 30 minutes incubation at 4°C in the dark. Cells were washed in FACS buffer and resuspended either in a 200µl of FACS buffer and collected, or fixed in 200µl of 2% paraformaldehyde (Sigma-Aldrich), kept in the dark at 4°C and collected as soon as possible.

2.11.3 Intracellular Foxp3 staining and Intracellular staining for GATA-3 and cytokines

For GATA-3 and intracellular cytokine staining (following polarisation) cells were harvested, washed in wash medium and restimulated using 50ng/ml of phorbol

myristate acetate [PMA (Sigma-Aldrich)], 1µg/ml ionomycin (Sigma-Aldrich) and 1/1000 dilution of stock brefeldin A (eBioscience) for 4 hours at 37°C. FoxP3 staining was carried out without additional stimulation. Cells were surface stained as described in section 2.11.2, followed by one wash in FACS buffer. Fixation/permeabilisation buffer (eBioscience) was prepared as per manufacturer's instructions, 400µl added to cell pellets and mixed well. Cells were incubated in the dark at 4°C for 30 minutes or overnight (both approaches having been shown previously in the host laboratory to be equally effective). Cells were washed in FACS buffer and FoxP3 antibody or isotype control diluted as shown in Table 3 in permeabilsation buffer (eBioscience). 50µl of antibody solution was added to cells followed by incubation in the dark for 30 minutes at 4°C. Cells were washed with FACS buffer, resuspended in 200µl of FACS buffer and acquired as soon as possible.

2.11.4 Flow cytometric data analysis

Flow cytometric data were collected on a FACS Calibur flow cytometer using CellQuest (Becton Dickinson) or LSR Fortessa with FACS DIVA (Becton Dickinson, UK). Data were analysed using FlowJo software (Treestar, USA).

2.12 Statistical analysis

Statistical advice was sought from Dr Rob Elton. Statistical analysis was performed using Prism software. Unpaired t-test or Mann-Whitney U tests were used when comparing two groups. To compare three or more groups a one-way analysis of variance (ANOVA) was used with Tukey's multiple comparison post test. The Kruskal-Wallis test with Dunn's multiple comparison post test was used to compare three or more groups where data were non-parametric. $p<0.05$ was considered significant for all tests.

Table 1: Overlapping peptide panel encompassing the sequence of OVA

Peptide Number	Positional Sequence	Amino Acid Sequence
1	1-15	GSIGAASMEFCFDVF
2	6-20	ASMEFCFDVFKELKV
3	11-25	CFDVFKELKVHHANE
4	16-30	KELKVHHANENIFYC
5	21-35	HHANENIFYCPIAIM
6	26-40	NIFYCPIAIMSALAM
7	31-45	PIAIMSALAMVYLGA
8	36-50	SALAMVYLGAKDSTR
9	41-55	VYLGAKDSTRTQINK
10	46-60	KDSTRTQINKVVRFD
11	51-65	TQINKVVRFDKLPGF
12	56-70	VVRFDKLPGFGDSIE
13	61-75	KLPGFGDSIEAQCGT
14	66-80	CDSIEAQCGTSVNVH
15	71-85	AQCGTSVNVHSSLRD
16	76-90	SVNVHSSLRDILNQI
17	81-95	SSLRDILNQITKPND
18	86-100	ILNQITKPNDVYSFS
19	91-105	TKPNDVYSFSLASRL
20	96-110	VYSFSLASRLYAEER
21	101-115	LASRLYAEERYPILP
22	106-120	YAEERYPILPEYLQC
23	111-125	YPILPEYLQCVKELY
24	116-130	EYLQCVKELYRGGLE
25	121-135	VKELYRGGLEPINFQ
26	126-140	RGGLEPINFQTAADQ
27	131-145	PINFQTAADQARELI
28	136-150	TAADQARELINSWVE
29	141-155	ARELINSWVESQTNG
30	146-160	NSWVESQTNGIIRNV
31	151-165	SQTNGIIRNVLQPSS
32	156-170	IIRNVLQPSSVDSQT
33	161-175	LQPSSVDSQTAMVLV
34	166-180	VDSQTAMVLVNAIVF
35	171-185	AMVLVNAIVFKGLWE
36	176-190	NAIVFKGLWEKTFKD
37	181-195	KGLWEKTFKDEDTQA
38	186-200	KTFKDEDTQAMPFRV
39	191-205	EDTQAMPFRVTEQES
40	196-210	MPFRVTEQESKPVQM
41	201-215	TEQESKPVQMMYQIG
42	206-220	KPVQMMYQIGLFRVA
43	211-225	MYQIGFRVASMASE
44	216-230	LFRVASMASEKMKIL
45	221-235	SMASEKMKILELPFA
46	226-240	KMKILELPFASGTMS
47	231-245	ELPFASGTMSMLVLL
48	236-250	SGTMSMLVLLPDEVS
49	241-255	MLVLLPDEVSGLEQL
50	246-260	PDEVSGLEQLESIIN

51	251-265	GLEQLESIINFEKLT
52	256-270	ESIINFEKLTEWTSS
53	261-275	FEKLTEWTSSNVMEE
54	266-280	EWTSSNVMEERKIKV
55	271-285	NVMEERKIKVYLPRM
56	276-290	RKIKVYLPRMKMEEK
57	281-295	YLPRMKMEEKYNLTS
58	286-300	KMEEKYNLTSVLMAM
59	291-305	YNLTSVLMAMGITDV
60	296-310	VLMAMGITDVFSSSA
61	301-315	GITDVFSSSANLSGI
62	306-320	FSSSANLSGISSAES
63	311-325	NLSGISSAESLKISQ
64	316-330	SSAESLKISQAVHAA
65	321-335	LKISQAVHAAHAEIN
66	326-340	AVHAAHAEINEAGRE
67	331-345	HAEINEAGREVVGSA
68	336-350	EAGREVVGSAEAGVD
69	341-355	VVGSAEAGVDAASVS
70	346-360	EAGVDAASVSEEFRA
71	351-365	AASVSEEFRADHPFL
72	356-370	EEFRADHPFLFCIKH
73	361-375	DHPFLFCIKHIATNA
74	366-380	FCIKHIATNAVLFFG
75	371-385	IATNAVLFFGRCVSP

Peptides all obtained from Peptide 2.0

Table 2: Antibodies used for detection of cytokines by ELISA

Cytokine Detected	Antibody	Clone	Stock Concentration	Dilution Used
IL-2	Rat anti-mouse IL-2*	JES6-1A12	0.5mg/ml	1:250
	Biotin conjugated anti-mouse IL-2	JES6-5H4	0.5mg/ml	1:1000
IL-4	Anti-mouse IL-4	11B11	0.5mg/ml	1:250
	Biotin conjugated anti-mouse IL-4	BVD6-24G2	0.5mg/ml	1:1000
IL-5	Anti-mouse IL-5*	TRFK5	0.5mg/ml	1:250
	Biotin conjugated anti-mouse IL-5*	TRFK4	0.5mg/ml	1:1000
IL-10	Anti-mouse IL-10	JES5-16E3	0.5mg/ml	1:500
	Biotin conjugated anti-mouse IL-10	JES5-2AS	0.5mg/ml	1:1000
IFN-γ	Anti-mouse IFN-γ	XMG1.2	0.5mg/ml	1:500
	Anti-mouse IFN-γ	R4-6A2	0.5mg/ml	1:1000
IL-13	Anti-mouse IL-13	eBio13A	0.5mg/ml	1:500
	Biotin conjugated anti-mouse IL-13	eBio1316H	0.5mg/ml	1:100

* indicates antibodies from BD bioscience, all other antibodies are from eBioscience.

Table 3: Antibodies used for flow cytometry

Antibody	Conjugate	Clone	Stock Concentration	Dilution
CD4	AF700 (Invitrogen)	MCD0429	0.5mg/ml	1:200
CD4	APC	RM4-5	0.2mg/ml	1:200
CD45.1	APC	A20	0.2mg/ml	1:200
CD45.1	PercpCy5.5	A20	0.2mg/ml	1:200
CD45.2	APC	104	0.2mg/ml	1:200
Vα2 TCR	PE	B20.1	0.1mg/ml	1:200
Vβ5.1/5.2 TCR	FITC	MR9-4	0.5mg/ml	1:200
CD62L	PE (BD)	MEL-14	0.2mg/ml	1:200
CD44	EF450	IM7	0.2mg/ml	1:200
FoxP3	APC	FJK-16a	0.2mg/ml	1:100
Rat IgG2a isotype control	APC	eBR2a	0.2mg/ml	1:100
IL-13	AF647	ebio13A	0.2mg/ml	1:100
Rat IgG1 isotype control	AF647	eBRG1	0.2mg/ml	1:200
IFN-γ	FITC	XMG1.2	0.5mg/ml	1:100
Rat IgG1 isotype control	FITC	eBRG1	0.2mg/ml	1:40
GATA-3	PE	TWAJ	20ul (0.06µg) /test	1:5
Rat IgG2b isotype control	PE	A95-1	0.2mg/ml	1:333

All antibodies are from eBioscience unless otherwise stated.

3 PIT using pOVA 323-339 does not significantly improve OVA-induced AAI in C57BL/6 mice

3.1 Introduction

Many previous AAI studies utilised BALB/c mice, which develop strong Th2 responses (Abbas et al., 1996). AAI protocols for the induction and assessment of disease used previously in the Schwarze lab have been based on BALB/c mice. Integral to this project, however, was the intention to carry out adoptive transfer studies using $CD4^+$ T cells from congenically labelled transgenic OT-II mice. These mice are on the C57BL/6 background and their $CD4^+$ T cells express TCRs reactive to the p323-339 peptide of OVA (hereafter referred to as pOVA) (Barnden et al., 1998). A further advantage of studying PIT in the C57BL/6 strain is the extensive breadth of transgenic mice available on this background e.g. FoxP3-DTR mice. This provides additional flexibility in terms of future mechanistic work based upon findings from this project. For these reasons it was decided at an early stage that this project would focus upon AAI in C57BL/6 mice.

OVA has been used extensively as a model allergen in murine models of AAI (Kearley et al., 2008, Hamelmann et al., 1999a) and was chosen for this project because:-

i) pOVA has previously been described as containing a $CD4^+$ T cell epitope for C57BL/6 mice (as well as for BALB/c mice) (Shimonkevitz et al., 1984, Llopiz et al., 2008, Barnden et al., 1998).

ii) of the availability of transgenic OT-II mice possessing $CD4^+$ TCRs reactive to pOVA (Barnden et al., 1998).

Work from the Anderton lab has previously shown the ability of soluble pOVA to tolerise $CD4^+$ OT-II cells (Konkel et al., 2010, Hochweller and Anderton, 2005). In those studies $CD4^+$ OT-II cells were adoptively transferred into naïve C57BL/6 mice prior to administration of intravenous soluble pOVA. Mice were then immunised with pOVA in Complete Freund's Adjuvant (CFA). In that context, pOVA PIT induced tolerance in $CD4^+$ OT-II cells *in vivo* so they no longer mounted an *in vitro* recall response to pOVA, in contrast to PBS treated controls. These findings were taken as a basis for this project. Importantly however, those data were limited towards a Th1, CFA-driven setting and to the assessment of tolerance following pOVA immunisation rather than immunisation with whole protein OVA. In order to optimise clinical relevance, it was important that this project assessed the effects of PIT on AAI induced with whole protein OVA. This would more accurately model allergic asthma in humans, which presumably develops in response to whole protein allergen, and would not impair the development of features such as OVA-specific IgE (known to develop towards multiple conformational epitopes within OVA) (Mine and Yang, 2007). Prior to the development of an adoptive transfer model assessing the effects of pOVA PIT on traceable pOVA-reactive $CD4^+$ OT-II cells in AAI, the effects of pOVA PIT on AAI were assessed in wild-type C57BL/6 mice. An initial approach assessing the effects of pOVA given intravenously prior to induction of AAI was taken; i.e. the same approach as that had previously induced OT-II $CD4^+$ T cell tolerance in the Anderton lab (Konkel et al., 2010, Hochweller and Anderton, 2005).

3.1.1 Aims

The aims of the experiments described in this chapter were therefore to:-

1. Induce AAI in C57BL/6 mice using OVA.
2. Characterise the disease and immunological readouts for this model.
3. Assess the effects of giving soluble pOVA intravenously, prior to OVA challenge and sensitisation, on the severity of AAI.

3.1.2 Experimental Approach

A variety of protocols exist for the induction of murine AAI using OVA. These often involve a sensitisation phase followed by an airway challenge phase (Takeda and Gelfand, 2009), although repeated airway challenges alone can also induce AAI (Nials and Uddin, 2008). Sensitisation phases may involve the use of Th2-promoting adjuvants such as alum (Nials and Uddin, 2008), but repeated sensitisation injections without adjuvant can also be effective (Janssen et al., 1999). Airway challenge regimes vary in terms of frequency, dose and route of administration e.g. intranasal, inhalation of aerosolised OVA or intratracheal approaches (Trujillo-Vargas et al., 2005, Hamelmann et al., 1999b). The Schwarze lab has previously induced AAI using protocols involving two intraperitoneal sensitisation injections of OVA adsorbed to alum followed by three intranasal OVA challenges (Schwarze et al., 1998). It was decided to follow this approach for the induction of AAI in C57BL/6 mice. OVA sensitisation with 10µg to 300µg has previously been used by others (Vultaggio et al., 2009, de Vries et al., 2009, Hellings et al., 2002) and challenge doses can be as high as 100µg (KuoLee et al., 2008). C57BL/6 mice tend to be a more difficult strain in which to induce an allergic Th2 phenotype, than for example BALB/c mice (Takeda et al., 2001). A dose of 100µg OVA was therefore initially chosen for sensitisation and 50µg for intranasal challenge, both are towards the higher end of the dosage spectrum used previously by others. The experimental approach taken in this chapter is shown in Figure 3.1.

3.2 Results

3.2.1 Sensitisation with OVA/alum and subsequent airway challenge induces features of AAI in C57BL/6 mice

C57BL/6 mice were given two intraperitoneal sensitisation injections of 100µg OVA adsorbed to alum, followed by three 50µg OVA intranasal challenges (the approach is shown in Figure 3.1). OVA sensitisation and challenge induced inflammatory changes in the lungs typical of AAI. Figure 3.2a shows that pulmonary inflammatory infiltrates were visible in the peri-vascular and peri-bronchiolar regions of OVA sensitised and challenged mice. Inflammatory infiltrates were not seen in mock sensitised and OVA challenged, OVA sensitised and PBS challenged or naïve controls (Figure 3.2a). Goblet cells were also present in the airways of OVA sensitised and challenged mice, and rarely found in controls (Fig. 3.2c). These inflammatory changes were quantified using a previously published inflammation scoring system (Leech et al., 2007a). Using this scoring system, Figure 3.2b demonstrates significantly increased inflammation scores in OVA sensitised and challenged mice, compared to controls. Figure 3.2d shows that, after OVA sensitisation and challenge, goblet cells were evident in the small airways and, to a greater extent, in the large airways (small and large airways have previously been defined in the Materials and Methods).

Total BAL cells were significantly increased in OVA sensitised and challenged mice compared to controls (Figure 3.3a). OVA sensitisation and challenge resulted in the presence of eosinophils within BAL, accounting for around 60% of total BAL cells (Figure 3.3b). Eosinophils were rarely seen in BAL obtained from controls, alveolar macrophages constituting the majority of BAL cells in these groups. In addition to a substantial increase in total numbers of eosinophils in BAL in OVA sensitised and challenged mice compared to controls (Figure 3.3c), lymphocytes were also increased compared to mock sensitised controls (Figure 3.3e). There was no significant change in the total number of macrophages in BAL between groups (Figure 3.3d). Neutrophils were rarely seen in BAL from any group and, therefore, total numbers have been omitted here.

3.2.2 mLN cells from OVA sensitised and challenged mice produce Th2 cytokines upon *in vitro* stimulation with OVA

To assess adaptive immune responses to OVA in this model, mediastinal lymph nodes (mLN) were harvested from mock sensitised OVA challenged or OVA sensitised and challenged mice, and cultured with increasing concentrations of OVA. mLN cells from OVA sensitised and OVA challenged mice, and not from controls, proliferated in response to OVA (Figure 3.4a). In a separate experiment, (such experiments being limited by the number of cells obtained from mLN), mLN cells from OVA sensitised and challenged mice versus naïve controls were cultured with increasing concentrations of OVA (Figures 3.4b-d). The presence of cytokines in supernatants after 72 hours of culture was determined using ELISA (Figure 3.4b-d). mLN cells from OVA sensitised and challenged mice produced the Th2 cytokines IL-4, IL-5 and IL-13 in a dose dependent manner in response to OVA, in contrast to mLN cells from naïve mice. Interferon-γ was below detection for both groups (data not shown). OVA specific-IgE was detected in serum from mice after OVA sensitisation and not in serum from mock sensitised controls (Figure 3.4e).

During optimisation of the AAI protocol, the experimental approach shown in Figure 3.1 was also carried out using 30µg of OVA as the intranasal challenge dose. Pulmonary inflammation scores and the presence of goblet cells were similar irrespective of whether 30µg or 50µg was used (data not shown). Both doses significantly increased the total numbers of BAL cells compared to controls, but the increase was greatest using the higher challenge dose and there was a trend for the percentage of eosinophils within BAL to be greater in the 50µg challenged group (data not shown). 50µg challenge dose was thus used in all subsequent experiments.

3.2.3 Induction of AAI in C57BL/6 mice does not result in measurable changes in lung function in contrast to BALB/c mice

Lung function can be assessed in mice using plethysmography. Invasive and non-invasive plethysmography techniques are available, however invasive measurements are currently the "gold standard" (Glaab et al., 2007). Invasive plethysmography enables the determination of resistance of the lung (Rl) and pulmonary dynamic compliance (Cdyn). Mice with AAI have been found to have increased Rl and reduced Cdyn in response to bronchoconstrictors such as methacholine (MCh), compared to non-AAI controls (Hoymann, 2007).

To assess lung function using invasive plethysmography in this model, AAI was induced in C57BL/6 mice as shown in Figure 3.1. 24 hours after the final airway challenge, mice were terminally anaesthetised, intubated surgically using a cannula inserted into the trachea and mechanically ventilated. Rl and Cdyn at baseline and in response to inhaled MCh were determined. Much of the lung function data available from previous studies on AAI is based on BALB/c mice (Hoymann, 2007). BALB/c mice can differ in their plethysmographic responses to MCh compared to C57BL/6 mice (Adler et al., 2004), therefore AAI was additionally induced in BALB/c mice to enable comparison of lung function between the two strains.

