

***A Streptococcus pneumoniae*-based
immunoregulatory therapy for asthma**

Submitted in total fulfilment of the requirements of the degree of

Doctor of Philosophy.

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Discipline of Immunology and Microbiology

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DECLARATION

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. I give consent to this copy of my thesis, when deposited in the University Library, being made available for loan and photocopying subject to the provisions of the Copyright Act 1968.

I hereby certify that this thesis is in the form of a series of published papers of which I am a joint author. I have included as part of the thesis preface a written statement signed by each co-author, endorsed by the Deputy Head of Faculty Research, attesting to my contribution to the joint publications.

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Alison Thorburn

30/07/10

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ABSTRACT

Asthma is a chronic inflammatory disease of the airways that affects over 300 million people worldwide. The disease is characterised by episodes of breathlessness, coughing, wheezing and airway hyperresponsiveness (AHR). Asthma results from a dysregulation in immunity that is underpinned by a cohort of effector T cell populations including T helper (Th)1, Th2, Th17 and natural killer T (NKT) cells. These effector T cells produce numerous inflammatory cytokines and chemokines that induce eosinophil influx, mucus hypersecretion and AHR. Antigen presenting cells play key roles in priming these responses. Regulatory T cells (Tregs) are essential for suppression of aberrant immune responses and maintenance of immune homeostasis. Both the number and function of Tregs is impaired in asthmatics, compared to healthy individuals. This reinforces the importance of Tregs in regulating a balanced immune response.

Microbial agents have been associated with increased or decreased risk of asthma. Microbial agents that have been associated with decreased asthma risk are under intense investigation for their potential utilisation in therapeutic strategies for asthma.

Streptococcus pneumoniae vaccination has been associated with decreased asthma-related hospitalisations in children and the elderly. Furthermore, early mouse studies observed that *S. pneumoniae* infection attenuated blood eosinophilia during parasitic infection. More recent studies have shown that both live and ethanol killed *S. pneumoniae* suppress the development of allergic airways disease (AAD) in mice, including eosinophil recruitment to the lungs, Th2 cytokine release, mucus hypersecretion and AHR. Therefore *S. pneumoniae* has the potential for development into a novel immunotherapy for asthma.

To examine this concept we first investigated the capacity of human *S. pneumoniae* vaccines, which were developed to prevent *S. pneumoniae* infection, to suppress AAD in mouse models (Chapter 2). In the next study, and in order to determine which components were required for *S. pneumoniae*-mediated suppression of AAD, *S. pneumoniae* components were tested for their capacity to suppress AAD (Chapter 3). Two potential *S. pneumoniae*-based immunotherapies were identified: the conjugate vaccine and the combination of type 3 capsular polysaccharide and pneumolysin (T3P+Ply). These *S. pneumoniae* immunotherapies suppressed the development of AAD when administered before, during and after sensitisation. Importantly, *S. pneumoniae* immunotherapy also attenuated established AAD. This demonstrated that *S. pneumoniae* immunotherapy has potential for therapeutic use in the prevention and/or treatment of asthma.

To determine the mechanisms involved in *S. pneumoniae*-mediated suppression of AAD a number of investigations were performed. Tregs were shown to be induced by *S. pneumoniae* immunotherapy. Furthermore, anti-CD25 antibody-mediated depletion of Tregs reversed the effect of immunotherapy. Hence, Tregs were required for immunotherapy-mediated suppression of AAD. In the third study, Tregs were shown to be induced in a biphasic manner to suppress immune responses and AAD through a broad range of mechanisms (Chapter 4). Together, these studies have identified potential and novel *S. pneumoniae* immunotherapies for asthma and determined the mechanism of action that underpins suppression of AAD.

PREFACE

Peer reviewed publications

These publications are not presented in this thesis but included in the Appendix.

- **Thorburn, A.N.** and Hansbro, P.M. Harnessing regulatory T cells to suppress asthma: From potential to therapy. *Am. J. Respir. Cell Mol. Biol.* 2010. In press. (Appendix I)
- **Thorburn, A.N.**, Preston, J.A., Starkey, M.R., Beckett, E.L., Horvat, J.C., Wade, M.A., Beagley, K.W., Gibson, P.G., Foster, P.S., Hansbro, P.M. *Streptococcus pneumoniae* infection suppresses the progression of allergic airways disease. *Eur. Resp. J.* 2010. In press. (Appendix II)
- **Thorburn, A.N.**, Hansbro, P.M., Gibson, P.G. Pneumococcal vaccines for allergic airways diseases. *Exp. Opin. Biol. Ther.* 2009;9(5):1-9. (Appendix III)

Publications prepared for submission

These publications form the basis of this thesis.

- **Thorburn, A.N.**, O'Sullivan, B.J., Thomas, R., Kumar, R., Foster, P.S., Gibson, P.G., Hansbro, P.M. Pneumococcal conjugate vaccine-induced regulatory T cells protect against allergic airways disease. Submitted to *Thorax* (Under secondary review). Presented in Chapter 2 of this thesis.
- **Thorburn, A.N.**, Foster, P.S., Gibson, P.G., Hansbro, P.M. Pneumococcal-based therapy suppresses natural killer T cells and allergic airways disease by inducing regulatory T cells. Submitted to *J. Allergy Clin. Immunol.* Presented in Chapter 3 of this thesis.

- **Thorburn, A.N.**, Foster, P.S., Gibson, P.G., Hansbro, P.M. Pneumococcal components induce regulatory T cells that mediate immune deviation and suppression to attenuate the development of allergic airways disease. Prepared for submission to *J. Immunol.* Presented in Chapter 4 of this thesis.

Published abstracts

- **Thorburn, A.N.**, Foster, P.S., Gibson, P.G., Hansbro, P.M. *Streptococcus pneumoniae* vaccine, Prevenar, induces regulatory T cells and prevents allergic airway disease. *Am. J. Respir. Crit. Care Med.* 2010;181:A5599.
- **Thorburn, A.N.**, Foster, P.S., Gibson, P.G., Hansbro, P.M. Development of a *Streptococcus pneumoniae* – based immunoregulatory therapy for asthma *Am. J. Respir. Crit. Care Med.* 2010;181:A5588.
- **Thorburn, A.N.**, Foster, P.S., Gibson, P.G., Hansbro, P.M. Induction of regulatory T cells by a novel immunoregulatory therapy suppresses the development of allergic airways disease. *J. Immunol.* 2010;184:97.10.
- **Thorburn, A.N.**, Foster, P.S., Gibson, P.G., Hansbro, P.M. CD25 (IL-2R α)-dependent induction of adaptive regulatory T cells by a novel immunoregulatory therapy (IRT) suppresses allergic airways disease. Australasian Society for Immunology 39th Annual Scientific Meeting: Delegate Book. Gold Coast, QLD. 2008.
- **Thorburn, A.N.**, O'Sullivan, B.J., Thomas, R., Foster, P.S., Gibson, P.G., Hansbro, P.M. Prevenar suppresses allergic airways disease through the induction of regulatory T cells. *Am. J. Respir. Crit. Care Med.* 2009;179:A2228.
- **Thorburn, A.N.**, Foster, P.S., Gibson, P.G., Hansbro, P.M. *Streptococcus pneumoniae* vaccine, Prevenar, utilizes Tregs to suppress asthma. *J. Immunol.* 2009;182:140.2.

- **Thorburn, A.N.**, O'Sullivan, B.J., Thomas, R., Foster, P.S., Gibson, P.G., Hansbro, P.M. Prevenar suppresses allergic airways disease through the expansion of regulatory T cells. *Respirology*. Darwin, NT. 2009.
- **Thorburn, A.N.**, Foster, P.S., Gibson, P.G., Hansbro, P.M. *Streptococcus pneumoniae* vaccine Prevenar: A potential therapy for the suppression of asthma. *Am. J. Respir. Crit. Care Med.* Toronto, ONT. 2008.
- **Thorburn, A.N.**, Foster, P.S., Gibson, P.G., Hansbro, P.M. *Streptococcus pneumoniae* vaccine Prevenar: A potential therapy for the suppression of asthma. *Respirology*. Melbourne, VIC. 2008.
- **Thorburn, A.N.**, O'Sullivan, B.J., Thomas, R., Foster, P.S., Gibson, P.G., Hansbro, P.M. Suppression of allergic airways disease by the *Streptococcus pneumoniae* vaccine Prevenar through the induction of regulatory T cells. Australasian Society for Immunology 38th Annual Scientific Meeting: Delegate Book. Canberra, ACT. 2008.

Research Higher Degree candidate Alison Thorburn contributed wholly to the publications/prepared manuscripts that form the basis of this thesis, as listed in the preface. This contribution involved the development of the projects, generation and analysis of experimental data and completion of the manuscript in collaboration with the other authors.

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ABBREVIATIONS

AAD	Allergic airways disease
AHR	Airways hyperresponsiveness
ALI	Acute lung injury
AlPO ₄	Aluminium phosphate
ANOVA	Analysis of variance
APC	Antigen presenting cell
ATCC	American type culture collection
AUD	Australian dollar
BAL	Bronchoalveolar lavage
BCG	Bacillus calmette-guerin
CbpA	Choline binding protein A
CCL	Chemokine ligand
CCR	Chemokine receptor
CD	Cluster of differentiation
cDNA	Complementary DNA
cfu	Colony forming units
CJ	Conjugate
COPD	Chronic obstructive pulmonary disease
CpG-ODN	CpG-oligodinucleotide
CREB	Cyclic adenosine monophosphate response element-binding
CRM ₁₉₇	Cross-reactive material 197

CTLA-4	Cytotoxic T lymphocyte antigen-4
CW	Cell walls
DC	Dendritic cell
DNA	Deoxyribonucleic acid
DPPE-PEG	di-palmitoyl-phosphatidyl-ethanolamine polyethylene glycol
Ebi3	Epstein-barr virus induced gene 3
ECP	Eosinophil cationic protein
EDN	Eosinophil-derived neurotoxin
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EPO	Eosinophil peroxidase
FACS	Fluorescent-activated cell sorting
Fc	Fragment crystallisable
FCS	Fetal calf serum
FGL2	Fibrinogen-like protein 2
FITC	Fluorescein isothiocyanate
FoxP3	Fork-head box protein 3
GFP	Green fluorescent protein
GITR	Glucocorticoid-induced TNFR-related protein
GM-CSF	Granulocyte macrophage colony stimulating factor
GPR	G protein-coupled receptor
h	Hour(s)
HBSS	HANKS balanced salt solution

HDM	House dust mite
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HO	Heme oxygenase
i.m.	Intramuscular
i.n.	Intranasal
i.p.	Intraperitoneal
i.t.	Intratracheal
i.v.	Intravenous
ICAM-1	Intercellular adhesion molecule-1
ICOS	Inducible T cell co-stimulator
IDO	Indolamine 2,3-dioxygenase
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
KC	Keratinocyte chemoattractant
LAG	Lymphocyte-activation gene
LFA	Lymphocyte function-associated antigen
LPS	Lipopolysaccharide
LT	Leukotrienes
LytA	Autolysin
M	Molar
MAL	MyD88-adaptor-like
MBP	major basic protein

MCP-1	Monocyte chemotactic protein-1
mDC	Myeloid DC
mg	Milligram
MHC	Major histocompatibility complex
min	Minute
MIP2	Macrophage inflammatory protein 2
MLN	Mediastinal lymph node
mM	Milimolar
mRNA	Messenger RNA
MyD88	Myeloid differentiation factor 88
n.s.	Not significant
NFAT	Nuclear factor of activated T cells
NF- κ B	Nuclear factor κ B
ng	Nanogram
NK	Natural killer
NKT	Natural killer T
Nrp	Neuropilin
$^{\circ}$ C	Degrees celcius
OVA	Ovalbumin
OVAp	OVA peptide
PAMP	Pattern associated molecular pattern
PBMCs	Peripheral blood mononuclear cell
PD-1	Programmed death 1

pDC	Plasmacytoid
PDGF	Platelet-derived growth factor
pg	Picogram
Ply	Pneumolysoid
PMA	Phorbol myristate acetate
PPR	Pattern recognition receptor
PS	Polysaccharide
PsaA	Pneumococcal surface adhesin A
PspA	Pneumococcal surface protein A
R	Receptor
RA	Rheumatoid arthritis
RANTES	Regulated upon activation, normal T cell expressed and secreted
Rel	Reticuloendotheliosis
RNA	Ribonucleic acid
Rory-t	RAR-related orphan receptor gamma-thymus
RT-PCR	Reverse transcriptase-polymerase chain reaction
RV	Rhinovirus
SEM	Standard error of the mean
Smad	Mother against decapentaplegic homolog
SpsA	<i>Streptococcus</i> secretory IgA binding protein
STAT	Signal transducer and activator of transcription
T3P	Type 3 polysaccharide

TARC	Thymus and activation-regulated chemokine
T-bet	T-box expressed in T cells
TCR	T cell receptor
TGF	Transforming growth factor
Th	T-helper
TIR	Toll/IL-1R
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TNFRS	Tumour necrosis factor receptor superfamily
Tr1	Treg 1 subtype
Tr3	Treg 3 subtype
TRAF	TNF receptor associated factor
TRAM	TRIF-related adaptor molecule
Treg	T regulatory cell
TRIF	TIR-domain-containing adapter-inducing interferon- β
USD	United States dollar
VEGF	Vascular endothelial growth factor
xg	Times g-force
α CD25	Anti-CD25 antibody
α Gal-Cer	α -galactosylceramide
α TGF- β	Anti-TGF- β antibody
μ g	Microgram
μ l	Microlitre

CHAPTER 1

INTRODUCTION

This chapter presents the current understanding of the pathogenic mechanisms and immune responses involved in asthma. The relationship between microbial exposure and asthma risk is reviewed. This leads to a discussion on the potential use of *Streptococcus pneumoniae* as a novel therapy for asthma, which is followed by the hypothesis and the aims that are investigated in this thesis.

1.1 ASTHMA

1.1.1 Asthma: An overview

Asthma is a common respiratory disease that affects people of all ages. The prevalence of asthma is particularly high in Western countries such as Australia, New Zealand, parts of Europe and North America (1). In these areas, asthma affects up to 1 in 10 adults and 1 in 7 children (2). In contrast, the prevalence of asthma in some third world countries may be as low as 2 %. In the USA, the prevalence of asthma increased 75 % between 1980 and 1994 (3). In Australia, the prevalence of asthma doubled in children between 1980 and 1990 (4). Most recent data indicate that the prevalence of asthma has plateaued in the countries with the highest rates. However, as a result of population growth in these countries, the absolute number of asthma cases continues to increase. Nevertheless, asthma is still increasing in countries with intermediate asthma rates. Furthermore, when children migrate from a country with low incidence to a country with high incidence, they have an 11 % chance of developing asthma (5). Hence, there are major environmental influences that predispose an individual to asthma. As developing countries, such as India and China, become more westernised incidence of asthma in these countries is expected to increase. Our understanding of the factors that predispose to asthma may aid in development of interventions that will reduced the burden of disease.

As well as morbidity, the financial costs of asthma are considerable. It has been estimated at \$USD 12 billion in the USA (1994), 17.7 billion EURO in Europe and \$AUD 720 million per annum (6-8).

Taken together, these facts demonstrate that the development of an effective therapy against asthma is a substantial world healthcare need.

1.2 THE PATHOGENESIS OF ASTHMA

1.2.1 Asthma pathogenesis: An overview

Asthma is an allergic airways disease (AAD) that is characterised by episodes of breathlessness, coughing, wheezing and airways hyperresponsiveness (AHR) (9). AHR is the increased sensitivity of the airways toward non-specific stimuli and causes the airways to constrict. These symptoms may lead to respiratory distress and even death. Predisposing factors, such as genetic make-up, may confer susceptibility and environmental factors may promote expression of the disease. Asthma is an acute on chronic disease, where exacerbation of chronic morbidity characterises an asthmatic attack. These manifestations are the result of early and late allergic responses to allergens or inducing agents.

1.2.2 Early phase response

The early or immediate phase response in asthma results from an overt reaction to stimuli such as allergens in a sensitised individual. Common allergens include house dust mite (HDM), pollen, grasses, moulds, animal dander and food products. Mast cells play a key role in this early phase response. Upon secondary exposure to allergen, allergen specific immunoglobulin (Ig)E binds to both allergen and FcεRI receptors on the surface of mast cells. Cross-linking of the IgE/FcεRI complex results in mast cell activation. Activated mast cells release preformed mediators such as histamine, heparin and serine proteases as well as newly formed lipid mediators including prostaglandins, leukotrienes and cytokines. These mediators have a number of downstream effects on airway structural elements and result in bronchoconstriction, mucus hypersecretion, cellular inflammation, vascular leakage and oedema. Notably, asthmatics have 2-6 fold higher mast cell numbers in their airways compared to healthy individuals and total

serum IgE correlates with the severity of asthma symptoms and AHR (10, 11). Furthermore, anti-IgE therapy (Omalizumab) has proven to be effective for allergic disease when administered in conjunction with corticosteroids (12). These studies provide support for the role of mast cells in asthma and highlight their importance in disease.

1.2.3 Late phase response

The more persistent late response is mediated by leukocytes such as memory T helper (Th)₂ cells, which are responsible for persistent IgE production. Eosinophils and neutrophils are also more characteristic of the late stage allergic response (13). Together, these cell types mediate the chronicity of disease by initiating airway changes and remodelling (14). Chronic inflammation drives airway remodelling, mucus hypersecretion and AHR, which leads to acute or chronic obstruction of the airways and the clinical symptoms of asthma.

1.3 IMMUNE RESPONSES IN ASTHMA

1.3.1 Immune response

The immunopathology underlying bronchial inflammation in asthma is driven by cluster of differentiation (CD)₄⁺ Th cells. This was identified in studies which showed that the elimination of CD₄⁺ cells prevented the development of allergic disease in mouse models (15).

It is now known that aberrant and dysregulated Th cell responses drive the disease pathology (16). Naïve T cells require two signals to mature into functional Th cells. Firstly, naïve T cells must recognise antigenic peptides bound to major

histocompatibility complex (MHC) class II molecules through interaction with the T cell receptor (TCR) and CD3. Secondly, naïve T cells must activate the pathways that lead to co-stimulation through CD28 interaction with CD80 or CD86 on professional antigen presenting cells (APC). This event is a protective measure to ensure that the T cells are responding to foreign or harmful antigens. If co-stimulation does not occur, the T cells become anergic and eventually apoptose.

Naïve T cells, therefore, “check” and usually “ignore” harmless antigenic peptides. Failure to distinguish between harmful and harmless antigens, leads to the activation of inappropriate Th cell responses. Th0 cells may differentiate into three major effector T cell subtypes Th1, Th2, Th17 (Figure 1.1). The development of each subtype is determined by the signals that are received during maturation. Furthermore, each subtype has a signature transcriptional and cytokine profile which mediates their function. While we understand the phenotype of their mature form, the mechanisms that determine the nature and eventual generation of different Th cell subtypes are largely undefined. Evidence suggests that it may be dependent upon the type of APC, concentration of antigen, or the presence of cytokines. Each subset then plays an important and defined role in subsequent immune responses.

1.3.2 Effector T cells and their roles in asthma

1.3.2.1 Th1 cells

In the presence of interleukin (IL)-12 Th0 cells differentiate into Th1 cells. A major cytokine released by Th1 cells is interferon (IFN) γ (Table 1.1). IFN- γ increases IL-12 production by dendritic cells (DCs) and macrophages, which in turn amplifies the Th1 response *via* a positive feedback loop (17). IFN- γ also inhibits IL-4 and thus preserves Th1 responses at the expense of Th2 responses (18).

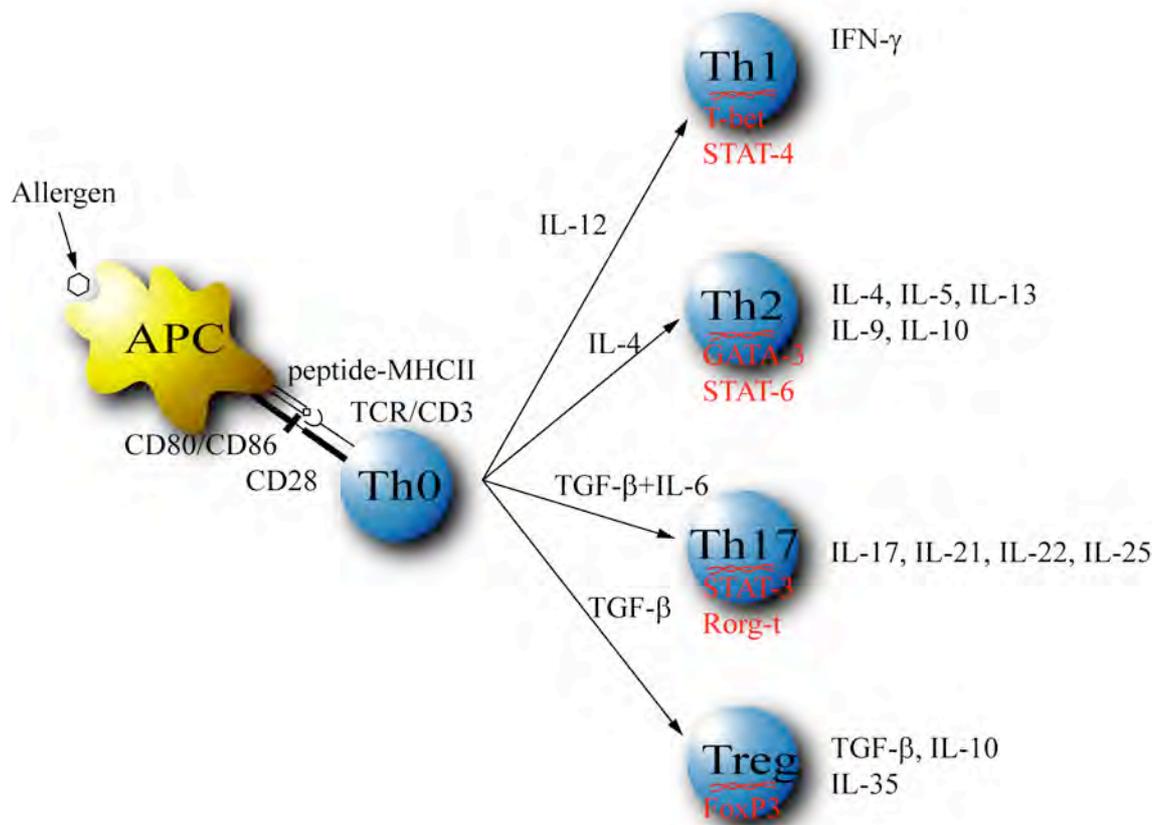


FIGURE 1.1. T cell subtypes. Antigen presenting cells (APCs) present antigen peptide on major histocompatibility complex (MHC) II to the T cell receptor (TCR) on T helper (Th)0. Co-stimulation may occur *via* CD80/CD86-CD28 interactions. Th0 cells differentiate under different conditions into Th1, Th2, Th17 or regulatory T cells (Treg) cells. Each T cell subtype may be identified by specific transcriptional and cytokine profiles. T-bet, T-box expressed in T cells; STAT, signal transducer and activator of transcription; Ror γ -t, RAR-related orphan receptor gamma-thymus; IL, interleukin; TGF- β transforming growth factor beta.

TABLE 1.1. Cytokines and their function in asthma

CYTOKINES	EFFECTOR T CELL SUBTYPE	FUNCTION
IFN- γ	Th1	Potent activator of macrophages Increases IL-12 in a positive feedback loop Inhibits IL-4 and the differentiation of Th0 into Th2 cells
IL-4	Th2/NKT	Induces differentiation of Th0 into Th2 in a positive feedback loop Induced B class switching to IgE Suppresses Th1 responses Upregulates MHCII
IL-5	Th2/NKT	Stimulates B cells to increase Ig secretion Activates eosinophils
IL-13	Th2/NKT	Induces B cell class switching to IgE Induces AHR, goblet cell metaplasia and mucus hypersecretion
IL-10	Th2/NKT	Enhances B cell survival, proliferation, and Ig secretion Down-regulates the expression IFN- γ Stimulates mast cells
IL-9	Th2	Stimulates cell proliferation and prevents apoptosis
IL-17	Th17	Induces the production of cytokines (IL-6, IL-1 β , TGF- β , TNF- α , GM-CSF), chemokines (IL-8, MCP-1), and prostaglandins Induces airway remodelling Attracts neutrophils
IL-21	Th17	Induces cell division and proliferation Regulates Ig class switching
IL-22	Th17	Stimulates inflammatory responses
IL-25 (IL-17E)	Th17	Induces the production of the cytokines IL-4, IL-5, IL-13, IL-8 Induces NF- κ B activation

TNF, tumor necrosis factor; GM-CSF, granulocyte macrophage colony stimulating factor; MCP-1, monocyte chemotactic protein-1; NF- κ B, nuclear factor- κ B

IFN- γ has pro-inflammatory activity in the lower airways and moderate to severe asthmatics have strong Th1 cell and IFN- γ responses during disease (19, 20). Furthermore, IFN- γ has been shown to induce AHR in mouse models of acute and chronic disease (21, 22). IFN- γ also amplifies Th intermediate responses which induce AHR (23). Therefore the induction of a Th1 response may contribute to disease pathogenesis in asthma.

1.3.2.2 Th2 cells

In the presence of IL-4 Th0 cells differentiate into Th2 cells. Th2 cells also release IL-4, which amplifies the Th2 response *via* a positive feedback loop (18).

Together, the Th2 cytokines IL-4, IL-5, IL-9, IL-10 and IL-13 induce the recruitment and activation of eosinophils, pulmonary inflammation, mucus hypersecretion, B cell isotype switching and AHR (Table 1.1). Animal studies have demonstrated a role for Th2 cells in AAD, since their elimination prevents the development of disease (24). Memory Th2 cells play an important role in the pathogenesis of asthma (25). They reside in the lungs and upon allergen exposure reactivate inflammation and exacerbation of disease.

Interestingly, studies have shown that AAD may continue to develop in the absence of the antigen specific activation of Th2 cells (26). Furthermore, AHR and eosinophilic inflammation may occur in the absence of IgE (27). These studies suggest that AAD is a complex and diverse disease that may be mediated by a variety of immune factors and pathways.

1.3.2.3 Th17 cells

Th17 cells are the most recently recognised member of the Th cell family (28). In the presence of IL-6 and transforming growth factor (TGF)- β Th0 cells differentiate into Th17 cells. In addition, IL-23 plays a role in expansion and maintenance of the Th17 cells population (29). Th17 cytokines play a major role in neutrophil chemotaxis and amplification of the inflammatory response by promoting inflammatory cytokine secretion (Table 1.1). They are negatively regulated by IFN- γ and IL-4 (30).

Bronchial biopsies from patients during acute episodes of severe asthma are infiltrated with Th17 cells (31). Furthermore, sputum and bronchoalveolar lavage (BAL) from asthmatics show increased levels of IL-17 (32).

Studies using animal models have also established that Th17 cells and their cytokines are major inducers of neutrophilic, eosinophilic and steroid resistant airway inflammation (33, 34). Hence, Th17 cells play an important role in the pathogenesis of asthma.

1.3.3 Natural killer T (NKT) cells and their roles in asthma

Another effector T cell type, NKT cells, are a unique subset of T cells, which respond to glycolipids presented by CD1d (instead of peptide presentation by MHCII) and secrete large amounts of Th2 cytokines (35). NKT cells are a heterogeneous group of cells that share properties of both T cells and NK cells. The most well characterised subset of CD1d-dependent NKT cells express an invariant TCR α chain. These cells are conserved between humans and mice and are implicated in many immunological processes.

NKT cells have been detected at high levels in the sinus mucosa and sputum of asthmatics compared to healthy individuals (36, 37). However, others have disputed these findings (38). Nevertheless, animal studies have identified a potential requirement

for NKT cells in AAD, largely in the induction of AHR, particularly IL-4/IL-13 producing NKT cells (39).

Although allergen-specific Th2 and NKT cells have many phenotypic similarities (e.g. expression of CD4 and production of Th2 cytokines) they also have cell specific activities. For example, Th2 cytokine release from the different cells is activated under different conditions, they have differential sensitivity to corticosteroids, and respond to different classes of antigen (proteins versus glycolipids) (40). Therefore, it has been proposed that Th2 cells and NKT cells interact synergistically to induce asthma (41). It is also possible that NKT and Th2 cells may have different and distinct roles in different subgroups of asthmatics.

1.3.4 Antigen presenting cells and their roles in asthma

APCs such as DCs and macrophages have critical roles in antigen presentation, initiation and maintenance of allergic disease. Immature APCs constantly sample the environment through pattern recognition receptors (PRRs) and upon maturation, upregulate cell-surface receptors such as CD80, CD86, and CD40. These receptors act as co-receptors in T cell activation and greatly enhance the APCs ability to activate T cells.

Both myeloid and plasmacytoid DCs (mDCs and pDCs respectively) are increased in the airways of asthmatic patients after allergen challenge, suggesting potential roles in allergic inflammation (42). *In vitro* studies have shown that allergen pulsed DCs from asthmatics stimulate IL-4 production, promoting Th2 differentiation whereas those from healthy controls induce IFN- γ production (43). Hence, DCs from asthmatics are primed to induce pro-asthmatic responses. Furthermore, since IFN- γ suppresses IL-4 and the differentiation of Th2 cells, healthy individuals are potentially

protected against the development of AAD. DCs from asthmatics display differential chemokine receptor expression, which contributes to an altered migratory pattern and allergic asthma. For example chemokine ligand (CCL)2 (monocyte chemotactic protein 1 (MCP-1)), CCL17 (thymus activation regulated chemokine (TARC)) and CCL22 (macrophage derived chemokine (MDC)) which promote homing and function of DCs are upregulated in asthma (44-47). Since DCs bridge the innate and adaptive immune system, they play a fundamental role in driving the disease process.

1.3.5 PRRs

PRRs recognise structurally conserved molecules known as pathogen-associated molecular patterns (PAMPs). Toll-like receptors (TLRs), Nod-like receptors, C-type lectin receptors and retinoic acid inducible gene-like helicases are PRRs that are known to play important roles in innate immunity and the function of TLRs are the best understood (48).

To date, 13 TLRs have been identified in mammals (10 in humans, 12 in mice) (49). Four adapter molecules are known to mediate TLR signalling, these are myeloid differentiation factor 88 (MyD88), MyD88-adapter-like (MAL)/tumour necrosis factor receptor associated factor (TRAF), toll/IL-1R-domain-containing adapter-inducing interferon- β (TRIF) and TRIF-related adaptor molecule (TRAM). These adapter molecules activate pathways that amplify signals and ultimately lead to the induction of the genes involved in inflammatory responses and the induction of type 1 interferon or co-stimulatory molecules. TLR interactions also define the fate of cells by activation of pathways that lead to cell proliferation, differentiation or apoptosis.

TLRs are expressed by a broad range of human cells including primary airway and alveolar type II epithelial cells, fibroblasts, airway smooth muscle, mast cells and

DCs (50-53). TLR ligation and pathway activation are also known to contribute to both the development of allergic sensitisation and early phase responses in asthma. DCs have also been shown to discriminate between allergens, and have different means of processing and presentation, therefore inducing a specific response to that allergen dependent upon the specific TLR ligation (54-56). Differential activation of TLRs, during exposure to microbial agents and in asthma, may lead to pro-regulatory or pro-asthmatic responses (57). Nevertheless, the distinct TLRs and TLR-signalling pathways that differentially regulate or promote the asthma phenotype remain unclear.

1.3.6 Eosinophils

Eosinophils differentiate from myeloid precursor cells in response to IL-3, IL-5 and granulocyte macrophage colony-stimulating factor (GM-CSF). After maturation, eosinophils infiltrate the blood and migrate to the lung in response to chemokines such as CCL11 (eotaxin-1), CCL24 (eotaxin 2), CCL5 (Regulated upon activation, normal T cell expressed and secreted (RANTES)) and leukotrienes (LT, e.g. LTB₄). Eosinophils have numerous effector functions and are capable of inducing tissue damage and dysfunction in the lung. These effects are mediated by the release of the granule proteins: major basic protein (MBP), eosinophil cationic protein (ECP), eosinophil peroxidase (EPO) and eosinophil-derived neurotoxin (EDN). Other factors that are released include reactive oxygen species (e.g. superoxide), lipid mediators (e.g. eicosanoids such as leukotrienes), enzymes (e.g. elastase), growth factors (e.g. TGF- β , vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF)) and cytokines (e.g. IL-1, IL-2, IL-4, IL-5, IL-6, IL-8, IL-13 and tumour necrosis factor (TNF) α).

Eosinophils play major roles in asthmatic responses and have been directly associated with disease severity (58). Both eosinophilic and non-eosinophilic (predominantly neutrophilic) phenotypes of asthma have been identified, which suggests that eosinophils are not the only markers for asthma (59). Animal studies have shown that the release of MBP and EPO are not sufficient to produce the asthmatic phenotype (60). Nevertheless, eosinophils have antigen presenting capacity and may play an important role in the development of pulmonary inflammation (61).

1.4 REGULATORY T CELLS (TREGS)

In healthy individuals Tregs play essential roles in regulating inflammatory responses and maintaining homeostasis and deficiencies in Tregs have been associated with asthma.

1.4.1 Tregs and asthma

The number of Tregs is reduced in the blood and/or sputum of atopic individuals and Tregs from atopic individuals have reduced capacity to suppress effector T cells and Th2 cytokines (62-66). Furthermore, chemotactic signals for Tregs, such as those in the CCL1 pathway, may be defective in asthmatics (67).

The requirement for Tregs in the effective suppression of asthmatic responses has been demonstrated using animal models of AAD. Adoptive transfer of CD4⁺CD25⁺ Tregs into sensitised mice prior to antigen challenge or during established disease suppresses AAD (68-70). These observations provide strong evidence that an effective regulatory response, which is controlled by Tregs, may be required to prevent the development and progression of asthma.

1.4.2 Characterisation of Tregs

Tregs may be characterised by the expression of many phenotypic and functional markers (71, 72). Two distinct populations of Tregs have been identified, that are termed natural and inducible, although other subtypes have also been identified (Table 1.2). Classically Tregs are characterised by CD4, CD25 and Foxp3 expression. CD25 is expressed on the majority (>90 %) of Foxp3⁺ T cells. However, Foxp3 is widely recognised as the “master of regulation” of Tregs since expression directly parallels their suppressive capacity, survival and stability. Many other markers have also been associated with Tregs and their function (Table 1.3). These markers have enabled the further delineation of the subtypes, activation and function of Tregs that may be important in different disease states. The ongoing identification of additional suppressive mechanisms and markers indicates that numerous other Treg subtypes or functions are likely to exist.

1.4.3 Mechanisms of suppression

Tregs utilise various mechanisms to modulate inflammatory responses (71). These mechanisms can be classified into three different categories that involve soluble factors, cell-contact and deprivation of growth factors (Table 1.4). The regulatory effects of Tregs on effector cell responses include: cell cycle arrest and inhibition of proliferation; induction of apoptosis; and suppression of cytokine release, DC maturation or antigen presentation and co-stimulation.

TABLE 1.2. Treg population origins and Treg subtype characteristics

TREG POPULATION	ORIGIN
Natural Tregs	Arise in the thymus by interaction with self-antigens
Inducible Tregs	Arise from naïve T cells in the lymphoid tissue after encounter with foreign antigen Generated under the influence of IL-2 and TGF- β
TREG SUBTYPES	CHARACTERISTICS
Tr1	High levels of IL-10 with or without TGF- β
Tr3	Release TGF- β

Adapted from Thorburn & Hansbro 2010 (71) (Appendix I).

TABLE 1.3 Treg markers and their association

MARKER	ASSOCIATED WITH	REF.
FoxP3	Suppression	(71)
CD69	A unique subset	(73)
CD103, TNFR2, CD101, CD45RB	Increased activation status	(74-77)
GITR, ICOS Activin A	Clonal expansion	(78-80)
IL-9	Enhanced suppressive function	(81)
HO-1, GPR83	Generation of Tregs	(82, 83)
Retinoic acid	Homing and differentiation	(84)
CD62L	Homing state	(85)
LFA-1	Induction and function	(86)
OX40/CD134, PD-1	Inhibition of suppression	(87, 88)
CD127	Low expression on Tregs	(89)
CD137/4-1BB	Survival	(90)

FoxP3, Fork-head box protein 3; TNFR, tumor necrosis factor receptor; GITR, glucocorticoid-induced TNFR-related protein; ICOS, inducible T cell co-stimulator; HO, heme oxygenase; GPR, G protein-coupled receptor; LFA, lymphocyte function-associated antigen; PD, programmed death.

Adapted from Thorburn & Hansbro 2010 (71) (Appendix I).

TABLE 1.4. Mechanisms of Treg-induced suppression

MECHANISM	FUNCTION	REF.
1. Soluble factors		
IL-10	Prevents the synthesis of pro-inflammatory cytokines Down-regulates the production of effector T cell cytokines, antigen presentation and co-stimulatory molecule expression	(91)
TGF- β	Prevents T cell proliferation and differentiation Inhibits B cell proliferation and apoptosis Inhibits macrophage proliferation and function Maintains Treg function and promotes the differentiation of Tregs.	(92)
IL-35	Direct suppression of target effector cell function	(93)
FGL2	Down-regulates DC function Limits activation of naïve T cells Induces apoptosis of B cells	(94)
Granzyme A	Induces perforin dependent apoptosis	(95)
Granzyme B	Induces perforin dependent or independent manner apoptosis	(96, 97)
Adenosine	Suppresses effector cell function by binding the A2A receptor Reduces DC function and co-stimulatory marker expression	(98)
2. Cell contact		
Galectin-1	Induces cell cycle arrest or apoptosis by binding to effector cells	(99)
CTLA-4	High affinity CD28 homologue; blocks co-stimulation and suppresses co-stimulatory molecule expression Stimulates the production of IDO-producing immunosuppressive DCs	(100)
LAG-3	High affinity CD4 homologue; suppresses the priming and differentiation of effector T cells	(101)
Nrp-1	Prolongs the interaction with DCs and reduces antigen	

presentation to naïve T cells (102)

3. Deprivation of growth factors

IL-2 Essential for both Treg and effector cell function (103)

Tregs compete with effector T cells for IL-2, effector T cells are deprived of stimulation leading to apoptosis

FGL2, Fibrinogen-like protein 2; CTLA-4, cytotoxic T-lymphocyte antigen 4; IDO, indoleamine 2,3-dioxygenase; LAG, lymphocyte-activation gene; Nrp-1, Neuropilin-1.

Adapted from Thorburn & Hansbro, 2010 (71) (Appendix I).

Many of the suppressive mechanisms utilised by Tregs have been widely proposed to involve the release of immunosuppressive factors such as IL-10 and TGF- β , which have been shown to mediate suppression *in vitro* (104, 105). However, both *in vivo* and *in vitro* experiments have shown that neutralising antibodies against IL-10 and TGF- β fail to abrogate suppression (106, 107). In addition, mice deficient in IL-10 or TGF- β have Tregs that possess normal function (108, 109). Other studies using transwell experiments demonstrate that cell-contact is required in some settings and not in others (110). These disparities, along with the recognition of many additional mechanisms of suppression have caused confusion and controversy in the field. It is possible that the mechanisms of suppression that are involved in individual situations are dependent on the disease state, nature of immune response, eliciting agent, immunological make-up of the host and Treg proximity from target cells. Elucidation of the requirements for the initiation of suppression will further the understanding of the contextual importance of each mechanism.

