STEM CELL AND BIOLOGICAL INTERVENTIONS TO TREAT ALLERGIC AIRWAY DISEASE

By

Heather Kavanagh

B.Sc.

A thesis submitted to

The National University of Ireland, Maynooth for the degree of

Doctor of Philosophy

NUI MAYNOOTH

Ollscoil na hÉireann Má Nuad

Department of Biology

Institute of Immunology

National University of Ireland, Maynooth

February 2010

Research Supervisor: Dr. Bernard Mahon

TABLE OF CONTENTS

DECLARATION OF AUTHORSHIP

I certify that the work presented herein is, to the best of my knowledge, original, resulting from research performed by me, except where acknowledged otherwise. This work has not been submitted in whole, or in part, for a degree at this or any other University.

_____ _____

Heather Kavanagh B.Sc. Date

SUMMARY

The aim of this work was to investigate immune modulation with a particular focus on airway inflammation and allergic pathogenesis. This was probed in a model of pathogen driven immunomodulation (*B. pertussis*), and two models of therapeutic intervention namely immunisation (attenuated *B. pertussis*, BPZE1) or using a candidate cell therapy approach (mesenchymal stem cells, MSC).

This work demonstrated that, in contrast to virulent *B. pertussis*, an attenuated, candidate vaccine strain of *B. pertussis*, BPZE1, did not enhance but rather reduced allergen-driven airway pathology supporting findings that suggest allergic asthma is linked not just to a $CD4^+$ T cell profile, but also to the degree of airway damage at the time of priming. The second approach sought to ascertain whether a candidate stem cell therapy could modulate immunity *in vivo* to a degree that was sufficient to suppress allergic lung inflammation. The work presented here demonstrated that adult bone marrow-derived allogeneic MSC actively prevent the induction of allergen driven pathology in a murine model. Prior stimulation of MSC with IFN-γ increased their protective effect. An increase in IL-10 as a result of MSC delivery suggested a role for MSC in T_{reg} induction. The immune mechanisms by which MSC confer protection in this model were probed and demonstrated that MSC generate the expansion of T_{reg} subsets, $CD4^+$FoxP3 and $CD8^+$ γ/δ T cells, in the lung. Moreover, expansion of T_{reg} was proven to exert a functional effect as depletion of these cells resulted in a negation of the protective effect of MSC. In an experimental asthma model, where mice devoid of naturally occurring T_{reg}, delivery of MSC fails to impair the development of allergic airway pathology and class switching to IgE.

A reduction in airway eosinophilia in the absence of regulatory T cells suggested an alternative mechanism of protection employed by MSC. Pre-incubation with MSC inhibited the expression of important adhesion markers on eosinophils which most likely affected their

ability to migrate as demonstrated by the inhibitory effect on their chemotactic migration. These data suggests a critical role for soluble factor secretion by MSC contributing to the reduction of airway eosinophilia. Collectively, these data provides a fundamental insight into novel therapeutic approaches that can profoundly influence the allergic airway response.

PUBLICATIONS

Patents:

EP Patent Application No. 09 305 371.8.2009. "Vaccine for prophylaxis or treatment of an allergen driven airway pathology". INSERM, Institut Pasteur de Lille, N.U.I. Maynooth.

Peer Reviewed Publications:

1. **Heather Kavanagh**, Cariosa Noone, Emer Cahill, Karen English, Camille Locht, Bernard P. Mahon. Attenuated *Bordetella pertussis* vaccine strain BPZE1 modulates allergen induced immunity and prevents allergic pulmonary pathology in a murine model. (2010). Clinical and Experimental Allergy. *In press.*

2. **Heather Kavanagh** and Bernard P. Mahon. Mesenchymal stem cells reduce allergen driven airway inflammation in an allogeneic murine model. *Submitted for publication.*

3. **Heather Kavanagh** and Bernard P. Mahon. Mesenchymal stem cells attenuate eotaxin and RANTES-induced chemotaxis of human eosinophils. *In preparation*

ABBREVIATIONS

BALF	Bronchoalveolar lavage fluid
BSA	Bovine serum albumin
CD	Cluster of differentiation
CFU	Colony forming unit
DC	Dendritic cell
DMEM	Dulbecco's modified eagle media
DNA	Deoxyribonucleic acid
EBAO	Ethidium bromide acridine orange
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence activated cell sorting
FCS	Fetal calf serum
FITC	Fluorescein Isothiocyanite
FOXP3	Forkhead box P3
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GM-CSF	Granulocyte macrophage colony-stimulating factor
HBSS	Hanks buffered saline solution
HGF	Hepatocyte growth factor
H&E	Haemotoxylin and Eosin
HRP	Horseradish peroxidise
IFN	Interferon
Ig	Immunoglobulin

IL	Interleukin
mAb	Monoclonal antibody
MEM	Minimum essential medium
MFI	Mean fluorescent intensity
MHC	Major histocompatibility complex
MSC	Mesenchymal stem cell
OVA	Ovalbumin
PAS	Periodic Acid Schiff
PBMC	Peripheral blood mononuclear cell
PBS	Potassium phosphate buffer
PE	Phycoerytherin
PenH	Enhanced pause
PI	Propidium iodide
RNA	Ribonucleic acid
RPMI	Roswell park memorial institute
RT	Room temperature
T_H	T helper
TMB	Tetramethly benzidine
TNF	Tumor necrosis factor
T_{reg}	Regulatory T cell
VCAM	Vascular cell adhesion molecule
VEGF	Vascular endothelial growth factor

ACKNOWLEDGEMENTS

Mam and Dad. There is no-one luckier than me to have parents like you. I'm delighted to finally provide you with a photo opportunity at a conferring ceremony to which I don't need to be dragged by the ear.

Dan. You're a legend. You were my rock and all the sentimental stuff throughout this whole thing, but above all else, you were my very own psycho-therapist. You steered me clear of the strait-jacket and premature ageing and for that I'll always love you.

Bernie. Thanks for giving me the most amazing opportunity and for keeping the faith in my abilities when mine wavered or escaped me altogether.

Emer, Laura, Cariosa and Deirdre. Thanks so much for the support, friendship and humour. Ye are little stars and made working in the Cellular Lab an absolute pleasure.

Niamh. Thanks for the escapism and the laughter. I'd be lost without ya.

Past members, now doctors of Cellular Immunology, Karen E, Karen S, Ciaran and Declan. Thanks a million for helping me out with the whole immunology bit which was kind of important.

"I feel a very unusual sensation – if it's not indigestion, I think it must be gratitude"

Benjamin Disraeli

CHAPTER 1

INTRODUCTION

1.1 THE CAPACITY TO ADAPT: THE EVOLUTIONARY BENEFIT CONFERRED BY MAMMALIAN IMMUNITY

Adaptation is the hallmark of mammalian and vertebrate immunity. A combination of exquisite specificity and powerful immunological memory distinguishes adaptive immunity from non-vertebrate defence based on the innate responses (Burnet, 1968). Adaptive immunity confers a powerful selective advantage allowing protection against pathogens and the opportunity for protective immunisation (Baumgarth, 2005). To achieve this within the coding capacity of the mammalian genome requires clonal selection and an elegant gene recombination system first predicted by Dreyer and Bennett (Dreyer, 1965) and shown by Tonegawa in 1976 (Hozumi, 1976). The drawback, however, is such that an approach requires an editing and selection system that limits the possibility for autoimmunity, allergy or transplant rejection (Sakaguchi, 1995). Consequently, control or modulation of immunity is a central feature of the adaptive immune response. A variety of inhibitory mechanisms and pathways are employed to control both the innate and adaptive immune response as well as to ensure immune tolerance and homeostasis (Fehervari, 2004). In a broad sense, such inhibitory pathways appear to be highly specialised and compartmentalised (Hori, 2003). However, this complexity presents both opportunities and challenges in targeting these pathways in the treatment of immune-mediated diseases. The development of clinically applicable strategies to harness the potency of inhibitory pathways for therapeutic purposes remains a major challenge. The purpose of this work was to employ novel non-chemotherapeutic approaches in an attempt to disrupt the pathogenesis of an inappropriate immune response (allergic asthma) and to understand the underlying mechanisms of efficacy.

1.1.1 THE INTERPLAY BETWEEN IMMUNE PROTECTION AND REGULATION

The adaptive immune system is composed of many interdependent processes and cell types which collectively protect the body from viral, parasitic, bacterial and fungal infections. It links specific antigen recognition to innate effector systems and provides provision for immunological memory. Although protection against infection is the prime benefit of immunity (Ahmed, 1996), pathologies are also associated with misdirected immune responses against antigens. Abnormal immune responses in the absence of infection occur in allergy or hypersensitivity, (where the antigen may be an innocuous foreign substance), in autoimmune disease, (where the response is to self antigen), or in graft rejection, (where the antigen is the non-autologous cell). In order to induce desirable and specific responses, immunisation is critical. This may invoke antibody-mediated immunity that links to effector functions such as complement activation, neutralisation or opsonisation (van de Winkel, 1991) and/or the cell-mediated response where host cells present antigenic epitopes via polymorphic MHC molecules to induce cytotoxicity (via $CD8^+$ cytotoxic T lymphoctes (CTL)) and coordination functions via $CD4^+$ helper T cells. As the coordination function of the T cell is central to the appropriateness and efficacy of adaptive immunity, it is this response which will be a major focus of this work.

1.1.2 $CD4^+$ T HELPER CELLS: CONDUCTING THE IMMUNE ORCHESTRA

Early discoveries of acquired immune-deficiencies induced by HIV have dramatically shown the important co-ordinating function of $CD4^+$ T cells in adaptive immunity (Koup, 1994). Antigen presentation by antigen presenting cells (APC, such as DC) to $CD4^+$ T cells results in activation and differentiation of these cells into several specific functional subsets (Mosmann, 1986). Each subset is defined by its' specific cytokine secretion profile and the functional effects they direct (summarised in Table 1.1). Th1 cells orchestrate protection

from intracellular pathogens and are defined by the production of IFN-γ, IL-2, TNF-α, TNF-β and lymphotoxin (Mosmann, 1989). They are the principle effector of pro-inflammatory reactions, delayed-type hypersensitivity and cell-mediated immunity against intracellular pathogens such as *Mycobacterium tuberculosis* and *Listeria sp*. In contrast, Th2 cells are characterized by the production of IL-4, IL-5, and IL-13 and mediate protection from extracellular parasites (Mosmann, 1989; Medoff, 2008). Unlike Th1 immunity, the Th2 response is often associated with humoral responses which produce high levels of pathogen-specific immunoglobulin generated to neutralise foreign organisms. Consequently, Th2 responses are important for resistance against extracellular pathogens such as helminths (Sher, 1992).

Th17 cells are a more recently described subset. These cells are characterised by the production of IL-17 and are associated with the clearance of extracellular bacteria and fungi (Bettelli, 2007; Bettelli, 2008). They are responsible for, or participate in, the induction of many organ-specific autoimmune diseases. These cells have critical roles in host defence against extracellular bacteria and also play pivotal roles in the pathogenesis of autoimmune disease. Th17 cells produce a range of cytokines including TNF, IL-17A, IL-17F, IL-21 and IL-22. Recent advancements in T cell biology have also discovered a variety of suppressive or regulatory T cell (T_{reg}) subsets that either develop as a normal part of the immune response (naturally occurring T_{reg}) or in response to antigenic stimulation (adaptive T_{reg}) (Bluestone, 2003). Naturally-occurring T_{reg} constitutively express high levels of surface CD25. These cells are characterized by the expression of IL-10 and TGF-β and can actively suppress proliferation and cytokine production from other effector T cells (Fehervari, 2004). They owe their suppressive phenotype, in part, to the expression of high levels of forkhead transcription factor FoxP3, as removal of FoxP3 diminishes their ability to suppress, whilst addition of FoxP3 to CD25 negative effector T cells confers suppressive function upon them

(Fontenot, 2003; Hori, 2003; Wan, 2007). Recent studies have shown that TGF-β, in the presence of IL-4 re-programmes Th2 differentiation and leads to the development of a new population of Th9 cells that produce IL-9 and IL-10 (Dardalhon, 2008). Th9 cells lack suppressive function and promote tissue inflammation. Adoptive transfer of Th9 cells into recombination-activating gene 1-deficient mice induced colitis and peripheral neuritis (Dardalhon, 2008). Follicular helper T cells (T_{fh}) cells can develop independently of other effector T cell subsets but almost certainly also derive from T cell types such as Th1, Th2 or Th17 cells. T_{fh} cells provide help to B cells and support antibody class switching in germinal centres and are mostly defined by their follicular localisation, which is dictated by the expression of CXC chemokine receptor 5 (CXCR5).

Although classically viewed as distinct lineages, studies have called into question whether helper $CD4^+$ T cell subsets are really distinct lineages, and if so how plastic are they in modifying their committed state (O'Shea, 2010). There are now abundant examples of this flexibility in terms of cytokine production (Zhou, 2009). For example, IL-10 was first recognised as a Th2-type cytokine; however, it is known that Th1, Th17 and T_{reg} can all produce IL-10. Similarly, T cells that express FoxP3 and RORγt, transcription factor associated with Th17 cell differentiation, have also been described. $FoxP3^+T_{reg}$ that become IL-17 producers can express the Th1 cell-associated transcription factor Tbet or make IFN-γ (Xu, 2007). Table 1.1 summarises each CD4 T helper cell and their functions.

Table 1.1 Summary of T cell subsets.

T cell subset	Cytokines produced	Transcription factor	Polarising Signals	Effector Function
Th1	IFN-γ, IL-2, LTα	Tbet/Stat4	IL-12, IFN-γ	Macrophage activation, autoimmunity
Th2	IL-4, IL-5, IL-13, IL-25, Amphiregulin	GATA-3/Stat5	IL-4, IL-2	Extracellular parasites, allergy and asthma
T_{reg}	TGF-β, IL-35, IL-10	FoxP3/Stat5	TGF-β, IL-2	Immune tolerance, regulation of immune responses, lymphocyte homeostasis
Th17	IL-21, IL17a, IL-17, IL-22	RORγt/Stat3	TGF-β (IL-1), IL-6, IL-21, IL-23	Extracellular bacteria, fungi Autoimmunity
Th9	IL-9, IL-10	Not known	TGF-β, IL-4	Tissue inflammation
T_{fh}	IL-21, IL-6	BCL6	CXCR5, IL-6, IL-21	Autoimmunity
Th22	IL-22, TNF-α, IL-13	RORC	IL-6, TNF-α	Inflammatory skin disorders

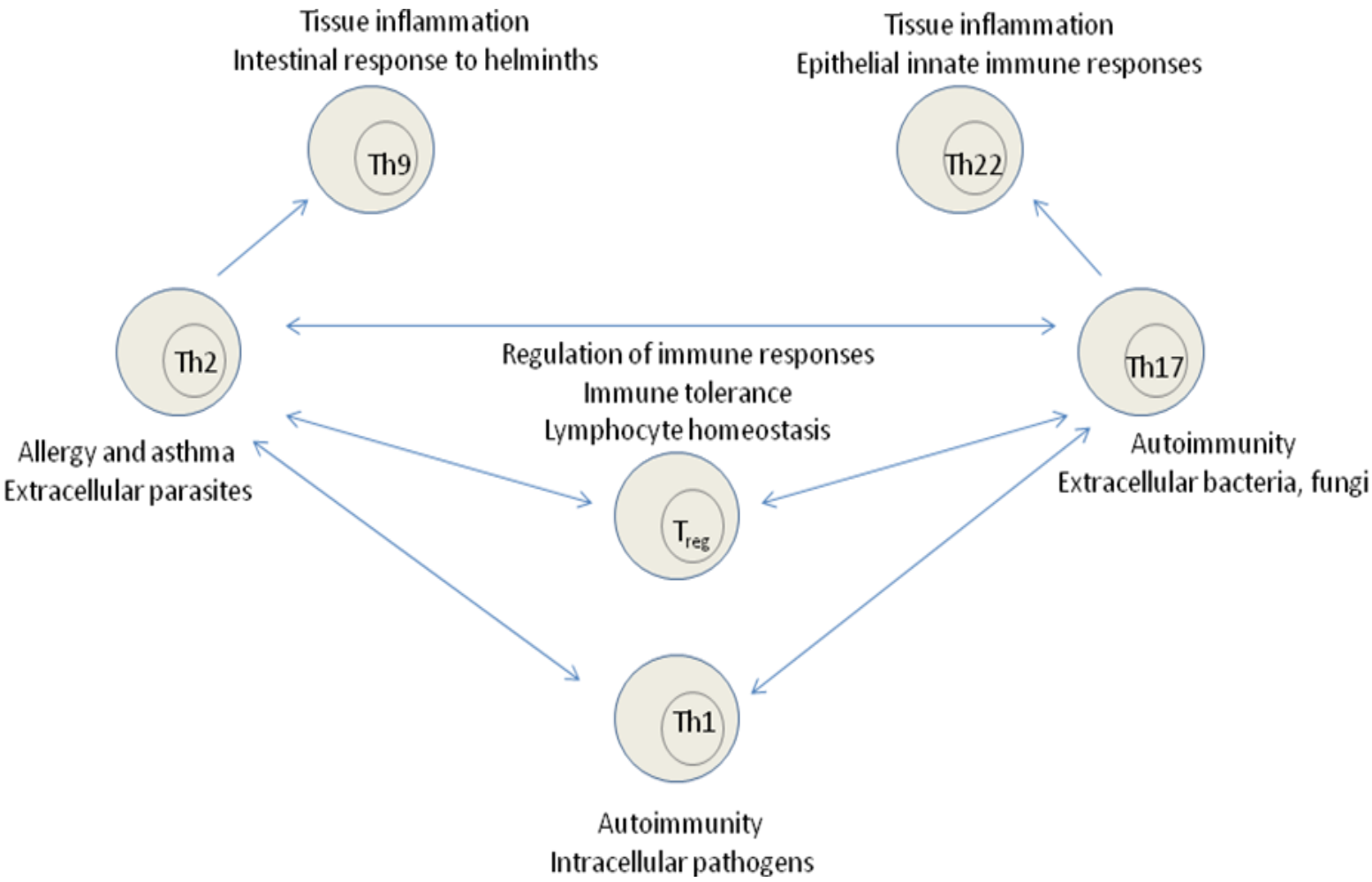


Figure 1.1 Summary of CD4 T helper cell fates and their functions

1.2 INFLAMMATION, REMODELLING AND REPAIR IN THE AIRWAYS

At the exposed external surfaces of the body such as the airways, the epithelium and immune system interact to defend against the numerous potentially harmful physical, chemical and biological agents present in the environment (Eder, 2006). The human lung is comprised of a large surface area for gas exchange, and is forty times the surface of the next largest epithelial organ, the skin (Warburton, 1998). It presents an estimated area of $100m^2$ that comes into contact with approximately 10,000L of environmental air during normal breathing each day (Elad, 2008). The bronchial epithelium acts as a semi-permeable barrier between the external environment and the inner bronchus, and is, therefore, particularly vulnerable to damage. There exists an array of cellular defences designed to limit the continual and inevitable threat of damage arising from the interaction between insult, and the tissue's molecular and cellular components. The cells of the innate, adaptive immune system and epithelium produce mediators that constitute airway inflammation (Holgate, 2000; Hackett, 2007).

Insult or disruption to the mature airway epithelium, as a result of inhaled particles, pathogens or inflammatory respiratory disease, must be repaired without delay. In the absence of repair, extensive changes in the architecture of the airway walls occur. Damaged cells are shed into the airway lumen, and the basement membrane becomes exposed. Epithelial cells at the edge of the "wound" or damaged area proliferate, flatten and migrate over the denuded basement membrane in order to protect the exposed airway (Erjefalt, 1997; Sacco, 2004). This process, termed "remodelling" of the epithelial structure is accompanied by goblet cell hyperplasia, immune cell recruitment, proliferation of fibrocytes (fibrosis) and proliferation of smooth muscle cells which leads to extracellular matrix (ECM) deposition. These processes all function to 'seal' the exposed site of injury (Erjefalt, 1997), re-establish

epithelial integrity and eventually restore normal airway function (Sacco, 2004) if appropriately controlled.

The airway epithelium is not inert in this process however. In asthma, the airway epithelium is a major source of cytokines and chemokines that are strongly implicated in maintaining asthmatic inflammation including IL-5, RANTES, eotaxin, macrophage chemotactic peptides and TSLP which are all potent stimuli for polarising the immune response towards Th2 (Bals, 2004), thus demonstrating how the epithelium can provide a microenvironment to sustain ongoing Th2-like inflammation (Bals, 2004). Following injury of the lung epithelium, the regeneration and repair process begins rapidly where damage recruits the cells involved in regeneration, inducing their proliferation, differentiation and recruitment to the site of injury (Sacco, 2004). The repairing epithelium sees a transitory squamous metaplasia followed by progressive re-differentiation in order to restore a pseudostratified and mucociliary epithelium (McDowell, 1979). During chronic inflammatory diseases, restoration of the epithelial tissue architecture by the above mechanism is diminished. It is suggested that the 'normal' epithelial repair processes may be overwhelmed and alternatively contribute to the pathogenesis of inflammatory airway disease (Erjefalt, 1997). Increased ECM deposition, immune cell infiltration, fibroblast and airway smooth muscle cell components, airway obstruction and a decrease in airway function are all common clinical pathophysiological features found in many inflammatory lung diseases including asthma, pulmonary fibrosis and adult respiratory distress syndrome (Holgate, 2000). These abnormal pathophysiologies remain unresolved, and may result in permanent loss of airway function and chronic disease.

1.2.1 ASTHMA AND ALLERGIC DISEASE

Asthma is one of the most common chronic inflammatory diseases and affects an estimated 300 million people worldwide (Galli, 2008). The pathogenesis of allergic asthma remains unclear; however, the current understanding is that allergic asthma involves two components: an aberrant remodelling response and the expansion of $CD4^+$ Th2 allergen driven inflammation. Genetic predisposition, coupled with environmental influences, appear to affect the regular suppression of Th2-mediated responses (Holgate, 2008). It has been hypothesised that abnormalities in the maturation of the lung during foetal and neonatal development may also render the airways more susceptible to environmental allergens, favouring polarisation towards the Th2 phenotype and thus, predisposing the individual to atopy and asthma (Holgate, 2000; Wark, 2005). Allergen-driven production of IL-4, IL-5 and IL-13 are typical of allergic pathologies and the secretion of such Th2-cytokines initiates isotype class-switching of B cells towards IgE and recruitment of eosinophils to the airways (Holgate, 2008). An important and consistent feature of chronic asthma is the production of excess mucus that blocks peripheral airways and is difficult to expectorate. The pathophysiology of asthma is complex and influenced by several factors, including genetic susceptibility (Finkelman, 2010), the nature of the antigen that initiates the response and the possible co-exposure with bacteria or viruses which stimulate the innate immune response (Wenzel, 2006).

1.2.2 THE PATHOLOGY ASSOCIATED WITH ASTHMA

Histopathological studies in asthmatic patients have established that asthma is a disease involving the central and peripheral airways (Saetta, 2001). The process includes structural (remodelling, thickening of airway wall) and cellular changes (inflammation at the airway wall). There is considerable overlap between the airway remodelling, and airway inflammation. Airway remodelling encompasses numerous alterations to the

airways including epithelium detachment (Naylor, 1962), an increase in the mass of airway smooth muscle, goblet cell and submucosal gland enlargement (Carroll, 1993), airway fibrosis, decreased cartilage integrity (Haraguchi, 1999), proliferation in blood vessels and airway edema (Sumi, 2007). These all contribute to a thickening of the airway wall. Goblet cell metaplasia involving the conducting airways (Ordenez, 2001) is common, however in chronic persistent disease, goblet cells also spread down to the more peripheral airways where they normally do not exist (Shimura, 1996). In addition, submucosal glands are larger and contain a greater proportion of mucin compared with serous cells (Green, 2010). These changes are linked to an ongoing chronic inflammatory process that involves the differentiation of $CD4^+$ T cells leading to the migration and activation of eosinophils, neutrophils and mast cells (Swain, 1990; Metcalfe, 1997). Recent data suggest that airway remodelling is not a direct effect of Th2 cytokine secretion, but due to abnormal injury and repair mechanisms arising from a genetic susceptibility to innocuous environmental allergens (Holgate, 2000; Wark, 2005; Holgate, 2007). The epithelial-mesenchymal trophic unit (EMTU) is thought to become reactivated due to over-expression of TGF, EGF and other nerve growth factors, thus accounting for airway remodelling. This has sparked debate over whether development of allergic asthma is due to a primary abnormality or whether it is acquired later in life.

Airway hyperresponsiveness (AHR) refers to the exaggerated response of the airways to external stimuli and is a key feature of asthma, although it is not exclusive to asthma, as AHR can present in other chronic airway diseases including cystic fibrosis (van Haren, 1995) and chronic obstructive pulmonary disorder (Postma, 1998). AHR is measured by assessing the lung response to inhaled stimuli, such as histamine or methacholine. The development of AHR is still poorly understood. Airway remodelling and inflammation contribute to AHR however none are directly implicated in its' onset (Wilder, 1999).

Airway inflammation is a multicellular process where an overabundance of eosinophils is the most striking feature (Kay, 2005). Eosinophils contribute to the allergic response by selective migration to specific sites within inflamed tissue through interactions between adhesion molecules on their surface and counter-ligands on tissue structures. Following accumulation at sites of inflammation, activated eosinophils degranulate to release cationic proteins and lipid mediators associated with local tissue damage and airway hyperreactivity (Gleich, 1988; Djukanovic, 1990). Ultrastructural studies have demonstrated extensively degranulated eosinophils in the airway tissue during active disease (Djukanovic, 1990; Erjefalt, 1998). The release of mediators such as histamine and cysteinyl leukotrienes from eosinophils and mast cells, trigger bronchoconstriction, airway oedema and mucus secretion, collectively contributing to the chronic inflammatory response which is fundamental to asthma pathogenesis (Holgate, 2008).

1.2.3 THE IMMUNE RESPONSE IN ASTHMA

A fundamental feature of asthma is the ability to recognise innocuous environmental allergens and respond to this by generating Th2 cytokines. The dominant type of T-cell present in asthmatic airways is Th2 (Anderson, 2002), and it is the secretion of Th2 cytokines that results in the recruitment of mast cells, basophils and eosinophils. These cell types collectively contribute to the persistent chronic inflammatory response. This results from engagement of T cells with allergen-loaded antigen presenting dendritic cells (DC) (Mellman, 2001). A network of DC are located under the airway epithelium where they can survey the airways for invading pathogens and inhaled antigens (Holt, 1990; Huh, 2003). This process is intensified by IgE bound to high affinity receptors on the surface of antigen-presenting cells (Kitamura, 2007) including basophils which are now seen as key APC in this condition (Maddur, 2009; Sokol, 2009). When sufficiently stimulated, airway APC such as DC

migrates to the draining lymph node to induce T cell responses (de Jong, 2005). The skew towards the production of Th2 lymphocytes largely depends on the secretion of IL-12 by DC. IL-12 has an essential role in Th1 development and has therefore also been considered as a potential therapy for asthma (Kuipers, 2004). However, whilst IL-12 can counteract Th2 sensitisation, it can also contribute to maximal expression of allergic airway disease post-sensitisation phase, exhibiting enhanced recruitment of $CD4^+$ T cells and eosinophils combined with upregulation of Th2 cytokines (Meyts, 2006). Once sensitised, T cells migrate back to the airways to the site of antigen presentation under the influence of chemokines including CCL11, CCL24, CCL26, CCL7, CCL13, CCL17 and CCL22, where they secrete a range of inflammatory cytokines such as IL-4, IL-5, IL-6, IL-9 and IL-13 (Kay 2006). Macrophages, dendritic cells, smooth muscle and epithelial cells produce IL-1β and $CD4^+$ T cells further enhance antigen induced T cell proliferation through the production of IL-2 (Anderson, 2002; Schmitz, 2003; Dragon, 2006).

It is thought that adjuvant signals from airway epithelium, generated in response to inhaled stimuli, influence the migration and maturation state of DC and T cells, and helps determine whether a particular antigen will trigger a T cell-mediated inflammatory response (Herrick, 2003; Huh, 2003; Piggott, 2005). After the initial sensitisation to an allergen, re-exposure results in a rapid exacerbation of allergic airway inflammation. Inhaled allergen binds to preformed IgE and IgG in the airways and stimulates mast cells and macrophages to release histamine and produce leukotrienes, TNF, and Th2 cytokines (Djukanovic, 1990). Not only are cytokines involved in maintaining the chronic inflammatory process, they are also responsible for the initiation of the early stages of this process. It is not simple to classify the numerous cytokines that are potentially involved in asthma because of their pleiotropic nature and overlapping properties. The early-phase of an asthmatic reaction is

initiated within minutes, and is subsequently followed several hours later by a late-phase reaction characterised by an influx of eosinophils and memory T cells into the airways.

1.2.4 T CELL SUBSETS IN ASTHMA - BEYOND TH2

Depending on the nature of the stimulus, naïve T cells differentiate into T_h1, T_h2, T_h17, the recently discovered T_h9 effector cells or indeed regulatory cells. The role of Th2 cells in controlling and perpetuating chronic inflammation in asthma has received much attention and has been described above. Zhao *et al* have recently suggested a link between Th17 cells and the pathogenesis of allergic asthma (Zhao, 2010) where increasing levels of these cells correlated with increasing severity of the disease in asthmatic patients. Levels of IL-17 mRNA were increased in the sputum of asthmatics (Bullens, 2006), whilst an increase in the production of IL-17 in allergic asthmatic individuals was also observed in response to allergic stimuli (Barczyk, 2003, Hashimoto, 2005, Molet, 2001). Similar results have been seen in mice, where sensitisation induced a strong Th17 response in combination with airway inflammation, thus demonstrating a role for IL-17 in allergic airway inflammation (He, 2009). However, a dual effect of IL-17 in allergic asthma has also been demonstrated which shows that it is essential during sensitisation in order to establish disease, but negatively regulates established allergic responses via inhibition of DC and chemokine synthesis (Schnyder-Candrian, 2006). Mucosal IL-17 administration inhibited asthma by reducing the production of IL-5 and the chemokines TARC and eotaxin, which control eosinophil recruitment and asthma (Schnyder-Candrian, 2006). The potential role of regulatory T cells in asthma is discussed below.

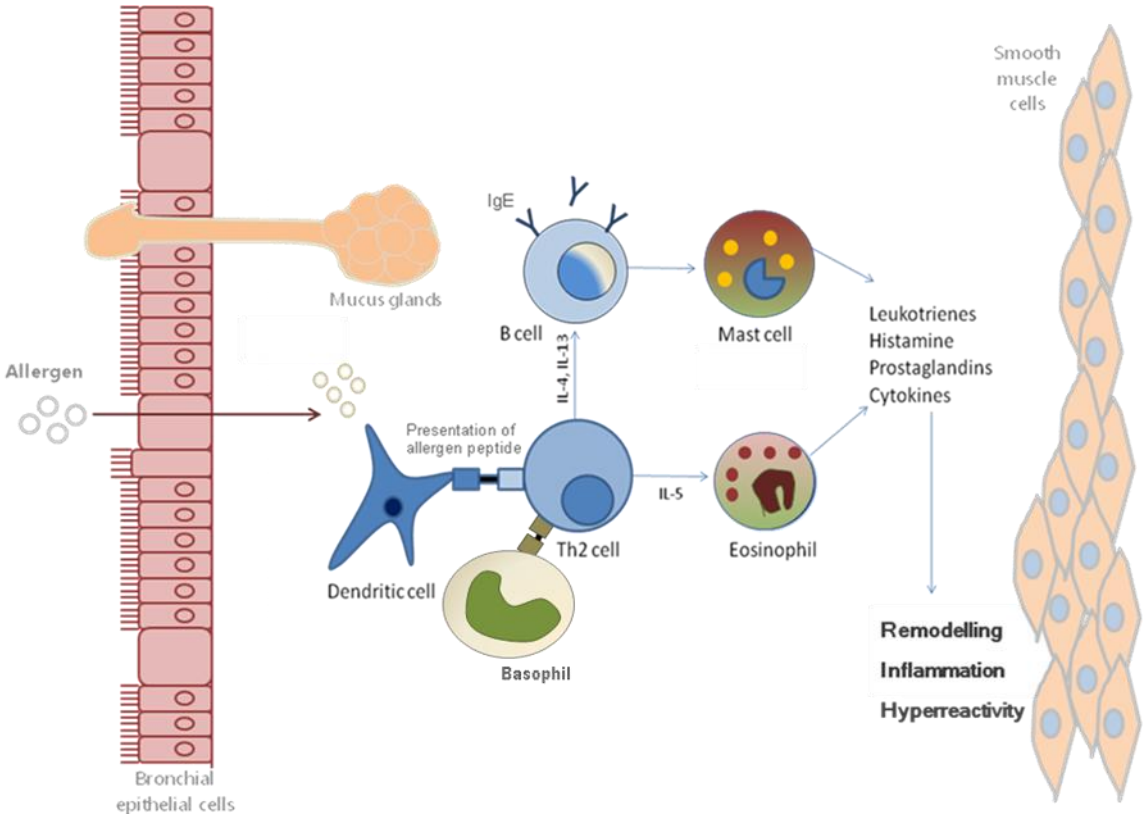


Figure 1.2 Inflammatory cells involved in allergic asthma. Repeated chronic allergen exposure leads to bronchial inflammation, lung remodelling and hyperreactivity. Following allergen uptake and processing by antigen-presenting cells (APC), allergen peptides are presented via MHC class II to Th2 T cells contributing to the secretion of Th2 cytokines. This leads to the activation of mast cells by allergen-specific IgE and the migration of eosinophils through the bloodstream to the lung mucosa, where they induce bronchial epithelium damage via the secretion of cytotoxic by-products. Th2 cytokine-dependent inflammation and epithelial activation results in airway remodelling, characterized by thickening of the basement membrane, mucus hypersecretion and smooth muscle cell proliferation.

1.3 THERAPEUTIC INTERVENTION IN ASTHMA

Asthma cannot be cured by any current medication; however symptoms can be suppressed and alleviated. Pharmacotherapy remains the cornerstone of treatment (British Thoracic Society, 2008) but once the drug is discontinued, the disease manifestations are likely to return (Guilbert, 2006). Table 1.2 provides an overview of the medications currently used in the management of asthma. Inhaled corticosteroids (ICS) are general anti-inflammatory agents that specifically target airway inflammation, whereas β-agonists are bronchodilators with a different but complementary mechanism of action that target bronchoconstriction of the airway smooth muscle (Lulich, 1988). Short-acting β-agonists provide immediate symptom relief, whilst long-acting β-agonists provide more sustained respite and are generally used in combination with ICS therapy Leukotriene modifiers block the leukotriene pathway (proinflammatory lipid mediators that promote airway smooth muscle contraction, among other inflammatory activities) and demonstrate both anti-inflammatory and broncho-dilating activity. Omalizumab, a recombinant humanised monoclonal anti-IgE antibody binds specifically to free IgE blocking the subsequent downstream cascade of inflammatory events (Hanania, 2008). However, long-term usage of pharmaco or chemotherapies does not appear to induce fundamental changes in immune responsiveness or alter the natural history of asthma (Bisgaard, 2006; Guilbert, 2006). Therefore, the need to develop strategies to permanently reverse immunologic reactivity and chronic airway inflammation has become increasingly important.

Medication class	Examples	Mechanism	Mode of administration
Long-term control mechanisms			
ICS	Budesonide, ciclesonide	Anti-inflammatory	Inhaled once or twice daily
LABAs	Salmeterol, formoterol	Bronchodilatory	Inhaled twice daily
Leukotriene modifiers	Montelukast, zileuton	Anti-inflammatory & bronchodilatory effects	Oral
Immunomodulators			
Anti-IgE	Omalizumab	Immunomodulatory/anti-inflammatory	Subcutaneous injection
Mast cell stabilisers	Cromolyn/nedocromil	Anti-inflammatory	Inhaled 4 times daily
Methylxanthines	Theophylline	Bronchodilatory (may have mild anti-inflammatory effects	Oral
Quick-relief agents			
SABAs	Albuterol, levalbuterol	Bronchodilator	Inhaled every 6 hr
Anti-cholinergics	Ipratropium bromide	Bronchodilator	Inhaled every 6 hr
Oral corticosteroids		Anti-inflammatory	Oral

Table 1.2 medications currently used in the management of asthma

1.3.1 IMMUNOTHERAPY

Specific-immunotherapy (allergen-SIT) has been used as a desensitising therapy for almost a century. The first study to inhibit grass pollen allergy by subcutaneous injection of grass pollen extract was demonstrated in 1911 (Noon, 1911). Allergen-SIT involves the administration of repeated and increasing doses of the sensitising allergen. Continuous treatment establishes a state of anergy in peripheral T cells. This T cell tolerance is characterised by the generation of allergen-specific T_{reg} cells which can suppress proliferative and cytokine responses (Francis, 2003; Akdis, 2006) which represents an essential step in the healthy immune response to allergen. Peripheral T cell tolerance is initiated by the increased autocrine effect of allergen-specific T_{reg} that produce high levels of anti-inflammatory cytokines IL-10 and TGF-β (Akdis, 1998; Jutel, 2003). Novel and promising SIT vaccines use recombinant proteins, peptides and hybrid allergens in an effort to improve efficacy and safety. The use of short peptide epitopes that do not allow for IgE cross-linking work to bypass the typical IgE-binding process, targeting instead pinocytic and phagocytic antigen uptake mechanisms in dendritic cells and macrophages (Akdis, 2001). This induces T cell tolerance involving T_{reg} without the effector cell degranulation effect that occurs as a result of IgE-facilitated antigen presentation. As a result, this allows higher doses of allergen to be administered without the risk of anaphylaxis (Akdis, 2001).

1.3.2 INFECTION AND ASTHMA: THE HYGIENE HYPOTHESIS

The reasons for the increasing prevalence of allergic respiratory diseases in developed countries and in undeveloped countries that develop a Western lifestyle remain unclear (Eder, 2006). However, the reported increase in atopy inversely correlates with a steady decline in the extent to which people in Western societies are exposed to infectious diseases such as

whooping cough, measles, tuberculosis, and influenza (Cookson, 1997). Allergic diseases appear to increase with advancing socioeconomic development and occur more frequently in industrialized countries than in developing areas (Eder, 2006). Whereas, a higher level of exposure to pathogens associated with an agricultural lifestyle prevent the development of allergic disorders in children (Von Ehrensetin, 2000). The lack of stimulation of Th1 cells in response to viral or bacterial infection with a subsequent deviation to Th2 immune responses founded the biological basis of the hygiene hypothesis, first theorised by Strachan in 1989, suggesting an association between an increasing prevalence of allergic diseases with decreased exposure to infectious agents in early childhood (Strachan, 1998). The reduced exposure to infection was linked to numerous variants including; a reduction in family size, less exposure to pets, and "higher standards of personal cleanliness".

Viral but not all bacterial respiratory infections precipitate reactive airway symptoms (Johnston, 1995). Lipopolysaccharide (LPS) or endotoxin is a major component of the outer membrane of ubiquitous gram-negative bacteria. Gram-negative infections constitute a significant proportion of clinical respiratory tract infections among children in early life, thus, there is ongoing and chronic environmental exposure to gram-negative organisms and their products. Gereda *et al* reported the first direct *in vivo* evidence that environmental exposure to LPS early in life (i.e. before polarised Th cell responses are established) protects against allergen sensitisation (Gereda, 2000). This study found significantly lower concentrations of LPS in the homes of allergen-sensitised infants when compared to those of non-sensitised infants and that this reduction was associated with a decrease in IFN-γ-producing Th1 cells. Since $CD4^+$ Th2 cells co-ordinate some allergies, it was suggested that the induction of counterbalancing responses might prevent the subsequent development of atopic disease (Strachan, 2000; Romagnani, 2004). LPS was suggested as a potential regulator of the allergic response. Microbial exposure activates innate immune pathways that alter Th1, Th2

and T_{reg} responses. This results in the suppression of T helper 2 cell expansion, and a consequent inhibition of isotype switching to IgE. Thus, the triggering of normal postnatal maturation through exposure to infections and/or commensal microbial stimuli, particularly in the gastrointestinal tract (Sudo, 1997), helps to skew the immune response away from allergic phenotype and towards the normal non-atopic immune response (Liu, 2001).

1.3.3 ASTHMA AS A DISEASE OF ABSENT REGULATION

Regulatory T cells have been recognised as playing a major role in asthma (Suto, 2001; Strickland, 2006). The primary function of regulatory T cells (T_{reg}) is to control immune responsiveness. Sakaguchi *et al* demonstrated that depletion of $CD4^+CD25^+$ T cells in mice resulted in the development of autoimmune pathology, which was prevented by the re-introduction of this cell type (Sakaguchi, 1995). T_{reg} are active in several disease models and have been shown to reverse established inflammation (Akbari, 2002; Najafian, 2003; Kearly, 2008). Naturally occurring T_{reg} are thymic-derived and are thought to play an important part in immune tolerance (Sakaguchi, 2005). A second population of $CD4^+CD25^+$ T_{reg} can be induced and expanded both *in vitro* and *in vivo* through antigenic stimulation (Bluestone, 2003). Both subsets have been shown to regulate immune response through IL-10 and TGF-β-dependent mechanisms (Jutel, 2003). The role of T_{reg} in asthma remains controversial with conflicting reports (Suto, 2001; Jaffar, 2004), however the general consensus is that expansion of T_{reg} is inversely related to lung inflammation and disease progression (Sakaguchi, 2007). In the final phase of chronic inflammation, the induction of antibody production by B cells occurs; eosinophils, neutrophils, mast cells and macrophages become activated and exert individual effector functions; airway remodelling, loss of cell function and apoptosis. As all of these events require the activation of effector T cells, peripheral tolerance is considered vital for a healthy immune response and represents a

mechanism to overcome chronic inflammation mediated by these T-cell subsets (Parijs, 1998).

The precise function and mechanism of action of each regulatory T cell subset has yet to be determined, however major subsets of T_{reg} including $CD4^+CD25^+$ Foxp3 T_{reg}, $CD8^+$TCRγδ and IL-10-producing T_{reg} have demonstrated protective effects in models of allergic airway inflammation. There is also evidence that the numbers or function of these subsets may be deficient in patients with atopic allergic disease (Grindbacke, 2004; Ling, 2004; Isogai, 2007), suggesting that these T cells have the potential to suppress pathogenic Th2 responses and that this suppression may be defective or overridden in asthmatic patients. Animal models have also allowed specific mechanisms of suppression by T_{reg} in allergic asthma to be identified (Lloyd, 2001). Transfer of antigen-specific T_{reg} from DO11.10 T cell-receptor transgenic mice into sensitised BALB/c mice prevented the development of airway hyperresponsiveness and Th2-mediated inflammation (Kearly, 2005). This suppression of allergic airway responses was dependent on IL-10 production from recipient T cells, but not from the transferred $CD4^+CD25^+$ T_{reg}. IL-10 is a cytokine with well-documented anti-inflammatory and immunoregulatory activities that acts to inhibit the synthesis of pro-inflammatory cytokines, chemokines (Fiorentino 1991) and eosinophils (Takanaski, 1994). In sensitised animals, IL-10 is effective in suppressing the inflammatory response to allergen (Zuany-Amorim, 1995).

Little is known about $CD8^+$ regulatory T cells. However, several studies have suggested that $CD8^+$γδ T cells may be influential in the $CD8^+$ T cell-mediated regulation of airway inflammation (Lahn, 1999; Isogai, 2007). These cells show tropism for mucosal epithelial cells, which is of potential importance in the epithelial repair process observed in chronic inflammatory conditions such as asthma (Wisnewski, 2001). Lahn *et al* found that while a deficiency in γδ T cells was associated with a reduction in inflammation within the

lungs, TCR-$\delta^{-/-}$ mice develop a higher level of AHR in response to antigen challenge than wild-type controls (Lahn, 1999). This indicates that these regulatory cells exert their effects downstream of AHR as opposed to acting at the level of inflammation. $CD8^{+}$ γδ T cells from naïve donor rats adoptively transferred into sensitised animals reduced BAL Th2 cytokine levels and were accompanied by a reduction in late-phase AHR and eosinophilia, partly via an IFN-γ- dependent pathway (Isogai, 2003; Isogai, 2007). These findings suggest the possibility that altered or defective $CD8^{+}$γδ T cell function may be a partial contributor to some forms of atopic asthma.

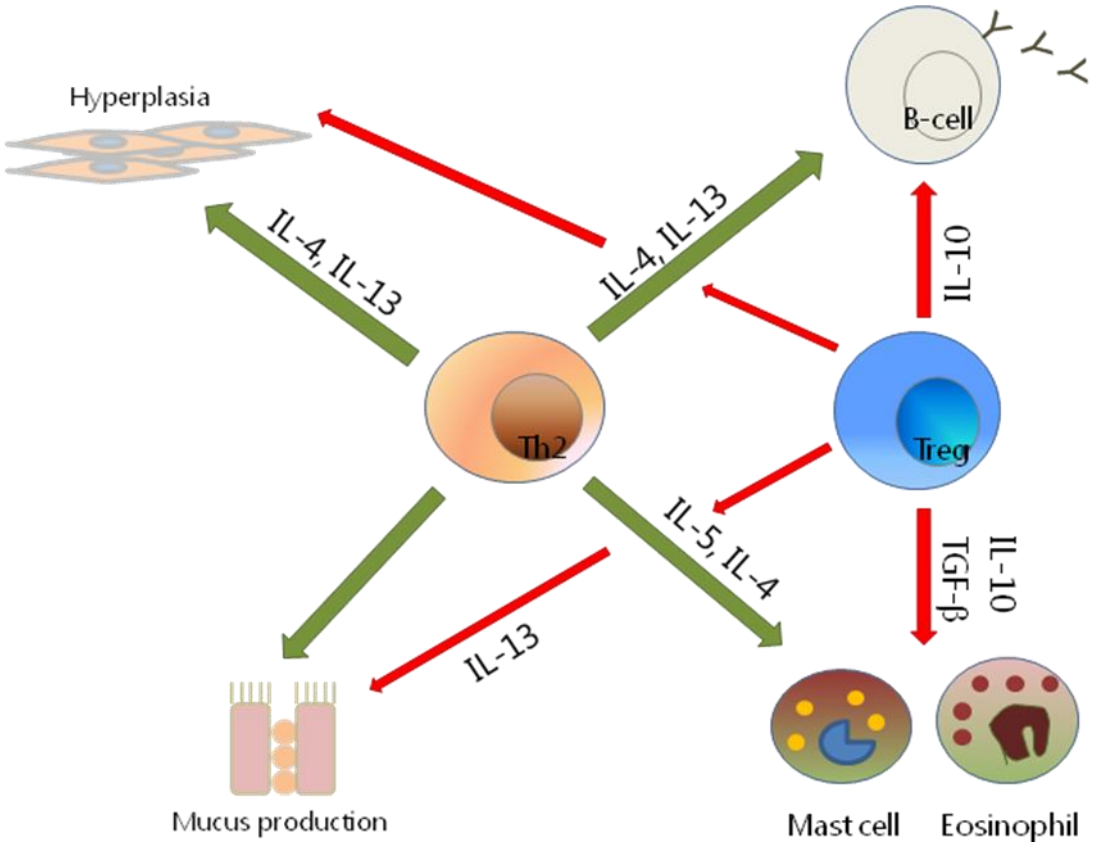


Figure 1.3 T_{reg} and the asthmatic airway. T_{reg} exert effects on multiple manifestations of allergic airway inflammation by regulating the activities of effector Th2 cells. T_{reg} secrete IL-10 and TGF-β which directly suppress IgE production and inhibit effector cells including eosinophils, mast cells and basophils. Also, Th2 cells are suppressed by T_{reg}, thus inhibiting the production of cytokines including IL-4, IL-5 and IL-13. These cytokines play critical roles in the differentiation and survival of mast cells, eosinophils and mucus-producing goblet cells. Green line indicates stimulation, red line indicates suppression.

1.3.4 MURINE MODELS OF ASTHMA

Animal models are useful in defining immunological mechanisms of asthma and allergic diseases. Mice do not spontaneously develop asthma, but using different exposure models certain features concordant with allergic asthma can be induced (Taube, 2004). Induction of disease by systemic immunisation with allergen in combination with adjuvant, e.g. aluminium hydroxide, leading to allergen-specific Th2 immunity, is the most common method used in mouse models (Takeda, 1997). Sensitised animals can then be exposed to respiratory challenge, via inhalation, intranasal or intratracheal routes, with allergen which results in an initial, but transient neutrophilic inflammatory response (Taube, 2003). This is followed by sustained lung eosinophilic infiltration, elevated levels of antigen-specific serum IgE, mucus hypersecretion and inflammation, increased airway hyperreactivity and an increase in Th2 cytokine levels which are all hallmarks of the human disease. Mouse models mimic clinically important human disease and allow the investigation of cellular and soluble mediators that can modulate the allergic immune response.

1.3.5 VACCINATION AND ASTHMA

The association between vaccination and the risk of atopic disease was first proposed by Odent *et al* in 1994 (Odent, 1994). Since then, studies have suggested that immunisation can play both protective and inflammatory roles, depending on the type of vaccine and the age at which it is administered (Farooqi, 1998; Martignon, 2005; Bernsen, 2003; Destefano, 2002; Garcia-Marcos, 2005; Gruber, 2008). There is a consensus that common vaccines including tetanus, diphtheria and pertussis are protective against airway hyperresponsiveness and allergen-induced sensitisation. Grüber *et al* demonstrated that vaccination of mice with a combined dose of diphtheria and tetanus prior to ovalbumin sensitisation, exhibited a statistically significant decrease in airway inflammation, and also in the production of

allergen-induced IgE. Further, Martignon *et al* demonstrated a reduced risk of atopic disease in those vaccinated against diphtheria, tetanus and pertussis compared with a control proband (Martignon, 2005). These findings concur with those of other investigators where a weak protective effect against asthma after pertussis vaccination was observed **(**Gruber, 2006). One particular study, Nilsson *et al*, demonstrated an 8% reduction in the risk of atopic disease in a randomised trial. Further studies (Henderson, 1999; Destefano, 2002) showed no significant difference in the incidence of early wheezing or later onset wheezing as a result of whole cell *pertussis* vaccination in the first 6 months of life. Two independent population-based studies published in 2005 reported a possible atopy-protective effect of immunization (Garcia-Marcos, 2005; Martignon, 2005). *B. pertussis* infection has been shown to exacerbate airway pathology in a murine model of allergen driven-inflammation despite induction of Th1 immunity (Ennis 2004). In contrast, systemic immunisation with Th1-inducing whole-cell pertussis vaccines inhibits allergic airway responsiveness (Ennis, 2005). Similarly, the acellular pertussis vaccine protects against *B. pertussis*-induced exacerbation of allergic asthma, but does induce IL-13 both at a systemic and local level (Ennis, 2005).

1.4 *BORDETELLA PERTUSSIS*, A WELL ADAPTED HUMAN RESPIRATORY PATHOGEN

B. pertussis is a Gram-negative bacteria and the causative agent of whooping cough, a severe respiratory disease that remains responsible for significant morbidity and mortality in infants worldwide (He, 2008). The illness begins with colonisation of the respiratory tract, followed by the catarrhal stage. This is the most contagious phase where infected individuals present with conjunctival irritation and a slight cough. After 7-10 days, the paroxysmal stage ensues and can last from 2 to 6 weeks (Heininger, 2001). It consists of the characteristic cough where a series of rapid forced expirations is followed by an inspiratory gasp, or "whoop". In neonates and young infants, the disease may present with apnoea and cyanosis

rather than coughing. The disease is most severe and life-threatening at this age with more recurrent complications, including pneumonia, seizures, encephalopathy and secondary respiratory infections (Smith, 2000). In non-fatal cases, mucus hypersecretion is a common pathological characteristic. The pathology from post-mortem observations includes epithelial and ciliary damage, bronchopneumonia and pulmonary edema.

1.4.1 VIRULENCE FACTORS OF *B. PERTUSSIS* AND IMMUNOMODULATION

Bordetella pertussis colonises the respiratory tract using numerous antigenic components, which are involved in the pathogenicity of the bacterium. *Bordetella* produces protein toxins, including pertussis toxin (PT), adenylate cyclase toxin (ACT), filamentous hemagglutinin (FHA), dermonecrotic toxin (*dnt*), and non-protein toxins, such as endotoxin and tracheal cytotoxin (TCT) which are fragments of the *Bordetella* peptidoglycan (Locht 1999). In addition, it produces numerous adhesion factors whose function is to attach the bacteria to the ciliated epithelium of the upper and lower respiratory tract. FHA is primarily involved in mediating this adherence to host cells (Babu, 2001). It also triggers several immune modulatory responses including the secretion of both inflammatory and anti-inflammatory cytokines by macrophages (McGuirk, 2000) as well as promoting the generation of regulatory T cells that suppress protection Th1 responses during infection (McGuirk, 2000). FHA exhibits a number of binding activities, including carbohydrate, heparin sulphate and integrin binding (Locht, 1993). However, more recent studies have suggested the contribution of multiple factors to the binding process, including ACT. Secreted ACT modifies a heparin-inhibitable carbohydrate binding domain of FHA which ultimately enhances its' binding ability to cultured lung epithelial cells (Perez, 2006). ACT is required to initiate infection; however, pertussis toxin is required for bacterial colonisation (Khelef, 1994). Comparable to PT, ACT has two distinct domains which possess different

functions. The first conducts enzymatic action, whilst the second has the ability to haemolyse red blood cells (Zaretzky, 2002). ACT binds to, and penetrates host cells which suppress their bactericidal functions by converting cellular ATP to cAMP. This uncontrolled cAMP signalling can also drive immature dendritic cells into a semi-mature state, which may hijack them to shape the local adaptive immune response towards tolerance of the pathogen (Vojtova, 2006). ACT-deficient mutants of *B. pertussis* are more efficiently phagocytosed by human neutrophils (Mobberly-Schuman, 2005) and achieve lower bacterial loads in a mouse model of infection when compared to wild-type strains (Carbonetti, 2005). ACT can suppress anti-bactericidal activity through inhibition of chemotactic migration, phagocytosis and the production of pro-inflammatory cytokines including TNF-α and IL12p70 (Ross, 2004; Boyd, 2005; Spensieri, 2006). This results in the promotion of bacterial colonisation. Recently, it has been shown that ACT can interfere with DC functionality, reducing protective Th1 immunity and triggering a polarisation towards a Th17 response (Fedele, 2010).

Both tracheal cytotoxin and dermonecrotic toxin, described below, contribute to the toxic effects observed during the disease. Tracheal cytotoxin is responsible for destroying the ciliated tracheal epithelial host cells through the production of IL-1α and nitric oxide (Flak, 2000). It also exerts deleterious effects on neutrophils (Cundell, 1994). It is therefore believed to be involved in the bouts of coughing which is characteristic of the disease. PT has long been associated with the systemic responses related with pertussis infection, including lymphocytosis, hypoglycemia and histamine sensitivity (Munoz, 1981). It is produced exclusively by *B. pertussis* and ADP-ribosylates several heterodimeric G proteins in mammalian cells, inhibiting many cell signalling events through G protein coupled receptors. Infection with PT-deficient mutants of *B. pertussis* did not lead to airway infection demonstrating its' significant contribution to bacterial growth in the respiratory tract and

inhibition of resident airway macrophages (Carbonetti, 2004; Carbonetti, 2007; Carbonetti, 2003). PT also inhibits early neutrophil influx to the airways after infection by suppressing the early production of the neutrophil-attracting chemokines KC, LIX and MIP-2 by airway macrophages and epithelial cells (Andreasen, 2008). This toxin exerts numerous immunosuppressive effects including the inhibition of serum antibody responses to *B. pertussis* antigens following infection (Mielcarek, 1998; Carbonetti, 2004), reduction of MHC class II molecules on the surface of monocytes and the modulation of surface markers on DC (Martino, 2006). Therefore, it has been suggested that PT promotes and prolongs *B. pertussis* infection by multiple mechanisms, exerting effects on both innate and adaptive immunity.

1.4.2 PERTUSSIS VACCINES

To date, two specific types of pertussis vaccines have been employed in infant immunisation programmes. Whole-cell pertussis vaccine (Pw) is composed of a suspension of killed B. *pertussis* cells. Pertussis infection rates decreased dramatically with the introduction of Pw in combination with diphtheria and tetanus toxoid. It has demonstrated an efficacy of ~80% and has been effective in markedly reducing the incidence rates in countries with good immunisation coverage (Burton, 2009). However, due to fact that undesirable components such as endotoxin cannot be eliminated during whole-cell vaccine production, an acceptable level of potency is inevitably associated with a greater incidence of adverse effects (Manclark, 1984). Fever, redness, swelling and pain at the site of injection are common. More severe complications including encephalopathy and permanent brain damage led to safety concerns. Therefore, in spite of conferring good, long-lasting immunity, the degree of reactogenicity associated with it prompted the development of acellular pertussis vaccines (Pa) (Deloria, 1995) which was as efficacious as the whole-cell vaccine but had a much better

profile as far as the mild-to moderate adverse reactions were concerned. The acellular vaccine contains purified, detoxified pertussis antigens, typically including those extracted from *B. pertussis* organisms, as well as those produced by genetic recombinant technology (Singh, 2006). Four antigens were identified as suitable for inclusion in such vaccines: PT, FHA, pertactin, and fimbrial antigens serotype 2 and 3. The vaccine may contain one or all of the above components. Repeated administration of Pa can cause extensive swelling at the site of injection (Rennels, 2003). In approximately 5 % of the cases, this swelling involves almost the entire limb and lasts for more than a week. Although the mechanism of this swelling has not been characterised yet, it has been proposed to be due to an Arthus hypersensitivity reaction caused by high antibody levels induced by the primary immunisation (Robbins, 2005). Whilst the replacement of first generation whole-cell vaccines by new acellular pertussis vaccine in many countries has significantly reduced the systemic adverse reactions observed with whole-cell vaccines, it has not abolished the need for repeated vaccination to achieve protection.

1.4.3 AN ATTENUATED *B. PERTUSSIS* VACCINE, BPZE1

Recently, a genetically-attenuated live vaccine against *B. pertussis*, BPZE1, has been developed as a candidate neonatal vaccine (Mielcarek, 2006). This live recombinant *B. pertussis* strain induces strong local and systemic immune responses upon intranasal delivery. Administration via the nasal route mimics natural infection and promotes long-lasting immunity in children from 1 month of age (Mascart, 2003). Three virulence factors have been targeted for attenuation; pertussis toxin, tracheal cytotoxin and dermonecrotic toxin. Attenuated BPZE1 lacks the *dnt* gene, reduced tracheal cytotoxin and produces inactive pertussis toxin. Genes encoding these toxins were deleted or replaced with genetically inactivated analogues in order to induce protection, without the severe pathology associated

with wild-type infection (Mielcarek, 2006). As described in Section 1.4.1, TCT is responsible for the destruction of ciliated cells in the trachea of infected hosts. The gene, AmpG, is responsible for internalising TCT into the cell wall of the bacteria where it is used in its' biosynthesis. *B. pertussis* AmpG was replaced with E. coli AmpG, resulting in a strain that expressed less than 1 % residual TCT activity. PT is a major virulence factor in the pathogenesis of *B. pertussis* infection; however it is composed of an enzymatically active moiety, S1, which is also a critical protective antigen. Therefore, PT was replaced with a mutated version, coding for an enzymatically inactive toxin. Finally, allelic exchange was used in order to delete DNT, a virulence factor produced by bacteria belonging to the genus *Bordetella* (Babu, 2001).