Significant changes in Cdyn and Rl were found in BALB/c mice with AAI compared to controls at a dose of 6mg/ml MCh (Figure 3.5a,b). Although a similar trend was also seen at 12mg/ml MCh, this did not reach significance. This is likely to be due to increased variation in Rl in the BALB/c AAI group at the 12mg/ml MCh dose which was noted to coincide with increased mucous production, which may have impaired air flow. Significant changes in Cdyn were also seen at 12mg/ml of MCh in BALB/c mice with AAI compared to naïve controls (Figure 3.5b). Importantly, no differences in either Rl or Cdyn in response to MCh were seen in C57BL/6 mice with AAI compared to controls (Figure 3.5c,d).

Significant changes in lung function in C57BL/6 mice with AAI are sometimes only seen at high MCh doses (Takeda et al., 2001). Invasive plethysmography was therefore repeated using OVA sensitised and challenged C57BL/6 mice versus mock sensitised controls, this time using MCh doses up to 50mg/ml (Figure 3.6a,b). Again no differences in Rl or Cdyn were detected. It was concluded that induction of AAI using this model did not induce measurable differences in lung function in C57BL/6 mice compared to controls; hence lung function was not assessed in later experiments.

3.2.4 pOVA PIT given intravenously prior to the induction of AAI does not improve disease in C57BL/6 mice

The administration of soluble pOVA was next applied to the AAI model as shown in Figure 3.1. The same route (intravenous) and dose (500µg) of soluble pOVA as had been successfully used previously in the Anderton lab to induce tolerance of pOVA-reactive $CD4^+$ OT-II cells (Konkel et al., 2010, Hochweller and Anderton, 2005), were chosen. Mice therefore received 500µg pOVA, or PBS as a control, intravenously 7 days prior to the induction of AAI. The results are shown in Figures 3.7 and 3.8.

There was no significant difference in total BAL cells between mice that received pOVA PIT and those that did not (Figure 3.7a). There was no significant difference in the total number of eosinophils or lymphocytes in the BAL of pOVA PIT treated mice compared to PBS treated controls (Figure 3.7c,e). Significantly fewer macrophages were present in BAL from mice that received pOVA PIT, compared to PBS treated controls (Figure 3.7d). pOVA PIT did not significantly alter levels of OVA-specific IgE, IgG1 or IgG2a in serum (data not shown). pOVA PIT did not significantly alter lung inflammation scores (Figure 3.8a), nor the percentage of goblet cells in small airways (Figure 3.8b). The experiment described in Figure 3.1 was carried out twice – in both experiments findings were consistent with those shown in Figure 3.7 and 3.8. The only exception was that the significant reduction in macrophage numbers in BAL from pOVA PIT treated versus PBS treated mice

(Figure 3.7d) was not found in the repeat experiment (data not shown). Splenocytes from pOVA PIT treated mice versus splenocytes from PBS treated controls were cultured with increasing concentrations of OVA. No significant differences in proliferation (Figure 3.9a) or the production of IL-5 (Figure 3.9b), measured in supernatants by ELISA, were found between the two groups.

3.2.5 Splenocytes from OVA sensitised mice do not mount a measurable proliferative or IL-5 response to pOVA *in vitro*

It was unclear from the data described above, whether the lack of improvement in AAI seen after pOVA PIT was because tolerance of pOVA-reactive $CD4^+$ T cells was not being induced. So far, *in vitro* recall responses had only been assessed to OVA and not to pOVA. This meant it was possible that $CD4^+$ T cells responding to other potential epitope(s) within OVA were still capable of driving OVA-induced AAI in this model, despite tolerance being induced to pOVA. The experimental protocol in Figure 3.1 is 39 days long. In order to try to assess whether or not tolerance to pOVA was being achieved it was decided to examine *in vitro* recall responses of splenocytes after OVA sensitisation but prior to challenge, thus reducing the length of experiments. Mice were given either pOVA PIT or PBS intravenously 7 days before OVA sensitisation. 7 days after sensitisation, splenocytes were cultured with increasing concentrations of pOVA or OVA and proliferation and IL-5 production were assessed. Multiple experiments were conducted involving either one or two OVA sensitisations. Importantly, although splenocytes from mice that had been sensitised using OVA/alum (+/- PIT), proliferated to OVA (Figure 3.10), as found previously following OVA challenge (Figure 3.9), proliferation could not reliably be detected in response to pOVA, regardless of whether mice had received pOVA PIT or PBS (Figure 3.10). In addition, while IL-5 was produced by splenocytes from pOVA PIT treated and PBS treated mice in response to OVA (Figure 3.11a), no IL-5 was produced by splenocytes from either group in response to pOVA (Figure 3.11b). Hence, because no *in vitro* recall response to pOVA could be detected after OVA sensitisation, no conclusion could be drawn as to whether or not pOVA PIT had induced tolerance of pOVA-reactive $CD4^+$ T cells.

Figure 3-1: Experimental approach for the experiments described in Chapter 3

Figure 3-2: OVA sensitisation and challenge induces pulmonary inflammation consistent with AAI

Figure 3-3: OVA sensitisation and challenge induce changes in BAL consistent with AAI

Figure 3-4: Effects of OVA sensitisation and challenge on mLN recall responses and serum OVA-specific IgE

Figure 3-5: Comparison of BALB/c and C57BL/6 lung function responses to MCh using invasive plethysmography

Figure 3-6: No significant differences in lung function are detected in C57BL/6 mice with AAI even at high doses of MCh

Figure 3-7: pOVA PIT prior to induction of AAI does not significantly alter BAL readouts

Figure 3-8: pOVA PIT prior to induction of AAI does not significantly alter pulmonary inflammation

Figure 3-9: pOVA PIT does not significantly alter *in vitro* recall responses of splenocytes to OVA on D32

Figure 3-10: Following OVA sensitisation, proliferation was detectable in splenocytes in response to OVA but not to pOVA, irrespective of pOVA PIT treatment

Figure 3-11: Following OVA sensitisation, splenocytes produce IL-5 in response to OVA but not to pOVA, irrespective of pOVA PIT treatment

3.3 Discussion

3.3.1 How does this AAI model compare to others?

3.3.1.1 Histological and immunological readouts

The histological findings shown in Figure 3.2a-d both in terms of cellular infiltrate into the lungs and the presence of goblet cells, are consistent with other AAI models (Finkelman and Wills-Karp, 2008). The total numbers of BAL cells after OVA sensitisation and challenge (Figure 3.3a) and the concentrations of Th2 cytokines produced upon *in vitro* recall of mLN cells to OVA (Figure 3.4) are within the ranges seen by previously by others (Leech et al., 2007a, Takeda et al., 2001, Zhou Y et al., 2001). The percentage of eosinophils within BAL (Figure 3.3b) is high, but similar to some other studies in C57BL/6 mice (Hamelmann et al., 1999b), which have a tendency for greater eosinophilia within BAL after induction of AAI compared to BALB/c mice (Wilder et al., 1999, Takeda et al., 2001). Indeed, numbers of BAL eosinophils have been reported to be up to 10 times greater in C57BL/6 mice than in BALB/c mice after OVA sensitisation and challenge (Morokata et al., 1999).

3.3.1.2 Lung function measurements

Non-invasive whole body plethysmography in which pressure changes and timing of respiration are used to derive the “enhanced pause” parameter known as PenH (Adler et al., 2004, Glaab et al., 2007), can be used as a measure of lung function in mice. However, although PenH changes can be significantly increased after induction of AAI (Takeda et al., 1997), concerns have been raised surrounding its use. Adler et al found that PenH changes do not necessarily correlate with changes in pulmonary resistance detected using invasive plethysmography (Adler et al., 2004), particularly in the case of C57BL/6 mice. Strain-specific differences in reflex responses to irritants such as MCh and how these alter breathing patterns are also likely to play a role in differences in PenH readings between strains (Adler et al., 2004). For these reasons, invasive plethysmography is currently the “gold-standard” approach to lung function testing in mice and was therefore chosen for use in this project.

During invasive plethysmography, resistance of the lung (Rl) and dynamic compliance (Cdyn) are derived from pressure, flow and volume measurements during mechanical ventilation of anaesthetised, intubated mice (Glaab et al., 2007). Rl indicates airway narrowing and/or resistance within the lung parenchyma and Cdyn indicates the elasticity of the lung tissue (Glaab et al., 2007). A feature of allergic asthma in humans is AHR - increased constriction of the airways upon encountering non-specific stimuli such as bronchoconstrictive agents (Cohn et al., 2004). The induction of AAI in mice can also induce AHR in response to bronchoconstrictive agents such as MCh, causing Rl to increase and Cdyn to decrease (Glaab et al., 2007, Zhang et al., 2009). Consistent with this, significant changes were found in Rl and Cdyn in OVA sensitised and challenged BALB/c mice at relatively low MCh doses (Figure 3.5a,b). However, no changes in Rl or Cdyn were detected in OVA sensitised and challenged C57BL/6 mice (Figure 3.5c,d). These data are similar to those of Wilder et al who also found significant differences in Rl in response to MCh in OVA sensitised and challenged BALB/c mice but not in C57BL/6 mice (Wilder et al., 1999). Others have also found no difference between Rl in C57BL/6 mice with AAI versus controls (Zhou et al., 2008). C57BL/6 mice have previously been described as hyporeactive to bronchial provocation (Levitt et al., 1990), although in Figure 3.5 both naïve C57BL/6 mice and those with AAI do display Rl and Cdyn changes in response to MCh, there is just no significant change in the AAI group compared to controls. However, changes in Rl and Cdyn have been found in some AAI models in C57BL/6 mice (Albertine et al., 2002). Takeda et al found that significant changes in Cdyn and Rl in C57BL/6 mice with AAI only occurred at high doses of MCh (Takeda et al., 2001), but this was not found here (Figure 3.6).

It is seems clear that strain differences exist between C57BL/6 and BALB/c mice in terms of lung function changes in AAI, and the data found here are consistent with this (Figure 3.5). It is interesting that the lack of change seen in Rl and Cdyn in C57BL/6 mice with AAI compared to controls occurs despite significant pulmonary

inflammation and eosinophilia within the BAL (Figure 3.2 and 3.3). The requirement for eosinophils for AHR in mouse AAI models remains unclear (Akuthota et al., 2011). AHR can develop in eosinophil deficient BALB/c mice with AAI (Humbles et al., 2004), however the number of eosinophils in BAL has been found to correlate with MCh induced changes in Rl and Cdyn in BALB/c mice but not in C57BL/6 mice (Takeda et al., 2001). Takeda et al found that the distribution of eosinophils within lung tissue after induction of AAI differed between strains and hypothesised that the relative paucity of eosinophils in peri-bronchial regions in C57BL/6 mice compared to BALB/c mice may be implicated in the less severe lung function changes seen in this strain (Takeda et al., 2001).

Inter-strain differences in lung function are also likely to reflect differences in lung structure, composition and baseline lung function. Differences in elastic recoil of the lungs between strains could affect Cdyn (Takeda et al., 2001) and differences in baseline physiological readings such as respiratory rate have been demonstrated between strains (Flandre et al., 2003). Recently, Thiesse et al used micro-computerised tomography to elegantly demonstrate variations in the structure of the lung in different mouse strains (Thiesse et al., 2010). Airways at certain points within the bronchial tree were found to be wider in C57BL/6 mice compared to BALB/c mice. Additonally, certain airways were noted to display bulging in C57BL/6 mice which was not seen in BALB/c mice, leading the authors to hypothesise that reduced elasticity of the C57BL/6 lungs may partly account for this. Therefore, it may be that current methods of measuring lung function, predominantly developed using BALB/c mice, are less able to detect AHR in C57BL/6 mice on account of factors such as differences in lung structure and composition.

3.3.2 Why does pOVA PIT not improve disease in this model?

The results presented in Figures 3.7 and 3.8 demonstrate that pOVA PIT given prior to OVA sensitisation and challenge does not improve AAI. So how can these results be accounted for? Only a few previous studies have examined the effects of pOVA PIT in OVA-induced AAI (Janssen et al., 1999, Janssen et al., 2000a, Janssen et al.,

2000b, Barbey et al., 2004) and it is valuable to discuss these in the context of the data shown in this chapter.

The findings of Janssen et al have similarities to those in Figure 3.7. They showed, in a model utilising BALB/c mice, that administering pOVA PIT subcutaneously after OVA sensitisation and prior to OVA challenge, led to more severe AAI compared to PBS treated controls (Janssen et al., 1999). In particular, AHR and the number of eosinophils in the BAL were increased in mice given pOVA PIT. Despite the results from the Janssen et al study, it was still possible that applying pOVA as shown in Figure 3.1 could improve disease because of several key differences in approach.

Firstly, Janssen et al administered pOVA PIT subcutaneously rather than intravenously. pOVA PIT exacerbated AHR and BAL eosinophilia (Janssen et al., 1999). In contrast, treatment with subcutaneous OVA (in the absence of adjuvant) reduced the severity of AAI. Furthermore, administering OVA intranasally increased the severity of AAI, whereas intranasal pOVA PIT did not alter disease severity (Janssen et al., 2000a). In that study Janssen et al found, using adoptive transfer of OVA-reactive T cells, that subcutaneous OVA induced a strong $CD4^+$ OVA-reactive T cell response which quickly occurred within peripheral lymph nodes and spleen, whereas subcutaneous pOVA, intranasal OVA or intranasal pOVA all led to slower, more localised OVA-reactive $CD4^+$ T cell responses (Janssen et al., 2000a). This led them to conclude that tolerance induction was favoured by a "strong, synchronized and systemic T cell response" (Janssen et al., 2000a), consistent with findings from others (Kearney et al., 1994). Since intravenous administration of soluble pOVA has previously been shown to induce rapid, systemic pOVA-reactive $CD4^+$ T cell responses (Kearney et al., 1994), and to induce tolerance prior to pOVA/CFA immunisation (Konkel et al., 2010, Hochweller and Anderton, 2005), this implied that administering pOVA intravenously prior to induction of AAI as in Figure 3.1, would induce a systemic pOVA-reactive $CD4^+$ T cell response and improve disease, but this was not found to be the case (Figures 3.7, 3.8). Thus, administration of

soluble pOVA, in a dose and route known to induce rapid and systemic activation of pOVA-reactive $CD4^+$ T cells, did not improve AAI.

Yet, induction of systemic T cell responses to soluble peptide does not always result in tolerance - Barbey et al, in a model utilising BALB/c mice, found that intranasal administration of pOVA and subsequent subcutaneous OVA/alum sensitisation, did not induce tolerance (assessed using in *vitro recall* responses to OVA), whereas treatment with intranasal OVA did induce tolerance (Barbey et al., 2004). In contrast to the findings of Janssen et al, they found that tolerance induced by intranasal OVA induced OVA-reactive $CD4^+$ T cell responses primarily in draining lymph nodes, whereas intranasal pOVA induced a systemic response but did not induce tolerance to OVA (Barbey et al., 2004).

A second important difference between the approach used in the Janssen et al study and that shown in Figure 3.1, is the nature of the $CD4^+$ T cells that are being targeted. Janssen et al chose to give pOVA PIT after mice had been sensitised to OVA and prior to OVA challenge, rather than prior to sensitisation as here. Much of the evidence demonstrating the ability of soluble peptides to induce $CD4^+$ T cell tolerance involves giving peptide prior to immunisation/sensitisation (Hochweller et al., 2006b). Hence, the majority of $CD4^+$ T cells encountering peptide will be encountering antigen for the first time – i.e. they will be naïve. Some evidence suggests that it may be easier to tolerise naïve $CD4^+$ T cells using peptide than to tolerise effector or memory T cells that have already responded to antigen (Liu and Wraith, 1995, Kurts, 2010). The heightened disease in the Janssen et al study may have been on account of pOVA activating effector/memory pOVA-reactive $CD4^+$ T cells rather than tolerising them. Administering pOVA PIT prior to OVA sensitisation as in Figure 3.1 might then have been predicted to induce tolerance on account of the naïve status of the pOVA-reactive $CD4^+$ T cells. However despite this, no improvement in AAI was seen here (Figure 3.7,3.8).

A third difference between the Janssen et al study and the experiments shown in Figure 3.7-3.9 are differences in mouse strains, since BALB/c mice were used in that study, in contrast to the C57BL/6 mice used here. It was possible that the predominance of eosinophils seen after pOVA may have been influenced by the tendency of BALB/c mice to skew towards Th2 responses (Abbas et al., 1996), and therefore might not be replicated in C57BL/6 mice. However, Figure 3.7 shows that despite the use of C57BL/6 mice in this study, there was no improvement in AAI disease readouts, including eosinophilia in BAL, following PIT.

Janssen et al found that maximal *in vitro* recall responses of lung draining LN from OVA sensitised and challenged BALB/c mice were 30% lower to pOVA than to OVA (Janssen et al., 1999). Interestingly, while IL-5 and IL-4 were found in OVA stimulated cultures, only IL-5 was detected from pOVA stimulated cultures, leading them to conclude that this might be implicated in the increased BAL eosinophilia in pOVA treated mice. In initial *in vitro* recall experiments (such as the ones shown in Figure 3.4 and Figure 3.9), responses were only assessed to OVA and not to pOVA. In Figure 3.10 and 3.11, however, no proliferative or IL-5 recall response to pOVA could be elicited from splenocytes from OVA sensitised mice (regardless of whether or not they had received pOVA PIT). This meant that no conclusions as to whether or not pOVA PIT had reduced the pOVA-reactive $CD4^+$ T cell response could be drawn. The lack of an *in vitro* response of splenocytes to pOVA may have been because in these experiments, mice were only sensitised to OVA and not challenged prior to assessment of pOVA recall responses (with the aim of shortening experimental time courses). Yet, Sun et al were similarly unable to demonstrate pOVA recall responses from splenocytes of OVA sensitised and challenged BALB/c mice (Sun et al., 2010).