1.4.4 Treg-mediated suppression in asthma

Many cell types involved in asthma are subject to Treg-mediated suppression (Figure 1.2). Tregs may suppress the priming of an allergic response and/or directly suppress terminally differentiated effector cells.

Tregs employ a number of different strategies to attenuate effector T cell responses during the development of AAD (Table 1.4). Tregs may attenuate the establishment of stable contacts between APCs and naïve T cells, inhibit APC activity or promote suppressive factors that prevent effector T cell development. These processes result in the suppression of immune response priming and prevent the development of adverse effector T cell responses.

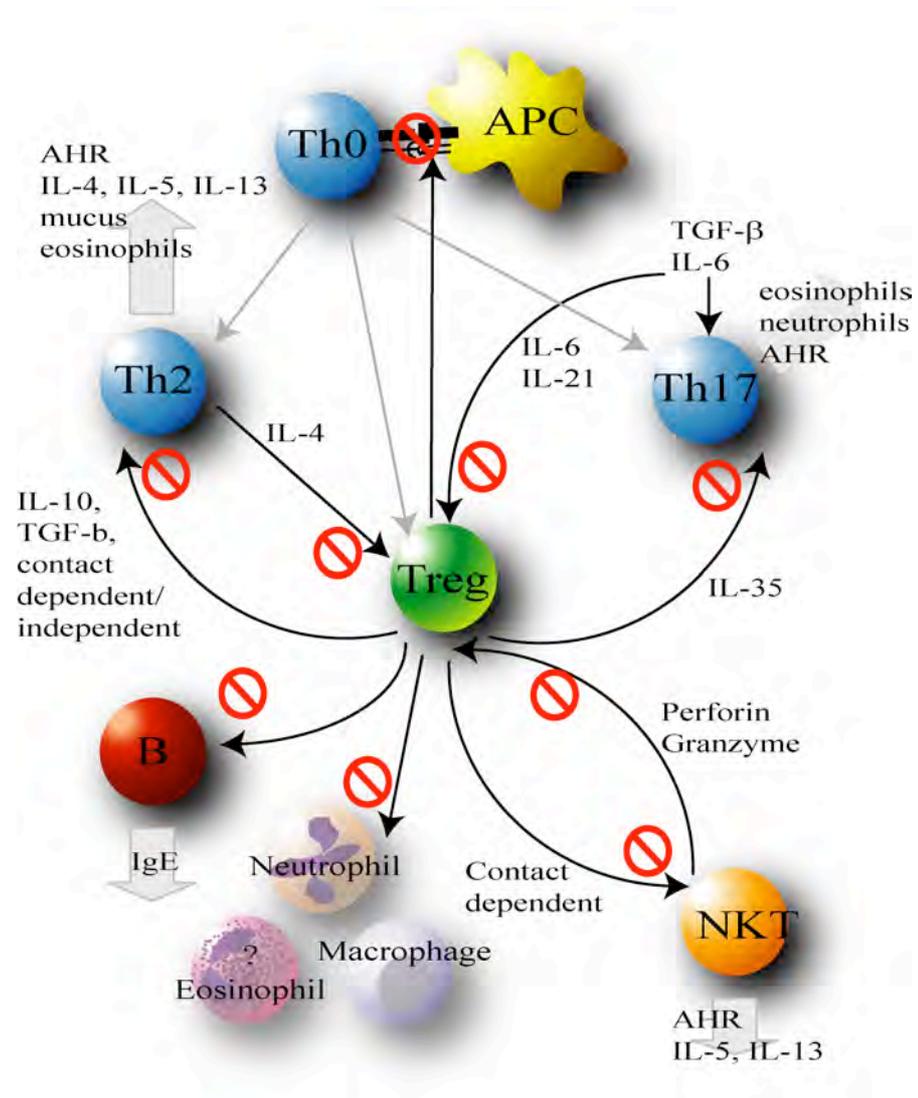


FIGURE 1.2. The role of Tregs in asthma. Treg mediated immunoregulation is crucial in preventing the dysregulated immune responses that drive the initiation, progression and exacerbation of asthma. By regulating Th2, Th17 and NKT cells, APCs, B cells and inflammatory cells Tregs prevent the development of allergic inflammation, IgE release, mucus hypersecretion and AHR. From Thorburn & Hansbro, 2010 (71) (Appendix I).

The function of terminally differentiated Th1, Th2 and Th17 cells may also be suppressed. This occurs through soluble or cell-mediated mechanisms. Suppression of Th cells results in attenuated Th cytokine release, leading to reduced cellular inflammation, B cell isotype switching and hallmark features of asthma. NKT proliferation, cytokine release and cytotoxic effects of NKT cells may also be suppressed by Tregs, in a cell contact dependent manner (111). Interestingly, NKT cells from asthmatics but not healthy controls have the ability to be cytotoxic toward Tregs, highlighting an important interplay between these cell types (112). In the absence of Th2 cell involvement, Tregs have been shown to suppress B cells, through close contact mediated release of granzyme and perforin (113, 114). Therefore, Tregs have the potential to prevent IgE release, which may attenuate mast cell mediated inflammation. Furthermore, IL-10 has been shown to induce IgG4 isotypes that are protective against the development of IgE and allergic disease in healthy individuals (115). Interestingly, the innate function of neutrophils and macrophages has also been shown to be suppressed by Tregs (116, 117). Hence, the broad array suppressive mechanisms by Tregs offer multiple angles of defence against the development and progression of the asthma.

1.5 MICROBIAL AGENTS AND ASTHMA

1.5.1 Microbial agents and asthma: An overview

The “Hygiene Hypothesis” suggests that the lack of exposure to microbial agents increases the susceptibility to allergic disease. In addition, the hypothesis suggests change in modern lifestyle, improved hygiene and increased antibiotic use has lead to decreased exposure to certain infections in westernised countries and may have

contributed to the increase in asthma and allergic disease. Recently, this hypothesis was investigated by comparing the effects of different rearing environments on host gene expression (118). In this study, pigs were housed either, outdoors or indoors and under isolation. Inflammatory genes were more highly expressed in pigs housed in isolation compared to those housed outdoors. This suggests that early life environment has significant effects on the developing immune response. Notably, this study did not provide data for the effect on Tregs. Further studies may provide additional insights into the development of effective strategies to reduce the burden of asthma. A key element that may be related to the development of asthma is exposure to microbial agents. Indeed, exposure to certain microbes may either increase or reduce the risk of asthma.

1.5.2 Microbial agents that increase asthma risk

It is commonly recognised that viral infections such as a cold and flu can increase airway constriction and exacerbate asthma in an asthmatic individual. It is estimated that the majority of respiratory viral infections implicated are human rhinoviruses (RVs) (119). The mechanisms of RV-induced asthma exacerbations are poorly understood. Atypical bacteria such as *Mycoplasma pneumoniae* and *Chlamydia pneumoniae*, have also been associated with exacerbation and predisposition to asthma (92). It is possible that vaccination to prevent these infections may be effective in suppressing the induction of asthma exacerbations.

1.5.3 Microbial agents that reduce asthma risk

Epidemiological evidence shows that asthma prevalence is inversely proportional to exposure to certain microbial agents (120, 121). More specifically, tuberculin reactivity

and *Bacillus calmette-guerin* (BCG) vaccination have been associated with reduced asthma risk (122, 123). This has led to studies which show that immunomodulatory therapy with BCG in animal models leads to reductions in allergic responses (124-126). However, the efficacy of mycobacterial immunization in preventing allergy in human trials has been varied (127-131). In murine models, mycobacteria has been shown to induce a Th1 response and several studies have shown that this leads to the suppression of the Th2 responses involved in atopic disease (132, 133).

Bacterial motifs, CpG oligodeoxynucleotides (CpG-ODNs) have also been tested in clinical trials for their capacity to attenuate allergic diseases. However, CpG-ODNs have had mixed results and limited efficacy in clinical trials, when administered prior to allergen challenge in asthma (134). CpG-ODNs also induce a Th1 response and have proven effective in suppressing allergic inflammation in mouse models (135). Furthermore, CpG-ODNs have been shown to suppress Treg function (136, 137). The effects of CpG-ODNs on Tregs may explain the limited efficacy in clinical trials.

Helminths including *Nippostrongylus brasiliensis*, *Schistosoma mansoni*, *Toxiplasma gondi* as well as bacteria including *Lactobacillus reuteri* and *Streptococcus pneumoniae* have been associated with the suppression of AAD (Table 1.5). This suggests that certain microbial agents may offer protection against asthma. The mechanism of action identified in these studies highlight a role for either the induction of Th1 cells or Tregs for suppression of AAD.

1.6 S. PNEUMONIAE

1.6.1 S. pneumoniae epidemiology

TABLE 1.5. Microbial agents and mechanisms that have been associated with reduced asthma risk

MICROBIAL AGENT	MECHANISM	REF.
<i>Bifidobacterium lactis</i>	Associated increase in TGF- β	(138)
CpG-ODNs	Th1 inducing	(139)
	Treg inducing	(140)
<i>Helicobacter pylori</i>	Unknown	(141)
<i>Heligmosomoides polygyrus</i>	Involves/dependent on IL-10	(142, 143)
<i>Lactobacillus reuteri</i>	Unknown, conflicting results; no change or increased IL-10	(144)
<i>Lactobacillus rhamnosus</i>	Associated increase in TGF- β	(138)
<i>Litomosoides sigmodontis</i>	Associated increase in TGF- β , however, blocking had no effect	(145)
	Suppression of IL-10	(145)
<i>Mycobacterium bovis</i>	Th1 inducing	(146)
<i>Mycobacterium vaccae</i>	Dependent on IL-10 and TGF- β	(147)
<i>Nippostrongylus brasiliensis</i>	Involved IL-10	(148)
<i>N. brasiliensis</i> products	Independent of TLR-2, TLR-4, IFN- γ , and IL-10	(149)
<i>S. pneumoniae</i>	Unknown, not IL-10 or TGF- β mediated, likely to be cell-contact mediated	(150, 151)
<i>Schistosoma japonicum</i>	Associated increase in IL-10	(152)
<i>Schistosoma mansoni</i>	Independent of IL-10, likely to be cell-contact mediated	(153)
<i>Toxoplasma gondii</i>	Associated increase in IL-10	(154)

Adapted from Thorburn & Hansbro, 2010 (71) (Appendix I).

Invasive *S. pneumoniae* infections are the sixth leading cause of death worldwide (155). *S. pneumoniae* is a common respiratory pathogen, and is the predominant cause of community-acquired pneumonia in children and adults (156). It also frequently causes otitis media, septicemia and meningitis. *S. pneumoniae* is a member of the human commensal flora and asymptotically colonises the nasopharynx of up to 60 % of healthy children and 30 % of healthy adults. Rate of carriage depends on age, environment and other respiratory tract infections. Antibiotics are commonly used to clear infection and vaccines, which induce antibodies toward *S. pneumoniae* capsular polysaccharides, are used to provide preventative measures.

1.6.2 *S. pneumoniae* characteristics and components

S. pneumoniae is an extracellular, gram positive diplococcus with a thick polysaccharide capsule that permits resistance to phagocytosis (Figure 1.3). A number of virulence factors also allow *S. pneumoniae* to evade the immune system (Table 1.6).

1.6.2.1 Capsule

S. pneumoniae has more than 90 different capsular serotypes, which have more recently been classified into 46 serogroups (157, 158). The capsule composition and thickness determines virulence by conferring resistance to phagocytosis. Due to the many different capsular types, country specific serotype frequencies and serotype conversion, the capsule poses a serious obstacle to the design of a universal vaccine.

1.6.2.2 Cell wall

The cell wall is estimated to contain more than 100 proteins of which 6 are known to play a role in pathogenesis and virulence (Table 1.6). The cell wall also contains

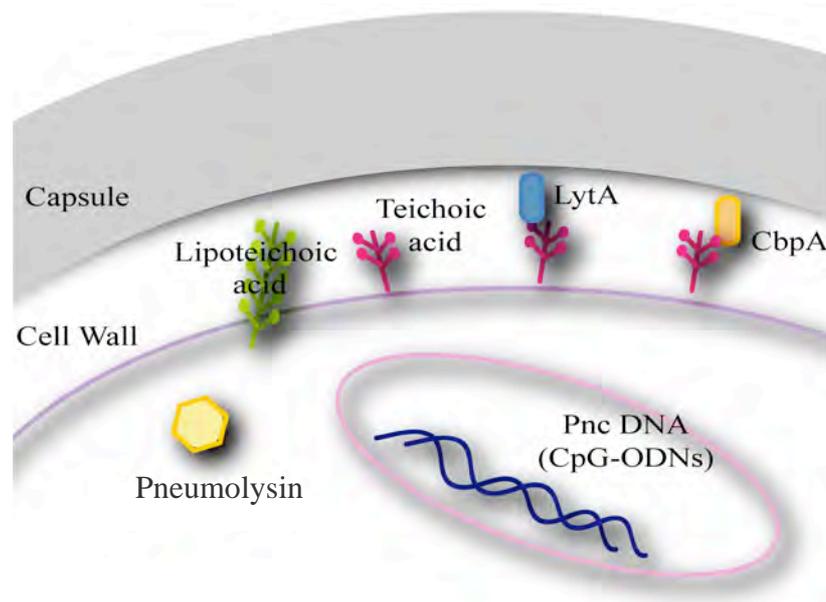


FIGURE 1.3. Pneumococcal structure. A thick polysaccharide capsule surrounds the cell wall, which contains many different components including lipoteichoic acid, teichoic acid and cell wall proteins, for example autolysin (Lyt)A and choline binding protein (Cbp)A. Illustration also shows the pneumococcal exotoxin, pneumolysin and pneumococcal (Pnc) DNA containing CpG-ODNs. Adapted from Tuomanen *et al.*, 1999 (159).

TABLE 1.6. The role of *S. pneumoniae* virulence factors

VIRULENCE FACTOR	ROLE
Polysaccharide capsule	<p>Fails to activate alternative complement pathway</p> <p>Confers resistance to phagocytosis</p> <p>Evades opsonisation by complement</p> <p>Low level of immunogenicity for some serotypes</p>
Cell wall	<p>Primarily role in inflammation</p> <p>Activates alternate complement pathway and anaphylotxin production</p> <p>Enhances vascular permeability and leukocyte activation</p>
Pneumolysin	<p>Cytolytic</p> <p>Inhibits cilia</p> <p>Disrupts epithelium</p> <p>Inhibits lymphocyte proliferation</p> <p>Inhibits bactericidal activity and proliferation of polymorphonuclear cells</p> <p>Inhibits antibody synthesis</p> <p>Activates complement</p>
Virulence proteins	
PspA	Inactivates complement
LytA	Lyses bacteria to release pneumolysin and cell wall components
CbpA	Cleaves host extra-cellular matrix
CbpG	Assists with adhesion
SpsA	Invades Ig receptor expression cells
PsaA	Participates in bacterial adhesion through metal binding lipoprotein
IgA1 protease	Counteracts host mucosal defence mechanisms
Neuraminidase	Exposes host cell receptors for <i>S. pneumoniae</i>

Psp, pneumococcal surface protein; Lyt, autolysin; Cbp, choline binding protein; Sps, *Streptococcus* secretory IgA binding protein; Psa, pneumococcal surface adhesin.

Adapted from Alonso DeVelasco *et al.*, 1995 (160) and Gosink *et al.*, 2000 (161).

peptidoglycan, teichoic acid and phosphorylcholine. Studies have shown that heat killed un-encapsulated *S. pneumoniae* can be protective against infection in mice, suggesting that cell wall components may be important for mounting immune responses against infection (162). The immunogenicity and protective capacity, against infection, of a number of *S. pneumoniae* proteins are under investigation in animal models (163).

1.6.2.3 Pneumolysin

Pneumolysin is thiol-activated cytotoxin released upon cell lysis (164). This exotoxin damages alveolar cells, inhibits ciliary beating of the respiratory epithelium and reduces migration of phagocytes. Pneumolysin is able to induce the release of cytokines from immune cells, suppress lymphocyte and antibody production and the bactericidal activity of neutrophils. Antibodies against pneumolysin are able to neutralise its toxic effects and block the activation of complement. Pneumolysin also has T cell epitopes and is recognised by TLR4 (165). Studies indicate that although pneumolysin is highly conserved across strains, it is more effective as a protein carrier for capsular polysaccharides rather than a “stand alone” vaccine candidate (166).

1.6.3 Immune response to *S. pneumoniae*

S. pneumoniae induces immune responses that promote the clearance of the bacteria. Secondary effects of these immune responses on simultaneously occurring disease states are possible but poorly understood. *S. pneumoniae* expresses many PAMPs that activate TLRs, such as TLR2 and TLR4, and MyD88 on innate cells, such as neutrophils, macrophages and DCs (167-170). These cells are able to phagocytose and destroy the bacteria and direct the production of antibodies. Anti-capsular antibody is protective against and able to clear *S. pneumoniae* infections (171, 172). T cell help is

required to induce class switching by B cells and induce memory B cells, which enhances defence against infection upon secondary encounter (173, 174). Infection may also induce T cell memory, however, the nature of the T cell response induced by *S. pneumoniae* is controversial and dependent on the infecting strain and the genetic background of the infected individual. Notably, unlike other Gram-positive bacteria, *S. pneumoniae* infection also induces the production of Tregs (175).

1.6.4 *S. pneumoniae* vaccines

Due to antibiotic resistance, the development of an effective *S. pneumoniae* vaccine has been, and remains, a major research priority. The basis of current vaccines relies on the properties of capsular polysaccharides that induce an antibody response that clears the infection. However, these vaccines are not completely effective due to the capsular complexity of the bacteria. An effective vaccine based on invariable and conserved structures is urgently needed.

1.6.4.1 Polysaccharide vaccine

A polysaccharide vaccine (trade name Pneumovax® 23) has been used for many years and contains a mixture of 23 different polysaccharide types (1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19F, 19A, 20, 22F, 23F, 33F; 25 µg of each; total 575 µg) dissolved in saline containing 0.25 % phenol. These 23 types cover approximately 90 % of serotypes associated with *S. pneumoniae* infections in the USA (176). The polysaccharide vaccine is recommended for routine vaccination for persons aged 50 years or older and children over 2 years with predetermined risk.

This vaccine induces the production of T cell independent antibodies and is relatively ineffective in young children, the elderly and the immunocompromised. The

vaccine is poorly immunogenic, resulting in a low antibody response of largely low-affinity IgM. The lack of conventional T cell help results in failure to induced antibody affinity maturation, isotype switching or immunological memory.

1.6.4.2 Conjugate vaccine

More recently, a conjugate vaccine (trade name Prevenar®) has been produced that contains a mixture of 7 polysaccharide types (4, 9V, 14,18C, 19F and 23F 2 µg of each and 6B 4 µg; total 16 µg) conjugated to diphtheria cross-reactive material 197 (CRM₁₉₇) carrier protein and adsorbed on aluminium phosphate (0.5 mg) in saline. Recently, a 13-valent form (addition of serotypes 1, 3, 5, 6A, 7F, and 19A; total 30.8 µg) was developed. Although not compulsory, the conjugate vaccine (7-valent) has been highly recommended for six week to 9 year olds and is commonly administered in industrialised countries.

The conjugate vaccine (7-valent) is almost completely effective against infection in infants due to the induction of a T cell dependent immune response that leads to an increase in antibodies, development of immunological memory and maturation of the immune response (177). Unlike the polysaccharide vaccine, the conjugate vaccine has been shown to induce DC driven T cell priming (178).

As a result of the use of the conjugate vaccine, prevalence of invasive *S. pneumoniae* disease has decreased significantly in children up to 17 years of age (179). Whether the conjugate vaccine provides protection for a longer period of time is unknown. Despite the conjugate vaccine being protective against a number of serotypes, it has been shown to induce capsule serotype switching. Less common strains are beginning to emerge and over time this vaccine will become less effective. This is a major concern because it will change the landscape of the disease.

1.6.4.3 New vaccines

Genomic advances and the sequencing of multiple *S. pneumoniae* genomes have led to an initial “proof of concept” approach to developing protein-based vaccines. Common *S. pneumoniae*-specific proteins have been identified as potential vaccine candidates that offer cross-protection between stains of different serotypes. Furthermore, protein in combination with DNA was shown to produce enhanced antibody responses and protective immunity against pneumococcal infection in mouse models (180). It is likely that the future of *S. pneumoniae* vaccine development will follow from this type of integrative approach.

1.7 S. PNEUMONIAE AND ASTHMA

1.7.1 Clinical associations

Studies of the association between *S. pneumoniae* vaccination and asthma show protective effects against asthma risk. Unfortunately however, there are few studies since pneumococcal vaccination is recommended for asthmatics, to prevent *S. pneumoniae* infection. One study showed an association of *S. pneumoniae* vaccination, with the 23-valent polysaccharide vaccine, with reductions in the number and severity of hospitalisations of elderly patients (181). In another study, pneumococcal vaccination was associated with a 30 % reduction in asthmatic episodes in children over a two year period (182). Furthermore, when combined with antibiotic therapy a 56 % reduction in asthmatic episodes was observed. The authors proposed that vaccination was protective against asthma since *S. pneumoniae* exacerbations were prevented.

However, we suggest that *S. pneumoniae* vaccination may modulate the immune response and that this reduces the asthma risk.

The affect of *S. pneumoniae* infection on asthma risk has not been widely investigated. Children born to asthmatic mothers and colonised with *S. pneumoniae* during the first month of life were more likely to develop asthma (183). Given their predisposition to asthma, this study may suggest that asthmatics are more susceptible to *S. pneumoniae* infection. Indeed, several studies have shown that asthmatics may be more susceptible to *S. pneumoniae* infection (184-186). Furthermore, asthmatics have reduced immune responses to *S. pneumoniae* and low *S. pneumoniae* IgG responses, particularly asthmatic children (187). This may result in asthmatics being more susceptible to virulent strains and pathogenic infection. These observations also indicate that asthmatics may benefit from exposure to *S. pneumoniae* vaccination, which may promote immune modulation and reduce susceptibility to asthma.

There is also evidence that asthmatics are at no greater risk of acquiring *S. pneumoniae*-induced diseases (188). A small study of 47 adult patients identified *S. pneumoniae* in chronic obstructive pulmonary disease (COPD) patients and not asthmatics (189). Therefore, it is also possible that lack of exposure to mild or asymptomatic *S. pneumoniae* may promote asthma.

Together, these data suggest *S. pneumoniae* may modulate the immune response to promote immune regulation and reduce asthma risk. We speculate that asthma may result from reduced immune responses to *S. pneumoniae* or lack of exposure to *S. pneumoniae*. This suggests that there may be the potential for early intervention with *S. pneumoniae* to promote immune regulation that may prevent asthma and reduce susceptibility to infection. Specifically designed studies need to be performed to provide a greater understanding of the relationship between *S. pneumoniae* and asthma.

1.7.2 Animal models

Studies of eosinophil responses in animals have shown that *S. pneumoniae* infection can suppress the number of eosinophils in circulation (190-193). Specifically, David Bass showed that after gastric parasitic infection with *Trichinella spiralis*, mice developed blood eosinophilia and that infection with *S. pneumoniae* abrogate this effect (193). These studies were the first to suggest that *S. pneumoniae* infection may be protective against the development of eosinophilia. Since eosinophils are primary effector cells in the late-phase inflammatory responses in allergic disease the ability of *S. pneumoniae* infections to decrease the influx of these cells may be important in the suppression of asthma and eosinophilic diseases.

Recently, we have demonstrated that *S. pneumoniae* infection suppresses hallmark features of AAD including eosinophils in BAL, blood and peribronchial tissue, release of Th2 cytokines from mediastinal lymph nodes (MLNs), goblet cell hyperplasia and AHR (151) (Appendix II). In another study, our group showed that ethanol killed *S. pneumoniae* had similar effects, suggesting that an active infection was not required for immune modulation (150). Together, this evidence suggests that pneumococcal components have the potential to be harnessed as an immunomodulatory therapy for asthma (194) (Appendix III).

Components that mediate the modulation of AAD remain to be determined. Furthermore, the mechanisms involved in suppression of AAD by *S. pneumoniae* components are unclear.

1.8 THERAPEUTICS FOR ASTHMA

1.8.1 Current therapeutics

Current therapeutic strategies for asthma include preventer medications, generally corticosteroids, coupled with the use of short-term fast-acting bronchodilators. Other widely used medications include long acting beta agonists, leukotriene receptor agonists, mast cell stabilisers and anti-IgE. Despite being quite effective therapies, there is still considerable ongoing morbidity related to asthma. In part, this is due to the complexity of the underlying causes of disease, however most relates to poor compliance to treatment. In addition, some patients may become resistant to currently available drugs. Therefore, a novel therapy that complements existing approaches, and may only be required at infrequent intervals may be beneficial.

1.8.2 Potential therapeutics

Based on our understanding of the immunopathology of asthma, many pathways and molecules have been identified as possible targets for asthma therapies. Targeting IgE, mast cells, pro-inflammatory cytokines or barrier function have been investigated as novel therapeutic strategies for asthma (195). However, the heterogeneity and contributions of multiple effector processes in asthma provides limitations for targeting one particular aspect of disease.

One novel therapeutic strategy that is under investigation is the use of peptides to target activation and inhibition of specific TLR responses (196). However, further investigation is required to determine the specific peptides, targets and pathways required for the development of an effective therapy.

1.8.2.1 Allergen-specific immunotherapy

Another therapeutic approach that has been recommended, but only for mild asthma, is allergen-specific immunotherapy. This method is particularly effective and involves the administration of increasing doses of a specific allergen to promote antigen specific Tregs that release IL-10 or TGF- β and inhibit allergen-specific Th2 responses (12, 197). However, this treatment must be tailored to the specific allergen and the patient must be monitored closely since serious side effects including anaphylaxis may occur. Further research is underway to improve safety and efficacy of this approach.

1.8.2.2 Tregs as novel therapeutics

Tregs have multi-targeting suppressing properties which heightens their potential for enhancement as an asthma therapy (71). This may be the most holistic approach to modulate the underlying causes of asthma.

One approach for the therapeutic use of Tregs in asthma is the expansion of Tregs *ex vivo* and adoptive transfer of these cells into an asthmatic individual. Another approach is the promotion of inducible Tregs. Glucocorticoid administration in conjunction with the active form of vitamin D ($1\alpha,25$ -dihydroxyvitamin D3 or calcitrol) has been shown to promote the induction of Tregs that release IL-10 (198). Importantly, this strategy is effective in patients that are refractory to steroid treatment and further studies are refining this strategy.

As presented in Table 1.4, many microbial agents have the potential to be harnessed for use as immunoregulatory therapies for asthma (71). However, the key non-infectious immunoregulatory components need to be identified.

1.8.3 Route of administration

The most efficacious route of administration of a therapy for asthma must be considered. Intramuscular delivery is the most common route of administration of many vaccines including the *S. pneumoniae* vaccines. While intramuscular administration is simple, efficient and suitable for all ages, this method of delivery lacks specificity toward the respiratory tract. Conversely, intranasal administration was shown to be a promising and superior method of delivery, compared to intramuscular administration, for the prevention of respiratory infections (199). Intranasal administration is respiratory tract-specific, however this method of delivery is difficult in children.

1.9 MOUSE MODELS OF AAD TO TEST NOVEL THERAPEUTICS

Mouse models of AAD are invaluable tools for investigating the effect of novel therapeutics on whole body systems *in vivo*. Several models of AAD have been established to investigate the different features of asthma, however no single model accurately represents all features of human disease. The most commonly used model is an acute model of AAD, which utilises ovalbumin (OVA) as the antigen and alum as the inducing adjuvant during the sensitisation phase followed by a subsequent period of airway challenge with OVA. However, other antigens such as house dust mite extract have been administered directly to the airways to induce AAD without the requirement of an adjuvant (200). Acute models have some characteristic features of asthma such as increased eosinophils in the BAL, blood and lung tissue; Th2 cytokine release from T cells; mucus hypersecretion; IgE production; and AHR (151). Chronic models involving long-term low dose challenge with OVA have also been established (201). Chronic models have additional features of asthma such as; increased intra-epithelial

eosinophils; airway remodelling, including sub-epithelial fibrosis; epithelial thickening; mucus cell hyperplasia; and AHR. Acute on chronic allergen exposure of animals also has characteristic features of acute exacerbations (202).

In addition to aiding the identification and development of a novel therapy for asthma, *in vivo* models may provide valuable tools to further our understanding of mechanisms underlying attenuation of disease. These models may facilitate the delineation of ‘real time’ events that are important for promoting an effective therapy for asthma.

1.10 HYPOTHESIS AND AIMS

Our analysis of the associations between *S. pneumoniae* and asthma have led us to develop the following hypothesis:

Immune modulation by *S. pneumoniae* may be utilised to develop an immunomodulatory therapy, as a novel approach to inducing regulatory T cells, to prevent and treat asthma.

To explore this hypothesis we:

1. Investigated the potential of currently available *S. pneumoniae*-based vaccines in the suppression and treatment of AAD
2. Investigated the potential of *S. pneumoniae* components in the suppression and treatment of AAD
3. Elucidated the mechanisms of immune modulation by *S. pneumoniae* immunotherapy

CHAPTER 2

PNEUMOCOCCAL CONJUGATE VACCINE-INDUCED REGULATORY T CELLS SUPPRESS THE DEVELOPMENT OF ALLERGIC AIRWAYS DISEASE

Chapter 2 investigates the potential for currently available *S. pneumoniae* vaccines to suppress the development of allergic airways disease. The role of regulatory T cells was assessed.

This paper has been submitted to *Thorax* and is under the second review.

2.1 ABSTRACT

Infections with some bacteria, including *S. pneumoniae*, have been associated with a reduced incidence of asthma. Components of *S. pneumoniae* may have the potential to modulate allergic inflammatory responses and suppress the development of asthma.

To determine if human *S. pneumoniae* vaccines have the potential to suppress asthma by elucidating their effect on AAD in mouse models.

AAD was induced in BALB/c mice by intraperitoneal sensitisation and intranasal challenge with OVA. Pneumococcal conjugate or polysaccharide vaccines were administered at the time of sensitisation or during established AAD. Hallmark features of AAD were assessed. Levels of Tregs were quantified by fluorescent-activated cell sorting and their immunoregulatory capacity was assessed using proliferation assays and anti-CD25 antibody treatment.

Intranasal administration of the conjugate vaccine, but not the polysaccharide vaccine, suppressed the hallmark features of AAD, including eosinophilic and T-helper 2-mediated inflammation; airway hyperresponsiveness; circulating IgE levels and mucus hypersecretion. Intramuscular administration of the conjugate vaccine had limited protective effects. The conjugate vaccine increased Tregs in the MLNs, lung and spleen. Furthermore, conjugate vaccine-induced Tregs had an enhanced capacity to suppress T effector responses. Anti-CD25 administration reversed the suppressive effects of the conjugate vaccine.

A currently available human conjugate vaccine suppresses the hallmark features of AAD through the induction of Tregs. Thus targeted administration may provide a novel immunoregulatory therapy for asthma.

2.2 INTRODUCTION

The prevalence of asthma varies inversely with the incidence of certain bacterial infections such as tuberculosis and typhoid (203). Furthermore, exposure to bacterial DNA motifs, such as CpG-oligodeoxynucleotides (ODNs), has been shown to suppress allergic inflammation by inducing a Th1 response in mouse models of asthma (204). However, utilisation of Th1 inducing agents to suppress AAD has not translated well into clinical application.

The development and progression of allergy and asthma may result from a lack of infection-induced regulatory T cell (Treg) expansion that leads to maladaptive immune responses and the development of disease. Indeed, several independent studies have linked impaired or altered Tregs with asthma (62, 64, 65, 67, 205). Many infectious agents have also been shown to induce Tregs and protect against AAD in murine models (71). The success of Treg driven suppression of AAD is due to the capacity of these cells to inhibit multiple effector arms of the immune responses involved in AAD. This includes attenuation of the function of Th2 cells, macrophages and DCs, NKT cells and B cells. Hence, suppression of allergic inflammation, by the induction of Treg responses may provide a mechanism for the suppression of disease.

In this regard, utilisation of immunoregulatory therapies that are based on bacterial components, which modulate allergic responses through induction of Tregs, may have benefits as therapeutic strategies for the suppression of asthma (71). Tregs are crucial in mediating protection against *S. pneumoniae* infection and are induced to substantially greater levels when compared to other Gram-positive bacteria (175). *S. pneumoniae* vaccination has been shown to reduce the incidence of asthma and associated hospitalisations in both children and the elderly (182, 206). Furthermore, *S. pneumoniae* infection has been associated with reduced eosinophilia and AAD in mouse

models (151, 193). In addition, we have shown that killed *S. pneumoniae* suppresses features of AAD in mouse models (150). These studies suggest that there is an opportunity for the development of a *S. pneumoniae*-based immunoregulatory therapy for asthma (reviewed in (194)).

A major hurdle in the development of a successful immunoregulatory therapy for asthma, based on an infectious agent, is the identification of effective suppressive factors. In this study we used *S. pneumoniae*-based vaccines, which are currently used in humans, to determine their efficacy in suppressing AAD in mouse models. Treatment with the conjugate vaccine resulted in attenuation of the expression of hallmark features of AAD through expansion of the Treg pool.

2.3 METHODS

2.3.1 Mice

Female BALB/c mice, aged 6-8 wk, were obtained from the Animal Breeding Facility at The University of Newcastle. Animals were given access to food and water *ad libitum* under specific pathogen free and controlled environmental conditions with a 12:12 hour light:dark cycle. All procedures were approved by the Animal Care and Ethics Committee of The University of Newcastle.

2.3.2 AAD model

The induction of AAD was performed as previously described (207). Mice were sensitised by intraperitoneal (i.p.) injection of OVA (day 0; 50 µg; Sigma-Aldrich, St. Louis, MO) with Rehydrogel (1 mg; Reheis, Berkeley Heights, NJ) in sterile saline (200 µl). Mice were challenged by intranasal (i.n.) droplet application of OVA under

isoflurane anesthesia (day 12-15; 10 µg in 50 µl sterile saline) and AAD was assessed 24 h after the final challenge (day 16). Control mice received saline sensitisation and OVA challenge. To recapitulate established disease, mice received OVA i.p. (day 0) followed by two sets of challenges (days 11-13 and 32-34).

2.3.3 Treatment

Mice were treated intranasally to directly condition the lung (33 µl) with 7-valent polysaccharide conjugate vaccine (Wyeth, Madison, NJ) or 23-valent polysaccharide vaccine (Merck & Co., Inc., Whitehouse station, NJ), with or without addition of CpG-ODN (10 µg, TCCATGACGTTCCCTACGTT; Geneworks, Thebarton, SA, Australia), i.n. under isoflurane anesthesia every 12 h for a total of three doses starting at the time of OVA sensitisation. To assess the effects of the conjugate vaccine in established AAD, mice were treated with the conjugate vaccine once or three times per week. To test the importance of route of delivery, mice received intramuscular (i.m.) administration of the conjugate vaccine (50 µl in each of the hind quadriceps). Where indicated, treatment was administered before or after sensitisation or mice received anti-CD25 (day -3, 100 µg in 200 µl saline i.p, PC61; eBioscience, San Diego, CA).

2.3.4 Assessment of cellular inflammation

Mice were euthanased by pentobarbitone overdose. Blood smears were prepared from whole blood (207). BAL was performed using two 1ml washes with HBSS (Trace Scientific, Noble Park, VIC, Australia) (207). BAL cells were centrifuged (400 xg, 7 min) and the pellet resuspended in red blood cell lysis buffer (on ice, 5 min). Cells were washed in HBSS and resuspended. Total cell numbers were enumerated using a hemocytometer. Cell suspensions were cytocentrifuged (300 xg, 10 min; Thermo

Fisher Scientific, Waltham, MA) and air-dried. Slides were stained with May Grunwald-Giemsa and differential leukocyte counts were determined with a total of 250 cells counted. All slides were assessed by a single examiner blinded to the treatment groups.

2.3.5 T cell cytokine release

MLNs were collected and pushed through 70 µm sieves. Spleens were pushed through coarse metal sieves prior to being passed through 70 µm sieves. Cells were centrifuged (400 xg, 7 min) and pellets resuspended in red blood cell lysis buffer (on ice, 5 min). Cells were washed in HBSS before being resuspended in RPMI media supplemented with 10 % FCS, 20 mM HEPES, 10 µg/ml penicillin/streptomycin, 2 mM L-glutamine, 50 µM 2-mercaptoethanol, 1 mM sodium pyruvate and 200 µg/ml OVA (207). Cell numbers were determined using a hemocytometer. Cells were cultured at 1×10^6 cells per well in 96-well U-bottomed plates (5 % CO₂, 37 °C) for 4 or 6 days, respectively, for lymph nodes or spleens, before supernatant collection and storage at -20 °C until required. Cytokine concentrations in cell culture supernatants were determined by ELISA (207). Serial dilutions of samples were analysed for IL-5, IL-13, IL-10, (BD Pharmingen, San Diego, CA), IFN-γ and TFG-β (R&D Systems, Minneapolis, MN).

2.3.6 Airways hyperresponsiveness

Airways hyperresponsiveness was assessed as previously described (150). Briefly, anaesthetised, tracheostomised mice were cannulated and connected to an inline aerosol and ventilator. Changes in airway function following challenge with increasing doses of aerosolised methacholine were assessed by analysis of pressure and flow waveforms and determination of airways resistance and dynamic compliance.

2.3.7 Serum antibodies

Whole blood was collected by cardiac puncture and allowed to clot at 4 °C overnight before centrifugation (12,000 xg, 10 min) and storage at -20 °C until use. Serial dilutions of sera were analysed by ELISA for total IgE (BD Pharmingen) and OVA-specific IgG1 (Southern Biotech, Birmingham, AL) using OVA (2 mg/well) as the capture antigen (150, 208).

2.3.8 Lung histology

Histology and determination of airway mucus secreting cells and tissue eosinophils were performed as described (150). Euthanised mice were bled out by severing the aorta in the lower abdominal cavity. Lungs were perfused with 0.9 % saline using a 19 G needle. Buffered formalin (1.5 ml, 10 %, Sigma-Aldrich) was injected into the trachea, before being tied off and lungs washed in 70 % ethanol. Lungs were embedded in paraffin, sectioned (4-6 µm) and stained with periodic acid Schiff (for mucus secreting cells) or chrome salt fixation (for eosinophils). Light microscopy was used to determine the mean number of mucus secreting cells around the airways and eosinophils adjacent to the basement membrane in 10 x 100 µm fields.

2.3.9 Flow cytometry

Single cell suspensions of MLNs and spleens were prepared, lung cells were prepared using the same method as used for spleens. 1×10^6 cells/well in a 96 well U-bottomed plate, were stained for surface-associated CD4, CD25, CD103, CTLA-4 (BD Pharmingen, San Diego, CA) and TGF-β1 (R&D Systems). After surface marker staining, fixation and permeabilisation, cells were stained intracellularly for Fork-head

box factor (FoxP3) according to the manufacturers' protocol (eBioscience) (151). Samples were analysed using a FACS Canto flow cytometer controlled by FACSDiVa software (version 4.1.1; BD Biosciences, Mississauga, Canada). Total cell numbers were determined by multiplying the total number of cells obtained by the total percentage of positive staining events.