1.4.4 MURINE MODELS OF PERTUSSIS

The most commonly used animal model for pertussis infection is the mouse aerosol induction model. This model has been used extensively for studies of *Bordetella pertussis* immunity and pathogenesis (Mills, 1997; Mills, 1998; Ennis, 2004; Skerry, 2009). One limitation of this particular model is the lack of the characteristic cough observed in the human condition. However, several important human infection manifestations are reproduced in this model including multiplication and clearance of the bacteria, confinement of the infection to the respiratory tract and an increased severity of disease in young animals. The newborn pig model of *B. pertussis* infection offers several advantages over murine challenge including laboured breathing, nasal discharges and bronchopneumonia (Elahi, 2005), however the practicality of large-scale studies make the mouse model more useful in the assessment of protective immune responses elicited by *B. pertussis* infection and vaccination (Mills, 2001). In addition, this model of *B. pertussis* infection in combination

with the OVA model of allergic airway inflammation has been well-characterised in previous studies (Ennis, 2004; Ennis, 2005a; Ennis, 2005b).

1.5. NOVEL IMMUNOMODULATORY THERAPIES & ASTHMA

As the immunopathology of asthma has become better defined, novel targets have been identified and agents developed for the treatment of asthma and allergic diseases. These include strategies involved in specifically inhibiting inflammatory mediators or attempting to alter the Th1-Th2 inflammatory balance. Stem cells represent great promise as a therapeutic tool in many diseases where they have been shown to slow or even block their progression. The capacity of stem cells to repair tissue creates huge potential for use in this area.

Stem cells and progenitor cells from adult tissues represent important potential for therapeutic intervention in a number of pathological conditions. Stem cells have the capacity for self-renewal and in the case of adult stem cells, can maintain their differentiation potential throughout the life of the organism. Adult stem cells were first identified in tissues where a high rate of cell turnover exists, such as bone marrow. The bone marrow is a complex mix of cell types including those from haematopoietic, mesenchymal and endothelial origin. Their primary role is the maintenance and repair and this capacity has offered extensive opportunities in a regenerative medicine setting. For instance, adult haematopoietic stem cells have been successfully used to reconstitute bone marrow following transplants for over 30 years (Bryder, 2006).

1.5.1 MESENCHYMAL STEM CELLS (MSC)

Mesenchymal stem cells (MSC) are non-haematopoietic, pluripotent, adult stem cells. Following transplantation of MSC into the bone marrow of non-obese diabetic-severe

combined immunodeficiency (NOD-SCID) mice, they have demonstrated the ability to differentiate into osteoblasts, myofibroblasts, pericytes, endothelial cells and bone-marrow stromal cells, all of which constitute the functional components of the haematopoietic stem cell niche (Muguruma, 2006). In this way, the MSC contribute to the maintenance of haematopoiesis through active interaction with haematopoietic cells. They are also fundamental for maintaining haematopoietic stem cells in a quiescent state in the bone marrow, until appropriately stimulated to differentiate and become released into the vascular system (Benvenuto, 2007; Dazzi, 2005).

Most MSC reside in the bone marrow, where they constitute 0.01- 0.001% of cells. However, they have been found in virtually all post-natal organs and tissues in the body, including the liver, adipose tissue, heart and synovium (da Silva Meirelles, 2006). Unlike haematopoietic cells, MSC are adherent cells and are easily expandable in culture. They are characterised based on their ability to differentiate into mesenchymal lineage cells including bone, fat, cartilage and smooth muscle cells (Krampera, 2003). Although no one specific cell marker has been identified, MSC do express CD44, CD29, CD105, CD73 and CD166. The absence of CD45, CD34, CD31, CD116, CD19 and glycosporin A distinguishes MSC from haematopoietic cells, endothelial cells, endothelial progenitors, monocytes, B cells and erythroblasts respectively (Stagg, 2007). Despite possessing various multi-lineage differentiation abilities, the main therapeutic application of MSC has been immune modulation. Unlike other stem cell types, MSC can not only evade immune response but also have the ability to mediate active suppression (Glennie, 2005; Corcione, 2006; Uccelli, 2006).

1.5.2 INFLUENCE OF MSC ON IMMUNE CELLS

MSC possess the unique ability to suppress immune responses, both *in vitro* and *in vivo*. It is for this reason that MSC have broad implications for therapy in chronic inflammatory diseases. MSC can indirectly inhibit various immune responses through the activation of T_{reg} (Augello, 2007; Selmani, 2008) and the inhibition of DC maturation (Chen, 2007; Ramasamy, 2007). MSC have also exhibited regulatory effects on a broad range of immune cells including T cells, B cells and NK cells (Glennie, 2005; Keating, 2006). The proliferative function of T cells stimulated with allogeneic splenocytes, peripheral blood mononuclear cells and mitogens including concanavalin A and anti-CD3 (Rasmusson, 2005; Ryan, 2007; English, 2008) is inhibited by MSC. This inhibitory effect is not MHC-restricted as it occurs in the presence of both autologous and allogeneic MSC. Studies have shown that MSC can induce a state of T-cell anergy, where T cells cannot proliferate or release cytokines in response to antigen presentation demonstrating direct control over T cell cycle division (Zappia, 2005). MSC have also exhibited inhibitory effects on cytotoxic T cells (Rasmusson, 2003). DC play a critical role in the initiation of an immune response, and have thus sparked interest as a potential target for suppression of these immune responses to alleviate/prevent allogeneic tissue rejection. During maturation, immature DC acquire the expression of co-stimulatory markers. MSC have been shown to inhibit the maturation of monocytes into DC *in vitro* (Jiang, 2005). They are also capable of inhibiting upregulation of CD1a, CD40, CD80, CD86 and HLA-DR during DC maturation, thereby maintaining DC in an immature state (Zhang, 2004). Similar studies investigated the effect by co-culturing allogeneic human MSC with LPS and demonstrated a 50 % inhibition of tumour necrosis factor (TNF-α) production by DC (Aggarwal, 2005). MSC can also induce regulatory antigen-presenting cells expressing IL-10 (Gur-Wahnon, 2007) indicating a polarisation of DC maturation towards a suppressor or inhibitory phenotype. In addition, MSC can affect the degree of respiratory burst of neutrophils and delay the spontaneous apoptosis of resting

and activated neutrophils via IL-6 production (Raffaghello, 2008). These data suggest the putative potent anti-inflammatory and immunoregulatory effect of MSC *in vivo*.

The precise mechanisms by which MSC exert their immunosuppressive effect are not clear, however both contact-dependent and soluble factor secretion mechanisms are thought to collaborate in the immunosuppression. Several soluble factors, (outlined in Table 1.2) have been reported to be involved in MSC-mediated immune regulation, including nitric oxide, indoleamine 2,3-dioxygenase (IDO) and prostaglandin E_2 (PGE_2). IFN-γ, either alone or in combination with TNF-α, IL-1α or IL-1-β, stimulates the production by MSC of nitric oxide synthase and of chemokines that attract T cells. T cell proliferation is inhibited through the subsequent production of nitric oxide (Ren, 2008). MSC also express IDO which breaks down tryptophan, an important amino acid required for T cell proliferation. A partial role for IL-10 had been suggested by Beyth and colleagues in human MSC inhibition of alloresponses (Beyth, 2005). Other examples of soluble factors constitutively expressed by MSC including transforming growth factor-β (TGF-β), hepatocyte growth factor (HGF), IL-10 and IL-6, contribute to the regulatory function of MSC. HGF has recently been shown to negatively regulate allergic airway inflammation and hyperresponsiveness in a murine model (Ito, 2007) via direct attenuation of eosinophil chemotactic function.

Table 1.3 (below) Soluble factors and enzymes secreted by MSC and putatively linked to immunomodulation.

Soluble factors/Enzymes	***Study***
Hepatocyte growth factor (HGF)	(Nicola, 2002)
Prostaglandin E2	(Chen, 2007)
TGF-β	(Nicola, 2002), (Ryan, 2007)
Indoleamine 2,3 dioxygenase (IDO)	(Meisel, 2004)
Nitric oxide	(Ren, 2008), (Sato, 2007)

1.5.3 CLINICAL APPLICATIONS OF MSC

MSC therapies to date have mainly focused on their ability to promote engraftment of haematopoietic stem cells, to block graft-versus-host-disease (GvHD) and to help promote structural repair of damaged tissues. Despite transplantation of very few MSC *in vivo* (<1%), MSC have exhibited significant improvement in the acute GvHD score (i.e. extent of damage ranging from rash to persistent nausea, vomiting, and anorexia), supporting their possible use as a therapy for immune-mediated disease. The ability of MSC to modulate immune responses *in vivo* was first investigated by Bartholomew *et al*, who demonstrated that administration of allogeneic MSC could prolong skin-graft survival in primates (Bartholomew, 2004). Since then, several studies have targeted MSC as a therapeutic tool in animal models of lung injury, myocardial infarction, graft-versus-host disease, kidney disease, diabetes and also some neurological disorders. Selective localisation of systemically delivered MSC has been demonstrated in models of kidney failure (Ezquer, 2008), myocardial infarction (Quevedo, 2009), and neurological injury (Bouchez, 2008). MSC possess the ability to roll and adhere in endothelial cells in a P-selectin and vascular cells

adhesion molecule-1-dependent manner allowing the migration into organs such as the lung, liver and spleen (Sackstein, 2008). Ortiz *et al* have shown significantly augmented engraftment of MSC in a pulmonary fibrosis model of lung injury in mice when compared to engraftment in the lung of normal mice (Ortiz, 2003). Likewise, Wu and co-workers demonstrated vigorous migration of MSC to the site of allograft rejection in a rat model, further highlighting their putative role in tissue repair during chronic rejection (Wu, 2003). A rat model of Parkinson's disease showed significant improvement in behaviour upon engraftment of rat MSC by intra-striatal injection (Bouchez, 2008). MSC also promoted the endogenous repair of pancreatic islet cells and renal glomeruli in a mouse model of streptozotocin-induced diabetes (Lee, 2006). Co-infusion of MSC and bone marrow cells inhibited the proliferation of β-cell-specific T lymphocytes from diabetic pancreas in a mouse model and this effect was not due to the reconstitution of the damaged islet cells (Urban, 2008). It is noteworthy that many of these studies demonstrated beneficial effects of MSC in the absence of engraftment and transdifferentiation. This suggests that the multi-potentiality of MSC is not critical for their immunosuppressive effect.

An increasing number of studies have demonstrated a functional role for MSC in animal models of acute lung injury. Systemic administration of MSC in a mouse model of bleomycin-induced pulmonary fibrosis decreased lung collagen accumulation and the level of matrix metalloproteinases (Ortiz, 2003). Further studies identified IL-1 receptor antagonist, expressed by MSC, as the mediator of this effect (Ortiz, 2007). This and numerous other studies suggest that MSC can have significant immunomodulatory effects in the lung, although the mechanisms by which they achieve this remain unclear.

Table 1.4 Effect of MSC therapy in animal models of disease

Disease model	*Model*	*Outcome*	*Reference*
Acute lung injury	Mouse	Syngeneic intrapulmonary murine MSC decrease the severity of endotoxin-induced acute lung injury and improve survival in mice	(Gupta, 2007)
Myocardial infarction	Rat	Syngeneic rat MSC demonstrated an anti-inflammatory role in ischemic heart disease	(Orlic, 2001)
Heart transplantation	Rat	Allogeneic rat MSC injected intravenously migrated to the heart during chronic rejection	(Wu, 2003)
Arthritis	Mouse	Allogeneic murine MSC prevented irreversible damage to bone and cartilage via Treg generation	(Augello, 2007)
Chronic lung injury	Mouse	Syngeneic murine-MSCs protect lung tissue from bleomycin-induced injury with anti-inflammatory effect	(Ortiz, 2007)
Graft-versus-host disease	Rat	Allogeneic rat-MSCs prevent lethal GVHD 66	(Ikehara, 2003)
Renal failure	Rat	Inhibition of pro-inflammatory cytokine production and induction of anti-apoptotic and trophic factors	(Imberti, 2007)
Multiple sclerosis (EAE)	Mouse	Syngeneic murine MSC reduced inflammatory infiltrates, demyelination and axonal loss	(Zappia, 2005; Gerdoni, 2007)
Diabetes	Mouse	Human MSC grafted kidney and pancreas in STZ NOD-SCID mice ameliorating diabetes and kidney disease	(Urban, 2008)
Retinal degeneration	Rat	Decreased retinal degeneration through anti-apoptotic and trophic molecules	(Inoue, 2007)

Table 1.5 Clinical trials using mesenchymal stem cells

Clinical trial	*Condition*	*Site/sponsor*	*Status*
MSC Transplantation in Decompensated Cirrhosis	Cirrhosis	University of Tehran	Recruiting
Safety Study of Adult Mesenchymal Stem Cells (MSC) to Treat Acute Myocardial Infarction	Myocardial infarction	Osiris Therapeutics, U.S.	Ongoing, but not recruiting
Phase I Clinical Trial of the Treatment of Crohn's Fistula by Adipose Mesenchymal Stem Cell Transplantation	Crohn's disease	"La Paz" University Hospital, Madrid, Spain	Ongoing, but not recruiting
Autologous Mesenchymal Stem Cells From Adipose Tissue in Patients With Secondary Progressive Multiple Sclerosis	Multiple sclerosis	Fundacion Progreso y Salud, Spain	Recruiting
Safety and Efficacy Study of Allogenic Mesenchymal Stem Cells to Treat Extensive Chronic Graft Versus Host Disease	GVHD	Guangdong General Hospital	Not yet open for participant recruitment
Prochymal® (Human Adult Stem Cells) Intravenous Infusion Following Acute Myocardial Infarction (AMI)	Myocardial infarction	Osiris Therapeutics	Recruiting
Prochymal® phase III Clinical Trial for the treatment of Steroid-Refractory Acute GvHD	GvHD	Osiris Therapeutics	Active, not recruiting
Prochymal® Phase iii Clinical Trial for the Treatment of Newly Diagnosed Acute GvHD	GvHD	Osiris Therapeutics	Completed
Prochymal® Phase III Crohn's Disease	Crohn's disease	Osiris Therapeutics	Active, not recruiting
OTI-010 for Graft-Versus-Host Disease Prophylaxis in Treating Patients Who Are Undergoing Donor Peripheral Stem Cell Transplantation for Hematologic Malignancies	GvHD	National Cancer Institute (NCI)	Ongoing, but not recruiting
Donor Mesenchymal Stem Cell Infusion in Treating Patients With Acute or Chronic Graft-Versus-Host Disease After Undergoing a Donor Stem Cell Transplant	GvHD	National Cancer Institute (NCI)	Ongoing, but not recruiting
Mesenchymal Stem Cells in Multiple Sclerosis (MSCIMS)	Multiple sclerosis	University of Cambridge	Ongoing, but not recruiting

1.6 AIMS & OBJECTIVES

This chapter has presented the evidence that the adaptive immune system plays a central role in protecting the organism against infections (such as *B. pertussis*) but can also be detrimental in conditions such as asthma. The control or modulation of immunity is therefore critical to the normal homeostasis of the organism but can also be exploited by infectious agents, or therapeutic intervention (immunisation or cell therapy). The overall goal of the work presented in this thesis was to investigate immune modulation with a particular focus on airway inflammation and allergic pathogenesis. This was probed in a model of pathogen driven immunomodulation (*B. pertussis*), and two models of therapeutic intervention namely immunisation (BPZE1) or using a candidate cell therapy approach (MSC). These were captured in the following specific aims:

i) To determine whether a candidate live, attenuated, neonatal vaccine modulated allergic airway pathology and how this compared to virulent infection with particular focus on immune modulatory mechanisms (Chapter 3).

ii) To determine the immunomodulatory effect of a potential cell therapy based on mesenchymal stem cells. This focused specifically on the establishment of an experimental system to confidently deliver reliable and reproducible MSC populations for use *in vivo* (Chapter 4).

iii) To ascertain whether a candidate MSC therapy could modulate immunity *in vivo* sufficient to suppress allergic lung inflammation (Chapter 5).

iv) To elucidate the immune mechanisms by which MSC confer protection (if any) in this model (Chapter 6).

In this way, a greater understanding of allergic disease and the factors that affect it may be obtained to provide fundamental insight to the hygiene hypothesis and novel therapeutic

approaches to treat asthma. Examination of immunomodulatory effects in these models will also contribute to a broader understanding of immune regulation in health & disease.

CHAPTER 2

MATERIALS & METHODS

2.1 MATERIALS

All chemicals used were from Sigma-Aldrich (Poole, Dorset, UK) unless otherwise stated. Reagents/materials are described in Tables 2.1 – 2.6.

2.2 CULTURE OF *BORDETELLA PERTUSSIS*

A stock aliquot of *B. pertussis* was thawed at 37 °C and 100 µl was aseptically transferred to a plate of Bordet-Gengou agar. The suspension was evenly spread across the plate using a sterile plate spreader and then sealed with parafilm (Pechiney Plastic Packaging Company, Chicago, IL). The plate was inverted and incubated at 37 °C for 3 days. After 72 h, 1 ml of supplement was added to 100 ml of S&S medium (section 2.2.3) in a 250 ml Erlemeyer flask and incubated for 20min at 37 °C. Using a sterile cell scraper, *B. pertussis* was harvested from the plate and transferred into the prepared flask. Cultures were agitated at 37 °C for a further 2-3 days to obtain culture dividing at log – this was ascertained by measurement of optical density at 580 nm.

2.3 ANIMAL STRAINS

The following mouse strains were used: FVB.Cg-Tg(GFPU)5Nagy/J (Jackson Labs, Bar Harbour, Maine, USA), BALB/c, 129 Sv/Ev, C57BL/6 (Harlan, Bichester, Oxon, UK). All mice were housed according to Dept. of Health (Ireland) guidelines.

2.4 ETHICAL APPROVAL

All procedures involving animals were carried out by licensed personnel (License no. B100/3999). Ethical approval was received from the ethics committee of NUI Maynooth.

2.5 AEROSOL INFECTION

An aerosol chamber was used for whole body aerosol exposure. Animals were placed in the chamber and a nebuliser for the bacterial suspension was attached to the chamber. The challenge inoculum 2 x 10^{10} of *B. pertussis* (BPSM or BPZE1) was administered directly into the aerosol chamber containing no more than 10 mice over a period of 15 min. Un-infected control mice were 'sham' sensitised with PBS (Zachariadis, 2006).

2.6 IMMUNISATION & AIRWAY DELIVERY OF OVA

Ovalbumin (OVA) (grade V, Sigma) was mixed with alum for intra-peritoneal (i.p.) delivery (Imject Alum, ThermoScientific, Rockford, IL, USA). Alum was mixed at a 1:2 ratio with a 100 μg/ml solution of OVA in sterile PBS, followed by vigorous shaking for at least 1h. 300 μl of this solution was administered per mouse using a 27 G needle on day 0, 7 and 14. On days 14, 25, 26 and 27, mice also received OVA by an intra-nasal route. This was performed by first, anaesthetising the mouse with isofluorane and then placing 150 μl of 50 μg/ml OVA diluted in sterile PBS on each nostril, allowing the solution to be inhaled. Control groups were sham sensitised with sterile PBS, i.p. and i.n.

2.7 MEASUREMENT OF LUNG FUNCTION

Unrestrained whole body plethysmography (WBP) was used to study the development of airway hyperresponsiveness after allergic sensitisation in mice. Airway responsiveness was assessed by methacholine-induced airflow obstruction from conscious mice using the Buxco whole-body plethysmography chamber in conjunction with the BioSystem XA software (Buxco Electronics, USA). Enhanced pause, or PenH, was used as a surrogate of airway responsiveness. PenH is an index of respiratory signal created by calculating the sum of the nasal air flow, and flow due to changes in the thoracic volume. Under normal conditions, these flows are out of phase and should largely cancel each other out, but

obstruction causes deviation in this signal. The instrument was calibrated by forcing 1ml of air into the chamber using a syringe and adjusting amplifier readouts until the signal was in range of detection (5.0 V to –5.0 V). The mouse was then placed in the chamber which was attached to two pneumotachographs. Baseline readings were collected upon aerosol delivery of PBS. This was followed by four increasing dosages of methacholine (3.3, 10, 30 and 50 mg/ml) each lasting 5 minutes.

2.8 BRONCHOALVEOLAR LAVAGE FLUID (BALF)

Mice were sacrificed by lethal injection sodium pentobarbitol to preserve the trachea, and the respiratory tract was exposed by dissection. An incision was made near the top of the trachea with a scalpel and a 20-gauge needle was inserted and fixed with suture thread around the trachea. BALF was obtained by flushing the lungs with 1.5 ml of cold PBS and withdrawing the fluid with a 3-way stop-cock syringe. BAL fluid was passed though a 10 K membrane centrifugal filter (Amicon Ultra, Millipore, Billerica, MA, USA) to concentrate the lavaged cells. Cells were recovered by centrifugation at 300 g for 5 min, and resuspended in PBS. Supernatants were collected for cytokine analysis and stored at -80 ˚C until use. 100 µl of BAL cells were cytospun at 10,000 rpm for 10 minutes and stained with Giemsa (Riedel-deHaen) according to manufacturer's instructions.

2.9 ASSESSMENT OF NEUTROPHIL ACTIVITY (MPO ASSAY)

BALF samples (30 µl) were added to 180 µl of PBS (pH 6.0) containing 0.167 mg/ml o-dianisidine dihydrochloride and 1 drop of hydrogen peroxidase. The change in absorbance was measured every 2-3 minutes using a plate reader (Labsystems) at 450 nm. Myeloperoxidase (MPO) activity was expressed as ΔAbs/min/U. A standard curve was

generated using serial dilutions of a human MPO standard with 5 U/ml as the highest concentration.

2.10 TISSUE PREPARATION

Lungs were processed for histology using an automated processor (Shandon Pathcentre, Runcorn, UK) which immerses the tissues in various fixative and dehydrating solutions: formalin, ethanol (x 6), xylene (x 3) (BDH AnalaR® Laboratory supplies, Poole, UK). The samples were embedded in paraffin wax using a Shandon Histocentre 2 (Shandon) and left to set overnight. 4 µm sections were cut using a microtome (Shandon Finesse 325, Thermo-Shandon, Waltham, MA, USA). The sections were placed in cold water containing ethanol followed by transfer to a hot water bath (42 °C) to smooth out folding. Prior to staining, tissue sections were heated to 56 °C for a minimum of 1 h to aid clearance of wax.

2.11 RESPIRATORY TRACT HISTOLOGY

Mice were culled and the lungs were removed and fixed in 10 % formalin (Sigma-Aldrich), embedded in paraffin, sectioned and stained with haematoxylin and eosin (H&E) for examination by light microscopy. Histopathological changes were graded according to a semi-quantitative scoring system as mild, moderate or severe, using the scoring devised by Ennis et al (Ennis, 2004).

2.11.1 HAEMATOXYLIN/EOSIN STAINING

Slide sections were immersed in 2 changes of xylene, 10 minutes each, followed by rehydration in 3 concentrations of ethanol ranging from 100 % to 80 %. The sections were then placed in 2 changes of water, followed by Harris Haematoxylin (Sigma) solution for 3 min. Slides were washed in water for 2 min, and then immersed in 1 % acid alcohol for 20

seconds, followed by another washing step. Slide sections were counterstained in Eosin Y (Sigma) for 3 min and washed again. Finally, the slides were put through a series of dehydration steps in ethanol concentrations ranging from 80 % to 100 % for 5 min each. Slides were mounted with DPX mountant (BDH) and examined under a light microscope.

2.11.2 ALCIAN BLUE & PAS STAINING

This is a combined method which uses both Alcian Blue and Periodic Acid-Schiff to demonstrate the full complement of tissue proteoglycans. Lung tissue slide sections were dipped in distilled water and then stained with Alcian blue for 15 min. Slides were washed in running tap water for 2 min, followed by brief washing in distilled water. Slides were stained with periodic acid (0.5 % aq) for 5 min, followed by a washing step in distilled water. Slides were then stained in Schiff reagent (Sigma) for 10 min and washed well in running tap water for 5 min. Slides were immersed in Discombes eosin for 10 min, followed by a washing step. Nuclei were stained with haematoxylin for 1 min followed by washing in running tap water for 2 min. Finally, the slides were washed in Scotts tap water, followed by a washing step in distilled water. Slides were mounted with DPX mountant and examined under light microscopy.

2.12 MEASUREMENT OF CYTOKINES

Bead array technology is a method for quantifying multiple cytokines simultaneously in a single sample volume. The technology is based on the same premise as enzyme-labelled immunosorbent assays (ELISA); however the bead array uses fluorescence, instead of colorimetric intensity for quantitation, and through the use of distinct beads labelled for different cytokines allows the simultaneous detection of multiple analytes from single small samples. Analysis of cytokines from BALF and splenocyte supernatant was carried out using

a Th1/Th2 Flow Cytomix mouse bead array kit (BenderMedSystems) and was analysed on a FACSCaliber flow cytometer (Becton Dickinson, Franklin Lakes). Standard curves and raw data were generated for each cytokine using FlowCytomix Pro 2.2 software (BenderMedSystems). Cytokines were quantified as per manufacturers' instructions. Briefly, samples, standards and blanks were added to 4 ml polypropylene FACS tubes (Falcon, BD Biosciences). Beads coated with primary antibodies for individual cytokines were mixed together and added to each tube. The beads are internally dyed with varying intensities of fluorescent dye corresponding to specific cytokines allowing for differentiation by flow cytometry. Biotin-conjugated secondary antibodies were added to all tubes and allowed to incubate for 2 h protected from light at room temperature. Following incubation, 1ml of FACS buffer was added to each tube and the tubes were centrifuged at 200 g for 5 min. Supernatant was discarded carefully and the wash step was repeated. Streptavidin-PE was added to the tubes and allowed to incubate at room temperature for 1 h and protected from light. This was followed by a wash step, which was repeated twice. The supernatant was discarded and each tube was resuspended in 500 µl of assay buffer. Cytokines were quantified based on the fluorescent intensity which was analysed on a flow cytometer.

2.13 MEASUREMENT OF OVA-SPECIFIC IGE

IgE was measured by ELISA. Plates (Nunclon, ThermoScientific, Rochester, NY) were coated with OVA (5 µg/ml) in carbonate coating buffer and left overnight at 4°C. The following day, plates were washed five times in PBS-Tween (0.05 %) and blocked with 10 % FCS in PBS. After 2 h at room temperature, plates were washed 3 times, and serum samples were applied (50 µl/well). Samples were left overnight at 4 °C or incubated at 37 °C for 1 hour. The plates were washed and biotinylated anti-IgE (2 µg/ml) was applied to each well. Following 2 h incubation at room temperature, the plates were washed and Streptavidin-

horseradish peroxidise (BD Pharmingen) was applied according to manufacturer's instructions and left for 30 min. The plates were washed again, and 100 µl of TMB was added to the wells. Reactions were allowed to proceed for 20 min, and O.D. at 450 nm was measured (Multiskan RC, Labsystems, Thermo, Waltham, MA, USA).

2.14 MEASUREMENT OF CELL VIABILITY (FLUORESCENT MICROSCOPY)

A small sample of cells was suspended in medium and diluted 1/10 in ethidium bromide and acridine orange (EB/AO). 20 µl was pipetted to a haemocytometer and live cells were counted using an epi-fluorescent light microscope.

2.15 MEASUREMENT OF CELL VIABILITY (FLOW CYTOMETRY)

Propidium iodide staining was measured by flow cytometry to determine cell viability. 1 x 10^6 cells were centrifuged at 600 g for 5min. Supernatant was discarded, and the pellet was resuspended in 1 ml of phosphate-buffered saline (PBS) at room temperature. A 2 µg/ml concentration of propidium iodide was added to the cells, and allowed to incubate at room temperature for 15 min. Analysis was carried out on a flow cytometer (FACSCalibur, California, USA).

2.16 ISOLATION OF TOTAL LUNG CELLS

Lungs were removed aseptically and transferred to a 15 ml tube containing complete RPMI. Using sterile tweezers, the lungs were transferred to a Petri dish and sectioned into small pieces using anatomical scissors. The small lung pieces were then digested in 10ml of collagenase digestion (500 U/ml) (Sigma) solution at 37 °C under constant horizontal shaking at 300 rpm. Following 1 h digestion, the lung was passed through a 40 µm sieve into a 50 ml tube using a sterile syringe top. 5 ml of medium was added and the digested lung solution

was centrifuged at 300 g for 5min. Supernatant was discarded and the pellet was resuspended in lysis buffer and incubated at room temperature for 5 min. Following incubation, the solution was centrifuged at 300 g for 5 min and the pellet resuspended in PBS. Lung cells were prepared for FACS analysis, as described in Section 2.20.

2.17 PERIPHERAL BLOOD MONONUCLEAR CELL (PBMC) ISOLATION

Human PBMC were isolated from buffy coat packs by density centrifugation using Lymphoprep. 50 ml of the buffy coat blood were added to 5 ml sterile 5 % w/v aq EDTA solution and diluted to 200 ml with complete Hank's Balanced Salt Solution (HBSS) (Invitrogen-Gibco) (containing 1 % v/v hi FCS (Biosera, Essex, UK) and 1 mM HEPES (Invitrogen-Gibco)). 40 ml of diluted blood were overlayed onto 10 ml Lymphoprep (Axis-Shield, Oslo, Norway) in a 50 ml tube ensuring no mixture of the solutions took place. Preparations were centrifuged at 400 x g for 25 min without acceleration or breaking. 20 ml of the resulting top layer of plasma were discarded from the tube, the remaining buffy layer was gently disturbed and the PBMC collected. Care was taken to not disturb the underlying erythrocyte layer. The collected PBMC were then centrifuged at 800 x g for 5 minutes. The supernatant was decanted and the cell pellet washed in 10 ml cHBSS, repeating the previous centrifugation step. Supernatant was again decanted and the cell pellet resuspended in 20 ml warm cRPMI (see Table 2.2). The cell suspension was then filtered through a 40 μm nylon mesh filter. Cells were maintained on ice until required.

2.18 $CD4^+$ T CELL ISOLATION

$CD4^+$ T cells were isolated from human PBMC using a MagCellect $CD4^+$ T cell negative selection isolation kit (R & D Systems) according to manufacturer's instructions. This kit employs a high affinity negative selection whereby undesired cells are bound by

biotinylated antibody and then captured by Streptavidin-conjugated magnetic particles. 10 ml of 1x MagCellect buffer was added to 20 x 10^7 cells. 200 µl of human $CD4^+$ T cell biotinylated antibody cocktail was added, mixed and incubated at 4 °C for 15 min. 250 µl of streptavidin was added to the cell suspension and incubated for 15 min at 4 °C. The volume was adjusted to 3 ml with buffer and mixed gently. Undesired cells were isolated from the sample by negative selection using a MagCellect Magnet incubated for 6 min at room temperature. Positive enrichment of the $CD4^+$ T cell population was achieved by aspirating the sample solution and placing the supernatant in a fresh 5 ml tube. Magnetic depletion was repeated and the purified cell population was prepared for flow cytometric analysis of CD4, CD3 and CD8 (Immunotools) expression.

2.19 MIXED LYMPHOCYTE REACTION (MLR)

Spleens were removed from mice aseptically into cRPMI. Splenocytes were isolated by homogenising the spleens through a 70 µm filter followed by resuspension in 8 ml of cRPMI containing 0.1 % v/v beta-mercaptoethanol. The homogenate was centrifuged at 1200 rpm for 7 minutes and resuspended in 2 ml of red blood cell lysis buffer solution (BioLegend, San Diego, CA, USA) for 10 min at room temperature. 2 ml of medium was added to the suspension to neutralise the lysis solution which was then centrifuged at 600 g for 5 min. Supernatant was removed and the cells were then resuspended in fresh cRPMI for counting using EB/AO. Splenocytes were seeded at a density of 1 x 10^6/ml and added to a 96-well plate in a final volume of 225 µl. For two-way mixed lymphocyte reactions, splenocytes from two MHC mismatched mice (typically BALB/C and C57/BL6) were co-cultured. The effect of MSC on lymphocyte proliferation was assessed by adding MSC to the MLR at a density of 1.5 x 10^5 cells/ml in a total volume of 225 µl. The cells were incubated at 37 °C for 72 h after which 100 µl of supernatant was carefully removed and stored at -20

°C for cytokine analysis. Medium supplemented with 5 μCi/ml of [^{3}H]-Thymidine (Amersham Biosciences, Buckinghamshire, England) replaced the removed supernatant and allowed to incubate for 6 h at 37 °C. Cultures were harvested using an automatic cell harvester (Micro96 Harvester, Skatron Flow Laboratories, Oslo, Norway) onto glass filter mats. The mats were dried and placed in plastic sample bags (Wallac– PerkinElmer, Waltham, MA, USA). 3 ml of β-scintillation fluid (Beta-Plate Scint, Wallac-PerkinElmer) was pipetted into the bag after which it was sealed and placed in a cassette. [^{3}H]-Thymidine incorporation was quantified by a β-scintillation counter (1450 MICROBETA Liquid Scintillation Counter; Wallac-Perkin Elmer) and results were expressed in counts per minute (cpm).

2.20 OVA-SPECIFIC T-CELL PROLIFERATION ASSAY

Splenocytes from mice were prepared as previously described (Section 2.17). The cells were seeded at a density of 1 x 10^6 cells/ml in a total volume of 225 μl. Concanavalin A (ConA)(5 ug/ml) was used as a positive control and increasing concentrations of OVA (20, 50 and 200 μg/ml) were added to selected cultures. Medium alone was added to negative control wells. Following incubation for 72 h, supernatants were removed and stored at -20 °C for cytokine analysis, and cultures received fresh medium. Cells were incubated for the final 6 h with 5 μCi/ml [^{3}H]-thymidine and proliferation was measured as radioactivity incorporated by liquid scintillation as described in 2.17.

2.21 ISOLATION AND CULTURE OF MURINE MESENCHYMAL STEM CELLS

Female mice were sacrificed by cervical dislocation. Femurs and tibias were removed under sterile conditions and stripped of connective tissue. The ends of each bone were removed and the bone marrow flushed out using a 27 G needle and syringe filled with

isolation medium (Table 2.1, p.73). Cells were then centrifuged at 600 g for 5 minutes, resuspended in complete isolation medium and plated in tissue culture flasks. Cells were maintained for a period of four weeks with medium changes every 3-4 days at 37 °C in 5 % CO_2 incubator. After this four-week period, cells were passaged (P1) and further cultured for two weeks at a low seeding density of 50 cells/cm^2 in complete isolation medium (cα-MEM expansion medium, Table 2.1, p. 72). The cells were then passaged and expanded twice (P2, P3) at a seeding density of 50 cells/cm^2 before use *in vitro* or *in vivo*.

2.22 CHARACTERISATION OF MSC BY FLOW CYTOMETRY

Cells were grown to 70-90 % confluence and split using 0.25 % trypsin/1 mM EDTA (Gibco, Carlsbad, CA) for 5 min at 37°C. An equal volume of medium (cα-MEM) was added to cells to neutralise the trypsin/EDTA (Gibco). Cells were centrifuged at 600 g for 5 min and resuspended in FACS buffer (PBS containing 1% FCS) for counting. Cells were resuspended in the appropriate volume of FACS buffer to yield a concentration of 1 x 10^5 cells/FACS tube in 50 µl. Fluorochrome or unconjugated antibodies (see Table 2.5) were incubated with the cells for 15 min at 4 °C. Cells were then washed in buffer and centrifuged at 300 g for 5 min. Supernatant was discarded and the cells requiring secondary labelling were allowed to incubate with the appropriate secondary conjugated antibodies for 15 min at 4 °C. All cells were washed in 2 ml of buffer, followed by centrifugation at 300 g for 5 min followed by resuspension in 300 µl of cell fixative. Cells were analysed for their cell surface expression of MHC class I, Sca-1, CD44, CD80, CD106, CD117, CD105, CD80, CD45 and CD90 by flow cytometry (FACSCalibur, California, USA) using CellQuest software (BD Biosciences, Oxford, UK).

2.23 CHARACTERISATION OF MSC DIFFERENTIATION CAPACITY

2.23.1 OSTEOGENESIS

MSC were seeded at a density of 1 x 10^3 cell/cm^2 in a 6-well plate in 2 ml cα-MEM. Once confluence had been reached (typically 7 days), cells were incubated in osteogenic differentiation medium (see Table 2.1). Fresh medium was added every 3-4 days for 21 days. At day 21, the medium was removed and the cells were washed in PBS and then fixed in 10 % (v/v) neutral buffered formalin for 20 min at room temperature. Formalin was removed and cells were washed in 2 ml of PBS. 1 ml of Alizarin Red (see Table 2.1) was added to the fixed cells and allowed to stain for 20 min. Excess stain was removed and the cells were washed with dH_2O. Finally, 1 ml of dH_2O was added to each well and cells were examined under the microscope for red deposits indicating the presence of osteogenesis.

2.23.2 CHARACTERISATION OF MSC ADIPOGENIC DIFFERENTIATION CAPACITY

MSC were seeded at a density of 1 x 10^3 cell/cm^2 in a 6-well plate (BD Falcon) in 2 ml cα-MEM. Once confluence had been reached (typically 7 days), cells were incubated in adipogenic differentiation medium (see Table 2.1) and fresh medium was added every 3-4 days for 21 days. At day 21, the medium was removed and the cells were washed in PBS and then fixed in 10 % neutral buffered formalin for 20 min at room temperature. Formalin was removed and cells were washed in 2 ml of PBS. 1 ml of Oil Red O (see Table 2.1) was added to the fixed cells and allowed to stain for 20 min. Excess stain was removed and the cells were washed with PBS. Finally, 1 ml of PBS was added to each well and cells were examined under the microscope for red fat globules indicating the presence of adipogenesis.

2.23.2 CHARACTERISATION OF MSC CHONDROGENIC DIFFERENTIATION CAPACITY

A pellet culture system was used for chondrogenesis differentiation. 2 x 10^5 cells were centrifuged in a 15 ml polypropylene tube. The pellet was cultured at 37 °C in 5 % CO_2 in

500 µl of chondrogenic medium (see Table 2.1). Fresh medium was added every 3-4 days for 21 days, after which the pellets were washed with Dulbecco's PBS. Purification of total RNA from cell cultures was performed using the RNeasy® Plus Mini kit (Qiagen, Crawley, UK) according to manufacturer's instructions, reverse-transcribed to cDNA and analysed for the expression of chondrogenesis markers aggrecan and collagen II using hotstart RT–PCR reaction (Promega, Southampton, UK) (Section 2.34). Glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) was detected as an internal control.

2.24 DELIVERY OF MSC TO MICE

Animals were held in conventional cages before and after procedure. Before infusion, cells were washed three times with sterile PBS and resuspended at a concentration of 5×10^5 cells/ml. The cells were delivered to the tail vein using a 27 G needle and a 1 ml syringe. Animals were then returned to their cages where they were monitored closely for the first hour, and at regular intervals daily for signs of distress or ill health. If distress or ill health was observed, animals were killed by cervical dislocation. At appropriate time points following infusion of MSC, animals were killed by cervical dislocation. Organs and serum were removed for analysis.

2.25 DEPLETION OF REGULATORY T CELLS *IN VIVO*

Mice were sensitised with 100 µg of OVA in alum on day 0, 7 and 14 according to the model described in Section 2.4. A moderate dose of cyclophosphamide (CY) (Sigma) was used in this study (Yung-Chang, 2006). Mice received 3 doses of 150 mg/kg CY intraperitoneally on day 8, 12 and 15. One group received 2 intravenous infusions of MSC via the tail vein on day 7 and 14. All groups were challenged with OVA (50 µg in 30 µl PBS) intranasally on day 25, 26 and day 27. Mice were culled on day 28. The effectiveness

of depletion was assessed by examination of regulatory T cell marker expression (CD25 and FoxP3) by flow cytometry.

2.26 DETECTION OF MALE CELLS IN FEMALE MICE BY FLUORESCENT *IN SITU* HYBRIDISATION (FISH)

FISH is a cytogenetic technique used to detect the presence or absence of DNA-specific sequences on chromosomes. A Y-chromosome-specific probe was used to detect male MSC transplanted into female recipients. Using sex-mismatched transplantation, the Y-chromosome of male donor MSC were detected with a Cy3 chromosome (Cambio 1200-YMC 3.02) paint probe. The probe hybridised with a prepared lung tissue section from recipient female mice. Tissue sections were dewaxed with xylene and subsequently rehydrated in several graded alcohols to water. The slides were incubated with sodium thiocyanate for 10 minutes at 80 ˚C and washed in PBS. Incubation in a pepsin solution was crucial prior to quenching in a glycine solution. Next, the tissue sections were fixed in 4 % paraformaldehyde for 2 min, washed with PBS and again dehydrated through graded alcohols. 13 µl of Cy3 Chromosome Y paint was dropped on the centre of the slide; a coverslip was mounted and sealed with rubber cement. The section was denatured at 60 ˚C and placed in a humid chamber to hybridise overnight at 37 ˚C. Slides were first washed in a formamide wash solution at 37 ˚C for 5 min three times, followed by a stringency wash solution at 37 ˚C for 15 min x 3, and finally in detergent wash solution at 37 ˚C for 10 min. Following 3 washes in PBS, the nuclei were counterstained with DAPI. Sections from un-transplanted females were used as negative controls for Y-chromosome staining.

2.27 CRYOSECTION AND IMMUNOFLUORESCENCE

Mice were sacrificed and the respiratory tract was exposed by dissection. A small incision was made in the trachea with a scalpel, and an 18 G needle inserted carefully to avoid damaging the trachea. A 1 ml syringe was filled with a 1:1 OCT (optimal control temperature): sucrose (10 %) solution, and fitted to the syringe. The lungs were slowly filled with OCT sucrose solution until they were expanded as much as possible. The lungs were dissected; the lobes were separated and placed in a disposable mould. The mould was filled with OCT (undiluted) until the lung lobes were fully covered, and snap frozen in liquid N_2 until OCT turned white in colour. Tissues were sliced into 5 μm sections on a cryostat (Shandon Cryotome, ThermoShandon, Waltham, MA) and stored at 80 ˚C until ready to examine by fluorescent microscopy.

2.28 RECOVERY OF CELLS FROM LIQUID NITROGEN

Cells were quickly thawed to 70 % liquid at 37 °C and transferred to a 15 ml tube. 8ml of warmed medium was added to the cells and centrifuged at 600 g for 5 min. Supernatant were discarded, and the pellet was resuspended in 2 ml of warmed medium for counting.

2.29 PARAFORMALDEHYDE-FIXED MSC

Cells were trypsinised and resuspended in 50 ml paraformaldehyde (0.5 % in PBS) for 20 min at room temperature. Cells were washed with 50 ml PBS three times and resuspended in PBS for counting using EB/AO.

2.30 ASSESSMENT OF CELL CHEMOTAXIS

Cell migration was examined using a 24-well Transwell culture chambers with 5.0 μm pore polycarbonate membrane insert (Corning Costar, Massachusetts, USA).

Recombinant murine CCL11 (eotaxin) (Immunotools) or recombinant murine RANTES (Immunotools) were diluted to 100 µM in serum-free medium (RPMI containing 1 % L-glutamine) and added to the lower chamber compartment in a final volume of 600 µl. Control wells contained medium alone. The polycarbonate membranes inserts were placed in the wells using sterile forceps and allowed to equilibrate with the chemokine at 37 °C in 5 % CO_2 for 10 min. 5 x 10^4 eosinophils (cell line, EOL-1) in a final volume of 100 µl were pipetted into the upper chamber. Following 4 h incubation, the membrane inserts were removed and cells that had migrated to the lower chamber were counted in five fields of view using a inverted microscope with a digital camera attached (Olympus, London, UK). Chemotactic index (CI) was calculated by normalising the cell migration in control wells to 1 and expressing wells containing the chemokine as fold increases of the control CI.

2.31 EXPRESSION OF EOSINOPHIL SURFACE MARKERS

To assess the effect of MSC on the expression of eosinophil adhesion markers, human MSC and human eosinophil cell line (EOL-1) were co-cultured in a 6-well plate (Nunc, Roskilde, Denmark) at a density of 5 x 10^4 and 3 x 10^5, respectively, for 48 h at 37 °C in 5 % CO_2. Co-cultures were prepared at four ratios of eosinophils:MSC (10:1, 5:1, 3:1 and 1:1). Eosinophils were carefully aspirated, leaving adherent MSC on the plate. The cells were centrifuged at 300 g for 5 min and resuspended in FACS buffer for viability counting (2.11). Cells were transferred to polypropylene tubes at a concentration of 5 x 10^4/tube in 50 µl of cold FACS buffer for incubation with appropriate antibodies. Cell surface expression of ICAM-1, ICAM-3, CD18 and L-selectin on eosinophils was examined by flow cytometry.

2.32 DETECTION OF APOPTOTIC CELLS

Apoptosis was detected by binding of Annexin V/Propidium iodide staining according to manufacturer's instructions (BD Biosciences). Cells were washed twice in PBS and resuspended in 1x binding buffer (BD Biosciences) at 1 x 10^5 cells/50 µl. 2.5 µl of Annexin V (BD Biosciences, Oxford, UK) and/or 5 µl of propidium iodide (BD Biosciences) were added to FACS tubes (Falcon, BD Biosciences) containing 50 µl of cell suspension and incubated for 15 min at RT in the dark. Cells were analysed for binding of Annexin V and/or propidium iodide by flow cytometry within 1 h.

2.33 RNA ISOLATION

Total RNA was extracted using TRIzol® Reagent (Invitrogen) according to the manufacturer's instructions. Briefly, 10^6 cells were lysed in 1ml TRIzol at room temperature for 5 min. 100 µl of RNA-grade chloroform (BDH) was added to the cells, mixed vigorously and incubated at room temperature for 5min. Samples were centrifuged at 12,000 g for 15 min at 4 °C. Two distinct layers resulted with RNA remaining in the clear, aqueous upper layer. 350-400 µl of RNA was carefully removed, ensuring the lower white DNA layer was not disturbed, and precipitated with 500 µl isopropanol (Riedel-de Haen). The samples were incubated at room temperature for 10 min and followed by centrifugation for 10 min at 4 °C. The resulting RNA pellet was washed with 1 ml 75 % v/v ethanol and centrifuged at 7,500 g for 5 min at 4 ^{o}C. The ethanol was aspirated and the RNA pellet was allowed to briefly air dry prior to resuspension in 30 µl RNase-free water (Promega, Southhampton, UK). The purity and concentration of RNA was determined using a spectrophotometer (Nanodrop 2000, ThermoScientific, Wilmington, DE, USA) which calculated the ratio of absorbance at 260 nm to 280 nm. A ratio between 1.8 and 2.0 indicated sufficient purity of the RNA. Samples outside this range were discarded.

2.34 DNase Treatment of RNA

RNA contaminated with genomic DNA was treated with DNase I (Invitrogen) as per manufacturer's instructions. 1 µl of DNase (Amplification grade) was added to 5 µl of RNA and incubated for 28 min at room temperature. The mix was then incubated at 65 °C for 10 min, followed by cooling on ice with 1 µl of 25 mM EDTA to inactivate the DNase. RNA was then reverse transcribed as detailed below (Section 2.35).

2.35 cDNA Synthesis

Total RNA was reverse transcribed using 25 U Superscript II (Invitrogen, Paisley, UK). Each reaction contained a 1x GoTaq reaction buffer (Promega), 2.5 mM $MgCl_2$ (Promega), 1 mM dNTP mix (Promega), 50 U/ml of ribonuclease inhibitor (Invitrogen) and 20 µg/ml Oligo (dT) $_{12\text{-}18}$ primer (Invitrogen) diluted in nuclease-free water (Promega). The conditions for cDNA synthesis were as follows: 25 ^{o}C for 10 min, 42 ^{o}C for 60 min, 95 ^{o}C for 5 min and 4 ^{o}C for 10 min. Quantification of cDNA was performed by measuring the absorbance value of the sample 260 nm. Samples were stored -20 ^{o}C until required.

2.36 Polymerase Chain Reaction (PCR)

PCR was used to determine the presence of specific DNA sequences (or mRNA following reverse transcription) using primers summarised in Table 2.5. Expression of the housekeeping gene, GAPDH, was used as a positive control. PCR reactions contained 2.5 mM $MgCL^{2+}$, 1 mM dNTP, 1x GoTaq reaction buffer, 40 U/ml Taq polymerase (Promega) and 0.4 pM of the appropriate primer pairs. The reaction mastermix was adjusted to a final volume of 24 µl with nuclease-free water. The PCR conditions were as follows: denaturation at 95 °C for 45 sec (10 min for first cycle), annealing for 45 sec (optimal annealing

temperatures are summarised in Table 2.6) and extension for 1 min at 72 °C. DNA products were resolved on a 1.3 % w/v agarose gel and detected by binding of ethidium bromide.

2.37 COMPLIANCE WITH GMO AND SAFETY GUIDELINES

All GMO/GMM work was performed according to approved standard operating procedures and recording protocols approved by the Environmental Protection Agency (Ireland). Safe working practices were employed throughout this study as documented in the Biology Department Safety manual NUI Maynooth.

2.38 STATISTICAL ANALYSIS

Student's T-test was used to measure the significance between groups. Probabilities less than 0.05 were considered significant. Values for all measurements were expressed as the mean ± standard error of the mean (SEM). Statistical analysis was performed using GraphPad Prism™ software (GraphPad, San Diego, CA). Comparison was made using the Kruskal Wallis test, or the Mann Whitney test as appropriate. Significance was denoted by p value <0.05.

CHAPTER 3

AN EXPLORATION OF THE "HYGIENE HYPOTHESIS" USING AN ATTENUATED STRAIN OF *BORDETELLA PERTUSSIS*

3.1 INTRODUCTION

Since $CD4^+$ Th2 cells represent a co-ordinating cell type in allergy, it has been suggested that the induction of counterbalancing responses might prevent the subsequent development of atopic disease (Strachan, 2000; Romagnani, 2004). According to this modification of Strachan's hygiene hypothesis, microbial exposure activates innate immune pathways that alter Th1, Th2 and T_{reg} responses (Romagnani, 2004) resulting in the suppression of Th2 cell expansion, and a consequent inhibition of isotype switching to IgE. Contrary to this hypothesis, several studies have suggested that viral and bacterial infections play a role in exacerbation of respiratory disease. Virulent *Bordetella pertussis* infection can exacerbate airway pathology (Mills, 1998; Ennis, 2004; Harju, 2006) and stimulate IgE responses in a murine model of allergen driven-inflammation despite induction of Th1 immunity (Nilsson, 1998; Ryan, 2000). However, systemic immunisation with Th1-inducing whole-cell pertussis (Pw) vaccines inhibits allergic airway responsiveness (Ennis, 2005) indicating that protection from allergen-driven pathology is not simply modulation of Th1/Th2 responses, but may also be associated with the degree of airway damage at the time of priming, such that allergen priming in the mucosae during breakdown of the airway epithelial mesenchymal unit may be an equally important factor (Holgate, 2000). A genetically-attenuated *B. pertussis* strain, BPZE1, has been developed as a live neonatal mucosal vaccine candidate against whooping cough (Mielcarek, 2006). This strain induces strong local and systemic immune responses upon a single intranasal delivery. This chapter explored the influence of the candidate vaccine on OVA-induced airway pathology in order to (1) test the hypothesis that priming during a period of respiratory damage is an important factor in the phenotypic shift from Th2 to Th1 allergen-specific responses and (2) ascertain the safety of this live vaccine candidate in a preclinical model.

3.2 STUDY DESIGN– A MODEL OF ALLERGIC AIRWAY INFLAMMATION

The murine model of ovalbumin driven airway inflammation was used to elucidate the effect of an attenuated *Bordetella pertussis* strain, BPZE1, *in vivo.* Systemic sensitisation to OVA followed by respiratory allergen exposure induces an acute allergic response in the airways and lungs. This model has been used previously to uncover the influence of infection on allergen-driven inflammation (Ennis, 2005; Ennis, 2005). This was probed using Th1-inducing virulent (airway damaging) and attenuated (non-damaging) bacterial strains. Mice were challenged with either virulent BPSM, or attenuated BPZE1, and sensitised to OVA at the peak of bacterial carriage (day 10) to investigate whether respiratory damage is an important factor in effecting a phenotypic shift from Th2 to Th1 allergen-specific responses. Section 3.3 summarises the experimental outline.

Doses of allergen and bacterial challenge were based on the prior work of Ennis *et al* (Ennis, 2005). A preliminary study was carried out in order to assess the efficacy of intranasal delivery of OVA with our without anaesthesia. Fully conscious or anaesthetised mice were administered trypan blue dye (0.4 % in PBS) intranasally. After 1h, the mice were culled. The lungs were dissected and examined for the presence of blue stain within the trachea and lungs. No stain was evident in the airways of non-anaesthetised mice, but deposited stain was seen in lungs of anaesthetised animals (Fig. 3.1). This suggested that intra-nasal delivery without anaesthesia likely resulted in the bulk of material being misdirected to the oesophagus and subsequently the gastro-intestinal tract. As a result, mice were routinely anaesthetised prior to intranasal delivery of OVA.

3.3 EXPERIMENTAL OUTLINE

Virulent *B. pertussis* (BPSM) or attenuated BPZE1, were cultured as described in Section 2.2 and delivered via aerosol to mice on day 0. Ten days later, at the height of

bacterial infection (Mills, 1998), mice were sensitised with OVA by intraperitoneal injection (100 μg) and injected again on day 24. On days 35, 36 and 37, mice were exposed to either saline or OVA via the intranasal (i.n.) route. Un-sensitised control mice received either PBS, BPSM or BPZE1 alone (Fig. 3.2). On day 38, airway hyperresponsiveness to a chemical irritant (methacholine) was assessed using whole-body plethysmography. On day 39, mice were bled by facial bleed for subsequent serum analysis. Mice were culled and BALF, spleen and lungs were sampled for subsequent analysis. BALF supernatants were collected and cytokine profiles were examined. Differential cell counts were performed to assess the extent of cellular infiltration. Splenocytes were cultured in the presence of exogenous OVA and their ability to mount a recall response was measured. Lungs were fixed in 10% formalin for histological analysis in order to examine the effect of an attenuated *B. pertussis* strain on OVA-induced airway pathology.

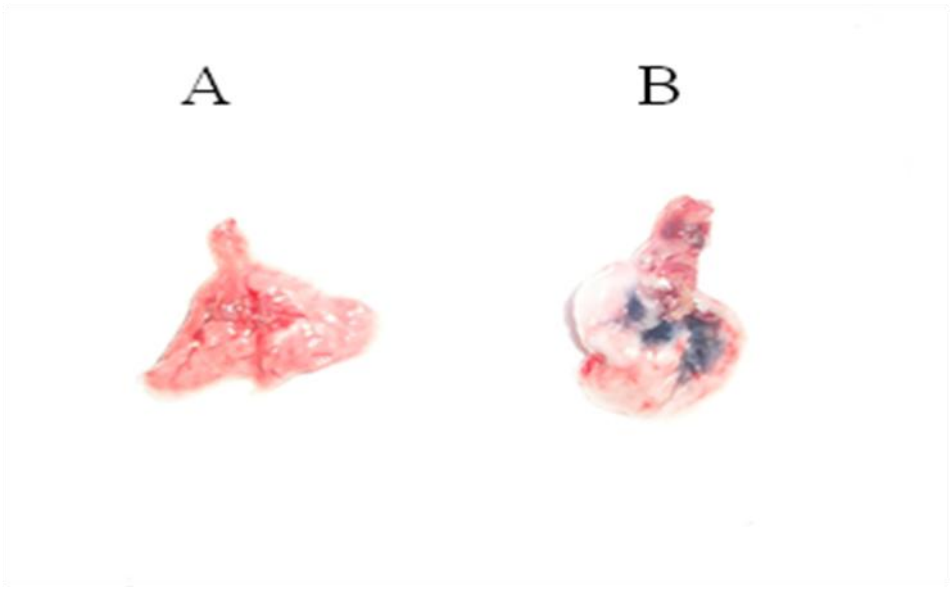


Figure 3.1 Anaesthesia during intranasal delivery of allergen is required for sufficient antigen delivery to the airways. Fully conscious (A) or anaesthetised (B) mice were administered 0.4 % trypan blue dye by placing 50 µl solution of each nostril and allowing it to be inhaled.

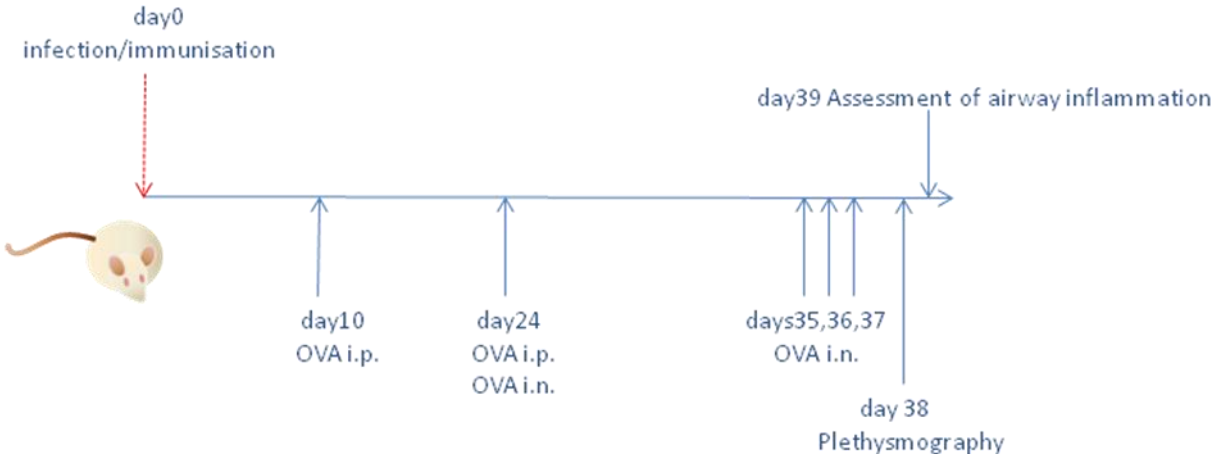


Figure 3.2 Experimental design. Groups of 6-8 week old female BALB/c mice (n=10) were challenged by aerosol with either an attenuated strain (BPZE1) or virulent (BPSM) *B. pertussis* on day 0. Control mice were sham infected by aerosol exposure to physiological saline. Mice were sensitised by intraperitoneal (i.p.) injection of either OVA or saline on days 10 and 24. On days 35, 36 and 37, mice were exposed to either saline or OVA via intranasal (i.n.) administration. Readouts were performed on day 39.

3.4 OPTIMISATIONS:

3.4.1 OVA-SPECIFIC IGE ELISA

Optimisation of an OVA-specific IgE assay was required in order to examine whether *B. pertussis* infection influenced IgE induction in a model of allergic airway inflammation. Three variables of the ELISA protocol were adjusted. Initially, milk powder and fetal calf serum (FCS) were compared as blocking buffers. Blocking with milk powder resulted in excessive background readings (Fig. 3.3A). Therefore, FCS was used in future experiments. Varying dilutions of Tween were used in order to determine the optimum buffer for washing the plate after serum incubation. The optimum was found to be 0.2 % Tween in PBS (Fig. 3.3B). Further, a 1/250 dilution of anti-mouse biotinylated IgE detection antibody and the use of a carbonate coating buffer instead of PBS proved most successful in this assay (Fig. 3.3D).