The lack of a demonstrable pOVA recall response following sensitisation with OVA implied that epitopes other than, or in addition to, pOVA might be important in the overall OVA-driven response in this model. A recent study by Yang et al gave support to this. In a model of OVA-induced food allergy in BALB/c mice, they

found that pOVA PIT given subcutaneously (using a multi-dose regime) at time-points after oral OVA sensitisation, led to worse anaphylaxis scores and increased histamine release upon oral OVA challenge, compared to mice that did not receive pOVA PIT (Yang et al., 2010). However, the same group have also recently identified three further potential $CD4^+$ T cell epitopes within OVA in addition to the epitope contained in pOVA, in the context of BALB/c mice (Yang and Mine, 2009). Treating mice with a combination of two of these peptides together with pOVA, significantly reduced anaphylaxis scores and histamine release upon subsequent oral OVA challenge (Yang et al., 2010).

In summary, there are several possibilities as to why pOVA PIT did not improve AAI in this model:-

i) Tolerance of pOVA-reactive T cells might not have been achieved in this model. The failure to detect pOVA recall responses from splenocytes of OVA sensitised mice means that this cannot be ruled out.
ii) An inherent property of the response to soluble pOVA is a (Th2) inflammatory response to OVA which can lead to enhanced disease.
iii) Additional OVA $CD4^+$ T cell epitopes might be important in driving OVA responses in C57BL/6 mice. Such epitopes are however likely to be different to those described by Yang et al since $CD4^+$ T cells in C57BL/6 mice will recognise peptide in the context of I-A^b MHC class II molecules rather than I-A^d as for BALB/c mice.

3.3.3 Concluding remarks

The findings in this chapter demonstrated that:-

- a model of OVA-induced AAI in C57BL/6 mice has been established.
- high dose pOVA PIT given intravenously prior to induction of AAI, did not reduce the severity of AAI.

The experiments described in subsequent chapters therefore sought to address:-

i) whether tolerance of pOVA-reactive $CD4^+$ T cells was being achieved (Chapter 4).

ii) whether additional $CD4^+$ T cell OVA epitope(s) are implicated in driving OVA-induced AAI in C57BL/6 mice and, if so, whether these are capable of improving AAI when given in the form of PIT (Chapter 5).

4 The effects of pOVA PIT on traceable pOVA-reactive $CD4^+$ T cells (OT-II cells) in models of Th2 sensitisation and AAI

4.1 Introduction

The data in Chapter 3 demonstrated that soluble pOVA given intravenously prior to the induction of OVA-induced AAI, did not improve disease (Figure 3.7,3.8). Since no measurable *in vitro* recall response to pOVA could reliably be elicited from splenocytes from OVA sensitised mice (Figure 3.10, 3.11), it was not possible to say from those experiments whether or not pOVA PIT had tolerised pOVA-reactive $CD4^+$ T cells. Yet the same application of soluble pOVA has previously induced tolerance of pOVA-reactive $CD4^+$ OT-II cells prior to immunisation with pOVA in CFA (Konkel et al., 2010, Hochweller and Anderton, 2005). It was thus important to determine whether or not pOVA PIT was inducing tolerance of pOVA-reactive $CD4^+$ T cells, as it was perhaps possible that pOVA-reactive $CD4^+$ T cells were less susceptible to tolerance induction in a Th2, compared to a Th1, setting.

Incorporating adoptive transfer of trackable $CD4^+$ OT-II cells (which have TCRs reactive towards pOVA), into models of OVA-driven AAI and allergic sensitisation, would enable direct assessment of the tolerising capacity of soluble pOVA on these cells in the context of Th2 mediated, OVA-driven disease. This would serve two main purposes. Firstly, if pOVA PIT was found to induce tolerance in such models, these would then provide a platform for mechanistic work involving PIT in allergic and AAI settings, based on the trackable nature of the OT-II cells. Secondly, if pOVA PIT was found to be capable of inducing tolerance of pOVA-reactive $CD4^+$ T cells in an allergic setting, this could imply that other OVA epitope(s), in addition to pOVA, were implicated in driving OVA-induced AAI; this could perhaps then account for the lack of therapeutic effect of pOVA PIT in OVA-induced AAI, seen in Chapter 3.

4.1.1 Aims

The experiments described in this chapter therefore aimed to:

i) Develop models of Th2 sensitisation and AAI which incorporated adoptive transfer of trackable pOVA-reactive (OT-II) $CD4^+$ T cells.
ii) Assess the ability of pOVA PIT to induce tolerance in pOVA-reactive (OT-II) $CD4^+$ T cells in settings of Th2 sensitisation and AAI.

4.1.2 Approach

Initially an approach involving adoptive transfer of naïve $CD4^+$ OT-II cells followed by pOVA PIT then OVA/alum sensitisation, was taken to examine the effects of pOVA PIT on pOVA-reactive $CD4^+$ T cells in the context of Th2 sensitisation. This model was then extended to include airway challenge, to assess the effects of pOVA PIT on pOVA-reactive $CD4^+$ cells in the context of AAI. But, as discussed below, problems in tracking naïve OT-II cells over long periods of time also necessitated the development of an alternative approach. This involved the adoptive transfer of Th2 polarised $CD4^+$ OT-II cells followed by OVA challenge. This model enabled the effects of pOVA PIT in the context of AAI driven by Th2 polarised OT-II cells, to be assessed.

4.2 Results

4.2.1 Adoptively transferred CD4$^+$ OT-II cells increase in frequency and become activated following OVA/alum sensitisation

The first step was to assess the ability to track adoptively transferred CD4$^+$ OT-II cells and assess their responses to *in vivo* OVA/alum sensitisation. The approach is shown in Figure 4.1a. Following intravenous transfer of 2x10^6 CD4$^+$ OT-II cells into C57BL/6 recipients, mice received either one or two OVA/alum sensitisations or PBS/alum as a control. 7 days after the first or second sensitisation, spleen and mLN were harvested and numbers and phenotype of OT-II cells were determined by flow cytometry.

The percentage of OT-II cells in the CD4$^+$ population (Figure 4.1b) and total CD4$^+$ OT-II cells (Figure 4.1c) in the spleen, were significantly increased seven days after either one [day 7 (D7)] or two [day 14 (D14)] OVA/alum sensitisations, compared to mock sensitised controls. Although OT-II cells were still significantly increased after two OVA sensitisations compared to mock sensitised controls, percentages and total numbers of OT-II cells were reduced by around 50% compared to after only one sensitisation (Figure 4.1b,c).

OT-II expression of CD62L (a cell surface molecule highly expressed on naïve and memory T cells) and CD44 (highly expressed on effector and memory T cells), was assessed (Figure 4.2a). The majority of CD4$^+$ OT-II cells prior to adoptive transfer were CD62L^{hi}CD44^{lo} - indicating a naïve phenotype (Figure 4.2b). Numbers of OT-II cells from spleens of mock sensitised mice were too low to accurately assess CD62L and CD44 expression. However, following OVA sensitisation, two populations of OT-II cells were identified encompassing CD62L^{lo}CD44^{hi} (associated with effector T cells) and CD62L^{hi}CD44^{hi} (associated with memory T cells) (Figure 4.2c-e). The frequency of these populations was not altered by the number of OVA

sensitisations (Figure 4.2d,e). mLN data mimicked those seen in the spleen at both time points (data not shown).

4.2.2 pOVA PIT prior to OVA/alum sensitisation reduces the frequency of CD4$^+$ OT-II cells in spleen and mLN following sensitisation

The effect of pOVA PIT on CD4$^+$ OT-II cells in the context of OVA/alum sensitisation was assessed. Figure 4.3 shows the experimental approach. In order to maximise detection of OT-II cells a short protocol was initially chosen involving only one OVA/alum sensitisation. Soluble pOVA or PBS as a control was given one day after adoptive transfer of CD4$^+$ OT-II cells. Mice received one OVA/alum sensitisation on D0. OT-II cells were assessed in spleen and mLN on D7 (Figure 4.4). Since OVA/alum is given via intraperitoneal injection, there was the possibility that OT-II cells may accumulate in the peritoneal cavity. To assess this, peritoneal wash-outs were also performed and the presence of CD4$^+$ OT-II cells assessed in wash-out fluid (Figure 4.4e,f). pOVA PIT significantly reduced the percentage of OT-II cells in the CD4$^+$ population and total numbers of CD4$^+$ OT-II cells in spleen and mLN, compared to mice that received PBS (Figure 4.4a-d). Although OT-II cells were found in the peritoneal cavity, these were also reduced in the pOVA PIT group (Figure 4.4e,f).

4.2.3 pOVA PIT prior to OVA sensitisation abrogates the *in vitro* recall response to pOVA with limited impact on the response to whole OVA

Functional responses of splenocytes following pOVA PIT were determined. Splenocytes were cultured with increasing doses of pOVA or OVA and the concentration of IL-2 measured in supernatants after 48 hours (Figure 4.5). No significant difference in the concentration of IL-2 was detected between pOVA treated or PBS treated groups in response to OVA (Figure 4.5a). High concentrations of IL-2 were detected after culturing splenocytes from PBS treated mice with pOVA (Figure 4.5b). This is likely to predominantly be due to responses of CD4$^+$ OT-II cells in the spleen (quantified previously in Figure 4.4b), responding to pOVA, since

previously no pOVA-induced proliferative response of splenocytes had been detected in OVA sensitised mice in the absence OT-II adoptive transfer (discussed in Chapter 3). Importantly, the IL-2 response to pOVA was abrogated in the pOVA PIT group (Figure 4.5b). This implied that pOVA PIT had induced tolerance of pOVA-reactive $CD4^+$ OT-II cells, abrogating their IL-2 response to pOVA. However, the effect on the OVA response was limited and not significantly different to PBS treated controls. This implied that host $CD4^+$ T cell responses to epitope(s) other than pOVA had been induced by OVA sensitisation.

Culture supernatants after 72 hours were also assessed for Th2 cytokines such as IL-5 – however these were below the limit of reliable detection (data not shown). A factor in this is likely to be that mice only received one OVA sensitisation in these experiments.

No significant differences in serum OVA-specific IgE were detected between pOVA PIT or PBS treated mice, although there was wide inter-group variation (Figure 4.6).

4.2.4 Tolerance of $CD4^+$ OT-II cells induced by pOVA PIT prior to sensitisation is likely to be primarily effected via deletion

Previous work in the Anderton lab had demonstrated that soluble pOVA effected tolerance of $CD4^+$ OT-II cells by inducing a rapid proliferative response followed by deletion (Hochweller and Anderton, 2005). It was important to assess whether pOVA PIT was also inducing tolerance via deletion of OT-II cells in this setting, particularly as the experiments described here were conducted in different animal facilities to those previously used by Hochweller et al.

The approach shown in Figure 4.7 was taken in order to assess OT-II cells over a time course following pOVA PIT. $CD4^+$ OT-II cells were labelled with CFSE prior to adoptive transfer to examine *in vivo* proliferation. Two and a half days after pOVA administration, $CD4^+$ OT-II cells in spleen had already undergone proliferation as

indicated by the reduction in CFSE intensity (Figure 4.8a). There was evidence of further rounds of proliferation having taken place by D5 and D10 (Figure 4.8a). The percentage of $CD4^+$ OT-II cells in the $CD4^+$ population (Figure 4.8b) and the total number of $CD4^+$ OT-II cells in spleen (Figure 4.8c) were significantly increased on D2.5 in the pOVA PIT group compared to the PBS treated group. $CD4^+$ OT-II cells in spleens of pOVA PIT treated mice subsequently declined on D5 and D10 - indicating deletion, consistent with the findings of Hochweller et al (Hochweller and Anderton, 2005). Interestingly however, the total number of $CD4^+$ OT-II cells in spleen on D10 were still significantly raised in the pOVA PIT group compared to PBS treated controls (Figure 4.8c).

Results from iLN were also assessed (Figure 4.9a-c). Data were consistent with findings from spleen, with the exception that by D10 there was no significant difference in the total number of $CD4^+$ OT-II cells in pOVA PIT and PBS treated groups (Figure 4.9c).

On D2.5, splenocytes from pOVA PIT treated mice proliferated strongly to all concentrations of pOVA tested (although this was still a pOVA dependent response, as culturing splenocytes with medium alone resulted in low levels of proliferation) (Figure 4.10a). Splenocytes from PBS treated mice did not proliferate to pOVA (Figure 4.10a). On D5 the proliferative response to pOVA in the pOVA PIT treated group had diminished and was equivalent to background levels by D10 (Figure 4.10a). A similar but less strong proliferative response pattern to OVA was also seen (Figure 4.10b).

Overall, these data indicate that, consistent with previous data from the Anderton lab (Hochweller and Anderton, 2005), $CD4^+$ OT-II cells undergo a proliferative burst shortly after pOVA administration (seen here both in iLN and spleen), followed by a contraction phase. OT-II cells were evaluated here in spleen and iLN only, therefore there is a possibility that OT-II cells could be present in non-lymphoid organs. This

has been found previously following OT-II transfer by Reinhardt et al (Reinhardt et al., 2001). However, in that study it was shown that following soluble pOVA administration deletion of OT-II cells in lymphoid tissues was mimicked in non-lymphoid tissues (Reinhardt et al., 2001). This is, therefore, also likely to be the case in the experiments shown here, although non-lymphoid tissues were not formally examined. These findings would therefore be consistent with the deletional paradigm of tolerance induction that has previously been proposed to predominant following high dose pOVA PIT (Hochweller and Anderton, 2005, Kearney et al., 1994).

4.2.5 FoxP3 expression in $CD4^+$ OT-II cells does not increase following pOVA PIT

Typically, 3-5% of $CD4^+$ OT-II cells were found to be $FoxP3^+$ prior to adoptive transfer (as shown in the example in Figure 4.11a). The percentage of host $CD4^+$ cells and $CD4^+$ OT-II cells were assessed on D2.5, D5 and D10 following pOVA PIT (Figure 4.11b). The number of OT-II cells in the group that received PBS was too low to accurately assess the percentage of $FoxP3^+$ OT-II cells (data not shown). Following pOVA PIT, the percentage of $CD4^+$ OT-II cells that were $FoxP3^+$ was lower on D2.5 than seen pre-transfer and did not increase thereafter (Figure 4.11c). Total numbers of $FoxP3^+$ $CD4^+$ OT-II cells fell from D2 to D10 (Figure 4.11d), reflecting the overall reduction in total $CD4^+$ OT-II cell numbers. The percentage of $FoxP3^+$ cells in the host $CD4^+$ population was not altered following pOVA PIT (data not shown).

4.2.6 $CD4^+$ OT-II cells are difficult to track over the time required for the full AAI model

So far, the tolerogenic effects of pOVA PIT on $CD4^+$ OT-II cells had only been demonstrated in the context of one OVA sensitisation. To extend this in order to examine the effects of pOVA PIT in the context of AAI, the approach shown in Figure 4.12 was taken. $CD4^+$ OT-II cells were transferred one day prior to soluble pOVA or PBS, and this was followed by the same protocol that had been used to induce AAI in wild type mice (without OT-II cell transfer) in Chapter 3. Very few

$CD4^+$ OT-II cells were detectable in mLN in either group (Figure 4.13a,b). In several mice from each group no OT-II cells were detectable in mLN (Figure 4.13a,b). There was no significant difference in lung inflammation scores (Figure 4.13c), the percentage of goblet cells in the airways (Figure 4.13d) or OVA-specific IgE (4.13e) between pOVA PIT or PBS treated groups. BAL readouts were not significantly different between the groups (Figure 4.14a-e)

Poor OT-II cell viability over the 40 day experiment described above most likely accounted for the low (even absent) numbers of OT-II cells in mLN even in some PBS treated mice. Different approaches were therefore taken to reduce the experimental length. The seven day period between pOVA PIT administration or PBS was kept constant. In two different experiments two OVA sensitisations were given either 7 or 14 days apart. $CD4^+$ OT-II cells in spleen were assessed 7 days after the last sensitisation. In both experiments however, OT-II cells could not consistently be detected and were often undetectable even in PBS treated groups (data not shown).

4.2.7 Adoptive transfer of $CD4^+$ OT-II cells, one OVA sensitisation followed by OVA challenge does not lead to an OT-II driven AAI phenotype

It therefore appeared that OT-II cells could not be reliably detected in the context of pOVA PIT or PBS followed by two OVA sensitisations. Data in Figure 4.1b,c had indicated that $CD4^+$ OT-II cell numbers were higher after one sensitisation compared to after two sensitisations. It was therefore postulated that adoptive transfer of $CD4^+$ OT-II cells followed by only one OVA sensitisation and then airway challenge may be sufficient to induce AAI and improve OT-II detection (this approach is shown in Figure 4.15). It was anticipated that an increased frequency of $CD4^+$ OT-II cells at the time of OVA airway challenge would lead to more severe AAI in comparison to mice that did not receive OT-II cell transfer. If so, then this $CD4^+$ OT-II driven AAI phenotype could then be utilised to assess the effects of pOVA PIT on $CD4^+$ OT-II cells in the context of AAI.

In previous experiments airway challenges were given intranasally. It was therefore possible that i) the exact dose of OVA reaching the lungs following intranasal challenge may vary between mice and ii) that OT-II cells were likely to track to nasal tissues as well as to the lung. To bypass these issues, it was decided to use an intratracheal approach in this, and all subsequent experiments. This more invasive approach meant that intratracheal challenges were given once every three days (see approach in Figure 4.15), compared to the consecutive intranasal challenges used previously. Importantly however, mice continued to receive a total of three airway challenges.

Previous experiments had also only assessed $CD4^+$ OT-II cells within mLN and spleen and not within lung tissue. Difficulties in detecting OT-II cells may therefore have been because they were mainly within the lung following OVA challenge. In this experiment, therefore, the left lung lobe was digested and OT-II cells detected using flow cytometry. The remaining lung was assessed histologically. Few $CD4^+$ OT-II cells were found in the left lung lobe or mLN after PBS challenge, whereas significant numbers were present in both tissues following OVA challenge (Figure 4.16a-d). Total numbers of $CD4^+$ OT-II cells in the spleen were not significantly different regardless of whether mice were challenged with PBS or OVA (Figure 4.16e,f).

BAL data from the same experiment are shown in Figure 4.17a,b. Importantly, very few eosinophils were detected in mice that received $CD4^+$ OT-II cells and OVA challenge (Figure 4.17b). In contrast, a higher percentage of eosinophils were detected in BAL from mice that had had OVA challenge in the absence of OT-II transfer (Figure 4.17b). Of note, the percentage of eosinophils in BAL in mice that did not receive $CD4^+$ OT-II cells constituted around 10% of total BAL cells - substantially lower than the percentage of eosinophils seen after two OVA sensitisations followed by OVA challenge in Chapter 3 (Figure 3.3b). Lung inflammation scores were low in all groups (Figure 4.17c) – similar inflammation

scores were found in OVA challenged mice regardless of whether or not they had had OT-II cell transfer.