2.3.10 Cell isolation

CD4⁺CD25⁺ and CD4⁺CD25⁻ effector cells were isolated using an AutoMACs Pro (Miltenyi Biotec, Auburn, CA) according to manufacturer's instructions to >90 % and >94 % purity respectively. Briefly, to enrich for CD4⁺ cells, microbead conjugated antibodies were used to deplete CD11c⁺, CD8⁺, CD11b⁺, CD45R⁺, CD49b⁺ and Ter-119⁺ cells. CD25⁺ cells were then isolated by positive selection to obtain CD4⁺CD25⁺ cells. CD4⁺CD25⁻ cells were further purified by positive selection with CD4⁺ microbead positive selection (151).

2.3.11 Cell suppression assay

Suppression assays were performed with 50×10^3 CD4⁺CD25⁻ cells and varying numbers of CD4⁺CD25⁺ cells (3, 6, 9 or 12×10^3) in RPMI (200 μ l, supplemented with 10 % FCS, 72 h, 37 °C) with anti-CD28 (1 μ g/ml; BD Pharmingen) and OVA peptide (1 μ g/ml) in 96 well U-bottomed plates. Cells were pulsed for the final 18 h of culture with [³H] thymidine (Amersham International Ltd., Amersham, UK) and enumerated using a microbeta counter (Wallac Microbeta TriLux, PerkinElmer, Waltham, MA) (151).

2.3.12 Data analysis

Data were analysed using GraphPad Prism (GraphPad Software, CA) and are represented as mean \pm SEM. One-way ANOVA with Dunnett's post test was used to determine significance between data with multiple comparisons. One-way repeated measures ANOVA and Bonferroni's post test was used to determine significance for AHR data. Unpaired Student's t test was used to determine differences between two groups. A p-value <0.05 was considered statistically significant. Where p is between 0.01-0.05 the numerical value has been provided.

2.4 RESULTS

2.4.1 Treatment with the conjugate but not polysaccharide vaccine suppresses the development of hallmark features of AAD

We first investigated the effect of pneumococcal vaccines on the development of AAD. Mice were treated with either the conjugate or polysaccharide vaccine, with or without CpG-ODN (as a positive control and potential adjuvant), at the time of OVA sensitisation, and the effect of vaccine treatment on hallmark features of AAD was assessed (Figure 2.1A).

Airway challenge of OVA-sensitised mice resulted in the induction of AAD, which was characterised by increased numbers of eosinophils in BAL fluid, OVA-induced IL-5 and IL-13 release from MLN T cells and airways hyperresponsiveness (AHR, Figure 2.1B-G). AAD was not induced in saline-sensitised OVA challenged mice (Figure 2.1B-G). Treatment of OVA-sensitised mice with the conjugate vaccine suppressed the development of hallmark features of AAD including; eosinophil numbers in BAL fluid, OVA-induced IL-5 and IL-13 release from MLN T cells and AHR (Figure 2.1A-G). The administration of CpG-ODN had no additional effect. By

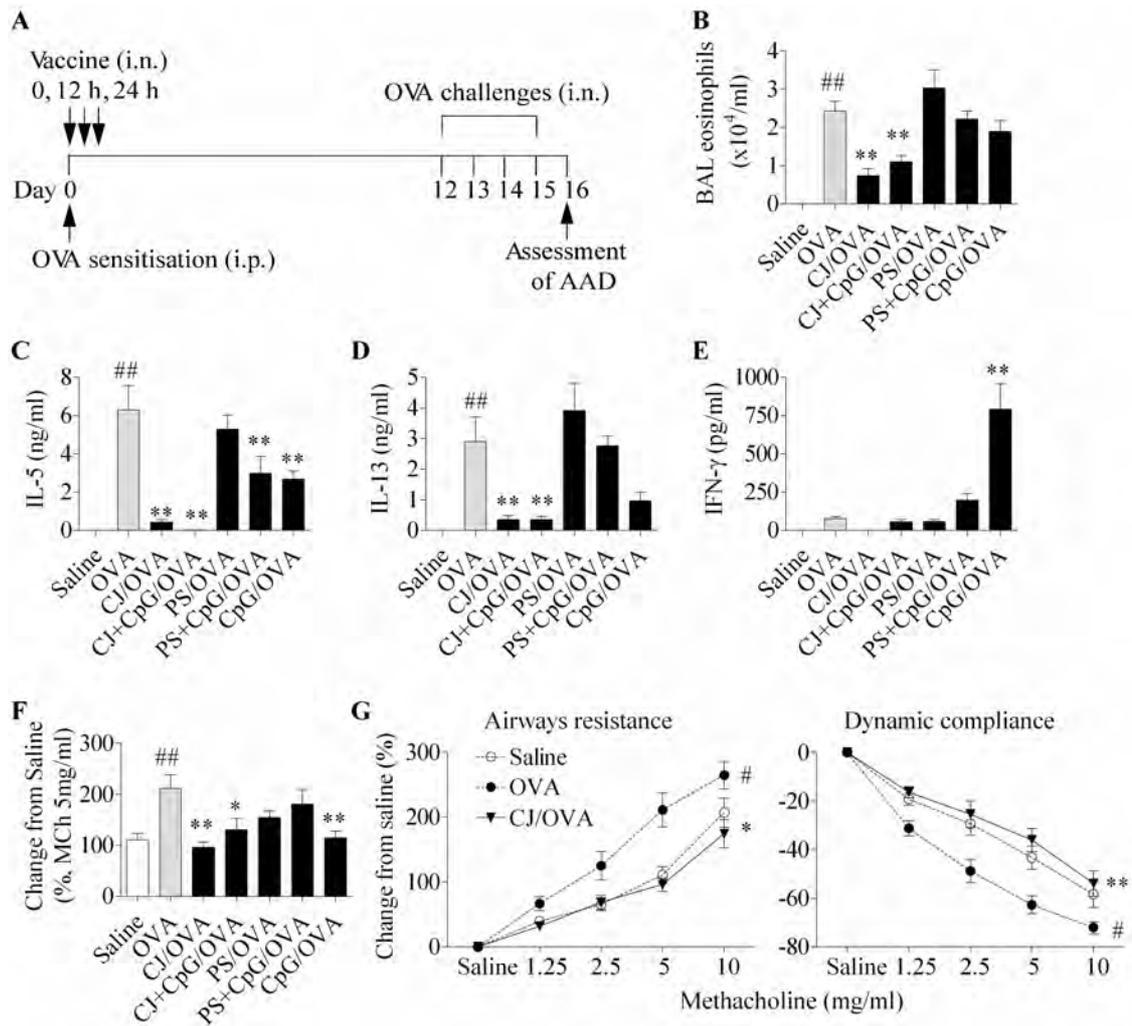


FIGURE 2.1. The effect of pneumococcal vaccines on AAD. (A) Experimental protocol to assess the effect of vaccines on the induction of AAD. AAD was induced by i.p. OVA sensitisation and i.n. OVA challenge. Controls received saline sensitisation and OVA challenge. Vaccine treatment was administered at the time of OVA sensitisation, in three doses every 12 h. Features of AAD were assessed 24 h after completion of the OVA challenge. The effect of conjugate (CJ) and polysaccharide (PS) vaccines, with and without CpG-ODNs, on (B) eosinophils in BAL, OVA-induced (C) IL-5, (D) IL-13 and (E) IFN- γ release from MLN T cells and (F) airways resistance at the representative dose of 5 mg/ml methacholine (MCh) reflective of full dose response curves. The effect of CJ on AHR is also shown as (G) full dose response

curves for airways resistance and dynamic compliance. Data represent mean \pm SEM from 6-8 mice. Significant differences between Saline-sensitised (Saline) and OVA-sensitised (OVA) controls are shown as # $p < 0.05$, ## $p < 0.01$. Significant differences between OVA-sensitised and vaccine treated OVA-sensitised mice are shown as * $p < 0.05$, ** $p < 0.01$. Significant differences for resistance and compliance are for the entire dose-response curves.

contrast, treatment with the polysaccharide vaccine had no effect on AAD (Figure 2.1A-F; see Figure 2.2 for all methacholine dose response curves). Administration of the polysaccharide vaccine with CpG-ODN attenuated OVA-induced IL-5 and IL-13 release, however, this was to the same level as CpG-ODN alone suggesting that the preventative effects were due to CpG-ODN. CpG-ODN treatment also suppressed the development of AHR, IL-5 and IL-13 ($p=0.0361$) but did not alter BAL eosinophil numbers. Unlike treatment with the conjugate vaccine, attenuation of AAD by CpG-ODN was associated with a substantial increase in OVA-induced IFN- γ release from lymph node T cells (Figure 2.1E).

2.4.2 Conjugate vaccine treatment before or after sensitisation suppresses the development of AAD

In addition to treatment at the time of sensitisation, the conjugate vaccine suppressed the development of AAD when administered before or after OVA-sensitisation (Figure 2.3). These data suggest that the effects of treatment persist and last for at least 26 days. The backbone components (aluminium phosphate ($AlPO_4$) + CRM₁₉₇) of the conjugate vaccine without *S. pneumoniae* capsular polysaccharides did not suppress the development of AAD (Figure 2.4).

2.4.3 Conjugate vaccine treatment suppresses established AAD

We then investigated whether administration of the conjugate vaccine would be effective in established AAD. A model of AAD was developed that involved sensitisation to OVA, followed by an initial challenge (days 11-13) to establish AAD and a second challenge (days 32-34) to recapitulate disease before the assessment of AAD (Figure 2.5A). The conjugate vaccine was administered during established AAD

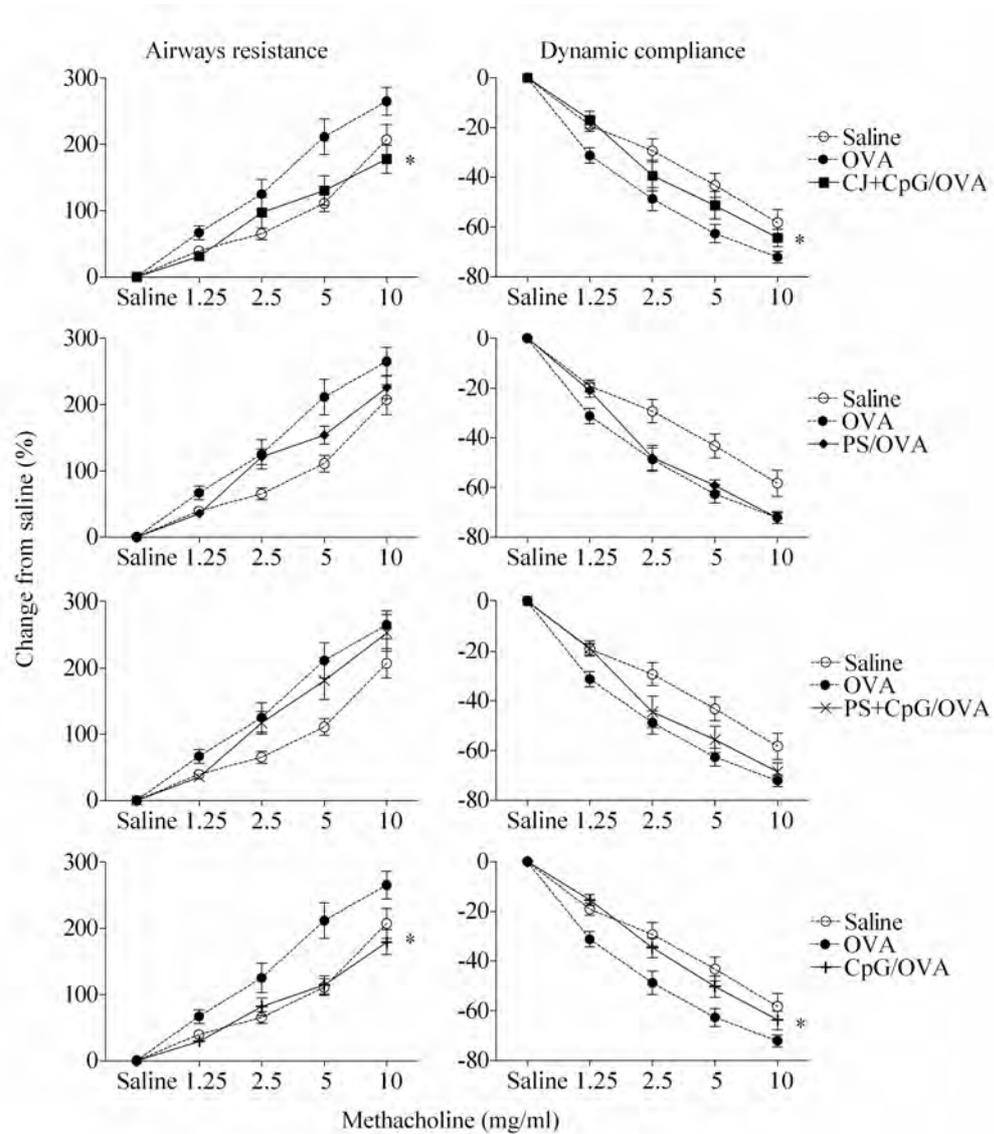


FIGURE 2.2. The effect of CJ and PS vaccines with or without CpG-ODN on AHR.

Data represent mean \pm SEM from 6 mice. Significant differences between OVA-sensitized mice and OVA-sensitized mice and vaccine treated OVA-sensitized mice are shown as * $p < 0.05$.

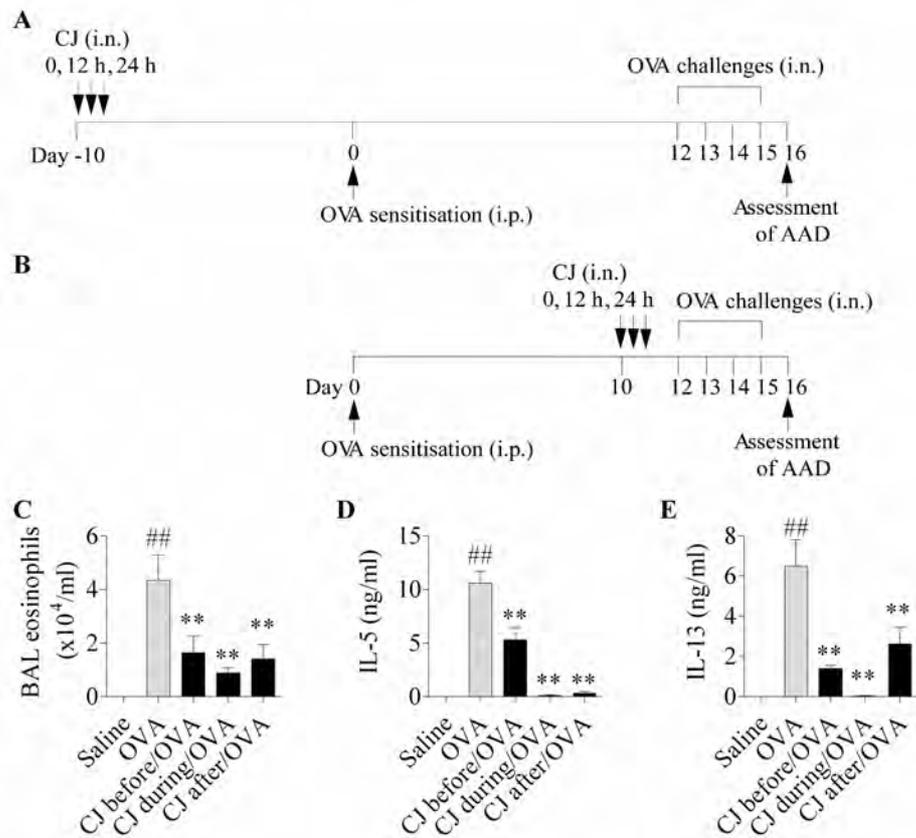


FIGURE 2.3. The effect of CJ treatment when administered before or after sensitisation. CJ treatment was administered as in Figure 2.1 during sensitisation, (A) before sensitisation or (B) after sensitisation in 3 doses every 12 h. The effect of treatment on (C) eosinophil numbers in BAL and OVA-induced (D) IL-5 and (E) IL-13 from MLN T cells. Data represent mean \pm SEM from 6 mice. Significant differences between Saline-sensitised and OVA-sensitised controls are shown as ## $p < 0.01$. Significant differences between OVA-sensitised mice and CJ treated OVA-sensitised mice are shown as ** $p < 0.01$.

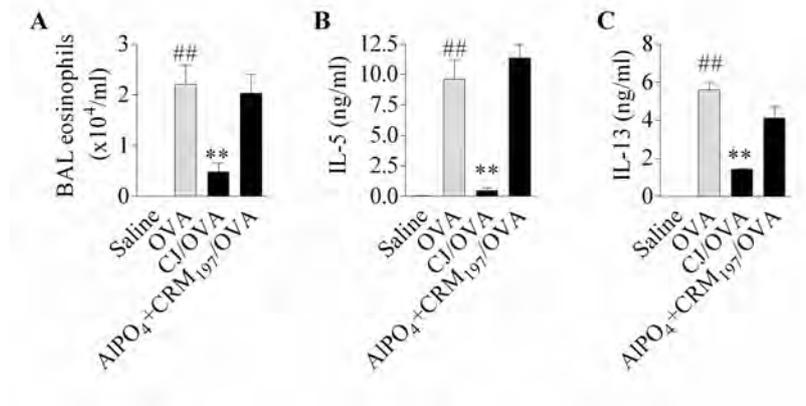


FIGURE 2.4. The effect of CJ backbone components AlPO₄+CRM₁₉₇. (A) eosinophil number in BAL and OVA-induced (B) IL-5 and (C) IL-13 release from MLN T cells. Data represent mean \pm SEM from 6 mice. Significant differences between Saline-sensitised and OVA-sensitised controls are shown as ## $p < 0.01$. Significant differences between OVA-sensitised mice and CJ backbone treated OVA-sensitised mice are shown as ** $p < 0.01$.

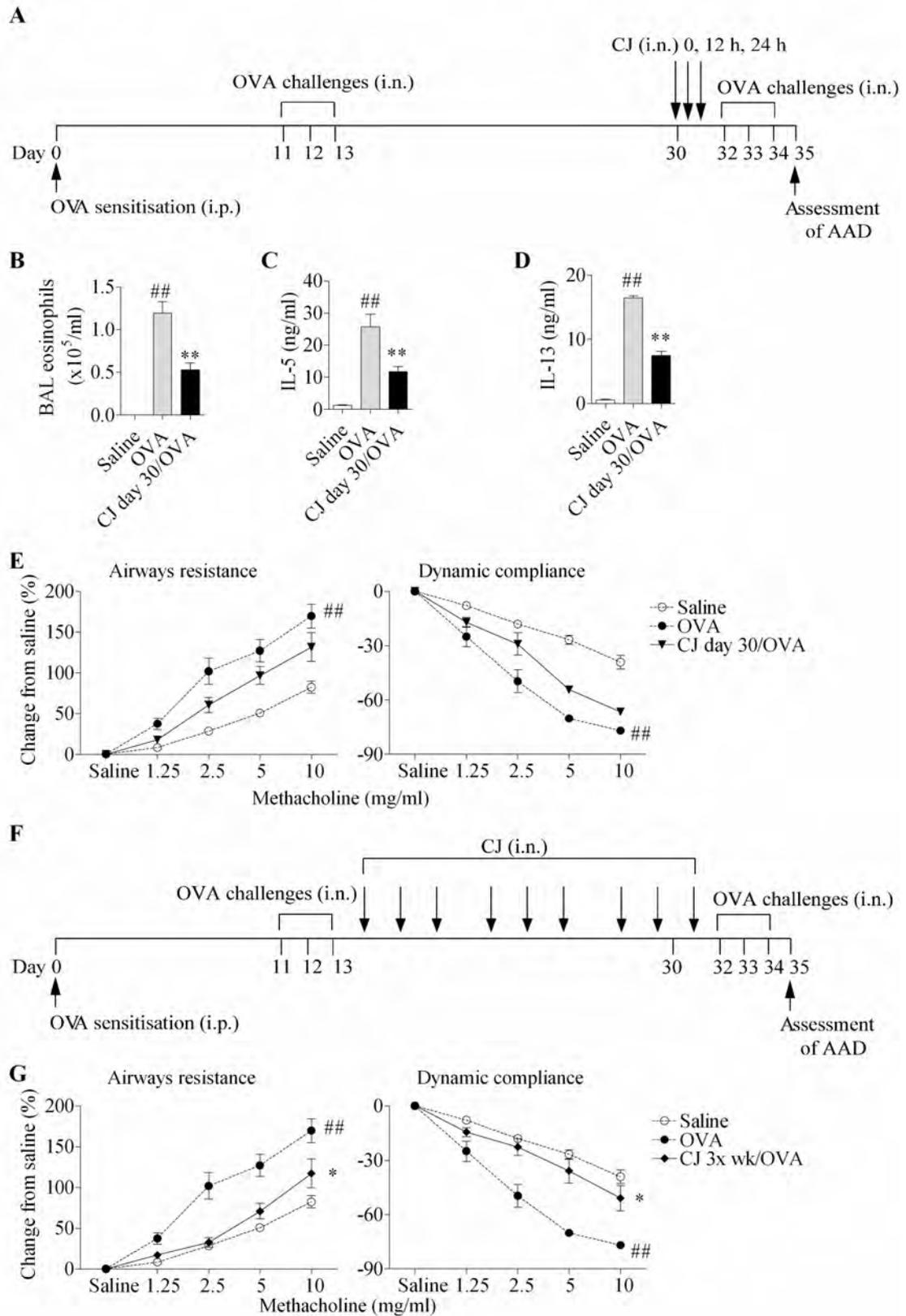


FIGURE 2.5. The effect of CJ treatment on established AAD. (A) Experimental protocol to assess the effect of CJ treatment on established AAD. AAD was established

(B) eosinophils in BAL, OVA-induced (C) IL-5 and (D) IL-13 release from MLN T cells and (E) AHR. (F) The effect of CJ treatment delivered three times per week commencing day 14 (totalling nine doses) on (G) AHR. Data represent mean \pm SEM from 6-8 mice. Significant differences between Saline-sensitised and OVA-sensitised controls are shown as # $p < 0.05$, ## $p < 0.01$. Significant differences between OVA-sensitised and CJ treated OVA-sensitised mice are shown as * $p < 0.05$, ** $p < 0.01$. Significant differences for resistance and compliance are for the entire dose-response curves.

on day 30. Administration of the conjugate vaccine suppressed eosinophils in BAL fluid and OVA-induced IL-5 and IL-13 release from MLN T cells (Figure 2.5B-D). However, this dosing regime did not suppress AHR in a statistically significant manner (Figure 2.5E). Therefore, the number of doses was increased by administering the conjugate vaccine three times per week, commencing one day after the initial challenge for a total of nine doses (day 14, 16, 18, 21, 23, 25, 28, 30 and 31, Figure 2.5F). Treatment with the conjugate vaccine three times per week suppressed AHR (Figure 2.5G).

2.4.4 Intramuscular administration of the conjugate vaccine had limited effects on the development of AAD

The conjugate vaccine is currently administered to humans to prevent *S. pneumoniae* infections *via* the i.m. route. Therefore, the effect of i.m. administration of the conjugate vaccine on the development of AAD was investigated (Figure 2.6A). I.m. administration of the conjugate vaccine suppressed eosinophil numbers in the BAL fluid and OVA-induced IL-5 and IL-13 ($p=0.0312$) release from splenic T cells, however, neither IL-5 or IL-13 release from MLN T cells nor AHR were suppressed (Figure 2.6B-G).

2.4.5 Conjugate vaccine treatment suppresses the development of systemic Th2 responses

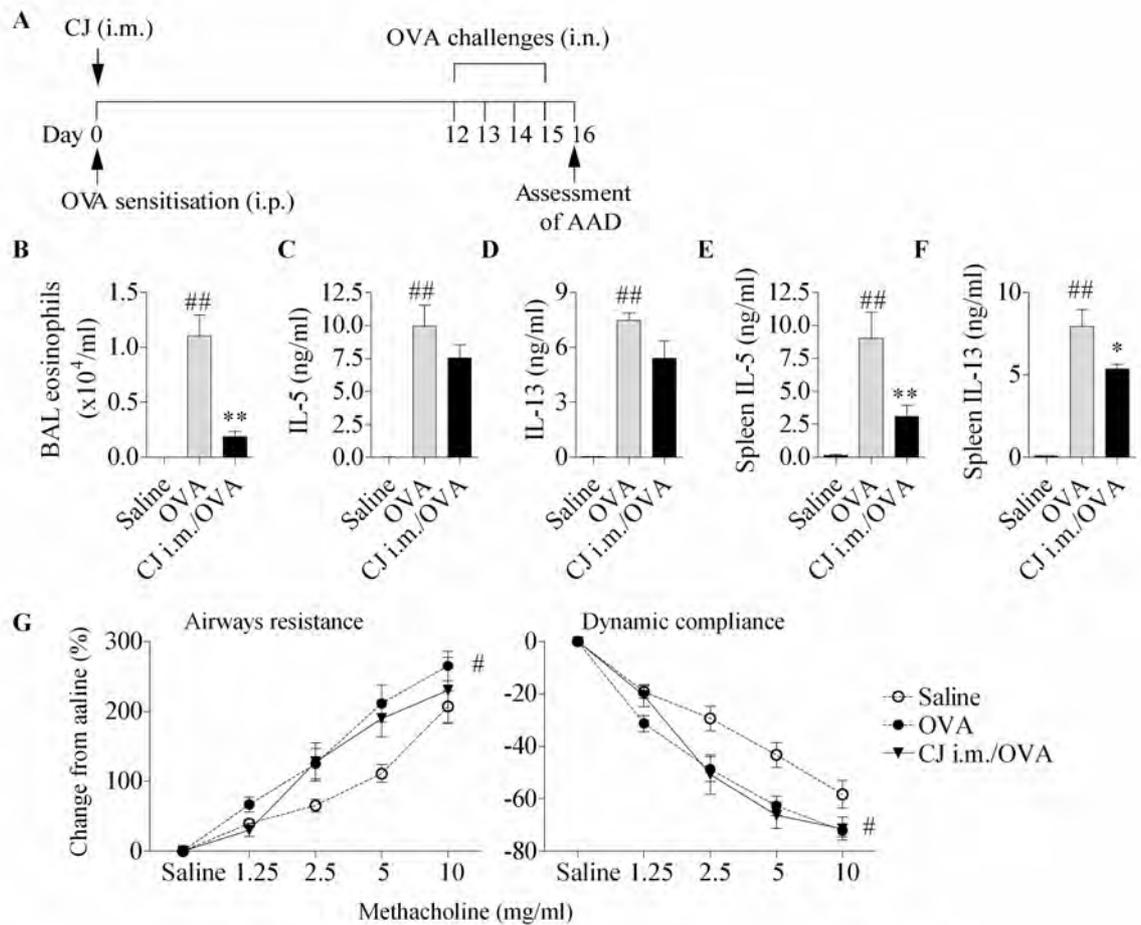


FIGURE 2.6. The effect of intramuscular administration of CJ on AAD. (A) CJ was administered by the i.m. route at the time of sensitisation. The effect of i.m. administration of CJ on (B) eosinophil numbers in BAL, OVA-induced (C) IL-5 and (D) IL-13 from MLN T cells, OVA-induced (E) IL-5 and (F) IL-13 from splenic T cells and (G) AHR were assessed. Data represent mean \pm SEM from 6-8 mice. Significant differences between Saline-sensitised and OVA-sensitised controls are shown as ## $p < 0.01$ and ### $p < 0.001$. Significant differences between OVA-sensitised mice and CJ treated OVA-sensitised mice are shown as * $p < 0.05$ and ** $p < 0.01$. Significant differences for resistance and compliance are for the entire dose-response curves.

We then further investigated the specific effects of intranasal administration of the conjugate vaccine on systemic Th2 responses, antibody responses and airway inflammation. The conjugate vaccine suppressed OVA-induced IL-5 and IL-13 cytokine release from splenic T cells (Figure 2.7A and B). The levels of circulating IgE ($p=0.0227$) and OVA-specific IgG1 in serum were also suppressed (Figure 2.7C and D). Histological analysis revealed that conjugate vaccine treatment reduced the accumulation of both mucus secreting cells and eosinophils in airway tissue (within 100 μm of the surface; Figure 2.7E and F). Conjugate vaccine treatment also suppressed the number of circulating eosinophils (Figure 2.7G).

2.4.6 Conjugate vaccine treatment induces Tregs

The suppression of AAD by the conjugate vaccine was not associated with an increased release of T cell derived IFN- γ in the MLNs. Therefore, we examined the role of Tregs in conjugate vaccine-mediated suppression in AAD. The number of CD4⁺CD25⁺FoxP3⁺ regulatory T cells in the MLNs, lungs and spleens were assessed after the induction of AAD (day 16). OVA-sensitised mice treated with the conjugate vaccine had significant increases in the total number of cells in the lymph nodes (Figure 2.8A). The percentage of CD4⁺ cells that were CD25⁺FoxP3⁺ in the lymph nodes, lung and spleen and the total number of CD4⁺CD25⁺FoxP3⁺ cells in the lymph nodes were increased compared to untreated OVA-sensitised mice (Figure 2.8B and C).

We then investigated whether the conjugate vaccine treatment induced CD4⁺CD25⁺FoxP3⁺ cells prior to OVA challenge. Again, treatment induced increases in the total number of cells in the lymph nodes (Figure 2.8D). The percentage of CD4⁺CD25⁺ cells that were FoxP3⁺ and the total number of CD4⁺CD25⁺FoxP3⁺ cells in the lymph nodes and lung were also increased (Figure 2.8E and F).

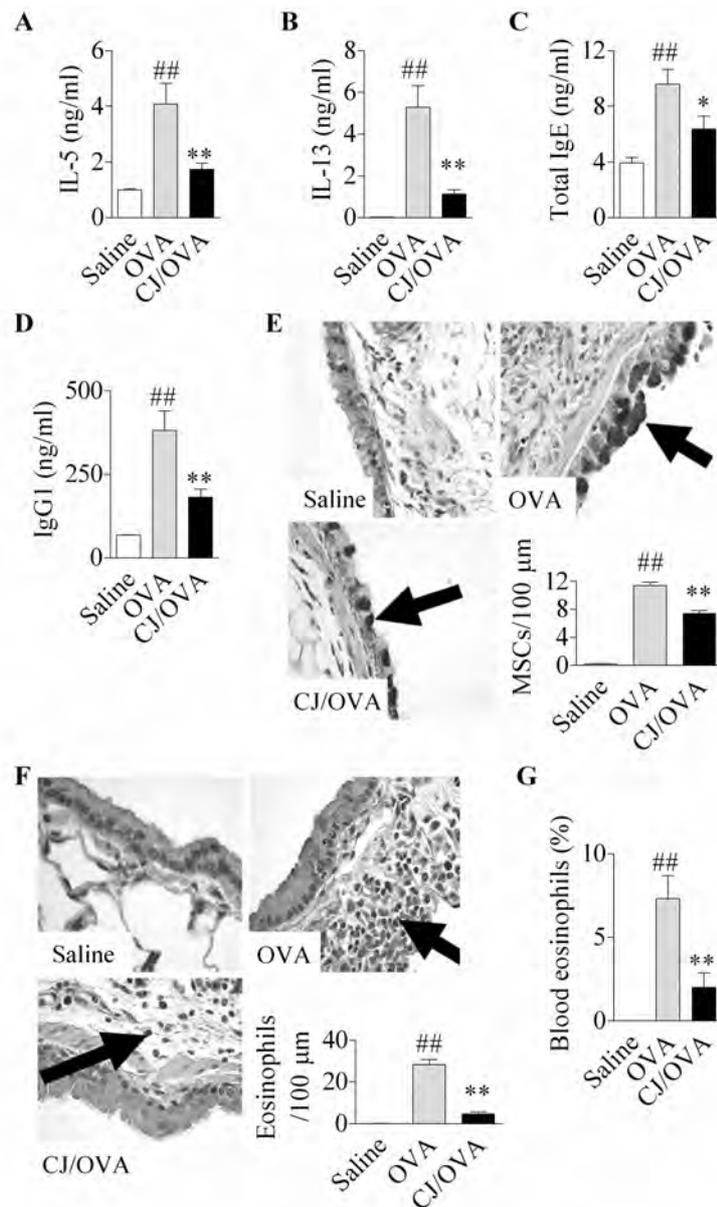


Figure 2.7. The effect of CJ treatment on additional features of AAD. OVA-induced (A) IL-5 and (B) IL-13 release from splenocyte T cells, serum levels of (C) total IgE and (D) OVA-specific IgG1, numbers of (E) mucus secreting cells (MSCs), numbers of (F) eosinophils in the airway tissue, and (G) eosinophils in blood. Arrows indicate MSCs or eosinophils in airway tissue. Data represent mean \pm SEM from 6-8 mice. Significant differences between Saline-sensitised and OVA-sensitised controls are shown as ## $p < 0.01$. Significant differences between OVA-sensitised mice and CJ treated OVA-sensitised mice are shown as * $p < 0.05$, ** $p < 0.01$.

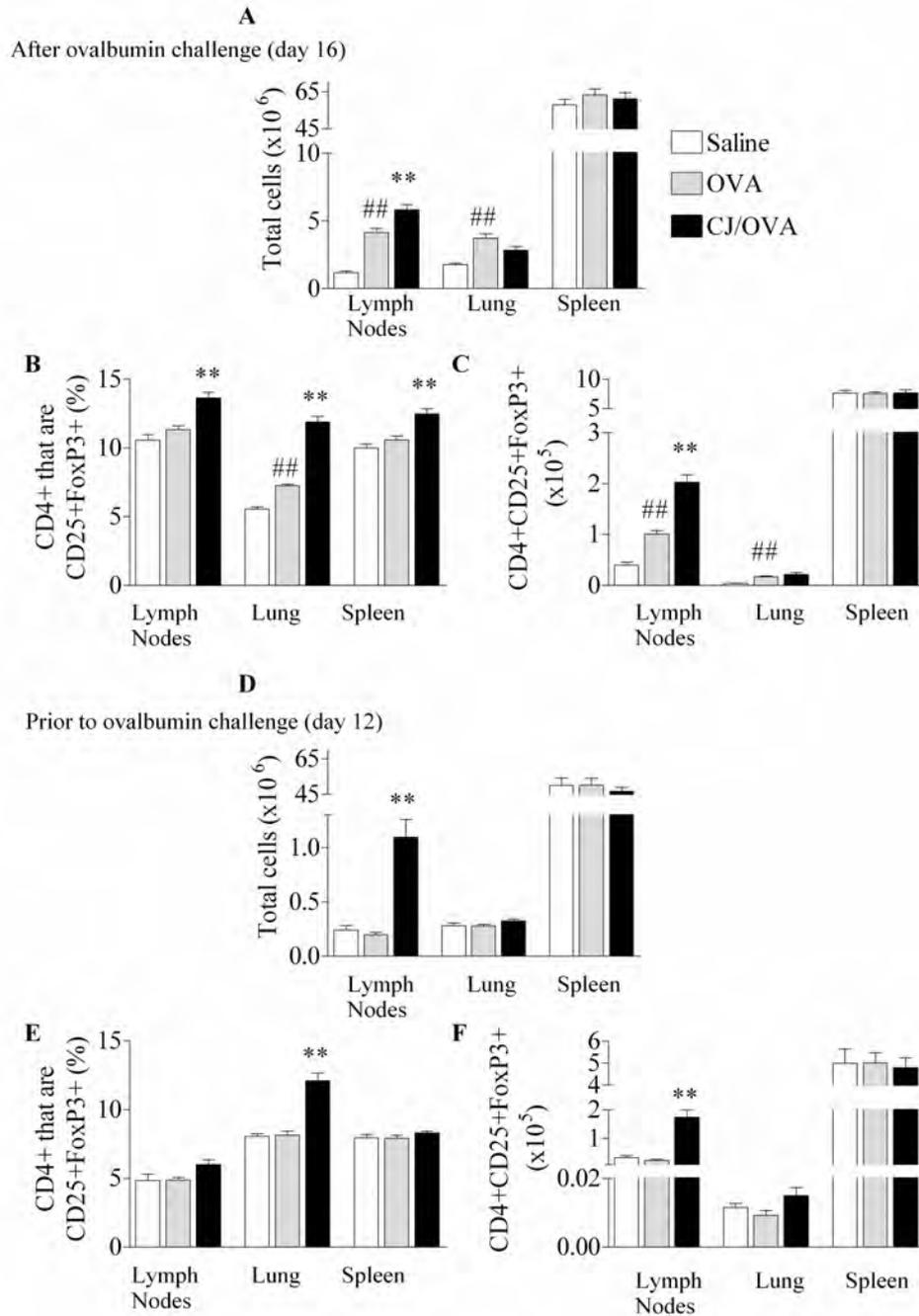


FIGURE 2.8. The effect of CJ treatment on Tregs. (A) total number of cells, (B) percentage of CD4+ cells that are CD25+FoxP3+ and (C) total CD4+CD25+FoxP3+ cells in the MLNs, lung and spleen in AAD. The effect of CJ treatment, assessed prior to OVA challenge on (D) total number of cells, (E) percentage of CD4+ cells that are CD25+FoxP3+ and (F) total CD4+CD25+FoxP3+ cells in the MLNs, lung and spleen.

Data represent mean \pm SEM from 6-8 mice. Significant differences between Saline-sensitised and OVA-sensitised controls are shown as ## $p < 0.01$. Significant differences between OVA-sensitised mice and CJ treated OVA-sensitised mice are shown as * $p < 0.05$, ** $p < 0.01$.

2.5.7 Conjugate vaccine treatment induces Tregs that have a greater suppressive capacity

IL-10 and TGF- β are immunosuppressive cytokines that may mediate the suppressive effects of Tregs. Therefore, the effect of conjugate vaccine treatment on the levels of expression of OVA-induced IL-10 and TGF- β release from MLN T cells was determined. Treatment suppressed OVA-induced IL-10 and TGF- β ($p=0.0224$) release from MLN T cells compared to untreated OVA-sensitised mice (Figure 2.9A and B). Furthermore, the percentage of CD4⁺CD25⁺ cells expressing membrane bound TGF- β remained unchanged (Figure 2.9C). Representative flow cytometric data are provided (Figure 2.10).

The levels of other markers, associated with the suppressive activity of Tregs, were also assessed. The conjugate vaccine treatment increased the expression of CD103 and cytotoxic T lymphocyte antigen-4 (CTLA-4, $p=0.0364$) by CD4⁺CD25⁺FoxP3⁺ cells in the MLNs (Figure 2.9D and E). The induction of AAD caused a statistically significant reduction in the proportion of FoxP3-expressing CD4⁺CD25⁺ cells, which was not observed in mice treated with the conjugate vaccine (Figure 2.9F).