3.4.2 COMPARISON STUDY OF MULTIPLEX BEAD ARRAY AND ELISA FOR CYTOKINE DETECTION

Due to the limited quantity of BALF supernatant resulting from the *in vivo* experiments, an alternative method for multiple cytokine quantitation was required in order to determine a broad cytokine profile from each sample. A comparison study to examine the similarity of results between multiplex bead array analysis and ELISA was carried out. Both ELISA and multiplex bead array assays were performed on supernatant samples from eight treatment groups resulting from a mixed lymphocyte reaction experiment. The resulting data were compared for their reproducibility. This study found a generally good agreement between the multiplex assay and ELISA. Whilst the bead array and ELISA assays yielded different absolute concentrations of cytokines, the cytokine levels followed similar qualitative patterns (i.e. similar trend), with Pearson correlation coefficients consistently above 0.85 for

IL-10 and IFN-γ, and 0.7 for IL-5 (Table 3.1). The interpretations of the degree of correlation (none, small, medium, large) are given in Table 3.2. Results were 2 to 10-fold lower for multiplex analysis when compared to ELISA determinations and there was poorer sensitivity in the case of IL-5 when compared to ELISA. Similar to other investigators (Chen, 1999; Khan, 2004; Young, 2008) multiplex bead array assays have demonstrated good correlations with ELISA, but poorer concurrence of quantitative values. However, these data indicated that the multiplex assay could be used as a sufficiently sensitive method to detect cytokines in splenocyte supernatant.

Table 3.1 Correlation between multiplex studies and ELISA of mouse cytokines.

Cytokine[1]	Pearsons correlation coefficient
[2]IL-5	0.692332
[2]IL-10	0.849009
[3]IFN-γ	0.89259

[1] Splenocyte supernatant from eight treatment groups were analysed for IL-5, IL-10 and IFN-γ using both ELISA and multiplex bead array methods. Each assay was performed according to the manufacturers' instructions. Standard curves were generated using reference concentrations supplied by each manufacturer. Pearson correlation coefficient was calculated as a measure of the correlation between two variables (ELISA and multiplex bead array).
[2]ELISA from R&D
[3]ELISA from Peprotech

Table 3.2 Summary of Pearson correlation coefficients

Descriptor of Correlation	Negative	Positive
None	-0.09 to 0.0	0.0 to 0.09
Small	-0.3 to -0.1	0.1 to 0.3
Medium	-0.5 to -0.3	0.3 to 0.5
Large	-1.0 to -0.5	0.5 to 1.0

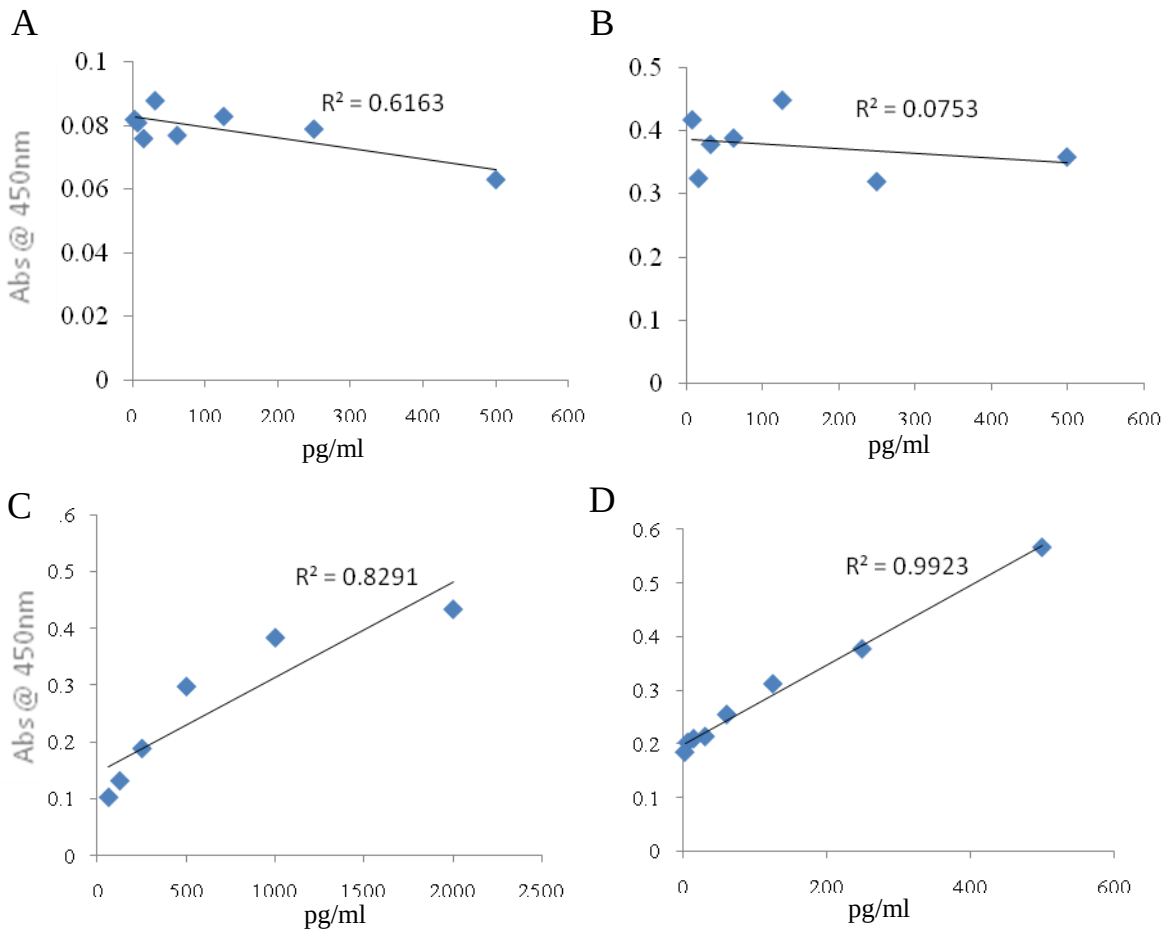


Figure 3.3 Optimisation of OVA-specific IgE assay. The sensitivity of an assay is given by the r^2 value of the standard curve. The closer the r^2 is to 1, the greater the sensitivity of the assay. (A) milk powder used as coating buffer, (B) low concentration of PBS-Tween wash buffer, (C) 1/200 dilution of biotinylated anti-IgE. (D) Optimum conditions included the use of carbonate coating buffer, a greater concentration of washing buffer and 1/250 dilution of biotinylated anti-IgE.

3.5 ATTENUATED *B. PERTUSSIS* PREVENTS OVA-DRIVEN ALLERGIC AIRWAY PATHOLOGY.

Virulent *B. pertussis* can exaggerate unrelated allergen priming in animal models (Ennis, 2004) and has been associated with exacerbation of allergy in humans (Harju, 2006). To assess the influence of the candidate BPZE1 vaccine on OVA-induced airway pathology, mice were challenged with either virulent BPSM, or attenuated BPZE1, and sensitised to OVA at the peak of bacterial carriage. As expected, non-sensitised groups exhibited no allergen-driven airway inflammation (Fig. 3.4). In the absence of infection, OVA-sensitised mice exhibited typical peribronchial and perivascular inflammation at day 39, which was not observed in control mice (Fig 3.5 A&B). At this time point, pathology due to virulent bacterial infection alone has resolved (Barnard, 1996; Skerry, 2009). Priming at the peak of virulent *B. pertussis* infection resulted in a more severe pathology when compared to non-infected sensitised mice, featuring strong perivascular inflammation and hypertrophied bronchiolar epithelium (Fig 3.5 C). In contrast, prior challenge with attenuated BPZE1 resulted in decreased peribronchial inflammation when compared to sensitised, BPSM-infected mice (Fig 3.5 C&D). Thus BPZE1 does not damage the airways, confirming earlier reports (Skerry, 2009), and thus differential challenge by BPSM or BPZE1 provides an opportunity to study priming in the presence or absence of airway damage.

Excessive mucus secretion from goblet cells is a characteristic feature of the allergic airway. An examination of lung tissues stained with PAS and Alcian blue demonstrated that prior immunisation with BPZE1 in OVA-sensitised mice reduced mucus secretion when compared to those sensitised to OVA alone (Fig. 3.6 G&H). In contrast, virulent BPSM-infection exacerbated goblet cell hyperplasia and mucus secretion in OVA-sensitised mice (Fig 3.6 D&E). Taken together these data show that unlike infection with virulent *B. pertussis*, administration of attenuated *B. pertussis* BPZE1 did not enhance, but reduced the

pathology associated with allergen sensitisation.

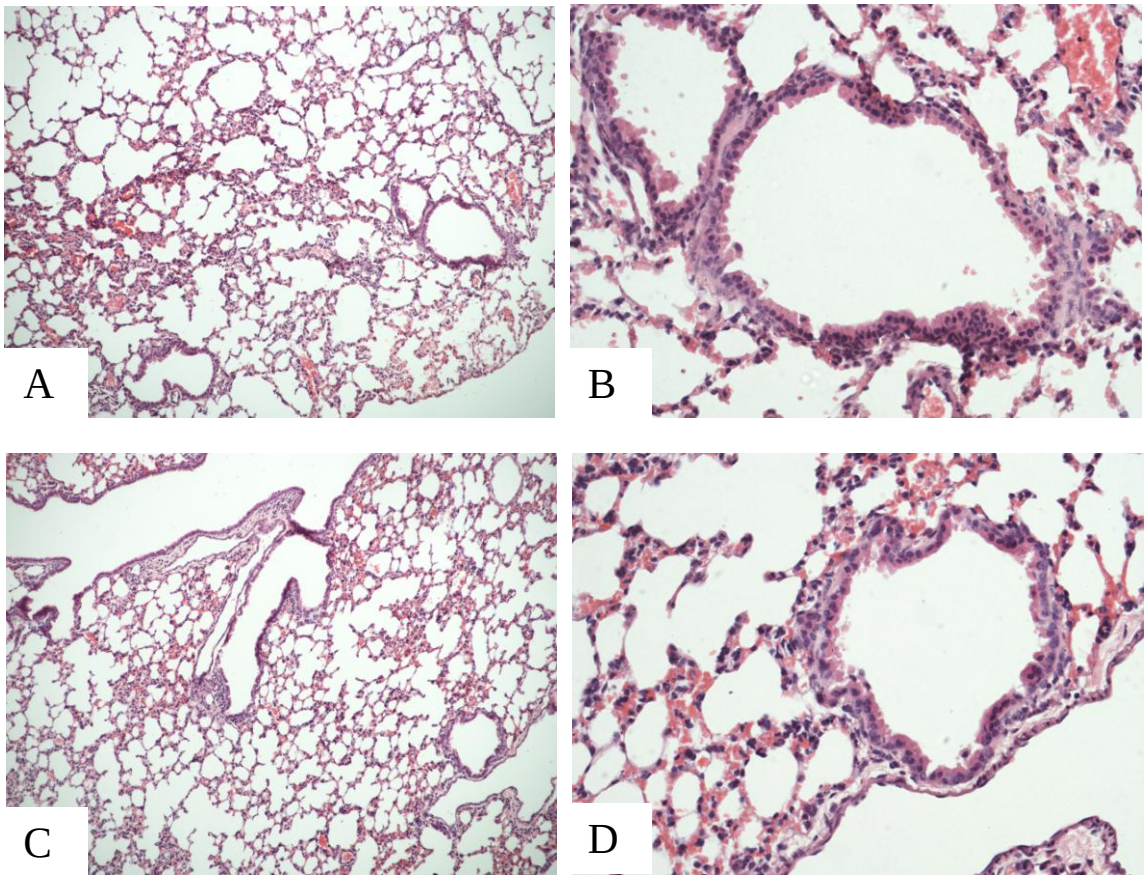


Figure 3.4 Non-sensitised mice exhibit no allergen-driven airway inflammation. Representative morphological changes at day 39 in bronchiolar transverse sections of lungs from (A&B) non-sensitised after immunisation with BPZE1 (C&D) Non-sensitised and infected with *B. pertussis.* All sections are representative of duplicate experiments; in each case at least five animals were assessed. Original magnification A & C x100, B & D x400.

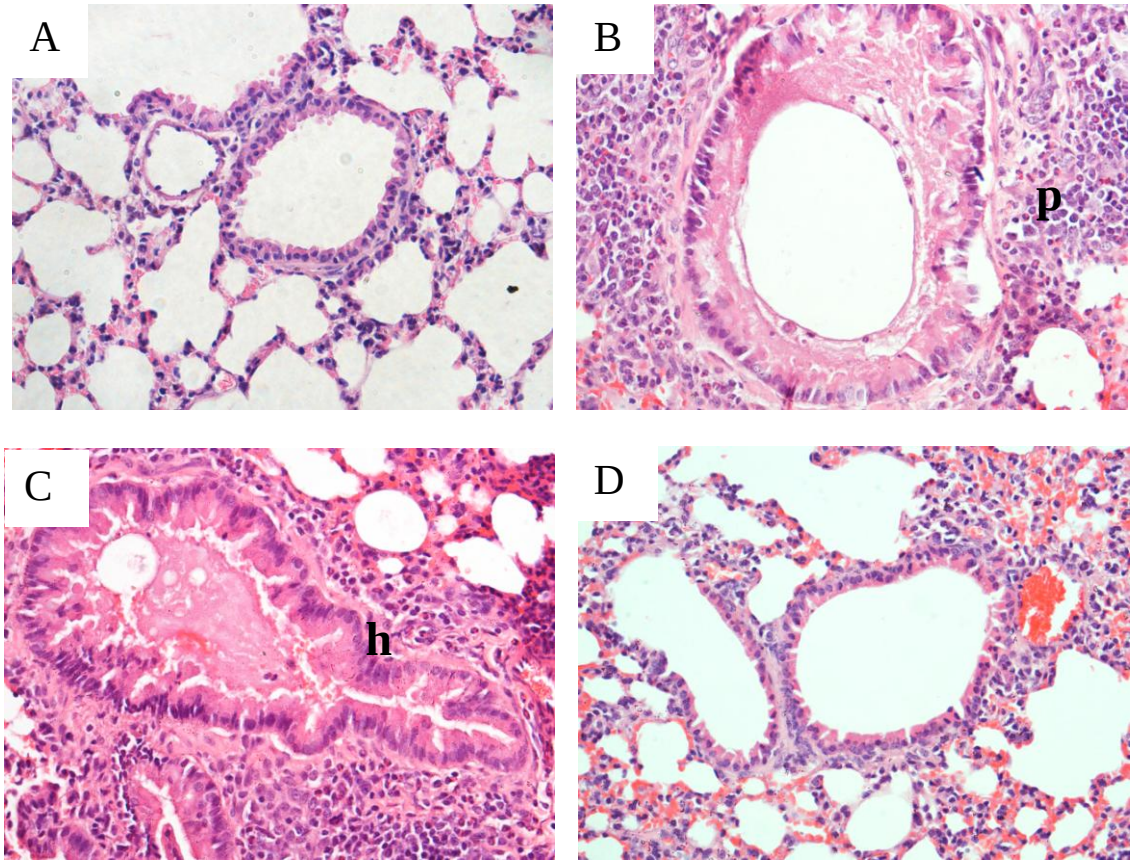
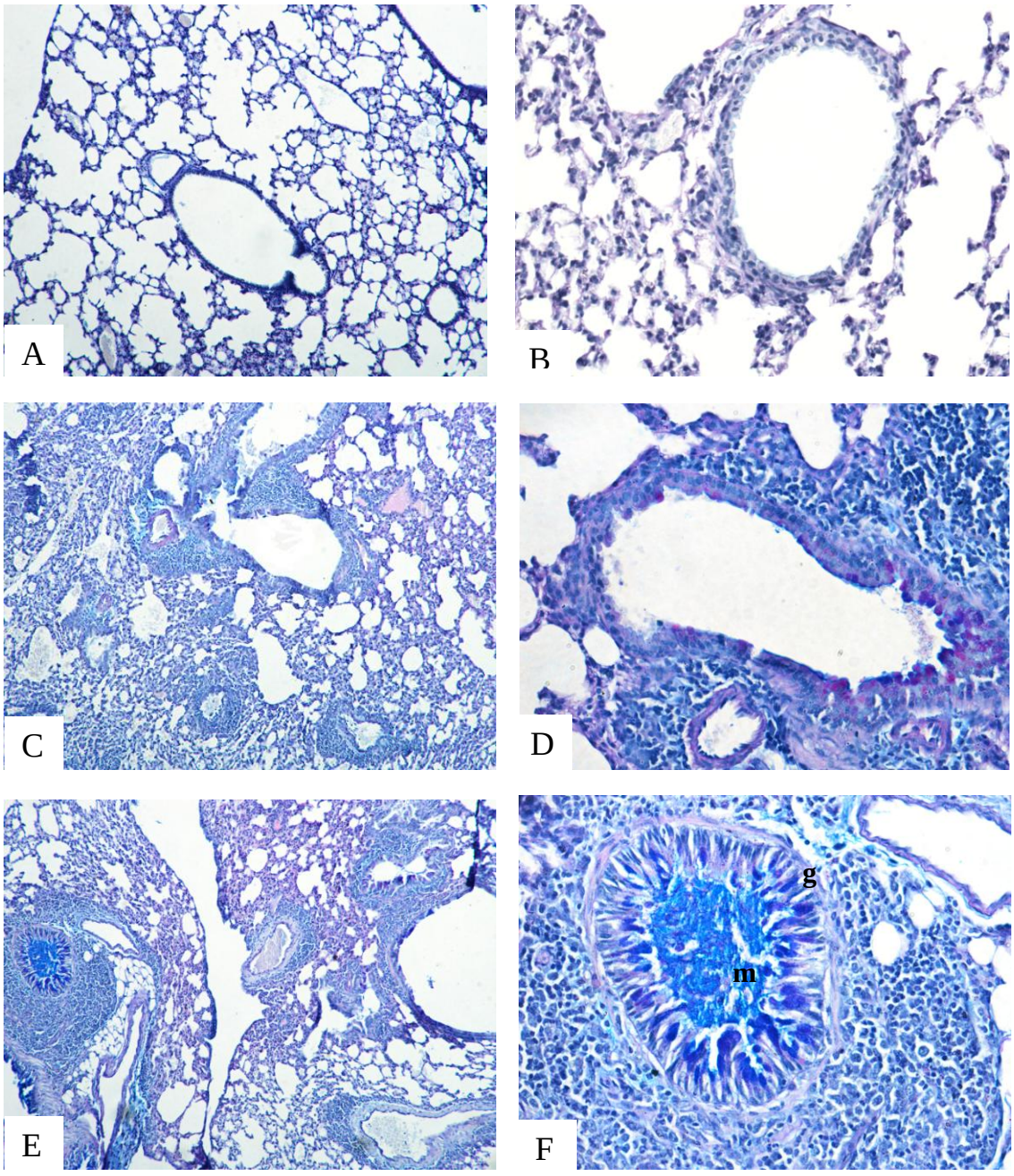


Figure 3.5 Attenuated *B. pertussis* BPZE1 reduces the severity of airway pathology induced by sensitising allergen. Representative morphological changes at day 39 in bronchiolar transverse sections of lungs from (A) Non-sensitised, (B) OVA-sensitised, (C) OVA-sensitised following infection with virulent *B. pertussis* SM, (D) OVA-sensitised following immunisation with BPZE1. Airway inflammation was detected using haematoxylin and eosin (H&E) staining of fixed lung sections. **p** and **h** indicate perivascular inflammation and bronchiolar epithelial hypertrophy, respectively. All sections are representative of duplicate experiments; in each case at least five animals were assessed. Original magnification A, B, C & D x400.


A
B
C
D
E
F
g
m

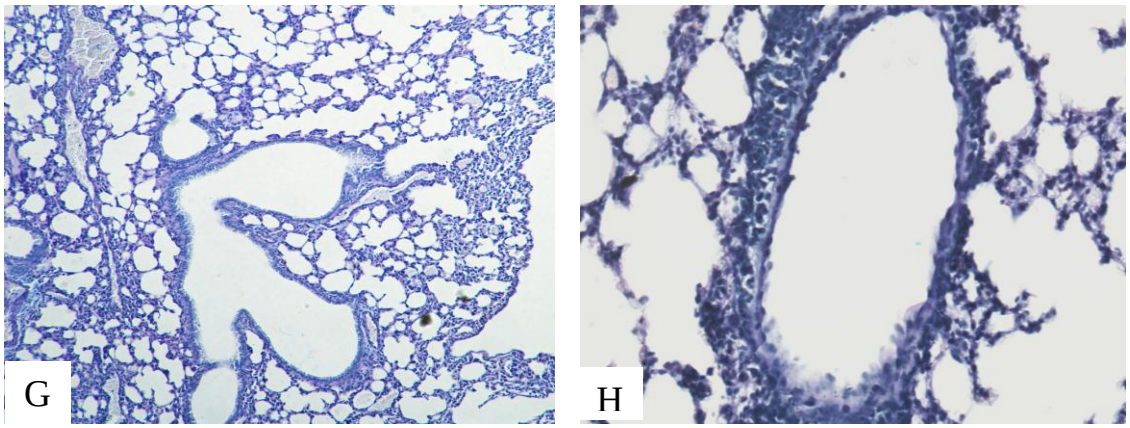


Figure 3.6 Attenuated *B. pertussis* BPZE1 reduces the severity of mucus hyperplasia to sensitising allergen. Representative morphological changes at day 39 in transverse sections of lungs (bronchiolar region) from (A & B) non-sensitised, (C & D) OVA-sensitised, (E & F) OVA-sensitised and infected with virulent *B. pertussis,* (G & H) OVA-sensitised and vaccinated with BPZE1. Airway inflammation was detected using combined Discombes/Alcian blue/PAS staining on lung sections. **g** and **m** indicate goblet cell hyperplasia and mucus secretion, respectively. All sections are representative of duplicate experiments; in each case at least five animals were assessed. Original magnification (A), (C), (E), (F) x100 and (B), (D), (F), (H) x400.

3.6 ATTENUATED *B. PERTUSSIS* BPZE1 PREVENTS OVA-DRIVEN ALLERGIC AIRWAY INFLAMMATION

Sensitisation and aerosol challenge of mice with ovalbumin results in airway eosinophilia and extensive lung damage analogous to that seen in asthma (Lukacs, 1994). Administration of live *B. pertussis* BPZE1 moderated the quality of the OVA-induced inflammatory influx to the respiratory tract. Control mice showed minimal cellularity in bronchoalveolar lavage (Fig. 3.7A & 3.8A), whereas OVA sensitisation/challenge resulted in significant infiltration by inflammatory cells (>2 x 10^6 cells, $p<0.05$) (Fig. 3.7B & 3.8B). The capacity for live attenuated *B. pertussis* BPZE1 to influence OVA-induced inflammatory influx to the respiratory tract was examined. OVA sensitisation resulted in characteristic inflammation with eosinophils, neutrophils, macrophages and lymphocytes detected in BALF, which was not seen in control mice (Fig. 3.7 & 3.8). Mice sensitised during virulent *B. pertussis* infection showed a similar pattern of inflammation. However, in marked contrast, immunisation with live attenuated BPZE1 prior to OVA sensitisation resulted in significantly reduced inflammatory infiltration of the airways ($p<0.05$) for all cells examined.

A key feature of inflammation in this model is OVA-driven allergic airway eosinophilia (Lukacs, 1994). However, immunisation with live attenuated BPZE1 prior to OVA sensitisation resulted in significant decrease in OVA-driven eosinophil infiltration of the airways ($p<0.05$) (Fig. 3.7D & 3.8D). The number of lymphocytes in the BALF was reduced by BPZE1 administration prior to OVA sensitisation/challenge, when compared to OVA treatment alone, whereas, no significant decrease was observed as a result of BPSM infection (Fig. 3.7B). Thus, a major finding of this study was that attenuated *B. pertussis* BPZE1 reduced the OVA-driven allergic airway inflammation typically seen in this model.

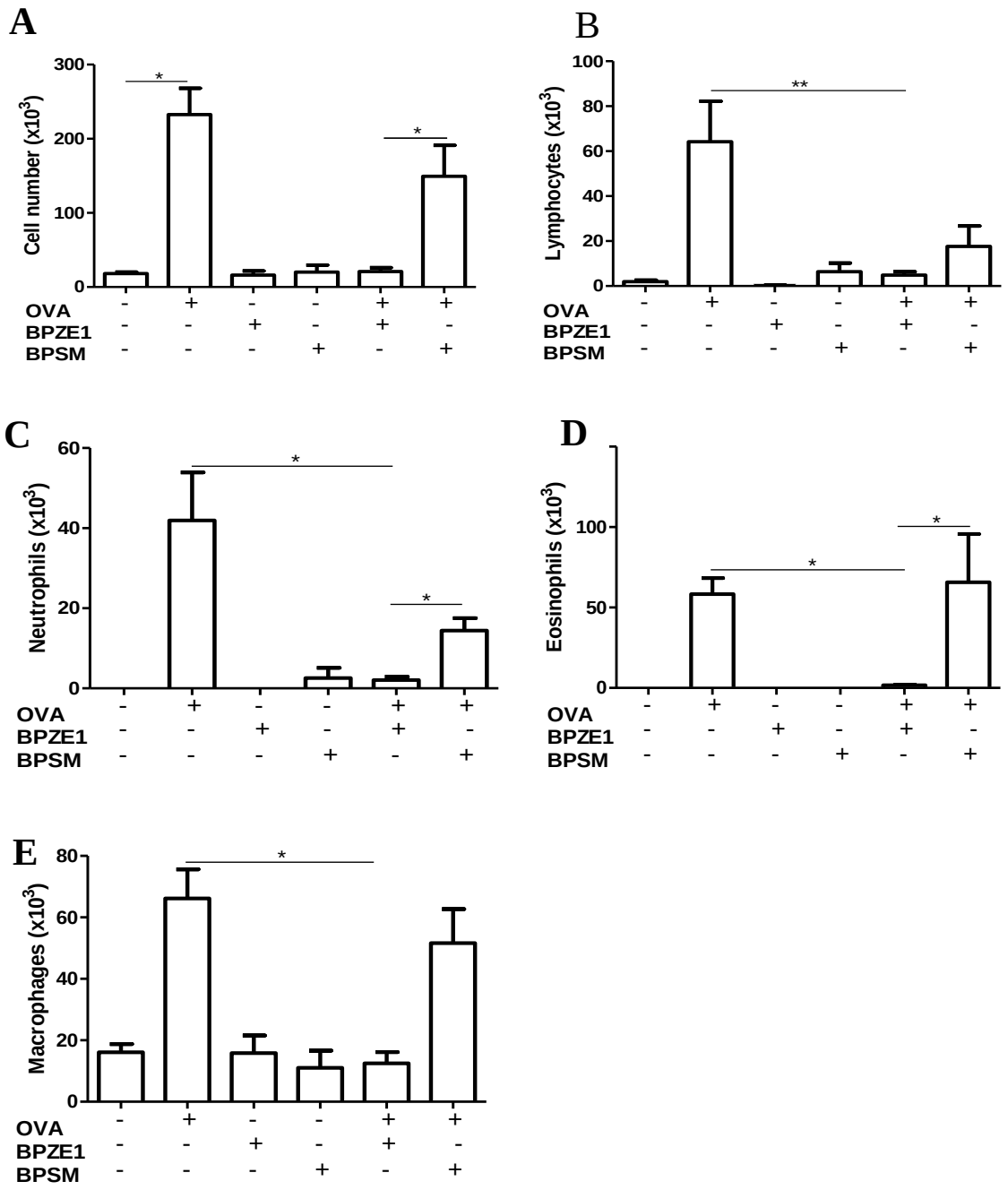


Figure 3.7 Attenuated *B. pertussis* BPZE1 reduces the cell infiltrate in BAL fluid. Effect of virulent *B. pertussis* infection, attenuated BPZE1 challenge and/or OVA sensitisation on BAL composition 24h after final OVA exposure. Negative controls were sham infected/sensitised with saline. BAL fluid was examined for the total cell number (A), or the presence of lymphocytes (B), neutrophils (C), eosinophils (D), or macrophages (E). The data are representative of two experiments; in each case, at least five animals were assessed. Results are expressed as mean ± S.E.M. of cell number. *$p < 0.05$.

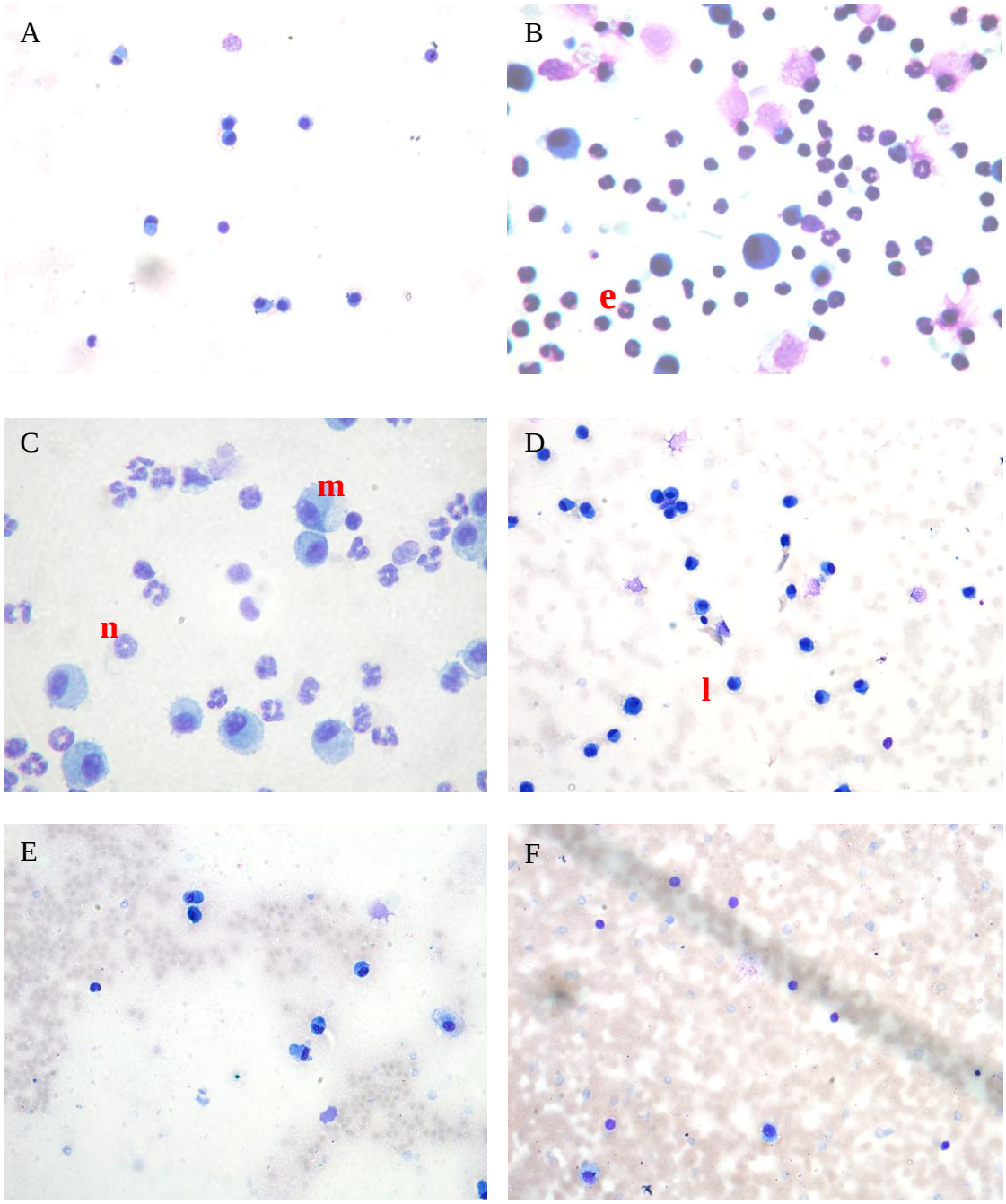


Figure 3.8 BALF was harvested on day 39 from (A) Non-sensitised (B) OVA-sensitised (C) OVA-sensitised and infected with *B. pertussis,* (D) OVA-sensitised and immunised with BPZE1, (E) Non-sensitised and immunised with BPZE1 and (F) Non-sensitised and infected with *B. pertussis.* Cells were stained with Giemsa according to manufacturers' instructions and examined microscopically for the presence of a number of cell types. e, m, n and l denote eosinophil, macrophage, neutrophil and lymphocyte, respectively. Magnification x 400.

3.7 ATTENUATED *B. PERTUSSIS* BPZE1 DOES NOT ENHANCE SERUM IgE RESPONSES TO SENSITISING ALLERGEN

OVA sensitisation in mice is known to induce IgE and a powerful specific Th2 response, whereas *B. pertussis* infection induces a strong Th1 response (Mills, 1993; Hamelmann, 1999). However, pertussis toxin alone can elevate IgE concentrations (Nilsson, 1998; Ryan, 2000). Therefore, it was important to explore whether BPZE1, producing a genetically inactivated pertussis toxin (Mielcarek, 2006), had an adjuvant effect or enhanced allergen-specific IgE. The influence of BPZE1 on allergic sensitisation was examined by measuring the concentration of OVA-specific IgE in serum from mice sensitised to OVA, infected with BPSM or BPZE1, or receiving combinations of these treatments (Fig. 3.9). Unlike non-sensitised controls, OVA sensitisation induced significant levels of IgE as expected. Allergen-specific IgE responses in mice exposed to attenuated BPZE1 prior to OVA sensitisation were not enhanced but were not significantly different to those receiving OVA alone. However, compared to mice infected with virulent BPSM in combination with OVA sensitisation, attenuated BPZE1 immunised mice exhibited a reduction ($p < 0.05$) in OVA-induced IgE. Therefore, unlike virulent *B. pertussis* SM, live attenuated *B. pertussis* BPZE1 delivered prior to allergen priming did not enhance or have an adjuvant effect upon the IgE response to allergen.

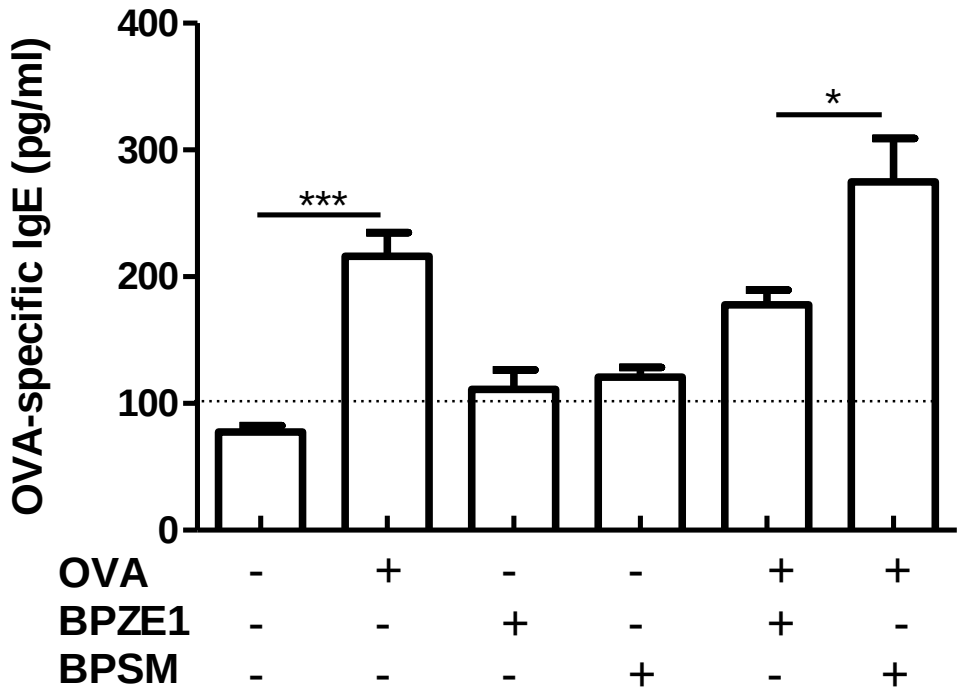


Figure 3.9 Attenuated *B. pertussis* BPZE1 does not enhance serum IgE responses to sensitising allergen. OVA-specific IgE in serum elicited in response to OVA sensitisation. Sera were collected on day 39 and OVA-specific serum IgE levels were measured by ELISA. The data presented are representative of two experiments; in each case, at least five animals were assessed, and each individual assessment was performed independently in triplicate. Concentrations below 100 pg/ml were considered negative. Results are expressed as mean antibody concentrations ± S.E.M. (*, $p < 0.05$, ***, $p<0.01$).

3.8 *B. PERTUSSIS* BPZE1 MODULATES RECALL CYTOKINE RESPONSES TO SENSITISING ALLERGEN

Pathology in the OVA model is driven by Th2 cytokines, in particular IL-5 and IL-13. However, there are two components to this; the first is the induction of IgE (Hamelmann, 1999; Sur, 1999) and classical atopic inflammation. The second is Th2-driven fibrosis/remodelling (Richter, 2001), which can be driven by IL-13 or antagonised by IFN-γ (Cohn, 2001; Wen, 2002). The observations here that allergen specific IgE was not significantly changed whilst pathology was reduced in mice immunised with BPZE1 prior to OVA sensitisation (Fig 3.5 & Fig 3.9) suggested that a mechanism of protection other than IgE reduction was involved here. To further investigate this, the influence of bacterial exposure on the pattern of allergen-induced cellular immune responses was characterised. OVA-specific cytokine production in both spleen cell preparations and BALF was assessed in the groups described above, in order to evaluate the influence of BPZE1 on allergen-induced priming. As expected, OVA sensitisation alone induced high levels of the Th2 cytokines IL-4, IL-5 and IL-13 (Fig. 3.10). Neither virulent BPSM nor attenuated BPZE1 alone induced any recall response to OVA. Virulent BPSM challenge prior to sensitisation did not induce a significant reduction in OVA-specific IL-5 or IL-13 and did not increase IFN-γ significantly (Fig. 3.10 & 3.11). However, a striking increase in IL-4 in BALF was observed when compared to those sensitised to OVA alone. In contrast, BPZE1 polarised the allergen response away from Th2-type cytokine production. Prior BPZE1 significantly reduced the levels of OVA-induced IL-5 ($p<0.005$), IL-13 ($p< 0.05$) and IL-4 ($c<0.005$). This was an immunomodulatory effect because suppression was not global; BPZE1 significantly increased IFN-γ in BALF and from splenocytes re-stimulated with OVA ($p< 0.05$) (Fig. 3.10 & 3.12). In summary, BPZE1 did not promote Th2 cytokine induction to allergen, but rather redirected this to a Th1 like response. This study demonstrated that BPZE1 shifted systemic

immune responses to OVA away from IL-4, IL-5 and IL-13 towards IFN-γ.

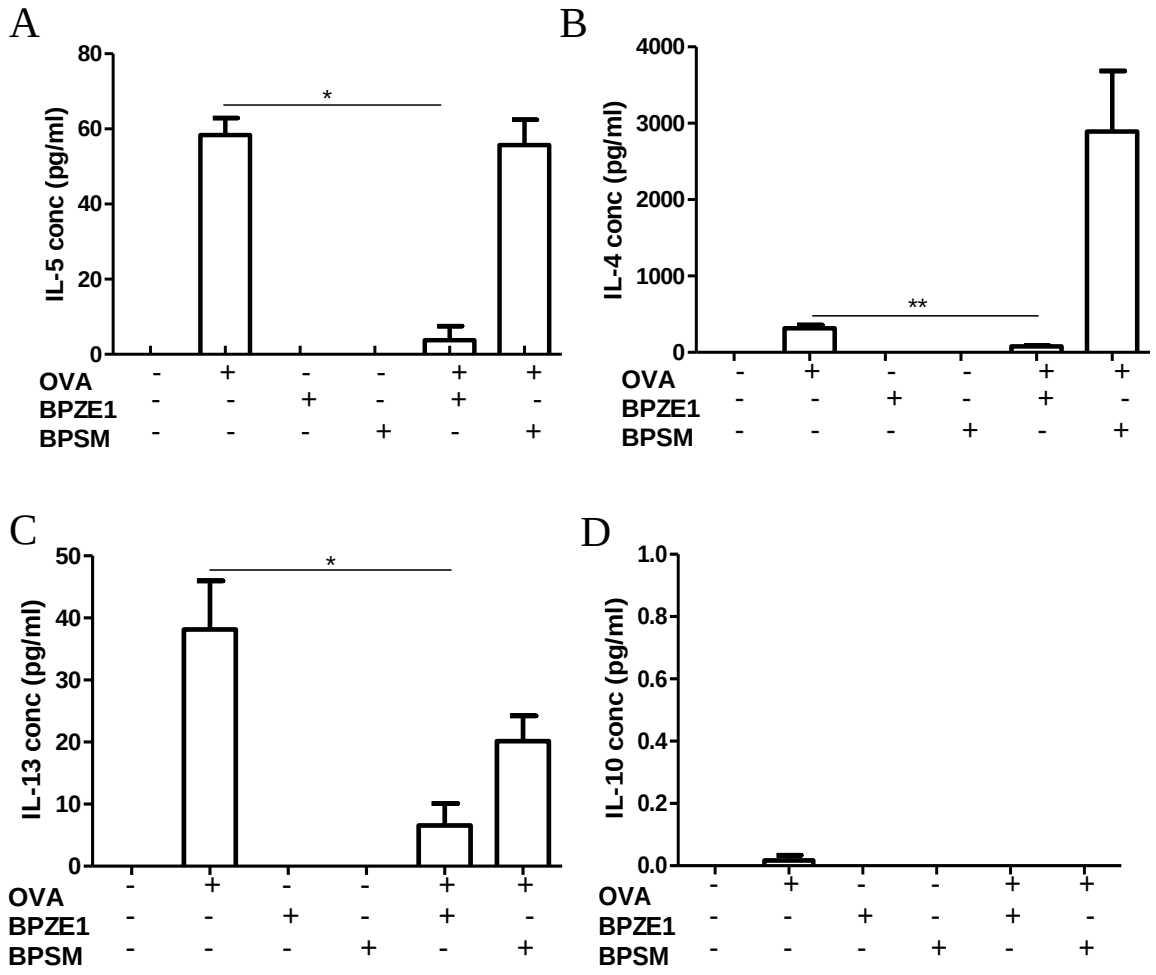


Figure 3.10 *B. pertussis* BPZE1 modulates recall cytokine responses to sensitising allergen in BALF. Cell-mediated immune responses in BALF, elicited by OVA sensitisation 10 days following prior exposure to attenuated (BPZE1) or virulent (BPSM) *B. pertussis* infection. BALF was harvested on day 39. Negative symbols indicate sham sensitisation or challenge with PBS. Cytokine responses from similar cultures are shown for (A) IL-5, (B) IL-4 (C) IL-13 and (D) IL-10. Responses are representative of duplicate experiments, each of which were determined independently from at least nine mice per group and are expressed as means ± S.E.M. $p < 0.05$.

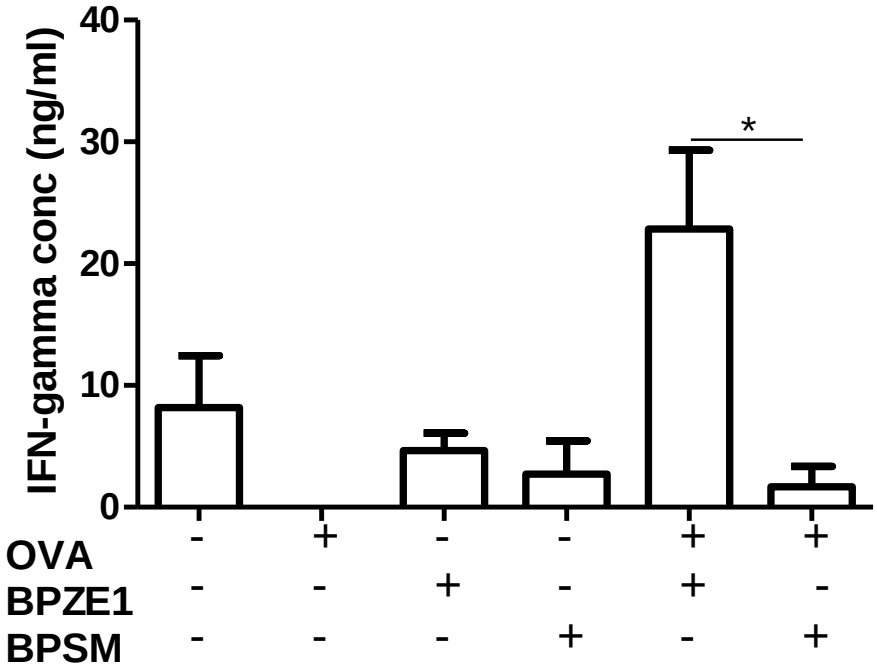


Figure 3.11 *B. pertussis* BPZE1 significantly increased IFN-γ production in BALF. BALF was harvested on day 39. Negative symbols indicate sham sensitisation or challenge with PBS. Responses are representative of duplicate experiments, each of which were determined independently from at least nine mice per group and are expressed as means ± S.E.M. $p < 0.05$.

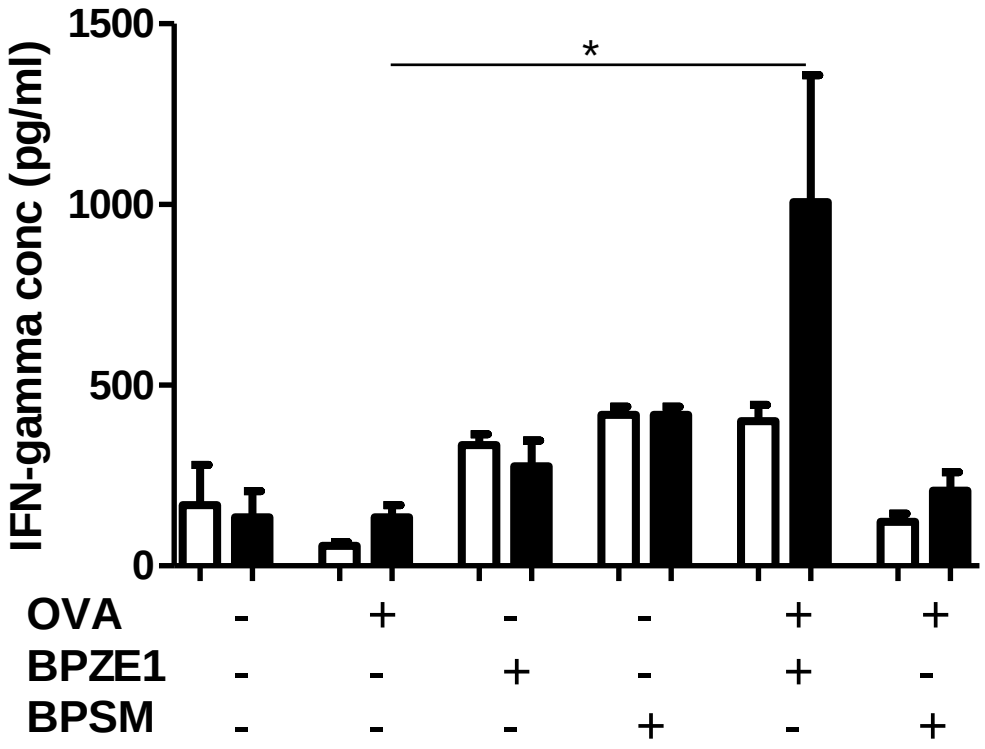


Figure 3.12 ***B. pertussis*** **BPZE1 significantly increased IFN-γ production from splenocytes re-stimulated with OVA.** Spleens were harvested on day 39 and processed as described in Section 2.13. Splenocytes were cultured in the presence of media alone (□) or OVA (200µg/ml) (■) for 72 h. Negative symbols indicate sham sensitisation or challenge with PBS. Responses are representative of duplicate experiments, each of which were determined independently from at least nine mice per group and are expressed as means ± S.E.M. $p < 0.05$.

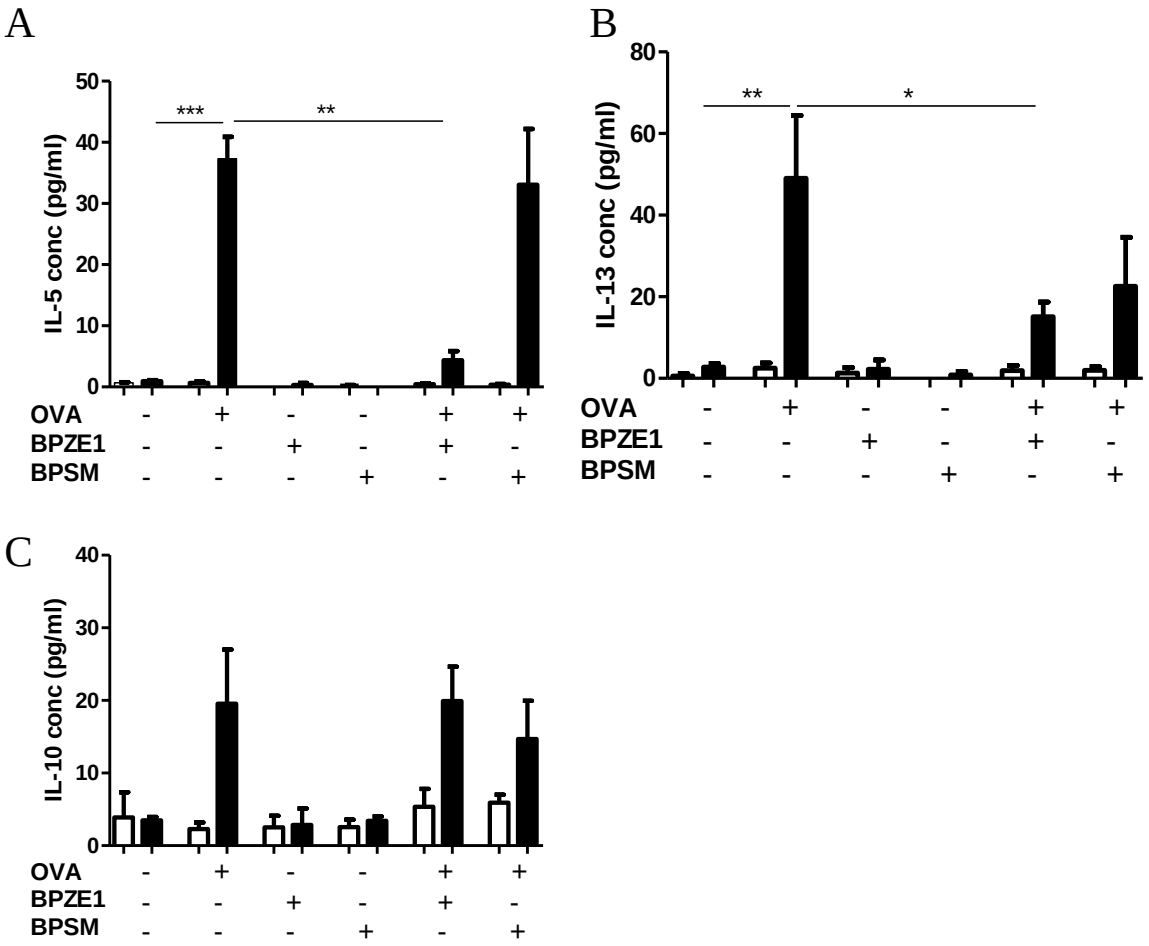


Figure 3.13 *B. pertussis* BPZE1 modulates recall cytokine responses to sensitising allergen from splenocytes re-stimulated with OVA. Cell-mediated immune responses from splenocytes restimulated with OVA elicited by OVA sensitisation 10 days following prior exposure to attenuated (BPZE1) or virulent (BPSM) *B. pertussis* infection. Cytokine responses from similar cultures are shown for (A) IL-5, (B) IL-13 and (C) IL-10. Spleens were harvested on day 39 and processed as described in Section 2.13. Splenocytes were cultured in the presence of media alone (□) or OVA (200µg/ml) (■) for 72 h. Negative symbols indicate sham sensitisation or challenge with PBS. Responses are representative of duplicate experiments, each of which were determined independently from at least nine mice per group and are expressed as means ± S.E.M.

3.9 ATTENUATED *B. PERTUSSIS* BPZE1 DOES NOT ENHANCE AIRWAY HYPERRESPONSIVENESS TO SENSITISING ALLERGEN

In further support of these observations, the influence of BPZE1 immunisation on lung function was examined using unrestrained whole-body plethysmography to measure PenH, a surrogate of airway hyperresponsiveness, following exposure to methacholine. As expected, sensitisation with OVA increased airway hyperresponsiveness (Fig. 3.14). Challenge with wildtype *B. pertussis* prior to OVA sensitisation demonstrated similar PenH values when compared to those sensitised to OVA alone. However, prior immunisation with BPZE1 resulted in significantly reduced bronchial hyperreactivity compared to those infected with virulent *B. pertussis*. PenH cannot assess direct pulmonary mechanics, but does reflect them (Lomask, 2006), therefore, the data needs to be interpreted with caution. Nevertheless, when combined with the histological and cell infiltration studies, these data strongly suggest that a live attenuated *B. pertussis* vaccine did not exacerbate OVA driven allergic airway inflammation.

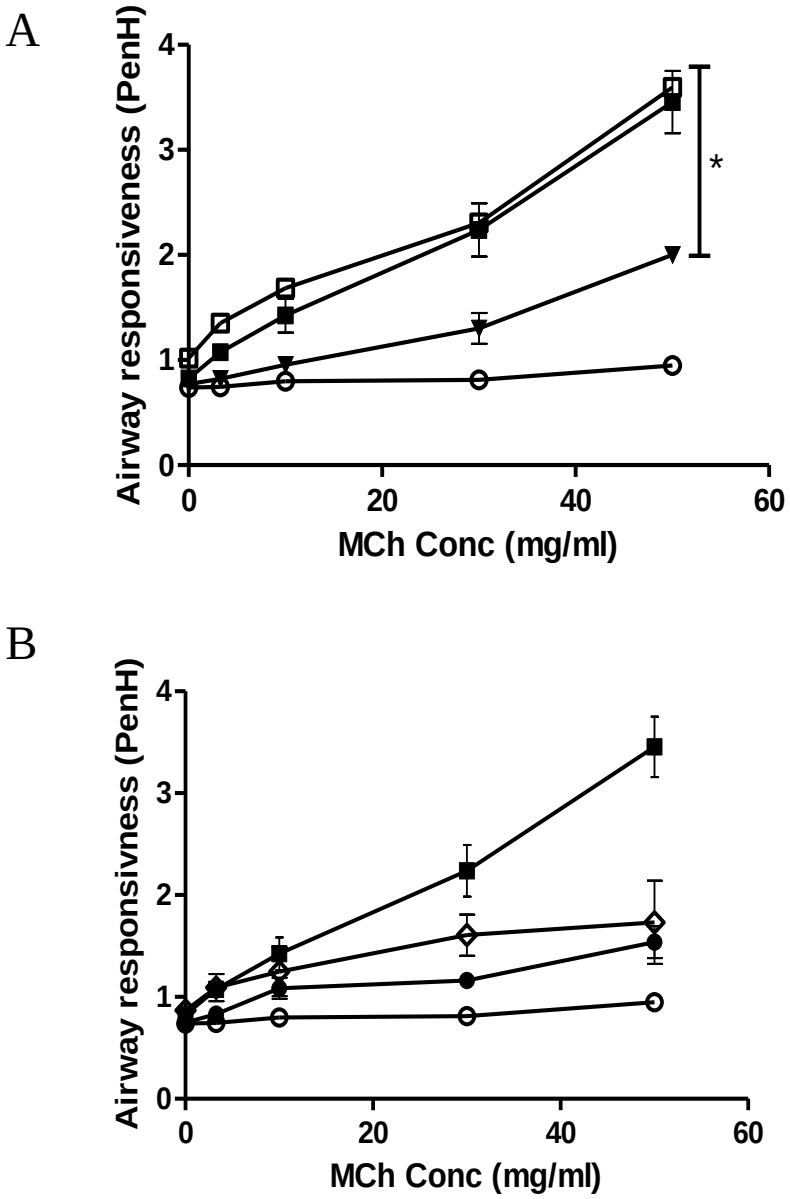


Figure 3.14 BPZE1 challenge prior to OVA sensitisation decreases airway hyperresponsiveness to sensitising allergen. Airway responsiveness was assessed on day 38 by methacholine induced airflow obstruction from conscious mice using whole-body plethysmography in conjunction with the BioSystem XA software (Buxco Electronics, USA) as described in Section 2.5. (A) Non-sensitised (-○-), OVA-sensitised (-■-), BPSM infected + OVA sensitisation (-□-) and BPZE1 challenge + OVA sensitisation (-▼-). (B) Non-sensitised (Control-○-), OVA-sensitised (-■-), non-sensitised BPSM infected (-◇-) and non-sensitised BPZE1 challenge (-●-). Results are expressed as mean enhanced pause (PenH) ± S.E.M. Where no error bars are visible, error bars are shorter than the size of the data point symbol.

3.10 ATTENUATED *B. PERTUSSIS* BPZE1 DOES NOT ENHANCE RECALL PROLIFERATIVE RESPONSES TO SENSITISING ALLERGEN

It was clear that attenuated BPZE1 has a radically different effect on allergen driven airway pathology when compared to virulent *B. pertussis*. The influence of bacterial exposure on the pattern of allergen induced modulation of the immune response was characterised. In addition to allergen-specific cytokine induction, the effect of BPZE1 on recall proliferative responses to exogenous ovalbumin was examined *ex vivo*. Splenocytes from OVA challenged but not saline challenged mice proliferated significantly in response to exogenously added antigen in a dose dependent manner (Fig. 3.15). Likewise, virulent BPSM induced a strong proliferative response to OVA ($p<0.001$). BPZE1 significantly reduced the levels of OVA specific proliferative responses when compared to OVA-sensitised mice ($p<0.05$). These data indicate that this attenuated *B. pertussis* strain did not have the adjuvant effect on recall OVA-specific proliferative responses, routinely associated with virulent *B. pertussis* (Ennis, 2004).

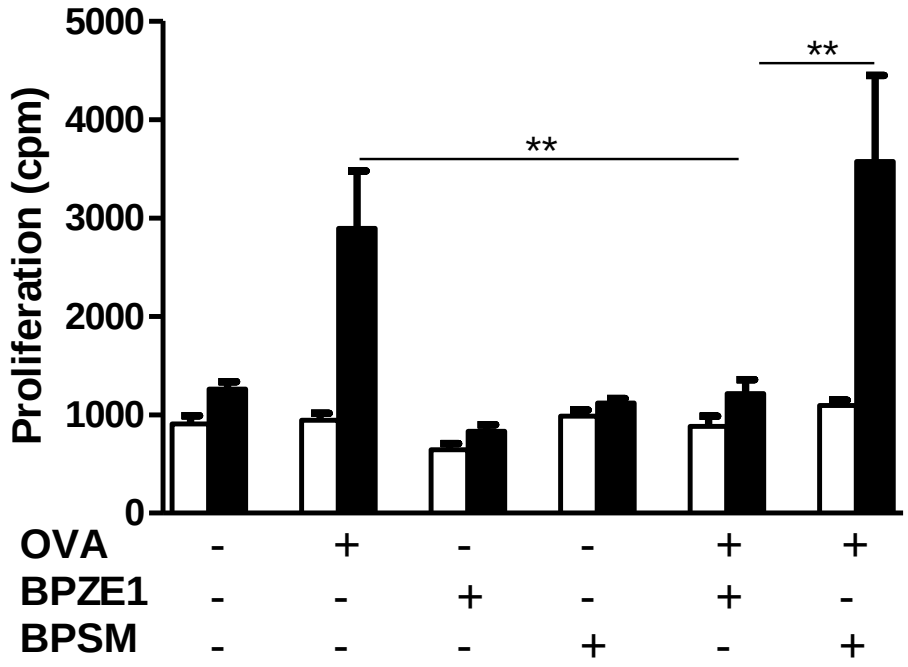


Figure 3.15 BPZE1 prevents recall proliferative response to exogenous allergen. Cell-mediated immune responses from splenocytes to OVA, elicited by OVA sensitisation 10 days following prior exposure to attenuated (BPZE1) or virulent (BPSM) *B. pertussis* infection. Spleens were harvested on day 39 and processed as described in Section 2.13. Splenocytes were cultured in the presence of medium alone (□) or OVA (200 µg/ml) (■) for 72 h. Subsequent proliferation was measured by 3H-thymidine incorporation (**, $p<0.05$). Negative symbols indicate sham sensitisation or challenge with PBS. Responses are representative of duplicate experiments, each of which were determined independently from at least nine mice per group and are expressed as means ± S.E.M.