These data indicated that although many $CD4^+$ OT-II cells were present in the lung following OVA challenge, they did not appear to be generating a Th2 effector response. Since significant eosinophilia was only detected in BAL from mice that had OVA challenge in the absence of OT-II cell transfer, this implied that the OT-II response to OVA was likely to be counteracting the host $CD4^+$ Th2 responses to OVA. One OVA sensitisation did not therefore appear to induce strong and/or stable Th2 polarisation of $CD4^+$ OT-II cells.

4.2.8 $CD4^+$ OT-II cells can be polarised to a Th2 phenotype *in vitro* and their phenotype is comparable to Th2 polarised DO11.10 $CD4^+$ cells

It was therefore important to examine whether OT-II cells could be polarised to a Th2 phenotype *in vitro*. The approach shown in Figure 4.18a was taken. $CD4^+$ cells from transgenic DO11.10 mice, which also have TCRs reactive to pOVA 323-339 but are on the BALB/c background, were included as a comparison.

Supernatants from OT-II and DO11.10 Th2 polarisation cultures both contained high concentrations of IL-13 (Figure 4.18b). IL-5 was present in supernatants from DO11.10 and OT-II cultures but a much higher concentration was detected in supernatants from DO11.10 cell cultures (Figure 4.18b).

$CD4^+$ cells were isolated after Th2 polarisation, rested for three days in IL-2, then cultured with irradiated strain-matched splenocytes and OVA or pOVA. Freshly isolated $CD4^+$ cells from naïve DO11.10 or OT-II mice were also cultured. Th2 polarised $CD4^+$ OT-II cells produced the most IL-2 upon culture with OVA, naïve OT-II cells had the second highest IL-2 response (Figure 4.18c). Th2 polarised and

naïve DO11.10 $CD4^+$ cells had similar IL-2 responses to OVA (Figure 4.18c). Concentrations of IL-2 produced in response to pOVA were above the limit of detection for all groups (data not shown).

IL-13 and IL-5 were produced by Th2 polarised OT-II and DO11.10 $CD4^+$ cells in a dose dependent manner to OVA (Figure 4.18d,e). In contrast to the lower IL-5 concentration in supernatant from OT-II Th2 polarisation cultures compared to DO11.10 Th2 polarisation cultures (Figure 4.18b), upon recall to OVA there was no inability of Th2 polarised $CD4^+$ OT-II cells to produce IL-5, compared to Th2 polarised DO11.10 $CD4^+$ cells (Figure 4.18e). IL-13 and IL-5 in Th2 DO11.10 and OT-II cultures were above limits of detection for all doses of pOVA tested (Figure 4.18 f,g). Naïve $CD4^+$ T cells from either OT-II or DO11.10 mice produced IL-13 more efficiently in response to pOVA than to OVA (Figure 4.18d,f).

Intracellular cytokine staining for Th2 cytokines was performed numerous times on $CD4^+$ T cells after 4 days of culture in Th2 polarising conditions. However, IL-5, IL-13 or IL-4 were not detected intracellularly (data not shown). However, IL-13 was detected intracellularly in $CD4^+$ OT-II cells after 6 days of culture in Th2 polarising conditions and not in cells cultured in Th1 or Th0 conditions (Figure 4.19a,b). There was also increased expression of the Th2-associated transcription factor GATA-3 in $CD4^+$ OT-II cells after 6 days of culture in Th2 polarising conditions (Figure 4.19c). GATA-3 expression was greater in the Th2 $CD4^+$ OT-II cells when compared to either Th0 or Th1 polarised $CD4^+$ OT-II cells, as evidenced by the increase in mean fluorescence intensity (Figure 4.19c). No IL-4 or IL-5 was detected in Th2 polarised $CD4^+$ OT-II cells by intracellular staining after 6 days of culture (data not shown).

In summary, naïve OT-II cells could be effectively polarised to a Th2 phenotype *in vitro*. Following Th2 polarisation $CD4^+$ OT-II cells produced more IL-2 in response to OVA compared to that produced by naïve $CD4^+$ cells, and *in vitro* recall responses to OVA and to pOVA were associated with the production of Th2 cytokines.

4.2.9 Adoptive transfer of Th2 polarised $CD4^+$ OT-II cells induces AAI following OVA airway challenge

Previous studies have shown that adoptive transfer of Th2 polarised pOVA-reactive $CD4^+$ cells followed by airway challenge induces features of AAI (Cohn et al., 1997, Jacobsen et al., 2008, Hansen et al., 1999) The approach shown in Figure 4.20 was used to assess whether OT-II Th2 cells, generated as shown in Figure 4.18, were capable of inducing AAI. $4.5x10^6$ OT-II cells were transferred, consistent with some other studies (Cohn et al., 1997).

Adoptive transfer of Th2 polarised $CD4^+$ OT-II cells followed by OVA challenge induced pulmonary inflammation (Figure 4.21a,b). Peri-vascular inflammation scores were significantly increased in the group that received Th2 polarised $CD4^+$ OT-II cells followed by OVA challenge compared to mice that received naïve $CD4^+$ OT-II cells and PBS challenge (Figure 4.21b). Regarding peri-bronchiolar inflammation, this was significantly increased in the group that received Th2 polarised $CD4^+$ OT-II cells followed by OVA challenge compared to the group that received Th2 polarised $CD4^+$ OT-II cells and PBS challenge (Figure 4.21b). No significant difference was found between the inflammation scores of mice that received Th2 polarised $CD4^+$ OT-II cells followed by OVA challenge compared to mice that received naïve $CD4^+$ OT-II cells and OVA challenge, although it would be interesting to assess whether this was still the case if the experiment was repeated with larger group sizes. Inflammation scores were higher than those seen previously in AAI induced without OT-II transfer (Figure 3.2b). Interestingly, transfer of $4.5x10^6$ naïve $CD4^+$ OT-II cells followed by OVA challenge also induced some pulmonary inflammation (Figure 4.21a,b). Goblet cells were seen in both groups that received OVA challenge regardless of whether the $CD4^+$ cells transferred were naïve or Th2 polarised (Figure 4.21 c,d). However, total BAL cells and the percentage and total numbers of eosinophils in BAL were only significantly raised following Th2 $CD4^+$ OT-II transfer and OVA challenge (Figure 4.22a-e). These data are compatible with some IL-13 but little IL-5 previously detected in supernatants from naïve $CD4^+$

OT-II cells after *in vitro* culture with OVA (Figure 4.18d,e). OVA-specific IgE was detectable in mice that received Th2 polarised $CD4^+$ OT-II cells and OVA challenge OVA, but not in controls (Figure 4.22f). This was surprising given the short timeframe of this experiment. However, OVA-specific IgE was not detected in subsequent similar experiments.

$CD4^+$ OT-II cells in the left lung lobe were significantly increased in the group that received Th2 $CD4^+$ OT-II and OVA challenge compared to all other groups (Figure 4.23a,b). The percentage of OT-II cells in the $CD4^+$ population in the left lung lobe was not significantly different between mice that received naive $CD4^+$ OT-II cells and OVA challenge versus mice that received Th2 $CD4^+$ OT-II cells and PBS challenge (Figure 4.23a).

4.2.10 pOVA PIT prior to OVA challenge significantly improves Th2 $CD4^+$ OT-II driven AAI

The effects of pOVA PIT on Th2 polarised $CD4^+$ OT-II driven AAI were examined using the approach in Figure 4.24. pOVA PIT prior to OVA challenge resulted in lower pulmonary inflammation scores (Figure 4.25a,b) and a lower percentage of goblet cells in small airways (Figure 4.25c,d) compared to PBS treated, OVA challenged controls. There were significantly fewer total BAL cells and significantly fewer eosinophils in BAL from pOVA PIT treated OVA challenged mice compared to PBS treated OVA challenged controls (Figure 4.26a-d). Examples of BAL cytospins from pOVA treated versus PBS treated OVA challenged mice are shown in Figure 4.26c where the reduction in the frequency of eosinophils in BAL following pOVA PIT can be seen. Total lymphocytes in BAL were also significantly reduced in the pOVA PIT treated OVA challenged group compared to the PBS treated OVA challenged group (Figure 4.26f). There was no significant difference in the total number of macrophages in BAL between groups (Figure 4.26e).

Reduced AAI severity was associated with a significant reduction in $CD4^+$ OT-II cells in the lung in pOVA PIT treated OVA challenged mice compared to PBS

treated OVA challenged mice (Figure 4.27a,b). Total $CD4^+$ cells in the lungs of PBS treated OVA challenged mice were also increased, and were greater than the total number of lung $CD4^+$ OT-II cells, indicating an infiltration of host $CD4^+$ cells into the inflamed lung (Figure 4.27c). The percentage of OT-II cells in the $CD4^+$ population and the total number of OT-II cells in the lung in the pOVA PIT treated group were not significantly different to PBS challenged controls (Figure 4.27a,b). There was no significant reduction in $CD4^+$ OT-II cells in the mLN of pOVA PIT treated OVA challenged mice compared to PBS treated OVA challenged mice (Figure 4.27d,e). There was no significant difference in the percentages or total numbers of OT-II cells in the spleen between groups (Figure 4.27g,h). Hence, improvements in disease in the pOVA PIT group were associated with a marked reduction in total $CD4^+$ OT-II cells in the lung, but no significant difference was found in total $CD4^+$ OT-II cells in the mLN and spleen between the two groups.

Prior to adoptive transfer, Th2 polarised $CD4^+$ OT-II cells consisted of a mixture of $CD62L^{lo}CD44^{hi}$ (associated with effector cells) and $CD62L^{hi}CD44^{hi}$ (associated with memory cells) populations (Figure 4.28a). On D8, the majority of $CD4^+$ OT-II cells in the left lung lobe displayed an effector phenotype in all groups (Figure 4.28b,c). There was no significant difference in the percentages of effector or memory cells in the $CD4^+$ OT-II population in lung, mLN or spleen between pOVA PIT treated OVA challenged or PBS treated OVA challenged mice (Figure 4.28b-g).

The concentration of IL-10 in BAL fluid was very low and did not differ significantly between groups (data not shown). In addition, in these experiments, OVA-specific IgE was not detected in any group (data not shown).

4.2.11 pOVA PIT does not induce FoxP3 expression in $CD4^+$ OT-II cells in Th2 $CD4^+$ OT-II driven AAI

FoxP3 expression in Th2 polarised $CD4^+$ OT-II cells was low (typically around 0.5%) at the time of adoptive transfer (Figure 4.29a). FoxP3 expression in $CD4^+$ OT-II cells in the lung remained low on D8 and was not significantly altered following

pOVA PIT with or without OVA challenge (Figure 4.29b,c). The expression of FoxP3 in host $CD4^+$ T cells was also unaffected (Figure 4.29d).

4.2.12 $CD4^+$ OT-II cells are increased in pOVA PIT treated mice compared to PBS treated mice prior to first challenge in Th2 OT-II driven AAI

The percentage of OT-II cells in the $CD4^+$ population in peripheral blood was assessed on D-1 i.e. one day prior to OVA challenge, and was significantly increased in pOVA PIT treated mice compared to PBS treated controls (Figure 4.30). It was possible that $CD4^+$ OT-II cells within peripheral LNs and spleen were at a lower frequency than seen in blood at this time point and also that the percentage of $CD4^+$ OT-II cells may have reduced (i.e. as a consequence of deletion) in the 24 hours before the first OVA challenge on D0. A separate experiment was therefore carried out to assess $CD4^+$ OT-II cells in blood, peripheral LNs and spleen and their functional responses on D0 prior to OVA challenge (see approach Figure 4.31).

The percentage of OT-II cells in the $CD4^+$ population in peripheral blood was still significantly higher in the pOVA PIT group compared to PBS treated controls on D0 (Figure 4.32a) and higher than seen on D-1 in the previous experiment (Figure 4.30). Percentages and total numbers of $CD4^+$ OT-II cells were also increased in iLN and spleen (Figure 4.32b-e).

Concentrations of IL-2 in supernatants from *in vitro* splenocyte cultures were very high in both groups, even in the absence of antigen (Figure 4.33a,b). Overall, more IL-2 was produced by splenocytes from PBS treated mice, although a dose dependent response to antigen was only seen in response to pOVA (Figure 4.33b). IL-5 production was similar in both groups, although again this was not solely an antigen dependent response (Figure 4.33c,d). Therefore, despite around five times as many $CD4^+$ OT-II cells present in spleens of pOVA PIT treated mice (Figure 4.32e), IL-2 and IL-5 production from splenocytes was not higher than from splenocytes from PBS treated mice (Figure 4.33a-d). Notably, IL-10 was produced by splenocytes

from pOVA PIT treated mice in a dose-dependent manner to pOVA (Figure 4.33f). This was not seen in cultures derived from PBS treated controls (Figure 4.33f). Whether this was on account of the greater frequency of OT-II cells in the pOVA PIT group, or the induction of, or a skewing towards IL-10 production by OT-II cells, is unclear from these experiments. No dose dependent IL-10 response to OVA was seen in either group (Figure 4.33e).

Figure 4-1: Adoptively transferred CD4$^+$ OT-II cells are increased after OVA sensitisation

Figure 4-2: Adoptively transferred CD4$^+$ OT-II cells become activated after OVA sensitisation

Figure 4-3: Experimental approach to assess OT-II cells after one OVA sensitisation +/- pOVA PIT

Figure 4-4: pOVA PIT prior to OVA sensitisation reduces the number of OT-II cells in spleen and mLN

Figure 4-5: pOVA PIT prior to OVA sensitisation abrogates the recall response to pOVA with limited impact on the IL-2 responsess to OVA

Figure 4-6: pOVA PIT prior to OVA sensitisation does not significantly alter OVA-specific IgE

Figure 4-7: Experimental approach to assess the fate of OT-II cells following pOVA PIT

Figure 4-8: pOVA PIT induces proliferation of OT-II cells in the spleen followed by deletion

Figure 4-9: pOVA PIT induces proliferation of OT-II cells followed by deletion in peripheral LN

Figure 4-10: Time course of OVA and pOVA proliferative recall responses following pOVA PIT

Figure 4-11: pOVA PIT does not increase FoxP3 expression in CD4$^+$ OT-II cells

Figure 4-12: Experimental approach for incorporating $CD4^+$ OT-II transfer into the AAI model +/- pOVA PIT

Figure 4-13: Difficulties in tracking adoptively transferred $CD4^+$ OT-II cells to D32

Figure 4-14: Adoptive transfer of naïve CD4$^+$ OT-II cells followed by pOVA PIT and induction of AAI does not alter BAL readouts on D32

Figure 4-15: Experimental approach examining the effects of naïve $CD4^+$ OT-II cell transfer, one OVA sensitisation and OVA challenge

Figure 4-16: CD4$^+$ OT-II cells are present in the lung after one sensitisation and OVA challenge

Figure 4-17: Adoptive transfer of CD4$^+$ OT-II cells, one OVA sensitisation followed by OVA challenge does not induce an OT-II driven AAI phenotype

Figure 4-18: CD4$^+$ OT-II cells can be polarised to a Th2 phenotype *in vitro*

Figure 4-19: IL-13 and GATA-3 are detectable intracellularly in CD4$^+$ OT-II cells after 6 days of culture in Th2 polarising conditions

Figure 4-20: Experimental approach for experiments examining the potential of Th2 polarised $CD4^+$ OT-II cells to induce AAI

Figure 4-21: Adoptively transferred Th2 polarised CD4$^+$ OT-II cells induce pulmonary inflammation consistent with AAI upon OVA airway challenge

Figure 4-22: Adoptive transfer of Th2 polarised CD4$^+$ OT-II cells followed by OVA challenge induces BAL changes consistent with AAI and can also induce OVA-specific IgE

Figure 4-23: Induction of AAI following adoptive transfer of Th2 OT-II cells is associated with increased $CD4^+$ OT-II cells in the lung

Figure 4-24: Experimental approach to examine the effects of pOVA PIT on Th2 OT-II driven AAI

Figure 4-25: pOVA PIT prior to OVA challenge improves pulmonary inflammation in Th2 OT-II driven AAI

Figure 4-26: pOVA IT prior to OVA challenge improves BAL readouts in Th2 OT-II driven AAI

Figure 4-27: Therapeutic effects of pOVA PIT in Th2 OT-II driven AAI are associated with fewer $CD4^+$ OT-II cells in the lung on D8

Figure 4-28: Assessing activation status of CD4$^+$ OT-II cells in the context of pOVA PIT in Th2 OT-II driven AAI

Figure 4-29: Characterisation of FoxP3 expression of $CD4^+$ OT-II and host cells after pOVA PIT in Th2 OT-II driven AAI

Figure 4-30: The percentage of OT-II cells in the $CD4^+$ population in blood is increased in pOVA PIT treated mice on D-1

Figure 4-31: Experimental approach for functional assessment of Th2 polarised CD4$^+$ OT-II cells 4 days after pOVA PIT

Figure 4-32: CD4$^+$ OT-II cells are elevated in blood, spleen and peripheral LN on D0 following pOVA PIT

Figure 4-33: Splenocytes harvested 4 days following pOVA PIT produce IL-10 in response to pOVA *in vitro*

Figure 4-34: Overview of the outcomes from the OT-II adoptive transfer models described in Chapter 4

4.3 Discussion

4.3.1 Overview of the experimental findings in this chapter

Figure 4.34a-d summarises the outcomes of the experiments involving adoptive transfer of naïve $CD4^+$ OT-II cells in this chapter. Although pOVA PIT induced tolerance of naïve $CD4^+$ OT-II cells in the context of one OVA/alum sensitisation, one sensitisation was insufficient to induce a reliable Th2 recall response (Figure 4.34a). However, OT-II cells were difficult to track in longer models (Figure 4.34b,c). It is probable that poor viability of naïve $CD4^+$ OT-II cells over the course of these experiments was a major contributory factor to these difficulties. Indeed, rapid attrition of naïve $CD4^+$ T cells has been reported by others, using a different transgenic system (Hu et al., 2001). Such problems are not associated with previously published models examining pOVA PIT after adoptive transfer of naïve OT-II or DO11.10 cells, because pOVA/CFA immunisations in these studies generate a strongly polarised immune response after only one immunisation (Kearney et al., 1994, Konkel et al., 2010). Furthermore, although the shortened approach shown in Figure 4.34d, consisting of one sensitisation and three challenges, enabled detection of OT-II cells in lung, LN and spleen; AAI was not induced, suggesting insufficient or unstable polarisation to a pathogenic Th2 phenotype. In contrast, in the model shown in Figure 4.34e, which incorporated Th2 polarised OT-II cells, AAI was induced, OT-II cells were easily trackable and pOVA PIT was found to reduce the severity of AAI.