We then hypothesised that as a result of the increased expression of CD103 and CTLA-4 and sustained expression of FoxP3, conjugate vaccine-induced Tregs would be more suppressive than those induced by OVA-sensitisation and challenge alone. Indeed, CD4⁺CD25⁺ cells isolated from the MLNs of conjugate vaccine treated mice suppressed CD4⁺CD25⁻ OVA-induced effector T cell proliferation to a greater extent when compared to untreated mice (approximately 12×10^3 CD4⁺CD25⁺ T cells from OVA-sensitised mice suppressed CD4⁺CD25⁻ effector T cells to an equivalent level to 1.5×10^3 CD4⁺CD25⁺ T cells from OVA-sensitised conjugate vaccine treated mice,

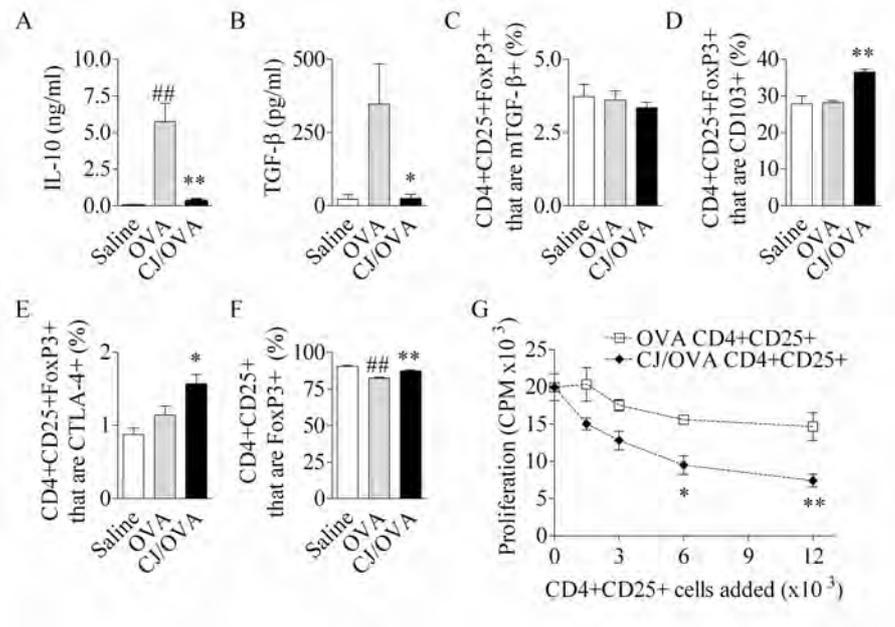


FIGURE 2.9. The effect of CJ treatment on Treg phenotype and suppressive capacity. OVA-induced (A) IL-10 and (B) TGF- β release from MLN T cells, the percentage of CD4+CD25+FoxP3+ cells expressing (C) membrane bound TGF- β (mTGF- β), (D) CD103 and (E) CTLA-4, and (F) the percentage of CD4+CD25+ that are FoxP3+. (G) Proliferation of CD4+CD25- cells cultured with varying numbers of CD4+CD25+ Tregs from OVA-sensitised or CJ treated OVA-sensitised mice and stimulated with OVA and anti-CD28. Data represent mean \pm SEM from 6-8 mice (A-F) or triplicate wells (G). Significant differences between Saline-sensitised and OVA-sensitised controls are shown # $p < 0.05$, ## $p < 0.01$. Significant differences between OVA-sensitised mice and CJ treated OVA-sensitised mice are shown as * $p < 0.05$, ** $p < 0.01$.

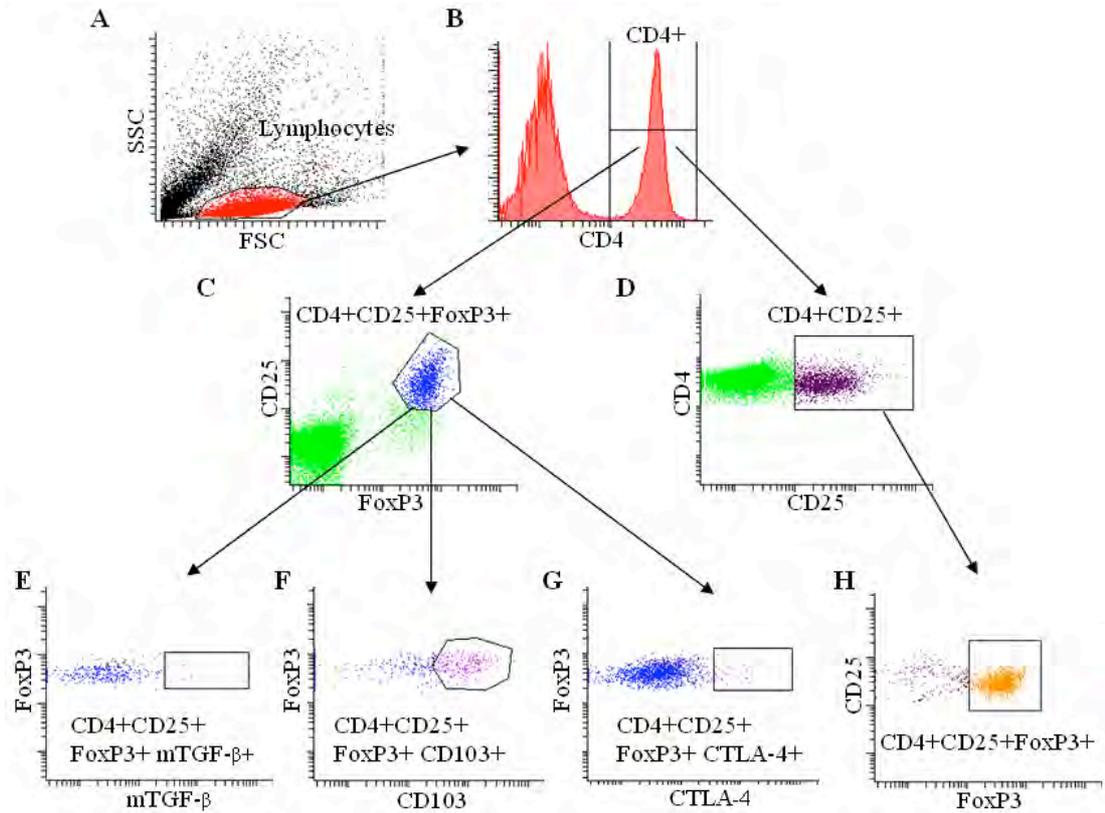


FIGURE 2.10. Representative flow cytometric data used in Treg phenotyping. (A) Lymphocytes were gated based on forward (FSC) versus side (SSC) scatter. (B) CD4+ cells were gated from the lymphocyte population. (C) CD25+FoxP3+ or (D) CD4+CD25+ populations were gated from the CD4+ population. (E) mTGF- β +, (F) CD103+, (G) CTLA-4+ populations were gated from the CD4+CD25+FoxP3+ population. (H) The percentage of CD4+CD25+ that were FoxP3+ was gated on the CD4+CD25+ population. 50,000 events were recorded for each sample.

Figure 2.9G). The suppression of OVA-induced T cell proliferation by Tregs also attenuated OVA induced Th2 cytokine release (IL-5 and IL-13, data not shown).

2.4.8 CD25 inactivation restores AAD following conjugate vaccine treatment

To confirm the role of conjugate vaccine-induced Tregs in suppressing AAD, Tregs were inactivated by the administration of anti-CD25. Anti-CD25 administration restored AHR to the levels observed with untreated OVA-sensitised and challenged mice (Figure 2.11A). Anti-CD25 treatment also restored eosinophil numbers in the BAL fluid and the levels of OVA-induced IL-5 and IL-13 released from MLN T cells (Figure 2.11B-D).

2.5 DISCUSSION

In this investigation we demonstrate that a *S. pneumoniae* conjugate vaccine, which is currently licensed for human use, suppresses hallmark features of AAD in mouse models, indicating its potential as an immunoregulatory therapy for asthma. Treatment with the conjugate vaccine reduced Th2 cytokine release, cellular inflammation, antibody production and AHR. These inhibitory effects were mediated by increased numbers and/or percentage of local and systemic Treg cells that had an enhanced suppressive capacity.

The efficacy of the conjugate vaccine in preventing AAD, compared to the lack of response to the polysaccharide vaccine, may be attributed to their different composition. The conjugate vaccine is composed of 7 types of pneumococcal polysaccharide that are conjugated to an immunogenic diphtheria toxoid, whereas the polysaccharide vaccine contains 23 serotypes of polysaccharide. The conjugate vaccine

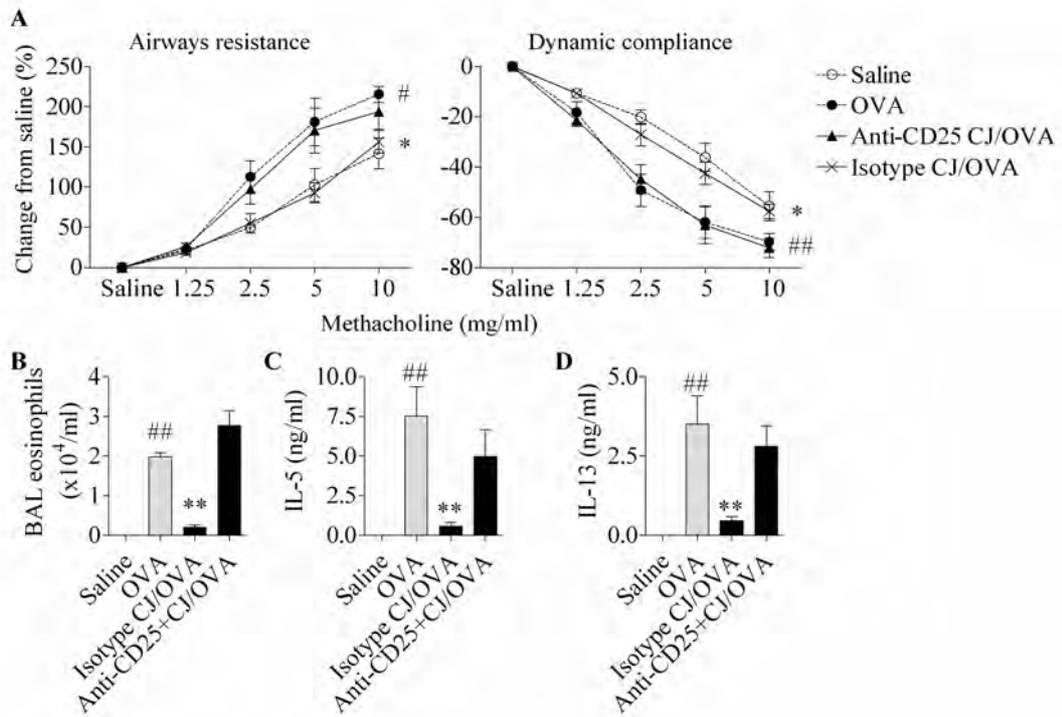


FIGURE 2.11. The effect of anti-CD25 antibody depletion of Tregs on CJ-induced suppression of AAD. (A) AHR, (B) eosinophil numbers in BAL and OVA-induced (C) IL-5 and (D) IL-13 release from MLN T cells. Data represent mean \pm SEM from 6 mice. Significant differences between Saline-sensitised and OVA-sensitised controls are shown as # $p < 0.05$, ## $p < 0.01$. Significant differences between OVA-sensitised mice and anti-CD25 and CJ treated OVA-sensitised mice are shown as * $p < 0.05$, ** $p < 0.01$.

induces DC-driven T cell priming and elicits both T cell and B cell dependent antibody response (178). By contrast, the polysaccharide vaccine induces T cell-independent antibody responses. Our results strongly suggest that to generate the induction of Tregs that suppress AAD, co-administration of polysaccharides with an immunogenic protein (conjugate vaccine) is required. Although polysaccharides alone (polysaccharide vaccine) are ineffective, the polysaccharide component of the conjugate vaccine is essential, since the backbone of the conjugate vaccine (AlPO₄+CRM₁₉₇) did not suppress AAD on its own. The conjugate but not the polysaccharide vaccine contains AlPO₄. It is unknown whether conjugate vaccine lacking AlPO₄ would suppress AAD. However, in other studies we have found that ethanol killed *S. pneumoniae*, in the absence of AlPO₄, effectively suppressed AAD (150). Therefore, it is likely that the AlPO₄ component is not necessary for the efficacy of the conjugate vaccine and that the conjugate vaccine without AlPO₄ would still be effective.

CpG-ODNs have been proposed as an immunoregulatory treatment for asthma. Indeed, CpG-ODNs can prevent the induction of many features of AAD in mouse models. However, CpG-ODNs induce a Th1 response and are associated with increased IFN- γ , which has pro-inflammatory activity in the lower airways that may increase the severity of AAD (19). IFN- γ has been shown to induce AHR in mouse models of acute and chronic disease and also amplifies Th intermediate responses that induce AHR (21-23). In our study, CpG-ODNs induced T cell IFN- γ release and not all features of AAD were attenuated. The conjugate vaccine was more effective at suppressing the features of AAD and without inducing IFN- γ release from T cells. Suppression was mediated by the induction of Tregs, which prevent the critical features of allergic inflammation. CpG-ODNs did not augment the effects of the conjugate vaccine. This is likely to be because the CpG-ODNs promote Th1 responses whereas the conjugate vaccine induces

its protective effects by the induction of Treg responses. CpG-ODN-induced IFN- γ release was prevented by the conjugate vaccine suggesting that the induction of a Th1 response by CpG-ODNs may be overcome or suppressed by conjugate vaccine-induced Tregs.

Importantly, intranasal administration of the conjugate vaccine was effective at suppressing established AAD. This provides support for the use of the conjugate vaccine in humans with established asthma. The conjugate vaccine may not offer instant relief during an asthma attack since the induction of Tregs is likely to take approximately 3-4 days. Hence, the conjugate vaccine is likely to be most effective when used prophylactically. Intramuscular administration of the conjugate vaccine had limited effects on AAD. This indicates that the vaccine must be delivered directly to the respiratory mucosa for greatest efficacy. We speculate that this may result from a requirement for the direct stimulation of DCs in the respiratory mucosa that subsequently induces enhanced suppression of AAD.

The conjugate vaccine promoted Treg expansion both during AAD and prior to OVA challenge. This indicates that the conjugate vaccine induces Treg expansion independently of OVA challenge. Tregs may be antigen specific or may have bystander effects that are independent of TCR engagement (71). We speculate that conjugate vaccine-induced Tregs are not antigen specific since they are also able to suppress anti-CD3/anti-CD28-induced stimulation of effector T cells (not shown). Further research is required to fully elucidate the mechanisms of the Treg-mediated suppression of AAD in this study.

Suppression of Th2 mediated responses occurs both locally and systemically, since Th2 cytokine release in the MLNs and spleen, levels of circulating IgE and IgG1, and eosinophils in the lung and blood were attenuated. It is possible that conjugate

vaccine-mediated suppression of local and systemic features of AAD commences with the induction of Tregs in the draining lymph nodes, which then traffic to the site of inflammation. Our data show that the conjugate vaccine increased the total number of Tregs in the lymph nodes prior to challenge, and a higher proportion of CD4⁺ cells were CD25⁺FoxP3⁺ in the lymph nodes, lung and spleen after challenge. Hence, in our system it is likely that expansion of the Treg pool occurs in the lymph nodes and that OVA challenge promotes Treg trafficking and the prevention and suppression of inflammation at distal sites. This agrees with a study by Tomura *et al.*, who showed that Tregs expand in the draining lymph nodes before moving to the inflammatory site at the skin (209). The extent of trafficking between the lymph nodes, lung and spleen in our study remains unknown. Lymph node Tregs express CCR7 and CCR6, whereas Tregs at the site of inflammation express CCR4 and CCR5, which may facilitate tracking of Treg migration (210). These markers may be used to study the movements of Tregs. Further investigation is required to understand conjugate vaccine-induced Treg trafficking and the chemokine receptors and ligands involved in the suppression of AAD.

A variety of Treg suppressive mechanisms toward effector responses have been reported and are characterised by the nature of immune response, eliciting agent, site of inflammation and genetic and immunologic background of the individual. IL-10 and TGF- β are soluble immunosuppressive cytokines that may be released by Tregs to mediate suppression. Notably, IL-10 is a pleiotropic cytokine and is also released by Th2 cells in AAD. In our study, the conjugate vaccine suppressed release of IL-10 and TGF- β from lymph node T cells, excluding their involvement in suppressing AAD. Our observation supports previous investigation in a model of AAD where a gastrointestinal nematode infestation suppressed AAD. Notably, adoptive transfer of nematode-induced

Tregs from IL-10^{-/-} mice transferred attenuation of Th2 responses and AAD (106). Recently, Pacifico *et al.*, have shown that *Schistosoma mansoni* antigens suppress AAD independently of IL-10 (211). Hence, IL-10 is not necessary for the attenuation of Th2 responses by Tregs. Others have also shown that suppression of effector T responses *in vitro* and *in vivo* does not require secretion of soluble factors, such as IL-10 or TGF- β by Tregs (68, 106, 212). Alternatively, Tregs may also mediate suppression through cell contact dependent mechanisms.

Notably, conjugate vaccine induced Tregs were more suppressive on a per cell basis. It is possible that CD4⁺CD25⁺ Tregs isolated from conjugate vaccine treated mice had fewer effector T cells which resulted in increased suppressive capacity. However, conjugate vaccine CD4⁺CD25⁺FoxP3⁺ cells had increased expression of the cell surface markers CD103⁺ and CTLA-4⁺, which are indicative of enhanced Treg functional capacity. CD103 is a marker of *in vivo* activated FoxP3⁺ Tregs and these cells have enhanced suppressive capacity (74). CTLA-4 expression inhibits CD80 and CD86 co-stimulation of T cells by DCs, and has recently been shown to be required for FoxP3⁺ Treg function *in vivo* (213). Thus an increase in CTLA-4⁺ Tregs with enhanced FoxP3 expression in conjugate vaccine-induced Tregs correlates with enhanced suppression of T cell proliferation. Asthmatics have reduced FoxP3 expression and glucocorticoids may be effective, in part, by up regulating expression (214, 215). Indeed FoxP3 expression is not up regulated in patients that are refractory to steroid treatment. In our study, induction of AAD resulted in a reduced proportion of CD4⁺CD25⁺ cells expressing FoxP3, however, treatment with the conjugate vaccine maintained expression at pre-antigen exposure levels. Taken together, these results indicate that conjugate vaccine treatment leads to combined increases in CD103 and CTLA4 and sustains the proportion of cells expressing FoxP3, resulting in a pool of

Tregs with greater functional immunosuppressive capacity. It is possible that other mechanisms are also involved in the increased suppressive capacity of Tregs after conjugate vaccine treatment, and further studies are underway to elucidate these mechanisms (47).

Administration of 100 µg anti-CD25 has been shown to deplete CD4+ cells that are CD25+ for up to 24 days (216). Furthermore, anti-CD25 have demonstrated an essential role for CD25+ Tregs in the suppression of AAD by *S. mansoni* antigens (211). In our study, anti-CD25 administration inhibited the effects of the conjugate vaccine on AAD, supporting the concept that conjugate vaccine-induced CD25+ Tregs are essential for the suppression of disease. Notably, the anti-CD25 method employed for depleting Tregs may also deplete CD25+ effector T cells, since CD25+ is also a marker of T cell activation. However, when anti-CD25 was administered before the conjugate vaccine complete restoration of AAD was achieved. This indicates that effector T cells were not affected and that Tregs were targeted using this method.

In addition to Tregs mediating the suppression of effector cell responses, the induction of Tregs may also result in deviation of T cell development away from the production of effector T cells. The induction of FoxP3 expression by naïve T cells fixes and stabilises their Treg lineage commitment, so that these cells can not develop into effector T cells (217). The induction of Tregs may also prevent the establishment of Th17 cells and Th17 mediated disease. In the presence of TGF-β naïve T cells are induced to develop into Treg cells whereas the combination of TGF-β and IL-6 promote Th17 cell differentiation. Treg inducing agents may block or prevent the involvement of IL-6 thereby promoting Treg induction and preventing the development of Th17 cells. In the context of AAD, blocking membrane bound IL-6R, with anti-IL-6R antibody, induced Tregs and resulted in the attenuation of AAD (218). Therefore in

addition to the direct suppression of effector T cell responses, conjugate vaccine induced-Tregs may also suppress AAD *via* deviation of T cell development away from the production of effector T cells.

In summary, we have shown that the *S. pneumoniae* conjugate vaccine suppresses the critical features of AAD. Furthermore, the expansion of vaccine-induced Tregs that have a greater functional capacity underpins the mechanism of suppression of AAD. Utilisation of the conjugate vaccine as a Treg inducing immunoregulatory therapy may provide a novel approach for the treatment of asthma.

CHAPTER 3

PNEUMOCOCCAL-BASED THERAPY SUPPRESSES NATURAL KILLER T CELLS AND ALLERGIC AIRWAYS DISEASE BY INDUCING REGULATORY T CELLS

Chapter 3 describes the testing of key *S. pneumoniae* components in order to develop an immunoregulatory therapy for allergic airways disease. The role of natural killer T cells and regulatory T cells was also examined.

This paper has been submitted to *The Journal of Allergy and Clinical Immunology*.

3.1 ABSTRACT

Asthma is an AAD caused by dysregulated immune responses and characterised by eosinophilic inflammation, mucus hypersecretion and AHR. NKT cells have previously been shown to contribute to AHR in some mouse models. Conversely, Tregs control aberrant immune responses and maintain homeostasis. Recent evidence suggests that *S. pneumoniae* has immunoregulatory properties that have the potential to be harnessed therapeutically for asthma. In order to transfer this potential into a successful therapy, the essential immunoregulatory components of the bacteria must be identified. In this study, we employed mouse models of AAD to identify the *S. pneumoniae* components that have suppressive properties. Our results show that when co-administered, type-3-polysaccharide and pneumolysoid (T3P+Ply) suppressed the development of eosinophilic inflammation, Th2 cytokine release, mucus hypersecretion and AHR. Importantly, T3P+Ply also attenuated features of AAD when administered during established disease. We show that NKT cells contributed to the development of AAD and that this effect was also suppressed by treatment with T3P+Ply. In addition, adoptive transfer of NKT cells induced AHR, which could also be reversed by T3P+Ply. Moreover, T3P+Ply-induced Tregs were essential for suppression of NKT cells and AAD. Collectively, our results show that the *S. pneumoniae* components T3P+Ply suppress AAD through the induction of Tregs that blocked the activity of NKT cells. These data suggest that *S. pneumoniae* components may be developed into an effective therapeutic strategy for the suppression of allergic asthma *via* the suppression of NKT cells.

3.2 INTRODUCTION

The prevalence of asthma continues to increase in westernised countries (219, 220). Th2 cells are critical for the pathogenesis of asthma and release of cytokines (IL-4, -5 and -13) that promote eosinophil influx, mucus hypersecretion and AHR (58). However, NKT cells are also now recognised for their importance in the pathogenesis of asthma (221). NKT cells are a small and unique population of lymphocytes that express both T and NK cell markers, release high concentrations of Th2 cytokines (35). The CD4⁺ IL-4 and IL-13 producing NKT cell subset is indispensable for the development of AHR in mouse models of AAD (38). The role of NKT cells in asthma is controversial, which may be due to the varying techniques used to identify NKT cells and non-uniform patient groups tested (222, 223). Mouse models provide a uniform disease state and are an excellent tool for investigating the role of NKT cells in AAD. The induction of both Th2 cells and NKT cells may be controlled by the function of Tregs, which suppress aberrant immune responses and maintain homeostasis (71). Asthmatics are known to have fewer and less functional Tregs and as a result, asthma is a state of dysregulated immunity (62, 65).

Some infectious agents predispose and/or exacerbate the severity of AAD such as asthma, where as others are protective (119, 139, 144, 147, 194, 224). Infectious agents that are protective may prevent or suppress AAD by modulating the immune system and are under investigation for therapeutic use. However, there are major limitations that need to be addressed before the potential of infectious agents can be transferred into therapeutic use (71). These include removal of the infectious capacity and simplification of the composition, whilst preserving the immunoregulatory capacity. Furthermore, an understanding of the mechanism of action is desirable in order to understand the implications and limitations of the therapy.

Early studies using mouse models showed that *S. pneumoniae* infection attenuated eosinophilic inflammation during parasitic infection (173). Using mouse models of AAD, we have previously shown that ethanol killed *S. pneumoniae*, suppressed the development of features of AAD including: eosinophils in BAL; Th2 cytokine (IL-5 and IL-13) release from MLN T cells; the number of mucus secreting cells; and airways hyperresponsiveness (150). In humans, *S. pneumoniae* immunisation has been associated with decreased asthma related hospitalisations in both children and the elderly, which may be the result of immune modulation (181, 182).

We hypothesised that particular *S. pneumoniae* components must be essential for suppression of AAD and their identification may enable the development of a simplified therapy. We selected four of the most immunostimulatory *S. pneumoniae* components and identified the most effective immunoregulatory combination. We investigated the immunoregulatory and suppressive properties of this combination in different mouse models of AAD. Their mechanism of action was characterised by assessing the effects on NKT cells and Tregs.

3.3 METHODS

3.3.1 Animals

Adult (6 week old) female BALB/c mice were maintained under specific pathogen free and controlled environmental conditions. All procedures were approved by the Animal Care and Ethics Committee of The University of Newcastle.

3.3.2 Models of AAD

Induction of AAD was performed as previously described (150, 200). For OVA-induced AAD, mice were sensitised to OVA (i.p.; day 0; 50 µg; Sigma-Aldrich, St. Louis, MO) with Rehydrogel (1 mg; Reheis, Berkeley Heights, NJ) in sterile saline (200 µl) and challenged by i.n. droplet application of OVA under isoflurane anesthesia (day 12-15; 10 µg in 50 µl sterile saline). To recapitulate established disease, mice received OVA i.p. (day 0) followed by two sets of challenges (days 11-13 and 33-34). Control mice received saline sensitisation and OVA challenge. For HDM-induced AAD, mice were sensitised to HDM (*Dermatophagoides pteronyssinus*) extract (i.n.; day 0, 1 and 2; 50 mg; Greer Labs, Lenoir, NC) in sterile saline (50 µl) and challenged with HDM (i.n.; day 14-17; 5 mg in 50 µl sterile saline). Mice were euthanased by pentobarbitone overdose 24 h after the final challenge. Where indicated, mice received anti-CD25 antibody (i.p; day -3; 100 µg in 200 µl saline, PC61).

3.3.3 *S. pneumoniae* and components

Ethanol killed *S. pneumoniae* (Type 3 strain ATCC NCO1265) was prepared as previously described (150).

Cell walls were purified as previously described with minor modifications (225). Briefly, unencapsulated *S. pneumoniae* (strain ATCC BAA-255/R6) was cultured overnight in heart infusion media. 5×10^8 cfu were ethanol killed (75%; 2 h on ice) and sonicated for 10 cycles. The suspension was digested with DNase (10 µg/ml; Promega, Mannheim, Germany) and RNase (50 µg/ml; Promega) for 1 h at 37 °C followed by treatment with trypsin-EDTA (100 µg/ml) and CaCl (10 mM) for 2 h at 37 °C. The digest was sedimented by centrifugation and resuspended in SDS (20 min; 90 °C) followed by 8 washes in sterile saline. Purified cell walls were stored at -20 °C until required.

Mice were administered killed *S. pneumoniae* (2×10^5 cfu), T3P (2 μ g; ATCC), cell walls (equivalent of 2×10^5 cfu), Ply (lipopolysaccharide (LPS)-free; 40 ng; University of Adelaide, Adelaide, SA, Australia) and/or CpG-Oligodinucleotides (ODNs; 10 μ g; TCCATGACGTTCTACGTT; Geneworks, Thebarton, SA, Australia) in sterile saline (30 μ l; every 12 h for three doses) by intratracheal (i.t.) administration under alfaxan intravenous (i.v.) anesthesia. To assess the effects of the T3P+Ply in established AAD, mice were treated with T3P+Ply three times per week (day 14, 16, 18, 21, 23, 25, 28, 30 and 31).

3.3.4 Assessment of cellular inflammation

Preparation and enumeration of blood and BAL leukocytes was performed as previously described (150, 207).

3.3.5 Cell preparation

Mediastinal lymph nodes were pushed through 70 μ m sieves to prepare single cell suspensions. Lungs and spleens were pushed through coarse metal sieves prior to being passed through 70 μ m sieves to prepare single cell suspensions. Samples were processed for use as previously described (150).

3.3.6 T cell cytokine release

Cells (5×10^6 per ml) were cultured in RPMI media supplemented with 10 % FCS, HEPES (20 mM), penicillin/streptomycin (10 μ g/ml), L-glutamine (2 mM), 2-mercaptoethanol (50 μ M), sodium pyruvate (1 mM) and OVA (200 μ g/ml) or anti-CD3 and antiCD28 (5 μ g/ml) as indicated) for 4 or 6 days (5 % CO₂, 37 °C) respectively, for lymph nodes or spleens, before supernatant collection and storage at -20 °C until

required. IL-5 and IL-13 concentrations in cell culture supernatants were determined by ELISA (BD Pharmingen, San Diego, CA).

3.3.7 Airways hyperresponsiveness

Airways hyperresponsiveness was assessed as previously described (151). Briefly, anaesthetised, tracheostomised mice were cannulated and connected to an inline aerosol and ventilator. Changes in airway function following challenge with increasing doses of aerosolised methacholine (1.25, 2.5, 5 and 10 mg/ml) were assessed by analysis of pressure and flow waveforms and determination of transpulmonary resistance and dynamic compliance.

3.3.8 Lung histology

Lungs were perfused, inflated, fixed, embedded, sectioned, and stained to enumerate airway mucus secreting cells and tissue eosinophils (151).

3.3.9 Flow cytometry

One $\times 10^6$ cells/well in a 96 well U-bottomed plate, were stained for α -GalCer-loaded CD1d (226), TCR β , CD4, CD25 (BD Pharmingen, San Diego, CA). Cells were permeabilised and stained for FoxP3 according to the manufacturer's protocol (eBioscience, San Diego, CA). Samples were analysed using a FACS Canto flow cytometer controlled by FACSDiVa software (version 4.1.1; BD Biosciences, Mississauga, Canada).

3.3.10 Adoptive transfer of NKT cells

Splenocytes were obtained from naïve mice and enriched for CD4⁺ cells using microbead conjugated antibodies to deplete CD11c⁺, CD8⁺, CD11b⁺, CD45R⁺, CD49b⁺ and Ter-119⁺ cells (AutoMACS Pro, Miltenyi Biotec, Auburn, CA). The microbead- fraction was labeled with α -GalCer-loaded CD1d-PE tetramer, washed, labeled with anti-PE microbeads, washed and positively selected for microbead⁺ cells. CD1d⁺TCR β ⁺ cells were subsequently purified by flow cytometry (FACS Aria, BD Biosciences) to >97 % purity. CD1d⁺TCR β ⁺ cells were administered by i.v. injection (day 10; 3×10^5 in 100 μ l saline).

3.3.11 Data analysis

Data were analysed using GraphPad Prism (GraphPad Software, CA) and are represented as mean \pm SEM. One-way ANOVA with Dunnett's post test was used to determine significance between data with multiple comparisons. One-way repeated measures ANOVA and Bonferroni's post test was used to determine significance for AHR data. A p-value <0.05 was considered statistically significant.

3.4 RESULTS

3.4.1 Identification of *S. pneumoniae* immunoregulatory components

We tested the immunoregulatory capacity of four major *S. pneumoniae* components. These were: 1. the capsular polysaccharide of the type 3 strain (T3P), since the type 3 serotype of *S. pneumoniae* was used in previous studies (146), 2. cell walls (CW) prepared from an unencapsulated *S. pneumoniae* strain and processed to remove non-cell wall components, 3. the detoxified derivative of *S. pneumoniae* pneumolysin, Ply,

and 4. CpG-ODNs, to represent bacterial DNA, known to have adjuvant properties and induce Th1 responses (15, 223).

To identify the *S. pneumoniae* immunoregulatory components we employed a widely used OVA-induced model of AAD. Different combinations of components were administered at the time of sensitisation to OVA and the effects on the induction of AAD were assessed (Figure 3.1A).

The development of AAD was characterised by increased numbers of eosinophils in the BAL and OVA-induced IL-5 and IL-13 release from MLN T cells (Figure 3.1B-D). As expected, killed *S. pneumoniae* suppressed the number of eosinophils in the BAL and OVA-induced IL-5 and IL-13 release from MLN T cells. When all four components (T3P+CW+Ply+CpG) were administered together, the number of eosinophils in the BAL and OVA-induced IL-5 and IL-13 release from MLN T cells was also suppressed. This indicated that immunoregulatory components responsible for suppressing the development of AAD were contained within the mix of the four components tested.

Suppression of the number of eosinophils in the BAL and OVA-induced IL-5 and IL-13 release from MLN T cells was achieved whenever T3P and Ply were administered together. Neither T3P alone nor Ply alone attenuated AAD, indicating that a synergistic action was necessary for suppression.

CpG attenuated the number of eosinophils in the BAL but had no effect on OVA-induced IL-5 and IL-13 release from MLN T cells. When CpG and T3P were administered together all these features of AAD were suppressed, however, not to the same magnitude as the T3P and Ply combination.

Cell walls alone also suppressed these features of AAD, however, their action was dampened in combination with most other components.

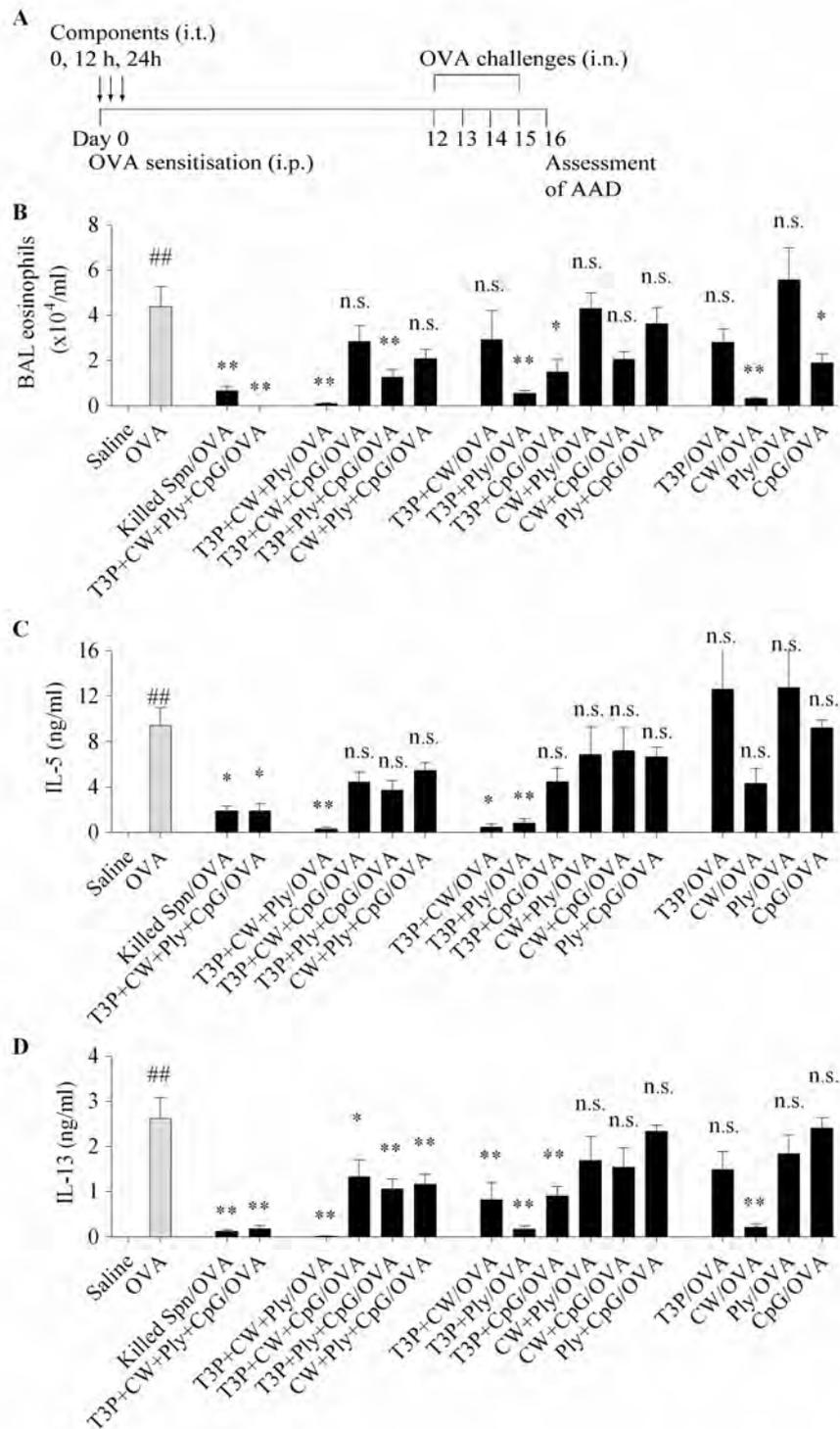


FIGURE 3.1. Identification of *S. pneumoniae* components that suppress AAD. (A) Experimental protocol: AAD was induced by i.p. OVA sensitisation and i.n. OVA challenge. Controls received saline sensitisation and OVA challenge. Component treatment was administered at the time of OVA sensitisation, in 3 doses every 12 h.

Features of AAD were assessed 24 h after the final OVA challenge. The effect of *S. pneumoniae* (Spn) components on (B) eosinophils in BAL and OVA-induced (C) IL-5 and (D) IL-13 release from MLN T cells. Data represent mean \pm SEM from 6-8 mice. Significant differences between saline-sensitised and OVA-sensitised controls are shown as ## $p < 0.01$. Significant differences between OVA-sensitised and component treated OVA-sensitised mice are shown as * $p < 0.05$ and ** $p < 0.01$. n.s. not significant.

3.4.2 Identification of *S. pneumoniae* components that suppress AHR

Combinations of components that suppressed the development of eosinophils in BAL and OVA-induced IL-5 and IL-13 release from MLN T cells were investigated for their capacity to suppress AHR.

The development of AAD was also characterised by enhanced AHR in terms of increased airways resistance and decreased dynamic compliance compared to saline sensitised mice (Figure 3.2). As expected, killed *S. pneumoniae* suppressed the development of AHR. Component combinations that had the greatest suppressive effects on eosinophil influx and Th2 cytokine release (Figure 3.1) were also effective at suppressing AHR. When T3P and Ply were administered together, with or without other components they suppressed the development of AHR.

The administration of cell walls alone also suppressed the development of AHR and showed potential as an immunoregulatory therapy for AAD (Figure 3.2). However, cell walls are complex mixtures of carbohydrates, peptidoglycan and proteins. Therefore, extensive additional research would be required to identify the active component(s). Consequently, the combination of T3P+Ply was chosen as a potential immunoregulatory therapy for AAD and was the focus for subsequent studies.