3.11 SUMMARY

This chapter explored the influence of the candidate vaccine on OVA-induced airway pathology in order to (1) test the hypothesis that priming during a period of respiratory damage is an important factor in the phenotypic shift from Th2 to Th1 allergen-specific responses and (2) ascertain the safety of this live vaccine candidate in a preclinical model. An attenuated strain of *B. pertussis*, BPZE1, did not enhance but reduced allergen-driven airway pathology. BPZE1 prevented OVA-driven inflammation of the airways, assessed by histological and qualitative analysis of mucus production. Furthermore, BPZE1 diminished the severity of allergen-driven inflammation in the lungs. Eosinophilic and neutrophilic infiltration was significantly reduced in BPZE1-immunized mice sensitised to OVA. Virulent BPSM enhanced IL-4 in BALF, whereas BPZE1 modulated allergen responses towards a Th1 phenotype, demonstrated by a marked increase in IFN-γ. Likewise, analysis of recall responses to allergen showed that BPZE1 modulated responses away from IL-5 and IL-13 towards IFN-γ. In contrast to virulent *B. pertussis*, BPZE1 demonstrated no adjuvant associated increase in allergen-specific IgE.

The present study supports findings that suggest allergic asthma is linked not just to $CD4^+$ T cell profile, but also to the degree of airway damage at the time of priming (Ryan, 2000). Here, we use virulent (airway damaging) and attenuated (non-damaging) bacterial strains to show that prior immunisation with BPZE1, that does not damage the airway epithelium (Skerry, 2009) results in a significant reduction in allergic pathology, compared to those mice sensitised whilst infected with virulent *B. pertussis* (Skerry, 2009). Since attenuation of BPZE1 is based on the genetic removal or attenuation of three major toxins, pertussis toxin, tracheal cytotoxin and dermonecrotic toxin (Mielcarek, 2006), this study suggests that one, or a combination, of the attenuated virulence factors in BPZE1 plays a role in the adjuvant effect observed with virulent *B. pertussis* strains. The key finding here is that

in contrast to virulent *B. pertussis*, attenuated BPZE1 did not exacerbate allergen-induced airway pathology in a murine model indicating that induction of a robust Th1 response in the absence of respiratory damage can counteract the pro-allergic responses of Th2 cells.

The increase of allergic diseases in the industrialised world has often been explained by a decline in infections during childhood, suggesting that bacterial and viral infections during early life direct the maturing immune system towards Th1, which acts to counterbalance the pro-allergic responses of Th2 cells (Strachan, 2000; Romagnani, 2004). This results in the suppression of Th2 cell expansion, and a consequent inhibition of isotype switching to IgE. Some versions of the hygiene hypothesis suggest that Th1-inducing infections may have an inhibitory effect on the development of atopy (Strachan, 2000). However, previous studies have demonstrated that the induction of Th1 immunity did not counterbalance Th2 driven allergy but rather altered the pathology (Hansen, 1999; Ennis, 2004). This has been clarified by work with microbial agents; for example virulent *B. pertussis* enhanced the severity of allergen driven airway pathology (Mills, 1998; Ennis, 2004) despite induction of Th1 immunity. In contrast, systemic immunisation with a Th1-inducing whole cell pertussis (Pw) vaccine inhibited allergic airway responsiveness (Ennis, 2005). These seemingly counter-intuitive findings suggest that protection from allergen-driven pathology is linked not just to $CD4^+$ T cell profile, but also to the degree of airway damage at the time of priming. This is more consistent with asthma models predicated on damage and the disruption of the epithelial mesenchymal unit (Holgate, 2000; Wark, 2005; Holgate, 2007; Swindle, 2009). These observations have an important public health implication as the need to understand the role of immunisation and prevalence of asthma is necessary for reassuring populations recruited to mass vaccination programmes.

Immunisation with Pw protects against *B. pertussis*-exacerbation of OVA-induced airway hyperresponsiveness in a murine model of allergic airway inflammation (Ennis 2005)

and there is no allergy-promoting effect in response to common childhood vaccines, including pertussis vaccines (Grüber, 2008). However, much less is known about protective effects of childhood vaccines against allergic asthma. Other investigators have shown that live attenuated vaccines, such as oral poliomyelitis vaccine or Bacillus Calmette-Guérin, inhibited the development of asthma and allergic disease (Martignon, 2005). The purpose of Chapter 3 was to test the hypothesis that priming during a period of respiratory damage is an important factor in the pathology of allergen-specific responses. This was probed using virulent (airway damaging) and attenuated (non-damaging) bacterial strains and in particular BPZE1 a candidate for use as a live, intranasal, single-dose neonatal vaccine against whooping cough (Mielcarek, 2006).

The present study supports findings showing that prior immunisation with BPZE1, which does not damage the airway epithelium (Skerry, 2009), results in significantly reduced allergic pathology, compared to those mice sensitised whilst infected with virulent *B. pertussis*. BPZE1 prevented OVA-driven inflammation of the airways, diminished the severity of allergen-driven inflammation in the lungs and significantly reduced airway eosinophilia. Unlike the virulent strains used in this study, BPZE1 modulated allergen responses away from Th2-like responses towards a Th1 phenotype, demonstrated by a marked increase in IFN-γ. BPZE1 also demonstrated no adjuvant associated increase in allergen-specific IgE. Taken together, these data demonstrate that attenuated BPZE1 acts as a powerful immunomodulator that suppresses allergen-driven pathology at both a local and systemic level, does not promote allergic sensitisation and may indeed be immunomodulatory.

The mechanisms underlying the beneficial influence of attenuated BPZE1 on allergen-driven pathology may be multiple and inter-linked. In other models, immune interaction between infection and allergen sensitisation critically depends on timing of challenges

(Peebles, 2001). Here, the challenge schedule was chosen to examine the effect of prior immunisation on allergen sensitisation and for consistency with previous reports (Ennis, 2004; Ennis, 2005a; Ennis, 2005b). However it will be important to examine the reverse scenario to assess the effect of immunisation on pre-existing allergen sensitivity, such as that seen using unmethylated CpG oligodeoxynucleotides which inhibited ragweed allergen-induced lung inflammation in pre-sensitised animals (Sur, 1999). As exposure to CpG oligodeoxynucleotides would be expected during challenge with BPZE1, the vaccine might also be predicted to inhibit pre-existing sensitivity.

Pathology in the OVA model is driven by Th2 cytokines, in particular IL-5 and IL-13. However, there are two components to this; the first is the induction of IgE (Hamelmann, 1999) and classical atopic inflammation. The second is Th2-driven fibrosis/remodelling (Richter, 2001), which can be antagonised by IFN-γ (Cohn, 2001; Wen, 2002). The observations here that allergen specific IgE was not significantly changed whilst pathology was reduced in mice immunised with BPZE1 prior to OVA sensitisation suggested that a mechanism of protection other than IgE reduction was involved here. Infection with virulent *B. pertussis* exaggerates the OVA-induced inflammatory influx to the respiratory tract, with an increase in eosinophils, and an associated increase in the severity of airway pathology. Administration of attenuated BPZE1 prior to allergen sensitisation significantly reduced inflammatory infiltration (Fig. 3.1). The inhibition of allergic airway pathology might be explained by the modulation of the key cytokines involved in remodelling. For example, airway mucus hypersecretion is in part driven by IL-13 and is a major pathophysiological feature of both allergic asthma (Wills-Karp, 1998) and whooping cough (Heininger, 2001). Therefore, it is not surprising that mucus production mirrored IL-13 levels in this study and was significantly reduced in sensitised mice previously exposed to BPZE1. This study demonstrated that BPZE1 shifted systemic immune responses to OVA away from IL-4, IL-5

and IL-13 towards IFN-γ (Fig. 3.12). IL-4 and IL-5 contribute to tissue damage and remodelling due to their function as mediators of eosinophil recruitment (Brusselle, 1994; Gleich, 2000). However, IFN-γ can directly suppress IL-4 gene expression through IRF-1 and 2, which bind to three distinct IL-4 promoter sites and act as transcriptional repressors (Elser, 2002). IFN-γ can also inhibit the expression of the eotaxin receptor (CCR3), an important inducer of eosinophil differentiation from hematopoietic progenitor cells (Lamkhioued, 2003).

Taken together, these data suggest that immune modulation in this model has its greatest impact on mucus secretion and remodelling rather than IgE induction. Since attenuation of BPZE1 is based on the genetic removal or attenuation of three major toxins, pertussis toxin, tracheal cytotoxin and dermonecrotic toxin (Mielcarek, 2006), this study suggests that one, or a combination, of the attenuated virulence factors in BPZE1 plays a role in the adjuvant effect observed with virulent *B. pertussis* strains. The protection against allergen-driven pathology and the modulation of the allergic immune response by BPZE1 seen here is consistent with some versions of the hygiene hypothesis. It might be that the key beneficial feature of BPZE1 is the combination of a Th1 skewed response, combined with the absence of induced airway pathology. This is supported by previous reports in which exacerbation of airway pathology to allergen was associated with allergen priming during a period of airway damage or remodelling (Gern, 2000; Ennis, 2004; Marsland, 2004). Fig. 3.16 illustrates the plausible mechanisms by which an attenuated *B. pertussis* strain, in the absence of damage, regulates the allergic inflammatory response via an IFN-γ-dependent pathway.

In both systems OVA tends to induce Th2 responses and remodelling, whereas infection tends to support IFN-γ and Th1. In the case of sensitisation during a Th1 infection but where no epithelial damage occurs (BPZE1), conventional immune modulation takes

place, thus BPZE1 reduces Th2 cytokine production, increases Th1 immunity, and inhibits IL-5 secretion and eosinophilia. Furthermore IFN-γ directly effects remodelling. In contrast when a breakdown in the mesenchymal-epithelial unit occurs (e.g. damage by virulent BPSM) in spite of IFN-γ production, there is an over-riding requirement to repair the airways. The fibrotic/remodelling process is exacerbated by the active pertussis toxin adjuvant effect on OVA-induced IL-13 and IL-4 which in turn promotes fibrosis. Thus there is an enhanced pathology despite induction of Th immunity.

The data presented here support the use of BPZE1 as a candidate vaccine even for populations where exposure to allergens and atopy is prevalent. Most current pertussis vaccination regimes require three doses, beginning at 2 months of age necessitating 6 months for optimal protection (Das, 2002). Therefore, there is a need for vaccines that induce strong protection against *B. pertussis* in neonates. BPZE1 has been developed as a candidate neonatal vaccine (Mielcarek, 2006) as it induces strong local and systemic immune responses upon intranasal delivery (Mielcarek, 2006). Administration via the nasal route mimics natural infection and is expected to promote long-lasting immunity in children from 1 month of age (Mascart, 2003). Live recombinant bacteria that have been adapted to the respiratory tract are attractive vehicles for the presentation of vaccine antigens to the respiratory mucosa. As a result, *B. pertussis* has been used successfully as a live bacterial vector in mouse models (Mielcarek, 2001). These combined benefits of BPZE1 as a potential protective agent against atopy makes it an attractive candidate as a neonatal vaccine against whooping cough and also a potential vehicle for vaccine delivery via the nasal route.

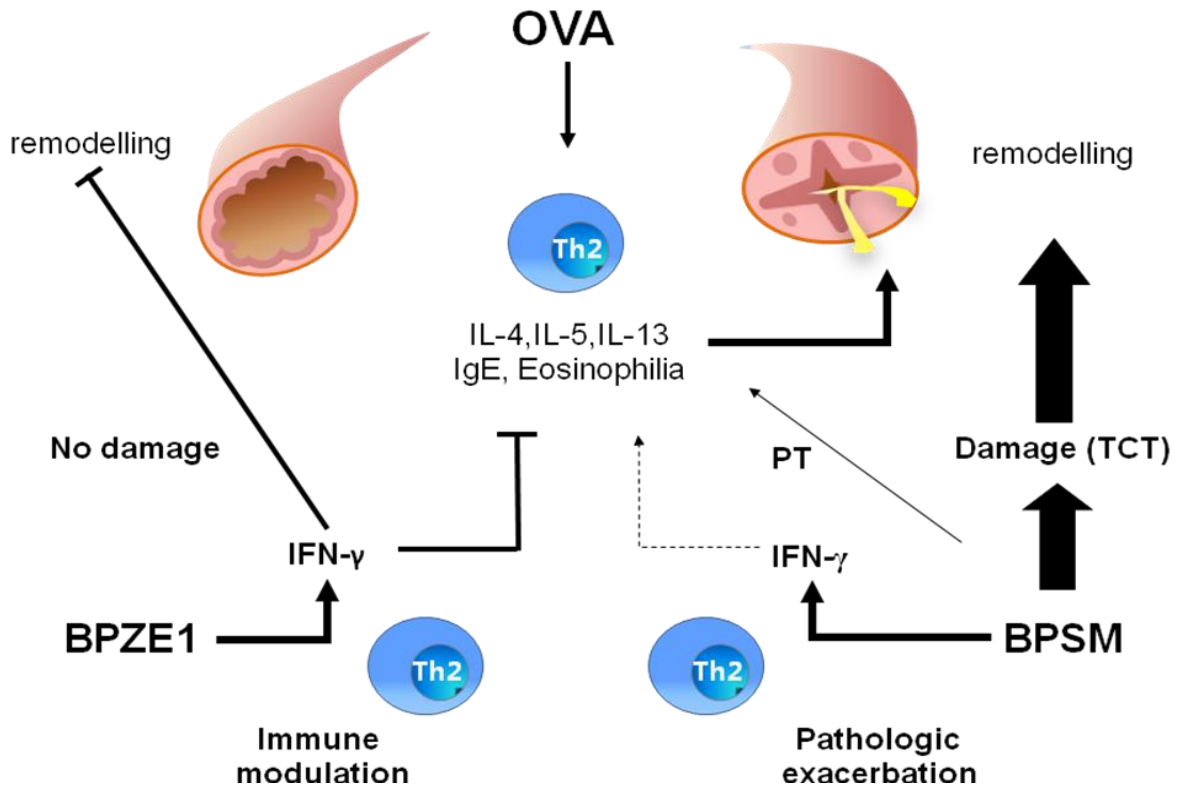


Figure 3.16 Schematic presentation of plausible mechanism by which attenuated *B. pertussis*, in the absence of damage, regulates the allergic inflammatory response via an IFN-γ-dependent pathway. In the absence of respiratory damage, BPZE1 increases Th1 immunity, reduces Th2 cytokine production and inhibits IL-5 secretion and eosinophilia which directly affects airway remodelling. In contrast when a breakdown in the mesenchymal-epithelial unit occurs (e.g. damage by virulent BPSM) in spite of IFN-γ production, the fibrotic/remodelling process is exacerbated by the active pertussis toxin adjuvant effect on OVA-induced IL-13 and IL-4 which in turn promotes fibrosis.

CHAPTER 4

THE ISOLATION AND CHARACTERISATION OF ADULT MESENCHYMAL STEM CELLS

4.1 INTRODUCTION

The overall goal of the work presented in this thesis was to investigate immune modulation with a particular focus on airway inflammation and allergic pathogenesis. In Chapter 3, this was probed using virulent (airway damaging) and attenuated (non-damaging) bacterial strains to test the hypothesis that priming during a period of respiratory damage is an important factor in the phenotypic shift from Th2 to Th1 allergen-specific responses. The immunomodulatory capacity of mesenchymal stem cells (MSC) suggests that they have a potential therapeutic value in a number of inflammatory and autoimmune diseases models (Lee, 2006; Bouchez, 2008). MSC are a heterogenous subset of stromal stem cells that can differentiate into cells of mesodermal lineage, such as adipocytes, osteocytes and chondrocytes (Horwitz, 2005). They possess potent anti-proliferative and anti-inflammatory effects supporting their possible use as a therapy for immune-mediated diseases (Krampera, 2003; Barry, 2005). The purpose of Chapter 4 was to determine the immunomodulatory effect of bone marrow-derived MSC with particular emphasis on the establishment of an experimental system. This was broken down into three objectives: (1) to establish and verify that the protocol for MSC isolation from bone marrow delivers reliable and homogenous MSC populations for potential cell therapy (2) to investigate the effect of exogenous IFN-γ on MSC-mediated immunosuppression *in vitro*, and (3) to examine the immunosuppressive capacity of MSC isolated from IFN-γ-receptor knockout mice.

4.2 ISOLATION OF ADULT MURINE MSC BY ADHERENCE TO PLASTIC

As bone marrow contains both haematopoietic and mesenchymal cell types, it was important to determine the cell type(s) isolated and the purity of those cells. Murine MSC were isolated from the femurs and tibiae of BALB/c female mice using plastic adherence and selective media, as described in Section 2.21. MSC isolated by adherence to plastic were

examined microscopically at each passage for changes in morphology. A homogeneous MSC population was evident at passage 3 with fibroblastoid spindle shaped morphology (Fig. 4.1). There was no evidence of spontaneous differentiation in these cultures. Subsequent passages were characterised by flow cytometry for expression of surface markers. Bone marrow-derived adherent cell layers comprise many different cell types including fibroblasts, hematopoietic progenitor cells, macrophages, endothelial cells, and adipocytes (Tavassoli, 1982; Zuckerman, 1983; Phinney, 1999). Typically, MSC isolated by other groups expressed CD9, CD29 and CD81 and variable levels of Sca-1, CD106 and CD44 but not CD11b, CD31, CD34, CD45, CD48, CD90, CD117 or CD135 (Baddoo, 2003). Although no specific marker has been reported for murine MSC, the expression of Sca-1 and CD44 and the lack of expression of CD45 and CD11b are typically used for the identification of MSC (Peister, 2004). Primary cultured cells from mouse bone marrow were immunophenotyped for expression of various cell surface antigens. CD44 and Sca-1 expression and absence of CD11b and CD45 was observed on the populations selected. Flow cytometric analysis demonstrated that the MSC population lacked detectable expression of MHC II. They were also negative for the haematopoietic markers CD45, CD11b and CD34 and the haematopoietic progenitor cell marker CD117. Passage 3 murine MSC expressed low levels of CD106 and CD80 (Fig. 4.2). The cells were also positive for CD105. The stability of expression was measured over passages 4-8 for all cell types. MSC characterised at each subsequent passage possessed a similar phenotype to that seen at passage 3. Thus the cells isolated were devoid of contaminating haematopoietic cells but showed stable expression of typical MSC markers (Fig. 4.1 and Table 4.1) (Baddoo, 2003; Peister, 2004).

MSC were also isolated from two other mouse strains (FV/BN and IFN-$\gamma R^{-/-}$), these had the same appearance and expression characteristics as the MSC described above (Fig. 4.2). A limited number of adult human MSC were also required. Human MSC were isolated

and expanded from aspirates of bone marrow from three different donors provided by collaborators at the National University of Ireland Galway (NUIG) and validated by flow cytometry (Table 4.2).

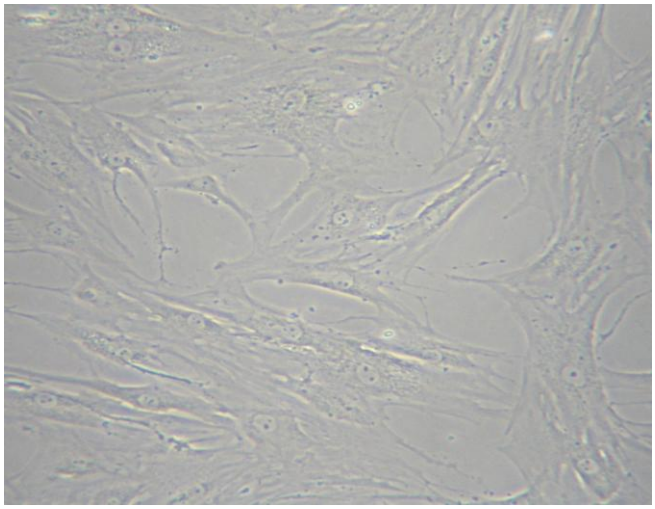


Figure 4.1 MSC exhibit a homogeneous morphology typical of stromal cells. Plastic adherent MSC were grown to near confluence and examined using phase contrast microscopy for morphological appearance at passage 3. Magnification x100.

Table 4.1 Summary of surface marker expression of mouse mesenchymal stem cells

Marker	Expression
MHC I	+
MHC II	-
Sca-I	+
CD11b	-
CD34	-
CD44	+
CD45	-
CD80	+
CD90	-
CD105	+
CD106	+
CD117	-

Table 4.2 Summary of surface marker expression of human mesenchymal stem cells

Marker	Expression
CD29	-
CD34	-
CD44	+
CD45	-
CD105	+
CD106	+
CD90	+
HLA-ABC	+
HLA-DR	-

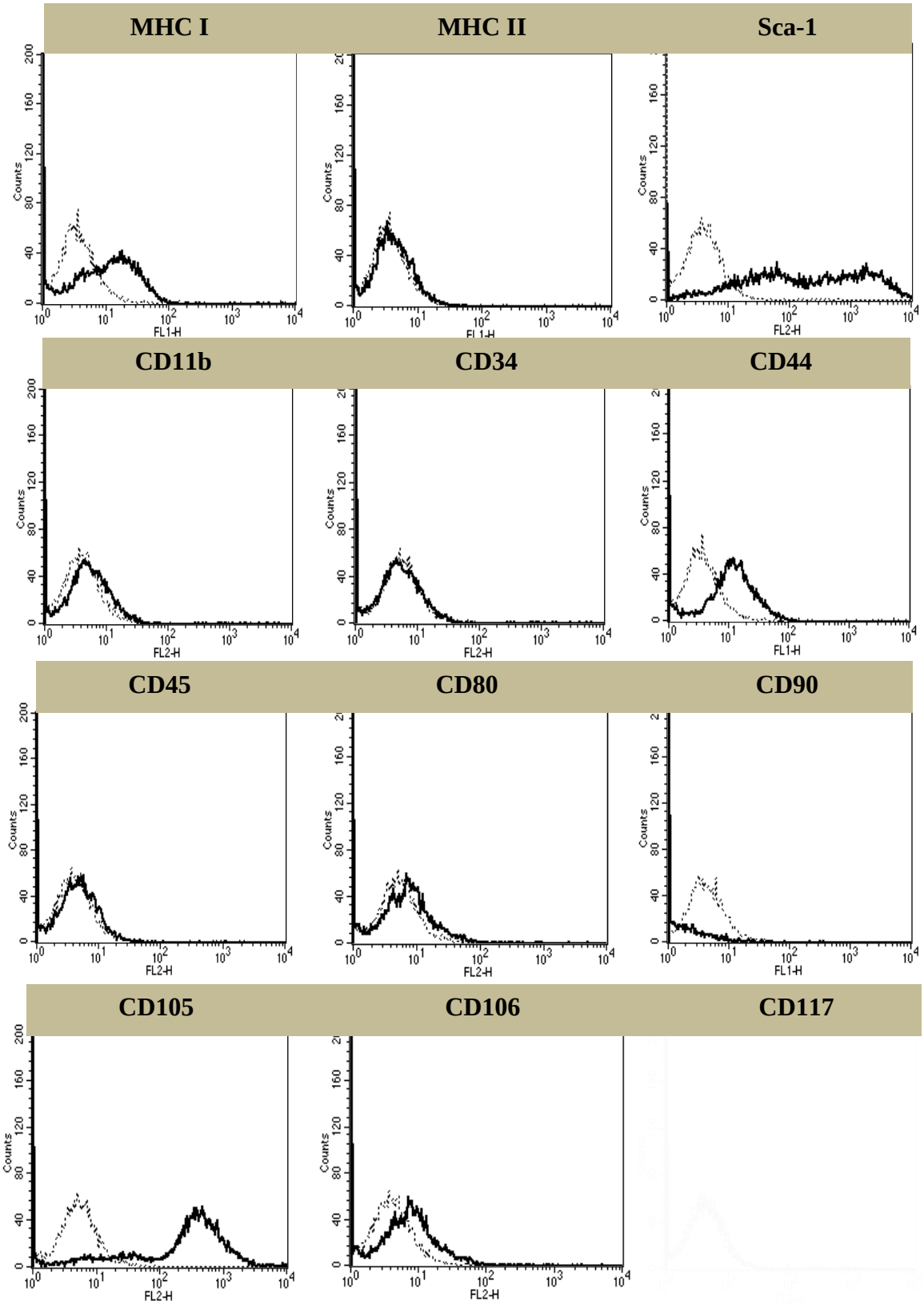


Figure 4.2 MSC isolated by adherence to plastic from FV/BN mice expressed MHC class I, Sca-1, CD44, CD80 and CD106 but not CD117, CD105, CD80, CD45 and CD90 at passage 3. MSC were characterised by flow cytometry for expression of a panel of markers (black) and corresponding isotype controls (dotted line) at passage 4. A minimum of 10,000 events were acquired for each marker.

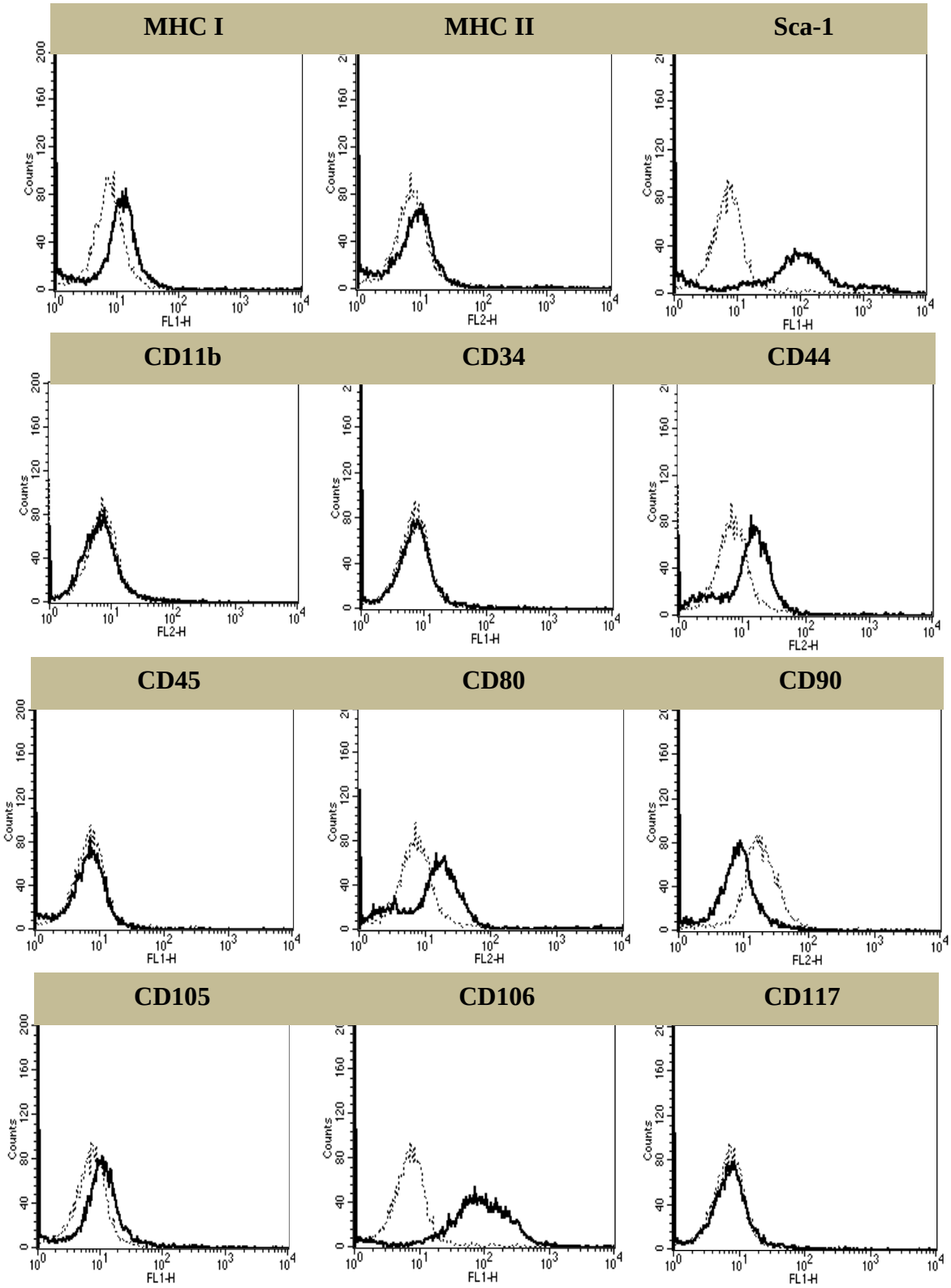


Figure 4.3 MSC isolated by adherence to plastic from IFN-γR$^{-/-}$ mice at passage 3 expressed MHC class I, Sca-1, CD44, CD90 and CD105 but not MHC class II, CD11b, CD34, CD45, CD117 or CD80. Plastic adherent MSC were characterised for expression of a number of markers (black) and corresponding isotype controls (dotted line) at passage 4. A minimum of 10,000 events were acquired for each marker.

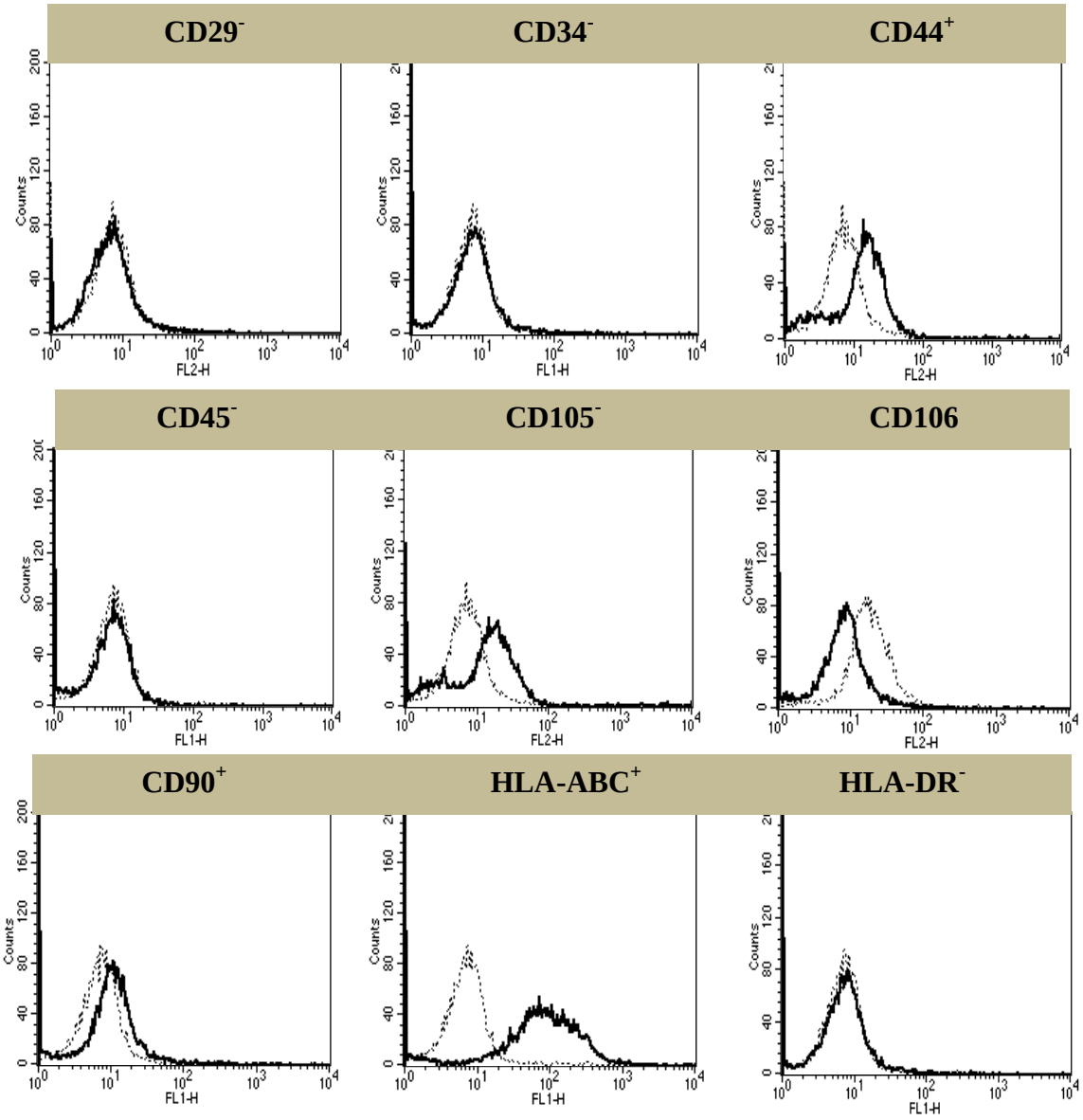


Figure 4.3 MSC isolated by adherence to plastic from human MSC at passage 3 expressed CD44, CD105, CD44, CD106, CD90 and HLA-ABC but not CD29, CDCD34, CD45 or HLA-DR. Plastic adherent MSC were characterised for expression of a number of markers (black) and corresponding isotype controls (dotted line) at passage 4. A minimum of 10,000 events were acquired for each marker.

4.3 DIFFERENTIATION CAPACITY OF MSC

An important characteristic of MSC is the ability to differentiate into multiple cell types, including cartilage, fatty tissue and bone. In order to investigate the stemness or differentiation potential of the isolated MSC, their ability to differentiate into adipocytes, chondocytes and osteoblasts was examined (Fig. 4.5). In addition, MSC were cultured for the same period without differentiation components and examined for the appearance of spontaneous differentiation. Adipocytes were visualised by staining with Oil Red O, osteoblasts by staining with Alizarin Red S and chondrocytes by Alcian blue staining. Control cells were stained with the corresponding dyes. Cells differentiated into adipocytes were identifiable by large lipid filled vacuoles. Adipocytes, chondocytes or osteoblasts were not observed in control cultures, indicating the absence of spontaneous differentiation (Fig. 4.5). These cells maintained their differentiation capacity through each passage. These data confirm the multilineage potential associated with MSC and their capacity to retain this function through each generation. The ability to attach to tissue culture plastic, their potential to differentiate along mesenchymal lineages, fibroblastic morphology, and the pattern of cell surface markers support the method of isolation used here as sufficient to obtain a pure and stable population of MSC for further study.

4.4 IMMUNOMODULATORY CAPABILITY OF MSC

MSC inhibit the proliferation of T cells induced by non-specific mitogens (Nicola, 2002; Ryan, 2007). Therefore, it was important to test for the presence of this characteristic feature prior to using the cells therapeutically. The immunosuppressive ability of MSC was assessed by carrying out mitogen driven proliferation assays. Splenocytes were cultured in the presence of the mitogen ConA in the presence or absence of MSC. The level of

proliferation was measured using ^{3}H thymidine incorporation. As expected, ConA stimulation induced significant splenocyte proliferation (Fig. 4.6), however, MSC significantly inhibited this (*, $p<0.05$). ConA stimulation is a powerful T cell mitogen, so this result indicated that MSC were capable of potent immunosuppression of lymphocyte activity.

MSC also possess the ability to suppress alloreactivity (Djouad, 2003; Krampera, 2003; Augello, 2005). This function was assessed by carrying out a two way murine mixed lymphocyte reaction (MLR). Splenocytes from two MHC-mismatched mice (D1 and D2 isolated from BALB/C and C57/BL6 mice, respectively) were co-cultured. The effect of MSC on lymphocyte proliferation was assessed by adding allogeneic MSC isolated from FV/BN mice to the MLR. The co-culture of allogeneic splenocytes (D1 & D2) induced significant proliferation; however, MSC effectively inhibited the proliferation of mismatched splenocyte T cells (Fig. 4.7). This capacity was retained over subsequent passages (4-7).

These data suggested that the MSC populations used here were capable of significantly reducing both alloantigen-driven and mitogen-driven proliferation in mixed lymphocyte reactions supporting their use as a therapeutic modality *in vivo*.

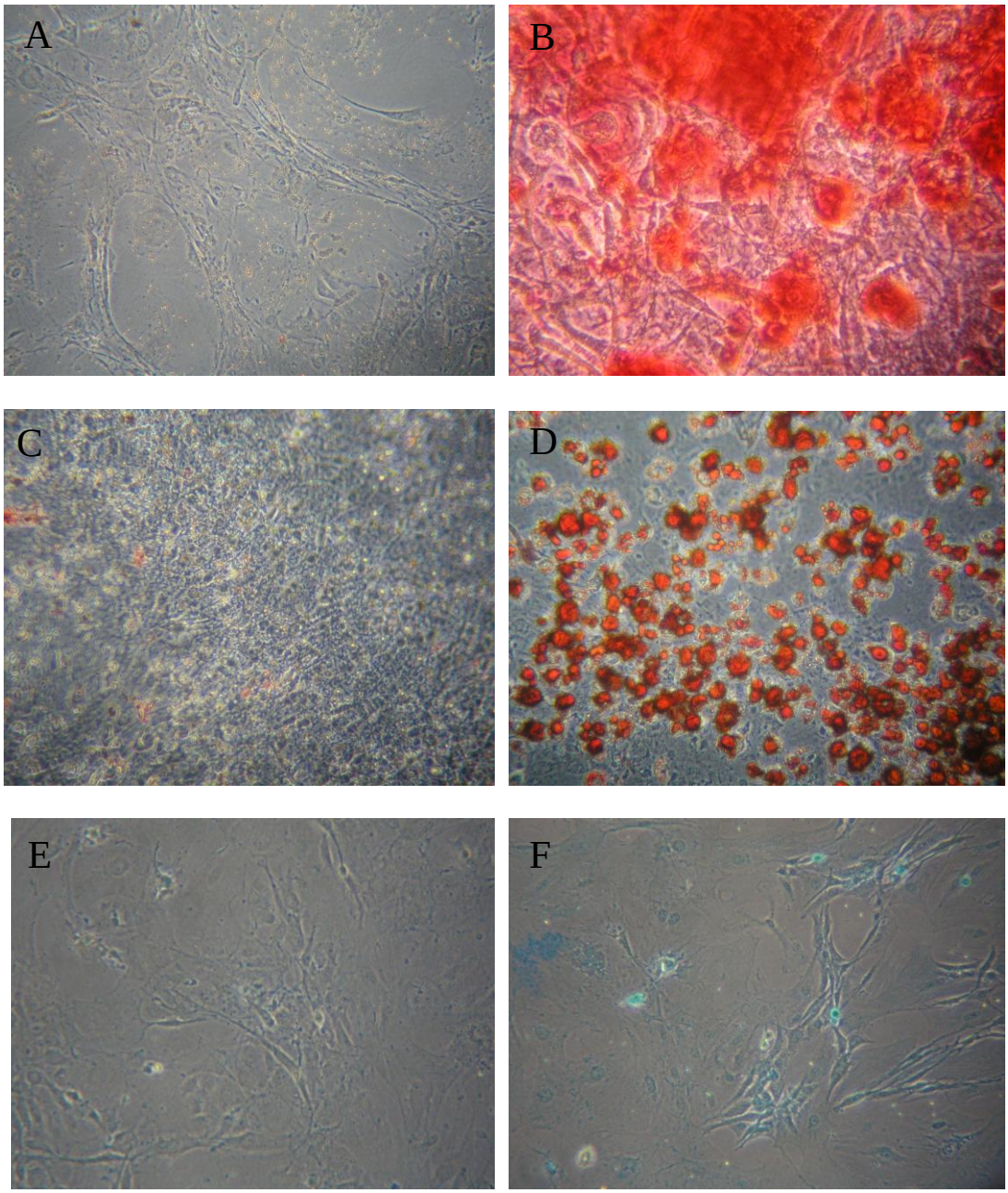


Figure 4.5 MSC differentiated along the chondrocytic, adipogenic and osteogenic lineages. MSC were seeded at a density of 1 x 10^3cells/cm^2 and cultured in normal media (A & C), osteogenic induction media (B) adipogenic induction media (D) or chondrogenic induction media (F) for 21 days. Adipogenesis was visualised by staining cells with Oil Red O. Osteogenesis was assessed using Alizarin Red S. MSC did not spontaneously differentiate into adipogenic (A) osteogenic cells (C) or chondrogenic cells (E) (when cultured for 21 days in normal expansion media without differentiation components. Magnification x 100.

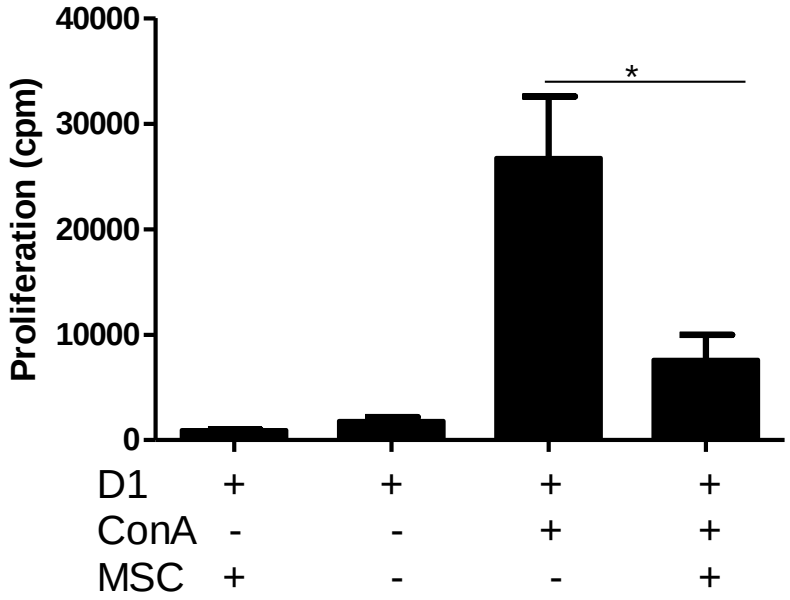


Figure 4.6 Allogeneic MSC inhibit mitogen driven proliferation. FV/BN MSC (1.5×10^4 /ml) were examined for their capacity to inhibit mitogen (ConA) driven proliferation of BALB/c (D1) splenocytes at passage 3. MSC significantly inhibited mitogen driven proliferation (*, $p<0.05$). Results are representative of three experiments, each performed in triplicate.

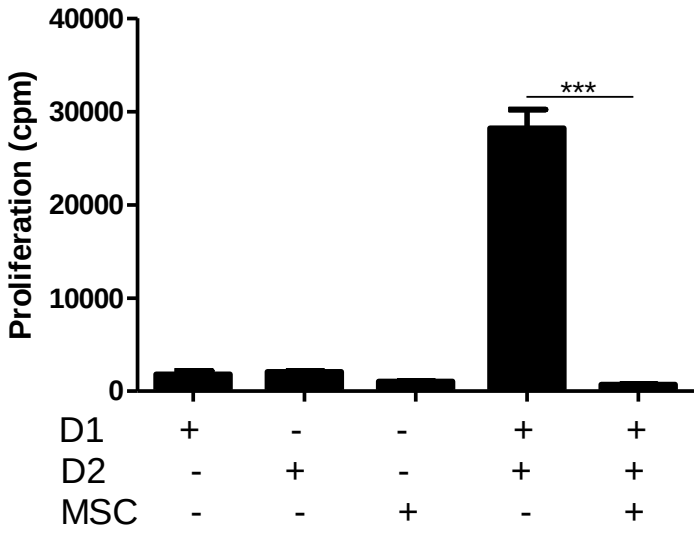


Figure 4.7 Allogeneic MSC inhibit allo-driven proliferation. MSC were examined for their capacity to inhibit allogeneic-driven proliferation of splenocytes in a two-way mixed lymphocyte reaction. FV/BN MSC (1.5×10^4) were co-cultured with splenocytes (2×10^5) from MHC-mismatched mice (BALB/c (D1) and C57BL/6 (D2) respectively). MSC significantly inhibited alloantigen driven proliferation (*$p<0.0005$). Results are representative of three experiments, each performed in triplicate.

4.5 IFN-GAMMA ENHANCES MSC-MEDIATED IMMUNOSUPPRESSION

In vitro studies have shown that MSC can suppress T lymphocyte proliferation more efficiently when triggered by cellular stimuli, non-specific mitogenic stimuli or antigen (Sheng, 2008, Phinney, 2006, Corcione, 2006). Therefore, pre-stimulation or "licensing" of MSC prior to *in vivo* administration could potentiate its' putative protective effect (Ryan, 2007; Polchert, 2008). Interferon-gamma (IFN-γ) is a major pro-inflammatory cytokine secreted by activated T cells (Krampera, 2006) and can strongly up-regulate co-stimulatory molecules (Cheng, 2007). The effect of pre-incubation with IFN-γ on MSC was examined in terms of enhancement of immunosuppressive ability. Allogeneic MSC (FV/BN) were cultured in the presence of recombinant mouse IFN-γ. The cells were washed and subsequently cultured in the presence of MHC-mismatched (BALB/c and C57/BL/6) splenocytes or mitogenic stimulus (ConA). IFN-γ enhanced the inhibitory effect of MSC compared to non-stimulated cells. This was the case in both mitogen-driven (Fig. 4.8 A) and allo-driven (Fig. 4.8 B) reactions. These data indicated that the immunosuppressive effect of MSC could be enhanced by pre-stimulation of MSC and supported previous findings that IFN-γ licensed MSC for novel functions. These data also supported the use of these licensed cells in *in vivo* models of disease.

4.6 IFN- GAMMA-SPECIFIC SIGNALING IS REQUIRED FOR IMMUNOSUPPRESSION BY MSC

To confirm that IFN-γ-specific signaling played a role in triggering the enhancement of immunosuppressive function of MSC, MSC isolated from IFN-γ receptor knockout mice ($IFN\gamma R^{-/-}$) were used in a co-culture with allogeneic splenocytes or ConA mitogen. Co-culture of mismatched splenocytes with $IFN\gamma R^{-/-}$ MSC failed to inhibit suppression (Fig. 4.9). Thus, lack of the IFN-γ receptor impairs the normal functionality of MSC to inhibit proliferation supporting a critical role for this cytokine in immune suppression. Interestingly,

IFNγR$^{-/-}$ MSC were capable of effective suppression of mitogen-driven T cell proliferation indicating that IFN-γ is not essential for inhibition of proliferation to occur (Fig. 4.10). To further investigate the role of IFN-γ in mitogen-driven proliferation, a more physiologically relevant approach which uses beads coated with anti-CD3 and anti-CD28 to stimulate T cells in a manner that partially mimics antigen specific stimulation by antigen-presenting cells was employed (Fig. 4.11). Here, MSC lacking IFN-γ receptor expression were not capable of effective suppression of mitogenic proliferation demonstrating the critical role of IFN-γ signalling in MSC-mediated inhibition of cognate allogeneic T cell responses.

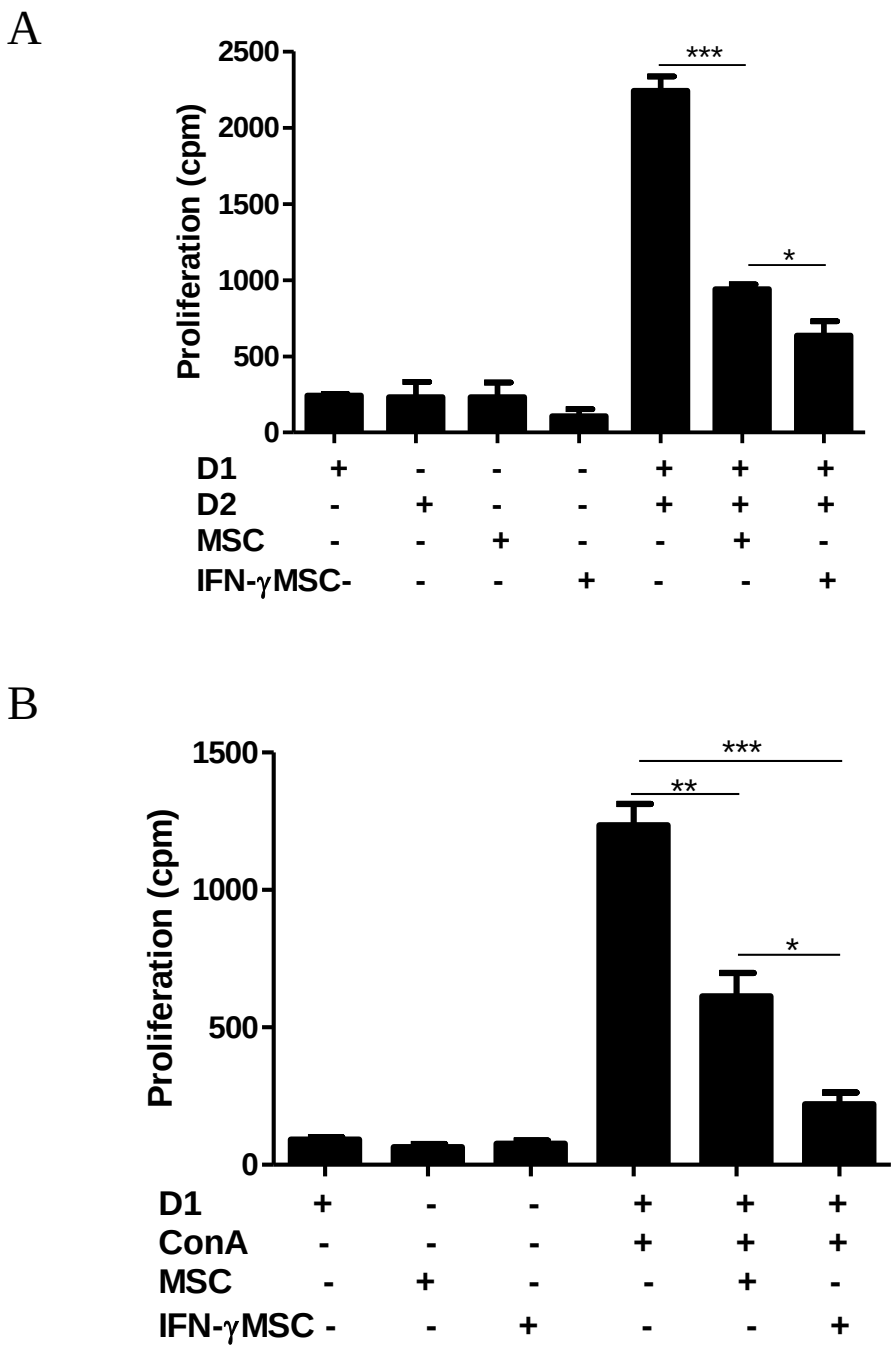


Figure 4.8 "Licensed" MSC enhance the immunosuppressive ability of MSC in allo- and mitogen-driven reactions. MSC cultured in the presence of 500U/ml of recombinant IFN-γ were added to an MLR at a density of 1.5 x 10^4/ml. The effect of prior stimulation on (A) allo-driven and (B) mitogen-driven proliferation was assessed by ^{3}H thymidine incorporation. Results are representative of three experiments, each performed in triplicate. (* $p<0.05$, ** $p<0.005$, *** $p<0.0005$).

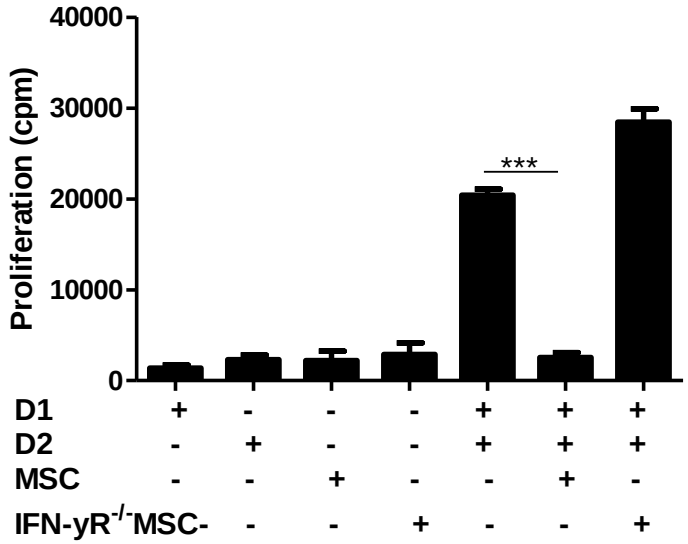


Figure 4.9 IFN-y receptor KO MSC fail to suppress allo-driven T cell proliferation. MSC isolated from IFN-γ receptor knockout mice were examined for their capacity to inhibit allogeneic-driven proliferation of splenocytes in a two-way mixed lymphocyte reaction. MSC (1.5 x10^4/well) were co-cultured with splenocytes (2 x 10^5/well) from MHC-mismatched mice (BALB/c (D1) and C57BL/6 (D2) respectively). MSC lacking the IFN-γ receptor did not retain the capacity to suppress allo-driven proliferation. Results are representative of three experiments, each performed in triplicate. ** p<0.005

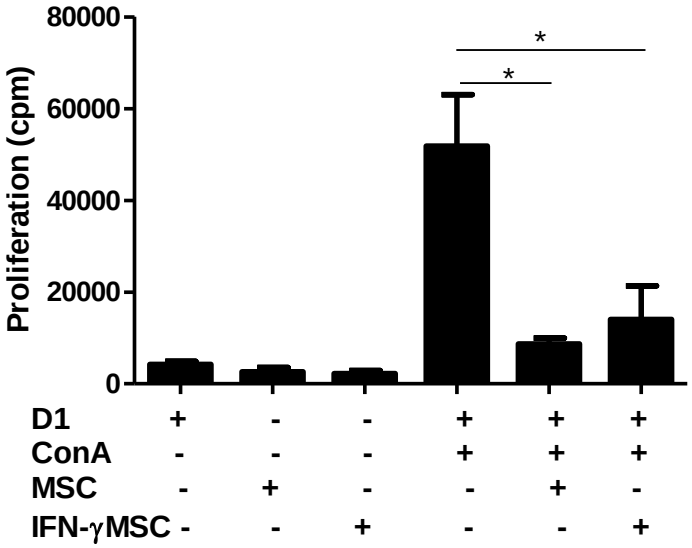


Figure 4.10 MSC retain the capacity to inhibit mitogen-driven proliferation in the absence of the IFN-γ receptor. MSC lacking the IFN-γ receptor (1.5 x 10^4/ml) were examined for their capacity to inhibit mitogen (ConA) driven proliferation of BALB/c splenocytes. Results are representative of three experiments, each performed in triplicate. * p<0.05

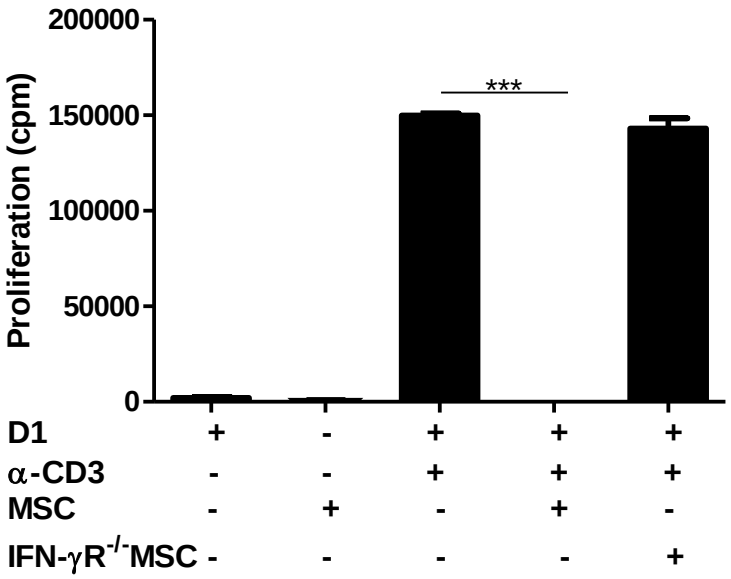
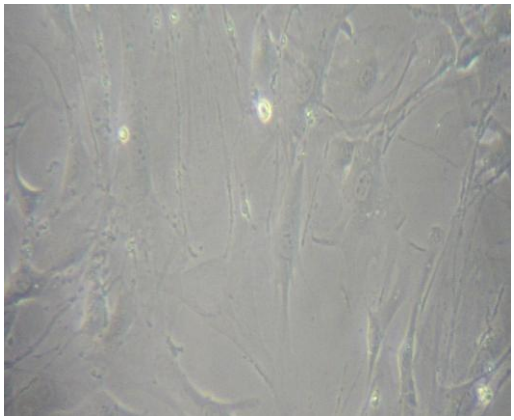


Figure 4.11. IFN-y receptor KO MSC fail to suppress anti-CD3 T cell proliferation. MSC isolated from IFN-γ receptor knockout mice were examined for their capacity to inhibit αCD3/CD28-driven proliferation of splenocytes. FV/BN MSC (1.5×10^4) or IFN-$\gamma R^{-/-}$ MSC were co-cultured with CD3/CD28. MSC lacking the IFN-γ receptor did not retain the capacity to suppress proliferation. Results are representative of three experiments, each performed in triplicate.

4.7 VIABILITY OF MSC IS NOT AFFECTED BY SHEARING FORCE

Prior to *in vivo* studies, it was important to ascertain whether the structural integrity and viability of MSC was affected by intravenous injection. The mechanical responses of mesenchymal stem cells to deformation were investigated to determine the effect of different gauge needles on cell viability. This study was carried out to demonstrate that shearing force was not a confounding factor. MSC (1×10^5) were passed through four needle sizes (23 G, 25 G, 27 G and 30 G) via syringe and viability assessed pre and post passage. Viability was assessed by viable cell counting (Chapter 2.14). The cells were then transferred to individual tissue culture flasks and incubated for 48 h in order to assess whether the cells retained their capacity to adhere to plastic. In addition, the effect on MSC structure and size was assessed by flow cytometry, where forward and side scatter identified any change in size or uniformity (Fig. 4.9). No significant effect was identified as a result of passing MSC through any size needle in this study.

A



B

MSC passage through needle of gauge	% Loss of MSC viability
23G	**0.9**
25G	**1.5**
27G	**0.9**
30G	**1.3**

Fig. 4.12 Viability of MSC was not significantly affected by passing through needle. MSC retained their capacity to adhere to plastic following passage through needle (A). No significant loss of viable cells was observed (B). Viability was assessed by counting with EB/AO before and after passage through needle.