A further outcome from the experiments in this chapter was that in the OVA/CFA setting, pOVA PIT tolerised the pOVA response with limited impact on the overall response to OVA (Figure 4.5). This suggested that other T cell epitope(s) in addition to pOVA were likely to be involved in the overall response to OVA.

This discussion will focus on the effects of pOVA PIT in the experiments involving the adoptive transfer of i) naïve $CD4^+$ OT-II cells, ii) Th2 OT-II cells, and then discuss the issues surrounding the potential for additional OVA T cell epitope(s).

4.3.2 Mechanisms of tolerance induction of naïve CD4$^+$ OT-II cells using pOVA PIT in the context of one OVA/alum sensitisation

Figures 4.3-4.5 indicate that pOVA PIT prior to one OVA/alum sensitisation induced tolerance of pOVA-reactive CD4$^+$ OT-II cells. This is evident from the reduction in CD4$^+$ OT-II cells in spleen and peripheral LN seven days after OVA sensitisation (Figure 4.4a-d), together with the abrogation of *in vitro* IL-2 production by splenocytes in response to pOVA (Figure 4.5b). These findings are consistent with previous data from the Anderton lab demonstrating the ability of pOVA PIT to induce tolerance of naïve CD4$^+$ OT-II cells prior to pOVA/CFA immunisation (Konkel et al., 2010, Hochweller and Anderton, 2005).

Previous studies using adoptive transfer of transgenic pOVA-reactive CD4$^+$ T cells (both DO11.10 cells into BALB/c mice as well as OT-II cells into C57BL/6 mice), have shown that intravenous pOVA PIT induces rapid proliferation of pOVA-reactive CD4$^+$ cells (Kearney et al., 1994, Hochweller and Anderton, 2005). The total number of the pOVA-reactive T cells tends to peak three days after pOVA PIT and this peak is followed by a contraction phase, most rapid 3-5 days after pOVA PIT (Kearney et al., 1994, Hochweller and Anderton, 2005). This has been proposed to occur due to the wholesale deletion of pOVA-reactive CD4$^+$ cells, as a consequence of insufficient upregulation of the costimulatory molecule CD40 on DCs, in conditions promoting tolerance (Hochweller and Anderton, 2005). Hence, although OT-II cells do upregulate the CD40 ligand (CD154) in response to the efficient TCR ligation provided by pOVA PIT, the lack of strong CD40-CD154 ligation results in a failure of OX40L upregulation on the DC (Hochweller and Anderton, 2005). This prevents the upregulation of the anti-apoptotic proteins Bcl-2 and Bcl-xL by the responding T cell, which is known to occur upon OX40L on the DC ligating to OX40 on the T cell (Hochweller et al., 2006a, Hochweller and Anderton, 2005, Rogers et al., 2001). Indeed, administering an OX40 agonist antibody 2 days after high-dose PIT has been found to prevent tolerance induction and inhibits the contraction phase, in a model utilising transgenic CD4$^+$ T cells reactive to

cytochrome c (Bansal-Pakala et al., 2001). The data in Figures 4.7-4.9 are consistent with a proliferative phase followed by a contraction phase. In the spleen, loss of $CD4^+$ OT-II cells had evidently begun by day 5 after pOVA PIT (Figure 4.8b,c). In the iLN however, such loss appeared to occur predominantly after day 5 and prior to day 10 (Figure 4.9b,c).

Despite this evidence supporting the deletional tolerance paradigm, total numbers of $CD4^+$ OT-II cells were still higher in the spleen 10 days after pOVA PIT compared to PBS treated mice (Figure 4.8c). By extrapolation, this means that the number of $CD4^+$ OT-II cells in spleens of pOVA PIT treated mice would also be raised compared to PBS treated controls on the day of OVA/alum sensitisation for the experiments shown in Figure 4.3-4.6. Therefore, in the pOVA PIT group, the reduction in the total number of $CD4^+$ OT-II cells seven days after OVA sensitisation and the abrogation of IL-2 response to pOVA (Figure 4.4b,d and Figure 4.5), occurs despite an increased number of $CD4^+$ OT-II cells in spleen at the time of OVA sensitisation. So why is this the case?

The *de novo* expression of FoxP3 by naïve $CD4^+$ T cells has been associated with tolerance induction in OVA-reactive (DO11.10) $CD4^+$ T cells when OVA is expressed as a self-antigen in recipient mice (Thompson et al., 2011), and after continuous delivery of very low dose peptide (Verginis et al., 2008). Importantly here, the percentage of $CD4^+$ OT-II cells that were $FoxP3^+$ did not change in response to pOVA PIT (Figure 4.11), indicating that OT-II cells are not converting to $FoxP3^+$ T regulatory cells following pOVA PIT. Pape et al have, however, previously shown that a small population of pOVA-reactive $CD4^+$ T cells, (DO11.10 cells in that study), do escape deletion following pOVA PIT, but remain in a state of anergy (Pape et al., 1998). Such cells had a reduced, but not abrogated, capacity to proliferate *in vivo* following pOVA/CFA immunisation (Pape et al., 1998). pOVA-reactive $CD4^+$ T cells that escaped deletion following pOVA PIT also displayed a reduced ability to produce IL-2 and TNF-α in response to pOVA stimulation *in vitro*

12 days after pOVA PIT (Pape et al., 1998). A similar anergic phenotype has also been found by others in similar studies (London et al., 2000).

In summary, the data in Figures 4.3-4.10 are consistent with pOVA PIT inducing a proliferative burst in naïve $CD4^+$ OT-II cells, followed by an extensive contraction phase. A population of $CD4^+$ OT-II cells escape deletion and are most likely to be in a state of anergy, consistent with previous studies.

4.3.3 Issues related to inducing tolerance of antigen-experienced T cells

One reason why the Th2 OT-II approach was not favoured initially was because recently activated or memory T cells are often thought to be more resistant to tolerance induction, compared to naïve T cells (Liu and Wraith, 1995, Kurts, 2010). Previously activated cells, particularly memory cells, can proliferate in response to low concentrations of antigen *in vitro* (London et al., 2000). Memory T cells also rely less on costimulation. For example, London et al found that *in vitro* proliferation of memory $CD4^+$ DO11.10 cells was unaffected when APC lacked CD40, and was only minimally reduced when APC lacked CD80 and CD86 (London et al., 2000). Memory and effector $CD4^+$ T cells, in contrast to naïve $CD4^+$ T cells, have also been found capable of responding to antigen presented by APC with low costimulatory ability, such as non-activated B cells (Croft et al., 1994). Hence, these reduced costimulatory requirements and heightened antigen sensitivity, may mean that PIT is less likely to induce tolerance of effector/memory T cells. Indeed, several studies have been unable to induce tolerance in predominantly memory T cell populations using OVA protein orally (Chung et al., 1999), intravenous high dose pOVA (London et al., 2000) or, perhaps unsurprisingly given the apparent lack of dependence on costimulation via CD40L, using CD40L blockade (Chen et al., 2004). Interestingly, other studies have been able to demonstrate tolerance in effector and/or memory populations. Nasreen et al found that transferring memory $CD4^+$ OT-II cells (generated *in vitro* after 7 days of culture with pOVA), into transgenic mice in which steady-state DCs expressed OVA, led to tolerance (Nasreen et al., 2010). In that

study, an initial proliferation phase was followed by a contraction phase, similar to that described here in respect to the response of naïve OT-II cells to soluble pOVA (Nasreen et al., 2010). Furthermore, Higgins et al found that high dose intravenous PIT induced tolerance of hemagglutinin (HA)-reactive transgenic $CD4^+$ cells, 6 days after infection with vaccinia virus expressing HA, i.e. when cells were likely to be of an effector phenotype (Higgins et al., 2002). Campbell et al were also able to demonstrate therapeutic improvement using PIT, given only one day after allergen airway challenge; again when many allergen-reactive effector T cells were likely to be present (Campbell et al., 2009). Therefore, although it may be more difficult, it is still possible to induce tolerance of antigen-experienced T cells, as found here in the experiments shown in Figure 4.25-4.26.

In summary, while pOVA PIT did not improve OVA-induced AAI in Chapter 3, pOVA PIT did improve disease when AAI was solely driven by Th2 polarised pOVA-reactive $CD4^+$ cells, even though these cells were antigen-experienced. A key question is whether tolerance induced by pOVA PIT in the Th2 transfer model is effected by the same mechanisms that are thought to be responsible for inducing tolerance of naïve $CD4^+$ OT-II cells i.e. predominantly deletional? To address this requires further discussion of the nature of the transferred Th2 cells in this model.

4.3.4 What is the activation status of the Th2 polarised OT-II cells upon transfer?

It is not possible from the experiments conducted in this chapter to specify the exact effector/memory status of the transferred Th2 OT-II population. However, the pre-transfer Th2 polarised OT-II population was $CD44^{hi}$ with a mixture of $CD62L^{lo}$ and $CD62L^{hi}$ expressing cells (Figure 4.28a). This could indicate a mixed effector/memory population (MacLeod et al., 2009, Sprent and Surh, 2002). The findings of Hu et al are particularly relevant here. They used a similar protocol to polarise transgenic $CD4^+$ T cells reactive to cytochrome c, to a Th2 phenotype (Hu et al., 2001). Cells were then adoptively transferred into MHC $II^{-/-}$ mice or rested for a

further 3 days in medium alone and then transferred (Hu et al., 2001). On the day of transfer, 57% of non-rested Th2 $CD4^+$ cells were actively proliferating compared to only 6% of cells after resting (Hu et al., 2001). Adoptive transfer of non-rested Th2 $CD4^+$ cells led to an expansion phase followed by a contraction phase, resulting in a long-lived population (Hu et al., 2001). In contrast, although transfer of rested Th2 $CD4^+$ cells also resulted in a long-lived population, this occurred in the absence of a proliferation/contraction phase (Hu et al., 2001).

The findings of Hu et al, together with the CD44/CD62L expression of the Th2 cells pre-transfer shown in Figure 4.28a, imply that:-

i) it is likely that the Th2 OT-II cells transferred here contained a mixture of effector and memory T cells, or at least cells that were capable of becoming memory T cells.

ii) Th2 OT-II cells were likely to have proliferated initially following transfer since no rest period was included in these experiments. This may be implicated in the high cytokine concentrations detected in cultures in the absence of antigen, 4 days after adoptive transfer (Figure 4.33).

4.3.5 How might the therapeutic effects of pOVA PIT in Th2 OT-II driven AAI be mediated?

It is important to note that OT-II mice used in this project were RAG sufficient and therefore could express endogenous TCR α and/or β chains. This could allow for the generation of pOVA-reactive TCRs with subtly different requirements for recognition of the pOVA I-A^b peptide-MHC complex. On this point it is worth considering the existence of so-called type B cells (cells that respond to exogenous peptide only and not to the same peptide derived from naturally processed protein) (Viner et al., 1996). In contrast, type A T cells can respond to their epitope either when processed from whole protein or when delivered in peptide form. Since OT-II

mice were originally derived following OVA immunisation this means that OT-II $CD4^+$ cells were originally type A cells (Barnden et al., 1998).

In the experiments shown in Figure 4.18-4.33 OT-II cells were polarised to a Th2 phenotype using pOVA, and not OVA, stimulation. It is therefore possible that under these conditions T cells expressing variant TCRs able to respond in a type B manner (i.e. to peptide but not protein) could have been preferentially expanded. In this project every OT-II mouse was screened for the appropriate Vα and Vβ TCR chains prior to use and both chains were found on around 90% of $CD4^+$ T cells (Appendix 8.1). While the remaining 10% of $CD4^+$ cells may recognize pOVA in a conformation different to that found after natural processing of OVA (as a consequence of α or β chain rearrangement), it seems unlikely that such cells would constitute a large proportion of the remaining 10% of $CD4^+$ T cells. The effect this may have on the type A/type B ratio of OT-II cells post polarization is thus likely to be small.

Perhaps most pertinently, it is clear from the experiments in this chapter that Th2 OT-II cells polarised using pOVA are capable of responding to OVA (as shown in Figure 4.18 *in vitro* and that disease is induced following OVA challenge in Figures 4.21-4.26). Thus, the polarised population is likely to contain a substantial population of type A cells (Viner et al., 1996).

The reduced severity of AAI in the pOVA PIT group was associated with a significant reduction in the total number of $CD4^+$ OT-II cells in the lung (Figure 4.27b). Interestingly, there was no significant difference in total number of $CD4^+$ OT-II cells in mLN (Figure 4.27e). These lung data would be compatible with pOVA PIT inducing deletion of $CD4^+$ OT-II cells (as proposed in the case of pOVA PIT and naïve OT-II cells), however the mLN findings would fit less well with this conclusion. Again, there was no evidence that effector cells were converting to $FoxP3^+$ cells following pOVA PIT (Figure 4.29).

The stark reduction in total $CD4^+$ OT-II cells seen in the lung and not in the mLN in the pOVA PIT group could also suggest:-

i) that pOVA PIT affects the ability of Th2 OT-II cells to migrate to the lung upon OVA challenge

and/or

ii) that pOVA PIT induces apoptosis of Th2 OT-II cells upon encountering OVA in lung.

These possibilities are further discussed below.

4.3.6 Does pOVA PIT inhibit the homing of Th2 $CD4^+$ OT-II cells to the lung during OVA challenge?

The data in Figure 4.27a,b indicate that Th2 OT-II cells are found within the lung even in mice that only received PBS challenge. The lungs were perfused with PBS via the heart prior to digestion so it is unlikely that this represents OT-II cells that were present in the blood. Furthermore, Figure 4.23a,b shows that following adoptive transfer of naïve OT-II cells and PBS challenge, very few OT-II cells were present in the lung. Data from Harris et al suggest that this could be the result of differences in CD62L expression between the transferred populations. In that study, transgenic $CD4^+$ T cells reactive towards cytochrome c were activated and polarised *in vitro* for 5-6 days which, similar to the findings shown in Figure 4.28a, induced $CD44^{hi}CD62L^{lo/med}$ and $CD44^{hi}CD62L^{hi}$ populations (Harris et al., 2002). 5 days after adoptive transfer (in the absence of airway challenge), transferred cells were found in lung and mLN (Harris et al., 2002). In the LN these were predominantly $CD44^{hi}CD62L^{hi}$, whereas those in the lung were predominantly $CD44^{hi}CD62L^{lo/med}$ (Harris et al., 2002). By labelling transferred cells with CFSE in that study, there was also evidence that airway challenge with moth cytochrome c induced proliferation of cytochrome c-reactive T cells present in the LN, but not in the lung (Harris et al.,

2002). However, both populations produced effector cytokines (Harris et al., 2002). A subsequent increase in T cells into the lungs following airway challenge, occurred as a result of cytochrome c-reactive T cells proliferating in draining LN and migrating to the lung, rather than proliferation of the cells that had initially homed to the lung following adoptive transfer (Harris et al., 2002).

Consistent with the findings of Harris et al, some Th2 OT-II cells appear to home to the lung following adoptive transfer prior to OVA challenge (Figure 4.27a,b). Thus, if pOVA PIT affected the migration of Th2 OT-II cells from the mLN to the lung, this could account for the findings in Figure 4.25-4.27, particularly since the percentage, and total number, of OT-II cells in the lung in the pOVA PIT OVA challenge group is very similar to the PBS challenged groups (Figure 4.27a,b). These findings are similar to the previously discussed Campbell et al study where PIT led to reduced numbers of Th2 $CD4^+$ cells in the lung, while the number of Th2 $CD4^+$ cells in mLN was not significantly different to controls (Campbell et al., 2009). It is very difficult to identify potential mechanisms that could inhibit Th2 cell migration from the mLN to the lung, because a multitude of chemokine-chemokine receptor interactions are implicated in the homing of Th2 cells to the lung (Medoff et al., 2008). There is also evidence of substantial redundancy in these mechanisms (Medoff et al., 2008), which appear to vary according to the activation status of the T cell (Tager et al., 2003). Two prime candidates are the chemokine receptors CCR4 and CCR8, both of which are induced upon Th2 differentiation (Medoff et al., 2008); however, the role of these in AAI is unclear. Some studies have found no absolute requirement for either CCR4 or CCR8 for homing of Th2 cells to the lungs and the development of AAI (Conroy et al., 2003, Mikhak et al., 2009). In contrast, Mikhak et al found that Th2 polarised OT-II cells deficient in CCR4 were reduced in number in the lung after OVA challenge, and that this was associated with a reduction in the severity of AAI (Mikhak et al., 2009). The chemokines CCL17 and CCL22 (both ligands for CCR4), were reduced in lung and BAL in the PIT treated group in the aforementioned Campbell et al study (Campbell et al., 2009). It is thus possible that if pOVA PIT had altered the chemokine receptor expression on Th2 cells and/or the

chemokine milieu in the lung, that this may account for the reduced severity of AAI in Figure 4.25-4.27.

4.3.7 Does pOVA PIT induce deletion of Th2 CD4$^+$ OT-II cells upon encountering OVA in the lung?

Another possibility is that pOVA PIT induces OT-II Th2 cells to become more susceptible to apoptosis upon encountering OVA. This could fit with the recognised phenomenon that cells encountering strong antigenic stimulation during particular stages of the cell cycle are very sensitive to apoptosis (Yu et al., 1996, Lenardo et al., 1999), and the likelihood that a substantial proportion of the transferred Th2 population proliferates following adoptive transfer [(Hu et al., 2001), and discussed above].