3.4.3 T3P+Ply immunoregulatory therapy suppresses the development of additional features of AAD

We then examined the capacity of T3P+Ply immunoregulatory therapy to suppress the induction of additional features of AAD. The development of AAD was characterised by increased levels of eosinophils in the blood and lung tissue, numbers of mucus secreting cells around the airways and OVA-induced IL-5 and IL-13 release from

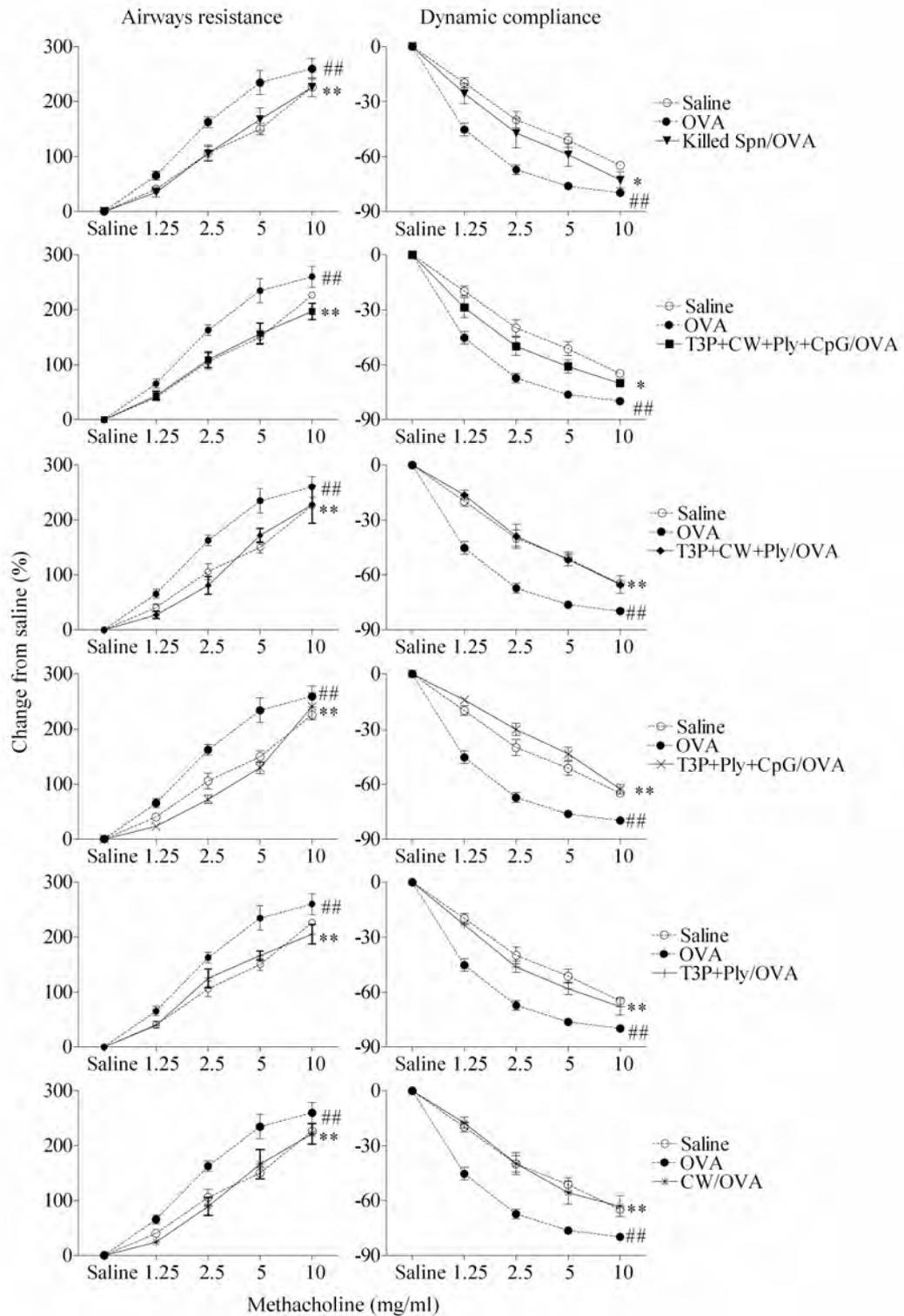


FIGURE 3.2. Selected *S. pneumoniae* components suppress AHR. Components that suppressed eosinophil influx into the airways and Th2 cytokine release in AAD were tested for their capacity to suppress AHR, in terms of airways resistance and dynamic

compliance. Data represent mean \pm SEM from 6-8 mice. Significant differences are shown for the entire dose-response curve between saline-sensitised and OVA-sensitised controls are shown as ## $p < 0.01$ and between OVA-sensitised and component treated OVA-sensitised mice as * $p < 0.05$, ** $p < 0.01$.

splenic T cells (Figure 3.3A-E). Administration of T3P+Ply during sensitisation suppressed all of these local and systemic features of AAD (Figure 3.3A-E).

We then investigated whether T3P+Ply could suppress the induction of AAD when administered prior to sensitisation (Figure 3.3F), or the progression of AAD when administered after sensitisation (Figure 3.3G). Administration of T3P+Ply before or after sensitisation suppressed the levels of eosinophils in the BAL and blood, and OVA-specific IL-5 and IL-13 release from MLN and splenic T cells (Figure 3.3H-M). These data suggest that T3P+Ply may be utilised for both the prevention and treatment of AAD.

3.4.4 T3P+Ply immunoregulatory therapy suppresses AAD when administered during established disease

We then investigated whether administration of T3P+Ply immunoregulatory therapy would be effective during established AAD. A model of AAD was developed that involved sensitisation to OVA, followed by an initial challenge (days 11-13) to establish AAD and a second challenge (days 33-34) to recapitulate disease before the assessment of AAD (Figure 3.4A). T3P+Ply was administered during established AAD commencing one day after the initial challenge for a total of 9 doses (day 14, 16, 18, 21, 23, 25, 28, 30 and 31, Figure 3.4A). Administration of the T3P+Ply during established disease suppressed eosinophils in the BAL, OVA-induced IL-5 and IL-13 release from MLN T cells and AHR (Figure 3.4B-E).

3.4.5 T3P+Ply immunoregulatory therapy suppresses the induction of HDM-induced AAD

To determine whether T3P+Ply could suppress AAD induced by other allergens, we

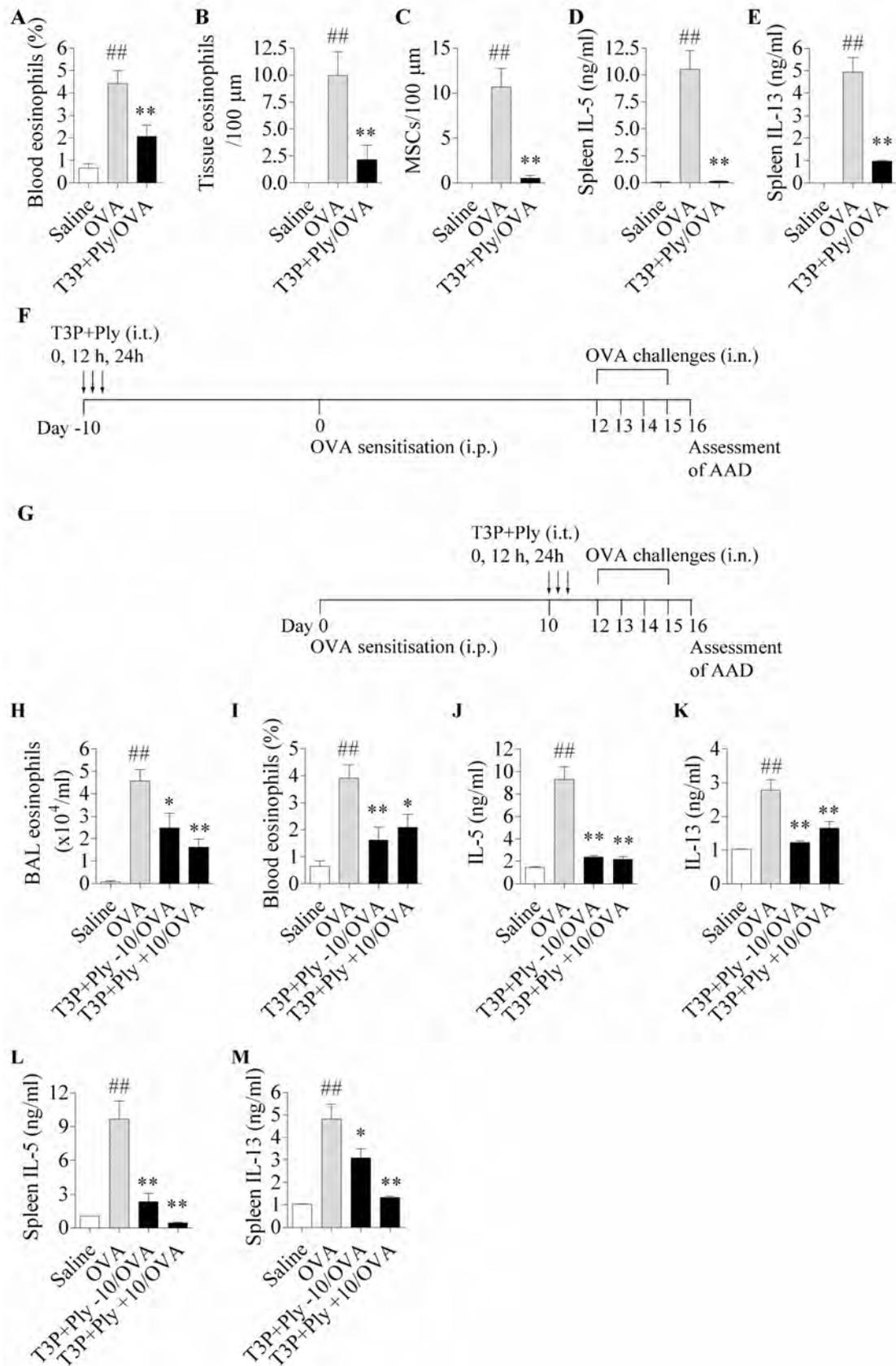


FIGURE 3.3. T3P+Ply suppress the development of additional features of AAD.

The effect of T3P+Ply treatment on the levels of eosinophils in (A) blood and (B)

airway tissue, (C) MSCs around the airways, and OVA-induced (D) IL-5 and (E) IL-13 release from splenic T cells. Experimental protocol used for administration of T3P+Ply (F) before and (G) after sensitisation. The effect of T3P+Ply administration before (T3P+Ply -10) and after (T3P+Ply +10) sensitisation on eosinophils in (H) BAL and (I) blood, OVA-induced (J) IL-5 and (K) IL-13 release from MLN T cells and OVA-induced (L) IL-5 and (M) IL-13 release from splenic T cells. Data represent mean \pm SEM from 6-8 mice. Significant differences between saline-sensitised and OVA-sensitised controls are shown as ## $p < 0.01$. Significant differences between OVA-sensitised and component treated OVA-sensitised mice are shown as * $p < 0.05$ and ** $p < 0.01$. n.s. not significant.

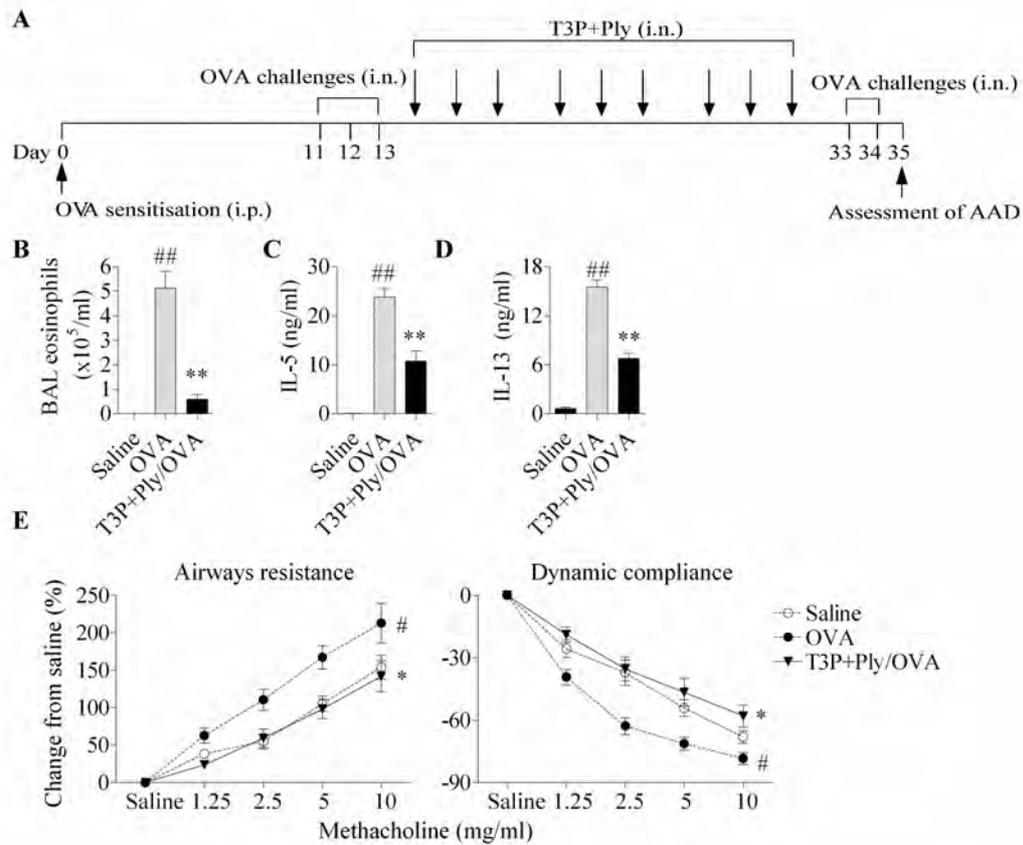


FIGURE 3.4. T3P+Ply attenuate AAD when administered during established disease. (A) Experimental protocol: AAD was established by i.p. OVA sensitisation followed by an initial i.n. OVA challenge and then a second challenge to recapitulate AAD. T3P+Ply were delivered three times per week commencing day 14 (totalling 9 doses). The effect of T3P+Ply treatment on (B) eosinophils in BAL, OVA-induced (C) IL-5 and (D) IL-13 release from MLN T cells and (E) AHR. Data represent mean \pm SEM from 6-8 mice. Significant differences between saline-sensitised and OVA-sensitised controls are shown as # $p < 0.05$ and ## $p < 0.01$. Significant differences between OVA-sensitised and component treated OVA-sensitised mice are shown as * $p < 0.05$ and ** $p < 0.01$. Significance for resistance and compliance are given for the entire dose response curve.

used a HDM model in which sensitisation was induced directly *via* the airways in the absence of an adjuvant (Figure 3.5A). The development of HDM-induced AAD led to increased numbers of eosinophils in the BAL and IL-5 and IL-13 release from MLN T cells (Fig 3.5B-E). Administration of T3P+Ply during sensitisation again suppressed all of these features (Fig 3.5B-E).

HDM-induced AAD also resulted in the development of AHR and administration of T3P+Ply suppressed the development of AHR (Figure 3.5F). These data provide support for the use of T3P+Ply as an immunoregulatory therapy for AADs.

3.4.6 T3P+Ply immunoregulatory therapy suppresses the accumulation of NKT cells in the lungs and NKT cell-induced AHR

Since AHR is a major physiological outcome of AADs and given that NKT cells are essential for AHR, we investigated the effect of T3P+Ply on the induction of NKT cells in our model of OVA-induced AAD. The induction of AAD resulted in increased numbers of CD1d+TCR β + (NKT) cells in the lung compared to saline sensitised mice (Figure 3.6A). Administration of T3P+Ply reduced the total number of CD1d+TCR β + cells in the lungs. T3P+Ply administration had no effect on the small number of CD1d+TCR β + cells in the MLNs (not shown).

To assess the capacity of T3P+Ply to suppress NKT function, we isolated NKT cells from naïve mice and adoptively transferred these cells into saline or OVA sensitised mice, prior to OVA challenge (Figure 3.6B and C). When NKT cells were adoptively transferred into saline sensitised mice, which were then challenged with OVA, AHR was increased compared to saline sensitised mice that did not receive NKT cells (Figure 3.6D). This confirmed that adoptive transfer of NKT cells induced AHR in our model. When NKT cells were adoptively transferred into OVA sensitised mice,

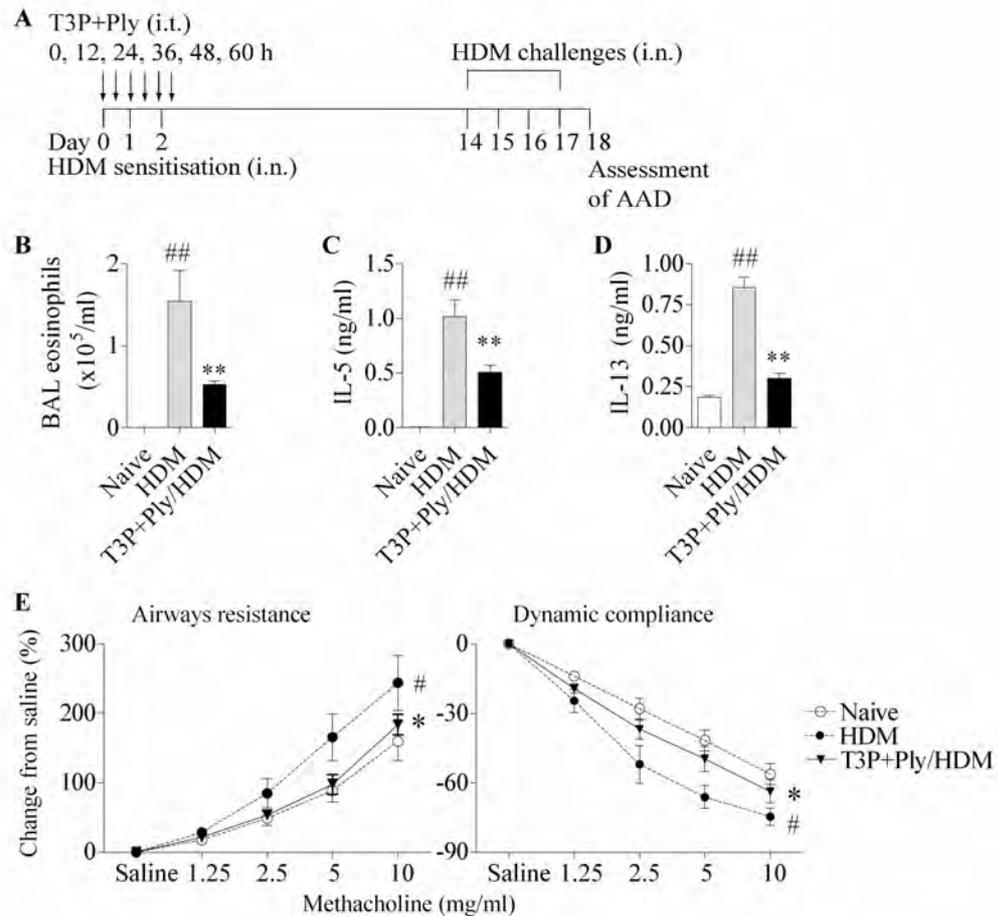


FIGURE 3.5. T3P+Ply suppress the development of HDM-induced AAD. (A) Experimental protocol: AAD was induced by three i.n. administrations of HDM followed by four i.n. challenges. Component treatment was administered throughout the sensitisation phase every 12 h for 6 doses. Features of AAD were assessed 24 h after the final HDM challenge: (B) eosinophils in the BAL, anti-CD3/anti-CD28 stimulated (C) IL-5 and (D) IL-13 release from MLN T cells and (E) AHR. Data represent mean \pm SEM from 6 mice. Significant differences between saline-sensitised and OVA-sensitised controls are shown as # $p < 0.05$ and ## $p < 0.01$. Significant differences between OVA-sensitised and component treated OVA-sensitised mice are shown as * $p < 0.05$ and ** $p < 0.01$. Significance for resistance and compliance are given for the entire dose response curve.

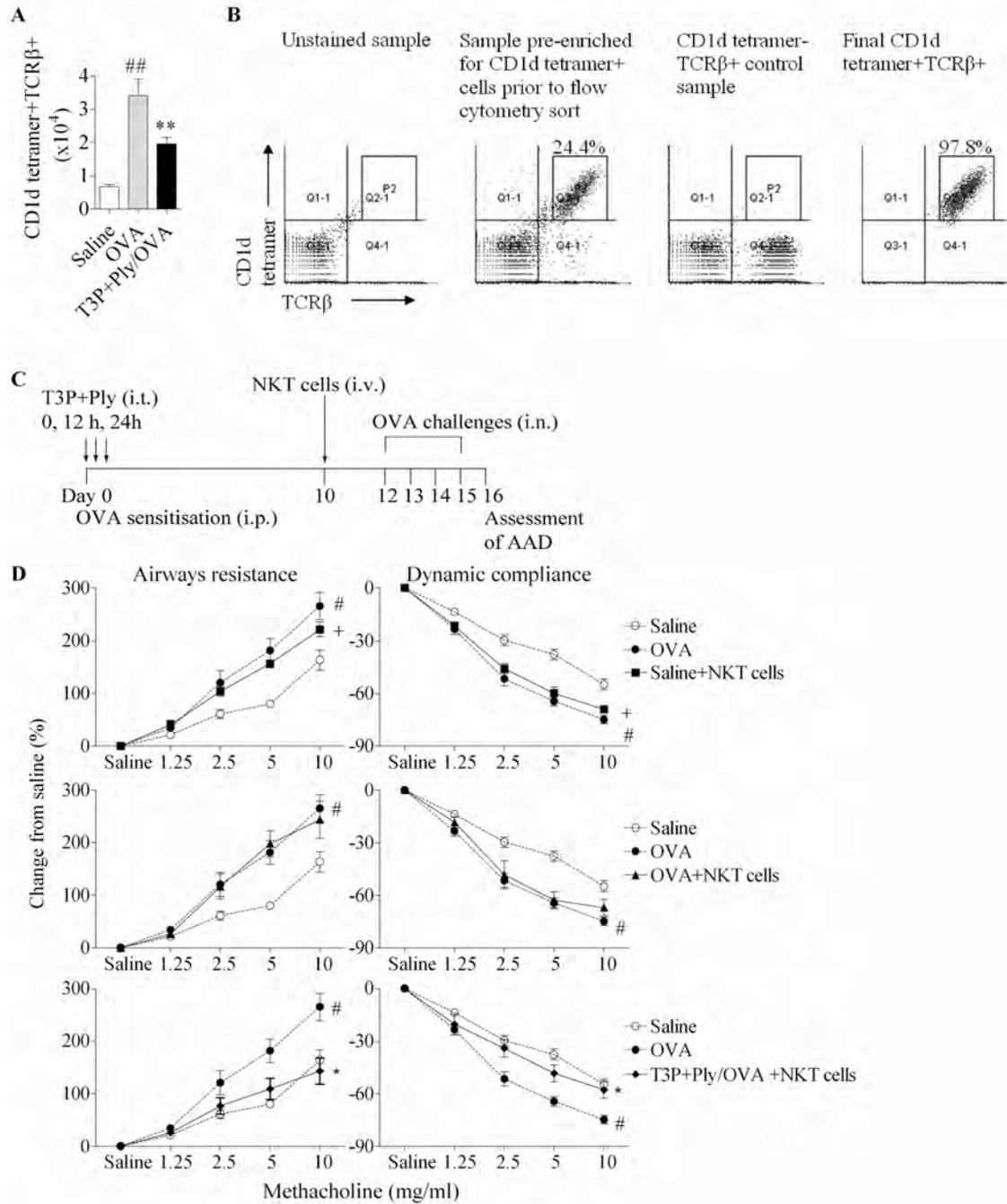


FIGURE 3.6. T3P+Ply suppress NKT cells in AAD. (A) Total CD1d+TCRβ⁺ in the lung after the induction of OVA-induced AAD. (B) Representative flow cytometry data for the isolation of CD1d+TCRβ⁺ cells showing the unstained, pre-sorted, control and final populations. (C) Experimental protocol for adoptive transfer: Similar to Figure 1 but mice received 3×10^5 CD1d+TCRβ⁺ (NKT) cells i.v. on Day 10. (D) The effect of NKT cell adoptive transfer and T3P+Ply treatment on AHR. Data represent mean \pm

SEM from 6-8 mice. Significant differences between saline-sensitised and OVA-sensitised controls are shown as # $p < 0.05$, ## $p < 0.01$. Significant differences between OVA-sensitised mice and T3P+Ply treated OVA-sensitised mice are shown as * $p < 0.05$ and ** $p < 0.01$. Significant differences between Saline and Saline+NKT cells are shown as + $p < 0.05$. Significance for resistance and compliance are given for the entire dose response curve.

which were then challenged, AHR remained at the same level. When NKT cells were adoptively transferred into T3P+Ply treated OVA sensitised mice, which were then challenged, AHR was suppressed. Together, these data indicate that T3P+Ply treatment is able to suppress the induction of NKT cells and also overcome NKT cell-induced AHR.

3.4.7 T3P+Ply-induced Tregs are required for the suppression of AAD

Since Tregs are known to regulate aberrant immune responses such as those that occur in AAD, we investigated a role for Tregs in T3P+Ply-mediated suppression of OVA-induced AAD. The development of AAD resulted in increased numbers of CD4+CD25+FoxP3+ Tregs in the lung compared to saline sensitised mice (Figure 3.7A and B). T3P+Ply treatment resulted in an additional increase in the number of CD4+CD25+FoxP3+ Tregs in the lung compared to untreated mice. Notably, T3P+Ply administration had no effect on the numbers of Tregs in the MLNs (not shown). This indicated that an induction of Tregs in the lung may play a role in suppressing the development of AAD.

To determine whether Tregs were required, for the attenuation of AAD by T3P+Ply, anti-CD25 (PC61) antibody was delivered prior to T3P+Ply administration. Anti-CD25 antibody depleted the number of CD4+CD25+FoxP3+ Tregs in the lungs to the level of the saline group (data not shown). Anti-CD25 administration reversed the protective effects of T3P+Ply on the level of eosinophils in BAL and blood, OVA-induced IL-5 and IL-13 release from MLN T cells and AHR compared to the relevant isotype control (Figure 3.7C-G). These data suggest that T3P+Ply-induced Tregs are required to suppress the development of AAD.

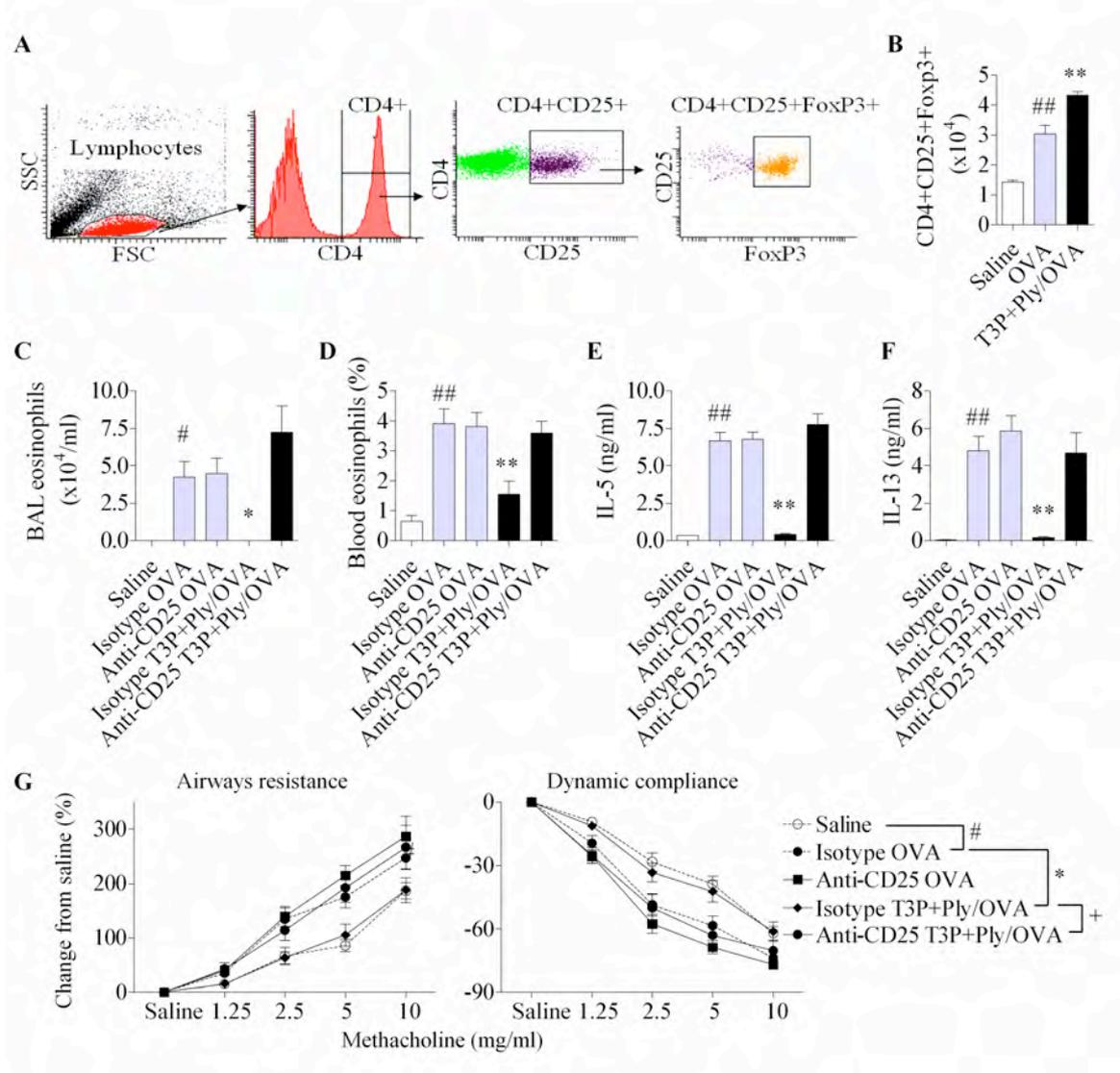


FIGURE 3.7. T3P+Ply induces Tregs that are required for the suppression of AAD.

(A) Representative flow cytometry data demonstrating gating strategy for CD4+CD25+FoxP3+ cells. (B) Total CD4+CD25+FoxP3+ cells in the lung after the induction of OVA-induced AAD. The effect of anti-CD25 administration on the suppressive effects of T3P+Ply treatment on eosinophils in (C) BAL and (D) blood, OVA-induced (E) IL-5 and (F) IL-13 release from MLN T cells and (G) AHR. Data represent mean \pm SEM from 6-8 mice. Significant differences between saline-sensitised and OVA-sensitised controls are shown as # $p < 0.05$ and ## $p < 0.01$. Significant differences between OVA-sensitised and component treated OVA-sensitised mice are shown as * $p < 0.05$ and ** $p < 0.01$. Significant differences between isotype and anti-

CD25 administration on T3P+Ply treatment are shown as + $p < 0.05$. Significance for resistance and compliance are given for the entire dose response curve.

3.4.8 T3P+Ply-induced Tregs are required to suppress the induction of NKT cells

We then investigated whether Tregs were required for the suppression of NKT cells and therefore AHR. Anti-CD25 depletion of Tregs reversed the capacity of T3P+Ply to suppress the induction of NKT cells (Figure 3.8). These data suggest that T3P+Ply treatment suppresses NKT cells, and AHR, through the induction of Tregs.

3.5 DISCUSSION

This study has identified a specific combination of *S. pneumoniae* components that may be developed into an effective immunoregulatory therapy for AAD. Using different models of AAD, we showed that the combination of *S. pneumoniae* capsular polysaccharide and pneumolysoid (T3P+Ply) suppressed the development of all hallmark features of AAD including; eosinophils in BAL, blood and tissue; Th2 cytokine (IL-5 and IL-13) release from MLN and splenic T cells; the number of mucus secreting cells around the airways; and AHR. Importantly, we showed that administration of T3P+Ply during established disease suppressed AAD. The suppressive effects of T3P+Ply were associated with a reduction in the numbers and function of NKT cells. Indeed, the adoptive transfer of NKT cells induced AHR, which was reversed by T3P+Ply treatment. This provided a direct link between the effect of T3P+Ply, suppression of NKT cells and attenuation of AHR. We also showed that T3P+Ply induced increased numbers of Tregs, which were required for the attenuation of AAD. Finally we showed that T3P+Ply-induced Tregs were required to suppress the induction of NKT cells.

T3P+Ply had synergistic effects since neither capsular polysaccharide (T3P) nor Ply alone suppressed AAD. Given that Ply is an immunogenic protein it is likely to

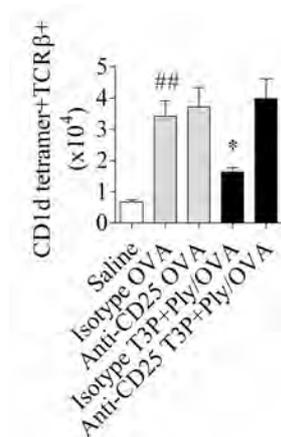


FIGURE 3.8. T3P+Ply-induced Tregs are required for the suppression of NKT cells. Total CD1d+TCRβ+ cells in the lung after the induction of OVA-induced AAD. Data represent mean ± SEM from 6-8 mice. Significant differences between saline-sensitised and OVA-sensitised controls are shown as ## p<0.01. Significant differences between OVA-sensitised mice and T3P+Ply treated OVA-sensitised mice are shown as * p<0.05.

provide adjuvant properties to T3P. CpG provided adjuvant properties to T3P, however, not to the same magnitude as Ply. Furthermore, the use of CpG-ODNs as an immunotherapy may also be problematic since they induce IFN- γ , which has damaging effects on the airways and may increase the severity of AAD (19). Cell walls contain non-capsular polysaccharide and components which may also act as an adjuvants (227). However, at this stage the use of cell walls as an immunotherapy for AAD is problematic due to their reversible action and complex composition. Polysaccharides alone are known to induce T cell-independent responses that are poorly immunogenic. Without T cell help, the development of antibody affinity maturation, isotype switching and immunological memory is limited (177). However, in the presence of an adjuvant, polysaccharides may elicit a T cell-dependent response and enhance antibody production. Others have shown that immunological protection against *S. pneumoniae* infection is T cell-dependent (175). Furthermore, the currently available *S. pneumoniae* 7-valent protein-conjugated vaccine is much more effective in protecting against pneumococcal infection compared to the 23-polysaccharide vaccine, which lacks the diphtheria toxoid adjuvant (229). Collectively, these observations indicate that T3P+Ply may suppress AAD through mechanisms of action that are T cell-dependent.

The adoptive transfer of NKT cells from naïve mice into saline sensitised mice and associated increase in AHR may be due activation of NKT cells *ex vivo*. It is possible that the isolation of NKT cells by α -GalCer-loaded CD1d tetramer activated the NKT cells and caused the associated increase in AHR. This caveat must be considered since the adoptively transferred NKT cells may not represent a steady state population. Nevertheless, the capacity for T3P+Ply treatment to suppress NKT cell numbers, function and therefore AHR has been clearly demonstrated.

Apart from *S. pneumoniae*, other infectious agents such as helminths, lactobacilli and mycobacterium have been shown to induce Tregs and suppress AAD in mouse models (144, 147, 152). Mycobacterial cell wall components (lipoglycans lipoarabinomannan and phosphatidylinositol mannan) have also been shown to suppress AAD, however their effect on AHR has not been determined (229). Furthermore, mycobacterial phosphatidylinositol mannoside has been reported to bind CD1d and activate human and mouse NKT cells, which may be a problem for the therapeutic use of mycobacterial components (230). Clinical application of heat killed *Mycobacterium vaccae* and its delipidated, deglycolipidated and arabinogalactan depleted derivative have not been successful in clinical trials (130, 231). Interestingly, *S. pneumoniae* induces more Tregs than other Gram-positive bacteria (175). Hence, T3P+Ply has the potential to be one of the most effective immunoregulatory combinations of bacterial components for attenuation of AAD.

The specific molecular pathway associated with recognition and/or uptake of T3P+Ply, which then mediates the induction of Tregs, remains to be elucidated. TLR2 signalling has been shown to expand the number of Tregs independent of APCs (232). Since T3P may engage with TLR2, the involvement of this pathway warrants further investigation. Notably, in the presence of TLR2 agonists, pneumolysin can activate macrophage responses (165). Whether this synergistic effect is involved in T3P+Ply-mediated suppression of AAD remains to be determined. Tregs may also be induced by indoleamine 2,3-dioxygenase (IDO), the rate-limiting enzyme for tryptophan catabolism produced by activated DCs (233). In humans, *S. pneumoniae* infection has been associated with high IDO levels (234). Hence, there is potential for T3P+Ply to utilise this pathway to induce Tregs. Alternatively, T3P+Ply may affect the expression of co-stimulatory and activation molecules on APCs and therefore suppress the induction of

AAD. The importance of these pathways and mechanisms in T3P+Ply-mediated suppression of AAD is yet to be determined.

The recognition of asthma as a Th2-driven disease has led to the development of a number of Th2 targeted therapies however, they have not been successful. Since NKT cells are required for the development of AHR, NKT cells may be important targets for a successful therapy. NKT cells are known to release Th1, Th2 and Th17 cytokines and chemokines, which enhance allergic inflammation and AHR (13). NKT cells also activate alveolar macrophages to produce IL-13 and IL-25, which are involved in the pathogenesis of asthma (235). The capacity of T3P+Ply to suppress the development of NKT cells in the lung highlights its potential as an effective therapy.

Importantly, to our knowledge, this is the first study to report the suppression of the numbers and function of NKT cells by inducible-Tregs in an *in vivo* model of AAD. The induction of Tregs by T3P+Ply was clearly required to suppress the development of AAD, since the depletion of Tregs by the administration of anti-CD25 reversed the protective effects of T3P+Ply. This protocol depletes the number of CD4⁺ cells that are CD25⁺ for up to 24 days ((216) and data not shown). This occurs through the neutralisation of CD25 (IL-2R α) and blockade of the signalling cascade induced by IL-2, which compromises the induction of FoxP3 and the development of Tregs.

Unlike conventional T cells, NKT cells require cell-cell contact mediated suppression as demonstrated by transwell studies (111). Furthermore, neutralisation of IL-10 and TGF- β showed that these cytokines were not required for suppression of NKT cells however intercellular adhesion molecule-1 (ICAM-1), which plays an important role in cell-cell contact, was necessary. Moreover, the suppression of NKT cells did not involve the inhibition of APCs. Together, this evidence suggests that Treg ligands, such as Galectin-1 which induces cell death of the target cell, may be involved

in Treg mediated suppression of NKT cells (99). If cell-cell contact is necessary for Treg mediated suppression of NKT cells, this suggests that Tregs need to be in the same location as NKT cells in order for effective suppression to occur. In our study, the increase in T3P+Ply-induced Tregs and associated decrease in NKT cells in the lung, but not lymph nodes, provides circumstantial evidence that cell contact mediated suppression may be occurring.

An interesting relationship between NKT cells and Tregs in asthma is beginning to emerge. A study by Nguyen and colleagues, demonstrated that NKT cells from asthmatics secreted higher levels of perforin and granzyme B and killed Tregs more effectively compared to healthy controls (112). This suggests that asthmatic NKT cells have increased toxicity toward Tregs which may contribute to the dysregulated immune response in asthmatics. In addition to higher total numbers of NKT cells, asthmatics have also been reported to have lower numbers of Tregs (62). It is therefore possible that there is an imbalance between Tregs and NKT cells that promotes allergic inflammation and pathogenesis in asthmatic individuals. T3P+Ply-induced Tregs and the associated suppression of NKT cells has the potential to restore this imbalance thereby providing an effective therapy for asthma.