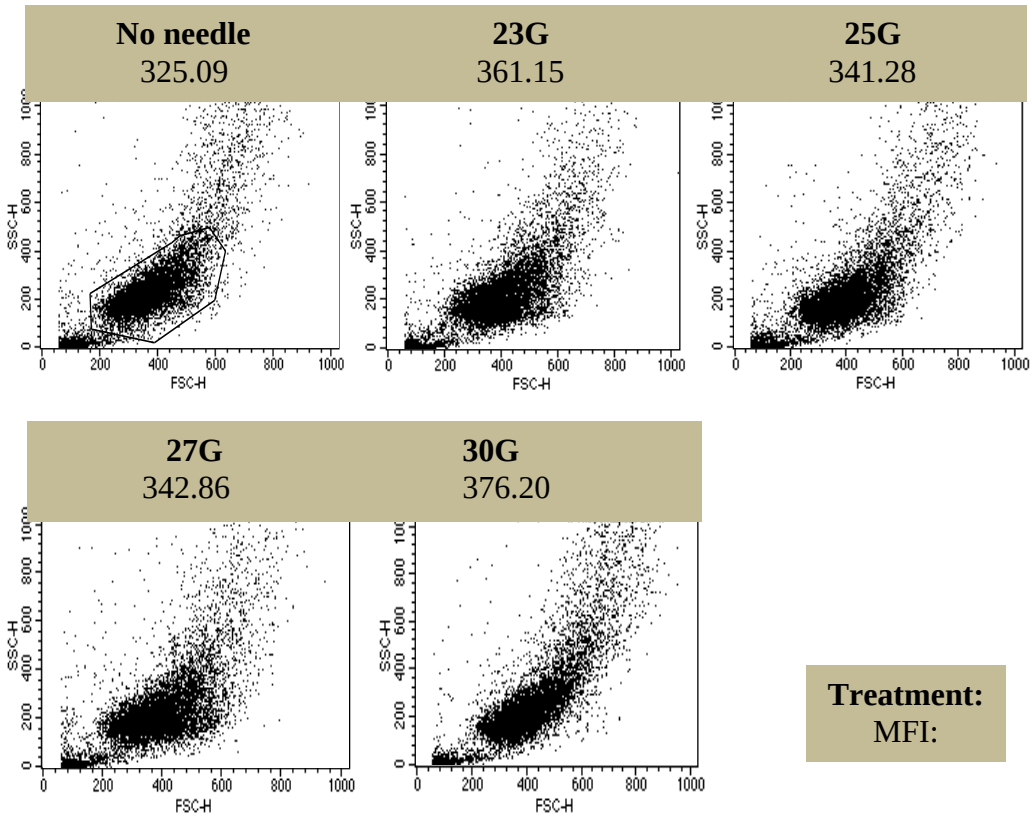


Figure 4.13 The physical characteristics of MSC are not affected by shearing force. MSC (1×10^6) were forced through four needle sizes (23 G, 25 G, 27 G, 30 G). The effect on MSC structure and size was assessed on a flow cytometer, plotting forward versus side scatter. Results are representative of three experiments.

4.8 SUMMARY

In Chapter 4, functional assays were carried out in order to address quality-control issues that might arise as a result of systemic delivery of MSC *in vivo*. These studies established the homogeneity of an MSC cell population with a hypo-immunogenic phenotype. Their "stemness" was defined by their ability to differentiate into adipocytes, osteoblasts and chondrocyte-like cells and by the surface marker phenotype of $CD34^{-}CD11b^{-}CD45^{-}$MHC class $II^{-}CD44^{+}Sca\text{-}1^{+}$MHC class I low following isolation from adult mouse bone marrow. Cells retained multi-lineage potential through each subsequent generation.

The immunosuppressive properties of MSC were confirmed by carrying out T-cell proliferation assays using a variety of stimuli including mitogens, anti-CD3, and allo-antigens. These demonstrated the ability of MSC to inhibit proliferation irrespective of the donor source. The method of delivery *in vivo* via intravenous injection was also examined to ensure that the structural integrity of the cells would not be compromised by shearing force through the needle. No significant effect on viability was identified as a result of passing the MSC through various needle gauges, suggesting that MSC will survive the processes needed for intravenous administration. The effect of exogenous IFN-γ on MSC-mediated immunosuppression *in vitro* was also investigated, demonstrating an enhancement of immunosuppressive function in both mitogen-driven and allo-driven reactions as a result of prior stimulation. These data verified the results of others (Polchert, 2008) by demonstrating the enhancing effect of pre-stimulation of MSC and indicated the importance of the inclusion of these licensed cells in further studies. A comprehensive analysis of this study can be found within the discussion section at the end of Chapter 6.

CHAPTER 5

THE INFLUENCE OF MSC ON ALLERGIC LUNG INFLAMMATION

5.1 INTRODUCTION

The current treatments for allergic asthma require suppression of immune effector mechanisms to alleviate symptoms. A fundamental intervention against the cause of asthma would require immune reprogramming of the response to innocuous environmental allergens. The immunomodulatory capacity of multipotent mesenchymal stem cells (MSC) suggests that they have a potential therapeutic value in asthma and other hypersensitive or autoimmune diseases. The ability of MSC to be readily isolated and expanded *ex vivo* (Chapter 4) makes them promising candidates for cellular immunotherapy. Extensive *in vitro* studies have demonstrated that, unlike other stem cell types, MSC can not only evade alloreactivity, but can also actively suppress broader immune responses (Bartholomew, 2004; Aggarwal, 2005; Barry, 2005). Preclinical models of autoimmune/inflammatory conditions such as transplant rejection (Le Blanc, 2004), rheumatoid arthritis (Augello, 2007), and multiple sclerosis (Constantin, 2009), have demonstrated a functional role for MSC *in vivo*.

It should also be noted that MSC are not a panacea, and have been unsuccessful as a cell therapy in some models (Nauta, 2006; Sudres, 2006). The reasons for such failures are not clear; however it may be that MSC were not sufficiently "primed" or "licensed" for their biological role and thus lacking appropriate surface markers for homing or secretion of soluble effector molecules. IFN-γ is a major pro-inflammatory cytokine produced by activated T-cells and plays an important role in priming the immunosuppressive property of MSC (Sheng, 2008). Chapter 4 alluded to a critical role for IFN-γ in MSC-mediated immunosuppression, thus it was possible that IFN-γ pre-stimulation of MSC would result in an improved cell therapy.

This study addressed the hypothesis that adult bone marrow derived-MSC would prevent the pathology associated with allergen-driven airway inflammation. This was tested using a combination of a well established model of OVA driven inflammation and a cell

therapy based on allogeneic MSC alone or pre-stimulated with IFN-γ. This chapter examined the influence of MSC delivered therapeutically on the key aspects of allergen driven inflammation including IgE induction, influx of inflammatory cells, modulation of cytokine profile and the effect on allergic airway pathology.

5.2 STUDY DESIGN - OVA MODEL OF ALLERGIC AIRWAY INFLAMMATION

The murine model of ovalbumin driven airway inflammation was used again to elucidate the possible immunosuppressive effects of MSC cell therapy *in vivo*. Allogeneic MSC from FVBN mice were isolated, cultured and characterised as described in Section 2.21. An additional group included IFN-γ-licensed MSC where cells were stimulated with 500U/ml murine recombinant IFN-γ 48h prior to intra-venous infusion. Mice were sensitised to OVA and received two intravenous treatments with allogeneic MSC. The first was administered on day 7 after initial allergen challenge (Fig. 5.1 and Chapter 3.3), and again on day 14, 6h after the initial respiratory challenge with OVA. The time-points for delivery of cells were chosen to mimic as much as possible other studies where delivery of MSC was carried out immediately after, or on the same day as disease induction. Additional control groups included (1) paraformaldehyde-fixed MSC and (2) interferon-γ "licensed" MSC, delivered to OVA-sensitised and non-sensitised groups. On day 28, mice were bled by facial bleed for subsequent serum analysis. Mice were culled and BALF, spleen and lungs were sampled for subsequent analysis. BALF supernatants were collected and cytokine profiles were examined. Differential cell counts were performed to assess the extent of cellular infiltration. Splenocytes were cultured in the presence of exogenous OVA and their ability to mount a recall response was measured. Lungs were fixed for histological analysis in order to examine the effect of MSC delivery on OVA-induced airway pathology.

Initially, a preliminary study was carried out in order to assess the efficacy of systemic intravenous delivery of MSC. Donor cell tracing was necessary to test whether transplanted donor cells were present in the lung following systemic delivery. Female mice were administered GFP-expressing transgenic MSC from male donors intravenously, via the tail vein. After 6,h, mice were culled. The lungs were dissected and snap frozen for subsequent sectioning (Chapter 2.27). Lung tissue sections were examined for GFP fluorescence. Although fluorescence was detectable (Fig 5.2) this was difficult to detect against background fluorescence. Therefore a fluorescent *in situ* hybridisation technique (FISH) was used for lung sections where female mice had received intravenous infusion of male MSC. A Y-chromosome-specific probe was used to detect male MSC transplanted into female recipients in mice (Chapter 2.26). Using gender-mismatched transplantation, the Y-chromosome of male donor MSC was detected with a paint probe. The probe hybridised with a prepared lung tissue section from the recipient female mice (Figure 5.2B). Sections from mice receiving female MSC were used as negative controls for Y-chromosome staining. Results from this study indicated that MSC were present in the lung 6 h after intravenous injection and confirmed that the techniques for use in this model resulted in at least some MSC locating to the site of pathology.

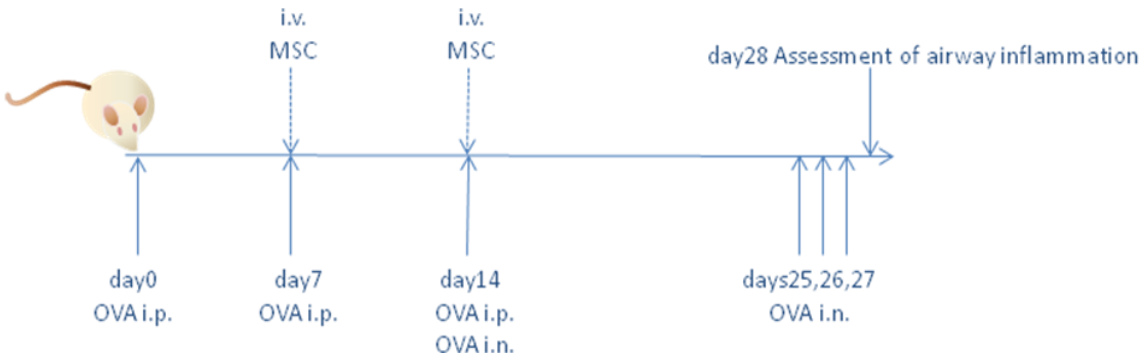


Figure 5.1. Groups of 6-8 week old female BALB/c mice (n=8) were sensitised by intraperitoneal (i.p.) injection of either OVA or saline on day 0, 7 and 14. On days 25, 26 and 27, mice were exposed to either saline or OVA via intranasal (i.n.) administration. Unsensitised controls received PBS. Mice received allogeneic MSC ($5x10^6$ /ml) by intravenous injection on day 7 and 14, 6 h after OVA i.p. /i.n. Readouts were performed on day 28. BALF, spleens and lungs were harvested for subsequent analysis. Mice were bled by facial bleed for serum analysis.

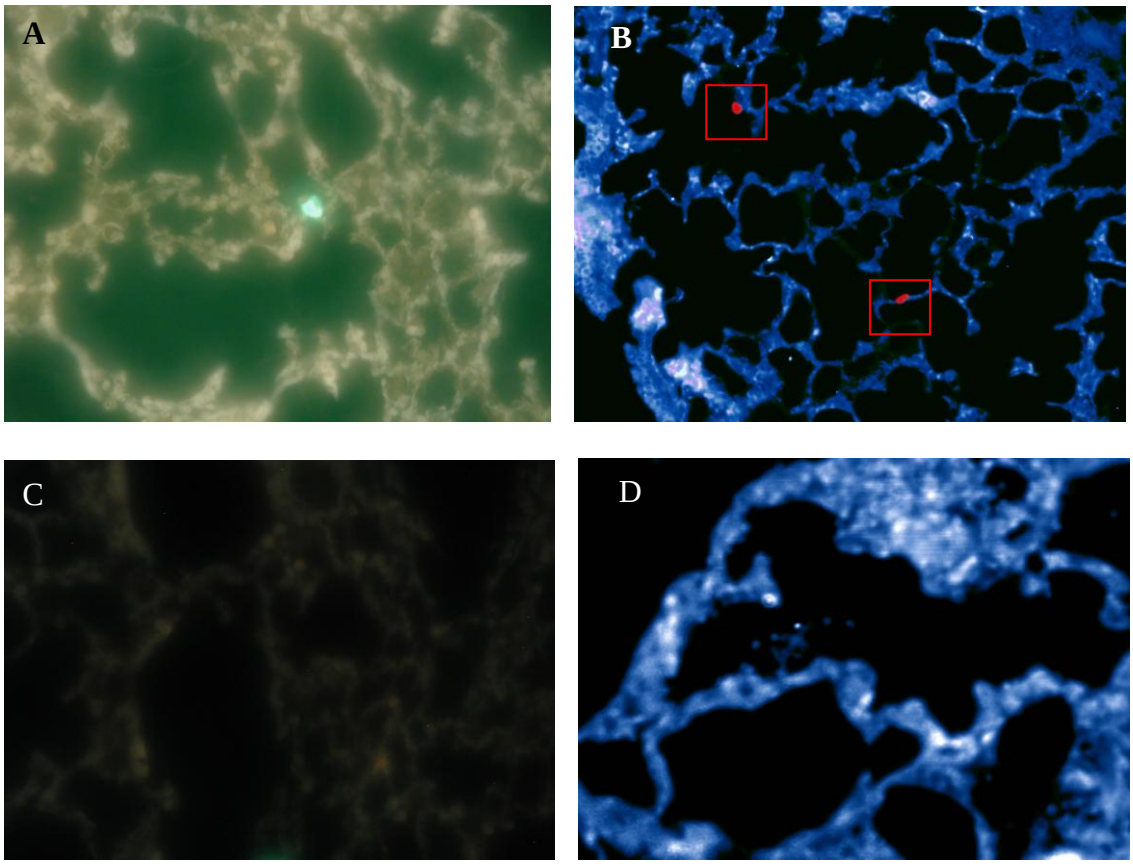


Figure 5.2. MSC are present in the lung following systemic intravenous delivery via the tail vein. (A) GFP-expressing transgenic MSC were delivered intravenously via the tail vein into BALB/c mice. (B) Donor cell tracing using FISH technology was carried out on lung tissue sections following intravenous delivery of male MSC into female recipients. After 6 h, mice were culled. The lungs were fixed for subsequent analysis and examination. No GFP-expressing cells or Y-chromosome staining was evident in controls (C) and (D) respectively. Magnification x 100.

5.3 ALLOGENEIC MSC REDUCE OVA-DRIVEN AIRWAY PATHOLOGY

Previous studies have demonstrated a role for MSC in effective intervention in lung diseases (Zhao, 2008; Cho, 2009; Lee, 2009). To assess the influence of MSC on OVA-induced airway pathology, histological analysis of lung tissue sections were carried out. As expected, non-sensitised groups exhibited no allergen-driven airway inflammation (Figure 5.3). No inflammation resulted from live allogeneic cell delivery; however inflammation was observed in those mice receiving fixed MSC (Fig. 5.4). In the absence of MSC treatment, OVA-sensitised mice exhibited typical peribronchial and perivascular inflammation at day 28, which was not observed in control mice (Fig 5.3 A & B). Delivery of PFA-fixed MSC resulted in a more severe pathology when compared to OVA-sensitised mice, displaying strong perivascular inflammation and bronchiolar epithelial hypertrophy (Fig 5.3 C). This phenomenon may be due to the fixed cells' inability to produce the soluble factors which would otherwise protect them from allo-rejection, suppress T-cell proliferation or, in this case, attenuate the allergic airway response. In contrast, live MSC treatment resulted in decreased peribronchial inflammation when compared to sensitised mice (Fig 5.3 C & D). It is important to note that this protective effect was more apparent in those mice that received MSC pre-treated with IFN-γ, suggesting that *in vitro* stimulation of allogeneic MSC can improve their ability to reduce pathology. This was most evident in the histopathological analysis of the lung tissues where mucus hypersecretion and peribronchial inflammation was significantly reduced (Fig. 5.4).

A consistent feature of asthma is the production of excess mucus, causing blockage of the peripheral airways (Young, 2006). In order to assess the effect of MSC on mucus hypersecretion, lungs were sectioned and stained with combined Discombes/Alcian blue and periodic acid-Schiff stain. MSC delivery reduced mucus metaplasia in allergen-sensitised

mice, consistent with the findings above (Fig. 5.5). In contrast, PFA-fixed MSC exacerbated goblet cell hyperplasia and mucus secretion in OVA-sensitised mice (Fig 5.4 D & E). Mucus secretion and goblet cell hyperplasia were greatly reduced in sensitised mice treated with IFN-γ pre-treated MSC, but were exacerbated in mice receiving fixed MSC (Fig 5.5). These data show that MSC treatment attenuated the pathology associated with allergen sensitisation and that prior activation of MSC enhanced this effect. Evidence is also provided for the importance of soluble-factor mediated protection by MSC as delivery of fixed MSC exacerbated allergic airway pathology in this model.

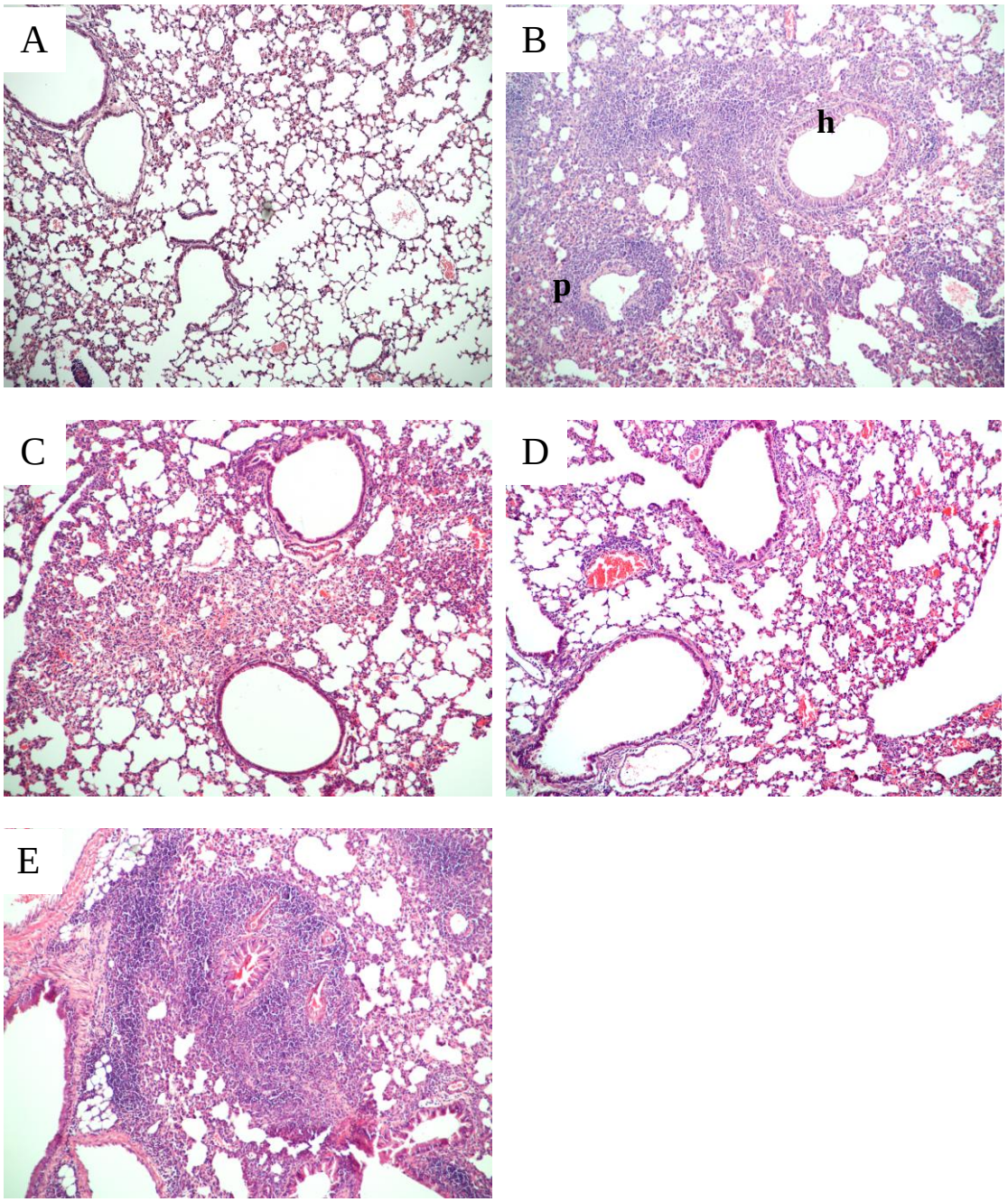


Figure 5.3 MSC reduce the severity of airway pathology induced by sensitising allergen. Representative morphological changes at day 28 in bronchiolar transverse sections of lungs from (A) Non-sensitised, (B) OVA-sensitised, (C) OVA-sensitised, MSC treated, (D) OVA-sensitised, IFN-γ-stimulated MSC treated, (E) OVA-sensitised, PFA-fixed MSC treated. Airway inflammation was detected using haematoxylin and eosin (H&E) staining of fixed lung sections. **p** and **h** indicate perivascular inflammation and bronchiolar epithelial hypertrophy, respectively. All sections are representative of duplicate experiments; in each case at least five animals were assessed. Original magnification A, B, C, D & E x100.

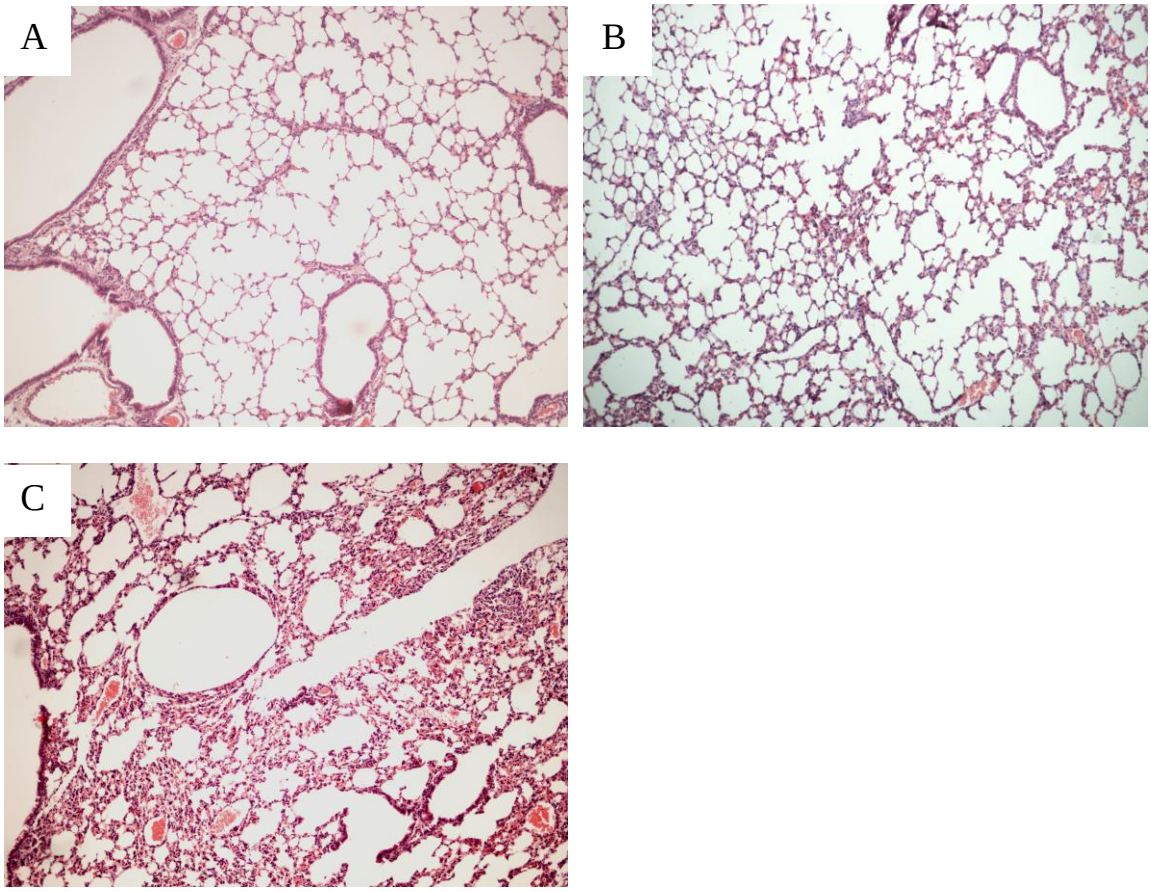
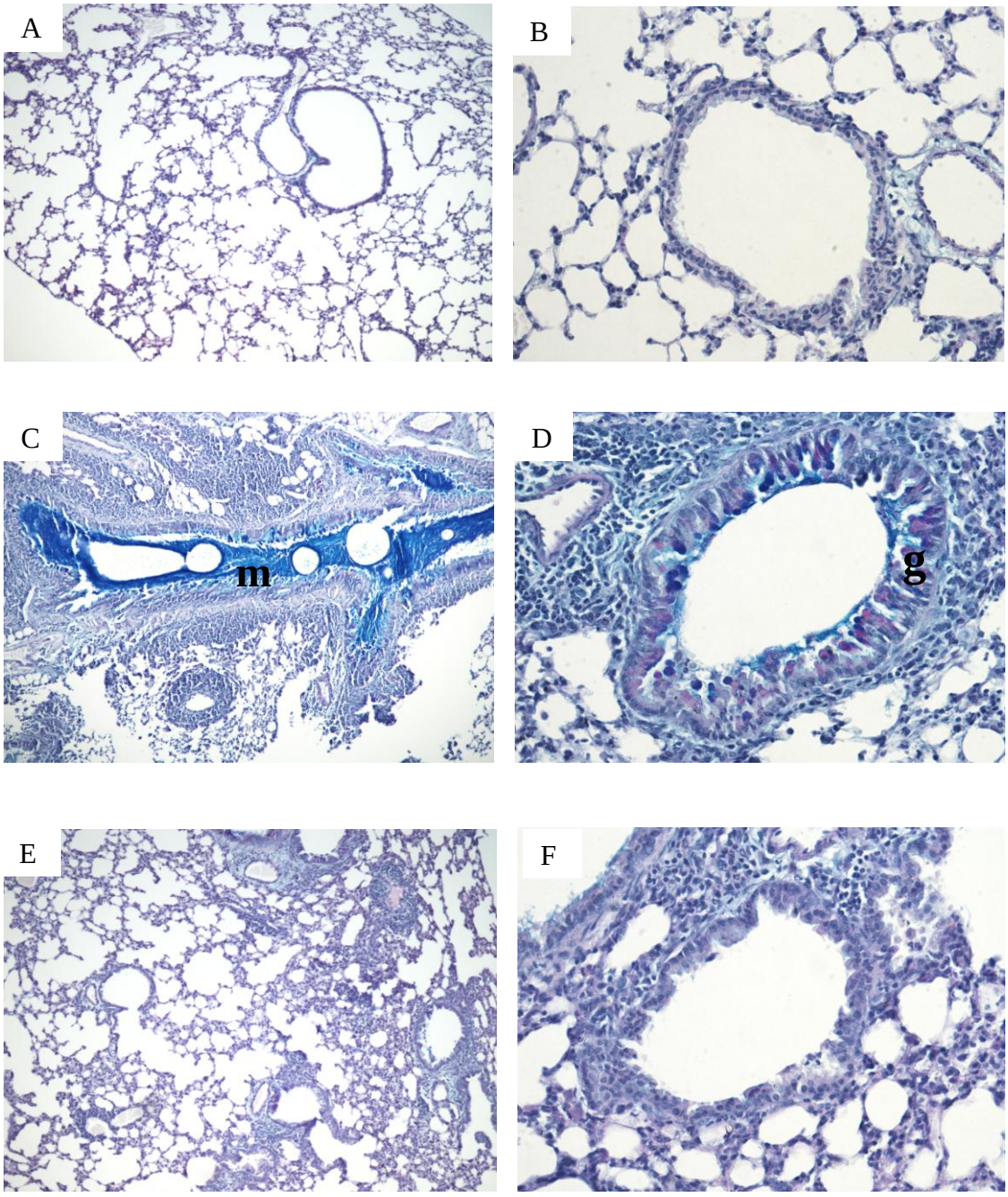


Figure 5.4 Non-sensitised groups do not display allergen-driven airway inflammation following delivery of live, but not fixed, MSC. Representative morphological changes at day 28 in bronchiolar transverse sections of lungs from (A) Non-sensitised, MSC treated (B) Non-sensitised, IFN-γ-stimulated MSC treated and (C) Non-sensitised, PFA-fixed MSC treated. All sections are representative of duplicate experiments; in each case at least five animals were assessed. Original magnification x100.


A
B
C
m
D
g
E
F

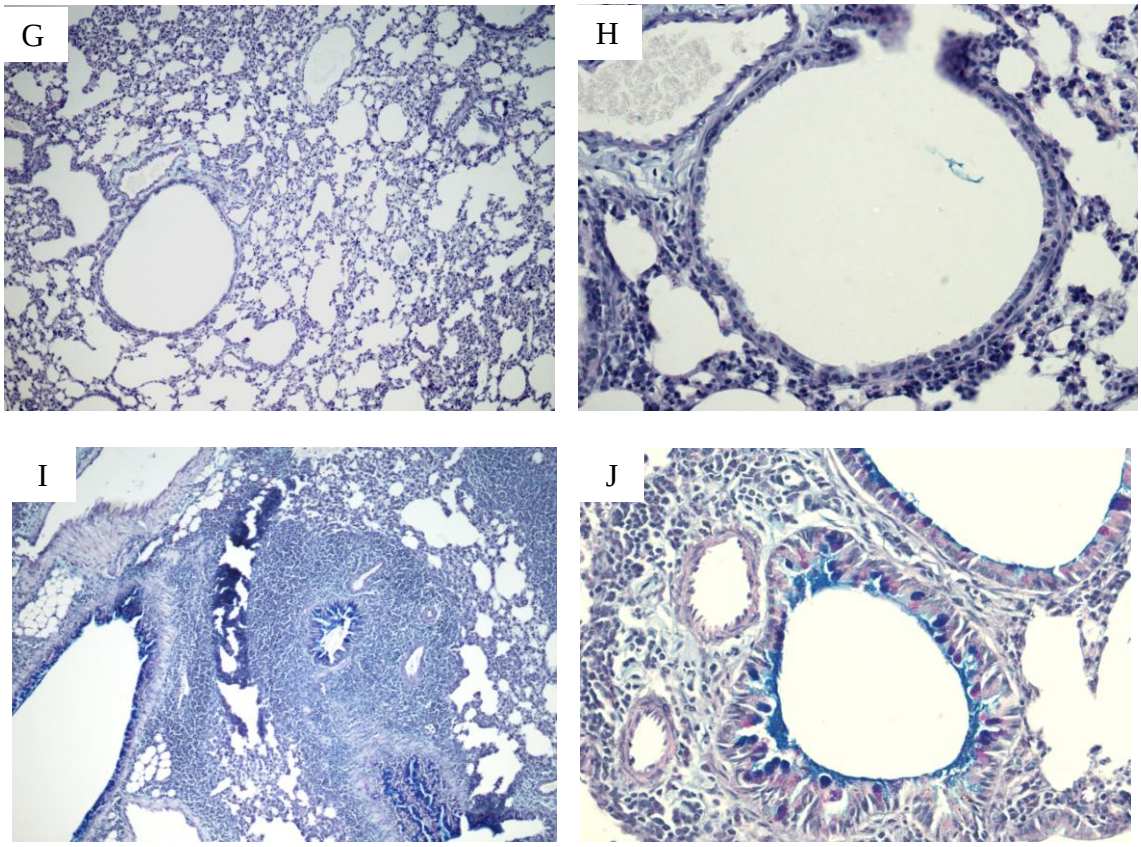


Figure 5.5 MSC reduce the severity of mucus hyperplasia to sensitising allergen. Representative morphological changes at day 28 in transverse sections of bronchioles from (A&B) non-sensitised, (C&D) OVA-sensitised, (E&F) OVA-sensitised, MSC infused, (G&H) OVA-sensitised, infused with IFN-γ-stimulated MSC, (I&J) OVA-sensitised, infused with PFA-fixed MSC. Airway inflammation was detected using combined Discombes/Alcian blue/PAS staining on lung sections. **g** and **m** indicate goblet cell hyperplasia and mucus secretion, respectively. All sections are representative of duplicate experiments; in each case at least five animals were assessed. Original magnification (A), (C), (E), (G) & (I) x100 and (B), (D), (F), (H) & (I) x400.

5.4 ALLOGENEIC MSC MODULATE THE INNATE RESPONSE

Extensive *in vitro* studies have shown that MSC can exert profound immunosuppressive effects via modulation of both cellular and innate immune pathways (Tse, 2003; Jiang, 2005; Spaggiari, 2006). For clarity, therefore, MSC therapy in this model was investigated in terms of the effect on (1) innate effector cells, (2) humoral immunity, (3) local cell-mediated immunity and (4) systemic cell-mediated immune responses.

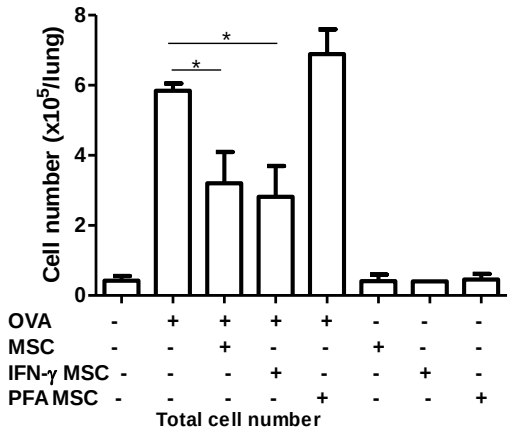
5.4.1 MSC REDUCE THE CELL INFILTRATE IN BAL FLUID

A cardinal feature of allergen driven airway inflammation is the elevated number of inflammatory cells, particularly eosinophils (Varney, 1992). Therefore, the influence of MSC cell therapy on the quantity and quality of cellular infiltration in allergen sensitised airways was examined. Control mice showed minimal cellularity in bronchoalveolar lavage (Fig. 5.6 & 5.8A), whereas OVA sensitisation resulted in significant infiltration by inflammatory cells (> 6 x 10^5 cells per lung) ($p<0.05$). Total cellular infiltration was decreased in OVA-sensitised mice that received MSC, whereas it remained high in sensitised animals treated with PFA-fixed MSC. BALF from control mice showed minimal cellularity (Fig. 5.6 & 5.7), whereas OVA sensitisation/challenge resulted in significant infiltration by inflammatory cells including eosinophils. However, airway eosinophilia was significantly reduced in OVA sensitised mice treated following MSC delivery ($p<0.05$). This effect was only evident upon delivery of live, but not fixed cells, as OVA-sensitised, PFA-fixed MSC treated groups exhibited a considerable increase in airway eosinophilia when compared to OVA sensitised mice (Fig. 5.6 B & 5.7 E). Similarly, delivery of MSC in sensitised mice resulted in a significant reduction in lymphocytes and macrophages. There were few remarkable differences in the numbers of neutrophils among the different groups (Fig. 5.6 C). Infiltration of neutrophils in combined MSC-treated/OVA sensitised mice was typically

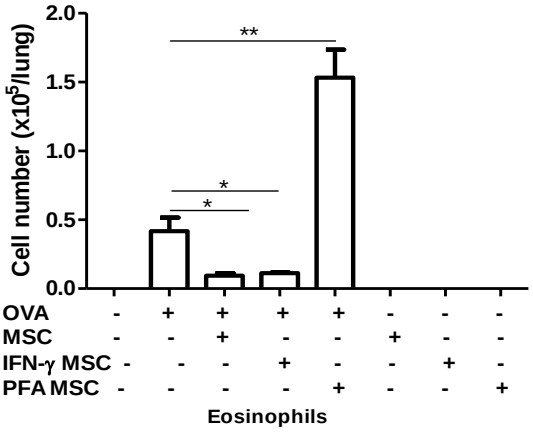
lower than OVA alone (Fig. 5.6 C). However, this did not achieve statistical significance in this study. Few differences were observed in those mice receiving activated MSC, when compared to those without prior IFN-γ stimulation with regard to infiltration of eosinophils, lymphocytes and neutrophils. However, the numbers of macrophages in this group were not unlike those seen in the OVA-sensitised alone group. These findings demonstrate that allogeneic MSC have wide ranging therapeutic influence on allergen driven airway inflammation, but this influence is attributable to living MSC and cannot be replaced by fixed cells.

Figure 5.6 (See below). MSC reduce the cell infiltrate in BAL fluid. Effect of MSC infusion and/or OVA sensitisation on BAL composition 24 h after final (i.e. day 29) OVA exposure. Negative controls were sham infected/sensitised with saline. BAL fluid was examined for the total cell number (A), or the presence of eosinophils (B), neutrophils (C) macrophages (D) or lymphocytes (E). The data are representative of two experiments; in each case, at least five animals were assessed. Results are expressed as mean ± S.E.M. of cell number. (*, $p < 0.05$, **, $p<0.01$).

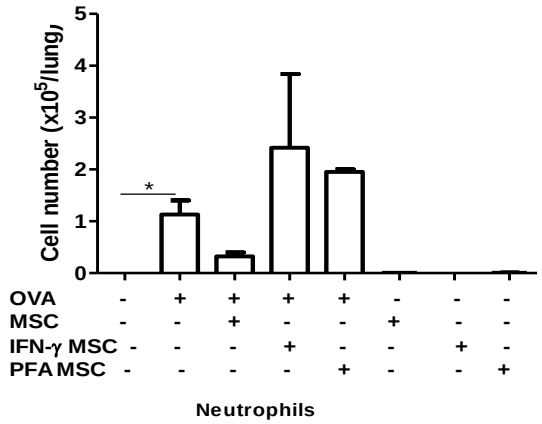
A


Cell number (x10^5/lung)
8
6
4
2
0
*
*
OVA - + + + + - - -
MSC - - + - - + - -
IFN-γ MSC - - - + - - + -
PFA MSC - - - - + - - +
Total cell number

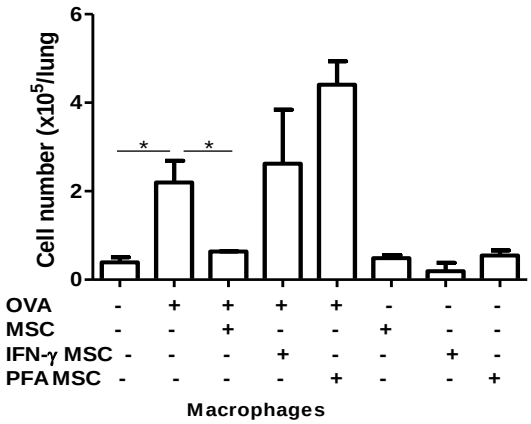
B


Cell number (x10^5/lung)
2.0
1.5
1.0
0.5
0.0
**
*
*
OVA - + + + + - - -
MSC - - + - - + - -
IFN-γ MSC - - - + - - + -
PFA MSC - - - - + - - +
Eosinophils

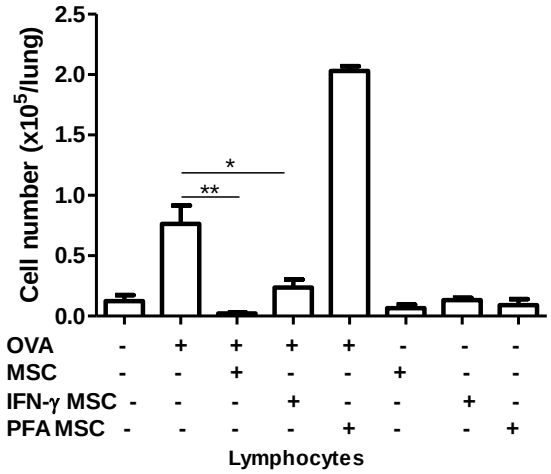
C


Cell number (x10^5/lung)
5
4
3
2
1
0
*
OVA - + + + + - - -
MSC - - + - - + - -
IFN-γ MSC - - - + - - + -
PFA MSC - - - - + - - +
Neutrophils

D


Cell number (x10^5/lung
6
4
2
0
*
*
OVA - + + + + - - -
MSC - - + - - + - -
IFN-γ MSC - - - + - - + -
PFA MSC - - - - + - - +
Macrophages

E


Cell number (x10^5/lung)
2.5
2.0
1.5
1.0
0.5
0.0
*
**
OVA - + + + + - - -
MSC - - + - - + - -
IFN-γ MSC - - - + - - + -
PFA MSC - - - - + - - +
Lymphocytes

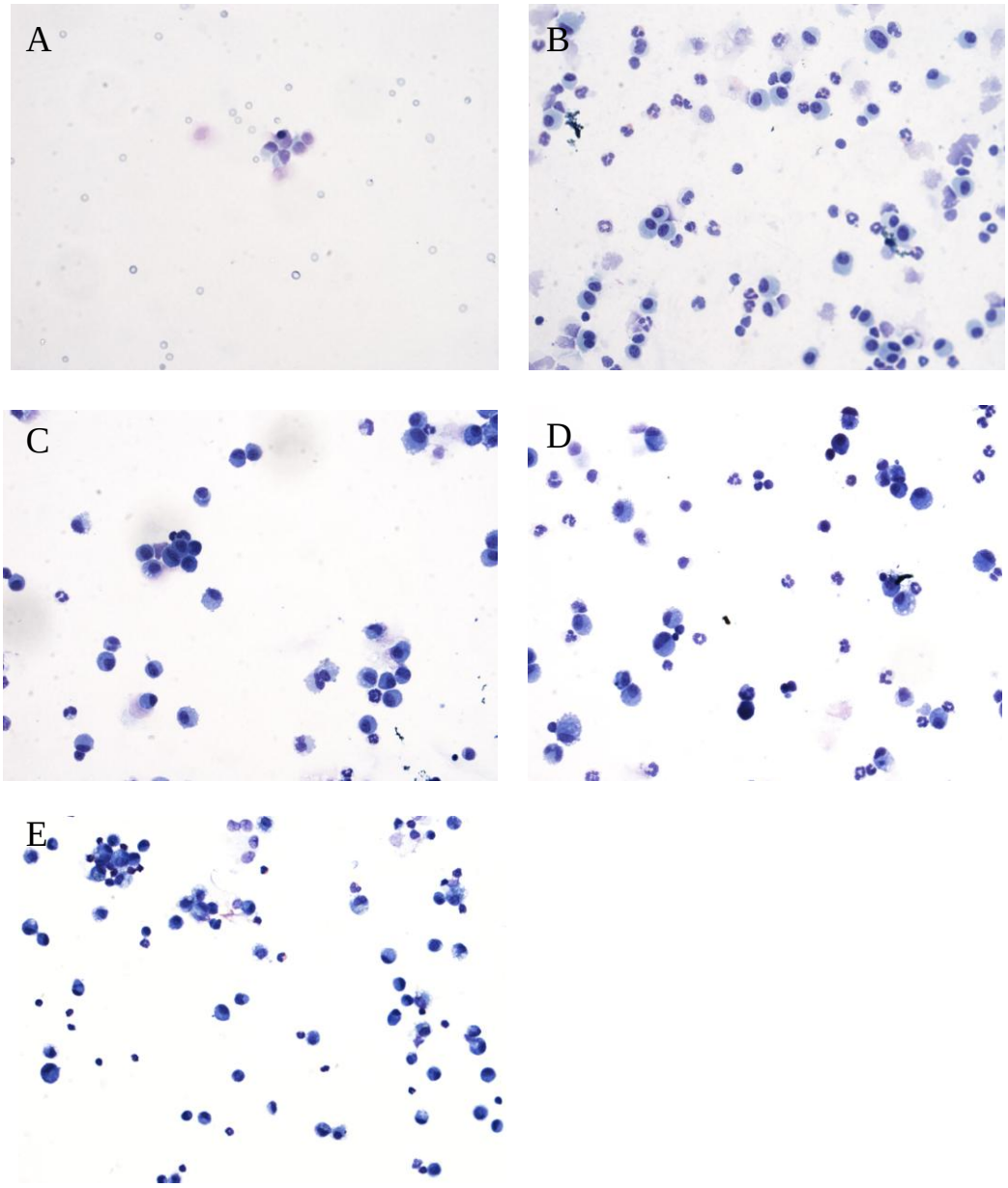


Figure 5.7 MSC reduced the numbers of cells in the airways of OVA sensitised mice. BALF was sampled on day 28 from (A) Non-sensitised (B) OVA-sensitised (C) OVA-sensitised, MSC infused, (D) OVA-sensitised, infused with IFN-γ-stimulated MSC, (E) OVA-sensitised, infused with PFA-fixed MSC. Cells were stained with Giemsa and examined microscopically for the presence of a number of cell types. Magnification x400.

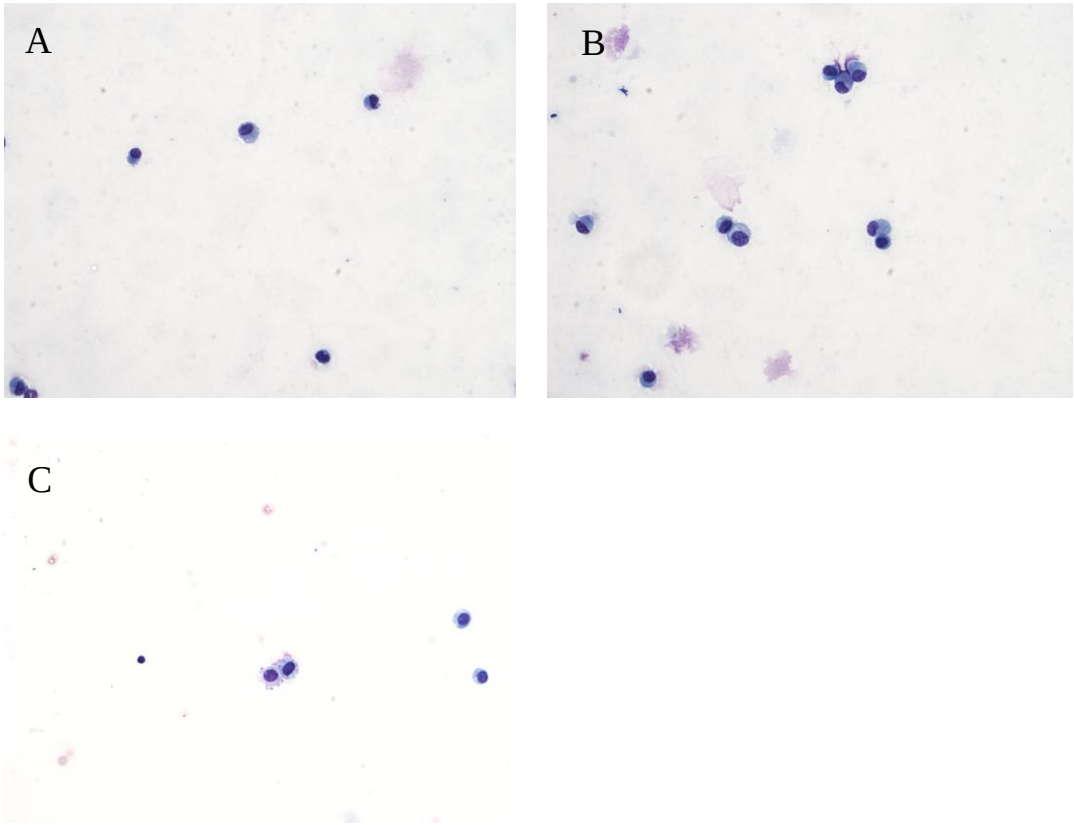


Figure 5.8 Non-sensitised mice exhibited no allergen-driven airway inflammation. Representative morphological changes at day 28 in bronchiolar transverse sections of lungs from (A) Non-sensitised and infused with MSC (B) Non-sensitised and infused with IFN-γ-stimulated MSC and (C) Non-sensitised and infused with PFA-fixed MSC. All sections are representative of duplicate experiments; in each case at least five animals were assessed. Original magnification x400.

5.4.2 MSC REDUCE MYELOPEROXIDASE ACTIVITY IN LUNGS OF OVA- SENSITISED MICE

Migration and accumulation of neutrophils is a characteristic feature of the host response to injury (Menezes GB 2008). Neutrophils release inflammatory mediators, including myeloperoxidase (MPO). Therefore the production of MPO is an indicator of local neutrophil activity. BALF was harvested from mice on day 28 (Fig. 5.9), and the cell free supernatants were analysed for the presence of MPO, as described in Section 2.9. The levels of MPO (Fig. 5.9) reflected the numbers of neutrophils observed in differential cell counts (Fig. 5.6). Lungs from non-sensitised control mice contained no, or baseline concentrations of MPO, whilst a considerable increase was demonstrated in OVA-sensitised BALF. MSC delivery resulted in a significant decrease in neutrophilic activity. Activation of MSC with IFN-γ prior to delivery in OVA-sensitised mice indicated a trend towards increased MPO activity; however this did not reach significance in either supernatant analysis or differential cell counts in BALF (Fig. 5.9 & 5.6 C).

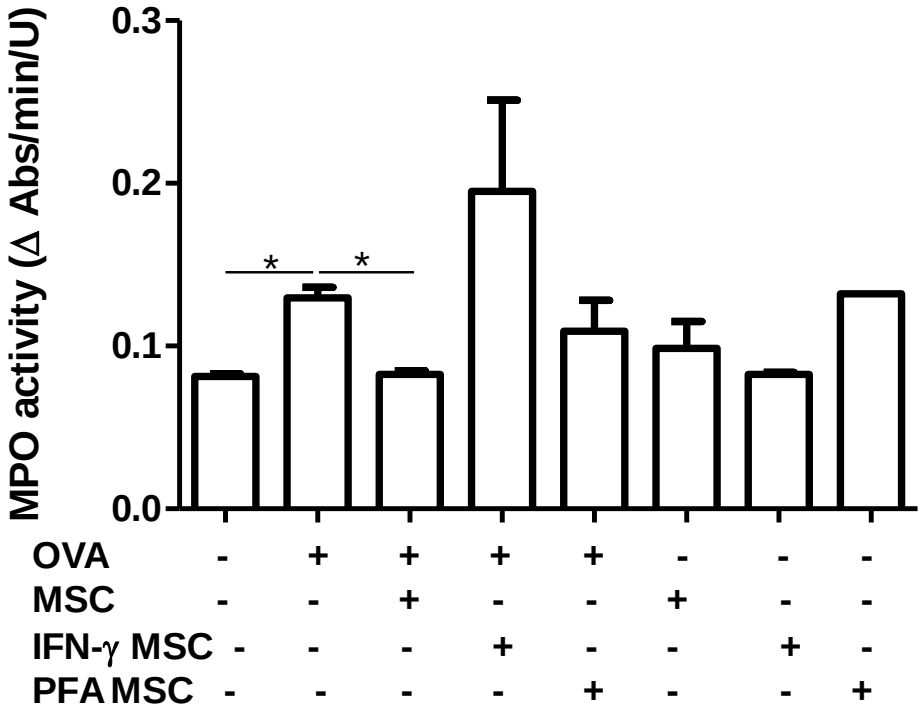


Figure 5.9 Live, but not PFA fixed MSC significantly reduced (MPO) activity in murine BALF. BALF on day 28 was analysed for MPO activity as described in section 2.9. The change in absorbance was measured at 450nm. MPO activity was calculated using a standard curve generated from human MPO. Results are expressed as the change in OD (absorbance) (ΔAbs/min/U protein). Responses are representative of duplicate experiments, each of which were determined independently from at least four mice per group and are expressed as means ± S.E.M. $p < 0.05$.

5.5 ALLOGENEIC MSC PREVENT ALLERGEN-DRIVEN INDUCTION OF IgE

IgE induction is a key feature of allergen driven pathologies and OVA sensitisation in mice is known to induce elevated levels of IgE combined with a powerful allergen-specific Th2 response (Hamelmann, 1999). MSC can inhibit B-cell proliferation and differentiation *in vitro* (Corcione, 2006). Therefore, their effect on the production of serum OVA-specific IgE was investigated here. Previous studies have investigated the modulatory effects of human MSC on the alloantigen-specific humoral response and shown that they are capable of suppressing allo-specific antibody production *in vitro* (Comoli, 2008). Therefore, the capacity for adult allogeneic bone marrow-derived MSC to influence IgE induction in this model was examined by measuring the concentration of OVA-specific IgE in serum from OVA-sensitised mice in which MSC were used therapeutically. Non-sensitised, MSC-treated mice demonstrated negligible levels of IgE, comparable with control groups. As expected, elevated serum levels of OVA-specific IgE were observed in those mice sensitised to OVA. Sensitised mice that received fixed MSC showed similar levels of OVA specific IgE. However, this effect was inhibited in OVA sensitised, MSC-treated mice ($p<0.05$) (Fig 5.10). Similar to histological data, the protective effect of MSC delivery was enhanced upon treatment with IFN-γ activated MSC ($p< 0.001$). These data demonstrate that cell therapy based on allogeneic MSC can suppress the induction of allergen-specific IgE detectable in serum.

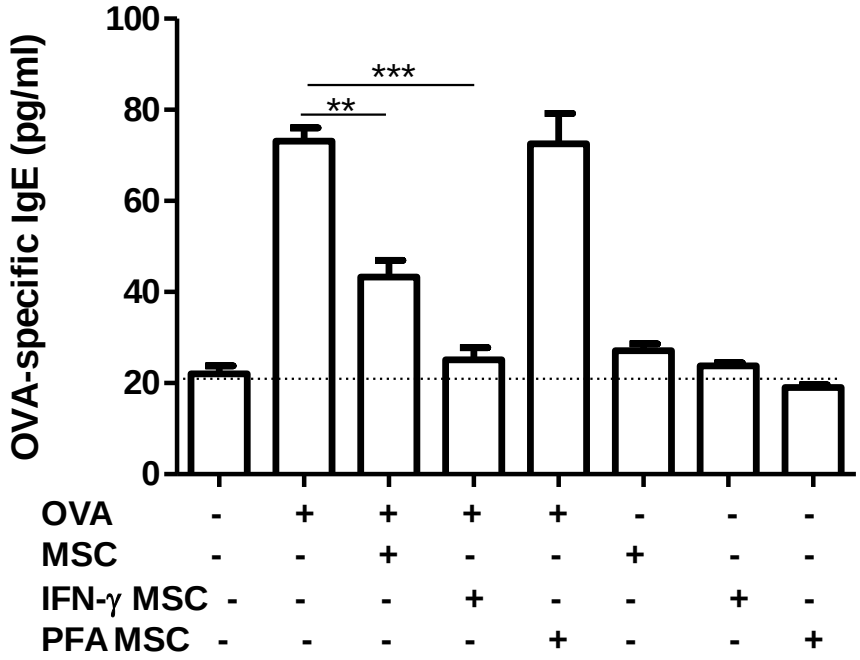


Figure 5.10 MSC reduce serum IgE responses to sensitising allergen. OVA-specific IgE in serum elicited in response to OVA sensitisation. Sera were collected on day 28 and OVA-specific serum IgE levels were measured by ELISA. The data presented are representative of two experiments; in each case, at least five animals were assessed, and each individual assessment was performed independently in triplicate. Concentrations below 20 pg/ml were considered negative. Results are expressed as mean antibody concentrations ± S.E.M. (**, $p < 0.01$, ***, $p<0.001$).

5.6 ALLO-MSC MODULATE LOCAL CELL-MEDIATED RESPONSES

The phenomena described above are interesting but might be explained by MSC mediated global, non-antigen specific, immune suppression or by MSC interference in immune induction. Therefore, the effect of allogeneic MSC on T cell mediated immunity was examined in this model. Particular attention was given to IL-4, IL-5 and IL-13 induction as these play well defined roles in allergen driven pathology.

5.6.1 ALLO-MSC MODULATE LOCAL CYTOKINE RESPONSES *IN VIVO*

The cytokine profile in the BALF from the mice described above was investigated. As expected, IL-4, IL-5 and IL-13 in both BALF and splenocyte supernatants were significantly higher in the OVA sensitised group when compared to those sham sensitised with PBS ($p < 0.05$). However, levels of IL-5 were significantly increased ($p < 0.05$) in the sensitised, MSC-treated group. The increase in IL-5 was surprising given the considerable decrease in eosinophilic lung infiltration and improvement in airway pathology. The typical damaging effects of IL-5 in acute lung inflammation may have been in part offset by the significant increase in IL-10 (Fig. 5.11 D) ($p<0.05$) and also the marked reduction in IL-4 (Fig. 5.11A) ($p<0.05$). IL-4 plays an important role in sustaining established IgE responses (Del Prete, 1988); therefore a decrease in its secretion can moderate the induction of IgE. Despite reducing circulating and sputum eosinophil numbers, systemic administration of anti-IL-5 antibody only partially reduces airway tissue eosinophils in asthmatic patients (Flood-Page, 2003), possibly because eosinophils lose IL-5 receptor from the cell surface as they enter the airways from circulation, and lose their dependence on IL-5 for survival (Liu, 2002). Several studies have also demonstrated a role for IL-10 in modulating the allergic immune response (Prigione, 2009). IL-10 plays a direct regulatory role in controlling the accumulation of eosinophils at inflammatory sites

by accelerating their apoptosis (Takanaski, 1994) but can also participate in the induction of regulatory T cells which in turn inhibit allergic airway inflammation (Grindbacke, 2004).

IL-13 was reduced in sensitised groups following delivery of MSC and "licensed" MSC (Fig. 5.11 B). This effect was offset upon delivery of fixed cells, indicating a crucial role for soluble factor secretion in MSC-mediated protection in this model. An increase in IL-5 was also observed in those mice treated with "licensed" MSC; however this was significantly less than those levels observed in groups treated with MSC without prior stimulation. Similarly, an increase in IL-10 was demonstrated in sensitised, IFN-γ licensed MSC treated mice, however this was also reduced when compared to "unlicensed" MSC-treated groups.

5.6.2 ALLOGENEIC MSC MODULATE LOCAL RECALL PROLIFERATIVE RESPONSES TO OVA

The influence on recall proliferative response to exogenous OVA was examined *ex vivo*. Mediastinal lymph nodes (MLN) from OVA challenged, but not saline challenged mice proliferated significantly in response to OVA, as expected (Fig. 5.12). MSC delivery in sensitised mice resulted in an increase in proliferation in response to OVA when compared to those sensitised to OVA alone. However, this effect was counteracted when MSC were activated prior to delivery. As MLN are the draining lymph nodes for the upper airways, the increase in BALF IL-5 and IL-10 in MSC-treated mice may explain the enhanced T cell response in these groups. Prior activation of cells does not cause an equivalent increase in these cytokines which is reflected in the absence of proliferative response in the MLN. Delivery of PFA-fixed MSC to OVA challenged mice prevented the inhibition of proliferation observed when live cells were delivered. Cytokine profile analysis of this group

adds further credence to this hypothesis as dramatic increases in IL-13 and IL-4 production was evident in BALF which again mirrored T cell proliferative responses to OVA *in vitro*.