Inadequate survival signals are also implicated in this respect. Recently, signalling through CX3CR1 (the receptor for the chemokine CX3CL1 - which is expressed by cells such as DCs and epithelial cells, and the production of which is known to be increased in inflammatory settings), was examined in AAI (Mionnet et al., 2010). In that study, the severity of AAI was found to be reduced in CX3CR1-deficient mice (Mionnet et al., 2010). In CX3CR1-sufficient mice, CX3CR1 was found to be upregulated on Th2 cells in the lung and BAL after the induction of AAI (Mionnet et al., 2010). CX3CR1-deficient allergen-reactive Th2 cells [polarised *in vitro* from naïve transgenic CD4$^+$ cells reactive towards the leishmania homolog of receptors for activated C kinase (LACK) antigen], were able to reach the lung in a model of LACK-induced AAI (Mionnet et al., 2010). However, these cells were more susceptible to apoptosis than CX3CR1-sufficient Th2 cells, during LACK airway challenges (Mionnet et al., 2010). CX3CR1-deficient Th2 cells were also susceptible to apoptosis in the mLN (Mionnet et al., 2010). It is interesting to speculate that if pOVA PIT impaired/abrogated the expression of chemokine receptors such as CX3CR1, this could lead to increased apoptosis of Th2 OT-II cells upon encountering OVA, although this hypothesis would fit less well with the lack of a significant reduction in OT-II cells in mLN in the pOVA PIT group (Figure 4.27d,e).

Another potential candidate is OX40. Croft et al found that costimulation via OX40 was required by memory Th2 cells (generated after 3 days *in vitro* with pOVA in Th2 promoting conditions, followed by 3-6 days rest), to induce AAI, in a model similar to that shown in Figure 4.20 using Th2 polarised OT-II cells (Salek-Ardakani et al., 2003). Although $OX40^{-/-}$ OT-II Th2 memory cells could proliferate and produce cytokines early upon pOVA stimulation *in vitro*, their survival was much reduced, particularly from day 2 onwards (Salek-Ardakani et al., 2003). *In vivo*, OX40 was increased on Th2 OT-II memory cells shortly after allergen challenge (Salek-Ardakani et al., 2003). Adoptive transfer of $OX40^{-/-}$ Th2 OT-II memory cells followed by OVA challenge, led to less severe AAI compared to that seen after transfer of $OX40^{+/+}$ Th2 OT-II cells, and was associated with fewer Th2 OT-II cells in mLN and lung (Salek-Ardakani et al., 2003). Therefore, despite their reduced costimulatory requirements, costimulation via OX40 did appear to be required for an efficient memory Th2 cell response in that study. It is possible that pOVA PIT may have impaired OX40 expression on Th2 polarised OT-II cells. This could account for the reduction in OT-II cells in the lung in Figure 4.27b; again however, such a mechanism would not appear to correlate with the apparent lack of reduction of OT-II cells in the mLN (Figure 4.27e).

4.3.8 Does pOVA PIT induce anergic and/or regulatory mechanisms in Th2 polarised OT-II cells?

Figure 4.33 shows that 4 days after pOVA PIT, the IL-2 produced by splenocytes from pOVA PIT treated mice in response to pOVA was reduced (yet still very high), compared to PBS treated controls (Figure 4.33b). A dose dependent response of splenocytes to OVA was not found in either group (Figure 4.33a), and it may be that background proliferation was already too strong to elicit this. IL-5 recall responses to pOVA were similar between the two groups (Figure 4.33c,d). Hence, despite around five times as many $CD4^{+}$ OT-II cells in spleens of pOVA PIT treated mice, these appeared less responsive (yet still responsive) to pOVA *in vitro*. This perhaps indicates that a degree of anergy has been induced. In addition, a dose dependent IL-

10 response to pOVA was detected from splenocytes in the pOVA PIT treated group (Figure 4.33f). This could simply be as a consequence of the increased number of OT-II cells present in the splenocyte cultures in the pOVA PIT group. Alternatively, it could imply that the induction of IL-10 may play a role in the therapeutic effects of pOVA PIT seen in this model, as has been proposed in other PIT studies (Campbell et al., 2009, Vissers et al., 2004). In either case, IL-10 production has not been switched off. It is thus possible that PIT-induced stimulation of Th2 OT-II cells may have led to the generation of IL-10 producing cells.

4.3.9 $CD4^+$ epitopes in addition to pOVA are likely to be implicated in the OVA-driven immune response

The abrogated IL-2 response to pOVA after OVA sensitisation in the pOVA PIT treated group compared to PBS treated controls indicates tolerance induction of $CD4^+$ pOVA-reactive T cells (Figure 4.5b). However, this was not associated with a significant reduction in the IL-2 response to OVA in the pOVA PIT group (Figure 4.5a). Importantly, IL-2 produced by splenocytes in response to OVA in Figure 4.5a will represent responses from $CD4^+$ OT-II cells and from host $CD4^+$ T cells as a consequence of OVA/alum sensitisation. Thus, despite pOVA PIT inducing tolerance of pOVA-reactive $CD4^+$ OT-II cells, it appeared that host $CD4^+$ T cells were responding to additional epitope(s) within OVA. Although Yang et al have recently described three potential additional $I\text{-}A^d$ $CD4^+$ T cell epitopes within OVA in BALB/c mice (Yang and Mine, 2009), to the author's knowledge, formal characterisation of OVA epitopes in $I\text{-}A^b$ restricted C57BL/6 mice, has not previously been reported. It was therefore likely that additional epitope(s) were involved in the $CD4^+$ T cell response to OVA in this model. If this were so, then the data shown in Figure 4.5 would suggest that pOVA PIT does not inhibit $CD4^+$ T responses to other potential OVA epitope(s) i.e. there is a lack of linked suppression. This could be a reason behind the lack of therapeutic effect of pOVA PIT in OVA-induced AAI in Chapter 3. These observations formed the basis for the experiments described in the next chapter.

4.3.10 Concluding remarks

In summary, the data in this chapter have demonstrated:-

- pOVA PIT given intravenously can tolerise naïve $CD4^+$ pOVA-reactive cells in the setting of one OVA sensitisation. This is likely to be effected primarily via deletional mechanisms.
- pOVA PIT reduces the severity of OVA-induced AAI when this is solely mediated by Th2 polarised pOVA-reactive $CD4^+$ cells.
- It is unclear how pOVA PIT effects tolerance in the Th2 OT-II AAI model. Possible mechanisms include impaired Th2 cell homing to the lung, apoptosis of Th2 cells upon encountering OVA in the lung and/or the induction of IL-10.
- Epitopes other than pOVA are likely to be implicated in the T cell response to OVA. pOVA PIT does not appear to induce linked suppression of responses directed towards these additional epitopes.

The experiments in the next chapter were therefore designed to further delineate T cell responses induced towards OVA in C57BL/6 mice.

5 Epitope mapping the T cell response to OVA in C57BL/6 mice and implications for PIT in OVA-driven AAI.

5.1 Introduction

The findings discussed in Chapters 3 and 4 implied that $CD4^+$ T cell epitopes in addition to pOVA may contribute to OVA driven responses in C57BL/6 mice. This could account for the lack of therapeutic effect seen using pOVA PIT in OVA-induced AAI in Chapter 3, despite the tolerogenic effects of pOVA PIT demonstrated on trackable pOVA-reactive $CD4^+$ T cells in Chapter 4. However, in Chapter 3, it was not possible to say whether or not tolerance of host $CD4^+$ pOVA-reactive T cells had occurred, because of the inability to reliably detect an *in vitro* recall response of splenocytes from OVA sensitised C57BL/6 mice to pOVA. It was therefore important to determine whether or not pOVA PIT could induce tolerance of (non-transgenic) pOVA-reactive $CD4^+$ T cells, and also to characterise any additional T cell epitopes implicated in OVA-induced T cell responses in C57BL/6 mice.

pOVA 323-339 was initially identified as an immunodominant $CD4^+$ T cell epitope in the context of $I\text{-}A^d$ (expressed by BALB/c mice) using OVA reactive T cell hybridomas and OVA fragments generated by trypsination (Shimonkevitz et al., 1984). C57BL/6 mice (which express $I\text{-}A^b$) also respond to pOVA (Barnden et al., 1998). In BALB/c mice $CD4^+$ T cell responses to pOVA are known not to constitute the full T cell response to OVA, and pOVA has been described as contributing between 25-60% of the $CD4^+$ T cell response to OVA (Renz et al., 1993, Janssen et al., 1999). In BALB/c mice, Yang et al recently found that PIT using pOVA together with two additional peptides, containing recently identified $CD4^+$ T cell epitopes (Yang and Mine, 2009), improved disease in a model of OVA-driven food allergy (Yang et al., 2010). This meant it was possible that a similar scenario was occurring in the experiments in Chapter 3. However, differences in MHC haplotypes between BALB/c and C57BL/6 mice meant that any additional $CD4^+$ T cell epitopes were unlikely to be the same as those described for BALB/c mice. To the author's knowledge, formal characterisation of the C57BL/6 T cell response to OVA has not

previously been carried out. Epitope mapping experiments were therefore designed, to identify additional epitopes within OVA, which could then be utilised for PIT.

5.1.1 Aims

This chapter aimed to:

i) Assess the *in vitro* recall response to pOVA by LN cells from C57BL/6 mice.
ii) Confirm that tolerance of pOVA-reactive T cells is induced following pOVA PIT in C57BL/6 mice.
iii) Characterise any additional T cell epitopes within OVA in C57BL/6 mice.
iv) To test tolerogenic efficacy of epitope(s) identified in (iii), either alone or in combination with pOVA, in the OVA-induced AAI model.

5.1.2 Approach

Initially, *in vitro* recall responses to OVA and to pOVA, and the effects of pOVA PIT, were assessed following OVA/CFA immunisation; since this is known to induce strong immune responses after one immunisation. Short-term T cell lines were then generated from peripheral LN of C57BL/6 mice immunised with OVA/CFA. These T cell lines were cultured with a panel of overlapping peptides which encompassed the full sequence of OVA and incorporated a 5 amino acid shift and a 10 amino acid overlap. Data from these experiments led to the synthesis of a peptide encompassing amino acids 263-278 from OVA (hereafter referred to as p263-278). The antigenicity of p263-278 and the relative contribution of T cell responses to this peptide to the overall OVA-induced T response, was examined. The effects of PIT using p263-278 alone and in combination with pOVA were then assessed in OVA-driven AAI.

5.2 Results

5.2.1 *In vitro* proliferative responses to pOVA do not constitute the full response to OVA in C57BL/6 mice

In order to characterise T cell responses to epitopes within OVA, a strong OVA induced immune response was required. The adjuvant CFA was therefore chosen for these experiments instead of alum. When LN cells were cultured from pOVA/CFA immunised mice, proliferative responses to OVA and to pOVA were similar to one another (Figure 5.1b). Figure 5.1c shows that one OVA/CFA immunisation induced a robust proliferative response of LN cells to OVA. Importantly, *in vitro* recall responses to pOVA were lower than to OVA (Figure 5.1c). It appeared that maximal proliferative responses were achieved in response to pOVA even at the lowest concentration tested, however this was still an antigen dependent response when compared to the low background proliferation seen upon culturing splenocytes with medium alone (Figure 5.1c, dotted line). It seems likely that the stronger immune response generated following OVA/CFA immunisation compared to OVA/alum immunisation, facilitates the detection of *in vitro* proliferative responses to pOVA, which are considerably lower than seen in response to OVA (Figure 5.1c). Furthermore, use of LN cells (which are more easily obtainable in large numbers following immunisation with CFA than after OVA/alum immunisation) and not splenocytes may also be a factor in the ability to detect proliferation in response to pOVA in these experiments. These data thus suggest, consistent with data from others in the context of BALB/c mice (Janssen et al., 1999, Yang and Mine, 2009), that T cell responses to pOVA do not constituent the full response to OVA. It was also noted during these experiments that responses to pOVA following OVA/CFA immunisation often varied between individual mice (data not shown). The extent to which T cell responses are directed towards pOVA following OVA/CFA immunisation therefore seems subject to some variability between individual mice.

5.2.2 pOVA PIT reduces but does not abolish the *in vitro* recall response to OVA following OVA/CFA immunisation in C57BL/6 mice.

pOVA PIT or PBS as a control were administered 7 days prior to OVA/CFA immunisation (the approach shown in Figure 5.2a). 10 days later, *in vitro* recall responses of LN cells to OVA and to pOVA were assessed (Figure 5.2b-d). Tolerance was induced towards pOVA in pOVA PIT treated mice, as shown by the lack of a detectable *in vitro* recall response to pOVA in this group (Figure 5.2b). Proliferative responses to OVA in the pOVA PIT treated group were reduced, but not abolished at higher antigen doses, compared to PBS treated controls (Figure 5.2b). However, at limiting doses of antigen pOVA PIT significantly reduced the response to OVA (Figure 5.2b) and the overall effect of pOVA PIT on the response to increasing concentrations of antigen was significantly reduced (Figure 5.2b). IFN-γ in culture supernatants from the PBS treated group was above the limit of detection for all concentrations of OVA tested, however, pOVA PIT resulted in a reduction in IFN-γ production in response to OVA since this was now within the limit of detection at lower concentrations of OVA (Figure 5.2c). Compatible with the proliferation data, IFN-γ was not detected in supernatants from LN cells from pOVA PIT treated mice in response to pOVA (Figure 5.2d). IFN-γ was produced (but to a variable degree) by LN from PBS treated mice in response to pOVA (Figure 5.2d). Overall, these data demonstrate that pOVA PIT given prior to OVA/CFA immunisation is capable of tolerising pOVA-reactive T cells in C57BL/6 mice. However, although this reduces the overall response to OVA it does not abolish it (Figure 5.2b,c). This meant that, despite the induction of tolerance of pOVA-reactive T cells following pOVA PIT, a significant response still remained towards OVA.

5.2.3 Epitope mapping the T cell response to OVA in C57BL/6 mice

In order to map the T cell response to epitope(s) contained within OVA in the context of C57BL/6 mice, short-term OVA-reactive T cell lines were generated as shown in Figure 5.3. A panel of 75 peptides was commissioned encompassing the amino acid sequence of OVA. These 15mer peptides incorporated a 5 amino acid

shift, and therefore a 10 amino acid overlap, to facilitate epitope mapping. OVA-reactive T cells were cultured with irradiated splenocytes (which acted as APC) and peptide. Responses to OVA, pOVA and medium alone were also included. Figure 5.4 shows the proliferative response of the OVA-reactive T cell line to each peptide in the panel. As predicted, proliferation occurred in response to peptides 321-335 and 326-340, representing a response to the T cell epitope contained within pOVA (Figure 5.4, blue bars). Proliferative responses were also seen in response to peptides 261-275 and 266-280 (Figure 5.4). Since these two peptides overlapped each other this strengthened the likelihood that they contained a T cell epitope within them. This experiment was conducted twice, once using 10µM of each peptide (Figure 5.4) and once using 2 µM of each peptide (data not shown). Data from these two experiments consistently showed proliferative responses to peptides 321-335 and 326-340, and 261-275 and 266-280. In addition, proliferation was also seen in response to peptide 26-40 (Figure 5.4). Low level proliferative responses were also consistently seen to peptide 301-315 (Figure 5.4) in both experiments, but not consistently in response to any other peptide. The proliferative response to an equimolar OVA concentration was around 6 times greater than seen to any individual peptide (Figure 5.4 – refer to figure legend for OVA response).

In summary, short-term OVA-reactive T cell lines proliferated in response to peptides spanning the pOVA sequence, and also in response to peptides 261-275 and 266-280. Proliferative responses were also consistently seen towards peptides 26-40 and 301-315.

5.2.4 A short term OVA-reactive T cell line responds to an epitope shared by peptides 261-275 and 266-280, in addition to responding to pOVA

In vitro recall responses of an OVA-reactive T cell line towards identified peptides of interest from the peptide panel were next determined. Dose-dependent proliferation was seen in response to pOVA and to peptides 261-275 and 266-280 (Figure 5.5). No proliferative response was seen in response to peptide 171-185, which was included

as a negative control (Figure 5.5). Although proliferative responses to peptide 26-40 and to peptide 301-315 were above background levels, this response was relatively low and did not appear to be dose-dependent (Figure 5.5). Furthermore, it had been noted upon reconstitution of the peptides that peptide 26-40 was not fully soluble, so it was hypothesised that this may play a role in the proliferation response elicted by this peptide. Interestingly, peptide 301-315 appeared toxic at high concentrations (indicated by the proliferative response to this peptide in Figure 5.5 and also noted during microscopic examination of cell cultures).

IFN-γ was produced by the T cell line in a dose-dependent fashion to OVA, pOVA and to peptides 261-275 and 266-280 (Figure 5.6). Overall, the proliferative and cytokine response to peptide 261-275 was lower than to peptide 266-280 (Figure 5.5 and Figure 5.6). IFN-γ was not detected in response to the negative control peptide 171-185, nor to 26-40 or 301-315 (Figure 5.6).

To ensure that the results described above were applicable to T cell responses following direct ex-vivo isolation, rather than specific to the short-term T cell line, recall responses of LN from OVA/CFA immunised mice were assessed in response to the peptides of interest (Figure 5.7). A similar pattern of response as had been seen using the short-term T cell line, was again seen.

Cumulatively, the above experiments led to the conclusion that immunisation with OVA led to T cell responses to an additional epitope shared by peptides 261-275 and 266-280.

5.2.5 p263-278 induces proliferation of LN cells from OVA/CFA immunised mice and reconstitutes the response seen to OVA when cultured together with pOVA

The core sequence shared by peptides 261-275 and 266-280 is 266-275. A 16mer peptide (263-278, hereafter referred to as p263-278), was thus synthesised to provide

three “flanking” residues on either side of this sequence. The sequence of p263-278 and the proposed T cell epitope contained within it is shown in Figure 5.8a. p263-278 induced proliferation of LN cells from OVA/CFA immunised mice (Figure 5.8c). A similar degree of proliferation was also seen in response to pOVA (Figure 5.8c). Interestingly, a combination of equimolar concentrations of pOVA and p263-278 appeared to almost fully reconstitute the recall response seen to OVA, as measured either by proliferation or by IFN-γ production (Figure 5.8c,d). Hence, it appeared that the T cell response to OVA was predominantly, if not entirely, directed towards epitope(s) contained within pOVA and p263-278.