Recently a CD1d-dependent antagonist, di-palmitoyl-phosphatidyl-ethanolamine polyethylene glycol (DPPE-PEG), has been shown to inhibit NKT cells and suppress AHR (236). However, DPPE-PEG had no effect on the development of OVA-specific Th2 responses. Hence, the induction of Tregs which also suppress other effector cells such as Th2 cells, may be a more global and effective strategy than direct targeting NKT cells.

In summary, we have shown that *S. pneumoniae* components T3P+Ply reverse the development of hallmark features of AAD. In addition, T3P+Ply suppress the number of NKT cells in the lung through the induction of Tregs. Due to the ability of

Tregs to suppress multiple effector arms of the immune response, this therapy has many advantages over current therapies and may be an effective therapeutic approach for asthma.

CHAPTER 4

PNEUMOCOCCAL COMPONENTS INDUCE REGULATORY T CELLS THAT MEDIATE IMMUNE DEVIATION AND SUPPRESSION TO ATTENUATE THE DEVELOPMENT OF ALLERGIC AIRWAYS DISEASE

Chapter 4 describes the investigation of the role of regulatory T cells in T3P+Ply-mediated suppression of allergic airways disease and the mechanisms that underpin suppression.

This paper is prepared for submission to *The Journal of Immunology*.

4.1 ABSTRACT

The induction of Tregs to promote immune regulation has potential as a therapeutic strategy for asthma. Recently, we identified key immunoregulatory components of *S. pneumoniae*, T3P+Ply, which suppress allergic airway disease (AAD) in mouse models. In order to elucidate the mechanism of suppression, we performed a thorough examination of the role of Tregs. BALB/c mice were sensitised to OVA i.p. and challenged i.n. days 12-15 to induce AAD. T3P+Ply was administered intratracheally at the time of sensitisation, in three doses (0, 12 h, 24 h). T3P+Ply treatment induced the early (36 h-4 day) expansion of Tregs in the MLNs and later (12-16 day) increase in numbers of Tregs in the lungs compared to untreated controls. Anti-CD25 antibody depletion of Tregs showed that T3P+Ply-induced Tregs during the early, but not the late phase, were CD25-dependent and required for suppression of AAD. T3P+Ply-mediated suppression required TGF- β during the early phase and prevented the induction of IL-6, and attenuated Th2 and Th17 responses. During AAD, T3P+Ply-induced Tregs in the lungs displayed a highly suppressive phenotype, which indicated an increased functional capacity. T3P+Ply also attenuated the expression of the co-stimulatory molecule CD86 on myeloid DCs and the number of DCs carrying OVA in the lung and MLNs. Therefore, bacterial components (T3P+Ply) induce Tregs in a biphasic manner that suppresses immune responses and AAD through a broad range of mechanisms.

4.2 INTRODUCTION

Asthma is an AAD that is characterised by airway inflammation and hyperresponsiveness to non-specific stimuli. The prevalence of asthma in westernised countries has doubled over the past three decades. Improvements in hygiene and vaccination regimes together with decreased exposure to infectious agents may be responsible for the increase in prevalence.

Asthma results from a dysregulation in immunity that is underpinned by a cohort of effector T cell populations including Th1, Th2, Th17 and NKT cells. Th2 and Th17 cells are particularly important in promoting the development, progression and exacerbation of disease (237). These effector T cells produce numerous inflammatory cytokines and chemokines that induce eosinophil influx, mucus hypersecretion and AHR. Since so many factors are involved in asthma pathogenesis, therapeutic strategies that target a single effector T cell response or inflammatory signal are unlikely to be successful. This suggests the need for a more global therapeutic approach. One such approach is to restore the dysregulated immune response through the induction of Tregs that suppress a broad array of pro-inflammatory factors (71).

Tregs are essential for maintaining homeostasis and preventing aberrant immune responses. They are characterised by the expression of the transcription factor FoxP3, which is essential for their suppressive effects. The induction of FoxP3 fixes and stabilises the Treg phenotype and prevents conversion or reversion into an effector T cell (217, 238). Tregs may be natural and constitutively present, or inducible and develop from naïve T cells. Hence, the induction of Tregs may result in deviation of T cell development away from the production of effector T cells. The more commonly reported role of Tregs is in suppressing pro-inflammatory cells. Tregs utilise a number of different regulatory mechanisms including: contact-mediated suppression, soluble

factor-mediated suppression, deprivation of inflammatory cell nutritional requirements and modulation of antigen presentation (71). The roles of immune deviation versus suppression in Treg-mediated suppression are poorly understood.

Asthmatics are known to have reduced numbers of Tregs and Tregs that with reduced functional capacity (62, 65). Tregs have the capacity to regulate pro-asthmatic responses, therefore harnessing Tregs may provide an effective therapeutic strategy for asthma (71).

We have previously shown that live or killed *S. pneumoniae* suppresses hallmark features of AAD in mouse models and has the potential to be manipulated into an immunoregulatory therapy for asthma (150, 151). *S. pneumoniae* suppression of AAD is mediated by the induction of Tregs. To develop a more defined *S. pneumoniae*-based immunoregulatory therapy for AAD, we have identified the key immunoregulatory components of the bacteria that are required for suppression. Two *S. pneumoniae* components, T3P+Ply, which when co-administered before, during or after sensitisation suppressed the hallmark features of AAD including: eosinophil infiltration of the airways, lung tissue and blood; IL-5 and IL-13 release from MLN and splenic T cells; mucus hypersecretion; and AHR. It remains unknown how bacterial components induce Tregs or how these Tregs mediate the suppression of AAD. In this study we investigate the *S. pneumoniae* components (T3P+Ply)-mediated induction of Tregs and mechanisms of suppression of AAD.

4.3 METHODS

4.3.1 Animals

Six-8 week-old female BALB/c mice were obtained from the Animal Breeding Facility at The University of Newcastle. BALB/c DO11.10 TCR transgenic mice were obtained from Australian BioResources, Moss Vale, NSW, Australia. All mice were maintained under specific pathogen free and controlled environmental conditions. Procedures were approved by the animal care and ethics committee of The University of Newcastle.

4.3.2 AAD

Induction of AAD was performed as previously described (150). Mice were sensitised to OVA (i.p.; day 0; 50 µg; Sigma-Aldrich, St. Louis, MO) with Rehydrogel (1 mg; Reheis, Berkeley Heights, NJ) in sterile saline (200 µl). Mice were challenged by i.n. droplet application of OVA (day 12-15; 10 µg in 50 µl sterile saline) under isoflurane anesthesia. To determine the capacity for DCs to take up OVA, mice received fluorescein isothiocyanate (FITC)-labeled OVA challenge (Invitrogen, Carlsbad, CA). Control mice received saline sensitisation and OVA challenge.

4.3.3 Immunoregulatory therapy

Mice were administered with T3P+Ply; type 3 polysaccharide (2 µg; ATCC) and pneumolysoid (LPS-free; 40 ng; University of Adelaide, Australia) in sterile saline (30 µl; every 12 h for three doses) by i.t. administration under alfaxan i.v. anesthesia. Where indicated, mice received anti-CD25 antibody (i.p; day -3; 100 µg; clone PC61; prepared in house; in 200 µl saline) or anti-TGF-β antibody (i.p; day 0-3; 25 µg; clone 1d11; eBioscience, San Diego, CA; in 200 µl saline). Controls received isotype control antibody.

4.3.4 Cell preparation

MLNs were pushed through 70 μm sieves and single cell suspensions from Lungs and spleens were prepared using the gentle MACS (Miltenyi Biotec, Auburn, CA) apparatus according to the manufacturer's protocol (151).

4.3.5 Flow cytometry

One $\times 10^6$ cells/well in 96 well U-bottomed plates, were stained for CD4, CD25 (BD Pharmingen, San Diego, CA), CTLA-4, CD103, CD11c, CD11b, F480, CD80, CD86, MHCII (Biolegend, San Diego, CA) and PDCA (Miltenyi Biotech) using fluorescently labelled monoclonal antibodies or isotype controls. Cells were permeabilised and stained intracellularly for FoxP3 according to the manufacturers' protocol (eBioscience) (151). For detection of IL-6 and IL-17, cells were stimulated with LPS (100 ng/ml) for 20 h or PMA (0.1 $\mu\text{g}/\text{ml}$) and ionomycin (1 $\mu\text{g}/\text{ml}$) for 6 h respectively. Brefaldin A (8 $\mu\text{g}/\text{ml}$) was added during the final 2 h of each culture. Samples and controls were analysed using a FACS Canto flow cytometer controlled by FACSDiVa software (version 4.1.1; BD Biosciences, Mississauga, Canada).

4.3.6 Assessment of cellular inflammation

BAL was performed as previously described and differential leukocyte counts were determined from a total of 250 cells (151).

4.3.7 T cell cytokine release

One $\times 10^6$ cells (from MLN, lung or spleen)/well in 96 well U-bottomed plates were cultured in RPMI media supplemented with 10 % FCS, HEPES (20 mM), penicillin/streptomycin (10 $\mu\text{g}/\text{ml}$), L-glutamine (2 mM), 2-mercaptoethanol (50 μM), sodium pyruvate (1 mM). Cells were stimulated with either OVA (200 $\mu\text{g}/\text{ml}$), OVA

peptide (10 µg/ml), anti-CD3 and anti-CD28 (5 µg/ml) or LPS (100 ng/ml) and cultured for 4 (MLNs and lungs) or 6 (spleen) days (5 % CO₂, 37 °C). Supernatants were collected and stored at -20 °C until required. Cytokine concentrations in cell culture supernatants were determined by ELISA (BD Pharmingen, San Diego, CA) (151).

4.3.8 Airways hyperresponsiveness

Airways hyperresponsiveness was assessed as previously described (151). Briefly, anaesthetised, tracheotomised mice were cannulated and connected to an inline aerosol and ventilator. Changes in airway function following challenge with increasing doses of aerosolised methacholine (1.25, 2.5, 5 and 10 mg/ml) were assessed by analysis of pressure and flow waveforms and airways resistance and dynamic compliance were determined.

4.3.9 Real-time PCR

For analysis of gene expression, total RNA was prepared from whole lung or MLNs by TRIzol extraction or from cell culture using a Purelink kit (Invitrogen) and cDNA was generated. Real-time RT-PCR was performed as previously described (239). Primer sequences: IL-2 F:CCTGAGCAGGATGGAGAATTACA, R:TCCAGAACATGCCG CAGAG. TGF-β F:CCCGAAGCGGACTACTATGCTAAA, R:GGTAACGCCAGGA ATTGTTGCTAT. CCR7 F:TGGTGGCTCTCCTTGTCATT, R:GTGGTATTCT CGC CGATGTAGT. IL-6 F:AGAAAACAATCTGAAACTTCCAGAGAT, R:GAAGACCA GAGGAAATTTTCAATAGG. IL-25 F:CTGAAGTGGAGCTCTGCATCTGTG, R:GT CCATGTGGGAGCCTGTCTGTAG. FoxP3 F:GGCCCTTCTCCAGGACAGA, R:GC TGATCATGGCTGGGTTGT. IL-10 F:CATTTGAATTCCTGGGTGAGAAG, R:GC CTTGTAGACACCTTGGT CTTGG. Ebi3 F:CCGCTCCCCTGGTTACTG, R:GTG

GCAATGAAGGACGTGGA. p35 F:GCCAGGTGTCTTAGCCAGTCC, R:GCCAGG
CAACTCTCGTTCTTG. IL-9 F:CATCCTTGCCTCTGTTTTGCTCTT, R:TCTTGATT
TCTGTGTGGCATTGGT. TLR2 F:TGTAGGGGCTTCACTTCTCTGCTT, R:AGACT
CCTGAGCAGAACAGCGTTT.

4.3.10 *In vitro* culture

One x 10⁶ DO11.10 splenocytes/well in 96 well U-bottomed plates were cultured in supplemented RPMI media (4 days, 5 % CO₂, 37 °C). OVA peptide (10 µg/ml; Invitrogen), T3P+Ply (6 µg and 120 ng, respectively) were added as indicated.

4.3.11 Data analysis

Data were analysed using GraphPad Prism (GraphPad Software, CA) and are represented as mean ± SEM. One-way ANOVA with Dunnett's post test was used to determine significance between data with multiple comparisons. Unpaired Student's t test was used to determine differences between two groups. One-way repeated measures ANOVA and Bonferroni's post test was used to determine significance for AHR data. p<0.05 was considered statistically significant.

4.4 RESULTS

4.4.1 T3P+Ply induces an early expansion of Tregs in the MLNs and a late increase in Tregs in the lung

A well established model of AAD was employed, where mice were sensitised to OVA i.p. and challenged i.n. days 12-15 later to induce AAD. T3P+Ply was administered i.t. at the time of sensitisation, in three doses (0, 12 h, 24 h; Figure 4.1A).

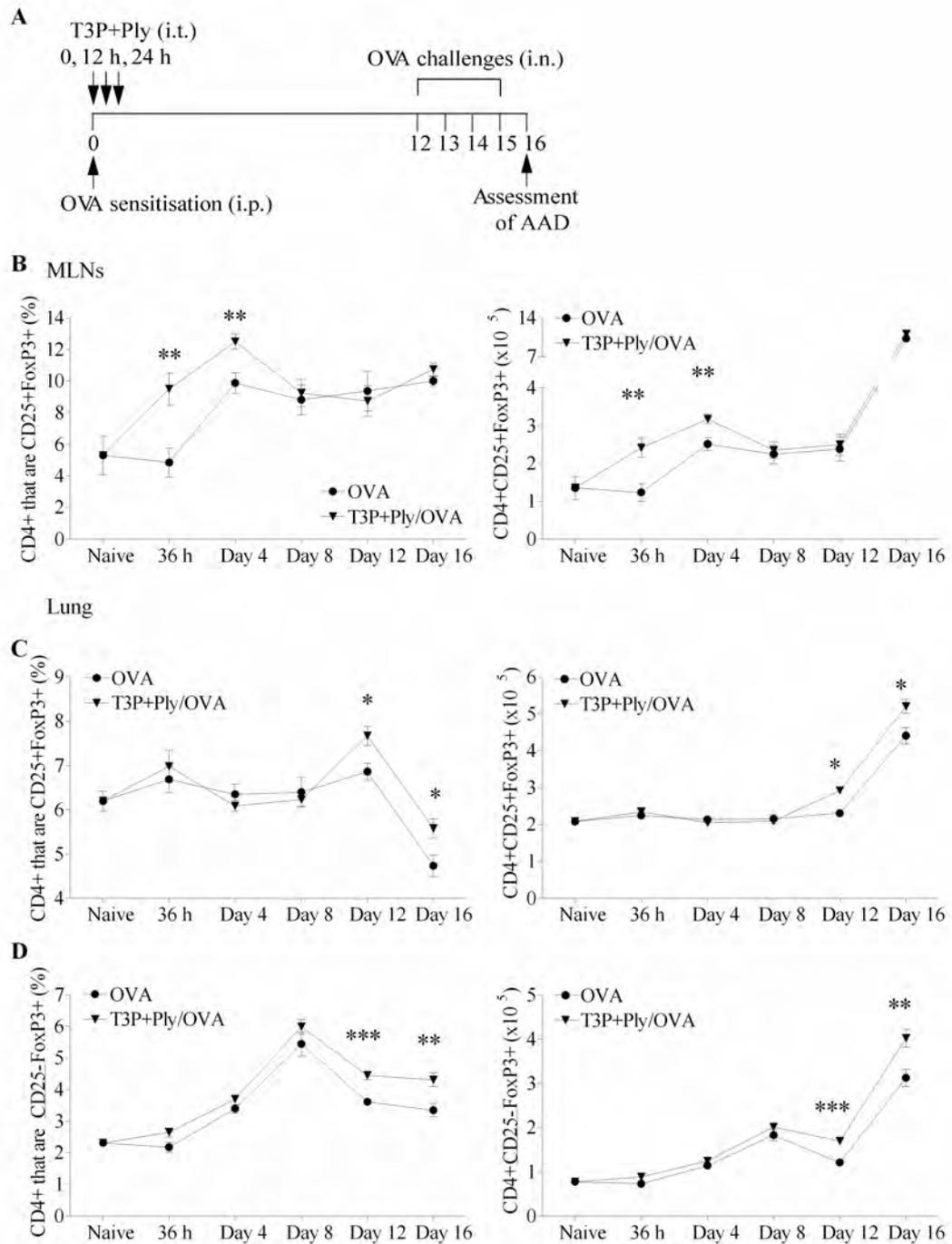


FIGURE 4.1. Time course analysis of T3P+Ply-induced Tregs in the MLNs and lung. (A) AAD was induced by i.p. OVA sensitisation and i.n. OVA challenge. Controls received saline sensitisation and OVA challenge. T3P+Ply treatment was administered at the time of OVA sensitisation, in 3 doses every 12 h. Features of AAD were assessed 24 h after the final OVA challenge. The percentage of CD4+ cells that were CD25+FoxP3+ and total number of CD4+CD25+FoxP3+ cells in the (B) MLNs

and (C) the lung. (D) The percentage of CD4+ cells that were CD25-FoxP3+ and total number of CD4+CD25-FoxP3+ cells in the lung. Data represent mean \pm SEM from 6-8 mice. Significant differences are shown as * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

To determine the capacity of T3P+Ply to induce Tregs we performed a time course analysis (0 h, 36 h, days 4, 8, 12 and 16) of Tregs in the MLNs and lung by flow cytometry.

In the MLNs, T3P+Ply treatment (T3P+Ply/OVA) increased the percentage of CD4⁺ cells that were CD25⁺FoxP3⁺ and the total number of CD4⁺CD25⁺FoxP3⁺ cells between 36 h and 4 days compared to the untreated (OVA) group (Figure 4.1B). T3P+Ply treatment did not alter the percentage or number of Tregs in the MLNs at other time points assessed. T3P+Ply treatment did not alter CD25⁻FoxP3⁺ cells in the MLNs (not shown).

In the lung, T3P+Ply treatment increased the percentage of CD4⁺ cells that were CD25⁺FoxP3⁺ and the total number of CD4⁺CD25⁺FoxP3⁺ cells between 12 and 16 days when compared to the untreated group (Figure 4.1C). T3P+Ply treatment also increased the percentage of CD4⁺ cells that were CD25⁻FoxP3⁺ and the total number of CD4⁺CD25⁻FoxP3⁺ cells between 12 and 16 days compared to the untreated group (Figure 4.1D). T3P+Ply treatment did not alter the percentage or number of Tregs at other time points assessed.

These results demonstrate that T3P+Ply enhances the early expansion of Tregs in the MLNs and a late increase in Tregs in the lung.

4.4.2 Anti-CD25 antibody depletes the number of CD4⁺ cells that are CD25⁺FoxP3⁺ in the MLNs and lungs

To investigate the role of early and late T3P+Ply-induced Tregs in mediating the suppression of AAD we first established a protocol to deplete Tregs using anti-CD25 (PC61) antibody. Anti-CD25 antibody was administered either 3 days prior to T3P+Ply treatment (day -3) or 3 days prior to OVA challenge (day +9). Tregs were assessed in

MLNs and lungs on days 4 and 16. In the MLNs, administration of anti-CD25 antibody on day -3 depleted the percentage of CD4⁺ cells that were CD25⁺FoxP3⁺ in the MLNs on day 4 (Figure 4.2A). Administration on either day -3 or +9 depleted the percentage of CD4⁺ cells that were CD25⁺FoxP3⁺ on day 16.

In the lung, administration on day -3 depleted the percentage of CD4⁺ cells that were CD25⁺FoxP3⁺ on day 4 (Figure 4.2B). Administration on either day -3 or +9 depleted the percentage of CD4⁺ cells that were CD25⁺FoxP3⁺ on day 16. These data show that administration depleted CD4⁺ cells that were CD25⁺FoxP3⁺ for at least 19 days.

Notably, CD4⁺ cells that were CD25⁺FoxP3⁺ were almost completely depleted on day 4 in both the MLNs and lung but began to replenish on day 16 (Figure 4.2A and B). Depletion of CD4⁺ cells that were CD25⁺FoxP3⁺ on day 16 in the MLNs was to similar levels as the saline-sensitised control group. Depletion of CD4⁺ cells that were CD25⁺FoxP3⁺ on day 16 in the lungs was to levels below the saline control group. Further assessment of Treg populations in the lung showed that administration of anti-CD25 antibody on day -3 had no effect on the percentage of CD4⁺ cells that were CD25⁻FoxP3⁺ in the lung on day 4 but suppressed these cells on day 16 (Figure 4.2C). Administration on day +9 had no effect on either day.

4.4.3 T3P+Ply induction of Tregs in the early phase induction is CD25-dependent and required for suppression of Th2 responses and AAD

We then employed these protocols to investigate whether T3P+Ply-induced Tregs were required for suppression of Th2 responses and AAD. The development of AAD was characterised by an increase in the number of eosinophils in the BAL, OVA-induced IL-5, IL-13 and IFN- γ release from MLN T cells, and AHR (isotype OVA groups,

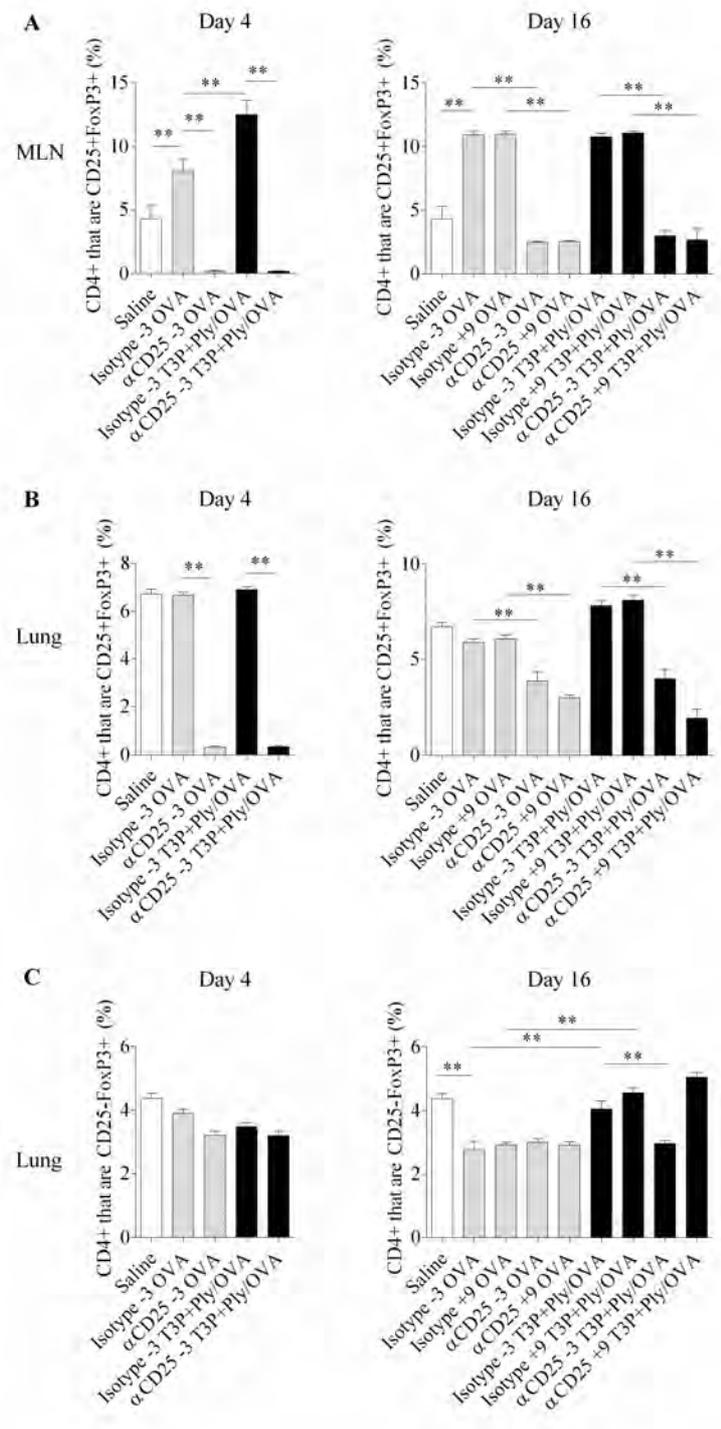


FIGURE 4.2. The effects of anti-CD25 antibody (α CD25) administration on Treg populations in the MLNs and lung when administered day -3 (-3) or day 9 (+9). The percentage of CD4+ cells that were CD25+FoxP3+ and total number of CD4+CD25+FoxP3+ cells in the (A) MLNs and (B) lungs on day 4 and 16. (C) The percentage of CD4+ cells that were CD25-FoxP3+ and total number of CD4+CD25-

FoxP3+ cells in the lung on Day 4 and 16. Data represent mean \pm SEM from 6-8 mice.

Significant differences are shown as ** $p < 0.01$.

Figure 4.3A-E). As expected, T3P+Ply treatment suppressed the development of eosinophils in the BAL; OVA-induced IL-5 and IL-13 release from MLN T cells; and AHR (isotype T3P+Ply/OVA groups). Notably, T3P+Ply and anti-CD25 had no effect on OVA-induced IFN- γ release from MLN T cells (Figure 4.3D). Administration of anti-CD25 on day -3 reversed the effect of T3P+Ply treatment on the development of eosinophils in the BAL, OVA-induced IL-5 and IL-13 release from MLN T cells, and AHR (α CD25 -3 T3P+Ply/OVA). However, anti-CD25 treatment on day +9 had no effect (α CD25 +9 T3P+Ply/OVA). These data suggest that early but not late T3P+Ply-induced Tregs are CD25-dependent and required for the suppression of Th2 responses and AAD.

4.4.4 T3P+Ply induced IL-2/IL-2R interactions may contribute to the suppression of AAD

Since the early population of CD25⁺ Tregs was required for T3P+Ply-mediated suppression of AAD, we examined the expression of factors involved in the induction of Tregs. Given that CD25 is the IL-2 alpha receptor, we assessed the role of IL-2 interactions in T3P+Ply-mediated suppression of AAD, by quantifying IL-2 gene expression in the lung. T3P+Ply treatment increased IL-2 gene expression on day 4, compared to the untreated group (Figure 4.4A). Therefore, IL-2 is likely to be important for the induction of Tregs since IL-2 expression was increased and blocking IL-2/IL-2R interactions with anti-CD25 depleted Tregs and reversed the effects of T3P+Ply.

4.4.5 T3P+Ply-induced TGF- β mediates the attenuation of Th2 responses and contributes to the suppression of AAD.

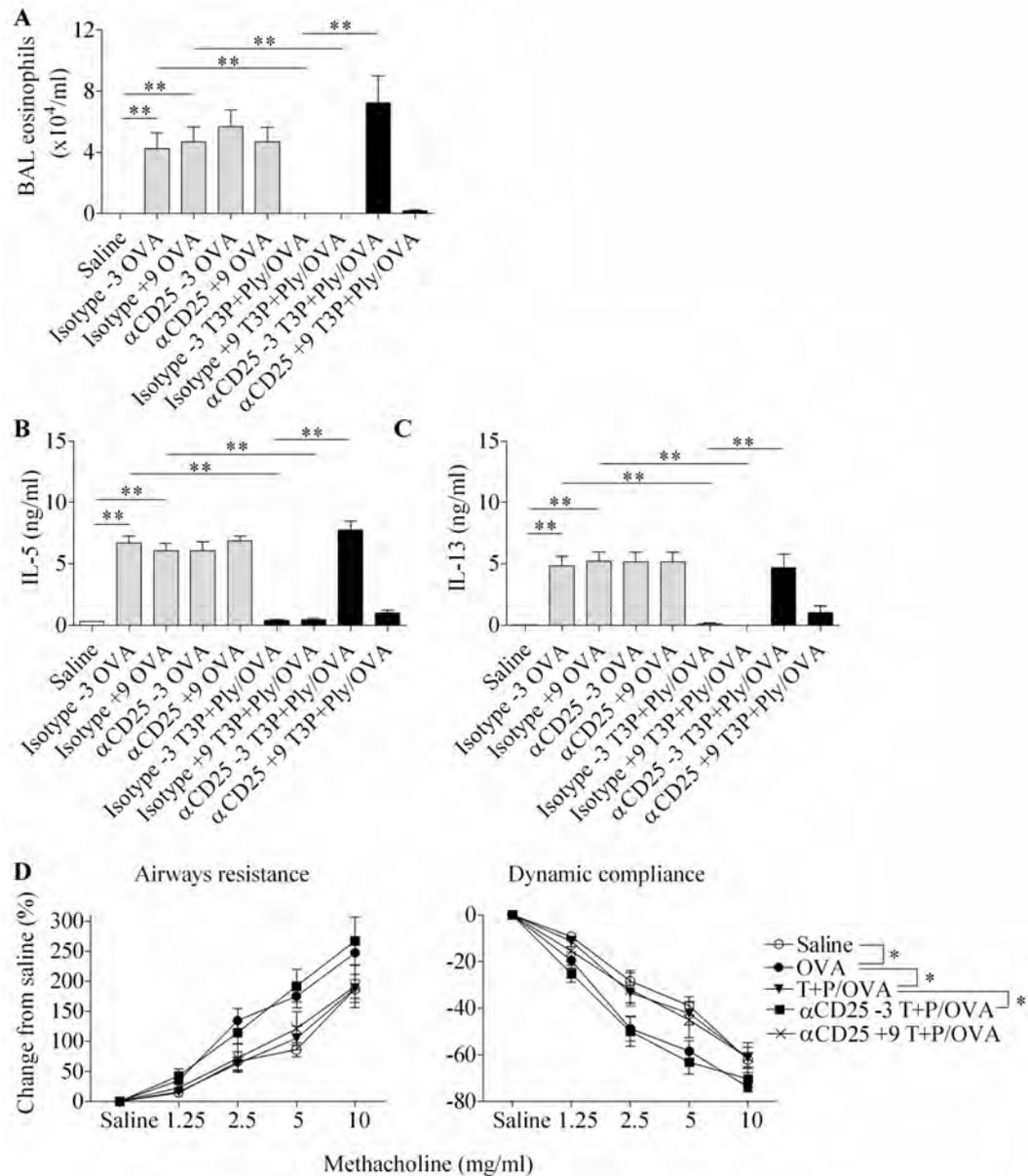


FIGURE 4.3. The effects of anti-CD25 antibody administration on T3P+Ply-mediated suppression of AAD. (A) The number of eosinophils in BAL, OVA-induced (B) IL-5 and (C) IL-13 release from MLN T cells and (D) AHR in terms of airways resistance and dynamic compliance. Data represent mean \pm SEM from 6-8 mice. Significant differences are shown as * $p < 0.05$ and ** $p < 0.01$. Significance for resistance and compliance are given for the entire dose response curve.

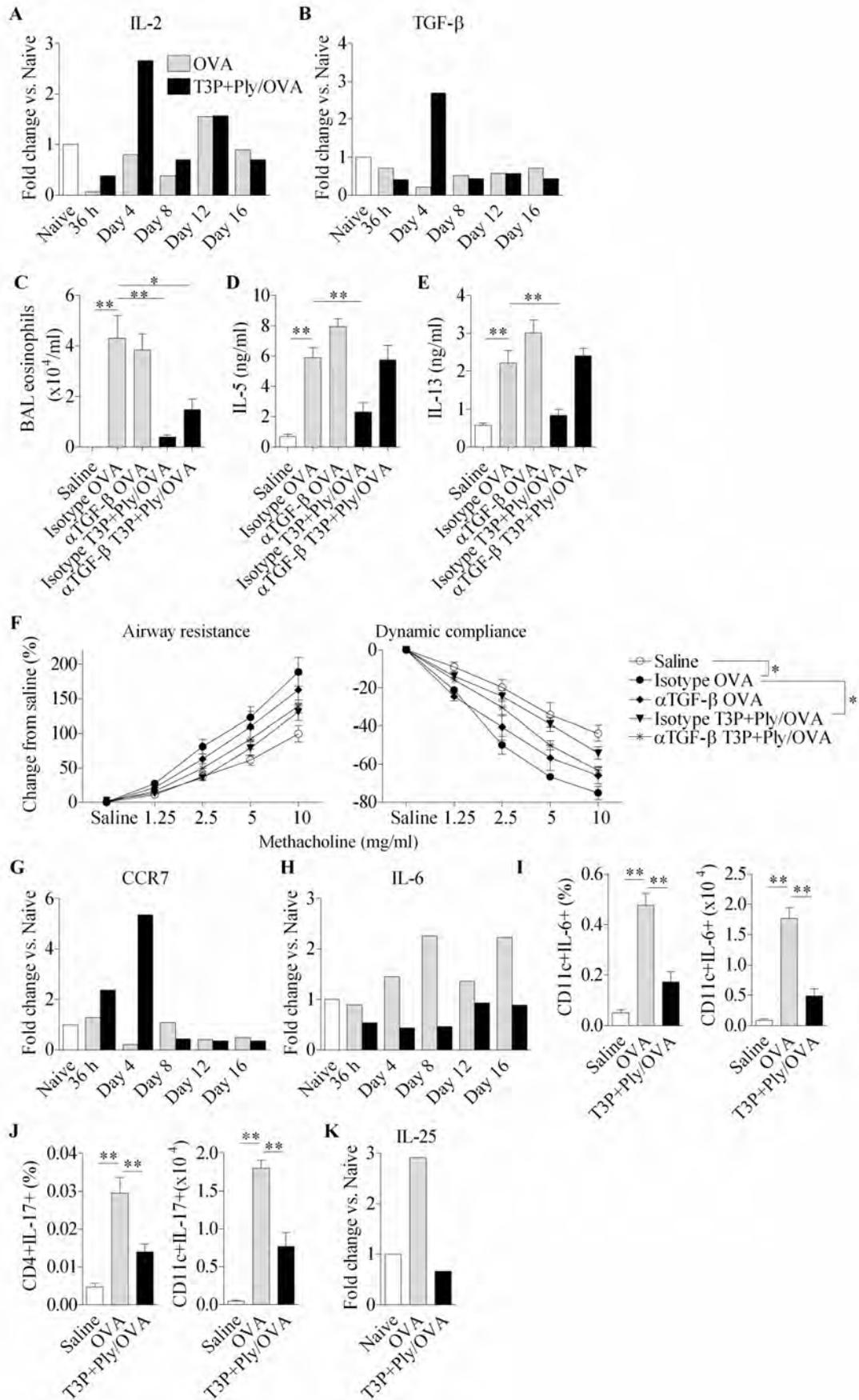


FIGURE 4.4. The effect of T3P+Ply on Treg-inducing factors and Th17 cells. (A) IL-2 (B) TGF- β mRNA expression in the lung. The effect of anti-TGF- β antibody (α TGF- β) administration on T3P+Ply-mediated suppression of (C) the number of eosinophils in BAL, OVA-induced (D) IL-5 and (E) IL-13 and (F) AHR. The effect of T3P+Ply on (G) CCR7 mRNA expression in the lung. The effect of T3P+Ply on (H) IL-6 mRNA expression, the percentage and total number of (I) CD11c+IL-6+ cells and (J) CD4+IL-17+ cells, and (K) IL-25 mRNA expression in the lung in AAD. For mRNA expression data are representative of 6 pooled RNA samples and data were confirmed in a repeat experiment. Where error bars are provided data represent mean \pm SEM from 6-8 mice. Significant differences are shown as * $p < 0.05$ and ** $p < 0.01$. Significance for resistance and compliance are given for the entire dose response curve.

TGF- β promotes the development of Tregs, is produced by Tregs and suppresses pro-inflammatory responses. Therefore, we assessed the role of TGF- β in T3P+Ply-mediated suppression of AAD by quantifying TGF- β gene expression in the lung. T3P+Ply treatment increased TGF- β expression on day 4 but not day 16, compared to the untreated group (Figure 4.4B). We further investigated the role of TGF- β using anti-TGF- β neutralising antibody. Administration of anti-TGF- β neutralising antibody on days 0-3 had no significant effect on T3P+Ply-mediated suppression of eosinophils in the BAL (Figure 4.4C). However, anti-TGF- β reversed the effect of T3P+Ply treatment on OVA-induced IL-5 and IL-13 release from MLN T cells (Figure 4.4D and E). In addition, anti-TGF- β partially reversed the effect of T3P+Ply treatment on AHR (Figure 4.4F). Therefore, TGF- β is required for T3P+Ply to achieve complete suppression of AAD.

Since the T3P+Ply-induced IL-2 and TGF- β in the lung correlated with the expansion of Tregs in the MLNs, we assessed CCR7 gene expression in the lung on day 4. T3P+Ply treatment increased CCR7 expression in the lung on day 4 compared to the untreated group (Figure 4.4G)

4.4.6 T3P+Ply suppresses the establishment of Th17 effector cell responses

Given that, in the presence of TGF- β , Tregs and Th17 cells may derive from a common progenitor in the absence or presence of IL-6 respectively, we investigated the effects of T3P+Ply on Th17 differentiation. T3P+Ply treatment inhibited IL-6 gene expression throughout the time course compared to untreated controls (Fig 4.4H). This correlated with a reduction in the percentage and total number of CD11c+IL-6+ cells in the lung in AAD compared to untreated controls (Figure 4.4I). T3P+Ply-induced increases in TGF-

β expression, decreases in IL-6 expression and CD11c+IL-6+ cells correlated with a reduction in the percentage and total number of CD4+IL-17+ cells and IL-25 expression in the lung in AAD compared to untreated controls (Figure 4.4J and K). These results suggest that T3P+Ply-induced Tregs suppress the induction and function of Th17 cells.

4.4.7 T3P+Ply suppresses the establishment of Th2 and Th17 effector cell responses *in vitro*

To confirm our *in vivo* observations, we used an *in vitro* system that utilised splenocytes from DO11.10 TCR transgenic mice. These mice have T cells that react specifically to the OVA 323-339 peptide (OVAp). DO11.10 splenocytes were cultured with or without T3P+Ply and OVAp and the effects on cytokine release and gene expression were assessed.

The presence of T3P+Ply suppressed OVAp-induced IL-5, IL-13, IL-4 and IL-17 release from splenocytes but had no effect on IL-10, compared to controls (Figure 4.5A-E). T3P+Ply induced TGF- β release irrespective of whether OVAp was present (Figure 4.5F). T3P+Ply treatment maintained the expression of FoxP3 (Figure 4.5G). Therefore, there is a correlation between the suppression of Th2 and Th17 responses and enhanced or maintained TGF- β and FoxP3 expression.