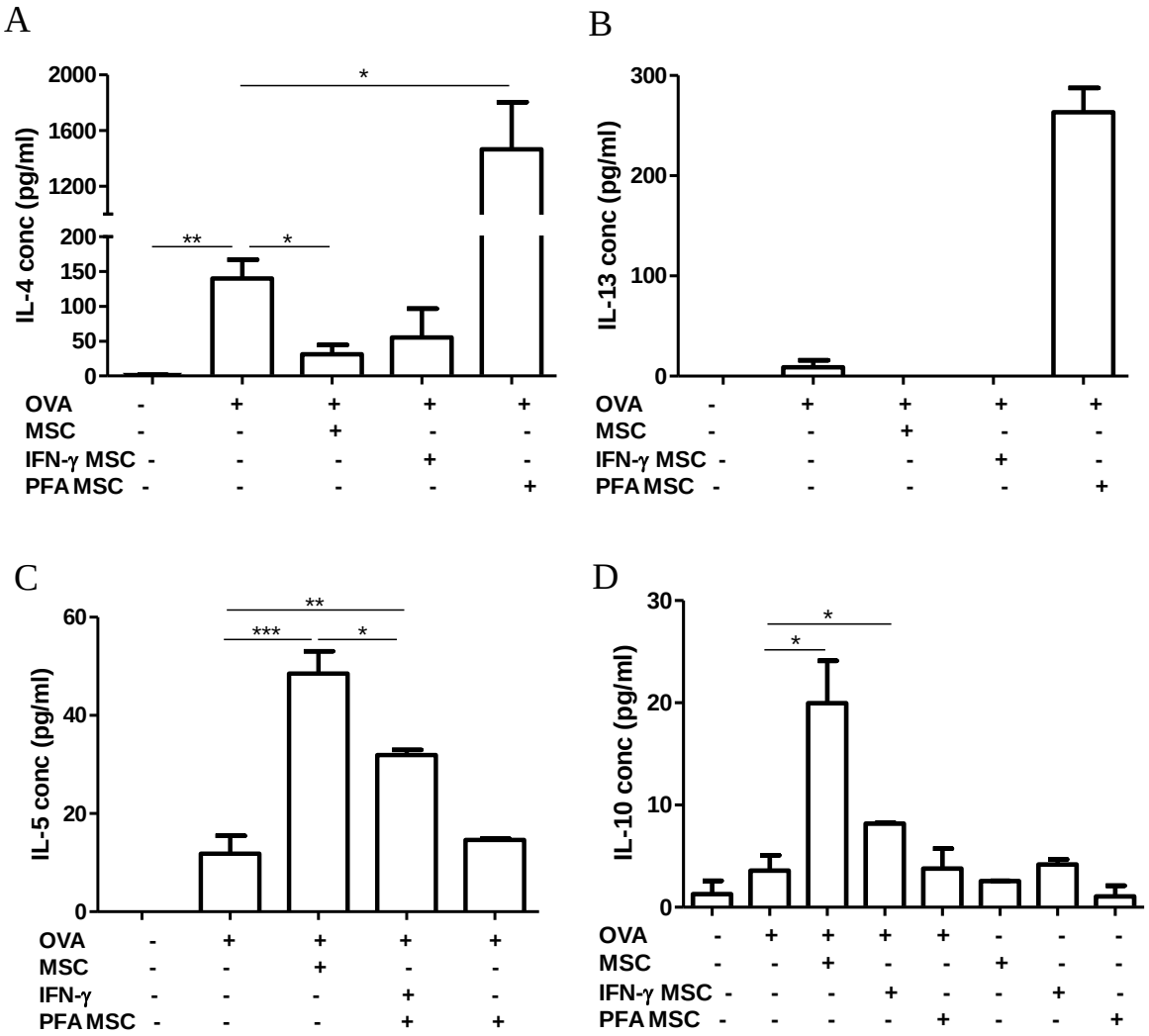


Figure 5.11 MSC modulate cytokine responses in BALF. Cytokines in BALF, elicited by OVA sensitisation following infusion with MSC. BALF was harvested on day 28. Negative symbols indicate sham sensitisation or challenge with PBS. Cytokine responses from similar cultures are shown for (A) IL-4, (B) IL-13 (C) IL-5 & (D) IL-10. Responses are representative of duplicate experiments, each of which were determined independently from at least nine mice per group and are expressed as means ± S.E.M. (*, $p < 0.05$).

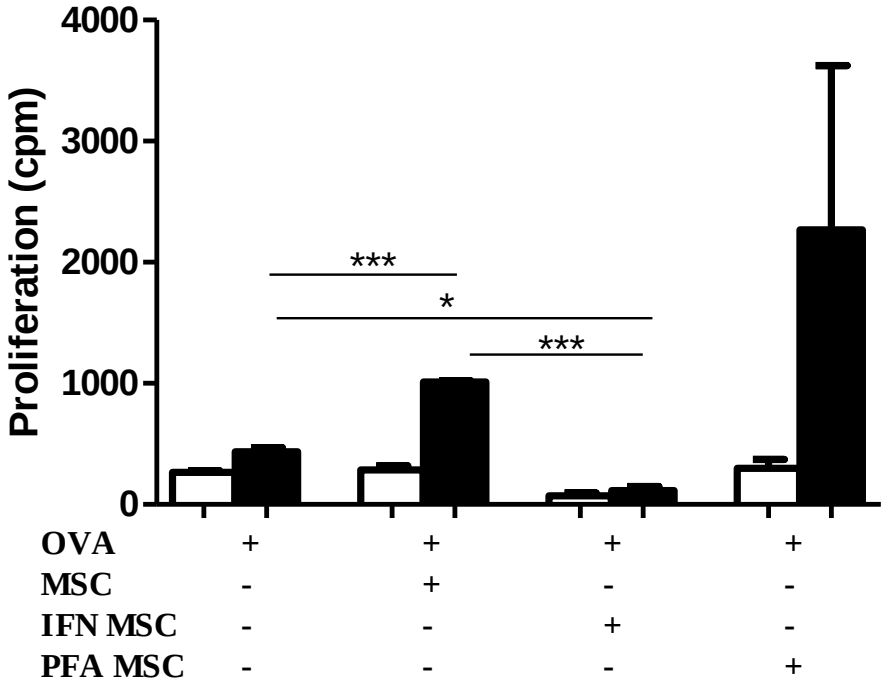


Figure 5.12 MSC modulate recall responses to sensitising allergen in local lymph nodes re-stimulated with OVA. Cell-mediated immune responses from mediastinal lymph nodes (MLN) to OVA elicited by OVA sensitisation. Lymph nodes were harvested on day 28 and processed as described in Section 2.17. Cells were cultured in the presence of media alone (□) or OVA (200µg/ml) (■) for 72h. Subsequent proliferation was measured by 3H-thymidine incorporation (*, $p<0.05$). No proliferation was detected from sham sensitised mice in MLN. Negative symbols indicate sham sensitisation or challenge with PBS. Responses are representative of duplicate experiments, each of which were determined independently from at least three mice per group and are expressed as means ± S.E.M.

5.7 ALLO-MSC MODULATE SYSTEMIC CELL-MEDIATED RESPONSES

In order to determine whether MSC were affecting local suppression at the site of injury only, systemic *in vivo* responses to MSC delivery were also investigated using supernatants from splenocyte preparations stimulated with OVA. Both cytokine profiles and proliferative responses were assessed. IL-4, IL-5 and IL-13 levels in splenocyte supernatants were significantly higher in the OVA sensitised group when compared to those sham sensitised with PBS. Unlike the increase in IL-5 seen in the BALF, MSC treatment did not significantly change the splenic IL-5 compared response to the OVA-sensitised group. Likewise, cytokine data did not concur between splenocyte preparations and BALF with regard to IL-4. A decrease in IL-4 production in the MSC-treated/OVA sensitised group (when compared to the OVA sensitised group) was observed in BALF, whereas an increase was demonstrated in splenocyte supernatant (Fig. 5.13 A). This effect was offset when MSC were activated prior to delivery. Fixed cells initiated a significant increase in the production of IL-4 when compared to those sensitised to OVA alone. As expected, an increase in IL-13 was demonstrated in OVA-sensitised mice when compared to those sham sensitised with PBS, however, no remarkable differences were observed upon MSC delivery. IL-10 production was markedly increased in both BALF and splenocyte supernatant (Fig. 5.11 D & 5.13 D). No significant induction/suppression of Th2-type cytokine responses were observed in sensitised, IFN-γ-stimulated MSC treated mice. These data suggest that MSC may be having a different effect locally (at the site of damage) when compared to the systemic response in the spleen.

The influence on recall proliferative response to exogenous OVA was examined *ex vivo*. Splenocytes from OVA challenged, but not saline challenged mice proliferated significantly in response to OVA, as expected. In contrast to T cell responses in MLN, proliferative responses from sensitised, MSC-treated mice were not significantly greater than

those seen in OVA-sensitised mice. However, no significant inhibitory effect on proliferation was observed in these groups. "Licensed" MSC prevented recall proliferative responses (Fig 5.14), whilst delivery of PFA-fixed MSC cells to OVA challenged mice prevented the inhibition of proliferation observed when stimulated cells were delivered.

These data have two implications. Firstly, figures 5.11 C & 5.13 A demonstrate that MSC-mediated immune suppression was not a global phenomenon as both BALF and OVA-driven recall cytokine responses were elevated by MSC in some instances. In addition, these data suggests the existence of one, or a combination of, functionally distinct pathways exploited by MSC to inhibit allergic airway inflammation, including possible immunomodulation via cytokine (IL-10) induction/suppression directly affecting immune cell infiltration and/or induction of regulatory T cells.

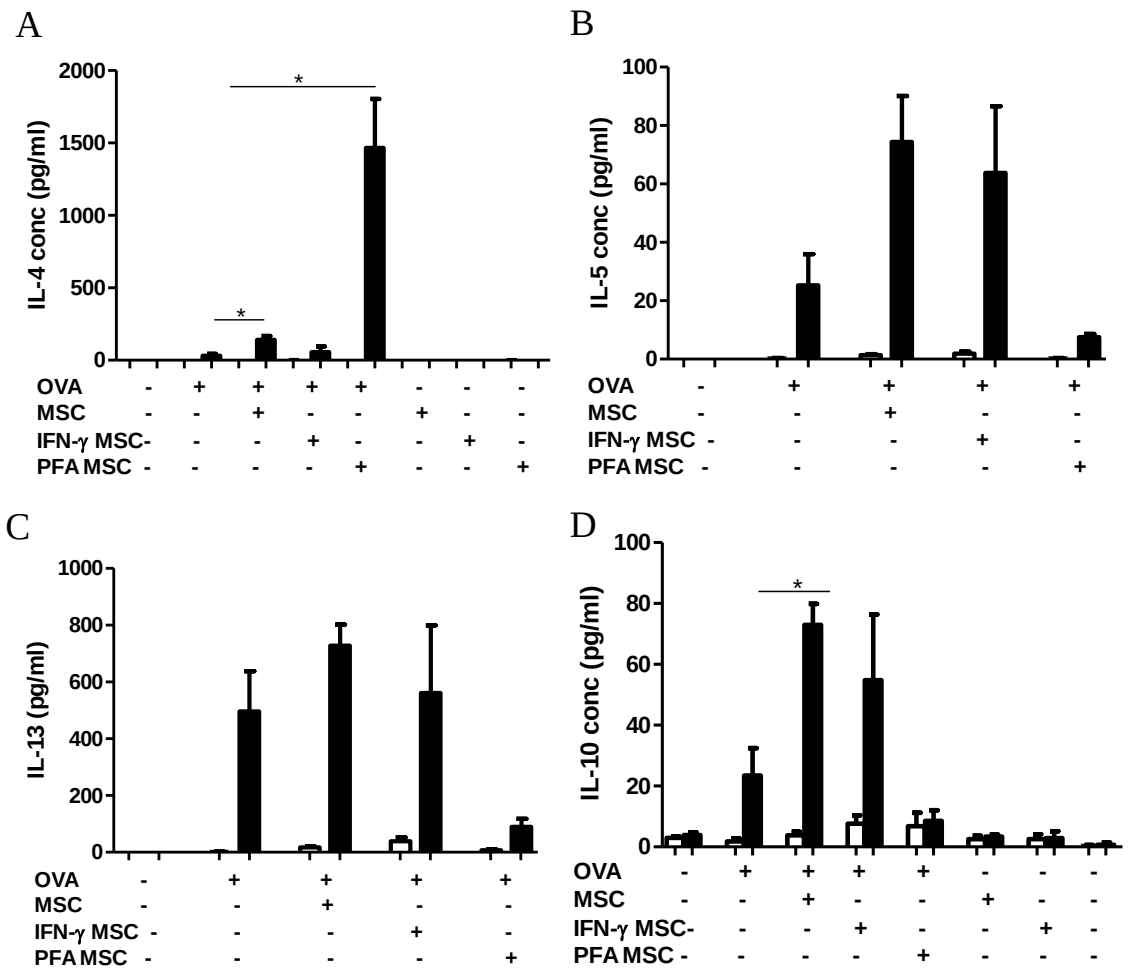


Figure 5.13 MSC modulate recall cytokine responses to sensitising allergen from murine splenocytes re-stimulated with OVA. Spleens were harvested on day 28 and processed as described in Section 2.19. Splenocytes were cultured in the presence of media alone (□) or OVA (200µg/ml) (■) for 72h. Negative symbols indicate sham sensitisation or challenge with PBS. Cytokine responses from similar cultures are shown for (A) IL-4, (B) IL-5 (C) IL-13 and (D) IL-10. Responses are representative of duplicate experiments, each of which were determined independently from at least nine mice per group and are expressed as means ± S.E.M. (*, $p < 0.05$).

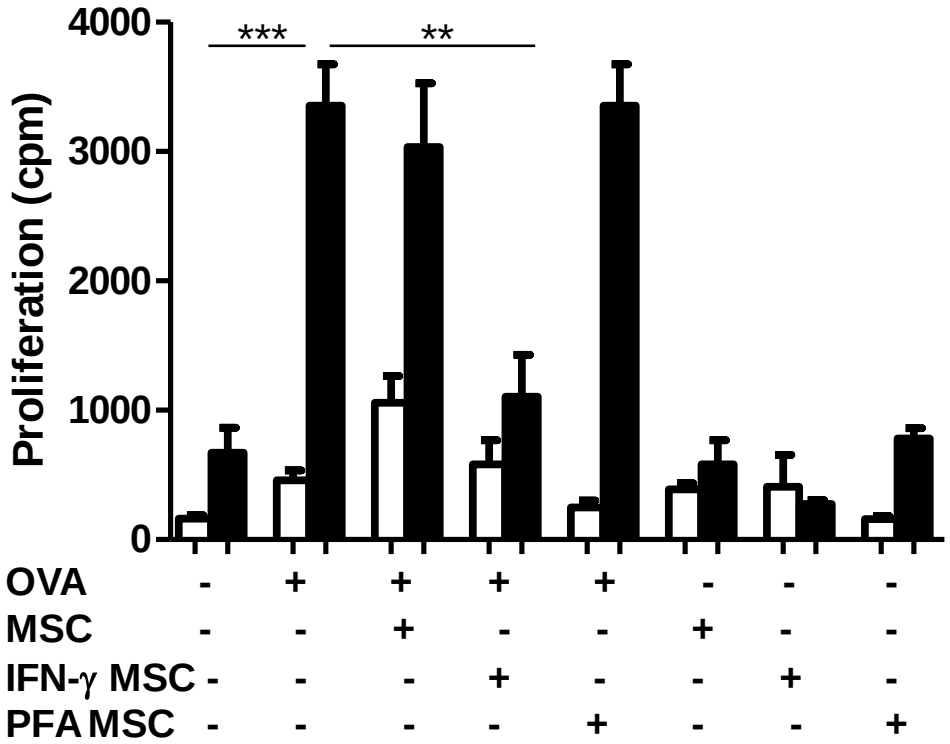


Figure 5.14 MSC modulate recall responses to sensitising allergen from splenocytes re-stimulated with OVA. Cell-mediated immune responses from splenocytes to OVA elicited by OVA sensitisation. Cytokine responses from similar cultures are shown for IL-5, IL-4, IL-13 and IL-10 in Figure 5.12. Spleens and lymph nodes were harvested on day 28 and processed as described in Section 2.19. Cells were cultured in the presence of media alone (□) or OVA (200µg/ml) (■) for 72 h. Subsequent proliferation was measured by 3H-thymidine incorporation (*, $p<0.05$). Negative symbols indicate sham sensitisation or challenge with PBS. Responses are representative of duplicate experiments, each of which were determined independently from at least nine mice per group and are expressed as means ± S.E.M.

5.8 PRE-STIMULATION OF MSC WITH IFN-GAMMA INDUCES HIGH LEVELS OF HGF

MSC express the immunosuppressive cytokines hepatocyte growth factor (HGF) and TGF-β at concentrations that can suppress allogeneic responses *in vitro* (Rehman, 2004; Barry, 2005). Moreover, co-culture with IFN-γ significantly increases the expression of these suppressive mediators (Ryan, 2007) supporting a role for IFN-γ in potentiating the immunosuppressive activities of MSC. HGF has recently been shown to negatively regulate allergic airway inflammation and hyperresponsiveness in a murine model (Ito, 2007) via direct attenuation of eosinophil chemotactic function. This led to the hypothesis that perhaps an increase in HGF production by "licensed" MSC effects an enhancement of protective function in this group. Therefore, the effect of prior stimulation of MSC on the secretion of HGF was investigated here. These data demonstrated a dramatic increase in the level of HGF in the conditioned media of MSC co-cultured with IFN-γ for 48h. This is an important finding as it supports the data observed in Chapter 4 which showed that "licensed" MSC exhibited a significant enhancement of protective function when compared to those without stimulation prior to delivery. Collectively, these data strongly indicate that enhanced production of HGF in "licensed" cells may contribute to their ability to inhibit allergic airway pathology when compared to un-stimulated cells.

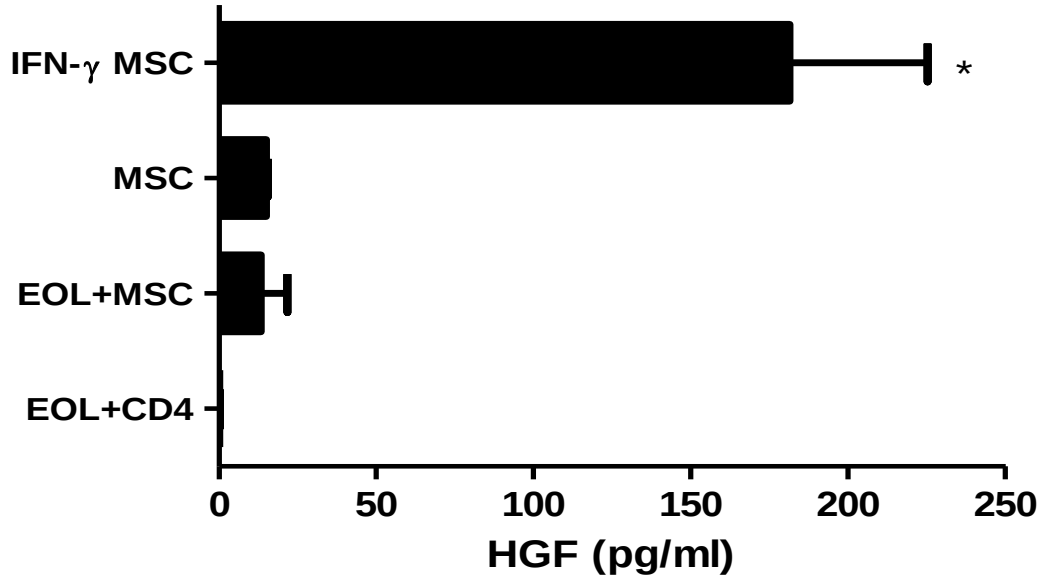


Fig. 5.15 Pre-stimulation with IFN-γ induces high levels of HGF secretion by MSC. 5 x 10^4 human MSC or IFN-γ-stimulated MSC were co-cultured with 3 x 10^5 human eosinophils (cell line, EOL-1) in the presence or absence of $CD4^+$ T cells for 48 h. Supernatants were analysed by ELISA for the presence of HGF.

5.9 SUMMARY

This study demonstrated that adult bone marrow derived allogeneic MSC actively prevent the induction of allergen driven pathology in a murine model and suggested a novel cell therapy for allergic human disease. Systemic administration of MSC protected the airways from OVA-induced pathology demonstrated by the reduction in BAL infiltration, airway pathology and OVA-specific IgE. MSC modulated cell-mediated responses at both a local and systemic level. Notable observations included a significant decrease in IL-4 combined with an increase in IL-10 in BALF and splenocyte preparations. Taken together, these findings demonstrate that MSC can reduce the intensity of the allergic airway inflammatory response through interactions with both innate and adaptive immunity. However, this inhibitory effect on airway inflammation is not mediated by global suppression as demonstrated by the increase in IL-5 in both the lung and spleen following MSC delivery. Prior stimulation of MSC with IFN-γ increased their protective effect in this model. This was most evident in the histopathological analysis of the lung tissues where mucus hypersecretion and peribronchial inflammation was significantly reduced. These data suggested that IFN-γ stimulation plays an important role in enhancing the efficacy of MSC in modulating the immune response in allergic airway inflammation. The significant increase in HGF, an established inhibitor of allergic airway inflammation, in the conditioned medium of IFN-γ-stimulated MSC suggests a role for this important cytokine in their protective effect.

Current data suggest that Th2 responses to allergens are suppressed by IL-10-producing regulatory T cells (Cottrez, 2000; Akdis, 2006; Meiler, 2008). However, the absence of IL-10 induction in IFN-γ-stimulated MSC-treated groups in BALF and splenocyte supernatant suggests an alternative mechanism of protection against allergic airway inflammation that is independent of IL-10 in this particular group. The induction of IL-10 seen here as a result of MSC delivery suggested a role for MSC in T_{reg} induction prompting

further investigation. A comprehensive analysis of this study can be found within the discussion section at the end of Chapter 6. Chapter 6 elucidates the mechanisms involved in the reduction of allergic airway pathology by MSC.

CHAPTER 6

MSC–MEDIATED PROTECTION AGAINST ALLERGEN DRIVEN AIRWAY PATHOLOGY REQUIRES REGULATORY T CELL POPULATIONS

6.1 INTRODUCTION

In Chapter 5, it was reported that the delivery of MSC reduced the pathology associated with allergic airway inflammation. As both global suppression and blocking of immune induction have been eliminated as potential mechanisms underlying this observation, three processes could explain these data (1) MSC induce regulatory T cells (T_{reg}) which suppress immunopathology; (2) MSC repair or regenerate the damaged airways ; (3) MSC prevent eosinophilia and thus prevent eosinophil mediated pathology. In Chapter 5, an increase in IL-10 was detected in bronchoalveolar lavage fluid and also spleen cell preparations of mice that received MSC (with OVA sensitisation/challenge) (Fig. 5.10 & 5.12), supporting the hypothesis that MSC induce the proliferation/differentiation of T_{reg} which in turn regulate inflammation through the production of IL-10. $CD4^+CD25^+FoxP3^+$ T_{reg} are critical for control of antigen-specific inflammation and their recruitment into the airways and can suppress allergic airway inflammation (Strickland, 2006). This chapter probed the mechanism of therapeutic efficacy of MSC. A set of experiments were designed to discriminate between these alternative hypotheses. This was structured in three parts; (1) by ascertaining whether there was an expansion of regulatory T cells upon MSC delivery, (2) establishing whether depletion of these suppressor cells would result in negation of the protective effect of MSC, (3) investigating whether MSC could directly inhibit eosinophil migration.

6.2 MSC CELL THERAPY INDUCES DEFINED REGULATORY T CELL SUBSETS *IN VIVO*

Cytokines produced by antigen-presenting cells (APC) and other cells play a critical role in the differentiation of regulatory T cells. APC are an important source of IL-10, and priming of naive T cells in the presence of this cytokine results in the differentiation of T_{reg}. An important finding of Chapter 5 was the significant induction of IL-10 as a result of MSC

delivery in both the spleen and lungs which warranted further investigation, as these data alluded to an alternative or additional mechanism to direct inhibition of allergic pathology via soluble factor secretion by MSC. Several regulatory T cell subsets exist; however two major subsets of T_{reg}, $CD4^+CD25^{hi}$ $Foxp3^+$ T_{reg} and $CD8^+\gamma\delta$ T cells have demonstrated protection in models of allergic airway inflammation (Lahn, 1999; Grindbacke, 2004; Ling, 2004; Isogai, 2007). This made a compelling case for examining MSC as cellular inducers of these regulatory T cells. First, it was necessary to investigate whether MSC could expand T_{reg} subsets implicated in regulating the allergic airway response. This was investigated by identifying T cell populations generated in response to MSC delivery in a model of allergic airway inflammation. Mice were sensitised with OVA on day 0, 7 and 14. Intravenous delivery of MSC was carried out on days 7 and 14, and respiratory challenge with OVA was performed on day 14. Spleens and lungs were harvested from mice 5 days after the last treatment with MSC (day 19). The generation and expansion of T_{reg} subsets was assessed in the lungs and spleens of OVA sensitised mice, and from those mice that also received MSC.

MSC therapy induced or expanded two populations of regulatory cells. A significant increase in $CD8^+\gamma\delta$ T cells was observed in lung and spleen preparations from MSC treated at day 19 when compared to non-treated, OVA-sensitised sensitised mice (Fig. 6.1). MSC also increase γ/δ $CD8^-$ cells in the lungs and in the spleen, most cells were either γ/δ^- $CD8^+$ or γ/δ^+ $CD8^-$ (Fig. 6.1). Furthermore, MSC induced or expanded a $CD4^+FoxP3^+$ population in both the lungs and spleen from sensitised, MSC-treated mice (Fig. 6.2 & 6.3). Intracellular Foxp3 expression was measured on a gated, viable, population. An increase in FoxP3 expression ($p < 0.05$) was observed in $CD4^+$ T cells from sensitised, MSC-treated mice. These data provide evidence that MSC induce distinct T_{reg} populations implicated in the suppression of the allergic airway inflammatory response.

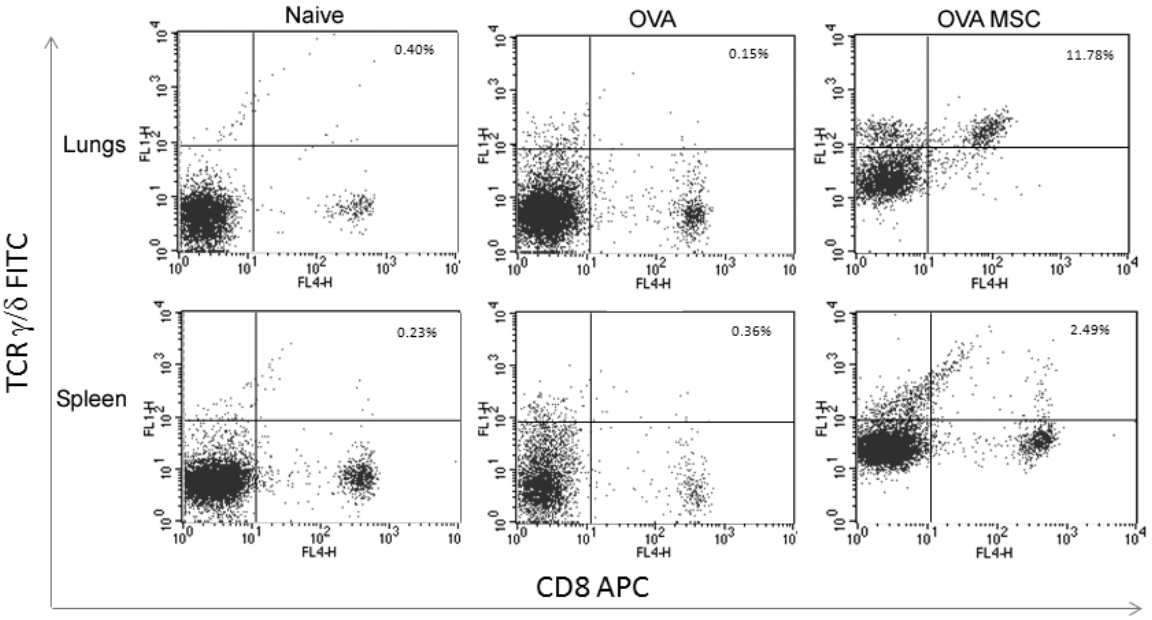


Figure 6.1 MSC cell therapy induced a population of CD8$^+$ γδ T cells. A population of viable lymphocytes was gated on and the percentage of cells expressing the γδTCR and CD8 on their surface was assessed in lungs and spleen of naïve, OVA-sensitised and OVA-sensitised, MSC-treated mice at day 19. Plots are representative of experiments performed at least three times.

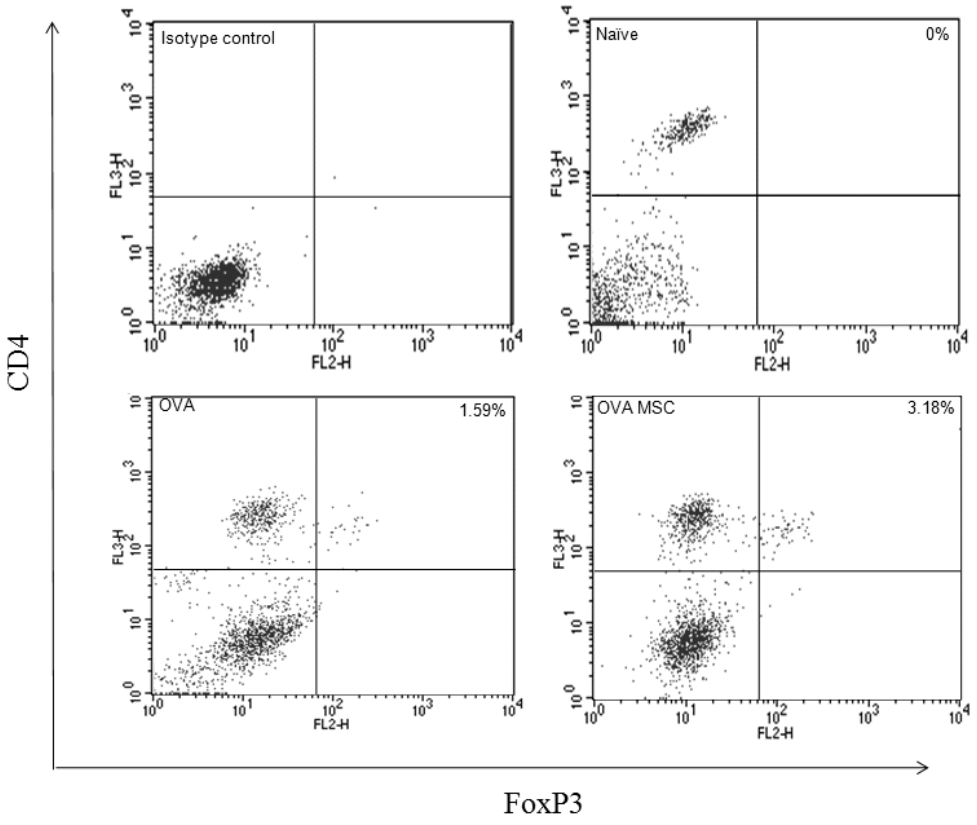


Figure 6.2 MSC cell therapy induced a population of $CD4^+$ $FoxP3^+$ T cells in the lungs of OVA primed mice. The OVA BALB/c allergic asthma model was used +/- MSC cell therapy (OVA MSC) on day 7 & 14. On d19, lungs were harvested and digested with collagenase/ DNase I for 1 h followed by intra/extracellular staining with PE-conjugated anti-FoxP3 and Cy5-conjugated anti-CD4.

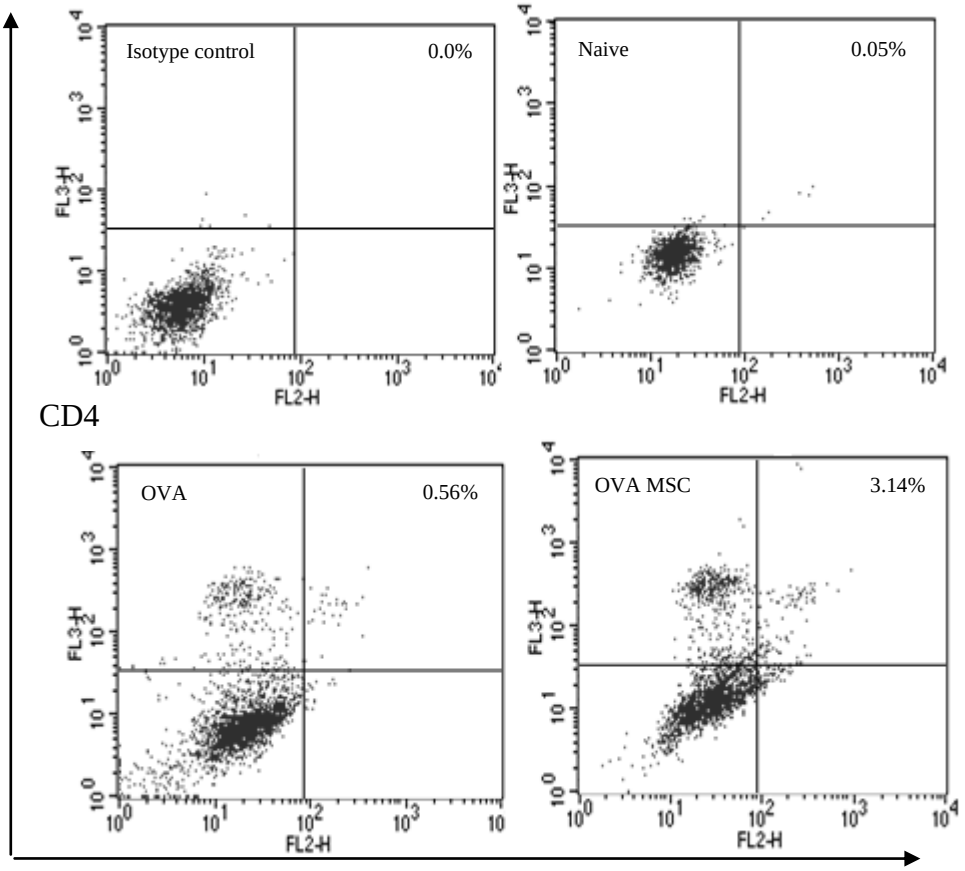


Figure 6.3 A population expressing $CD4^{+}FoxP3^{+}$ is increased in spleen following MSC delivery. Expression of CD4 and FoxP3 in splenocyte preparations isolated from OVA sensitised (OVA) or OVA-sensitised MSC treated (OVA MSC) mice at day 19. Spleens were harvested and cells were lysed and stained with PE-conjugated anti-FoxP3 and Cy5-conjugated anti-CD4 as above.

6.3 A LOW DOSE CYCLOPHOSPHAMIDE REGIMEN DEPLETES TREG CELLS IN THE MURINE OVA MODEL

The data in Chapter 5 demonstrated that MSC are of therapeutic benefit in an OVA model of allergic airway inflammation. In Section 6.2, it was shown that MSC induce regulatory T cell populations not seen in non-treated mice. Therefore it was possible that the protective effect of MSC demonstrated in this model was due to T_{reg} induction. A reductionist approach was therefore employed to see if MSC could support the beneficial effect in the absence of T_{reg}. In the absence of a knockout mouse, cyclophosphamide was used to deplete these suppressor cells *in vivo*. A monoclonal anti-CD25 antibody has been routinely used for depleting T_{reg} *in vivo* (Van Meirvenne, 2005; Boudousquie, 2009). However, due to the expression of CD25 on activated effector T cells, this approach could not be used as such cells would be potential targets for depletion by anti-CD25 mAB also. Consequently, the use of an anti-CD25 antibody to examine the role of T_{reg} mediated inhibition of airway inflammation by MSC was avoided and instead substituted with a model which depletes T_{reg} using cyclophosphamide. Cyclophosphamide (CY) is a cytotoxic, alkylating agent widely used as a chemotherapeutic agent (Aschan, 1999) that has a direct inhibitory effect on $CD4^+CD25^+$ $FoxP3^+$ T_{reg} by impairing the functionality of these cells, as well as depleting this population *in vivo* (Lutziak C 2005). It has been used in studies to examine the effect of T_{reg} depletion in various disease models (Uchida, 1994; Aschan, 1999; Yung-Chang, 2006). Absolute numbers of T_{reg} typically return to baseline levels approximately 10 days after CY administration in these models.

To validate this approach it was important to establish that CY could effectively deplete T_{reg} and determine for how long this could be achieved *in vivo*. Thus, the effect of CY treatment on the number and percentage of T-cell subsets in mice was examined in order to determine the optimum time points for CY administration in the OVA model and to test

the "leakiness" of the model. One group (Group A) received 1 i.p. injection of CY (150 mg/kg) and a second group received 2 i.p. administrations of CY (150 mg/kg) over a 10 day period. Both groups were first sensitised to OVA via intraperitoneal injection on day -4. Four days later (day 0), during T cell expansion (Lin C-H 2003), CY was delivered by i.p. to Group A and Group B. Group B received a second CY dose on day 6. At each time point (i.e. days 0, 4, 6 and 10) splenocytes were harvested and the effect of cyclophosphamide on the kinetics of regulatory T cell marker expression was examined. Expression of CD4, CD8, CD25 and FoxP3 was assessed by flow cytometry. The experimental outline is summarised in Fig. 6.4.

CY decreased the total number of splenocytes by ~50% at day 4 following treatment (Fig. 6.5). The decrease was maximal on day 4 and persisted for at least 2 days. Cell numbers returned to normal levels 10 days following CY administration. However, a decline in $CD4^+CD25^+$ and $CD4^+$FoxP3 expression was more profound (~85% decrease) and recovered more slowly than total splenocytes and $CD4^+$ cells (Fig. 6.5 & 6.6 A). Four days following CY treatment, the percentage of $CD4^+CD25^+$ and $CD4^+FoxP3^+$ in the spleen decreased dramatically and were only partially restored between days 6 and 10 (Fig. 6.6 C & 6.6 D). The decrease in $CD8^+$ T cell expression was not significantly reduced 4 days following CY treatment. The decrease in the numbers of $CD4^+$ cells was maximal on day 4 but began to increase thereafter; by day 10 after CY treatment, expression had returned to pre-treatment levels (Fig. 6.6 A). These data were similar to other studies suggesting that cyclophosphamide affects all $CD4^+$ T cells; however this affect was not sufficient to reduce allergic airway inflammation (Yung-Chang, 2006) and Fig. 6.9.

These data indicated that T_{reg} would not be depleted for a sufficient period over the course of the proposed experiment. Therefore an additional CY treatment was incorporated into the regimen. To assess that multiple doses of CY would repeatedly achieve depletion

one group of mice received a second intraperitoneal injection of CY on day 10. Analysis of this group demonstrated a significant reduction in $CD4^+CD25^+$ and $CD4^+$FoxP3 ($p < 0.05$) at day 4 (Table 6.1). These data highlighted the requirement for multiple administrations of CY in order to deplete T_{reg} cells for the duration of allergen sensitisation and challenge (i.e. 28 days) and informed the experimental outline in Section 6.5 below. This was consistent with previous studies showing the effect of CY treatment on regulatory T cell populations *in vivo* (Ikezawa, 2005; Lutziak, 2005). It showed that T_{reg} could be effectively eliminated from this model for the critical period between days 7 and 28 when cell therapy was considered to be having an effect. Thus a protocol was devised in which MSC could be delivered without allowing the induction of T_{reg} and provided sufficient information to determine suitable time-points for depleting T_{reg} in an OVA sensitisation model where therapeutic stem cells could be delivered.

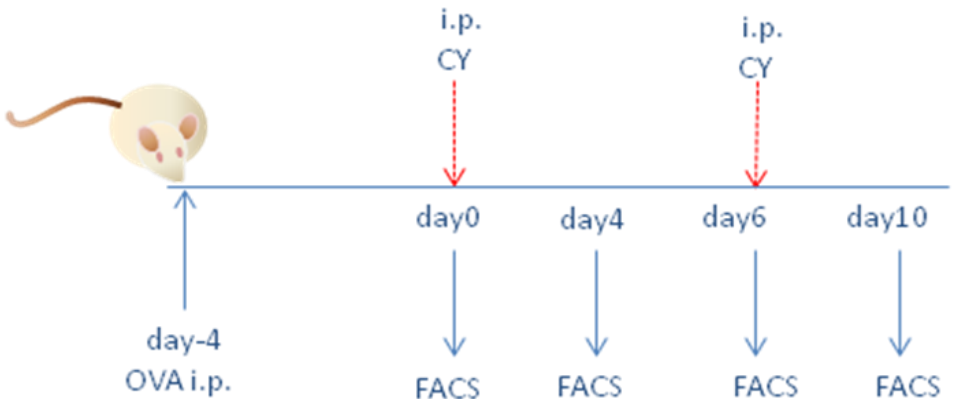


Figure. 6.4 Optimisation of cyclophosphamide-depletion of T_{reg}. Mice were sensitised with OVA via intraperitoneal (i.p.) injection on day 0. On day 4, cyclophosphamide was administered at a dose of 150 mg/kg i.p (n=4). Spleens were harvested on days 0, 4, 6 and 10. An additional group received a second dose of cyclophosphamide (150 mg/kg) on day 6. Spleens were harvested from this group on day 10. Expression of CD4, CD8, CD25 and FoxP3 on splenocytes was assessed by flow cytometry.

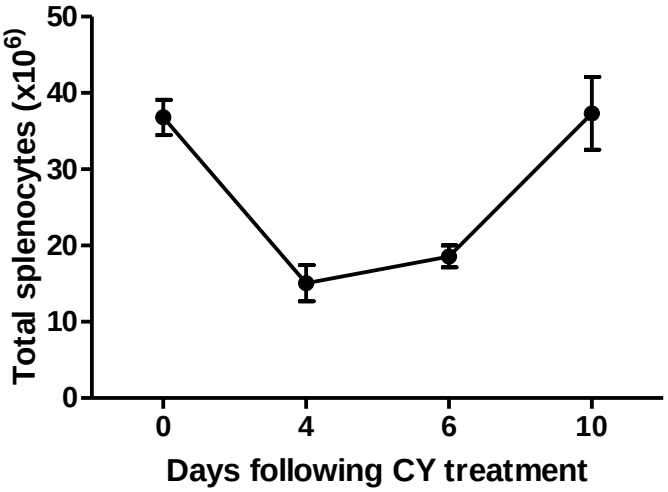


Figure 6.5 CY treatment reduced the number of spleen cells at day 4. Mice were sensitised with OVA i.p. (day -4) and then given 150 mg/kg of CY intraperitoneally at day 0. The effect on total cell number was assessed 4, 6 and 10 days following CY administration. Results are expressed as mean values ± S.D. obtained from groups of at least three mice and are representative of two experiments. *$p < 0.05$; **$p < 0.005$.

Table 6.1 CY decreases $CD4^+$, $CD4^+CD25^+$ and $CD4^+$FoxP3 expression in the spleen (Summary)

Marker	% Gated*				
	d0	**d4**	**d6**	**d10**	**d10 (2 CY)$^+$**
CD4	51.9 ± 2.61	20.9 ± 2.22	16.9 ± 7.55	46.2 ± 3.78	19.25 ± 4.62
CD8	12.8 ± 1.22	13.6 ± 1.88	8.9 ± 3.06	10.3 ± 0.69	11.65 ± 2.91
CD4FoxP3	3.03 ± 0.04	0.56 ± 0.01	0.83 ± 0.02	5.13 ± 0.12	0.22 ± 0.98
CD4CD25	4.72 ± 0.22	0.00 ± 0.03	0.56 ± 0.89	2.36 ± 1.67	0.87 ± 0.03

*Cells were gated on FSC and SSC to eliminate debris and dead cells. The % of cells gated expressing CD4, CD8, CD25 and FoxP3 were recorded. Results are expressed as mean values ± S.D. obtained from groups of at least three mice and are representative of two independent experiments. $^+$ indicates % at day 10 following a second administration of CY at day 6.

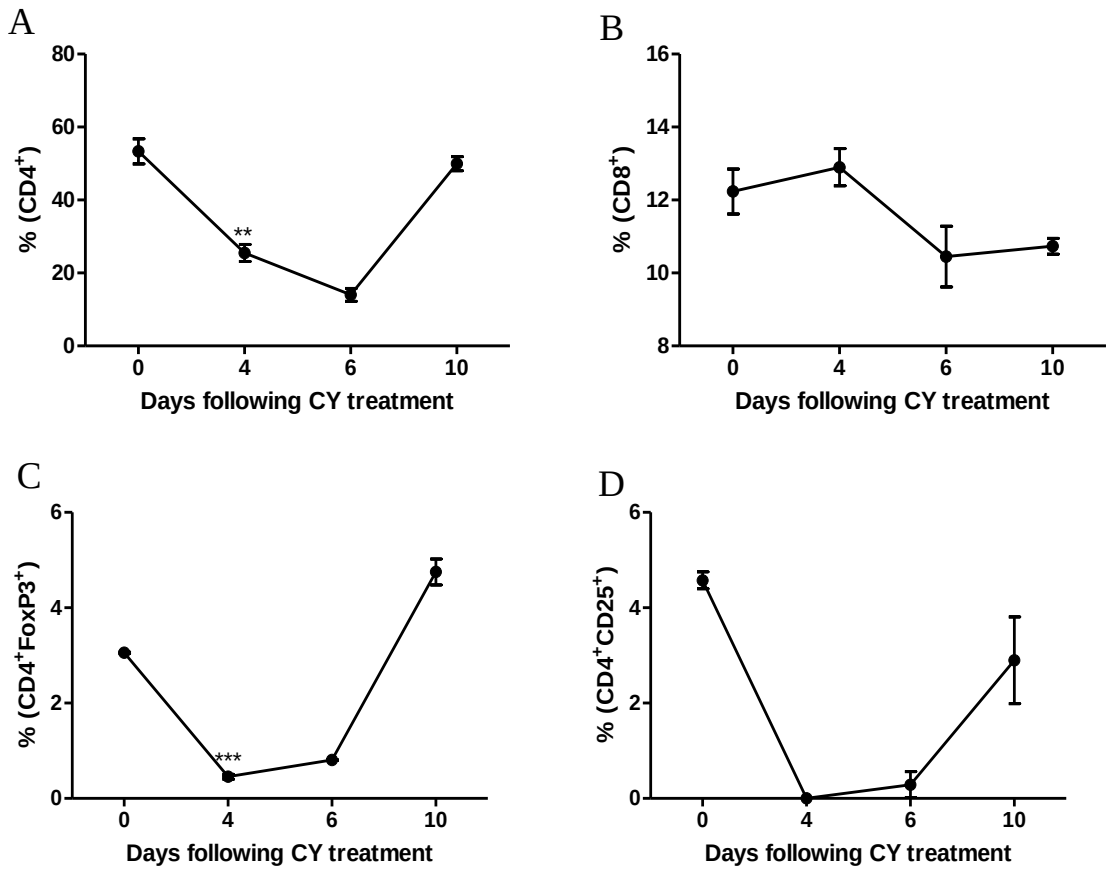


Figure 6.6 CY treatment reduced $CD4^+$ cells and depleted $CD4^+CD25^+$/ $CD4^+FoxP3^+$ cells in the spleen at day 4. The effect of CY treatment on (A) CD4, (B) CD8, (C) $CD4^+FoxP3^+$ & (D) $CD4^+CD25^+$. Mice were given 150 mg/kg of CY intraperitoneally at day 0. Spleen cells were isolated and stained with Cy5-conjugated anti-CD4mAb and APC-conjugated anti-CD8mAb and analysed by flow cytometry. Results are expressed as mean values ± S.D. obtained from groups of at least three mice and are representative of two independent experiments. *$p < 0.05$; **$p < 0.005$.

6.4 CY TREATMENT DID NOT AFFECT THE IMMUNOSUPPRESSIVE FUNCTION OF MSC

Adult stem cells, including MSC, express high levels of aldehyde dehydrogenase (ALDH) (Gentry, 2007). ALDH activity is important for multiple biological activities including drug-resistance to cytostatic agents including CY. However, it was important to investigate the potential cytotoxic effects of CY on MSC to exclude the possibility that CY was inducing apoptosis (or similar) and, as a result, negating the protective effect of the stem cells in this model. In order to examine the effect of CY treatment on MSC, a dose-response study was carried out where MSC were incubated with varying concentrations of CY in a 6-well plate for 48h. The ratios of MSC: CY were chosen to include the maximum exposure of MSC to CY that may occur *in vivo* (i.e. 0.5 x 10^6 MSC exposed to 150 mg/kg CY). The retention of immunosuppressive function was assessed by examining the ability of MSC to inhibit proliferation between MHC-mismatched splenocytes in a two-way mixed lymphocyte reaction. MSC retained their ability to inhibit proliferation in this assay at all ratios CY: MSC (Fig. 6.12). This study revealed that CY treatment exerted no detectable effect on the immunosuppressive capacity of MSC and supported previous findings (Gentry, 2007).

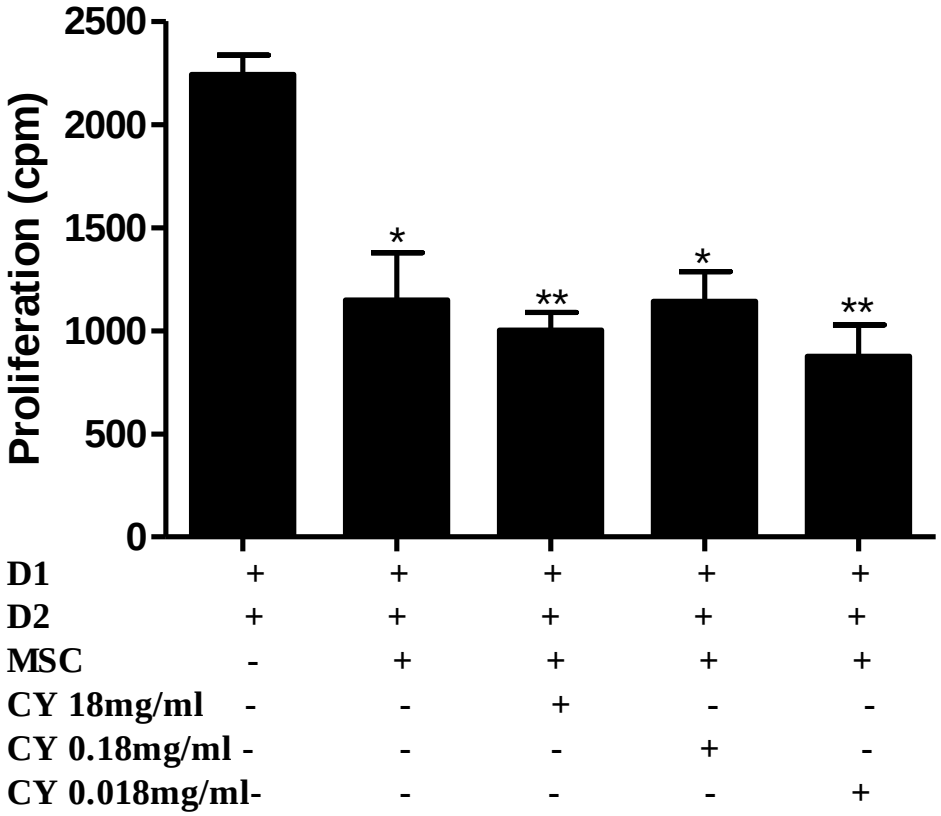


Figure 6.7 Cyclophosphamide does not affect the immunosuppressive function of MSC. MSC were co-cultured with three different concentrations of CY (18, 0.18 and 0.018 mg/ml) for 24 h in a 6-well plate. MSC were harvested from the plate and washed twice. For two-way mixed lymphocyte reactions, splenocytes from two MHC mismatched mice (D1 and D2, BALB/C and C57/BL6, respectively) were co-cultured. The effect of MSC on lymphocyte proliferation was assessed by adding MSC to the MLR at a density of 1.5 x 10^5 cells/ml. This experiment was only performed once. Results represent the mean (+/- SEM) of triplicate determinations.

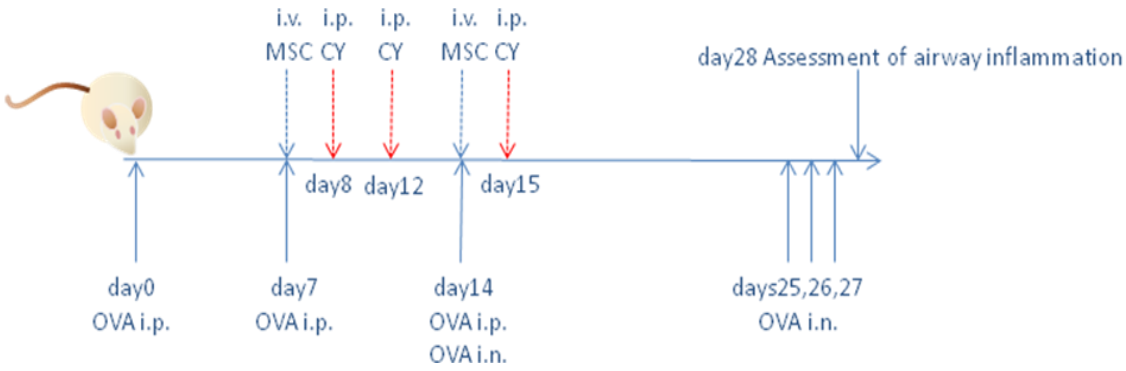


Figure 6.8 T_{reg} depletion in allergic airway inflammation model. Groups of 6-8 week old female BALB/c mice (n=8) were sensitised by i.p. injection with OVA (100 µg) on days 0, 7 and 14. On days 25, 26 and 27, mice were exposed to OVA (50 µg) via intranasal (i.n.) administration. Non-sensitised controls received PBS. Mice received allogeneic MSC (FV/BN) (5 x 10^6/ml) via intravenous injection on day 7 and 14, 6 h after OVA i.p./i.n. CY (150 mg/kg) was delivered via i.p. injection on day 8, 12 and 15. Readouts were performed on day 28. BALF, spleens and lungs were harvested for subsequent analysis. Mice were bled by facial bleed for serum analysis.

6.5 REGULATORY T CELLS PLAY ARE REQUIRED FOR MSC MEDIATED INHIBITION OF ALLERGIC AIRWAY INFLAMMATION

To investigate the hypothesis that MSC exert their immunosuppressive function via induction of T_{reg}, these suppressor cells were depleted using the protocol optimised above, in an ovalbumin-induced model of allergic airway inflammation (used previously in Chapter 3 & 5), and the effect of MSC delivery on airway pathology was examined. This approach would allow discrimination between two major mechanistic possibilities outlined in Section 6.1, because T_{reg} depletion should not interfere with potential repair function by MSC. Allergen sensitisation and MSC delivery was performed as described in Chapter 5. Briefly, mice were sensitised to OVA on days 0, 7 and 14 via i.p. injection, followed by intranasal challenge on day 14, 25, 26 and 27. Mice received MSC, or vehicle alone, intravenously on days 7 and 14. In addition, mice received three i.p. injections of CY on days 8, 12 and 15 (see experimental outline, Fig. 6.8) equivalent to the depletion timings optimised in Section 6.3. On day 28, mice were culled and the effect of MSC delivery on OVA-induced airway pathology in a T_{reg}-depleted model determined as previously described in Chapter 5.

Contrary to findings in Chapter 5 which demonstrated a significant protective effect by MSC, here, MSC could not alleviate pathology in T_{reg} depleted mice. Lung histology was assessed 24h after the last allergen challenge. In the absence of MSC treatment, OVA-sensitised mice (ovalbumin group), had significant cellular infiltration including perivascular and peribronchial inflammation (Fig. 6.9) demonstrating that the partial loss of $CD4^+$ cells (described in Section 6.3) does not impair induction of Th2-associated allergic airway pathology. The decrease in FoxP3 expression seen previously was consistent with the possibility that selective depletion of regulatory T cells contributes to the increase in the inflammatory response in CY treated mice. Sensitised, CY-treated mice that received MSC had a cellular infiltrate in the perivascular and peribronchial areas (Fig. 6.9). In periodic

acid-Schiff–stained sections, the CY/OVA group displayed mucus production but notably, delivery of MSC to T_{reg} depleted mice resulted in more pronounced levels of mucus production that partially plugged some airway lumina entirely (Fig. 6.10).

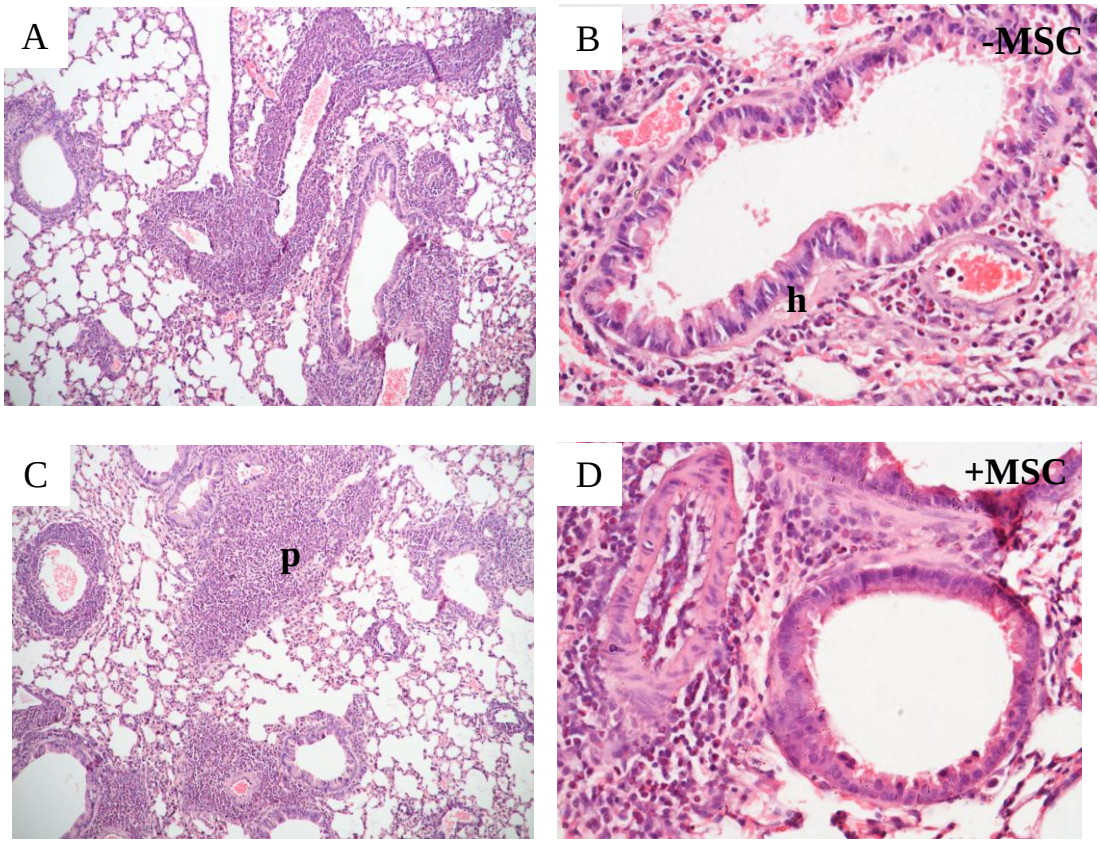


Figure 6.9 Transverse sections of lungs from OVA-sensitised, T_{reg} depleted mice (A&B), OVA-sensitised MSC treated/ T_{reg} depleted mice (C& D). Airway inflammation was detected using H&E staining on lung sections. **p** and **h** indicate perivascular inflammation and bronchiolar epithelial hypertrophy, respectively. All sections are representative at least three animals. Original magnification A and C x100; B and D x400.