5.2.6 The effects of giving pOVA and p263-278 in combination as PIT prior to the induction of OVA-induced AAI

Figure 5.9 shows the approach used to assess the effects of PIT using intravenous administration of p263-278, either alone or in combination with pOVA, upon OVA-induced AAI. There were no differences in peri-vascular or peri-bronchiolar lung inflammation scores between any of the groups (Figure 5.10a,b). There were no significant differences in the percentage of goblet cells in the airways or in total BAL cells between the groups (Figure 5.10c,d and Figure 5.11a). However, there were significantly fewer eosinophils in the BAL from pOVA+p263-278 PIT treated mice, compared to PBS treated controls (Figure 5.11c). In contrast, the number of eosinophils (and lymphocytes) in BAL was increased in the pOVA PIT group compared to the PBS group (Figure 5.11c,e). This is different to the findings following pOVA PIT in Chapter 3 – it is possible that differences in the route of airway challenge (intranasal versus intratracheal) may be implicated in these findings. There was also significantly less OVA-specific IgE detected in serum from the pOVA+p263-278 PIT treated mice, compared to PBS treated controls (Figure 5.12c). There was no significant difference in OVA-specific IgE levels from pOVA PIT or p263-278 PIT treated mice, compared to controls (Figure 5.12a,b). No significant difference in OVA specific IgG1 was detected in serum from PBS treated compared to pOVA+p263-278 PIT treated mice (data not shown). There was substantial inter-mouse variability between levels of OVA-specific IgG2a detected in serum from pOVA+p263-278 PIT treated mice (as indicated in Figure 5.12d).

Although at some (higher) serum dilutions the levels of IgG2a attained significance in this group versus PBS treated controls, the variability in IgG2a levels within the pOVA+p263-278 PIT group makes it difficult to draw a firm conclusion from these data (Figure 5.12d).

mLN cells were also harvested on D36, groups pooled and *in vitro* proliferative responses assessed to pOVA, p263-278 and OVA (Figure 5.13). mLN cells from the pOVA PIT group had an abrogated proliferative response to pOVA, although this had limited impact on the response to OVA (Figure 5.13a,c). The proliferative response to p263-278 was highest in the pOVA PIT group (Figure 5.13b). Proliferation in response to p263-278 was low in all other groups and did not appear to be reduced following p263-278 PIT (Figure 5.13b). The overall proliferative response to OVA in the pOVA + p263-278 PIT group was lower than seen in all other groups (Figure 5.13c). This could perhaps suggest that PIT using pOVA together with p263-278 can reduce (but not ablate) the response to OVA. As also found in Chapter 3, no recall response to pOVA (and in this case also to p263-278) could reliably be demonstrated from splenocytes from any group (data not shown).

Overall, PIT using a combination of pOVA and p263-278 significantly reduced the total number of eosinophils in the BAL and the level of OVA-specific IgE, but did not significantly improve other AAI parameters such as pulmonary inflammation scores or the percentage of goblet cells in the airways.

5.2.7 The effects of pOVA+p263-278 PIT on *in vitro* proliferation responses following OVA/CFA immunisation

A shorter experiment was carried out in order to further assess the effects of PIT using pOVA+p263-278 upon OVA responses, by administering PIT prior to OVA/CFA immunisation (this approach is shown in Figure 5.14a). Again, as seen previously in Figure 5.2, proliferation did not occur in response to pOVA in pOVA PIT treated groups (Figure 5.14b), indicating that tolerance had been achieved. This occurred regardless of whether pOVA PIT was given alone or in combination with

p263-278 (Figure 5.14b). Proliferation of LN cells from PBS treated mice in response to p263-278 was greater than seen to pOVA, implying that responses to p263-278 may be more dominant in this experiment (Figure 5.14a,b). PIT using p263-278 substantially reduced p263-278 proliferation but did not completely abolish it (Figure 5.14c). Surprisingly however, despite the reduction in p263-278 and pOVA responses following pOVA+p263-278 PIT, this had no effect on the overall response to OVA (Figure 5.14d). This is also unexpected given that cumulative cpm to pOVA alone and to p263-278 alone in the PBS treated group at 10µM of peptide, are similar to the cpm seen in response to 10µM OVA (Figure 5.4b-d). This observation together with the findings shown in Figure 5.8c, would be consistent with T cell responses towards pOVA and p263-278 constituting a major part of the T cell response to OVA. The lack of effect that inducing tolerance towards pOVA and p263-278 apparently had upon the overall response to OVA in Figure 5.14d, is therefore difficult to account for.

Figure 5-1: Proliferative responses to pOVA *in vitro* are demonstrable following OVA/CFA immunisation but do not constitute the full OVA response in C57BL/6 mice

Figure 5-2: pOVA PIT reduces but does not abrogate the *in vitro* recall response to OVA following OVA/CFA immunisation in C57BL/6 mice

Figure 5-3: Generating short-term T cell lines for use in peptide recall assays

Figure 5-4: Responses of an OVA-reactive short-term T cell line to a panel of overlapping peptides encompassing the sequence of OVA

Figure 5-5: Dose-dependent proliferation of an OVA-reactive short-term T cell line to pOVA and to peptide 261-275 and 266-280

Figure 5-6: Dose-dependent IFN-γ production from an OVA-reactive short-term T cell line to pOVA and to peptides 261-275 and 266-280

Figure 5-7: Dose-dependent proliferation of LN cells following OVA immunisation to pOVA and to peptides 261-275 and 266-280

Figure 5-8: pOVA in combination with p263-278 induces similar proliferation of LN cells from OVA/CFA immunised mice as seen in response to OVA alone

Figure 5-9: Experimental approach to assess the effects of PIT using pOVA+p263-278 prior to AAI induction in C57BL/6 mice

Figure 5-10: Assessment of the effects of PIT using p263-278 in combination with pOVA on pulmonary inflammation in OVA-induced AAI

Figure 5-11: Assessment of the effects of PIT using pOVA+p263-278 on BAL parameter in AAI

Figure 5-12: Assessment of the effects of PIT using pOVA+p263-278 on OVA-specific IgE on D36

Figure 5-13: *In vitro* proliferation responses of mLN cells appear to be reduced in response to OVA in mice that received pOVA+p263-278 PIT prior to induction of AAI

Figure 5-14: Assessment of the effects of PIT using pOVA+p263-278 prior to OVA/CFA immunisation on *in vitro* proliferation

5.3 Discussion

5.3.1 pOVA and p263-278 both contain immunodominant epitope(s) implicated in OVA-induced T cell responses in C57BL/6 mice

The experiments in this chapter have demonstrated that pOVA and p263-278 both contain T cell epitopes important for OVA-induced responses in C57BL/6 mice. Although there were previous difficulties in eliciting recall responses of splenocytes to pOVA following OVA/alum sensitisation, as discussed in Chapter 3, recall responses from LN cells to pOVA following OVA/CFA immunisation were demonstrated in this chapter (for example in Figure 5.1a). Such findings suggest that a strong *in vitro* recall response towards OVA, reflecting strong clonal expansion *in vivo*, is required in order to elicit a reliable *in vitro* response to pOVA. This is consistent with data from Sun et al, who found that splenocytes from OVA/alum sensitised mice did not proliferative *in vitro* in response to pOVA (Sun et al., 2010), and with the findings of Fifis et al, who showed that splenocytes responded to pOVA *in vitro* following a strong immunisation protocol comprising OVA, anti-CD40 and the viral mimetic (and TLR3 ligand), polyinosinic:polycytidylic acid (poly I:C), (Fifis et al., 2004).

Although T cell epitopes in addition to those contained within pOVA have recently been characterised in the context of I-A^d expressing BALB/c mice (Yang and Mine, 2009), to the author's knowledge, the findings in this chapter represent the first formal characterisation of T cell epitopes implicated in OVA-induced immune responses in C57BL/6 mice. In these experiments, responses to two overlapping peptides were found which shared the core sequence of 266-275, and subsequent experiments demonstrated responses to the 16mer peptide p263-278 (Figures 5.4-5.8). Following these experiments, more refined literature searches were carried out based upon the 263-278 sequence. The results of these searches unexpectedly highlighted that an OVA-derived epitope comprising the 265-280 sequence had been used previously by others, in a limited number of studies involving C57BL/6 mice. The first such study appears to be from Maecker et al. They examined cytotoxic T

cell responses following immunisation with a vector encoding a known $CD8^+$ T cell OVA epitope (SIINFEKL), together with the 265-280 epitope which they described as "the MHC class II-restricted T cell epitope of OVA" (Maecker et al., 1998). In that study, vector expression of 265-280 and the presence of $CD4^+$ T cells were both required to generate a strong cytotoxic T cell response to OVA (Maecker et al., 1998). Those findings are consistent with the data shown in this chapter. However, Maecker et al do not reference the 265-280 epitope so it is unclear from that paper where the information regarding this has been drawn. Following personal communication with the authors (Maecker HT and Levy S with Mackenzie KJ – personal communication), it appears that 265-280 was chosen using predication algorithms for peptide binding to $I\text{-}A^b$, and not following functional assessment. Subsequent studies, also focusing upon $CD4^+$ T cell influences on $CD8^+$ T cell responses, have also utilised 265-280, and also reference the initial Maecker et al study. In a recent publication by Mizukami et al, 265-280 is described as "a presumed helper epitope" (Mizukami et al., 2008). Again, the inclusion of the 265-280 epitope in a vaccine designed to induce cytotoxic T cell responses was found to enhance cytotoxic T cell responses to OVA-expressing tumour cells (Mizukami et al., 2008). Furthermore, in order to compare $CD4^+$ T cell responses in transgenic mice with glycosphingolipid storage disease to naïve controls, Gadola et al showed that IFN-γ was produced by splenocytes from OVA immunised C57BL/6 mice in response to the 265-280 peptide (Gadola et al., 2006). Proliferative responses were however not assessed in that study.

Thus, although $CD4^+$ response to the 265-280 sequence have previously been described in a limited number of studies, the epitope mapping experiments in this chapter have enabled formal characterisation of the T cell response to pOVA, and have demonstrated that the T cell response to OVA is predominantly directed towards epitope(s) within pOVA and p263-278.

5.3.2 What are the relative contributions of responses to epitope(s) within pOVA and p263-285 to the overall OVA-induced T cell response?

The findings described in this chapter enable some tentative predictions as to the relative contribution of pOVA and p263-278 directed T cell responses to the overall OVA-induced T cell response.

The proliferative response of OVA-derived T cell lines to pOVA was around 20% of that induced by OVA (Figure 5.4 and Figure 5.5 – note that the mean response to OVA is included in the figure legend). Experiments involving freshly isolated LN cells from OVA/CFA immunised mice show greater variability in terms of the pOVA–induced proliferative response as a percentage of the OVA response. Responses to 10µM pOVA ranged from around 20-50% of the responses to 10µM OVA in different experiments (Figures 5.2, 5.7, 5.8 and 5.14). This is comparable to BALB/c mice where pOVA induced T cell responses have previously been described as contributing to between 25-60% of the T cell response to OVA (Renz et al., 1993, Janssen et al., 1999).

Figure 5.8c indicates that the proliferative response of LN cells from OVA/CFA immunised mice to p263-278 comprised around 50% of that seen in response to OVA. Data from the PBS treated OVA/CFA immunised group in Figure 5.14c, are also consistent with this. Interestingly, in Figure 5.8c, the recall response of LN cells to equimolar concentrations of pOVA and p263-278 in combination, appeared to reconstitute the response to OVA. This would support the conclusion that T cell responses induced by OVA immunisation are predominantly directed towards epitope(s) contained within pOVA and p263-278. However, the data in Figure 5.14 do not fully support this, because despite evidence that tolerance was induced towards pOVA and p263-278 using pOVA+p263-278 PIT (Figure 5.14b,c), this did not substantially reduce the overall recall response to OVA (Figure 5.14d).

In summary, the majority of data in this chapter are consistent with the tentative estimation that responses directed towards epitope(s) within pOVA contribute to 20-50% and to p263-278 contribute to around 50%, of the overall OVA induced T cell response. More accurate quantification would require additional experiments, for example by assessing the peptide-induced responses of hybridomas generated following OVA/CFA immunisation (Robertson et al., 2000), or via peptide recall assays using enzyme-linked immunosorbent spot (ELISPOT) techniques.

5.3.3 The effects of pOVA+p263-278 PIT on OVA-driven immune responses and AAI

Given the apparent substantial contributions of pOVA and p263-278 responses to the overall OVA induced response, PIT using a combination of pOVA+p263-278 was predicted to induce tolerance of a substantial proportion of the OVA-induced T cell response, and reduce the severity of AAI. This was true for some AAI parameters; the total number of eosinophils in BAL was reduced in the pOVA+p263-278 PIT group compared to PBS treated controls (Figure 5.11c), and there was also a significant reduction in the levels of OVA-specific IgE in the serum of mice from the pOVA+p263-278 PIT group compared to PBS treated controls (Figure 5.12c). Furthermore, the proliferation of pooled mLN cells in response to OVA was lower in the pOVA+p263-278 PIT group compared to other groups (Figure 5.13c). However, pOVA+p263-278 PIT did not significantly reduce the percentage of goblet cells in the airways, nor did it lead to reduced pulmonary inflammation scores (Figure 5.10). Overall, the effects of pOVA+p263-278 PIT upon OVA-induced AAI were variable but encouraging, particularly when compared to the effects of PIT using pOVA or p263-278 alone, which did not reduce the severity of any AAI parameter or reduce the proliferation of mLN cells to OVA (Figure 5.10-5.13). Furthermore, in Figure 5.11c, the total number of eosinophils in BAL was actually increased in the pOVA PIT group compared to PBS treated controls.

The findings in Figure 5.14 demonstrate some important further points. Firstly, pOVA PIT and p263-278 successfully induced tolerance to themselves in the setting

of OVA/CFA immunisation, as indicated by the abrogated recall proliferation responses (Figure 5.14b,c). Secondly, neither peptide given alone as PIT induced linked suppression to the other peptide (Figure 5.14b,c). Additonally, in the OVA/CFA setting, pOVA+p263-278 PIT did not appear to reduce the overall recall proliferative response of LN cells to OVA (Figure 5.14d). This is different to the reduction in the recall proliferation response of mLN cells to OVA in the pOVA+p263-278 PIT group following the induction of AAI in Figure 5.13c. It is possible that the very strong stimulus provided by OVA/CFA immunisation could play a role in these differences, and this is further discussed below.

Taken together, the findings described in this chapter have some similarities with other studies, particularly with those of Yang et al (Yang et al., 2010). In that study, two OVA-derived peptides, previously identified by the same group as containing immunodominant OVA epitopes in the context of BALB/c mice (Yang and Mine, 2009), were used along with pOVA as PIT in a model of OVA-induced food allergy (Yang et al., 2010). PIT was given after oral sensitisation to OVA and prior to oral OVA re-challenge. PIT using the three peptides together, reduced allergic responses upon oral OVA re-challenge, indicating tolerance induction (Yang et al., 2010). However, when used alone, none of the three peptides reduced the allergic response to OVA, and PIT using only one of the peptides (147-161), increased disease severity (Yang et al., 2010). Therefore, in that model, the immunodominant OVA peptides had to be given in combination to exert therapeutic effects. Similarly in other studies pOVA PIT did not reduce OVA-induced responses (Janssen et al., 1999, Barbey et al., 2004), and in one study pOVA PIT exacerbated OVA-induced AAI (Janssen et al., 1999). Hence, it appears from the data described in this chapter and the findings of others (Barbey et al., 2004, Yang et al., 2010, Janssen et al., 1999), that PIT for OVA-induced allergic disease may only generate therapeutic effects when immunodominant peptides are used in combination. The lack of evidence for linked suppression suggested by the data in this chapter and by the findings of Yang et al (Yang et al., 2010), are likely, in part, to be implicated in this. This is in contrast to the linked suppression previously demonstrated in the Campbell et al PIT study (Campbell et al., 2009). IL-10 dependent mechanisms were found to be implicated in

tolerance induction in that study and attributed to the linked suppression that was found (Campbell et al., 2009). The induction of IL-10 may have been related to the intradermal and low dose PIT administration regime chosen in that study (Campbell et al., 2009) and is different to the high dose, intravenous, approach used here, which, as discussed in Chapter 4, is thought to lead to a predominantly deletional mode of tolerance (Hochweller and Anderton, 2005, Kearney et al., 1994).

The findings in this chapter have thus demonstrated that PIT using pOVA or p263-278 alone did not induce linked suppression and did not reduce the severity of OVA-induced AAI. However, PIT using pOVA and p263-278 in combination reduced, but did not ablate, some, but not all, of the parameters of OVA-induced AAI. In contrast, in the context of OVA/CFA immunisation, pOVA+p263-278 PIT did not appear to reduce the overall recall proliferation response of LN cells to OVA. So what reasons could account for these findings?

5.3.4 Why does pOVA+p263-278 PIT not ablate OVA-driven AAI, nor reduce the overall recall response to OVA following OVA/CFA immunisation?

There are several possibilities that could account for the limited effects of pOVA+p263-278 PIT on OVA-induced responses, seen in this chapter:-

i) Are “minor” OVA-derived T cell epitopes still capable of promoting OVA-induced responses?

It is possible that if T cell responses were additionally directed towards minor OVA-derived epitopes, this might not have been detected in the overlapping peptide experiments described in this chapter. If so, and in the absence of linked suppression, it is possible that responses to such epitopes could still generate an overall T cell response to OVA. This seems unlikely given the apparently substantial contribution of responses to pOVA and p263-278 to the overall OVA response, found in the majority of experiments in this chapter.

ii) Is incomplete tolerance induction and/or residual effector cytokine production by anergic T cells implicated?

If some pOVA and/or p263-278-reactive T cells escaped tolerance induction and remained pathogenic, then the continued restimulation of such cells to OVA in the AAI protocol (2 x OVA/alum sensitisation and 3 airway challenges) could be sufficient to generate OVA-induced AAI. Indeed, *in vitro* proliferation towards p263-278 was not completely abolished following p263-278 PIT (Figure 5.14c).

Another possibility could relate to anergic pOVA and p263-278-reactive T cell populations. These could have been induced following pOVA+p263-278 PIT if the same predominantly deletional and anergic mechanisms as were implicated in the experiments utilising naïve OT-II cells (discussed in Chapter 4) also occurred in the non-transgenic setting. Although anergic cells have substantially impaired proliferative and IL-2 producing capacities, the ability of anergic cells to generate effector cytokines in response to antigen can vary (Tanchot et al., 2001, Malvey et al., 1998, Schwartz, 2003). Hence, it is feasible that the multiple exposures to OVA during the AAI induction protocol could still be sufficient to generate Th2 cytokines from anergic cells and induce AAI.

iii) Do exogenous soluble pOVA and p263-278 target all populations of pOVA and p263-278-reactive T cells that are generated following endogenous processing of OVA?