4.4.8 T3P+Ply-induced Tregs in the lungs have a highly suppressive phenotype

Anti-CD25 experiments showed that CD25+ Tregs in the later phase were not required for suppression of AAD. Since CD25- Tregs remained at high levels they may play a role in suppression of AAD. Therefore we assessed the effects of T3P+Ply on the suppressive phenotype of Tregs, during AAD, using markers associated with functional capacity. T3P+Ply treatment enhanced the percentage of CD4+CD25+/-FoxP3+ cells

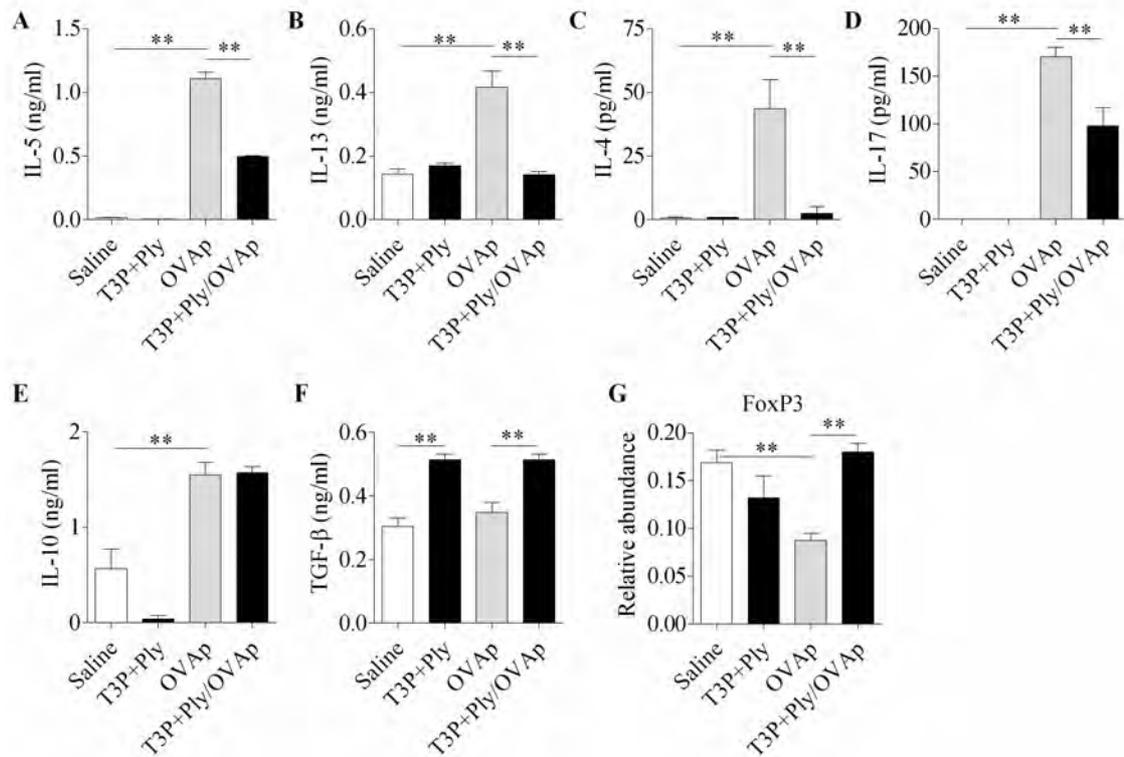


FIGURE 4.5. The effects of T3P+Ply treatment *in vitro*. OVA peptide (OVA)-induced (A) IL-5, (B) IL-13, (C) IL-4, (D) IL-17, (E) IL-10 and (F) TGF- β release, and (G) FoxP3 mRNA expression in OVA-stimulated OVA-TCR transgenic splenocytes *in vitro*. Data represent mean \pm SEM of triplicate wells. Significant differences are shown as ** $p < 0.01$.

that expressed CTLA-4 in the lung compared to the untreated group (Figure 4.6A). T3P+Ply also maintained the percentage of CD4⁺CD25^{+/-}FoxP3⁺CD103⁺ (Figure 4.6B). T3P+Ply treatment did not alter the expression of CD62L, GITR, or PD-1 on Tregs in the lung (not shown).

We then assessed the effects of T3P+Ply on the gene and protein levels of the immunosuppressive cytokines IL-10 and TGF- β in the lungs during AAD. T3P+Ply treatment suppressed both IL-10 gene expression in the lung and OVA-induced IL-10 release from lung T cells (Figure 4.6C). T3P+Ply treatment did not alter TGF- β gene expression or OVA-induced TGF- β release from lung T cells (Figure 4.4B and 4.6D).

Next, we assessed a panel of other markers associated with Treg suppressive capacity. T3P+Ply sustained Ebi3 gene expression and increased p35 gene expression, which together form the heterodimeric cytokine IL-35 (Figure 4.6E). T3P+Ply treatment was associated with increased IL-9 gene expression and a small increased in TLR2 expression (Figure 4.6F and G) compared to untreated controls.

4.4.9 T3P+Ply suppressed CD86 expression on mDCs and the number of DCs carrying OVA

Antigen presenting cells are essential mediators of immune responses. Therefore we investigated their role in T3P+Ply-mediated suppression of AAD. There was a decrease in CD86 expression on mDCs in the lung during AAD, compared to untreated controls (Figure 4.7A). This was the only statistical result from a time course analysis of pDCs (PDCA⁺CD11c⁺CD11b⁻), mDCs (CD11c⁺CD11b⁺) and macrophages (F480⁺CD11c^{+/-}) and their expression of MHCII, CD80 and CD86 performed on MLNs and lungs using flow cytometry (not shown). This suggests that many cell

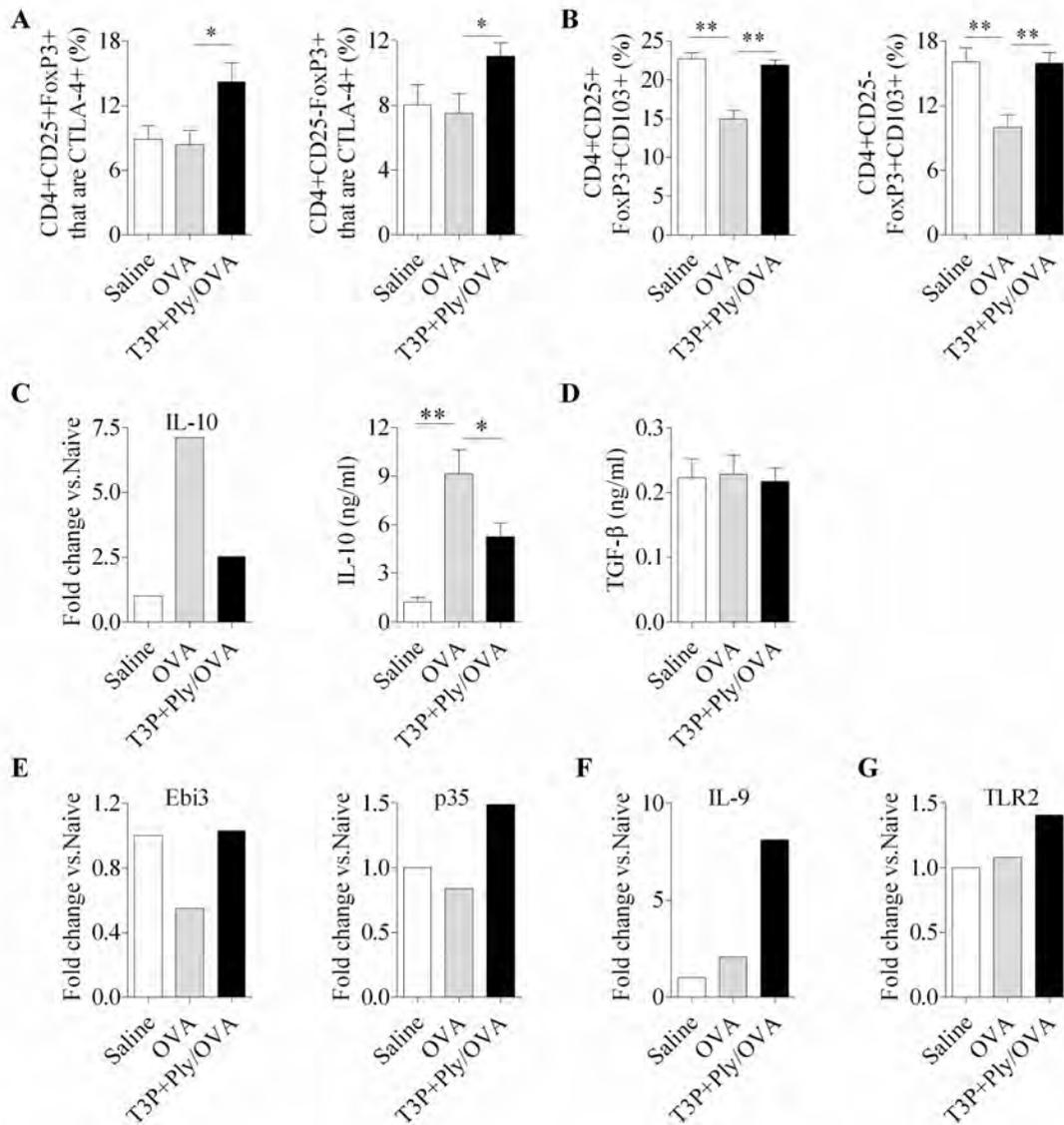


FIGURE 4.6. The effects of T3P+Ply treatment on Treg functional markers. The percentage of (A) CD4+CD25+FoxP3+ and CD4+CD25-FoxP3+ cells that were CTLA-4+. The percentage of (B) CD4+CD25+FoxP3+CD103+ and CD4+CD25-FoxP3+CD103+ cells. (C) IL-10 mRNA expression in the lung and OVA-induced IL-10 release from lung T cells. (D) OVA-induced TGF-β release from lung T cells. (E) Ebi3 and p35, (F) IL-9 and (G) TLR2 mRNA expression in the lung. Where error bars are provided data represent mean ± SEM from 6-8 mice. For mRNA expression data is representative of 6 pooled RNA samples and data was confirmed in a repeat experiment. Significant differences are shown as * p<0.05 and ** p<0.01.

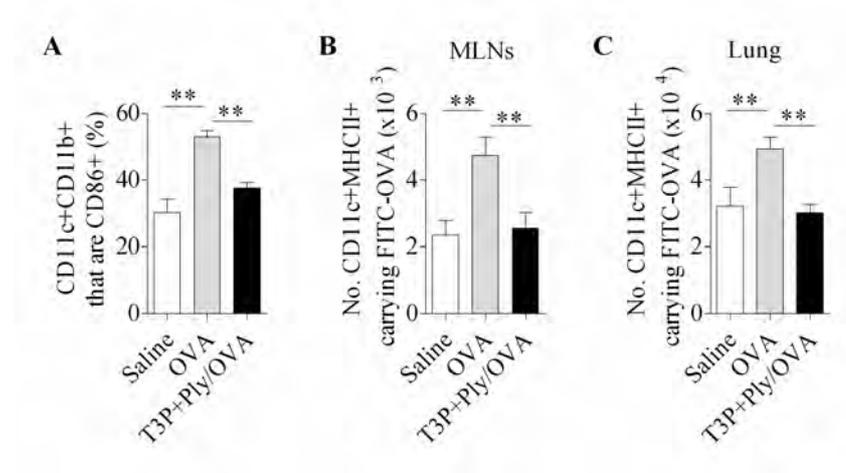


FIGURE 4.7. The effects of T3P+Ply treatment DCs. (A) the percentage of CD11c+CD11b+ cells that are CD86+ in the lung. The number of CD11c+MHCII+ DCs carrying FITC-labelled OVA in the (B) MLNs and (C) lung. Data represent mean \pm SEM of triplicate wells. Significant differences are shown as ** $p < 0.01$.

populations are unaffected by T3P+Ply treatment and that CD86 expression on mDCs may play a role in T3P+Ply-mediated suppression of AAD.

Next we assessed the capacity of DCs to carry OVA by administering FITC labelled-OVA during the challenge phase. T3P+Ply reduced the number of CD11c+MHCII+ cells carrying OVA in the MLNs and lungs compared to untreated controls (Figure 4.7B and C). The number of CD11c+MHCII+ cells carrying OVA in the MLNs was reduced by T3P+Ply treatment. This suggests that T3P+Ply reduces the capacity of DCs to carry OVA and promote AAD.

4.5 DISCUSSION

Here we show that the bacterial components T3P+Ply induced Tregs in a biphasic and CD25-dependent manner to suppress pro-inflammatory Th2 responses and AAD. The suppressive effects were mediated by increases in IL-2/IL-2R interactions and TGF- β , and Th17 responses were also suppressed. The induced Tregs had a highly suppressive phenotype and were associated with the suppression of DC function.

T3P+Ply treatment induced an early phase expansion of Tregs in the MLNs by 36 h, which continued to day 4. At least *in vitro*, under Treg inducing conditions naïve T cells take up to 5 days to differentiate into a functional FoxP3+ phenotype (240). Therefore, we hypothesise that there may be two different populations of Tregs in the MLNs during the early phase. The T3P+Ply-induced population detected after 36 h may be an innate-like population that has expanded in the MLNs. The T3P+Ply-induced population detected after 4 days may be a more mature and functional Treg phenotype, due to associated increases in IL-2, TGF- β . The lymph node homing

receptor CCR7 expression was also increased on day 4, which may also explain the increased number of Tregs in the MLNs.

Tregs expanded in response to OVA and may therefore be OVA-specific. However, in other studies we have shown that *S. pneumoniae*-induced Tregs also suppress anti-CD3/CD28 stimulated Th2 cell responses. Therefore, T3P+Ply treatment may prime Tregs to respond non-specifically to antigen challenge. Notably, although the changes in percentage and number of Tregs are small, Tregs potentially immunosuppressive and even small increases can have substantial effects.

Anti-CD25 depletion of Tregs may also deplete CD25⁺ effector T cells, since CD25⁺ is also a marker of T cell activation. However, anti-CD25 administration had no effect on the untreated allergic (α CD25 OVA) group and completely restored AAD indicating that effector T cells were not affected. This method of Treg depletion prevents the interaction of IL-2 with its receptor and inhibits the CD25 signalling pathway. This prevents the induction of FoxP3 and results in cell death.

Anti-CD25 administration on day -3 depleted T3P+Ply-induced CD25⁺ Tregs, which was required for suppression of AAD. Administration on day +9 also depleted T3P+Ply-induced CD25⁺ Tregs without affecting AAD. This may be because T3P+Ply-induced Tregs have matured and acquired a stable or fixed state, prior to administration at day +9. Indeed FoxP3 is known to fix and stabilise the Treg phenotype (217). We speculate that Tregs in the later stage are not dependent on CD25 and suppress AAD at the site of inflammation. Administration on day +9 did not affect T3P+Ply-induced CD25⁻ Tregs and this population may mediate the suppression of AAD in the absence of CD25⁺ Tregs during antigen challenge. This concept is supported by our data that shows increases in markers of a suppressive phenotype on CD25⁻ Tregs.

CD25 is the alpha chain of the IL-2 receptor and IL-2 provides important signals to Tregs for their development (103). Hence, the increase of IL-2 in the lungs on day 4 is likely to promote the early increase of Tregs in the MLNs. IL-2 is essential for the induction of TGF- β and expansion of CD4+CD25+FoxP3+ cells (241). In our study, IL-2:CD25 interactions and the induction of TGF- β were required for T3P+Ply-mediated suppression of AAD. Notably, TGF- β was not induced in the lungs by T3P+Ply during AAD. Therefore, it is likely that the induction of TGF- β in the lung on day 4 directs the expansion of Tregs in the MLNs. Therefore, TGF- β is required to initiate the response but not to sustain it.

The increase of Treg inducing factors IL-2 and TGF- β in the lungs was associated with increased lymph node homing receptor CCR7 gene expression. This receptor is involved in the homing of Treg to the lymph nodes *via* high endothelial venules (242). This suggests that Tregs are induced in the lungs and migrate *via* CCR7 to the MLNs where they are detected at higher numbers. Whether Tregs migrate to the lung on day 12 and 16, or a new population is formed, is unknown and further investigation is required.

Our results show that T3P+Ply treatment blocks increases in IL-6. This may promote the induction of Tregs and prevent the development of Th17 cells. Other studies have shown that blocking membrane bound IL-6R, with anti-IL-6R antibody induces Tregs and attenuates AAD (218). Since the Th17 cell cytokines IL-25 and IL-17 contribute to the inflammatory milieu in AAD, T3P+Ply may be effective in suppressing Th17 mediated pathogenesis in asthma.

Our *in vivo* data were supported by *in vitro* studies, which confirmed that T3P+Ply-induced Tregs suppressed Th2 and Th17 effector cell responses. FoxP3 has been shown to control NFAT and cause a deviation of differentiating effector T cells

into Tregs (243). This study supports our data, which show that FoxP3 expression inversely correlates with the development of effector T cells.

The second phase of induction of Tregs in the lungs, produced increases in CTLA-4 and CD103 expressing Tregs. These Tregs had a greater suppressive phenotype and potentially enhanced functional capacity. CTLA-4 is involved in the maintenance of T cell homeostasis and is required for the development and activity of Tregs (244). Treg-specific deficiency in CTLA-4 impairs the suppressive function of Tregs and Treg mediated down-regulation of CD80 and CD86 expression on DCs (100). Therefore, Tregs require CTLA-4 to suppress antigen presenting cell activation of T cells. CTLA4 expression is also a regulator of steady-state CD4⁺ FoxP3⁺ T cell homeostasis (245). CD103 is another marker associated with the functional capacity of Tregs and identifies a highly potent and functionally distinct population of Tregs, which are specialised for crosstalk with epithelial environments (246). Other studies have shown that CD103⁺ expression plays an important role in epithelial retention of Tregs through recognition of epithelial cadherin during infection (247). Furthermore, CD103⁺ Tregs (CD25^{+/-}) that express CTLA-4 suppress T cell proliferation in vitro and are protective in a mouse model of colitis (246). Hence, the T3P+Ply-mediated increase in Tregs expressing CTLA-4 and CD103 may promote increased functional capacity and immunosuppression in the Treg population.

IL-10 and TGF- β are immunosuppressive cytokines that may be released by Tregs to mediate suppression, which is dependent on the immunological context (71). IL-10 is a pleiotropic cytokine and is also released by Th2 cells in AAD. In our study, T3P+Ply suppressed IL-10 expression in the lung during AAD and TGF- β expression remained constant. This excluded their involvement in Treg-mediated suppression of AAD. Numerous studies have shown that Treg-mediated suppression of AAD does not

require IL-10 or TGF- β (68, 106, 211, 212). IL-35 is another, recently discovered, soluble factor and mediates close-contact suppression of effector cell responses by Tregs (93). It is a heterodimeric cytokine comprising or Epstein-Barr virus-induced gene 3 (Ebi3) and p35 (IL-12a), which is highly expressed by FoxP3⁺ but not resting CD4⁺ cells. Ebi3^{-/-} and IL-12a^{-/-} mice have Tregs with reduced suppressive capacity, which confirms the importance of IL-35 in Treg-mediated suppression (93). Our results showed that T3P+Ply treatment sustained Ebi3 and increased p35 gene expression, in whole lung tissue, suggesting that IL-35 may be involved in mediating suppression of AAD. IL-9 is also a pleiotropic cytokine most recognised for its role in Th2 mediated disease (248). However, IL-9 has also been reported to enhance the function of FoxP3⁺ regulatory T cells (81). In our study T3P+Ply also increased IL-9 expression in whole lung tissue, which may be involved in enhancing the function of Tregs since expression was associated with increases in other markers of functional capacity and suppression of effector T cell responses. The understanding of the relationship between Tregs and IL-9 is in its infancy and requires further investigation. TLR2 expression on Tregs has also been implicated in controlling the expansion and function of Tregs and TLR2 engagement promotes their survival (249, 250). It is possible that the T3P+Ply-induced increase in TLR2 expression, in whole lung tissue, may contribute to Treg function and stability. Together, these increases in markers associated with Treg suppressive phenotype and functional capacity may be important for the suppression of effector cell responses in the lung during AAD. The localisation of these Tregs in the lung during the late phase and the lack of immunosuppressive cytokines IL-10 and TGF- β , suggests that suppression occurs through contact/close contact-mediated mechanisms. Increases in numerous different Treg suppressive markers indicate that many independent mechanisms of suppression may be combining to attenuate AAD.

Pulmonary CD11c+CD11b+ mDCs play an important role in promoting Th2 responses during AAD (251). mDC activity is enhanced by expression of co-stimulatory (CD80, CD86) and activation (MHCII) molecules. In our study, T3P+Ply attenuated co-stimulatory molecule CD86 on mDCs. Therefore it is likely that T3P+Ply reduces the capacity of DCs to prime T cells, which would suppress AAD. It is possible that the T3P+Ply-mediated reduction in CD86 expression on mDCs is mediated by Tregs expressing CTLA-4. T3P+Ply-induced Tregs and suppression of AAD were also associated with reduction in the number of DCs containing OVA in the MLNs and lung. Therapeutic inhibition of DC function in AAD has been reported previously and proposed as a potential treatment for asthma (252). Alterations in both CD86 and the capacity for DCs to carry OVA, provides another indication that different mechanisms of suppression combine to attenuate AAD.

In summary, we have shown that a novel immunoregulatory therapy based on *S. pneumoniae* components, T3P+Ply, induces Tregs that suppress AAD. By taking a holistic approach to the analysis of T3P+Ply-induced Tregs we determined that the role of Tregs is biphasic, that suppression is mediated by numerous mechanisms and have enhanced the understanding of how Tregs are induced *in vivo*. This is the first study to demonstrate a dual role for Treg-mediated suppression of AAD. Our model continues to provide a valuable tool for examining the development of Tregs, their mechanisms of suppression and their therapeutic potential in AAD. Tregs may be a novel therapeutic strategy for the effective prevention and/or treatment of asthma.

CHAPTER 5

DISCUSSION

This chapter discusses the broader potential for a *S. pneumoniae*-based immunoregulatory therapy as a novel treatment for allergic airways disease, the implications of these studies and possible future directions.

5.1 SIGNIFICANT OUTCOMES

The clinical association between exposure to bacteria such as *S. pneumoniae* and the development of asthma remains poorly understood. We have used mouse models to investigate this association and assessed the affect of *S. pneumoniae* on AAD. Early studies showed that *S. pneumoniae* suppressed the development of hallmark features of AAD (146, 147). Here, we further these observations, and identified a currently available *S. pneumoniae* vaccine and the key *S. pneumoniae* components that suppress both the development of AAD and established AAD. Therefore, *S. pneumoniae*-based immunoregulatory therapies have potential as novel and effective strategies for prevention and/or treatment of asthma in humans. Suppression of AAD was dependent on the induction of Tregs, which had an increased functional capacity and suppressed the function of Th1, Th2, Th17 and NKT cells. *S. pneumoniae* immunoregulatory therapy-induced Tregs played a dual role in suppression of AAD. The induction of Tregs deviated the establishment of effector cell responses involved in AAD. These Tregs expressed markers that indicated they had a heightened suppressive phenotype and increased functional capacity. These data substantially enhance our understanding of the relationship between *S. pneumoniae* and asthma and the potential use of a *S. pneumoniae*-based immunoregulatory therapy for asthma.

5.2 *S. PNEUMONIAE* VACCINES AND THE SUPPRESSION OF AAD

We have demonstrated (Chapter 2) that targeted administration of the *S. pneumoniae* conjugate vaccine may provide a novel and effective immunoregulatory therapy for asthma.

5.2.1 Conjugate vaccine-mediated suppression of AAD

Interestingly, the conjugate vaccine, but not the polysaccharide vaccine suppressed AAD. Serotypes in the conjugate vaccine were also present in the polysaccharide vaccine, which suggested serotype differences could not explain the differential effects (section 1.6.4). The conjugate vaccine contained less polysaccharide by weight, suggesting that the amount of polysaccharide is unlikely to be responsible for the differential effects. Since the polysaccharide vaccine only induces an antibody response, the differential effects were probably due to the presence of the immunogenic protein (CRM₁₉₇) in the conjugate vaccine, which induced a T cell dependent response (177). This implicated the involvement of T cell mediated responses in the suppression of AAD. We also demonstrated that the backbone components of the conjugate vaccine have no effect on AAD. Together, these results indicate that synergistic effects of the combination of the polysaccharide and immunogenic protein are important in mediating suppression of AAD.

Other studies have demonstrated the potential use for microbial agents as therapeutic strategies in asthma but have not elucidated the effects on established disease (Table 1.5). An important part of this study involved the development of a new model of established AAD, in which AAD could be recapitulated by secondary challenge. The capacity for the conjugate vaccine to suppress established AAD is highly novel and provides encouraging evidence for the use of this therapy for asthma in humans.

The incidence of asthma in children has reduced slightly in recent times. Given our results, it may be speculated that the widespread use of the conjugate vaccine may be related to this reduced incidence of asthma in children. However, further research is required to investigate this possibility.

5.2.2 Conjugate vaccine: Route of administration and dose

We also demonstrated that the conjugate vaccine is most effective when administered directly into the airways (i.e. intranasally). Intranasal administration may provide a more targeted effect and could prime pulmonary antigen presenting cells (APCs) that may play important roles in mediating immunoregulation. However, we can not rule out the possibility that intramuscular administration of a higher dose of the conjugate vaccine may have enhanced efficacy in suppressing AAD. In the clinic, especially in children, intramuscular administration is a much easier and consistent method of delivery that is widely used. Whether the conjugate vaccine may be administered effectively *via* the intranasal route or other inhalation methods in humans remains to be determined.

Full spectrum dose dependent studies have not yet been performed. However, the conjugate vaccine was effective at suppressing AAD to a similar degree when 20 μ l, instead of 33 μ l, was administered i.n. per dose using the same protocol (data not shown). This suggests that the dose administered in these experiments is above the threshold of the immune response required for suppression of AAD.

Studies by others have shown that the administration of alum (contained within the conjugate vaccine) may cause aluminium allergy (253). This raises a safety issue for the administration of the conjugate vaccine directly into the airways. Administration of live or killed *S. pneumoniae* in the absence of alum suppresses AAD, therefore, alum-free conjugate vaccine is likely to be effective (150, 151).

5.3 *S. PNEUMONIAE* COMPONENTS AND THE SUPPRESSION OF AAD

5.3.1 T3P+Ply-mediated suppression of AAD

We have developed our own immunoregulatory therapy for AAD by identifying the key immunoregulatory *S. pneumoniae* components, type 3 polysaccharide and pneumolysoid, which are required for suppression of AAD (Chapter 3). This was a significant finding since the key components of many microbial agents associated with suppression of AAD have not been identified.

The requirement for co-administration of type 3 polysaccharide with pneumolysoid (T3P+Ply), drew similar comparisons to the *S. pneumoniae* vaccine study (Chapter 2). Again, the data showed that polysaccharide components must be administered with an immunogenic protein to have an effect on AAD. Furthermore, this suggests that the two components do not need to be conjugated. It is also not known whether the components act on the same cell or have independent but complementary effects. However, it is possible that conjugation would improve the efficacy of a T3P+Ply-based immunoregulatory therapy to induce Tregs and suppress AAD.

5.3.2 T3P+Ply-mediated suppression in different models of AAD

It was important to determine if T3P+Ply had effects on a model of AAD that did not use OVA as the allergen. Importantly, T3P+Ply also suppressed HDM-induced AAD. This model has the advantage of being induced in the absence of an adjuvant, therefore is more akin to the sensitisation process in human asthma.

5.4 MECHANISM OF SUPPRESSION OF AAD BY *S. PNEUMONIAE*

IMMUNOREGULATORY THERAPY

We then assessed the mechanisms of suppression of AAD. Although the conjugate vaccine is readily available its' molecular make-up is designed to prevent infection and not for therapeutic applications in asthma. We have determined that T3P+Ply are immunosuppressive components of *S. pneumoniae*. Therefore, T3P+Ply offer a novel and simplified form of *S. pneumoniae* immunoregulatory therapy that may be beneficial in asthma. For these reasons, further investigations into the mechanism of suppression was performed using T3P+Ply.

5.4.1 *S. pneumoniae* immunoregulatory therapy- induced Tregs

5.4.1.1 Depletion of Tregs

Anti-CD25 antibody depletion of Tregs showed a requirement for *S. pneumoniae* immunoregulatory therapy-induced Tregs in the suppression of AAD. Many previous studies have shown that anti-CD25 (PC61) depleted Tregs, which our data confirmed. It is important to note that we used the PC61 and not 7D4 isotype in this study. It has been reported that contrary to the current paradigm, anti-CD25 does not deplete Tregs but functionally inactivates them (254). However, the isotype used in the majority of this study was 7D4. The authors did not appreciate that the two anti-CD25 antibodies have very different effects (255). Recently the exact mechanism of Treg depletion by PC61 has been defined in mouse models and involves Fc γ RIII+ phagocytes (256). Indeed, functional blockade of Fc γ RIII expression *in vivo* inhibited PC61-mediated Treg depletion and Fc γ RIII -/- mice failed to mediate PC61 depletion of Tregs. This suggests that PC61-dependent phagocytosis of CD25+ cells is involved in the recognition and subsequent depletion of Tregs.

5.4.1.2 Induction of Tregs

The exact mechanism of *S. pneumoniae* recognition and the subsequent events that lead to the induction of Tregs is unclear. Our data show that the early production of TGF- β and IL-2 is important for the induction of Tregs. Other studies have shown that IL-2 is essential for TGF- β -mediated induction of Tregs (241). Furthermore, DCs have been shown to produce TGF- β and play an essential role in the expansion of Foxp3+ Tregs (105). Therefore, it is likely that these factors are induced by *S. pneumoniae*-mediated signalling pathways in DCs. Further investigation is required to understand the sequence of events.

Recently, Shafian *et al.* showed that the induction of Tregs after *M. tuberculosis* infection did not occur until *M. tuberculosis* was transported to the MLN (257). They also show that a very small number of Tregs have potent effects on delaying the priming of effector T cells in the lymph nodes and their accumulation in the lung. Our study (Chapter 4) shows a similar sequence of events. Our data indicate that T3P+Ply are taken to the MLNs, which may involve signalling through CCR7, and in the presence of IL-2 and TGF- β Treg expansion occurs. These Tregs, although low in numbers, have potent effects on delaying the priming of effector T cells in the lymph nodes. This may help to explain why the most suppressive effects by *S. pneumoniae* immunoregulatory therapy were achieved when it was administered within close proximity to OVA sensitisation. This may induce optimal prevention of effector T cell accumulation in the lung. It is perhaps not surprising that antigen must be taken to the lymph nodes in order for Treg expansion to occur since this is where naïve T cells are primed.

5.4.1.3 Mechanism of Treg-mediated suppression

Many Treg-mediated mechanisms of suppression have been identified (section 1.4.3). This has created controversy in the field, however it is generally accepted that in different immune conditions, different mechanisms prevail. It is likely that the immune system has evolved such a complex line of defence to address the diverse challenges of different disease states.

Our data demonstrate a dependence upon TGF- β during the induction of Tregs. However, TGF- β is unlikely to be involved in mediating immunosuppression at the site of inflammation since levels of TGF- β are either suppressed (with the conjugate vaccine) or remain the same (with T3P+Ply). Similarly, IL-10 is unlikely to have immunosuppressive effects at the site of inflammation, since levels of IL-10 are suppressed by *S. pneumoniae* immunoregulatory therapy. Both IL-10 and TGF- β dependent and independent mechanisms of Treg suppression have been identified (71). The assumption has been made that these Tregs are of different subtypes (Table 1.2). It is plausible that depending on the disease state, Tregs utilise different mechanisms of suppression. A simple example of this is provided by IL-10 and TGF- β deficient mice, where Treg suppressive function is unaffected by the absence of these genes (108, 109). Furthermore, both *in vivo* and *in vitro* experiments have shown that neutralising antibodies against IL-10 and TGF- β fail to abrogate suppression (106, 107, 211).

Instead, we showed that *S. pneumoniae*-induced Tregs expressed higher levels of markers associated with enhanced suppressive and functional capacity (CTLA-4; CD103; FoxP3; IL-35; IL-9; and TLR2). Multiple Treg cell functions may act either alone or synergistically, directly or indirectly at the site of antigen presentation to suppress immune responses (71).

As indicated by increased Ebi3 and p35, it is likely that IL-35 plays an important role in mediating suppression. This cytokine may be the primary soluble

immunosuppressive factor. Further investigation is currently limited by reagent availability, however the role of IL-35 will be investigated in future experiments.

We used suppression assays to demonstrate that Tregs isolated from conjugate vaccine treated mice suppressed OVA-induced and non-specific T cell proliferation to a similar degree. This may potentially indicate that the Tregs are non-antigen specific. However, it was also noted that Tregs in the lungs from T3P+Ply treated and untreated mice expand at the same rate during challenge. This could suggest that an OVA-specific population expands, or that DCs direct Treg responses regardless of T3P+Ply treatment but in a slightly different manner. Tregs are most likely having bystander effects on effector T cells. However, there may be both OVA specific and non-specific populations. Further investigation is required to understand the antigen specificity of Tregs.

5.4.2 Suppression of NKT cells

Few studies have investigated the relationship between NKT cells and Tregs. One study showed that Tregs suppress proliferation and cytokine (IFN- γ , IL-4, IL-13, and IL-10) production by all subsets of V α 24⁺ NKT cells in a contact-dependent APC independent manner (111). This provides evidence for Treg mediated suppression of NKT cells.

Another study showed that CD4⁺ NKT cells from asthmatics expressed higher levels of natural cytotoxicity receptors NKp30 and NKp46, and granzyme B and perforin (112). *In vitro* killing assays demonstrated that these NKT cells also had increased cytotoxicity against Tregs. This study has provided an explanation for the link between higher NKT cell numbers and lower Tregs numbers with decreased functional capacity in asthmatic individuals.

Our data correlates with the conclusions from these studies and furthers the understanding of the relationship between NKT cells and Tregs.

5.4.3 Suppression of Th1, Th2 and Th17 effector T cells

S. pneumoniae immunoregulatory therapy modulates the effects of the different T cell lineages in AAD. We suggest that this occurs by both deviation of the immune response from Th1, Th2 or Th17 and toward a Treg response and by the direct suppression of these cell types. This is the first report of the dual role of Tregs in a model system.

It is evident that the most influential T cell lineage is the Treg. The Treg has the capacity to suppress Th1 function and IFN- γ release, Th2 function and IL-4, IL-5, and IL-13 release and Th17 function IL-17 and IL-25 release. Hence, the signalling *via* these cytokines is suppressed and the multiple mechanisms by which they contribute to AAD are attenuated.

Deviation of the immune response towards Tregs prevents the initiation of effector cell responses and therefore affects all lineages of T cells. The most obvious lineage affected is the Th17 lineage since blockade of IL-6 promotes the induction of Tregs and prevents Th17 cell development. Th17 cells activate and recruit neutrophils. We do have data (not shown), that *S. pneumoniae* immunoregulatory therapy suppresses the number of neutrophils in the BAL during AAD. Furthermore, Th17 cells together with IL-23 have been shown to enhance Th2-cell mediated eosinophilic inflammation (33). The suppression of Th17 and Th2 cytokines by *S. pneumoniae* immunoregulatory therapy was associated with suppression of eosinophils in the BAL.

5.4.4 Effects on APCs

We observed some effects of *S. pneumoniae* immunoregulatory therapy on DCs during the suppression of AAD. The reduction of mDCs expressing CD86, together with increased expression of CTLA-4 implicates a role for Tregs in mediating the suppression of mDC co-stimulation and therefore AAD. This suggests another direct effect by Tregs on an effector cell type involved at the site of inflammation during AAD.

5.5 *S. PNEUMONIAE* IMMUNOREGULATORY THERAPY FOR ASTHMA

5.5.1 Conjugate vaccine versus T3P+Ply

The data presented show that there is a slight difference between the conjugate vaccine and T3P+Ply in regard to the induction of Tregs. The major difference is the sustained increase in the total number of Tregs in the MLN during AAD induced by the conjugate vaccine. This difference parallels increases in total cells in the MLNs with administration of the conjugate vaccine which does not occur with administration of T3P+Ply. The total amount of polysaccharide administered by the conjugate vaccine is 16 µg versus 6 µg by T3P+Ply. More polysaccharide combined with the conjugation to the immunogenic protein in the presence of AlPO₄ may explain these differences in Treg kinetics. If the effects of suppression of AAD are mediated by the synergistic effects of polysaccharide and protein, conjugation may enhance the effects on DCs on a per cell basis compared to random events required for the uptake of T3P+Ply by the same cell. Alternatively, conjugation may increase bystander responses to conjugated antigens. This suggests that the conjugate vaccine is a more potent inducer of Tregs, that the conjugate vaccine may be longer lasting and that conjugation may enhance the effects of T3P+Ply.

5.5.2 Routes of administration

As discussed, it is likely that a *S. pneumoniae* immunoregulatory needs to be administered to the airways for maximal suppression of AAD. In other studies (data not shown) we have compared different routes of delivery of T3P+Ply. Our data show that i.t. and i.n. administration of T3P+Ply suppress AAD to a similar degree. Like administration of the conjugate vaccine, i.m. delivery of T3P+Ply suppressed eosinophils in BAL and OVA-specific Th2 cytokine release from splenic T cells but had no effect on OVA-specific Th2 cytokine release from lymph node T cells or AHR. Since the transcutaneous (t.c.) route may be preferable for children, we investigated this route of administration. Mice were administered one dose of T3P+Ply t.c. by direct administration and absorption by the skin on a shaven part of their back. t.c. administration of T3P+Ply suppressed eosinophil numbers in the BAL, OVA-specific Th2 cytokine release from lymph node and splenic T cells, but had no effect on AHR. Whether this is dose dependent and suppression of AHR can be achieved at higher doses has not been determined.

5.5.3 Dosing strategies and requirements

Dose escalation studies are required to determine the optimal dose and dosing regime. Since the *S. pneumoniae* components suppress AAD through the induction of Tregs, Treg may be used as an indicator of dose efficacy.

It is likely that humans may require multiple therapeutic doses for the suppression of asthma. In other studies, we have used mouse models to investigate the effect of killed *S. pneumoniae* when administered throughout early life. These studies showed that administration of killed *S. pneumoniae* (i.n. 3-5 times per week) from the

first day of life suppressed the development of AAD induced in infant and adult mice. Mice did not display any adverse effects to the administration of numerous doses. Although this is a large number of doses in a short period of time, given the rapid development rate of a mouse (8 weeks old) versus human (20 years old), we speculate that this dosing regime equates to approximately once every three-six months in humans.

In addition, *S. pneumoniae* immunoregulatory therapy may not offer instant relief during an asthma attack since the induction of Tregs is likely to take approximately 3-4 days. Instead, *S. pneumoniae* immunoregulatory therapy is likely to be most effective when used prophylactically.

5.5.4 Harnessing Tregs for therapeutic use

The multi-targeting nature of Tregs allows for the regulation of numerous different effector arms of the immune response involved in asthma. The induction of Tregs to target effector responses may be the most holistic approach to modulate the underlying cause of disease. A major limitation of other studies is that the non-infectious key components of the microbial agent that induce Tregs have not been identified. Our studies successfully identified the key components of *S. pneumoniae* that are required for the induction of Tregs and suppression of AAD.

The translation of this approach needs to consider that a successful immunoregulatory therapy needs to overcome the existing Treg pool within an asthmatic individual. Since asthmatics have defective Tregs, the new pool needs to have normal/optimal functional capacity and chemotactic properties. Our data suggest that *S. pneumoniae*-immunoregulatory therapy can suppress established AAD. Furthermore, our model of AAD is characterised by a low number of Tregs and reduced

expression of CD103 and FoxP3 compared to the non-sensitised controls. Together, these data indicate that it may be possible for this therapy to overcome an existing Treg pool.