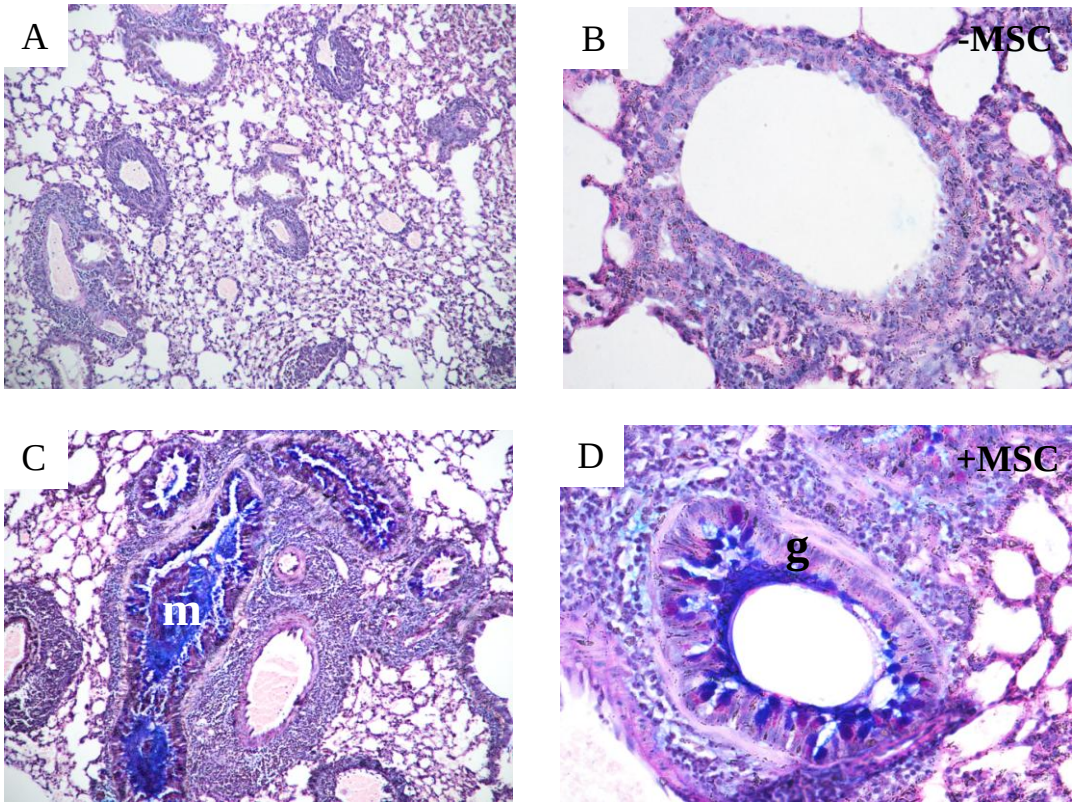


Figure 6.10 Representative morphological changes at 28 days in bronchiolar transverse sections of lungs from OVA sensitised-T_{reg} depleted mice without cell therapy (A&B), or with cell therapy (C&D). Airway inflammation was detected using combined Discombes/Alcian blue/PAS staining on lung sections. **g** and **m** indicate goblet cell hyperplasia and mucus secretion, respectively. All sections are representative of at least three animals. Original magnification A and C x100; B and D x400.

6.6 GENERATION OF T_{REG} BY MSC ARE REQUIRED FOR INHIBITION OF OVA-SPECIFIC IGE

Previously, a significant reduction in allergen-driven IgE was observed in mice receiving MSC following allergen sensitisation (Fig. 5.10). However, when mice were depleted of T_{reg} by cyclophosphamide, there was no observable difference in IgE in those that received MSC when compared to those that did not (Fig. 6.11). Consistent with previous studies (Su, 2006), CY treatment increased IgE production in OVA-sensitised mice (Fig. 6.11) compared to the levels seen in non CY treated (Fig. 5.10). Typical values in sensitised mice with no CY treatment were ~80 pg/ml; however, CY significantly increased OVA-specific IgE production to approximately 150 pg/ml. As CY selectively depletes T_{reg}, this suggests that IgE mediated allergy (atopic) conditions are moderated *in vivo* by the suppressive influence of constitutive T_{reg} in the OVA model and that T_{reg} are required for the MSC mediated reduction of IgE.

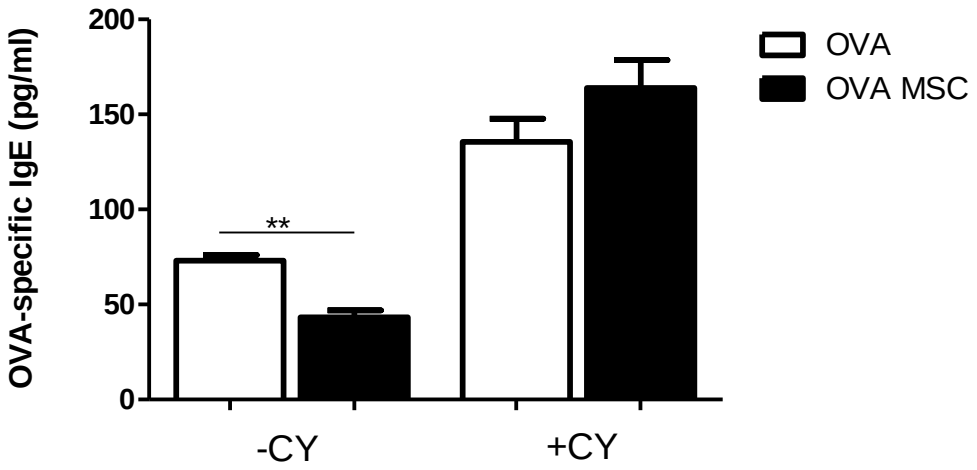


Figure 6.11 OVA-specific IgE in serum elicited in response to OVA sensitisation in T_{reg} depleted mice. Sera were collected on day 28 and OVA-specific serum IgE levels were measured by ELISA. The data presented are representative at least four animals, and each individual assessment was performed independently in triplicate. Results are expressed as mean antibody concentrations ± S.E.M.

6.7 MSC MEDIATE AIRWAY EOSINOPHILIA VIA A T_{REG}-INDEPENDENT PATHWAY

In Chapter 5, a reduction in BALF cellular infiltration was observed in those mice receiving MSC. This was evident in all cell types examined, including neutrophils, lymphocytes, macrophages and eosinophils (Fig. 5.6). In contrast, there was no significant differences in the immune cell types in the airways of allergen sensitised T_{reg} depleted mice in the presence or absence of MSC cell therapy, with one exception. A reduction in eosinophils was observed (Fig. 6.12 B). The degree of airway eosinophilia was significantly higher (>80 x 10^3 /lung) in all groups treated with CY when compared to those seen previously in OVA-sensitised groups with no CY treatment (>40 x 10^3 /lung) (see Chapter 5). This is consistent with studies that demonstrated a worsening of allergic airway pathology. However MSC treatment specifically reduced eosinophilia even in the absence of T_{reg} (Fig. 6.12 B). This observation was interesting as it indicated an alternative mechanism employed by MSC which directly inhibits airway eosinophilia, however its' main significance was that it allowed elimination of a hypothetical mechanism of MSC action. As a reduction in eosinophilia is retained in this model whilst the pathology is impaired it implies that reduced eosinophilia cannot account for the MSC mediated effects, and that these are linked to T_{reg} induction.

6.8 T_{REG} ARE REQUIRED FOR MSC MODULATION OF LOCAL AND SYSTEMIC CYTOKINE INDUCTION

In Chapter 5, MSC were shown to reduce local IL-4 and IL-13 in allergen primed mice, however, an increase in IL-5 production was observed. Examination of the cytokine profile of BALF from CY-treated, OVA-sensitised mice revealed no significant differences in IL-5, 13 or IL-4 between those receiving MSC, and those that did not (Fig. 6.13). A significant increase in IL-10 in MSC-treated mice was reported in Chapter 5 (Fig. 5.11 D &

5.12 D); however, when mice were depleted of T_{reg} using CY the opposite was true. There was a decrease in IL-10 in CY-OVA-MSC as a result of CY administration (Fig. 6.13 C). These data support the hypothesis that MSC generate regulatory T cells that suppress allergic airway responses *in vivo*.

As expected, incubation of splenocyte preparations from sensitised mice with exogenous OVA demonstrated a significant increase in proliferation (Fig. 6.14). This was also the case for sensitised mice receiving MSC, however the stimulation was significantly lower when compared to OVA-sensitised groups, suggesting that MSC mediated suppression may utilise additional (non T_{reg}) mechanisms which reduce proliferation but which are not sufficient to reduce pathology.

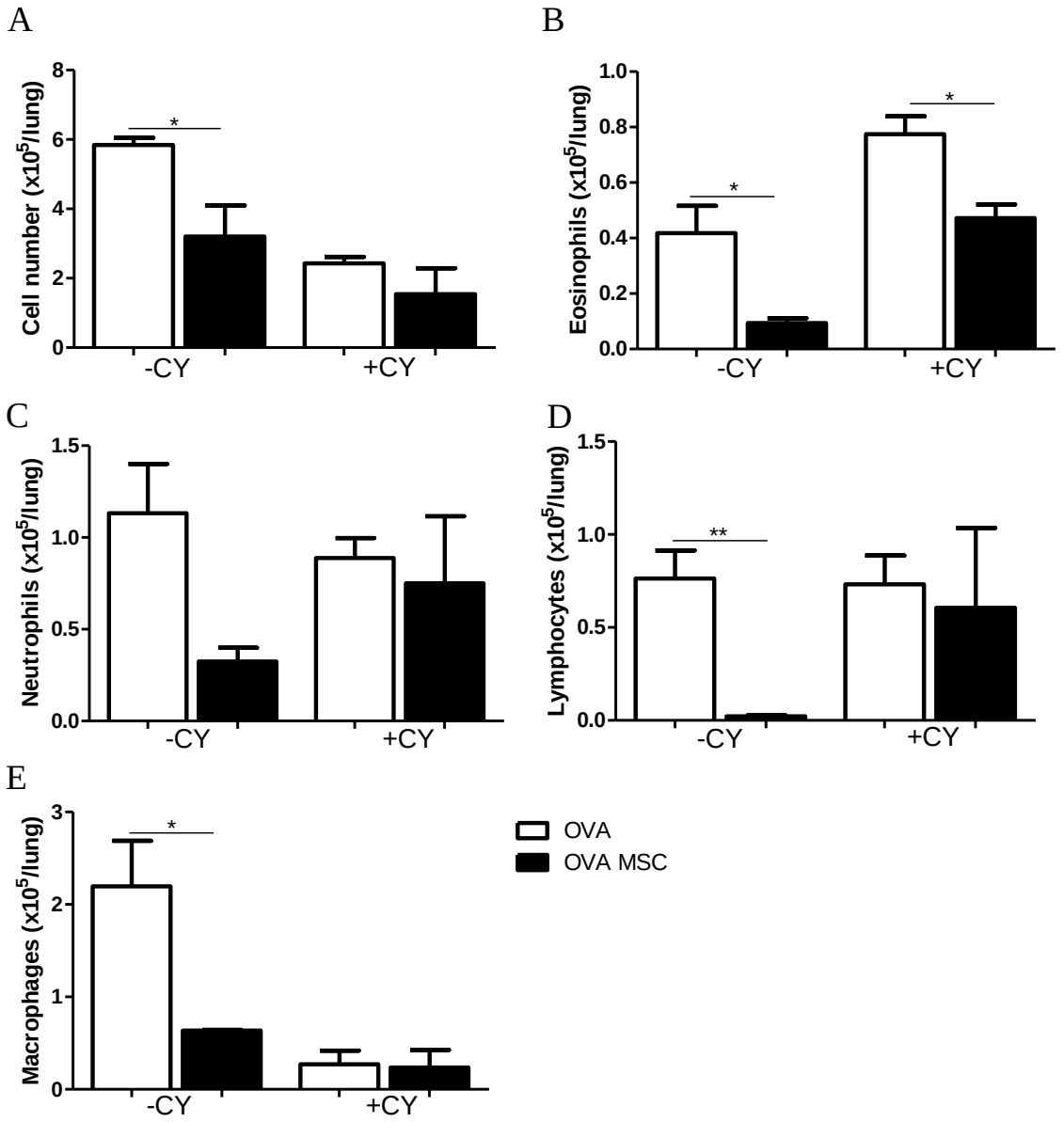


Figure 6.12 Depletion of T_{reg} does not affect MSC-mediated suppression of airway eosinophilia. Cellular composition of BAL from T_{reg}-depleted (CY-treated (150 mg/kg)) mice 24 h after final OVA exposure. Negative controls were sham infected/sensitised with saline. Lavages were examined for the total cell number (A), or the presence of eosinophils (B), neutrophils (C) lymphocytes (D) or macrophages (E). The data are representative of at least three animals. Results are expressed as mean ± S.E.M. of cell number. *$p < 0.05$.

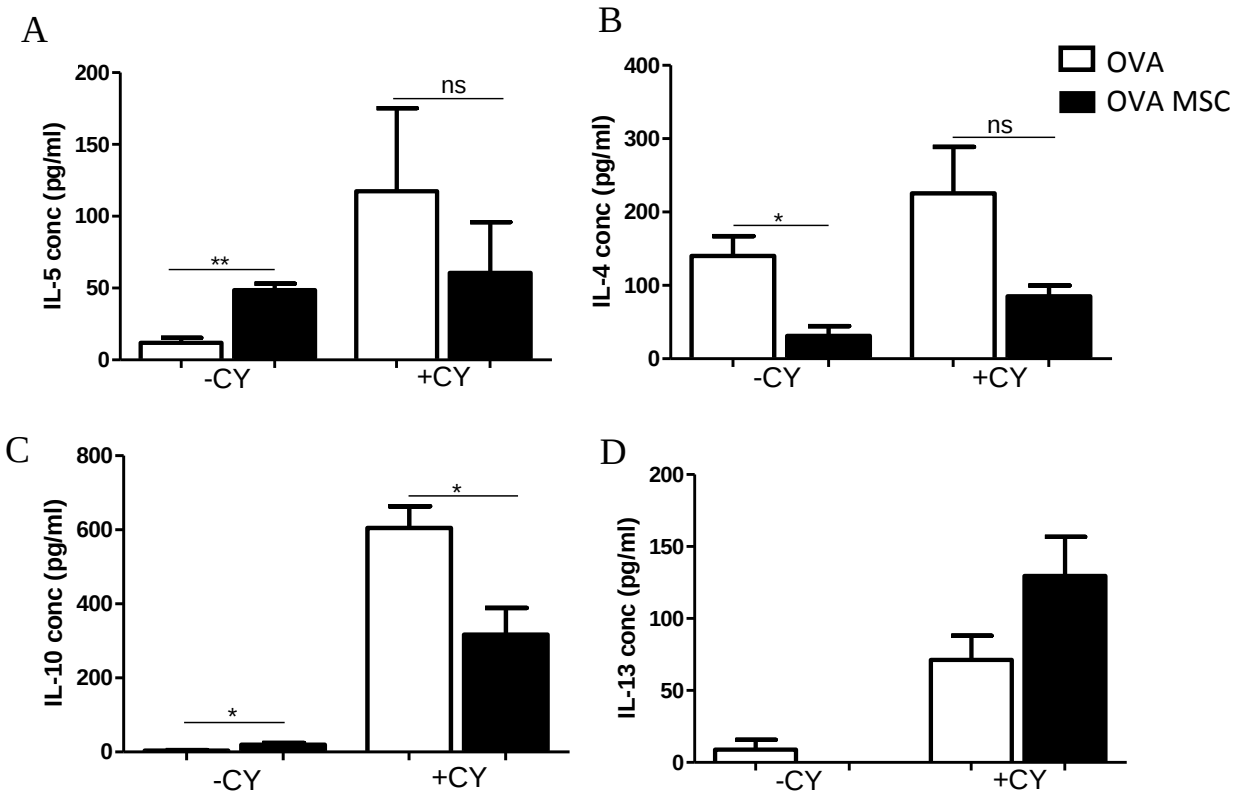


Figure 6.13 Depletion of T_{reg} resulted in a decrease in IL-10 production in sensitised, MSC-treated mice. Cytokine profile of BALF elicited by OVA sensitisation. Cytokine responses from similar cultures are shown for (A) IL-5, (B) IL-4, (C) IL-10 and (D) IL-13. Results are representative of responses determined independently from at least four mice per group and are expressed as means ± S.E.M. *p < 0.05.

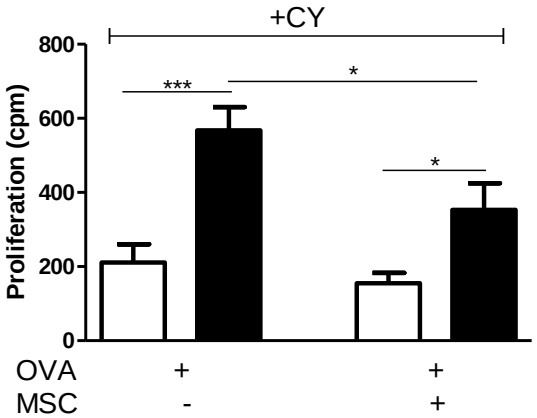


Figure 6.14 T_{reg} depletion impairs the inhibitory effect of MSC on T cell proliferation. Cells were cultured in the presence of media alone (☐) or OVA (200 µg/ml) (■) for 72 h. Subsequent proliferation was measured by 3H-thymidine incorporation (*, p<0.05). The data presented are representative at least four animals per group and are expressed as means ± S.E.M. *p < 0.05.

6.9 MSC DIRECTLY ALTER EXPRESSION OF ADHESION FACTORS ON EOSINOPHILS

In Section 6.8, MSC were shown to have a T_{reg}-independent effect on eosinophil recruitment to the airways. Despite a significant increase in IL-5, a reduction in airway eosinophilia was observed in mice receiving MSC in the absence of T_{reg} (Fig. 6.12). Therefore the ability of MSC to directly affect the function of eosinophils upon entering the airways was investigated. In this study, the functional effects of MSC on eosinophils were investigated *in vitro* in terms of modulating the chemotactic migration and expression of adhesion molecules, including intracellular adhesion molecule (ICAM-1), ICAM-3, L-selectin and leukocyte function-associated antigen-1 (CD11a/CD18).

Here, human MSC (hMSC) were used to investigate the putative direct affect on eosinophil function, as reagents for murine eosinophilia were not available. Human eosinophils (cell line EOL-1) were cultured in the presence of hMSC for 48h. This resulted in an inhibition of eosinophil expression of the adhesion molecules ICAM-1, ICAM-3, CD18 and L-selectin (Fig. 6.15 & 6.16). The experiment was carried out in a dose-dependent manner where four ratios of eosinophil to hMSC were used; 10:1, 5:1, 3:1 and 1:1. The percentage of ICAM-1 expressing human eosinophils decreased from 99.6% to 1%, and the mean fluorescent intensity (MFI) of the positive population decreased (59% to 17%) (Fig. 6.15 B). Likewise, the addition of hMSC strongly inhibited the expression of ICAM-3 and CD18 on eosinophils (Fig. 6.15 D & 6.16 B). A lesser but similar effect on L-selectin expression was observed, (Fig. 6.16 D). Inhibition of surface marker expression was only evident at high (1:1 eosinophil:MSC) ratios. Although this seemed non-physiologically relevant, the number of eosinophils in BALF following OVA sensitisation and challenge was approximately 0.5×10^5/lung. Since mice each received 0.5×10^6 MSC, the potential for MSC to come into contact with eosinophils in the lung is not impossible, however a soluble

effector seemed a more likely cause. As a consequence of these data, the ability of MSC to affect chemotactic migration of eosinophils *in vitro* was investigated.

6.10 MSC PREVENT EOTAXIN AND RANTES –INDUCED CHEMOTAXIS OF EOSINOPHILS

The potential of hMSC to regulate soluble factor-induced chemotaxis of eosinophils was investigated. Eotaxin has been implicated in many manifestations of allergic inflammation including airway eosinophilic infiltration (Conroy, 1997). Similarly, RANTES is a potent chemoattractant and activation factor for eosinophils (Russell, 1994). Eotaxin and RANTES-induced chemotaxis was measured using Transwell chamber plates, as described in Chapter 2.30. Human eosinophils (cell line, EOL-1) (5×10^4) were cultured with or without allogeneic hMSC (5×10^4) for 48 h. Human $CD4^+$ T cells were also included as an additional group where they were co-cultured with MSC and/or eosinophils, separated by a Transwell membrane, to investigate whether T cells were required for MSC to exert their inhibitory effects on eosinophil chemotaxis. Non-adherent eosinophils were aspirated from the wells leaving adherent MSC on the bottom. As expected, when compared with control groups (vehicle only), both eotaxin and RANTES induced eosinophil migration (Fig. 6.17 A & B). However, pre-incubation with MSC prevented migration of eosinophils in response to both chemokines. This effect was not seen when eosinophils were pre-incubated with $CD4^+$ T cells alone. Thus MSC blocking of eosinophil chemotaxis does not require $CD4^+$ cells.

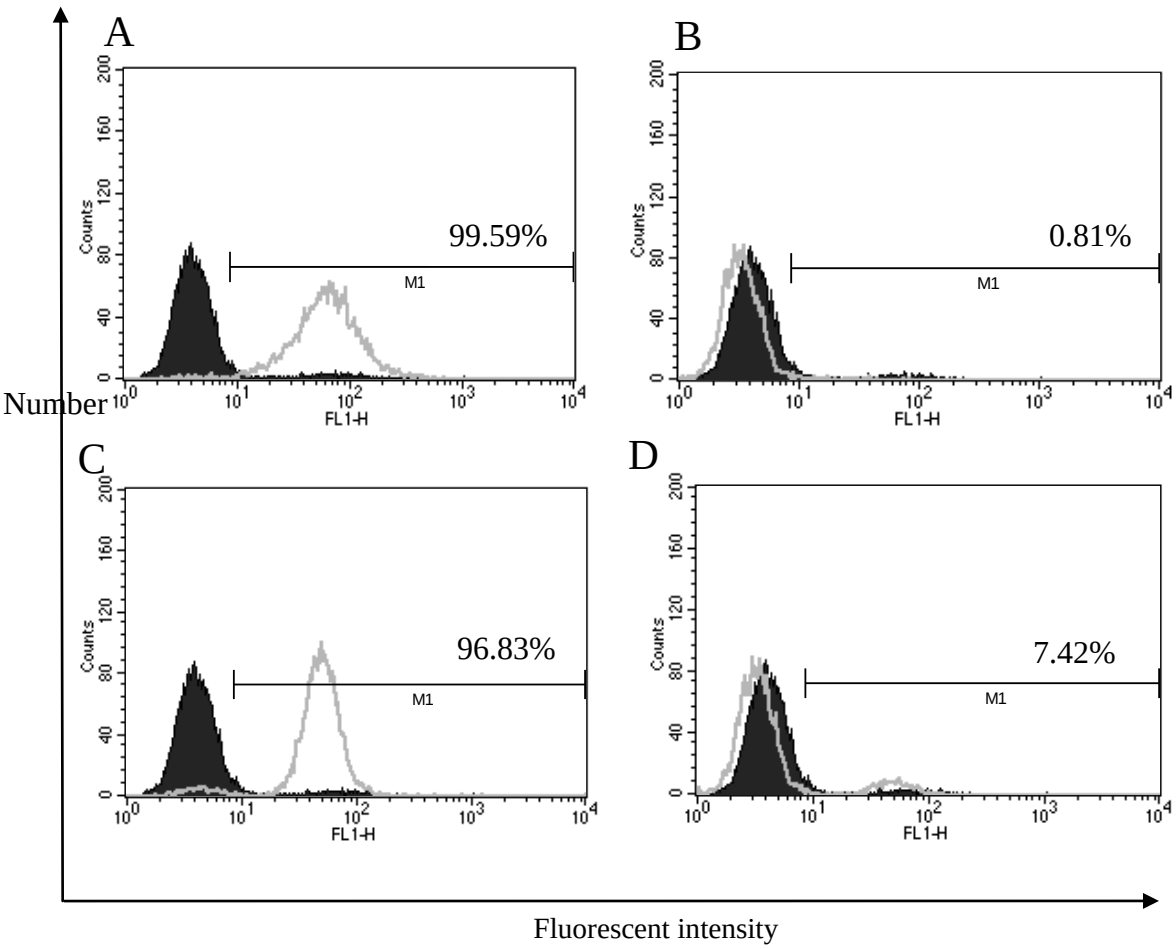


Figure 6.15 MSC directly inhibit the expression of ICAM-1 and ICAM-3 on eosinophils. Surface expression of (A&B) ICAM-1, (C&D) ICAM-3 on eosinophils (cell line, EOL-1) was determined by flow cytometry following co-incubation with hMSC (1:1) for 48 h. Non-adherent eosinophils were aspirated from the wells leaving adherent MSC. Expression was reduced when cells had been cultured in the presence of MSC (B) and (D). Plots are representative of experiments performed in triplicate. M line indicates the percentage of cells within that population.

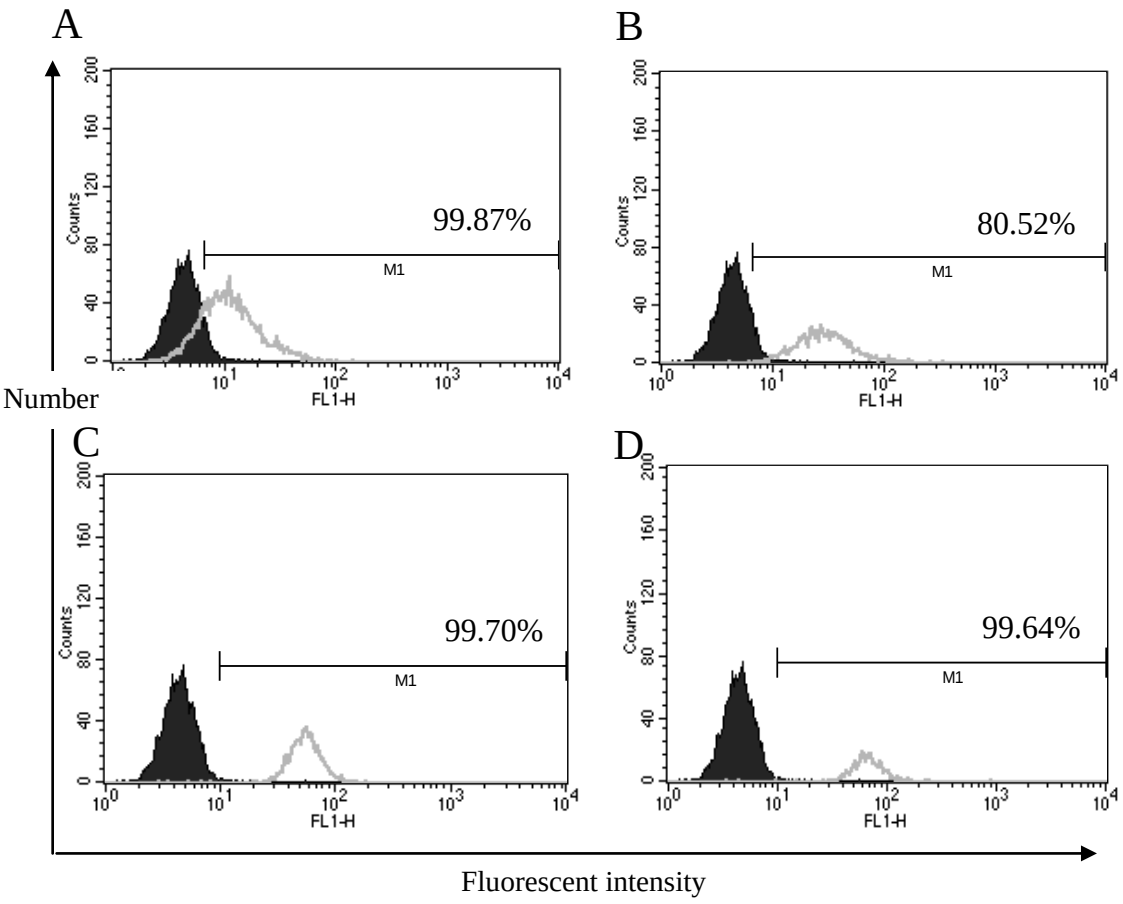


Figure 6.16 MSC reduce the expression of CD18 and L-selectin on eosinophils. Surface expression of (A&B) CD18, (C&D) L-selectin on eosinophils was determined by flow cytometry following co-incubation with hMSC (1:1) for 48 h. Expression was reduced when cells were cultured in the presence of MSC (B) and (D). Plots are representative of experiments performed in triplicate. M line indicates the percentage of positive cells within that population.

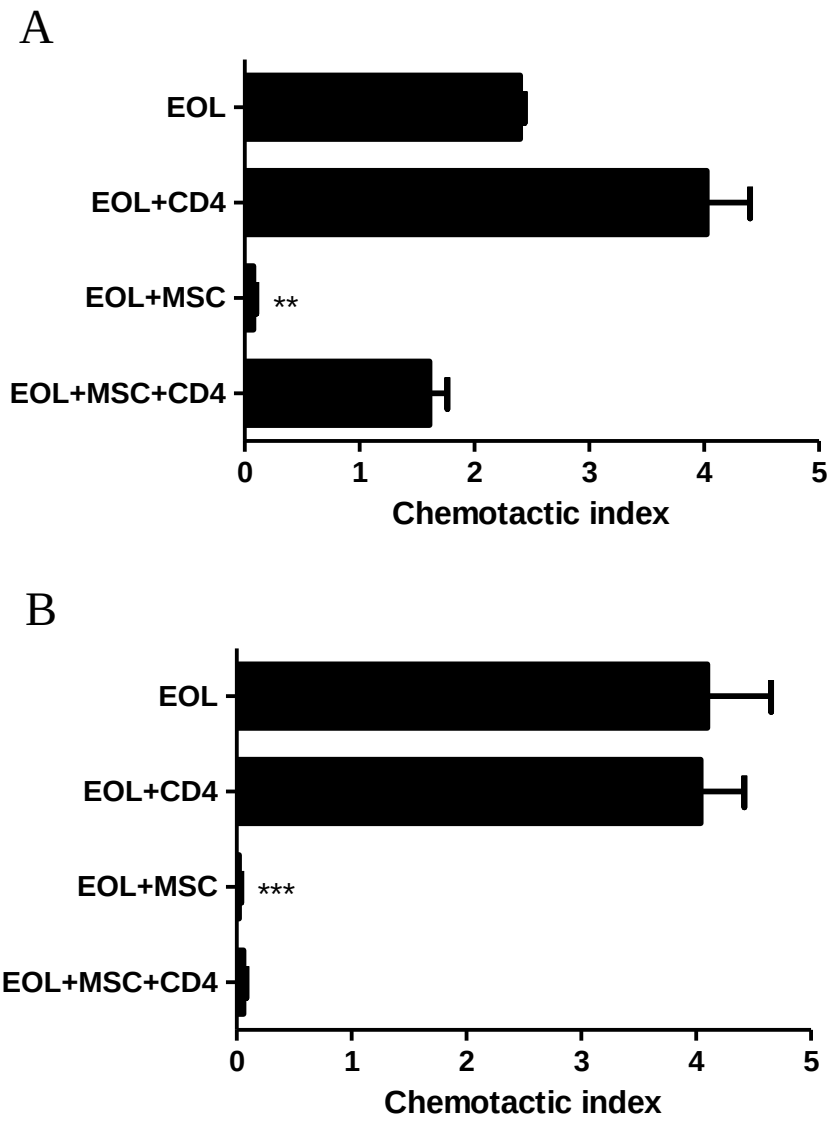


Figure 6.17 The effect of MSC on eosinophil chemotactic activity. Human eosinophils (5 x 10^4) (cell line, EOL-1) were pre-incubated with hMSC (1:1) for 48 h before isolation and addition of eosinophils to chemotaxis chambers. Chemotactic migration in response to (A) RANTES (100 µM) and (B) eotaxin (100 µM) is shown above. Data are expressed as a chemotactic index, calculated as the number of cells migrating in response to stimulus divided by the number of cells migrating in response to medium.

6.11 SUMMARY

Here the mechanism of action of an MSC-mediated cell therapy was determined. MSC cell therapy leads to the generation of peripheral $CD8^{+}\gamma/\delta$ and CD^{+}FoxP3 T_{reg}. Several mechanisms have been proposed for MSC-elicited immunosuppression including immune deviation, induction of anergy and suppression by T_{reg} (Maccario, 2005; English, 2008). In an experimental asthma model, where mice are devoid of naturally occurring T_{reg}, delivery of MSC fail to influence the development of allergic airway pathology and class switching to IgE. As neither eosinophilia nor other MSC functions are impaired in this model, the MSC effect can be attributed to T_{reg} induction. In summary, the work presented here developed models to address the three hypotheses defined at the beginning of this chapter and explained the protective effect seen in this model. Furthermore, the experiments herein identified a novel influence of human MSC on eosinophil function which although not wholly responsible for the cell therapeutic effect described in Chapter 5, nevertheless merits future exploration.

The capacity to therapeutically manipulate the immune response to allergens in order to restore or regulate normal immunity was examined further in subsequent chapters. Mesenchymal stem cells (MSC) are a heterogeneous subset of stromal stem cells that possess potent anti-proliferative and anti-inflammatory effects (Aggarwal, 2005; Barry, 2005; Uccelli, 2006; Comoli, 2008), data which support their possible use as a therapy for immune-mediated diseases. MSC can exert profound immunosuppressive effects via modulation of both cellular and innate immune pathways (Krampera, 2006). The present study addressed the hypothesis that adult bone marrow derived-MSC would prevent the pathology associated with allergen-driven airway inflammation. In Chapter 4, functional *in vitro* assays were carried out in order to address quality-control issues linked to systemic delivery of MSC *in vivo*. The capacity to actively inhibit immune responses is a characteristic feature of MSC. Data in Chapter 4

demonstrated the ability of MSC to inhibit allo-proliferation irrespective of the donor haplotype. This was important as functionally immunosuppressive MSC were central to the hypothesis that the pathology associated with allergen-driven airway inflammation could be prevented by cell therapy.

Several studies have provided evidence in support of the plasticity and immunomodulatory properties of MSC, as well as their ability to be recruited to sites of injury, where they can contribute to tissue repair. Selective localisation of systemically delivered MSC has been demonstrated in models of kidney failure (Ezquer, 2008), myocardial infarction (Quevedo, 2009), and neurological injury (Bouchez, 2008). Extensive *in vitro* studies have demonstrated that, unlike other stem cell types, MSC can not only evade alloreactivity, but also mediate active suppression of immune responses (Bartholomew, 2004; Ryan, 2007; English, 2008). Preclinical models of autoimmune/inflammatory conditions such as transplant rejection (Le Blanc, 2004), rheumatoid arthritis (Augello, 2007), and multiple sclerosis (Constantin, 2009), have demonstrated a functional role for MSC. Several studies have also demonstrated a functional protective effect of MSC in models of acute lung injury, including pulmonary fibrosis (Ortiz, 2003; Xu, 2007) and very recently allergic rhinitis (Cho, 2009). However, little was known about the mechanism of MSC-mediated immunomodulation and their clinical relevance in a model of allergic airway inflammation. Chapter 5 specifically addressed the hypothesis that adult bone marrow derived-MSC would prevent the pathology associated with allergen-driven airway inflammation. This was tested using a combination of the OVA-driven model of inflammation utilised in Chapter 3 and a cell therapy based on allogeneic MSC. This study demonstrated that adult bone marrow derived allogeneic MSC actively prevent the induction of allergen driven pathology in a murine model and suggests a novel cell therapy for allergic human disease. Systemic administration of MSC protected the airways from OVA-induced pathology, airway

eosinophilia and broader cellular inflammation. This effect was only evident upon delivery of live, but not fixed cells. A consistent feature of asthma is the production of excess mucus, causing blockage of the peripheral airways (Young, 2006). MSC delivery reduced mucus metaplasia in allergen-sensitised mice, consistent with the findings above. In contrast, PFA-fixed MSC exacerbated goblet cell hyperplasia and mucus secretion in OVA-sensitised mice airway pathology and OVA-specific IgE.

Previous studies have observed an increase in the efficacy of MSC immunosuppression *in vivo* when they are activated prior to delivery, a process termed "licensing" (Ryan, 2007; Polchert, 2008). This phenomenon was also investigated in Chapter 4 by examining the effect of exogenous IFN-γ on MSC-mediated immunosuppression *in vitro*. Stimulated MSC enhanced the inhibitory effect seen previously with un-stimulated MSC in both mitogen-driven and allo-driven reactions (Fig. 4.8). Furthermore, the inability of MSC isolated from IFN-γ-receptor knockout mice to suppress T-cell proliferation in a two-way mixed lymphocyte reaction demonstrated the importance of this cytokine in conferring immunosuppressive function to MSC (Fig, 4.9). These data verified the results of others (Ryan, 2007; Polchert, 2008) by demonstrating the enhancing effect of pre-stimulated MSC and indicated the importance of the inclusion of these licensed cells in further studies. In Chapter 5, prior stimulation of MSC with IFN-γ increased their protective effect. This was most evident in the histopathological analysis of the lung tissues where mucus hypersecretion and peribronchial inflammation was significantly reduced. These data suggest that IFN-γ stimulation can enhance the efficacy of live MSC in modulating the immune response in allergic airway inflammation. However, the reduction in pathology in those sensitised groups receiving non-stimulated MSC indicated that prior activation is not essential. Interestingly, the inhibitory effect of IFN-γ triggered by BPZE1 immunisation in Chapter 3 supports the

importance of this key cytokine in suppression of the allergic inflammatory response through either direct or indirect interactions.

The phenomena described above were interesting but could be explained by MSC-mediated global, non-specific immune suppression or by MSC interference in immune induction. Therefore, the effect of allogeneic MSC on T cell mediated immunity was examined in this model. Particular attention was given to IL-4, IL-5 and IL-13 induction as these play well defined roles in allergen driven pathology as evidenced by data from Chapter 3. OVA-sensitised mice exhibited a typical increase in BALF IL-5. However, levels of IL-5 were significantly increased in the sensitised, MSC-treated group in BALF when compared to OVA alone. This was surprising given the considerable decrease in eosinophilic lung infiltration and improvement in airway pathology (Fig. 5.3 & 5.6). The first conclusion from this was that MSC did not mediate global suppression or suppress antigen specific immune induction. The typical damaging effects of IL-5 in acute lung inflammation may have been countered in this instance by the significant increase in BALF IL-10 and also the marked reduction in IL-4. Several studies have demonstrated a role for IL-10 in modulating the allergic immune response (Prigione, 2009). IL-10 can inhibit critical steps in the initiation of the clinical manifestations of allergic airway inflammation, namely IgE dependent activation of mast cells and survival of eosinophils and can regulate the production of eosinophilotactic cytokines (Li, 2010). However, it has also been shown to play a direct regulatory role in controlling the accumulation of excessive eosinophils at inflammatory sites by accelerating their apoptosis (Takanaski, 1994). Furthermore, IL-10 can inhibit CCR5 (Patterson, 1999) on T cells, macrophages and dendritic cells, whose ligand is, amongst others, RANTES, and is a major eosinophil chemoattractant in the asthmatic lung (Venge, 1994). Despite reducing circulating and sputum eosinophil numbers, systemic administration of an anti-IL-5 antibody only partially reduces airway tissue eosinophils in asthmatic patients (Flood-Page, 2003).

This incomplete response may be because eosinophils lose their IL-5 receptor from the cell surface as they enter the airways from circulation, and, as a result, their dependence on IL-5 for survival (Liu, 2002). Therefore, in spite of the increase in IL-5 observed in MSC-treated group which may have initiated differentiation and migration of eosinophils, according to previous reports, this alone is not be sufficient to mobilise their accumulation in the lung. IL-4 plays an important role in sustaining established IgE responses (Del Prete, 1988); therefore a decrease in its secretion can lead to a reduction in IgE synthesis. IL-4 is required for adherence and activation of eosinophils in the lung and since IL-4 was significantly reduced in MSC-treated mice, this may have prevented the otherwise damaging pathology associated with OVA-driven inflammation.

Chapter 5 demonstrated that adult bone marrow-derived allogeneic MSC actively prevent the induction of allergen driven pathology in a murine model and suggested a novel cell therapy for allergic human disease and prompted further investigation of the mechanism/s employed by MSC in suppressing the allergen-driven response. MSC may exploit one, or a combination of, functionally distinct pathways to inhibit allergic airway inflammation, including (1) repair of damaged tissue, (2) induction of regulatory T cells or (3) immunomodulation via cytokine induction/suppression directly affecting immune cell infiltration. The third hypothesis is not consistent with the elevated IL-5 seen (Fig. 5.11 C) and therefore cannot pertain and so will not be discussed further. The possibility of a repair mechanism employed by MSC is not viable as the sole contributor to the MSC-mediated protective effect in this model since no inhibition of allergic airway pathology was demonstrated upon depletion of regulatory T cells in Chapter 6. .

The role of regulatory cells in MSC-mediated immunosuppression has been suggested by the expansion of $CD4^{+}CD25^{+}$ cells *in vitro* when cultured with MSC (Maccario, 2005; Prevosto, 2007; English, 2008; Wang, 2009). This created a compelling case for further

pursuing MSC as cellular tolerogens. First, it was necessary to confirm that MSC could feasibly mediate the expansion or proliferation of T_{reg} subsets that are important in regulating the allergic airway response. Several regulatory T cell subsets exist, including (1) $CD4^+CD25^+$, (2) $CD8^+CD28^-$, (3) $CD8^+TCR\gamma/\delta^+$, (4) $CD4^-CD8^-TCR\alpha/\beta^+$. There is evidence that the numbers or functions of these subsets are deficient in patients with atopic allergic disease (Grindbacke, 2004; Ling, 2004; Isogai, 2007). MSC induced $CD8^+TCR\gamma/\delta$ cells in the lung and spleen. $CD8^+$ T cells play important roles, either as effector or regulatory T cells, in several immunopathologic manifestations such as autoimmune diseases (Najafian, 2003; Brimnes, 2005), transplantation (Kato, 2010) and protection of the host against infectious diseases and cancer (Palmer, 2004; Nakamura, 2007; Gil, 2009). $CD8^+$ regulatory cells act through the production of suppressor cytokines (TGF-β or IL-10) or by inactivating dendritic cells (Rich, 1995; Gilliet, 2002). A growing body of evidence now exists suggesting that $CD8^+\gamma/\delta$ T cells are influential in $CD8^+$ T cell-mediated regulation of airway inflammation. A deficiency in this distinct subset is associated with a reduction in inflammation within the lungs (Lahn, 1999). Recent studies have also shown that γ/δ $CD8^+$ T cells adoptively transferred into sensitised rats reduced airway eosinophilia and hyperresponsiveness (Isogai, 2003; Isogai, 2007). The considerable increase in this distinct T cell subset upon MSC delivery is a novel finding and could, in part, explain the improvement in allergic airway pathology. However, as no induction of this distinct $CD8^+$ population was observed in OVA-sensitised mice, it could not be established that they were specifically depleted during CY treatment. Nevertheless, as CY can target proliferating T cells, it could be predicted that γ/δ $CD8^+$ T_{reg} were indeed inhibited following MSC delivery, thus suggesting their important protective effect in this model.

$CD4^+CD25^+$FoxP3-expressing T_{reg} suppress effector T cells both directly and via interactions with antigen-presenting cells, such as dendritic cells (Oderup, 2006). Recently,

using a FoxP3-specific conditional knockout of the IL-10 allele, it was demonstrated that IL-10 production by T_{reg} was required to control immune responses in the lung (Rubtsov, 2008). The current study revealed an increase in $CD4^{+}FoxP3^{+}$cells in the lungs of MSC-treated mice, prompting further investigation of these phenomena. T_{reg} were depleted using cyclophosphamide in the ovalbumin-induced model of allergic airway inflammation, and the effect of MSC delivery on airway pathology was examined. It was decided not to include a CY-treated, IFN-γ-stimulated MSC group here for clarity. There are limitations to the use cyclophosphamide over denileukin diftitox or an anti-CD25 monoclonal antibody to deplete T_{reg}. A monoclonal anti-CD25 antibody has been routinely used for depleting T_{reg} *in vivo* (McHugh, 2002; Van Meirvenne, 2005; Boudousquie, 2009). However, due to the expression of CD25 on activated effector T cells, they are thus potential targets for depletion by anti-CD25 mAB also. CY is widely used as an immunosuppressive drug and is reported to down-regulate T-cell derived IL-10 and TGF-β (Matar, 2001) and affect the functionality of T_{reg} (Lutziak, 2005). It has been used in studies to examine the effect of T_{reg} depletion in various disease models (Aschan, 1999; Itescu, 2002; Ghiringhelli, 2004).

No significant differences in pathology were observed in MSC-treated mice depleted of T_{reg} when compared with those sensitised with OVA alone. Likewise, the reduction in OVA-specific IgE seen previously with MSC-treatment was not observed here, suggesting the role of suppressor cells in MSC-mediated immunosuppression in this model. In spite of the inhibition of T_{reg}, a significant reduction in airway eosinophilia persisted in those treated with MSC. This is an important finding as it eliminates an eosinophil mediated effect on the MSC suppression of pathology. Thus these data suggest the existence of multiple mechanisms where the combination of T-cell-dependent and modes of protection are employed. Figure 6.18 outlines the putative mechanism involved in MSC-mediated

protection in allergic airway inflammation and illustrates the interplay between multiple mechanisms.

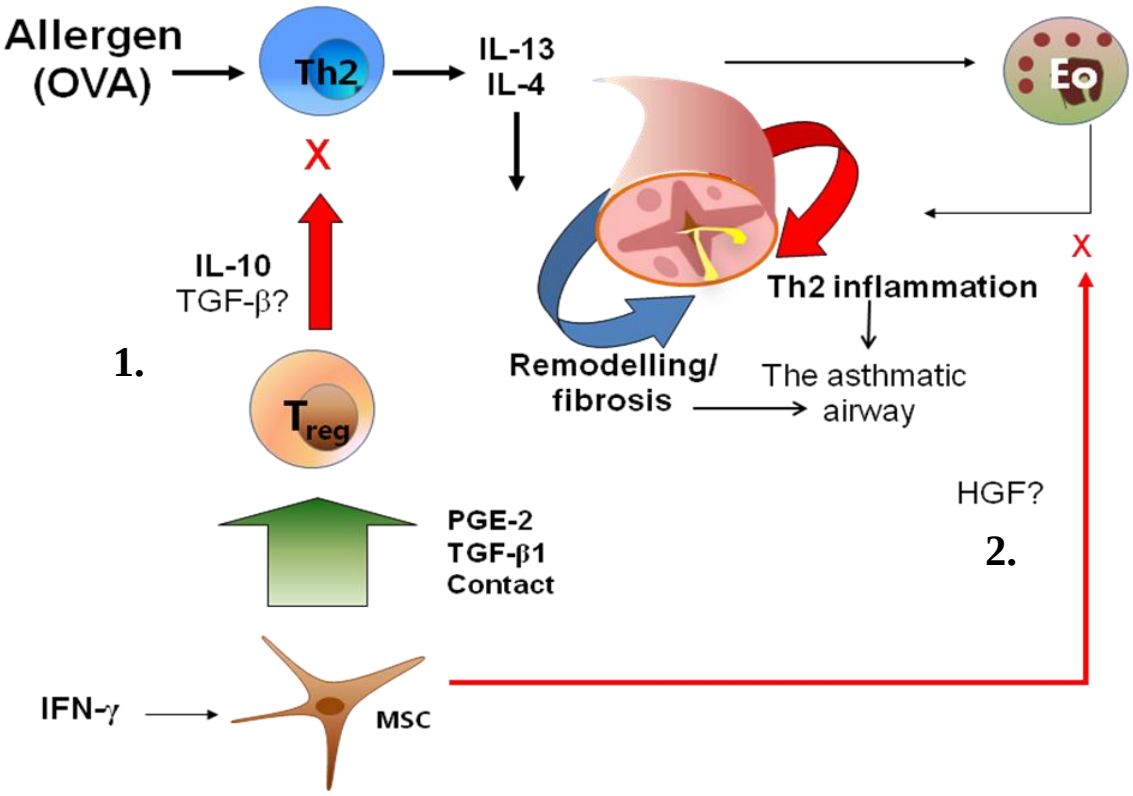


Figure 6.18 Schematic presentation of plausible mechanisms by which MSC regulates the allergen-driven response. (1) MSC can secrete a variety of soluble factors including TGF-β and PGE-2. Through a soluble factor or contact-dependent mechanism, MSC initiates the expansion of regulatory T-cells directly or indirectly via IL-10 secretion with a subsequent inhibition of the otherwise damaging effect of allergen sensitisation and respiratory challenge. (2) MSC directly inhibits airway eosinophilia through the secretion of HGF which can exerts a direct regulatory role on the function of eosinophils by inhibiting their chemotaxis.

This chapter also revealed a T_{reg} independent MSC-mediated effect on eosinophils that was not sufficient to alleviate airway pathology. In light of the reduction in airway eosinophilia in the presence of higher IL-5 secretion in MSC-treated mice, the possibility that MSC were exerting direct inhibitory effects on eosinophils and thus preventing their transmigration into the lung was investigated. The critical observation here was that MSC can efficiently inhibit or down-regulate key adhesion molecules on eosinophils, hindering their chemotactic ability (Fig. 6.17). Co-culture of T-cells with MSC and eosinophils was not essential for this to occur; demonstrating that indirect stimulation of T cells by MSC was not essential (e.g. IL-5, IL-4 are not involved). These data provide an explanation as to why eosinophilia could be reduced when IL-5 was elevated. Eosinophils express the integrin adhesion molecules CD11/18 complex which interacts with intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), respectively (Burke-Gaffney, 1998). However in Chapter 6 it was shown that human MSC interfered with expression of these adhesion molecules on human eosinophils (Fig. 6.16). Hepatocyte-growth factor (HGF) is a homeostatic mediator associated with the direct attenuation of allergic airway inflammation (Okunishi, 2009). It has recently been shown to exert a direct regulatory role on the function of eosinophils by inhibiting their chemotaxis (Ito, 2007). Since HGF is one of a variety of cytokines secreted by MSC (Ryan, 2007) it is reasonable to suggest that it plays a role here. Interestingly, co-culture with IFN-γ significantly increases the expression of these suppressive mediators (Ryan, 2007) supporting a role for IFN-γ in potentiating the immunosuppressive activities of MSC. HGF was significantly higher in conditioned media of pre-stimulated MSC when compared with MSC with no prior activation. The effect of IFN-γ in this model may therefore be related to its' ability to stimulate an increase in the production of HGF (Ryan, 2007). This may explain why the reduction in airway pathology was more evident in those sensitised mice receiving stimulated MSC when compared to those

treated with MSC in the absence of prior activation. This novel finding provides further insights into the mechanisms of MSC-mediated immune modulation *in vivo* and indicates that viable allogeneic MSC could be a therapeutic modality against allergen driven airway disease. It also suggests a role for IFN-γ- stimulated MSC whereby their efficacy is enhanced for use in a range of therapeutic treatments in human clinical trials.

The immunological properties associated with MSC would appear to target critical pathogenic features for the development of allergic asthma. A combination of T cell dysregulation, as well as the presence of inflammatory cytokine milieu in a coordinated fashion, leads to this failure to maintain tolerance to innocuous environmental allergens. MSC possess specific immunomodulatory properties that appear capable of protecting against this airway hyperresponsiveness through immune modulation and T_{reg} induction. Here, the immunoregulatory properties of MSC interfered effectively with the immune hyperresponsiveness in the course of allergen sensitisation, inhibiting the otherwise damaging effect of allergen sensitisation and respiratory challenge. The protective effect as a result of MSC therapy may have more far-reaching clinical implications in humans. For example, in atopic individuals with a genetic susceptibility for asthma or in patients who are unresponsive to current therapies, the use of stem cells may have more influential and sustained effects.

CHAPTER 7

DISCUSSION

Asthma is a chronic inflammatory disorder of the airways whose prevalence is steadily increasing in developed societies (Galli, 2008). New and improved anti-inflammatory drugs, particularly inhaled corticosteroids have improved the quality of life of patients, however, even the long-term usage of pharmacotherapies does not appear to induce fundamental changes in the immune responsiveness or alter the natural history of the disease (Bisgaard, 2006; Guilbert, 2006). Therefore, the need to develop strategies to permanently reverse immunologic reactivity and chronic airway inflammation has become recognised. Th2 cytokines, particularly IL-4, IL-5 and IL-13 initiate and potentiate the immune response in asthmatics (Kay, 2001). In allergen specific immunotherapy, it was first believed that Th1 cells such as IFN-γ played a beneficial role by down-regulating the Th2 response (Romagnani, 2004). However, further investigation has demonstrated that allergic airway inflammation is not simply a balance between Th1 and Th2 responses (Hansen, 1999). Regulatory T cells have emerged as key players in controlling the development of inflammation (Akbari, 2002; Kearly, 2008) and an improved understanding of these cells has led to the emergence of new therapeutic modalities that may provide a more long-term inhibition of immune hyperresponsiveness (Akdis, 2001; Jutel, 2003). This study examined therapeutic approaches to immune modulation, including the use of modified respiratory pathogens and stem cells, to define the mechanisms of action and to explore new ways to beneficially manipulate the immune response to aeroallergens.

Asthma is a multi-factorial disease which encompasses numerous manifestations including inflammation, repair and remodelling. Intriguingly, in both systems studied in this thesis, remodelling seemed to be more central to pathology than atopic inflammation. The observation that allergen-specific IgE was not significantly changed whilst pathology was reduced in mice immunised with BPZE1 demonstrates that the protective effect of immune modulation seen here had its greatest impact on mucus secretion and remodelling rather than

a reduction in IgE. Similarly, in the T_{reg}-depleted OVA model, a reduction in airway eosinophilia inhibition as a result of MSC therapy was not sufficient to modulate pathology. Such a finding is also consistent with human clinical studies in which reduced airway eosinophilia was achieved but this had little impact on pathology (Flood-Page, 2007). The implication of an alternative mechanism of action is that the biological therapies explored in this thesis could target complex multi-factorial diseases that involve both fibrotic and inflammatory processes or T_{reg} dependent and independent aspects, for example chronic GvHD. It may also provide novel approaches to treat currently intractable conditions where fibrosis is a major component such as scleroderma (Abraham, 2005) and idiopathic pulmonary fibrosis.

Many questions remain unanswered with regard to a biological therapy based on bacterial immunomodulation. For example, studies examining the protective role of mycobacteria and their components (e.g. Bacillus–Calmette–Guerin (BCG) immunisation) against the development of allergic diseases have demonstrated conflicting results (Krishna, 2002). Animal and experimental models have shown that exposure to Mycobacteria or mycobacterial proteins leads to a reduction in various allergic manifestations and although there are several epidemiological studies in support of this, they have not always been consistent. Nevertheless, intriguing evidence suggests that microbial immunomodulation does occur. Vaccination with whole-cell pertussis vaccine has been reported to protect against *B. pertussis* exacerbation of OVA-induced airway hyperresponsiveness in a murine model of allergic airway inflammation (Ennis, 2005b), however much less is known about protective effects of childhood vaccines against atopy. It might be that the key beneficial feature of BPZE1 is the combination of a Th1 skewed response, combined with the absence of induced airway pathology. This combined benefit makes BPZE1 an attractive candidate as a neonatal vaccine against whooping cough.

MSC also pose questions for cell therapy, however this has not impeded the more than 100 reported clinical trials currently using these cells. Foremost amongst these questions concerns the mechanism of therapeutic action. MSC are proposed to have beneficial function in numerous conditions, however it is rarely clear how the cells are functioning to achieve the benefit observed. This study is one of very few to actually demonstrate how MSC function *in vivo*. It is interesting to note that therapeutic improvement was linked not to a regenerative capacity (i.e. MSC did not seem to persist and differentiate into lung tissue) but rather it was through T_{reg} induction. Thus, the original claims by Caplan (Caplan 2006) and Phinney (Phinney 2007) that trophic influences are the major therapeutic mode of action is borne out by this study. A second problem arises from this lack of understanding; if the mode of action is not characterised, clinical release criteria and decisions around expansion and manufacturing quality cannot be based on rational or measurable indices (linked to efficacy) but rather these become a mere quality assurance exercise linked to whatever parameters are measurable. Therefore, it is not surprising that the recent Osiris trial using MSC (Prochymal III) against acute graft v Host disease was a failure (or partial in benefit). The cells used in that study were well characterised with regard to surface markers and differentiation capacity, but as the correlates of efficacy were unknown, these criteria were largely irrelevant. The approach adopted in this study, to examine and define the mechanism of action, is an essential step in the rational introduction of new stem cell therapies. In the absence of this approach, clinical trials will continue to encounter the failures seen with Prochymal.

The future for BPZE1 is promising, and the vaccine commences phase I human clinical trial in late 2010. Likewise MSC show a remarkable ability to improve currently intractable diseases, at least in model systems. The next key step for both approaches will rely on definition of their activities in scientific studies. Many asthmatics are refractory to

single or combined chemotherapies targeting single or at best a small number of inflammatory mediators. This work has shown that "biological" approaches based on cell therapies or attenuated microbes are effective modulators of the allergic response, providing a proof of principle in support for the eventual translation of novel biological cell therapies to humans.

CHAPTER 8

REFERENCES

ABRAHAM DJ, VARGA J. (2005) Scleroderma: from cell and molecular mechanisms to disease models. *Trends Immunol* 26:587-95.

AGGARWAL S. P. M. (2005). Human mesenchymal stem cells modulate allogeneic immune cell responses. *Blood,* 105: 1815-1822.

AHMED R G. D. (1996). Immunological memory and protective immunity: understanding their relation. *Science,* 272 (5258): 54-60.

AKBARI O. F. G., MEYER E. H. (2002). Antigen-specific regulatory T cells develop via the ICOS-ICOS-ligand pathway and inhibit allergen-induced airway hyperreactivity. *Nature Medicine* 8: 1024-1032.

AKDIS C. A. (2001). Bypassing IgE and targeting T cells for specific immunotherapy of allergy. *Trends Immunol,* 22: 175-178.

AKDIS C.A., AKDIS M, WUTHRICH B, BLASER K. (1998). Role of interleukin-10 in specific immunotherapy. *J Clin Invest,* 102: 98-106.

AKDIS M. (2006). Healthy immune responses to allergens: T regulatory cells and more. *Curr Opin Immuno,l* 18: 738-744.

ANDERSON G. (2002). The immunobiology of early asthma. *Med J Aust,* 177: S47-S49.

ANDREASEN C. N. (2008). Pertussis toxin inhibits early chemokine production to delay neutrophil recruitment in response to Bordetella pertussis respiratory tract infection in mice. *Infection and Immunity,* 76: 5139-5148.

ASCHAN J. C. S., HAGGLUND H, KLAESSON S, MATTSON J, REMBERGER M. (1999). Improved survival after bone-marrow transplantation for early leukaemia using busulfan-cyclophosphamide and individualized prophylaxis against graft-versus-host disease: a long term follow-up. *Clinical Transplantation,* 13: 512-519.

AUGELLO A., R. TASSO, ET AL. (2005). Bone marrow mesenchymal progenitor cells inhibit lymphocyte proliferation by activation of the programmed death 1 pathway. *Eur J Immunol,* 35(5): 1482-90.

AUGELLO A., NEGRINI S. M., CANCEDDA R, PENNESI G (2007). Cell therapy using allogeneic bone marrow mesenchymal stem cells prevents tisue damage in collagen-induced arthritis. *Arthritis Rheum,* 56(4): 1175-1186.

AUGELLO T. R., NEGRINI S. M., CANCEDDA R, PENNESI G (2007). Cell therapy using allogeneic bone marrow mesenchymal stem cells prevents tissue damage in collagen-induced arthritis. *Arthritis Rheum,* 56(4): 1175-1186.

BABU M. (2001). Virulence factors of *Bordetella pertussis*. *Current Science,* 80(12): 151201522.

BADDOO, M., K. HILL, ET AL. (2003). Characterization of mesenchymal stem cells isolated from murine bone marrow by negative selection. *J Cell Biochem,* 89(6): 1235-49.

BADDOO M, HILL K., WILKINSON R, GAUPP D, HUGHES C, KOPEN GC, PHINNEY DG (2003). Characterisation of mesenchymal stem cells isolated from murine bone marrow by negative selection. *J Cell Biochem,* 89: 1235-1249.

BALS R. (2004). Innate immunity in the lung: how epithelial cells fight against respiratory pathogens. *Eur Respir J,* 23: 327-333.

BARCZYK A, SOZANSKA E (2003). Interleukin-17 in sputum correlates with airway hyperresponsiveness to methacholine. *Respir Med,* 97: 726-733.

BARNARD A, MAHON B., WATKINS J, REDHEAD K, MILLS KHG (1996). Th1/Th2 cell dichotomy in acquired immunity to Bordetella pertussis: variables in the in vivo priming and in vitro cytokine detection techniques affect the classification of T cell subsets as Th1, Th2 or Th0. *Immunology,* 87: 372-380.

BARRY F.P., M. F., ENGLISH K (2005). Immunogenicity of adult mesenchymal stem cells: lessons from the fetal allograft. *Stem Cells Dev*, 14: 252-265.