Immunisation with an immunodominant peptide can induce T cell responses that are not found following immunisation with the whole protein. This can occur because immunisation with peptide enables “cryptic epitopes” to be displayed on MHC II – for example epitopes that are normally destroyed during processing of the whole protein (Sweenie et al., 2007, Anderton et al., 2002). Immunisation with peptide can also induce responses from T cells that can only respond to exogenously delivered peptide, but not to the same peptide when this is processed from whole protein (so-called Type B cells) (Unanue, 2002, Cirrito et al., 2001,

Viner et al., 1996). This is thought to occur as a result of conformational differences in peptide-MHC II complexes arising from differences in processing of exogenously delivered peptide versus the same peptide derived from a whole protein. For example, Viner et al demonstrated these effects in the context of immunisation with hen egg lysosyme. In that study, immunisation with protein stimulated a response from type A cells (cells that could recognise peptide when naturally processed from the whole protein). whereas immunisation with peptide could induce type A cells or type B cells (Viner et al., 1996). Therefore, if pOVA and p263-278 PIT favoured particular MHC II binding conformations, this could lead to tolerance induction of particular, but not all, pOVA and p263-278 reactive T cell populations. If the T cell populations that escaped tolerance induction via PIT could respond to OVA but not to exogenously processed peptides, this could perhaps account for the findings in Figure 5.14. This possibility has previously been suggested by others in the context of PIT in experimental autoimmune encephalomyelitis (EAE) (Anderton et al., 2002). Such studies, together with the results in Figure 5.14, thus demonstrate the complexity of designing peptides for therapeutic intervention and highlight the importance that such peptides must mimic the response to that peptide generated after natural processing from whole protein.

Another potential reason as to why pOVA+p263-278 PIT had limited impact on the response to OVA concerns post-translational modifications (PTM). It is known that during antigen processing amino acids within a peptide can undergo PTM which can alter the nature of the resulting T cell response [discussed in (Anderton, 2004)]. Many different potential PTM have been described which can be mediated through enzymatic activities within the APC (Anderton, 2004). For example, certain amino acids such as glutamine and asparagine can undergo deamidation (Anderton, 2004). Deamidation of glutamine to glutamic acid has been found to increase proliferative responses of gluten-reactive T cell lines derived from patients with coeliac disease, most likely as a consequence of increased MHC II binding affinity (Arentz-Hansen et al., 2000). Hydroxylation +/- glycosylation of certain amino acids can also influence the T cell response. In

a mouse model of collagen induced arthritis, collagen-reactive T cells were found to predominantly respond to an immunodominant collagen-II-derived peptide containing a hydroxlated and glycosated lysine residue (Corthay et al., 1998). PIT using this modified peptide was found to be much more effective than using an unmodified peptide (Backlund et al., 2002).

PTM arising during the OVA-induced immune responses described in this chapter, could have generated T cell responses that were not detected in the epitope mapping experiments. Potential candidates might, for example, be the asparagine residue in pOVA (at position 335, which could undergo deamidation), the threonine residues in p263-278 (at positions 265 and 268, which would be a candidate for hydroxylation and subsequent glycosylation), or the serine residues in p263-278 (positions 269 and 270), where post-translational phosphorylation could occur (Anderton, 2004). PTM of amino acids outwith the pOVA and p263-278 sequences could also be implicated. To investigate this further, T cell responses to deliberately modified OVA-derived peptides could be examined, however this would be strategically difficult as well as costly, on account of the wide variation in potential PTM. Intriguingly, PTM appear to be particularly associated with strong inflammatory responses, perhaps on account of potent APC activation (Scott et al., 2000, Herzog et al., 2005). It is interesting to speculate that PTMs could be more influential upon the nature of the T cell response in the OVA/CFA setting compared to the AAI setting.

5.3.5 Concluding Remarks

The conclusions that can be drawn from the findings in this chapter are, that:-

- pOVA and p263-278 both contain epitope(s) that are major constituents of OVA-induced immune responses in C57BL/6 mice.

- The contribution of T cell responses to epitope(s) within pOVA and p263-278 towards the overall OVA response have not been quantified. However, the majority of experiments in this chapter point to a significant contribution of pOVA and p263-278 T cell responses to the overall T cell response towards OVA.
- pOVA+p263-278 PIT induced tolerance to both peptides in the context of OVA/CFA immunisation, as determined by the abrogated recall proliferation response of LN cells. However, this had little impact on the overall OVA proliferative recall response in this model.
- PIT using either pOVA or p263-278 alone did not induce linked suppression of responses towards the other peptide.
- PIT using a combination of pOVA+p263-278 reduced the severity of some parameters of OVA-induced AAI, but did not abolish disease.

6 General Discussion

The findings from this thesis can be summarised as follows:-

- pOVA PIT given intravenously and in high dose prior to OVA sensitisation did not reduce the severity of OVA-induced AAI in C57BL/6 mice. However, the same pOVA PIT regime did induce tolerance of naïve $CD4^+$ OT-II cells in settings of OVA/CFA immunisation and one OVA/alum sensitisation; this was likely to be effected predominantly via deletional and anergic mechanisms.
- Difficulties were encountered when trying to incorporate adoptive transfer of naïve $CD4^+$ T cells into models of AAI. This was most likely due to poor viability of naive OT-II cells.
- A model of AAI induced by Th2 polarised OT-II cells was subsequently established. High dose, intravenous pOVA PIT given prior to OVA challenge, reduced the severity of AAI in that model. This was associated with a reduced number of OT-II cells in the lung, but not in mLN. Splenocytes from pOVA PIT treated mice, and not from PBS treated controls, produced IL-10 in response to pOVA.
- In C57BL/6 mice (in the absence of OT-II cell transfer), pOVA PIT in the OVA/CFA setting induced tolerance to pOVA, with limited impact on the response to OVA.
- Formal epitope mapping of T cell responses demonstrated that pOVA and p263-278 both contained T cell epitopes important for OVA-induced immune responses in C57BL/6 mice.
- In the OVA/CFA setting, pOVA+p263-278 PIT abrogated recall proliferation to pOVA and to p263-278, however, this had no substantial impact on the overall recall proliferation response to OVA. Furthermore, there was no evidence of linked suppression following pOVA or p263-278 PIT.
- PIT using a combination of pOVA+p263-278 given in high dose, intravenously prior to OVA sensitisation, had some efficacy in OVA-driven AAI – reducing some parameters, but not others.

Hence, the findings in this thesis support part (i) but not part (ii) of the hypothesis, since pOVA PIT induced tolerance to pOVA but did not induce linked-suppression, and did not reduce the severity of OVA-induced AAI. pOVA PIT used in combination with p263-278 reduced the severity of some features of OVA-induced AAI.

6.1 How can the findings in this thesis be most effectively utilised and built-upon to aid the clinical translation of PIT?

In the allergic setting, it is likely that the majority of allergen-reactive T cells will be antigen-experienced (Wambre et al., 2010, Bateman et al., 2006). Hence, the model developed in this thesis, using pOVA PIT in AAI driven by Th2 polarised OT-II cells, may hold particular relevance for the clinical translation of PIT. One intriguing aspect in this context was the preferential production of IL-10 by splenocytes from pOVA PIT treated mice (Figure 4.33). It is thus pertinent to consider how these data may fit with current concepts surrounding allergen-specific immunotherapy.

The immunoregulatory function of IL-10 is apparent [reviewed in (Moore et al., 2001)]. For example, the development of colitis in IL-10 deficient mice is thought to result from dysregulated immune responses in the gut (Kuhn et al., 1993). T cell production of IL-10 has been shown to be important in the natural induction of tolerance (Meiler et al., 2008) and has been attributed to therapeutic efficacy in several mouse and human allergen-specific immunotherapy studies (Vissers et al., 2004, Campbell et al., 2009, O'Neill et al., 2006). However, IL-10 producing regulatory T cells represent a heterogeneous population [discussed in (Hawrylowicz and O'Garra, 2005)]. The “Tr1” subset has been assigned to T cells that produce IL-10 in the absence of typical Th1 or Th2 signature cytokines (Barrat et al., 2002, Buer et al., 1998), whereas in other studies Tr1 cells may produce IL-10 in combination with other cytokines (Groux et al., 1997). Interestingly, IL-10 can be induced or preferentially expressed by effector T cells, such as Th1 cells, often in conditions of

chronic or strong antigenic stimulation [discussed in (Cope et al., 2011)]. Indeed, IL-10 was first cloned from a human T cell line that expressed IFN-γ^+ (as well as other cytokines such as IL-5) (Vieira et al., 1991). Recently, Jankovic et al found that IFN-γ^+ IL-10^+ effector T cells were present in mice infected with *Toxoplasma Gondii* (a protozoan parasite) (Jankovic et al., 2007). IL-10 is known to be required to prevent exaggerated Th1 responses and subsequent death in *Toxoplasma Gondii* infected mice (Gazzinelli et al., 1996). In the Jankovic et al study, even IL-10^- IFN-γ^+ cells from infected mice could be induced to produce IL-10 after additional *in vitro* stimulation (Jankovic et al., 2007). IFN-γ^+ IL-10^+ cells have similarly been found in other situations linked to strong and/or chronic Th1 cell stimulation both in humans and mice (Morita et al., 1998, Belkaid et al., 2001) and IL-10 appears to act via a negative feedback loop to regulate the Th1 response (Gabrysova et al., 2009). Th17 cells can also modulate their IL-10 production, and consequently their pathogenic potential, in response to variations in cytokine stimulation (McGeachy et al., 2007). The situation is perhaps more complicated for Th2 cells in light of their propensity to produce IL-10 (Mosmann et al., 1990). However, IL-10 production by memory Th2 cells upon restimulation *in vitro* is variable, but has been shown to be favoured by repeated *in vitro* stimulation of Th2 cells in the presence of IL-4, most likely as a result of epigenetic effects upon the IL-10 locus (Chang et al., 2007, Lohning et al., 2003).

The production of IL-10 by effector cells in situations of strong and/or chronic antigenic stimulation appears to represent an important self-regulatory mechanism to limit the immune response and subsequent damage to the host. Recently, Sabatos-Peyton et al have proposed a model in which these mechanisms could be harnessed to best effect in PIT (Sabatos-Peyton et al., 2010). This model is based on many studies from the Wraith group, several of which have utilised a model of EAE in transgenic mice which have TCRs reactive to the Ac1-9 peptide from myelin basic protein. Multi-dose intranasal PIT using an altered Ac1-9 peptide with high affinity for MHC II [known as Ac1-9 (4Y)], led to inhibition of IL-2 and IFN-γ from Ac1-9-reactive T cells, and induced IL-10 production (Burkhart et al., 1999). However, PIT using the wild-type Ac1-9 peptide, which has weak MHC II affinity, inhibited IL-2

and IFN-γ but did not enhance IL-10 production (Gabryšová and Wraith, 2010). The IL-10 production induced by PIT using the high affinity Ac1-9 (4Y) peptide was key to the enhanced therapeutic efficacy seen using this peptide in EAE (Gabryšová and Wraith, 2010). Such findings have led to the proposal that high signal strength favours the production of IL-10 producing peptide-reactive T cells, whereas low signal strength favours a predominantly anergic T cell response (Sabatos-Peyton et al., 2010).

This proposal can be cautiously interpreted in relation to the IL-10 production seen following pOVA PIT in the Th2 model in this thesis. Following Th2 polarisation, OT-II cells produced more IL-2, IL-13 and IL-5 in response to pOVA, compared to naïve OT-II cells (Figure 4.18). Hence the signal strength generated by applying pOVA PIT to the Th2 transfer model is likely to be high. This could thus favour IL-10 production in accordance with the Sabatos-Peyton et al model. Furthermore, it is probable that IL-2 and IL-5 production from OT-II cells following pOVA PIT was reduced on an individual cell basis. This could, therefore, also fit with the Sabatos-Peyton model where reductions in effector cytokine production are associated with increases in IL-10 production in situations of high signal strength. Further investigation would be needed to address these possibilities, for example by assessing IL-10 and signature Th2 cytokine production by OT-II cells following pOVA PIT on an individual cell level (e.g. by using intracellular cytokine staining). Notably, AAI was reduced, but not abolished, using pOVA PIT in the Th2 transfer model. This is probably due to the continued (but most likely reduced) production of Th2 cytokines by OT-II cells despite pOVA PIT. It is tempting to speculate that, in accordance with the Sabatos-Peyton model, multiple doses of pOVA PIT prior to airway challenge in the Th2 OT-II model may further reduce the severity of disease by increasing IL-10 production and hence regulating the remaining effector response.

6.2 Future work

6.2.1 Further mechanistic assessment of the effects of pOVA PIT in the Th2 OT-II model established in this thesis.

Additional avenues to investigate the role of IL-10 in the effects of pOVA PIT in the Th2 OT-II model have been outlined above. Other particular areas of interest include aspects such as the assessment of chemokine receptors on OT-II cells following pOVA PIT e.g. CCR4, CCR8, and the quantification of chemokines within the lung and BAL e.g. CCL17 and CCL22. Determining the expression of survival-associated molecules relevant in this setting such OX40 and CX3CR1 would also be justified, as would the quantification of OT-II cell death in the lung, e.g. by using terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) staining. Together these approaches would help to elucidate the mechanisms of tolerance induced by pOVA PIT in the polarised Th2 cell setting. It would also be of value to utilise the Th2 OT-II pOVA PIT model developed here to assess the susceptibility of different memory and effector T cell populations to tolerance induced by PIT (for example by cell-sorting Th2 polarised OT-II cells based on CD62L and CD44 expression and adoptively transferring these populations separately).

6.2.2 Can the efficacy of pOVA+p263-278 PIT in OVA-induced AAI be improved?

The lack of linked suppression in the pOVA+p263-278 model could be relevant to the limited therapeutic effect seen here, particularly if T cell responses to minor epitopes, or to peptides that have undergone PTM, are implicated. It would therefore be interesting to see whether efficacy could be enhanced by varying the route of delivery and/or the regime; for example by using regimes in accordance with other studies reporting either the induction of IL-10 and/or linked suppression or the induction of other regulatory mechanisms. Such an approach could include multi-dose regimes to favour IL-10 production as described above (Gabryšová and Wraith, 2010, Sabatos-Peyton et al., 2010) or alternatively very low dose regimes to favour the induction of regulatory T cells (Kang et al., 2007, Apostolou and von Boehmer, 2004). An intradermal approach, after sensitisation, has previously been found to

induce linked suppression (Campbell et al., 2009). Such an approach may also be effective here.

6.3 Concluding remarks

Antigen-experienced T cells are often thought difficult to tolerise, yet are probably more representative of T cell populations that are likely to predominate in allergic patients. Many previous PIT studies have focused on the effects of PIT on naïve T cells. In this thesis, pOVA PIT was found capable of inducing tolerance in polarised Th2 pOVA-reactive T cells, resulting in a significant reduction in disease severity; this model therefore holds particular importance for future PIT studies. Furthermore, to the author's knowledge, the data presented in this thesis also represent the first formal characterisation of T cell epitopes involved in OVA-induced T cell responses in C57BL/6 mice. The pOVA+p263-278 PIT model developed here, will serve as a valuable tool for the further assessment of PIT in AAI.

In summary, the findings in this thesis contribute to, and provide a mechanistic platform for, improved understanding of PIT, thus further aiding clinical translation.

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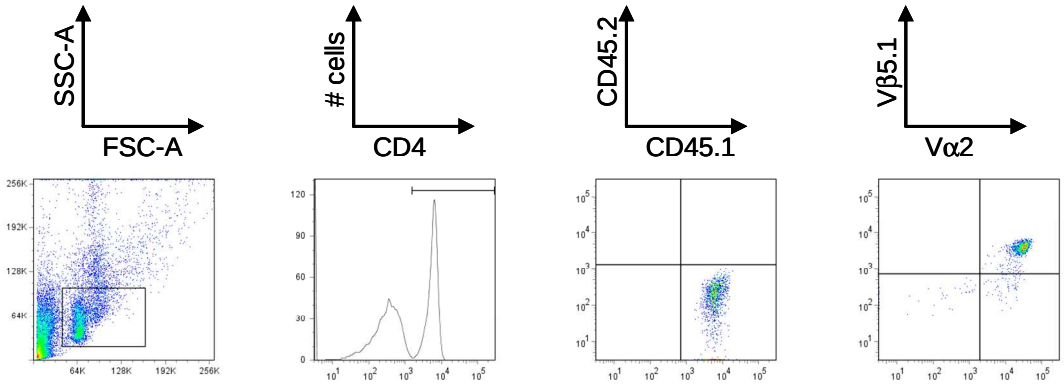
8 Appendix

8.1 Appendix 1 Phenotyping OT-II mice using flow cytometry



Blood samples were taken from OT-II mice at weaning and analysed using flow cytometry. $CD4^+$ T cells in blood were gated on and the prescence of theVβ5.1 and Vα2 TCR chains confirmed. The prescence of the congenic marker CD45.1, and the absence of the CD45.2 isoform (present in C57BL/6 mice), were also confirmed.

8.2 Appendix 2 Anaesthetic protocols

Table 4: Anaesthesia used for intratracheal instillations

Purpose	Drug	Stock Conc	Volume used	Dose/mouse	Route
Induction	Medetomidine hydrochloride (Pfizer Ltd)	1mg/ml	1ml	0.1ml/10g	i.p
	Ketamine (Fort Dodge Animal Health Ltd)	100mg/ml	0.75ml		
	0.9% w/v saline (Baxter Healthcare Ltd)		8.25ml		
Reversal	Atipamezole hydrochloride (Pfizer Ltd)	5mg/ml	0.4ml	0.1ml/10g	s.c
	0.9% w/v saline (Baxter Healthcare Ltd, Berkshire, UK)		0.6ml		

Table 5: Anaesthesia used for invasive plethysmography

Purpose	Drug	Stock Conc	Volume used	Dose/mouse	Route
Induction	Ketamine (Fort Dodge Animal Health Ltd)	100mg/ml	1.4ml	0.1ml/10g	i.p Repeat with a further 50% dose every 30 minutes if required
	Xylazine hydrochloride (Bayer Healthcare)	20mg/ml	0.7ml		
	0.9% w/v (Baxter Healthcare Ltd)		7.9ml		