Glucocorticoid administration in conjunction with the active form of vitamin D (1 α ,25-dihydroxyvitamin D3 or calcitrol) has also been shown to promote the induction of Tregs that release IL-10 (198). Importantly, this strategy is effective in patients that are refractory to steroid treatment and further studies are refining this strategy.

Adoptive transfer of *ex vivo* generated Tregs into asthmatic individuals is being considered as a therapeutic strategy. This method for harnessing the properties of Tregs raises a number of questions and problems. For example:

- Can functionally effective Tregs be generated?
- How many Tregs can be generated?
- How would Tregs be administered?
- How long would the effect of Tregs last?
- Is this treatment method sustainable?
- Would Tregs induce susceptibility to infection or enhance other diseases?

These major uncertainties indicate that an *in vivo* induction method may be more easily achievable.

In addition to the development of an immunoregulatory therapy for asthma, *in vivo* models that involve the induction of Tregs and suppression of AAD, may provide valuable tools that facilitate the further investigation of the characteristics, mechanisms and function of Tregs. These models may facilitate the delineation of ‘real time’ events that are important in the induction and enhanced suppressive function of Tregs.

New therapeutics based on our understanding of Treg function and the pathophysiology of asthma could have profound benefits for the care of asthmatics.

Hence, it is not surprising that the potential to harness the power of Tregs as an immunoregulatory therapeutic is of great interest. Through Tregs we have a multifactorial approach to a multifactorial disease, if only we can develop their potential into therapy.

5.6 FUTURE DIRECTIONS

Our studies have characterised the mechanisms underlying suppression of AAD by *S. pneumoniae* immunoregulatory therapy. However, additional studies are required to completely elucidate these mechanisms, which will be part of future studies.

5.6.1. Suppressive effects of *S. pneumoniae* immunoregulatory therapy on chronic features of AAD

In collaboration with Prof. Rakesh Kumar at the University of New South Wales, we have commenced studies into the effect of *S. pneumoniae* immunoregulatory therapy in experimental chronic asthma. In a model of chronic OVA aerosol challenge exposure 5 days per week for 4 weeks, post i.p. sensitisation, we administered killed *S. pneumoniae* intranasally twice per week. Killed *S. pneumoniae* suppressed the chronic airway inflammatory responses and remodelling. This suggests that *S. pneumoniae* immunoregulatory therapy may have the potential to suppress chronic features of asthma. Further experiments aim to elucidate the effects of T3P+Ply on chronic inflammation, investigate the potential for *S. pneumoniae* to reverse established chronic inflammation and determine the mechanism involved in suppression of chronic AAD.

5.6.2 Further investigation of the role of Tregs

5.6.2.1 Suppression of NKT cells

Our data demonstrate that an important interplay occurs between Tregs and NKT cells, which is required for T3P+Ply-mediated suppression of AHR by T3P+Ply. To date, only one other study has provided evidence for Treg mediated suppression by NKT cells. Furthermore, this study showed that Treg-dependent suppression of NKT cells requires cell-cell contact (111). To further our understanding of how Tregs suppress NKT cells in our study, it will be important to determine the type of cell-contact mediated suppression involved. NKT cells will be stimulated *in vitro* with α -galactosylceramide (α Gal-Cer) and varying numbers of Tregs added. Proliferation will be assessed by thymidine incorporation. Blocking antibodies, toward factors that may be involved in cell-contact mediated suppression will be added to co-cultures and the effect on proliferation assessed. Furthermore, the effects of Tregs from untreated allergic control mice and *S. pneumoniae* immunotherapy treated mice will be compared. This will allow us to elucidate the precise mechanism of Treg-mediated suppression in our system.

5.6.2.2 Suppression of Th cells

To further the understanding of Treg mediated deviation of effector T cell establishment and suppression lineage specific transcription factors should be assessed. Samples from MLN, lung and *in vitro* culture assays will be investigated for signal transducer and activator of transcription (STAT)3, STAT4, STAT6, T-box expressed in T cells (T-bet), transacting T cell-specific transcription factor GATA3, RAR-related orphan receptor gamma-thymus (Rory-t) and FoxP3. Analysis of gene expression of some transcription factors by quantitative RT-PCR is inconclusive, since phosphorylation is required for

signal activation. Therefore analysis of pathway activation will be performed using western blot.

5.6.2.3 Suppressive mechanisms

Given that our data indicates that immunoregulatory therapy induces Tregs that are more functional and suppressive, it is important to determine the role of each Treg factor. This will be carried out using blocking antibodies against each factor. For example, to investigate the role of CTLA-4 in mediating suppression, anti-CTLA-4 will be administered during the inflammatory stage (i.e. OVA challenge) to neutralise this marker. Similar methods will be used to determine the role of other factors.

Combinations of different neutralisation antibodies also have the potential to reveal the cooperative role of these different mechanisms of suppression.

Newly recognised Treg-specific markers are still being identified and their involvement in Treg-mediated suppression under different disease conditions remains to be determined. Recently, the role of tumour necrosis factor receptor superfamily (TNFRS)25 expression on Tregs has been investigated (258). Using an agonistic TNFRS25 antibody, the authors showed that TNFRS25 engagement expands CD4⁺Foxp3⁺ Tregs in mice with peak expansion between 4-5 days after administration. This is the first evidence of a physiological pathway that leads to Treg-specific expansion. Furthermore, Treg expansion by TNFRS25 stimulation inhibited the development of AAD (259). Notably, the TNFRS25 expanded CD4⁺Foxp3⁺ Tregs expressed higher levels of CD103. The authors also note that the expansion of Tregs was independent of CD80 and CD86 but dependent on IL-2 and MHCII (personal communication by Matthew Tsai, The University of Miami). Given the similarities between these observations and our data it would be interesting to determine whether

TNFRS25 engagement is involved in *S. pneumoniae* mediated expansion of Tregs and suppression of AAD.

The pathways and events that lead to the development and generation of Tregs is poorly understood. Recently, it was proposed that a FoxP3-specific enhanceosome containing c-Rel, p65, Nuclear factor of activated T cells (NFAT), mother against decapentaplegic homolog (Smad) and cyclic adenosine monophosphate response element-binding (CREB) promotes the generation of Tregs (260). Indeed, mice deficient in these factors had reduced Treg numbers. Binding of these factors to the FoxP3 promoter region and downstream of the promoter has been proposed as a possible strategy for manipulating Treg function. Investigating whether *S. pneumoniae* affects transcription of factors within the FoxP3 enhanceosome would be of interest.

5.6.2.4 Utilisation of new methods

Exciting new tools and techniques for investigating the role of Tregs are becoming more readily available. Genetically modified mice where the transcription of FoxP3 has been engineered to express green fluorescent protein. This enables tracking of FoxP3 expression by colorimetric analysis. We could employ these mice to further understand the kinetics of induction of Tregs and their subtypes.

Currently, this strain only exists on a C57BL/6 background strain. We could either recapitulate our data using C57/BL6 mice or generate green fluorescent protein (GFP)-FoxP3 expressing BALB/c mice. Given the substantial amount of data we have already generated using BALB/c mice it would be pertinent to generate these mice by backcrossing onto a BALB/s background. Furthermore, our data has been produced using female mice. X-inactivation occurs in female GFP-FoxP3 mice and so the number of GFP+FoxP3+ cells in the peripheral blood of females is approximately half

that of males (Jackson Laboratory Database). This needs to be considered in future experiments.

5.6.3 Further investigation of the role of APCs

5.6.3.1 APC uptake of *S. pneumoniae* components

Further investigations should assess the APCs involved in the uptake of type-3 polysaccharide and pneumolysin. By labelling each component and OVA with a different fluorochrome the interactions with different cell types can be visualised by real-time fluorescent microscopy. Similar protocols in combination with flow cytometry may be used to assess activation status and cytokines release. These studies would lead to a more definitive understanding of which cells are activated by the components and suppress inflammatory responses to OVA.

5.6.3.2 PRRs

PRRs may play an important role in the recognition of *S. pneumoniae* by APCs. In additional studies (not shown) the role of MyD88, TLR2 and TLR4 in *S. pneumoniae* mediated suppression of AAD was investigated using TLR deficient mice. The data showed that killed *S. pneumoniae* required MyD88 and TLR4 to suppress eosinophils in BAL, OVA-specific IL-5 and IL-13 release from splenic T cells and AHR. TLR2 was also required for the suppression of AHR. We also showed that *S. pneumoniae* upregulated TLR2 and TLR4 gene expression in the lungs 24 hours after administration. Furthermore, *S. pneumoniae* sustained an upregulation of TLR4 expression during AAD. A limitation of these data was that AAD was not induced to maximal levels in TLR2 and 4 deficient mice. Nevertheless, these data indicate that TLR2 and 4 are involved in *S. pneumoniae*-mediated suppression of AAD. *S. pneumoniae*

polysaccharide may be recognised by TLR2 and pneumolysin is recognised by TLR4. The role of TLRs in *S. pneumoniae*-mediated suppression of AAD may be investigated by administration of anti-TLR antibody during the administration phase. Neutralisation of TLRs during this phase will allow us to clarify the role of TLR signalling in *S. pneumoniae*-mediated suppression of AAD.

5.6.3.3 Nuclear factor (NF)- κ B

Upon activation, NF- κ B translocates into the nucleus and stimulates the expression of genes involved in cellular function. In asthma, NF- κ B plays a central role in the maintaining persistent inflammation (261). Indeed, maturation of human monocyte-derived DCs in the presence of asthmatic serum has been associated with high levels of NF- κ B (262). Furthermore, Ad κ B α M-mediated blockade of NF- κ B resulted in increased apoptosis and decreased expression of DC co-stimulation markers and T cell proliferation. Together, these studies indicate an important role for NF- κ B in promoting inflammation in asthma.

Reticuloendotheliosis (Rel)B is a member of the NF-KB family and plays an essential role in DC differentiation and maturation. Furthermore, RelB binding to RelA renders NF- κ B inactive. We hypothesise that *S. pneumoniae* immunoregulatory therapy may induce RelA or RelB to block NF- κ B activity. This may be investigated using studies that employ quantitative DNA binding, ELISA and confocal microscopy techniques.

5.6.3.4 Activation of a suppressive DC phenotype

Since no population changes in the absolute number of DC populations were identified in our studies, we propose that intrinsic changes may be important in mediating *S.*

pneumoniae suppression of AAD and that Tregs may induce IDO in DCs *via* CTLA-4/B7 interactions, thus conferring immunosuppressive activity upon DCs (263).

Preliminary data (not shown) demonstrates that T3P+Ply treated mice have higher levels of IDO in their lungs during AAD compared to untreated groups. These data, in parallel with the increased expression of CTLA-4 on Tregs suggest that IDO may play an important role in *S. pneumoniae*-mediated suppression of AAD. Further studies could confirm the levels of IDO by measurement of kynurenine using enzymatic assays.

5.6.4 Chemokines and migration pathways

Evidence suggests that specific chemokine signalling pathways that may enhance Treg recruitment to the airways are defective in asthmatics. For example both chemokine C motif ligand and receptor (XCL1 and XCR1) expression are reduced in asthmatic Tregs compared to healthy controls (264). This study also showed that asthmatic Treg incubation with recombinant human XCL1 significantly increased Treg-mediated suppression. Hence, XCL1 may play an important role in reversing disease *via* Tregs.

Our data (Chapter 4) indicates an association between Treg expansion in the lymph nodes and CCR7 expression in the lungs. Although this may suggest a role for CCR7 in the trafficking of cells from the lungs to the lymph nodes, this observation needs to be confirmed by blocking CCR7 using anti-CCR7 antibody. Further investigation into other chemokines and chemokine receptors involved would allow for a greater understanding of the entire process leading to *S. pneumoniae*-mediated suppression of AAD. Initial studies would involve quantitative RT-PCR analysis of MLN and lung RNA for a panel of chemokines. Highly purified Tregs from treated and untreated mice could be compared for cell specific differences by RT-PCR and flow cytometry. Chemotactic factors known to be important for Treg chemotaxis could be

investigated *in vivo* using anti-chemokine antibodies to determine their involvement in *S. pneumoniae*-induced Treg-mediated suppression of AAD.

5.6.5 Potential use of *S. pneumoniae* immunoregulatory therapy for other inflammatory conditions

Tregs play important roles in promoting homeostasis and suppressing aberrant responses in a number of other disease conditions. Therefore *S. pneumoniae*-induced Tregs may have potential for therapeutic application in other inflammatory conditions.

Recently, Tregs have been shown to play an important role in the resolution of acute lung injury (ALI) (265). This study showed that adoptive transfer of Tregs into T cell deficient mice after the induction of ALI by LPS normalised resolution of disease. Similarly, depletion of Tregs in wild-type mice, delayed the resolution of ALI. These data suggest that Tregs may be a potential target for treating ALI, for which there are no specific therapies currently available.

In preliminary studies, we have investigated the effect of T3P+Ply on the development of LPS-induced ALI. T3P+Ply suppressed the development of neutrophils in the BAL (not shown). This was associated a decrease in the levels of mouse neutrophil chemoattractants macrophage inflammatory protein (MIP)2 and keratinocyte chemoattractant (KC) in the lung. Further studies could assess the effects on physiological features of ALI.

Tregs are altered during the development of autoimmune diseases such as rheumatoid arthritis (RA) (266). RA patients have increased numbers of Tregs that are less functional. Thus, the induction of a more functional population of Tregs may be an effective therapeutic strategy. Since, T3P+Ply-induced Tregs are more functional, this may identify a possible therapeutic strategy for restoring Treg function in RA.

5.6.6 Assessing the effect of *S. pneumoniae* immunoregulatory therapy using clinical samples

In order to assess the potential for translation into humans, it may be important to perform experiments on human samples. Our *in vitro* studies using mouse splenocytes shows that T3P+Ply suppresses the development of antigen stimulated Th2 responses. Similar experiments could be carried out using human peripheral blood mononuclear cells (PBMCs) and airway epithelial cells stimulated with allergen. T3P+Ply could be added to allergen stimulated cells and supernatants assessed and RNA assayed for pro-inflammatory markers. Many outcomes would be obtained from these experiments including but not limited to measurement of Treg inducing factors, FoxP3 gene expression, Treg proliferation and pro-inflammatory cytokines. Furthermore, samples from healthy and asthmatic individuals may be compared to determine whether Tregs of similar functional capacity can be induced from PBMCs from asthmatics.

5.6.7. Translation of *S. pneumoniae* immunoregulatory therapy into the clinic: Clinical trials

Tracking the health of children born to asthmatic mothers in relation to administering the conjugate vaccine would potentially allow for associations between conjugate vaccine administration and asthma. However, the conjugate vaccine is now part of the immunisation schedule for infants. Hence, prospective studies are no longer possible.

Our data suggest that multiple doses are required and that the i.m. route, which is the current route of conjugate vaccine delivery, is not as effective as other routes. Therefore a specifically designed study needs to be performed. Such a study would examine the efficacy of conjugate vaccine administration in asthmatics. Studies would

determine if the conjugate vaccine improves clinical markers of asthma. Measurements would include; Treg responses, induced sputum eosinophils, exhaled nitric oxide, as an additional marker of eosinophilic inflammation, asthma control score, asthma quality of life score, spirometry, asthma exacerbations, maintenance oral corticosteroid doses, AHR and T cell responses to allergen.

Such a study would allow us to determine if conjugate vaccine administration has immunoregulatory effects in refractory asthmatics and may lead to additional investigations into the use of a *S. pneumoniae*-based immunoregulatory therapy for asthma in humans.

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APPENDIX

This section contains two first author reviews that were published in relation to this thesis, and a co-first author paper, as outlined in the preface.

APPENDIX I

Thorburn, A.N. and Hansbro, P.M. Harnessing regulatory T cells to suppress asthma:
From potential to therapy. *Am. J. Respir. Cell Mol. Biol.* 2010. In press.

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APPENDIX II

Thorburn, A.N., Preston, J.A., Starkey, M.R., Beckett, E.L., Horvat, J.C., Wade, M.A., O'Sullivan, B.J., Thomas, R., Beagley, K.W., Gibson, P.G., Foster, P.S., Hansbro, P.M. *Streptococcus pneumoniae* infection suppresses allergic airways disease by inducing regulatory T cells. *Eur. Respir. J.* 2010. In press.

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APPENDIX III

Thorburn, A.N., Hansbro, P.M., Gibson, P.G. Pneumococcal vaccines for allergic airways diseases. *Exp. Opin. Biol. Ther.* 2009;9(5):1-9.

Expert Opinion

1. Introduction
2. Exposure to infectious agents and asthma risk
3. Spn and asthma risk
4. Spn vaccination and asthma
5. Spn and eosinophil responses
6. Phases of asthma
7. Immunopathology of asthma (and allergic sensitization)
8. T regulatory cells and asthma pathogenesis
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12. Spn immunoregulatory therapy: proof-of-concept
13. Effect of current vaccines
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Pneumococcal vaccines for allergic airways diseases

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Background: Asthma is a common global health problem. Environmental exposures such as bacteria may protect against asthma development. **Objective:** This review aims to examine the possible protective role of pneumococcal infection and vaccination in asthma. **Methods:** A review of known experimental biology and human epidemiology relating to asthma and pneumococcal infection was performed. **Results:** Pneumococcal infection can modulate components of allergic airways disease such as airways hyper-responsiveness and airway eosinophilia. Exposure to killed pneumococcus can reproduce these effects and the mechanism may involve control by T regulatory cells. **Conclusions:** Pneumococcal immunoregulatory therapy is a potentially important approach to asthma management that requires further evaluation in well-designed research studies.

Keywords: allergy, asthma, eosinophil, immunoregulatory, infection, *Streptococcus pneumoniae*, T regulatory cell, vaccine

Expert Opin. Biol. Ther. (2009) 9(5):621-629

1. Introduction

Asthma is a common illness throughout the world, with a significant geographic variation in prevalence. In Australia and parts of Europe and North America the prevalence is high [1], with rates of current asthma affecting up to 1 in 10 adults and 1 in 7 children. This contrasts with some African countries where the prevalence can be as low as 2% [1]. There has also been a dramatic rise in the prevalence of asthma. While the most recent data indicate a stabilization of asthma prevalence in the countries with the highest rates, asthma still appears to be increasing in countries with intermediate asthma rates. A study using objective confirmation of asthma, that was independent of physician's diagnosis, found there had been at least a doubling of asthma prevalence in school age children in Eastern Australia between 1980 and 1990 [2], that has subsequently stabilized [3]. Migration studies also demonstrate that children can develop asthma when they move from a low-prevalence country to a country with high asthma prevalence. Adolescents who migrated to Australia from countries with low asthma prevalence experienced an 11% increase in the prevalence of wheezing for every year of residence in Australia [4]. These observations demonstrate that there are significant environmental influences that affect asthma, and indicate a pressing need to understand these changes and develop interventions that will reduce the burden of illness from asthma.

The mechanisms underlying asthma involve immune-mediated airway inflammation, with evidence of both genetic and environmental modulation of asthmatic responses. Genetic factors confer susceptibility, and interact with environmental exposures to permit expression of the disease. The recent world-wide changes in asthma prevalence imply significant environmental effects, and also hold out the possibility that modification of exposures or the subsequent immune responses, termed immunoregulatory therapy, may reduce the burden of asthma. A key

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exposure that may be related to the development of asthma is exposure to microbial species. Exposure to certain bacteria may either increase or reduce asthma risk, and we have proposed that infection with the respiratory pathogen *Streptococcus pneumoniae* may be protective against asthma. This review will examine the evidence for this proposal from human epidemiological studies and from intervention studies in model systems.

2. Exposure to infectious agents and asthma risk

Epidemiological studies have revealed that the increase in the prevalence of asthma has been associated with a reduction in exposure to infectious agents throughout life. For example, Jones *et al.* [5] collated data to show that those countries with the highest risk of childhood bacterial infection had the lowest risk of asthma. Specifically, there was a high ratio of deaths from asthma compared with those from tuberculosis in countries with a high asthma prevalence, whereas countries with a low asthma prevalence had a low ratio [5].

Further support for the association between exposure to infectious agents and asthma risk is provided by studies of antibiotic use and asthma. Infants who are given antibiotics in the first year of life have a greater risk of subsequently developing childhood wheezing [6,7]. The mechanisms of this association may involve modulation of intestinal microflora by antibiotic use [8], and studies are now trying to establish if the association is a causal relationship or a confounding effect [9].

Other epidemiological studies have shown a relationship between tuberculin reactivity in populations and reduced asthma risk [10], and between neonatal BCG vaccination and reduced asthma risk [11]. This has led to studies showing that immunomodulatory therapy with Bacille Calmette-Guérin (BCG) in animal model systems leads to reductions in allergic responses. The efficacy of mycobacterial immunization in preventing allergy in human trials has been varied, with decreases in the severity of atopic dermatitis but no effect on allergic asthma. Successful results were also dependent on the age of study subjects [12-17]. Exposure to bacterial DNA sequences, termed CpG oligodeoxynucleotides (CpG-ODN) can also modulate inflammatory responses. CpG stimulation of lymphocytes results in the development of potent T-helper-1 (Th1)-biased immune responses that have proven effective in suppressing allergic inflammation in mouse models [18]. These observations are now being tested in preclinical studies.

These studies demonstrate that there may be specificity in the protective antibacterial responses. The determinants of this specificity are not known but potentially involve the bacterial species used, the dose of the active moieties (e.g., endotoxin) and the pattern-recognition receptors that are engaged by the agent used. The studies also show that there is a need to be cautious in the interpretation of results

since translation of successful results in model systems may not be replicated in human trials [19], and also, unwanted adverse inflammatory effects may result from immune deviation. For example, neutrophilic airway response has been observed with high doses of CpG, which is consistent with the induction of Th1 responses by CpG [20]. The potential efficacy of pneumococcal vaccination for asthma needs to be considered against this background.

3. Spn and asthma risk

Streptococcus pneumoniae (Spn) is a common respiratory pathogen, and is the predominant cause of community-acquired pneumonia in children and adults. It also frequently causes otitis media, septicemia and meningitis. Invasive Spn infections are the sixth leading cause of death worldwide. Spn infection has not previously been widely implicated in the development and exacerbation of asthma. One recent study suggested that neonatal (first month of life only) exposure to commensal Spn was associated with an increased asthma risk [21]. Asthma has also been observed as an independent risk factor for invasive Spn disease [22-24]. Talbot *et al.* [22] conducted a nested case control study of 635 people with invasive pneumococcal disease and 6350 controls. Asthma was significantly overrepresented in the cases with invasive pneumococcal disease, such that people with asthma were found to have a 2.4-fold increased odds of having invasive pneumococcal disease compared with people without asthma. The risk was further increased in severe asthma, where there was an almost fourfold increase in the annual incidence of invasive pneumococcal disease compared with controls without asthma. A serotype analysis of the invasive pneumococcal isolates revealed that 56% of isolates were covered by the polyvalent conjugate vaccine, and 86% were covered by the 23-valent polysaccharide vaccine. Twenty-nine percent of isolates were covered only by the polysaccharide vaccine. This study raises important questions about the relationship between asthma and Spn and highlights the need for further studies of mechanisms and treatment of Spn in asthma.

4. Spn vaccination and asthma

Studies of Spn vaccination that include asthmatics also provide an opportunity to examine the interaction between Spn and asthma. These studies are few in number since asthmatics are seldom recommended as a target group in vaccination schedules using pneumococcal vaccines. Vaccination of elderly patients with a pneumococcal polysaccharide vaccine was associated with a reduction in the number and severity of hospitalizations for asthma in a retrospective study [25]. The vaccine used in this study was the 23-valent polysaccharide vaccine. A Cochrane systematic review [26] examined the effect of pneumococcal vaccination in asthma and identified three potential studies. One study [27] was a randomized controlled trial that examined the effect of pneumococcal

vaccination (and several other interventions) in children with asthma and recurrent (over four episodes in a year) otitis media. The study reported that the children treated with pneumococcal vaccination had a 30% reduction in asthma episodes over a 2-year period.

5. Spn and eosinophil responses

The inflammatory process in asthma that is induced by allergen exposure typically involves increases in eosinophils in several body compartments, including the airway, circulation and bone marrow. Eosinophilia is typical of allergic asthma. Experimental studies of eosinophil responses have observed that Spn infection can suppress circulating eosinophil counts [28-31]. When a pneumococcal abscess was induced in an experimental model of parasitic eosinophilia due to trichinosis, there was a rapid fall in circulating eosinophils that was unrelated to adrenal activity.

6. Phases of asthma

The potential for immunoregulatory intervention in asthma can be classified into three distinct phases: phase 1 intervention occurs prior to the development of allergic sensitization; phase 2 intervention occurs after sensitization and before the development of clinical asthma; and phase 3 intervention is given during active asthma, for example, during an asthma exacerbation.

Immunoregulatory therapy for the first phase corresponds to primary prevention of asthma and is designed to reduce sensitization to allergens that provoke asthma. Sensitization involves an interaction between innate and acquired immune responses. When a susceptible host is exposed to an allergen, the allergen is processed and presented to the immune active cells (typically T helper cells) by antigen presenting cells, such as dendritic cells, and there is a subsequent expansion of allergen specific T lymphocytes that exhibit a T-helper-2 (Th2) cytokine secretion profile, and generation of allergen-specific IgE by activated/matured B cells [32]. Sensitization to the allergens associated with asthma usually occurs in infancy and childhood. Sensitization to ingested allergens occurs during infancy, and aeroallergens induce sensitization in mid to late childhood. Immunoregulatory therapy to prevent sensitization would need to be administered in early childhood in order to be successful, and represents an example of primary disease prevention. There are several challenges to this approach, including the limited responsiveness to vaccines during infancy.

Once sensitized, there is a latency period before the clinical expression of asthma. During this period a person has evidence of allergen-specific IgE but does not have symptoms of asthma. This is termed the atopic state, and in western and developed countries up to 40% of the population are sensitized (atopic) to aeroallergens. This is an attractive target period for immunoregulatory therapy since subjects can be

identified by screening for allergen specific IgE and are generally in a vaccine-responsive period of life [33]. Intervention during this phase may be considered secondary prevention of asthma.

The third period for consideration of immunoregulatory therapy is during active asthma. As described above, up to 1 in 10 adults and 1 in 7 children have current asthma. Most children (>80%) and many adults (60%) have current asthma occurring in association with atopic sensitization. These people present to doctors for care and management strategies aimed at improving current clinical status and preventing future disease related events, a phase termed tertiary prevention [34].

7. Immunopathology of asthma (and allergic sensitization)

The immunopathology of asthma is characterised by the infiltration of eosinophils and T-lymphocytes into the airway, which is associated with increased mucus production, vascular exudation, and epithelial injury and epithelial desquamation. There is in addition a series of changes in airway structural elements such as smooth muscle that involve hypertrophy, hyperplasia and infiltration of mast cells into the muscle. The inflammatory response can be heterogeneous, with some patients demonstrating a relatively eosinophil-poor inflammatory pattern that is more commonly seen in severe adult asthma. The production of the Th2 cytokines IL-4, IL-5 and IL-13 by activated CD4⁺ T-lymphocytes is instrumental in disease pathogenesis in allergic asthma and mediates features of pathophysiology including IgE production, eosinophilia, mucus hypersecretion and airway hyperresponsiveness (AHR). While asthma is mainly Th2 driven, responses involving Th1 cells and IFN- γ are induced in severe asthmatics [35]. The mechanisms of how IFN- γ contributes to the immunopathology of asthma are unclear, but may involve the induction of apoptosis of bronchial epithelial cells [36].

8. T regulatory cells and asthma pathogenesis

Several independent studies have linked impaired or altered T regulatory cells (Tregs) in patients with asthma compared to healthy individuals [37-45]. Tregs have the ability to suppress effector responses involved in the pathogenesis of asthma and therefore have been considered as potential therapeutic targets for upregulation. IL-10 and TGF- β have been highlighted as important mediators of suppression. Indeed, corticosteroids prevent the protective effects of Tregs by inhibiting IL-10 [46] and membrane-bound TGF- β is a candidate for predicting the severity of asthma [47]. Adoptive transfer of CD4⁺CD25⁺ Tregs in animal models of allergic airways disease (AAD) has reinforced their potential as a therapeutic agent [48]. Studies in allergic patients treated with immunotherapy inducing Tregs have been associated with suppression of allergy [49,50]. Therefore the administration

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of a targeted immunotherapy that reconstitutes the immune response through induction of Tregs is an attractive therapeutic for suppression of asthma.

9. Immune responses to Spn

Spn induces innate as well as adaptive (antibody and T-cell) immune responses.

Spn contains numerous pathogen-associated molecular patterns that activate toll-like receptors (TLRs), TLR-2, TLR-4 and the adaptor protein myeloid differentiation primary response gene 88 (MyD88) on innate cells including neutrophils and macrophages [51-54]. These phagocytic cells enter the infected area and are effective at phagocytosing and destroying the bacteria but these responses may also result in the suppression of Th2 responses and antagonize eosinophil influx in AADs.

It has been known for many years that anti-capsular antibody production is sufficient to protect against- and clear Spn infections [55,56]. However, help from T cells is necessary for the production and maturation of antibodies as well as class switching and the induction of memory of B cells [57,58]. T cell memory is also induced by infection, however the nature of the T cell response induced by infection is controversial and is dependent on the strain of Spn and the genetic background of the infected individual. Recently it has been shown that IL-17A is released from T cells upon infection. The activities of this cytokine include neutrophil activation and chemotaxis in concert with TLR-mediated IL-23 release from dendritic cells (DCs) [59]. This reinforces the neutrophil influx induced by innate responses and may further contribute to the suppression of AADs. Importantly, unlike other Gram-positive bacteria, Spn infection also induces the production of Tregs [60]. These Tregs may be important in protection against infection by controlling inflammatory responses. A consequent of the production of these Tregs may be the attenuation of Th2 responses in asthma [61].

Therefore, both innate and adaptive immune responses are induced by Spn infection that may suppress Th2 responses and AADs and asthma by multiple different mechanisms.

Although Spn infection and vaccine development are a clear research focus and investigated world wide, knowledge of the mechanisms underlying immunoregulation is in its infancy. Detailed elucidation of the mechanisms involved is the focus of in-depth studies in our laboratories.

10. Models to test the hypothesis

The assessment of Spn immunoregulatory therapy requires initial evaluation in model systems. This involves development of a model of allergic sensitization, a model of non-lethal Spn infection, and then a combined allergen-Spn model. There are several murine models that can be used to investigate different features of asthma, however, no single model

accurately represents all of the features of human disease. These models use an antigen, often ovalbumin, with a Th2-inducing adjuvant to induce sensitization and allergic responses. Other antigens such as cockroach extract or house dust mites may also be used, which may not require the co-administration of adjuvants [62,63]. Models have been developed using variations in mouse strain, and modifications of timing, route, dose and method of antigen administration. These effects are assessed using a variety of methods to examine tissue inflammatory and repair responses as well as accompanying physiological changes.

The closest approximations of chronic asthma and acute exacerbations of chronic asthma use long-term low dose aerosol challenge with ovalbumin [64,65]. Chronic models have characteristics of chronic inflammation accompanied by the recruitment of intra-epithelial eosinophils as well as features of airway remodelling similar to that observed in asthmatics including sub-epithelial fibrosis, epithelial thickening and mucus cell hyperplasia/metaplasia. This model also exhibits characteristic physiological changes of AHR to inhaled bronchoconstrictors. A single moderate-level antigen challenge induces an exacerbation that is characterised by an acute inflammatory component in the distal (intrapulmonary) airways with enhanced recruitment of CD3⁺ T cells, as well as exaggerated AHR. This can be superimposed on the chronic asthma model in order to mimic the *in vivo* situation of acute on chronic allergen exposure and subsequent responses.

Use of chronic models takes a long time and is labour-intensive and, therefore, other acute models are used extensively to model acute phases of disease [66,67]. Typically an antigen such as ovalbumin is administered systemically, which induces the development of potent Th2 responses. Adaptive immune responses to this sensitization are allowed to mature before rechallenge with cognate antigen occurs. This attracts memory Th2 cells into the lung, which release Th2 cytokines including IL-4, -5 and -13 that, in turn, promote the influx of eosinophils into the airways and the development of mucus secreting hyperplasia and metaplasia. These are models of acute broncho-pulmonary inflammation [67] and allergic inflammatory responses that together induce the development of AHR.

11. Non-lethal Spn models

Most mouse models of Spn infection are lethal models, which are used to test the efficacy of vaccines and treatments on serious invasive Spn disease. In order to investigate the effects of Spn infection on asthma using mouse models, non-lethal models of Spn infections are required. These have been developed using different strains of mice and use either lower doses of highly pathogenic strains [68] or less virulent strains [69]. These non-lethal models involve the development of acute lung infection over 24 – 48 h, which may involve septicaemia. This induces peribronchial and

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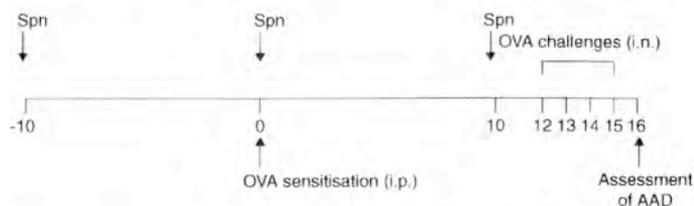


Figure 1. Model of acute allergic airways disease (AAD) and intervention with *Streptococcus pneumoniae* (Spn). Groups are sensitized intra-peritoneally (i.p.) with ovalbumin (OVA) in a T helper type 2 (Th2)-inducing adjuvant. Th2 cells are allowed to develop and AAD is precipitated 12 days after sensitization by four intra-nasal (i.n.) OVA challenges without adjuvant. AAD is assessed 1 day later. Spn exposure can occur before (Phase I), during (Phase II) or after (Phase III) sensitization.

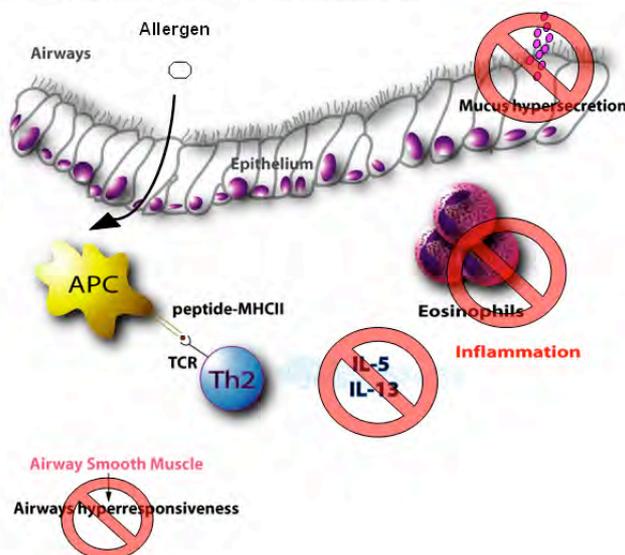


Figure 2. Effects of *Streptococcus pneumoniae* (Spn) on allergic airways disease. Spn suppresses hallmark features of allergic airways disease including airways hyperresponsiveness, T-helper type 2 (Th2) cytokines IL-5 and IL-13, eosinophils and mucus hypersecretion. APC: antigen-presenting cell

perivascular inflammation and the influx of leukocytes and neutrophils into the lungs, which subsequently resolve the infection.

These models can be combined with allergic sensitization models with infection before (Phase I), during (Phase II) or after (Phase III) sensitization (Figure 1). Thus the development of these non-lethal Spn infection models has allowed the investigation of the effects of infection on the development and progression of allergic airways disease.

12. Spn immunoregulatory therapy: proof-of-concept

The potential for the use of Spn as an immunotherapeutic agent for asthma has been investigated in proof-of-concept

trials [67]. These studies used killed Spn delivered intratracheally to mice at different times relative to sensitization in acute AAD. In order to investigate both prevention and treatment Spn was administered before or during sensitization or before challenge of sensitized mice. Administration at these different time points mirrors the three phases of allergic asthma described above.

Treatment before, during or after sensitization had generally the same effects and suppressed ovalbumin-induced Th2 cytokine release from lung-draining lymph node T cells, eosinophil influx into the airways, lung tissue and blood, mucus secreting cell hyperplasia and AHR (Figure 2). Furthermore these effects were largely reproduced using an extended model of AAD (24 days versus 12 days between sensitization and challenge). This study clearly demonstrates the potential

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for the use of Spn as an immunotherapy for AADs and extensive studies are currently underway to further investigate these possibilities.

13. Effect of current vaccines

As a result of the differences in their composition, the currently available 23-valent polysaccharide vaccine (PSv) and 7-valent conjugate vaccine (Cv) elicit different immune responses. PSv generates a T-cell-independent response which is poorly immunogenic, resulting in a low antibody response of largely low-affinity IgM. The lack of conventional T-cell help results in failure to induce antibody affinity maturation, isotype switching or immunological memory [70]. Polysaccharide protein conjugation enables Cv to elicit a T-cell dependent immune response and enhanced antibody production. Therefore, Cv promotes affinity maturation, isotype switching and induction of immunological memory [71]. Unlike PSv, Cv has been shown to induce DC-driven T cell priming [72].

Despite the knowledge of current vaccine efficacy against infection, it is not known whether they may provide an immunoregulatory therapy against AAD. To date, no polysaccharide based vaccine, such as PSv, has been shown to suppress AAD, suggesting that low antibody production is incapable of suppression of the Th2 response. Due to the complex pathogenesis of asthma, we propose that the strong immune response elicited by Cv may encompass the modulatory effects previously shown by killed Spn.

Apart from capsular polysaccharide, additional bacterial components may be involved in modulation of the immune response leading to the suppression of AAD. Identification of the components involved in suppression of AAD by Spn is required to create a specific and targeted immunoregulatory therapy for suppression of AAD. Furthermore, the use of different component combinations will allow us to understand the important underlying modulatory events. The potential for Spn components to work in an independent or synergistic manner in suppression of AAD is unknown.

14. Expert opinion

Asthma is a common disease of increasing global significance. There is a need to understand the genetic and environmental influences that modulate the expression of asthma. Environmental bacteria such as Spn may be able to modulate the expression of asthma, and if so, this effect could be therapeutically useful, using Spn vaccines as immunoregulatory therapy. Future work is needed to identify the efficacy of this approach in experimental systems and to identify the key components of Spn that are responsible for this effect, as well as discovering the mechanism of the effect. Epidemiological studies using existing databases could take advantage of current pneumococcal vaccine strategies to collate human data of the effect of pneumococcal vaccines on asthma prevalence. Once these studies have been completed, a randomized controlled trial of pneumococcal immunoregulatory therapy for asthma is needed to determine the clinical benefits of this approach. Exploiting the beneficial immunoregulatory effects of environmental bacteria represents an exciting approach to address a disease of immense and increasing importance to global human health.

15. Conclusion

Studies indicate that despite the availability of effective pharmacotherapy, asthma remains a common disease with an increasing burden of illness. There is a need to look for other treatment approaches. Reduced exposure to bacteria or their products is associated with increased asthma, and exposure to bacteria, either by vaccination or infection may be protective against asthma. Spn is a potentially useful immunoregulatory therapy for asthma. There is a clear path for future research to evaluate this possibility using model systems, epidemiological studies and clinical trials of available and novel pneumococcal vaccine products.

Declaration of interest

P Gibson has received financial support from NHMRC.

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