BARTHOLOMEW A, SIATSKAS M (2004). Mesenchymal stem cells suppress lymphocyte proliferation in vitro and prolong skin graft survival in vivo. *Experimental Hematology*, 30: 42-48.

BAUMGARTH N, HERZENBERG LA (2005). Inherent specificities in natural antibodies: a key to immune defence against pathogen invasion. *Springer Semin Immun*, 26 (4): 347-362.

BENVENUTO F, GERDONI E, GUALANDI F, FRASSONI F, PISTOIA V, MANCARDI G, UCCELLI A (2007). Human mesenchymal stem cells promote survival of T cells in a quiescent state. *Stem Cells* 25: 1753-1760.

BETTELLI E K., KUCHROO V (2007). Th17: the third member of the effector T cell trilogy. *Curr Opin Immunol,* 19: 652-557.

BETTELLI E, OUKKA M, KUCHROP V (2008). Induction and effector functions of Th17 cells. *Nature,* 114: 1209-1217.

BEYTH, S., BOROVSKY, ET AL. (2005). Human mesenchymal stem cells alter antigen-presenting cell maturation and induce T-cell unresponsiveness. *Blood,* 105(5): 2214-9.

BISGAARD H, LOLAND L (2006). Intermittent inhaled corticosteroids in infants with episodic wheezing. *N Engl J Med,* 354: 1998-2005.

BLUESTONE JA.(2003). Natural versus adaptive regulatory T cells. *Nat Rev Immunol,* 3: 253-257.

BOUCHEZ G, VOURC'H P, GARREAU L, BODARD S, RICO A, GUILLOTEAU D, CHARBORD P, BESNARD JC, CHALON S (2008). Partial recovery dopaminergic pathway after graft of adult mesenchymal stem cells in a rat model of Parkinson's disease. *Neurochemistry International*, 52(7): 1332-1342.

BOUDOUSQUIE C, BARBIER N, SPERTINI F (2009). CD4+CD25+ T cell depletion impairs tolerance induction in a murine model of asthma. *Clinical and Experimental Allergy,* 39: 1415-1426.

BOYD AP, CONROY H, MAHON N, LAVELLE EC, MILLS KH (2005). Bordetella pertussis adenyate cyclase toxin modulates innate and adaptive immune responses: distinct roles for acylation and enzymatic activity in immunomodulation and cell death. *J Immunol* 175: 730-738.

BRIMNES J, DOTAN I, SHAO L, NAKAZAWA A, MAYER L (2005). Defects in CD8+ regulatory T cells in the lamina propria of patients with inflammatory bowel disease. *J Immunol,* 174(9): 5814-5822.

BRUSSELLE GG, TAVERNIER JH, VAN DER HAYDEN JG, CUVELIER CA (1994). Attenuation of allergic airway inflammation in IL-4 deficient mice. *Clinical Exp Allergy,* 24(1): 73-80.

BRYDER, D., J. ROSSI, ET AL. (2006). Hematopoietic stem cells: the paradigmatic tissue-specific stem cell. *Am J Pathol,* 169(2): 338-46.

BULLENS MA, COTEUR L, DILISSEN E, HELLINGS PW, DUPONT LJ, CEUPPENS JL (2006). IL-17 mRNA in sputum of asthmatic patients: linking T cell driven inflammation and granulocytic influx. *Respiratory Research,* 7(135): 1-9.

BURKE-GAFFNEY A, H. P. (1998). A CD18/ICAM-1-dependent pathway mediates eosinophil adhesion to human bronchial epithelial cells. *Am J Respir Cell Mol Biol,* 19: 408-418.

BURNET F (1968). Evolution of the immune process in vertebrates. *Nature,* 218: 426-430.

BURTON A, LAUTENBACH B, GACIC-DOBO M, NEILL M, KARIMOV R, WOLFSON L, JONES G, BIRMINGHAM M (2009). WHO and UNICEF estimates of national

infant immunisation coverage: methods and processes. *Bull World Health Organ,* 87: 535-541.

CAPLAN A, DENNIS J (2006). Mesenchymal stem cells as trophic mediators. *J Cell Biochem*, 98: 1076-1084.

CARBONETTI N, ANDREASEN C, BUSHER N (2005). Pertussis toxin and adenylate cyclase toxin provide a one-two punch for establishment of Bordetella pertussis infection of the respiratory tract. *Infection and Immunity,* 73: 2698-2703.

CARBONETTI N, ANDREASEN C, DUDLEY E, MAYS RM (2004). Suppression of serum antibody responses by pertussis toxin after respiratory tract colonization by *Bordetella pertussis* and identification of an immunodominant lipoprotein. *Infection and Immunity,* 72: 3350-3358.

CARBONETTI N, MAYS RM WORTHINGTON ZE (2003). Pertussis toxin plays an early role in the respiratory tract colonization by Bordetella pertussis. *Infection and Immunity,* 71: 6358-6366.

CARBONETTI N, VAN ROOIJEN N, AYALA VI (2007). Pertussis toxin targets airway macrophages to promote Bordetella pertussis infection of the respiratory tract. *Infection and Immunity,* 75: 1713-1720.

CARROLL N, MORTON A, JAMES A (1993). The structure of large and small airways in nonfatal and fatal asthma. *Am Rev Respir Dis*, 147: 405-410.

CHEN L, YUE H, HAN Q, CHEN B, SHI M, LI J, YOU S, SHI Y, ZHAO RC (2007). Effects of human mesenchymal stem cells on the differentiation of dendritic cells from CD34+ cells. *Stem Cells Dev,* 16: 719-731.

CHEN, JD WILSON, E CROWTHER, TZEGGAI K, JE BISHOP, R VARRO (1999). Simultaneous quantification of six human cytokines in a single sample using microparticle-based flow cytometric technology. *Clinical Chemistry,* 45: 1693-1694.

CHENG X, VENTURA E (2007). The PD-1/PD-L pathway is up-regulated during IL-12-induced suppression of EAE mediated by IFN-gamma. *J Neuroimmunol,* 185: 75-86.

CHO KS, PARK HY, JUNG JS, JEON SG, KIM YK, ROHY HJ (2009). Immunomodulatory effects of adipose tissue-dervied stem cells in an allergic rhinitis mouse model. *Stem Cells,* 27(1): 259-265.

COHN L, NIU N, HOMER R, BOTTOMLY K (2001). IL-4 promotes airway eosinophilia by suppressing IFN-gamma production: Defining a novel role for IFN-gamma in the regulation of allergic airway inflammation. *Journal of Immunology,* 166(4): 2760-2767.

COMOLI P, MACCARIO R, AVANZINI MA, MARCONI M, GROFF A, COMETA A, CIONI M, PORRETTI L, BARBERI W, FRASSONI F, LOCATELLI F (2008). Human mesenchymal stem cells inhibit antibody production induced *in vitro* by allostimulation. *Nephrology Dialysis Transplantation,* 23(4): 1196-1202.

CONROY D, RANKIN S, PALFRAMAN R, COLLINS P, GRIFFITHS-JOHNSON D, WILLIAMS T (1997). The role of the eosinophil-selective chemokine, eotaxin, in allergic and non-allergic airways inflammation. *Mem Inst Oswaldo Cruz,* 92: 183-191.

CONSTANTIN G, ROSSI B, ANGIARI S, CALDERAN L, ANGHILERI E, GINI B, BACH SD, MARTINELLO M, BIFARI F, GALIE M, TURANO E, BUDUI S, SBARBATI A, KRAMPERA M, BONETTI B (2009). Adipose-derived mesenchymal stem cells ameliorate chronic experimental autoimmune encephalomyelitis. *Stem Cells,* 27(10): 2624-2635.

COOKSON WOCM. (1997). Asthma: an epidemic in the absence of infection. *Science,* 275: 41-42.

CORCIONE, A. (2006). Human mesenchymal stem cells modulate B-cell functions. *Blood,* 107: 367-372.

COTTREZ F, COFFMAN RL, GROUX H (2000). T regulatory cells 1 inhibit a Th2-specific response *in vivo*. *J Immunol,* 165: 4848-4853.

CUNDELL DR, TAYLOR GW, GOLDMAN WE, FLAK T, COLE PJ, WILSON R (1994). Effect of tracheal cytotoxin from Bordetella pertussis on human neutrophil function in vivo. *Infection and Immunity,* 62(639-643).

DA SILVA MEIRELLES L, NARDI NB (2006). Mesenchymal stem cells reside in virtually all post-natal organs and tissues. *J Cell Sci* 119: 2204-2213.

DARDALHON, V. (2008). IL-4 inhibits TGF-beta-induced FoxP3 cells, and together with TGF-beta generates IL-9+IL-10+FoxP3(-) effector T cells. *Nature Immunol,* 9: 1347-1355.

DAS, P. (2002). Whooping cough makes global comeback. *Lancet Infect Dis,* 2(6): 322.

DAZZI, F., RAMASAMY, ET AL. (2006). The role of mesenchymal stem cells in haemopoiesis. *Blood Reviews,* 20(3): 161-171.

DE JONG E, KAPSENBERG M (2005). Dendritic cell-mediated T cell polarisation. *Springer Semin Immunopathol,* 26: 289-307.

DEL PRETE G, PARRONCHI P, CHRETIEN I, TIRI A, MACCHIA D, RICCI M, BANCHEREAU J, DE VRIES J AND ROMAGNANI S (1988). IL-4 is an essential factor for the IgE synthesis induced in vitro by human T cell clones and their supernatants. *The Journal of Immunology,* 140(12): 4193-4198.

DELORIA MA, DECKER MD, ENGLUND JA, STEINHOFF MC, PICHICHERO ME, RENNELS MB, ANDERSON EL, EDWARDS KM (1995). Association of reactions after consecutive acellular or whole-cell pertussis vaccine immunisations. *Pediatrics,* 96(3): 592-594.

DESTEFANO F, KRAMARZ P, TRUMAN B, IADEMARCO M, MULLOOLY JP, JACKSON L, DAVIS R, BLACK S, SHINEFIELD HR, MARCY M, WARD JI, CHEN R (2002). Childhood vaccinations and risk of asthma. *The Pediatric Infections Disease Journal*, 21(6): 498-504.

DJOUAD, F., PLENCE F, ET AL. (2003). Immunosuppressive effect of mesenchymal stem cells favors tumor growth in allogeneic animals. *Blood*, 102(10): 3837-44.

DJUKANOVIC R, WILSON JW, BEASLEY CR, TWENTYMAN OP, HOWARTH RH, HOLGATE ST (1990). Mucosal inflammation in asthma. *Am Rev Respir Dis*, 142: 434-457.

DJUKANOVIC R, BRITTEN KM, WILSON SJ, WALLS AF, ROCHE WR, HOWARTH PH, HOLGATE (1990). Quantitation of mast cells and eosinophils in the bronchial mucosa of symptomatic atopic asthmatics and healthy control subjects using immunohistochemistry. *Am Rev Respir Dis*, 142(863-867).

DRAGON S, YAND J, UNRUH H, HALAYKO AJ, SOUSSI-GOUNNI (2006). IL-17 enhances IL-1beta mediated CXCL-8 release from human airway smooth muscle cells. *Am J Physiol Lung Cell Mol Physiol*, 292: 1023-1029.

DREYER WJ, (1965). The molecular basis of antibody production: a paradox. *Proc Natl Sci USA*, 54: 864-869.

EDER W, VON MUTIUS E (2006). The asthma epidemic. *N Engl J Med*, 355: 2226-2235.

ELAD D, KECK T (2008). Air-conditioning in the human nasal cavity. *Respiratory Physiology and Neurobiology*, 163(1-3): 121-127.

ELAHI S, KORZENIOWSKI J, BUCHANAN R, O'CONNOR B, PEPPLER MS, HALPERIN SA, LEE SF, BABIUK LA, GERDTS V (2005). Infection of mewborn piglets with Bordetella pertussis: a new model for pertussis. *Infection and Immunity*, 73(6): 3636-3645.

ELSER B, KOCK S, GIAISI M, KIRCHHOFF S, KRAMMER PH, LI-WEBER M (2002). IFN-gamma represses IL-4 expression via IRF-1 and IRF-2. *Immunity*, 17: 703-712.

ENGLISH K, B MAHON BP (2008). Murine mesenchymal stem cells suppress dendritic cell migration, maturation and antigen presentation. *Immunology Letters*, 115(1): 50-58.

ENGLISH K, RYAN. J., TOBIN L, MURPHY MJ, BARRY FP, MAHON BP (2008). Cell contact, prostaglandin E_2 and transforming growth factor beta 1 play non-redundant roles in human mesenchymal stem cell induction of CD4+CD25^{high}forkhead box P3+ regulatory T cells. *Clinical and Experimental Immunology*, 156: 149-160.

ENNIS DP, CASSIDY. J., MAHON BP (2005a). Acellular pertussis vaccine protects against exacerbation of allergic asthma due to Bordetella pertussis in a murine model. *Clin Diagn Lab Immunol*, 12(3): 409-17.

ENNIS DP, CASSIDY. J., MAHON BP (2005b). Whole-cell pertussis vaccine protects against Bordetella pertussis exacerbation of allergic asthma. *Immunol Lett*, 15(97(1)): 91-100.

ENNIS DP, CASSIDY. J., MAHON BP. (2005). Acellular pertussis vaccine protects against exacerbation of allergic asthma due to Bordetella pertussis in a murine model. *Clin Diagn Lab Immunol*, 12(3): 409-17.

ENNIS DP, CASSIDY J, MAHON BP. (2004). Prior Bordetella pertussis infection modulates allergen priming and the severity of airway pathology in a murine model of allergic asthma. *Clin Exp Allergy*, 34(9): 1488-97.

ERJEFALT JS, GREIFF L, KORSGREN M, GIZYCKI M, JEFFERY PK, PERSSON CGA (1998). Cytolysis and piecemeal degranulation as distinct modes of activation of airway mucosal eosinophils. *J Allergy Clin Immunol*, 102: 286-294.

ERJEFALT JS, P. C. (1997). Airway epithelial repair: breathtakingly quick and multipotentiall pathogenic. *Thorax*, 52: 1010-1012.

EZQUER FE, PARRAU DB, CARPIO D, YANEZ AJ, CONGET PA (2008). Systemic administration of multipotent mesenchymal stromal cells reverts hyperglycemia and prevents nephropathy in type 1 diabetic mice. *Biol Blood Marrow Transplant*, 14(6): 631-640.

FAROOQI I., HOPKIN J.M. (1998) Early childhood infection and atopic disorder. *Thorax*, 53L 927-932.

FEDELE G, PALAZZO R, NASSO M, CHONG CHEUNG GY, COOTE JG, AUSIELLO CM (2010). Bordetella pertussis commits human dendritic cells to promote a Th1/Th17 response through the activity of adenylate cyclase toxin and MAPK-pathways. *Plos One*, 5(1): 8734.

FEHERVARI Z. (2004). CD4+ Tregs and immune control. *J Clin Invest*, 114: 1209-1217.

FINKELMAN FD, B. J., VERCELLI D, ROTHENBERG ME (2010). Key advances in mechanism of asthma, allergy and immunology in 2009. *Journal of Allergy and Clinical Immunology*, 125(2): 312-318.

FIORENTINO DF, MOSMANN TR, HOWARD M, O'GARRA A (1991). IL-10 inhibits cytokine production by activated macrophages. *J Immunol*, 147: 3815-3822.

FLAK TA, ENGLE JT, GOLDMAN WE (2000). Synergistic epithelial responses to endotoxin and a naturally occuring muramyl peptide. *Infection and Immunity*, 68: 1235-1242.

FLOOD-PAGE PT, KAY AB, ROBINSON DS (2003). Eosinophil's role remains uncertain as anti-interleukin-5 only partially depletes numbers in asthmatic airways. *AM J Respir Crit Care Med*, 167: 199-204.

FONTENOT JD, RUDENSKY AY (2003). FoxP3 programs the development and function of CD4+CD25+ regulatory T cells. *Nat Immunol*, 4: 330-336.

FRANCIS JN, DURHAM SR (2003). Induction of IL-10+CD4+CD25+ T cells by grass pollen immunotherapy. *J Allergy Clin Immunol,* 111(1255-1261).

GALLI SJ, PILIPONSKY AM (2008). The development of allergic inflammation. *Nature,* 454: 445-454.

GARCIA-MARCOS L, CANFLANCA IM (2005). BCG immunisation at birth and atopic diseases in homogenous population of Spanish schoolchildren. *Int Arch Allergy Immunol,* 137: 303-309.

GENTRY T, WINSTEAD L, DEIBERT E, FIORDALISI M, BALBER A (2007). Simultaneous isolation of human BM hematopoietic, endothelial and mesenchymal progenitor cells by flow sorting based on aldehyde dehydrogenase activity: implications for cells therapy. *Cytotherapy,* 9(3): 259-274.

GERDONI, E. (2007). Mesenchymal stem cells effectively modulate pathogenic immune response in experimental autoimmune encephalomyelitis. *Ann Neurol,* 61: 219-227.

GEREDA JE, THATAYATIKOM A, STREIB JE, PRICE MR (2000). Relation between house-dust endotoxin exposure, type 1 T cell development and allergen sensitisation in infants at high risk of asthma. *Lancet,* 355: 1680-1683.

GERN, J. (2000). Viral and bacterial infections in the development and progression of asthma. *J Allergy Clin Immunol,* 105: S497-502.

GHIRINGHELLI F, SCHMITT E (2004). CD4+CD25+ regulatory T cells suppress tumor immunity but are sensitive to cyclophosphamide which allows immunotherapy of established tumors to be curative. *Eur J Immunol,* 34: 336-344.

GIL M, BAMBACH BJ, ROKITA H, KOZBAR D (2009). Targeting a mimotope vaccine to activating Fcgamma receptors empowers dendritic cells to prime specific CD8+ T cells responses in tumor-bearing mice. *J Immunol,* 183(10): 6808-6818.

GILLIET M, L. Y. (2002). Generation of human VF8 T regulatory cells by CD40 ligand-activated plasmacytoid dendritic cells. *J Exp Med,* 195: 695-704.

GLEICH, G. (2000). Mechanisms of eosinophil-associated inflammation. *J Allergy Clin Immunol,* 105 651–663.

GLEICH GJ, FUJISAWA T, VANHOUTTE PM (1988). The eosinophil as a mediator of damage to respiratory epithelium: a model for bronchial hyperreactivity. *J Allergy Clin Immunol,* 81: 776-881.

GLENNIE S, DYSON PJ (2005). Bone marrow mesenchymal stem cells induce division arrest anergy of activated T cells. *Blood,* 105: 2821-2827.

GREEN FHY, JAMES A, MCPHEE LJ, MITCHELL I, MAUAD T (2010). Increased myoepithelial cells of bronchial submucosal glands in fatal asthma. *Thorax,* 65: 32-38.

GRINDBACKE H, ANDERSON AC, SURI-PAYER E, RAK S, RUDIN A. (2004). Defective suppression of Th2 cytokines by CD4CD25 regulatory T cells in birch allergies during birch pollen season. *Clinical and Experimental Allergy*, 34: 1364-1372.

GRÜBER C, HILL D, BAUCHAU V (2008). Early atopic disease and early childhood immunization--is there a link? EPAAC Study Group. *Allergy,* 63(11): 1464-72.

GUILBERT TW, ZEIGER RS (2006). Long-term inhaled corticosteroids in preschool children at high risk of asthma. *N Engl J Med,* 354(19): 1985-1997.

GUPTA, N. (2007). Intrapulmonary delivery of bone marrow-derived mesenchymal stem cells improves survival and attenuates endotoxin-induced acute lung injury in mice. *J Immunol*, 179: 1855-1863.

GUR-WAHNON D, BEYTH S, LIEBERGALL M, RACHMILEWITZ J (2007). Contact-dependent induction of regulatory antigen-presenting cells by human mesenchymal stem cells is medaitedvia STAT3 signalling. *Experimental Hematology*, 35: 426-433.

HACKETT TL, K. D. (2007). The role of epithelial injury and repair in the origins of asthma. *Curr Opin Allergy Clin Immunol*, 7: 63-68.

HAMELMANN E, OSHIBA A, GELFAND EW (1999). Role of IgE in the development of allergic airway inflammation and airway hyperresponsiveness-a murine model. *Allergy*, 54(4): 297-305.

HANANIA, N. (2008). Trageting airway inflammation in asthma: current and future therapies. *Chest*, 133(4): 989-998.

HANSEN G, DEKRUYFF RH, UMETSU DT. (1999). Allergen-specific Th1 cells fail to counterbalance Th2 cell-induced airway hyperreactivity but cause severe airway inflammation. *J Clin Invest*. 103(2).

HARAGUCHI M, SHIRATO K (1999). Morphometric analysis of bronchial cartilage in chronic obstructive pulmonary disease and bronchial asthma. *AM J Respir Crit Care Med*, 158: 1005-1013.

HARJU T H., NOKSO-KOIVISTO J , KORHONEN T , RÄTY R , HE Q , HOVI T , MERTSOLA J , BLOIGU A , RYTILÄ P, SAIKKU P (2006). Pathogenic bacteria and viruses in induced sputum or pharyngeal secretions of adults with stable asthma. *Thorax*, 61(579-584).

HE Q, M. J. (2008). Factors contributing to pertussis resurgence. *Future Microbiol*, 3: 329-339.

HE R, YOON J, OYOSHI MK, MACGINNITIE A, GOYA S, FREYSCHMIDT E-J, KENZIE ANJ, UMETSU DT, OETTGEN HC, GEHA RS (2009). Exaggerated IL-17

response to epicutaneous sensitisation mediates airway inflammation in the absence of IL-5 and IL-13. *J Allergy Clin Immunol,* 124(5): 761-770.

HEININGER, U. (2001). Recent progress in clinical and basic pertussis research. *Eur J Immunol,* 160: 201-213.

HENDERSON J, GRIFFITHS M, HARVEY I, GOLDING J (1999). Pertussis vaccination and wheezing illnesses in young children: prospective cohort study. *British Medical Journal,* 318: 1173-1176.

HERRICK C, B. K. (2003). To respond or not to respond: T cells in allergic asthma. *Nat Rev Immunol,* 3: 405-412.

HOLGATE, S. (2007). Epithelium dysfunction in asthma. *J Allergy Clin Immunolm* 120: 1233-1244.

HOLGATE S, WILSON S, ROCHE W, DAVIES D (2000). Bronchial epithelium as a key regulator of airway allergen sensitisation and remodelling in asthma. *AM J Respir Crit Care Med,* 162: 113-117.

HOLGATE, S. T. (2008). Pathogenesis of Asthma. *Clinical and Experimental Allergy,* 38: 872–897.

HOLGATE ST, LACKIE PM, WILSON SJ, PUDDICOMBE SM, LORDAN JL. (2000). Epithelial-mesenchymal interactions in the pathogenesis of asthma. *J Allergy Clin Immunol,* 105(2): 193-204.

HOLT P, S.,OLIVERY J, HOLT B, MCMENAMIN P (1990). A contiguous network of dendritic antigen-presenting cells within the respiratory epithelium. *Int Arch Allergy Appl Immunol,* 91: 155-159.

HORI S, SAKAGUCHI S (2003). Control of regulatory T cell development by the transcription factor FoxP3. *Science,* 299: 1057-1061.

HORWITZ, E. (2005). Clarification of the nomenclature for MSC: The International Society for Cellular Therapy position statement. *Cytotherapy,* 7: 393-395.

HOZUMI N, T. S. (1976). Evidence for somative rearrangement of immunoglobulin genes coding for variable and constant regions. *Proc Natl Sci USA,* 73(10): 3628-3632.

HUH J, JAHNSEN F (2003). Bidirectional interactions between antigen-bearing respiratory tract dendritic cells (DCs) and T cells precede the late phase reaction in experimental asthma: DC activation occurs in the airway mucosa but not in the lung parenchyma. *J Exp Med,* 198: 19-30.

IKEHARA, S. (2003). A novel strategy for allogeneic stem cell transplantation: perfusion method plus intra-bone marrow injection of stem cells. *Exp Hematol,* 31: 1142-1146.

IKEZAWA Y, TAMURA C, TAKAHASHI K, MINAMI M, IKEZAWA Z (2005). Cyclophosphamide decreases the number, percentage and the function of CD25+CD4+ regulatory T cells, which suppress the induction of contact hypersensivity. *Journal of Dermatological Science,* 39: 105-112.

IMBERTI, B. (2007). Insulin-like growth factor-1 sustains stem cell mediated renal repair. *J Am Soc Nephrol,* 18: 2921-2928.

INOUE, Y. (2007). Subretinal transplantation of bone marrow mesenchymal stem cells delays retinal degeneration in the RCS rat model of retinal degeneration. *Experimental Eye Research,* 85: 234-241.

ISOGAI S, FRASER RS, TAHA R, HAMID Q, MATIN JG (2007). Interferon-gamma-dependent inhibition of late allergic airway responses and eosinophilia by CD8+gammadelta T cells. *Immunology,* 122(2): 230-238.

ISOGAI S, MAGHNI K, RAMOS-BARBON D, TAHA R, YOSHIZAWA Y (2003). The effects of CD+gamma delta T cells on late allergic airway responses and airway inflammation in rats. *J Allergy Clin Immunol,* 112(3): 547-555.

ITESCU S, LIETZ K (2002). Intravenous pulse administration of cyclophosphamide is an effective and safe treatment for sensitised cardiac allograft recipients. *Circulation,* 105: 1214-1219.

ITO W, KANEHIRO A, KATO H, YAMAGUCHI K, UEKI S, KAYABA H, CHIBARA J (2007). Hepatocyte growth factor attenuates eotaxin and PGD2-induced chemotaxis of human eosinophils. *Allergy,* 62: 4150422.

JAFFAR Z, ROBERS K (2004). CD4+CD25+ T cells regulate airway eosinophilic inflammation by modulating the Th2 cell phenotype. *J Immunol,* 172: 3842-3849.

JIANG XX, LIU B, ZHANG SX, WU Y, YU XD, MAO N (2005). Human mesenchymal stem cells inhibit differentiation and function of monocyte-derived dendritic cells. *Blood,* 105(10): 4120-4126.

JOHNSTON SL, SANDERSON G, SMITH S, LAMPE F (1995). Community study of role of viral infections in exacerbations of asthma in 9-11-year-old children. British *Medical Journal,* 310: 1225-1229.

JUTEL, M. (2003). IL-10 and TGF-beta co-operate in the regulatory T cell response to mucosal allergens in normal immunity and specific immunotherapy. *Eur J Immunol,* 33(1205-1214).

KATO K, KUICK R, MINEISHI S, HEXNER E, FERRARA JL, EMERSON SG, ZHANG Y (2010). Identification of stem cell transcriptional programs normally expressed in embryonic and neural stem cells in alloreactive CD8+ T cells mediating graft-versus-host disease. *Biol Blood Marrow Transplant,*

KAY, A. (2001). Allergy and allergic disease. *N Engl J Med,* 344: 30-37.

KAY, A. (2005). The role of eosinophils in the pathogenesis of asthma. *Trends Mol Med,* 11: 148-152.

KAY, A. (2006). The role of T lymphocytes in asthma. *Chem Immunol Allergy,* 91: 59-75.

KEARLY J, B. J., ROBINSON DS, LLOYD CM (2005). Resolution of airway inflammation and hyperreactivity after in vivo transfer of CD4+CD25+ regulatory T cells is interleukin 10 dependent. *J Exp Med,* 202: 1539-1537.

KEARLY J, LLOYD CM (2008). CD4+CD25+ regulatory T cells reverse established allergic airway inflammation and prevent airway remodelling. *J Allergy Clin Immunol*, 122: 617-624.

KEATING, A. (2006). Mesenchymal stromal cells. *Current Opinion in Hematology,* 13(6): 419-425.

KHELEF N, VARGAFTIG B, GUISO N. (1994) Characterisation of murine lung inflammation after infection with parental *Bordetella pertussis* and mutants deficient in adhesions and toxins. *Infection and Immunity*, 62(7): 2893-2900.

KOUP R., SAFFRIT J.T., CAO Y., ANDREWS CA., MCLEOD G., BORKOWSKY W., FARTHING C., HO DD. (1994) Temporal association of cellular immune responses with the initial control of viremia in primary human immunodeficiency virus type-1 syndrome. *Journal of Virology*, 68 (7): 4650-4655.

KRAMPERA M, ANGELI R, PASINI A, LIOTTA F, ANDREINI A, SANTARLASCI V, MAZZINGHI B, PIZZOLO G, VINANTE F, ROMAGNANI P, MAGGI E, ROMAGNANI S, ANNUNZIATO F. (2006). Role for interferon-gamma in the immunomodulatory activity of human bone marrow mesenchymal stem cells. *Stem Cells*, 24(2): 386-398.

KRAMPERA, M., S. GLENNIE, ET AL. (2003). Bone marrow mesenchymal stem cells inhibit the response of naive and memory antigen-specific T cells to their cognate peptide. *Blood*, 101(9): 3722-9.

KRAMPERA M, DYSON J, SCOTT D, LAYLOR R, SIMPSON E, DAZZI F (2003). Bone marrow mesenchymal stem cells inhibit the response of naïve and memory antigen-specific T cells to their cognate peptide. *Blood,* 101: 3722-3729.

KRISHNA MT, SS SALVI. (2002) Could administration of bacille Calmette-Guerin vaccination at birth protect from the development of asthma and allergic diseases in the western world? Has this question been adequately investigated? *Pediatr. Allergy Immunol* 13:172.

KUIPERS H,HIJDRA D (2004). Dendritic cells retrovirally over-expressing IL12 induce strong Th1 response to inhaled antigen in the lung but fail to revert established Th2 sensitisation. *J Leukocyte Biol,* 76: 1028-1038.

LAHN M, TAKEDA K, JOETHAM A, SCHWARZE J, KOUMLHLER, O'BRIEN R, GELFAND E, BORN W (1999). Negative regulation of airway responsivenss that is dependent on gamma delta T cells and independent of alphabeta T cells. *Nature Medicine*, 5: 1150-1156.

LAMKHIOUED B, HAMID Q, MANSOUR N, DELESPESSE, RENZI PM (2003). The CCR3 receptor is involved in eosinophil differentiation and is up-regulated by Th2 cytokines in CD34+ progenitor cells. *J Immunol*, 170: 537-547.

LE BLANC K, SUNDBERG B (2004). Treatment of severe acute graft-versus-host disease with third party haploidentical mesenchymal stem cells. *Lancet*, 363: 1439-1441.

LEE JW, GUPTA N, SERIKOV V, MATTHAY MA (2009). Allogeneic human mesenchymal stem cells for treatment of E. coli endotoxin-induced acute lung injury in the ex vivo perfused human lung. *Proceedings of the National Academy of Science U.S.A.*, 106(38): 16357-16362.

LEE RH, SREGER R, SPEES JL, PULIN AA, OLSON SD, PROCKOP DJ (2006). Multipotent stromal cells from human marrow home to and promote repair of

pancreatic islets and renal glomeruli in diabete NOD/scid mice. *Proceedings of the National Academy of Science U.S.A.,* 103: 17438-17443.

LI X, HUANG H, ZHANG X, TOWN J, DAVIS B, COCKCROFT DW, GORDON JR (2010). Induction of type 2 T helper cell allergen tolerance by IL-10-differentiated regulatory dendritic cells. *Am J Respir Cell Mol Biol* 42(2): 190-199.

LIN C-H. (2003). Efficient expansion of regulatory T cells *in vitro* and *in vivo* with CD28 superagonist. *Eur J Immunol,* 33: 626-638.

LING EM, NGUYEN XD (2004). Relation of CD4+CD25+ regulatory T cell-suppression of allergen-driven T-cell activation to atopic status and expression of allergic disease. *Lancet,* 363: 608-615.

LIU AH, (2001). Endotoxin: friend or foe? *Allergy Asthma Proc,* 22(338-340).

LIU LY, BATES ME, VRTIS RF, GERN JE, KITA H (2002). Decreased expression of membrane IL-5 receptor alpha on human eosinophils, II: IL-5 down-modulates its receptor via a proteinase-mediated process. *Journal of Immunology,* 169: 6459-6466.

LLOYD CM, COYLE AJ, GUTIERREZ-RAMOS JC (2001). Mouse models of allergic airway disease. *Adv Immunol,* 77(263-295).

LOCHT C, MENOZZI D, RENAULD G (1993). The filamentous haemagglutinin, a multi-facted adhesion produced by virulent *Bordetella*. *Molecular Microbiology,* 9: 653-660.

LOCHT C. (1999) Molecular aspects of Bordetella pertussis pathogenesis. *Internatl Microbiol,*2:137-144.

LOMASK, M. (2006). Further exploration of the PenH parameter. *Exp Tox Pathol,* 57: 13-20.

LUKACS NW, CHENSUE SW, KUNKEL SL (1994). Interleukin-4-dependent pulmonary eosinophil infiltration in a murine model of asthma. *American journal of respiratory cell and molecular biology,* 10: 526-532.

LULICH KM, PATERSON JW (1988). Beta-adrenoceptor function in asthmatic bronchial smooth muscle. *Gen Pharmacol,* 19: 307-311.

LUTZIAK C, DE PASCALIS R, KASHMIRI S, SCHLOM J, SABZEVARI H (2005). Inhibition of CD4+CD25+ T regulatory cell function implicated in enhanced immune response by low-dose cyclophosphamide. *Blood,* 105: 2862-2868.

LUTZIAK ME, DE PASCALIS R, KASHMIRI SV, SCHLOM J, SABZEVARI H. (2005). Inhibition of CD4+CD25+ T regulatory cell function implicated in enhanced immune response by low-dose cyclophosphamide. *Blood,* 105: 2862-2968.

MACCARIO R, MORETTA A, COMETA A, COMOLI P, MONTAGNA D, DAUDT L, IBATICI A, PIAGGIO G, POZZI S, FRASSONI F, LOCATELLI F (2005). Interaction of human mesenchymal stem cells with cells involved in alloantigen-specific immune response favors the differentiation of CD4+ T-cell subsets expressing a regulatory/suppressive phenotype. *Haematologica,* 90(4): 516-525.

MANCLARK CR, (1984). Pertussis. *Bacterial vaccines,* : 69-106.

MARSLAND BJ, KOPF M, LE GROS G. (2004). Allergic airway inflammation is exacerbated during acute influenza infection and correlates with increased allergen presentation and recruitment of allergen-specific T-helper type 2 cells. *Clin Exp Allergy,* 34(8): 1299-306.

MARTIGNON G, ANNESI-MAESANO I. (2005). Does childhood immunization against infectious diseases protect from the development of atopic disease? *Pediatr Allergy Immunol,* 16(3): 193-200.

MARTINO A, AURRICHIO G, COLIZZI V, BALDINI PM (2006). Influence of pertussis toxin on CD1a isoform expression in human dendritic cells. *J Clin Immunol,* 26: 153-159.

MASCART F, MALFROOT A, HAINAUT M, PIÉRARD D, TEMERMAN S, PELTIER A, DEBRIE AS, LEVY J, DEL GIUDICE G, LOCHT C. (2003). Bordetella pertussis infection in 2-month-old infants promotes type 1 T cell responses. *J Immunol,* 170(1): 1504-9.

MATAR P, GERVASONI S, SCHAROVSKY O (2001). Down-regulation of T-cell-derived IL-1 production by low-dose cyclophosphamide treatment in tumor-bearing rats restores *in vitro* normal lymphoproliferative response. *In. Immunopharmacol*, 1: 307-319.

MCDOWELL EM, SCHURCH W, TRUMP BF (1979). The respiratory epithelium: VII. Epidermoid metaplasia of hamster tracheal epithelium during regeneration following mechanical injury. *J Natl Cancer Inst,* 62: 995-1008.

MCGUIRK P. (2000). Direct anti-inflammatory effect of a bacterial virulence factor: IL-10-dependent suppression of IL-12 production by filamentous hemagglutinin from Bordetella pertussis. *Eur J Immunol,* 30: 415-422.

MCHUGH RS. (2002). Cutting Edge: depletion of CD4+CD25+ regulatory T cells is necessary, but not sufficient, for induction of organ-specific autoimmune disease. *J Immunol*, 168: 5979-5983.

MEDOFF B, LUSTER A (2008). T cell trafficking in allergic asthma: the ins and outs. *Annu Rev Immunol,* 26: 205-232.

MEILER F, KLUNKER S, RUCKERT B, AKDIS CA, AKDIS M (2008). In vivo switch to IL-10-secreting T regulatory cells in high dose allergen exposure. *J Exp Med,* 24(205): 2887-2898.

MEISEL R, LARYEA M, GOBEL U, DAUBENER W, DILLO D (2004). Human bone marrow stromal cells inhibit allogeneic T-cell responses by indoleamine 2,3-dioxygenase-mediated tryptophan degradation. *Blood*, 103: 4619-4621.

MELLMAN I, S. R. (2001). Dendritic cells: specialised and regulated antigen processing machines. *Cell*, 106: 255-258.

MENEZES GB, PEREIRA-SILVA PE, KLEIN A, CARA DC, FRANCHISI N (2008). Differential involvement of cyclooxygenase isoforms in neutrophils migration in vivo and in vitro. *European Journal of Pharmacology*, 598(1-3): 118-122.

METCALFE DD, MEKORI YA (1997). Mast cells. *Physiol Rev*, 77: 1033-1079.

MEYTS I, H. P., HENS (2006). IL-12 contributes to allergen-induced airway inflammation in experimental asthma. *J Immunol*, 177: 6460-6470.

MIELCAREK N, LOCHT C (2001). Nasal vaccination using live bacterial vectors. *Adv Drug Deliv Rev*, 51: 55-70.

MIELCAREK N, RAZE D, BERTOUT J, ROUANET C, YOUNES AB, CREUSY C, ENGLE J, GOLDMAN WE, LOCHT C. (2006). Live attenuated B. pertussis as a single-dose nasal vaccine against whooping cough. *PLoS Pathog*, 2(7): e65.

MIELCAREK N, REMOUE F, ANTOINE R, CAPRON A, LOCHT C (1998). Homologous and heterologous protection after single intranasal administration of live attenuated recombinant *Bordetella pertussis*. *Nat Biotechnol*, 16: 454-457.

MILLS, K. (2001). Immunity to *Bordetella pertussis*. *Microbes Infect*, 3: 655-677.

MILLS K.H.G., R. M., RYAN E., MAHON .B.P. (1997). A murine model in which protection correlates with pertussis vaccine efficacy in children reveals complementary roles for humoral and cell-mediated immunity in protection against Bordetella pertussis. *Infection and Immunity*, 66: 594-602.

MILLS KH, M. B., RYAN E, MAHON BP (1998). A respiratory challenge model for infection with Bordetella pertussis: application in the assessment of pertussis vaccine potency and in defining the mechanism of protective immunity. *Developments in biological standardization*, 95: 31-41.

MILLS, K. H. G., A. BARNARD, J. WATKINS, K. REDHEAD. (1993). Cell-mediated immunity to Bordetella pertussis: role of Th1 cells in bacterial clearance in a murine respiratory infection model. *Infect and Immunity,* 61: 399–410.

MOBBERLY-SCHUMAN P, W. A. (2005). Influence of CCR3 (CD11b/CD18) expression on phagocytosis of *Bordetella pertussis* by human neutrophils. *Infection and Immunity,* 73: 7317-7323.

MADDUR M.S., KAVERI S.V., BAYRY J. (2009) Basophils as antigen presenting cells. Trends in Immunology, 31(2): 45-48.

MOLET S, DAVOINE F, NUTKU E, TAHA R, PAGE N, OLIVENSTEIN R, ELIAS J, CHAKIR J (2001). IL-17 is increased in asthmatic airways and induces human bronchial fibroblasts to produce cytokines. *J Allergy Clin Immunol,* 108: 430-438.

MOSMANN T, BOND MW, GIEDLIN MA, COFFMA RA (1986). Two types of murine helper T cell clone. 1. Definition according to profiles of lymphokine activities and secreted proteins. *J Immunol,* 136: 2348-2357.

MOSMANN T, C. R. (1989). Th1 and Th2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annu Rev Immunol,* 7: 145-173.

MUGURUMA, Y. (2006). Reconstitution of the functional human hematopoietic microenvironment derived from human mesenchymal stem cells in the murine bone marrow compartment. *Blood*, 107: 1878-1887.

MUNOZ JJ, BERGMAN RK, SADOWSKI PL (1981). Biological activities of crystalline pertussigen from Bordetella pertussis. *Infection and Immunity,* 33: 820-826.

NAJAFIAN N, SALAMA AD, ZHU B, BENOU C, YUAN X (2003). Regulatory functions of CD8+CD28- T cells in an autoimmune disease model. *J Clin Invest,* 112(7): 1937-1048.

NAKAMURA Y, URBAN J, SHERIDAN B, GIERMASZ A, NISHIMURA F, SASAKI K, CUMBERLAND R, MUTHUSWAMY R, MAILLIARD RB, LARREGINA AT, FALO LD, GOODING W, STORKUS WJ, OKADA H, HENDRICKS RL, KALINSKI P (2007). Helper function of memory CD8+ T cells: heterologous CD8+ T cells support the induction of therapeutic cancer immunity. *Cancer Research,* 67(20): 10012-10018.

NAUTA AJ, KRUISSELBRINK AB, LURVINK EG, WILLMZE R, FIBBE WE (2006). Donor-derived mesenchymal stem cells are immunogenic in an allogeneic host stimulate donor graft rejection in a nonmyeloablative setting. *Blood,* 108: 2114-2120.

NAYLOR, B. (1962). The shedding of the mucosa of the bronchial tree in asthma. *Thorax,* 17: 69-72.

NICOLA, M. D. (2002). Human bone marrow stromal cells suppress T-lymphocytes proliferation induced by cellular and nonspecific mitogenic stimuli. *Blood,* 99: 3838-3843.

NILSSON L, GRANSTRÖM M, BJÖRKSTÉN B, KJELLMAN NI. (1998). Pertussis IgE and atopic disease. *Allergy,* 53(12): 1195-201.

NOON, L. (1911). Prophylactic inoculation against hay fever. *Lancet,* 177: 1572-1573.

ODENT M.R., CULPIN E.E., KIMMEL T. (1994) Pertussis vaccination and asthma: Is there a link? *JAMA,* 272: 592-593.

ODERUP C, MAKOWSAK A, CILIO CM, IVARS F (2006). Cytotoxic T lymphocytes antigen-4-dependent down-modulation of costimulatory molecules on dendritic cells in CD4+CD25+ regulatory T cell-mediated suppression. *Immunology,* 118: 240.

OKUNISHI K, OKASORA T, NAKAGOME K, IMAMURA M, HARADA H, MATSUMOTO T, TANAKA R, YAMAMOTO K, TABATA Y, DOHI M (2009). Intratracheal delivery of hepatocyte growth factor directly attenuates allergic airway inflammation in mice. *Int Arch Allergy Immunol*, 149(1): 14-20.

ORDENEZ C, WONG H, FERRANDO R, WU R, HYDE D (2001). Mild and moderate asthma is associated with airway goblet cell hyperplasia and abnormalities in mucin gene expression. *AM J Respir Crit Care Med*, 163: 517-523.

ORLIC, D. (2001). Bone marrow cells regenerate infarcted myocardium. *Nature*, 410: 701-705.

ORTIZ LA, FATTMAN, C, PANDEY AC, TORRES G, GO K, PHINNEY DG (2007). Interleukin 1 receptor antagonist mediates the anti-inflammatory and anti-fibrotic effect of mesenchymal stem cells during lung injury. *PNAS*, 104(26): 11002-11007.

ORTIZ LA, MCBRIDE C, GAUPP D, BADDOO M, KAMISKI N, PHINNEY DG (2003). Mesenchymal stem cell engraftment in lung is enhanced in response to bleomycin exposure and ameliorates its fibrotic effects. *Proceedings of the National Academy of Science U.S.A.*, 100(14): 8407-8411.

O'SHEA J, PAUL WE. (2010) Mechanisms underlying lineage commitment and plasticity of helper CD4+ T cells. *Science*, 327: 1098-1102.

PALMER, D. (2004). Vaccine-stimulated, adoptively transferred CD8+ T cells traffic indiscriminately and ubiquitously while mediating specific tumor destruction. *J Immunol*, 173: 7209-7216.

PARIJS LV, (1998). Homeostasis and self-tolerance in the immune system: Turning lymphocytes off. *Science*, 280(5361): 243-248.

PATTERSON B, ANDERSSON J, SULLIVAN Y, SU F, JIYAMAPA D, BURKI Z, LANDAY A (1999). Regulation of CCR5 and CXCR4 expression by type 1 and type

2 cytokines: CCR5 expression is downregulated by IL-10 in CD4-positive lymphcytes. *Cinical Immunology,* 91: 254-262.

PEEBLES RS, COLLINS R, JARZECKA K, FURLONG J, MITCHELL D, SHELLER J, GRAHAM BS. (2001). Immune interaction between respiratory syncytial virus infection and allergen sensitisation critically depends on timing of challenges. *Journal of Infectious Diseases,* 184(11): 1374-1379.

PEISTER A, LARSON BL, HALL BM, GIBSON LF, PROCKOP DJ (2004). Adult stem cells from bone marrow (MSCs) isolated from different strains of inbred mice vary in surface epitopes, rates of proliferation and differentiation potential. *Blood,* 103: 1662-1668.

PEREZ V, CAN DER POL W, YANTOMO O, RODRIGUEZ M (2006). Adenylate cyclase influences filamentous haemagglutinin-mediated attachment of Bordetella pertussis to epithelial alveolar cells. *FEMS Immunol Med Microbiol*, 48: 140-147.

PHINNEY DJ (2007). Biochemical heterogeneity of mesenchymal stem cell populations: clues to their therapeutic efficacy. *Cell Cycle*, 6: 2884-2889.

PHINNEY DG, ISAACSON RL, PROCKOP DJ (1999). Plastic adherent stromal cells from the bone marrow of commonly used strains of inbred mice: variations in yield, growth and in differentiation. *Cell Biochem,* 72: 570-585.

PIGGOTT D, XU L (2005). MyD88-dependent induction of allergic Th2 responses to intranasal antigen. *J Clin Invest,* 115: 459-467.

POLCHERT D, DOUGLAS G, KIDD M, MOADSIRI A, REINA E, GENRICH K, MEHROTRA S, SETTY S, SMITH B, BARTHOLOMEW A (2008). IFN-gamma activation of mesenchymal stem cells for treatment and prevention of graft *versus* host disease. *European Journal of Immunology*, 38(6): 1745-1755.

POSTMA DS. (1998). Characteristics of airway hyperresponsiveness in asthma and chronic obstructive pulmonary disease. *AM J Respir Crit Care Med,* 158: 187-S192.

PREVOSTO C, CANEVALI P, RAFFAELLA ZOCCHI M, POGGI A (2007). Generation of CD4+ or CD8+ regulatory T cells upon mesenchymal stem cell-lymphocyte interaction. *Haematologica,* 92(7): 881-888.

PRIGIONE I, TOSCA MA, SILVESTRI M, PISTOIA V, CIPRANDI G, ROSSI GA (2009). Interferon-gamma and IL-10 may protect from allergic polysensitisation in children: preliminary evidence. *Allergy*,

QUEVEDO HC, OSKOUEI BN, FEIGENBAUM GS, RODRIGUEZ JE, VALDES D, PATTANY PM, ZAMBRANO JP, HU Q, MCNIECE I, HELDMAN AW, HARE JM (2009). Allogeneic mesenchymal stem cells restore cardiac function in chronic ischemic cardiomyopathy via trilineage differentiating capacity. *Proceedings of the National Academy of Science U.S.A.,* 106(33): 14022-14027.

RAFFAGHELLO, L. (2008). Human mesenchymal stem cells inhibit neutrophil apoptosis: a model for neutrophil preservation in the bone marrow niche. *Stem Cells,* 26: 151-162.

RAMASAMY R., FAZEKASOVA H., LAM E.W.F., SOEIRO I., LOMBARDI G., DAZZI F. (2007) Mesenchymal stem cell inhibit dendritic cell differentiation and function by preventing entry into the cell cycle. *Transplantation,* 83(1): 71-76.

RASMUSSON I, SUNDBERG B, LE BLANC K (2003). Mesenchymal stem cells inhibit the formation of cytotoxin T lymphocytes, but not activated cytotoxic T lymphocytes or natural killer cells. *Transplantation,* 76: 1208-1213.

RASMUSSON I, SUNDBERG, ET AL. (2005). Human mesenchymal stem cells affect IgG production induced by lipopolysaccharide, cytomegalovirus and varicella zoster virus in human spleen. *Biology of Blood and Marrow Transplantation,* 11(2): 69-70.

REHMAN J, LI J (2004). "ecretion of angiogenic and antiapoptotic factors by human adipose stromal cells. *Circulation,* 109: 1292-1298.

REN, G. (2008). Mesenchymal stem cell-mediated immunosuppression occurs via concerted action of chemokines and nitric oxide. *Cell Stem Cell,* 2: 141-150.

RENNELS, M. (2003). Extensive swelling reactions occuring after booster doses of diphtheria-tetanus-acellular pertussi vaccines. *Semin Pediatr Infect Dis,* 14: 196-198.

RICH S, LEE HM, LIN J (1995). Transforming growth factor beta 1 costimulated growth and regulatory function of staphyloccocal enterotoxin B-responsive CD8+ T cells. *J Immunol,* 155: 609-618.

RICHTER A, LORDAN JL, BUCCHIERI F, WILSON SJ. (2001). The contribution of interleukin (IL)-4 and IL-13 to the epithelial-mesenchymal trophic unit in asthma. *American Journal of Respiratory Cell and Molecular Biology,* 25(3): 385-391.

ROBBINS J, TROLLFORS B, SATO H, SATO Y (2005). The diphtheria and pertussis components of diphtheria-tetanus toxoids-pertussis vaccine should be genetically inactivated mutant toxins. *Journal of Infectious Diseases,* 191: 81-88.

ROMAGNANI, S. (2004) Immunologic influences on allergy and the TH1/TH2 balance. *J Allergy Clin Immunol,* 113(3): 395-400.

ROSS PJ, MILLS KH, BOYD AP (2004). Adenylate cyclase toxin from Bordetella pertussis synergises with lipopolysaccharide to promote innate interleukin-10 production and enhances the induction of Th2 and regulatory T cells. *Infection and Immunity*, 72: 1568-1579.

RUBTSOV Y, CHI E (2008). Regulatory T cell-derived interleukin-10 limits inflammation at environmental interfaces. *Immunity,* 28: 546-558.

RUSSELL J, H. D. (1994). RANTES is a chemotactic and activating factor for human eosinophils. *Pediatrics,* 94(2): 259-260.

RYAN EJ, KJELLMAN N, GOTHEFORS L, MILLS KH. (2000). Booster immunization of children with an acellular pertussis vaccine enhances Th2 cytokine production and serum IgE responses against pertussis toxin but not against common allergens. *Clin Exp Immunol*, 121(12): 193-200.

RYAN JM, BARRY. F., MURPHY JM, MAHON BP (2007). "Interferon-gamma does not break, but promotes the immunosuppressive capacity of adult human mesenchymal stem cells." Clinical and Experimental Immunology 149(2): 353-363.

S-H YOUNG, JR ROBERTS, AD ERDELY, PC ZEIDLER-ERDELY (2008). Performance evaluation of cytometric bead assays for the measurement of lung cytokines in two rodent models. *Journal of Immunological Methods*, 331(1-2): 59-68.

SACCO O, SABATINI F, SALE R, DEFILIPPI AC, ROSSI GA (2004). Epithelial cells and fibroblasts: structural repair and remodelling in the airways. *Paediatr Respir Rev*, 5(Suppl A): S35-40.

SACKSTEIN, R. (2008). Ex vivo glycan engineering of CD44 programs human multipotent mesenchymal stromal cell trafficking to bone. *Nature Medicine,* 14(2): 181-187.

SAETTA M, T. G. (2001). Airway pathology in asthma. *Eur Respir J,* 18: 18S-23S.

SAKAGUCHI, S. (2005). Naturally arising FoxP3-expressing CD25+CD4+ regulatory T cells in immunological tolerance to self and non-self. *Nature Immunol,* 6: 345-352.

SAKAGUCHI S, P. F. (2007). Emerging challenges in regulatory T cell function and biology. *Science,* 317: 627-629.

SAKAGUCHI S, ASANO M, ITOH M, TODA M (1995). Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J Immunol*, 155: 1151-1164.

SATO K, OH I, MEGURO A, HATANAKA K, NAGAI T, MUROI K, OZAWA K (2007). Nitric oxide plays a critical role in suppression of T-cell proliferation by mesenchymal stem cells. *Blood,* 109(228-234).

SCHMITZ N, KOPT M (2003). The IL-1 receptor is critical for Th2 cell type airway immune response in a mild but not in a more severe model. *Eur J Immunol,* 33: 991-1000.

SCHNYDER-CANDRIAN S, COUILLIN I, MERCIER I, BROMBACHER F, QUESMIAUX V, FOSSIEZ F, RYFFEL B, SCHNYDER B (2006). Interleukin-17 is a negative regulatory of established allergic asthma. *J Exp Med,* 1-11.

SELMANI Z, ZIDI B, FAVIER B, GAIFFE E, OBERT L, BORG C, SAAS P, TIBERGHIEN P, ROUAS-FREISS N, CAROSELLA ED, DESCHASEAUX F (2008). Human leukocyte antigen-G5 secretion by human mesenchymal stem cells is required to suppress T lymphocyte and natural killer function and to induce CD4+CD25highFoxP3+ regulatory T cells. *Stem Cells*, 26: 212-222.

SHENG H, JIN Y, ZHANG Q, ZHANG Y, WANG L, SHEN B, YIN S, LIU W, CUI L, NINGLI L (2008). A critical role of IFN-gamma in priming MSC-mediated suppression of T cell proliferation through up-regulation of B7-H1. *Cell Research,* 18: 846-857.

SHER A (1992). Regulation of immunity to parasites by T cells and T cell-derived cytokines. *Annu Rev Immunol,* 10: 385-409.

SHIMURA S, HARAGUCHI M, SHIRATO K (1996). Continuity of airway goblet cells and intraluminal mucus in the airways of patients with bronchial asthma. *Eur Respir J*, 9: 1395-1401.

SINGH M, L. K. (2006). Whooping cough: the current scene. *Chest*, 130: 1547-1553.

SKERRY CM, CASSIDY. J., ENGLISH K, FEUNOU-FEUNOU P, LOCHT C, MAHON BP (2009). A live attenuated Bordetella pertussis candidate vaccine does not cause

disseminating infection in IFN-gamma receptor knockout mice. *Clinical and Vaccine Immunology,* 169(1344-1351).

SMITH C, V. H. (2000). Early infantile pertussis: increasingly prevalent and potentially fatal. *Eur J Pediatr,* 159: 898-900.

SOCIETY, B. T. (2008). British Guideline on the Management of Asthma. *Thorax,* 63(S4): 1-121.

SOKOL C.L., ET AL. (2009) Basophils function as antigen presenting cells for allergen-induced T helper type 2 responses. *Nature Immunology*, 10: 713-720.

SPAGGIARI GM, BECCHETTI S, MINGARI MC, MORETTA L (2006). Mesenchymal stem cell-natural killer cell interactions: evidence that activated NK cells are capable of killing MSCs, whereas MSCs can inhibit IL-2-induced NK-cell proliferation. *Blood,* 107: 1484-1490.

SPENSIERI F, FAZIO C, NASSO M, STEFANELLI P (2006). *Bordetella pertussis* inhibition of interleukin-12 (IL-12) p70 in human monocyte-derived dendritic cells blocks IL-12 p35 through adenylate cyclase toxin-dependent cyclic AMP induction. *Infection and Immunity*, 74: 2831-2838.

SS KHAN, D REDA, AF SUFFREDINI, JP MCCOY (2004). Multiplex bead array assays for detection of soluble cytokines: comparisons of sensitivity and quantitative values among kits from multiple manufacturers. *Cytometry B Clin Cytom,* 61(1): 35-39.

STAGG, J. (2007). Immune regulation by mesenchymal stem cells: two sides to the coin. *Tissue Antigens,* 69(1): 1-9.

STRACHAN, D. (2000). Family size, infection and atopy: the first decade of the "hygiene hypothesis". *Thorax,* 55(Suppl): S2-10.

STRICKLAND DH, ZOSKY GR (2006). Reversal of airway hyperresponsiveness by induction of airway mucosal CD4+CD25+ regulatory T cells. *Journal of Experimental Medicine,* 203: 2649-2460.

SU Y-C, COOLEY M, SEWELL W (2006). Cyclophosphamide augments inflammation by reducing immunosuppression in a mouse model of allergic airway disease. *Journal of Allergy and Clinical Immunology,* 117(3): 635-641.

SUDO N, TANAKA K, AIBA Y, KUBO C, KOGA Y (1997). The requirement of intestinal bacterial flora for the development of an IgE production system fully susceptible to oral tolerance induction. *J Immunol,* 159-: 1739-1745.

SUDRES M, TRENADO A, GREGOIRE S, CHARLOTTE F, LEVACHER B, LATAILLADE JJ, BOURIN P, HOLY X, VERNANT JP, KLATZMAN D, COHN JL (2006). Bone marrow mesenchymal stem cells suppress lymphocyte proliferation in vitro but fail to prevent graft-versus-host disease in mice. *J Immunol,* 176: 7761-7767.

SUMI Y, H. Q. (2007). Airway remodelling in asthma. *Allergol Int,* 56(4): 341-348.

SUR S, CHOUDHURY BK, SUR N, ALAM R, KLINMAN DM (1999). Long-term prevention of allergic lung inflammation in a mouse model of asthma by CpG oligodeoxynucleotides. *Journal of Immunology,* 162(10): 6284-6293.

SUTO, A. (2001). Role of CD4(+)CD25(+) regulatory T cells in T helper 2 cell-mediated allergic inflammation in the airways. *J Exp Med,* 194: 1349-1359.

SWAIN SL, ENGLISH M, HUSTON G (1990). IL-4 directs the development of Th2-like helper effectors. *J Immunol,* 145: 3796-3806.

SWINDLE EJ, DAVIES DE (2009). Breakdown in epithelial barrier function in patients with asthma: identification of novel therapeutic approaches. *J Allergy Clin Immunol,* 124: 23-24.

TAKANASKI S, XING Z, O'BYRNE P, DOLOVICH J, JORDANA M (1994). Interleukin 10 inhibits lipopolysaccharide-induced survival and cytokine production by human peripheral blood eosinophils. *Journal of Experimental Medicine,* 180: 1711-1715.

TAKEDA K, JOETHAM A, SCHULTZ LD, LARSEN GL, IRVIN CG, GELFAND EW (1997). Development of eosinophilic airway inflammation and airway hyperresponsiveness in mast cell-deficient mice. *J Exp Med*, 186: 449-454.

TAUBE C, GELFAND EW (2004). Insights into the pathogenesis of asthma utilising murine models. *Int Arch Allergy Immunol*, 135: 173-186.

TAUBE C, RHA YH, TAKEDA K, JOETHAM A, PARK JW, BALHORN A, TAKAI T, POCH KR, NICK JA, GELFAND EW (2003). Transient neutrophil infiltration after allergen challenge is dependent on specific antibodies and FcgammaIII receptors. *Journal of Immunology*, 170(4301-4309).

TAVASSOLI M, T. K. (1982). Morphological studies on long-term culture of marrow cells: characterisation of the adherent stromal cells and their interactions in maintaining the proliferation of hemopoeitic stem cells. *Am J Anat*, 164: 91-111.

TSE WT, BEYER WM, EGALKA MC, GUINAN EC (2003). Suppression of allogeneic T-cell proliferation by human marrow stromal cells: implications in transplantation. *Transplantation,* 75: 389-397.

UCCELLI A, PISTOIA V (2006). Immunoregulatory function of mesenchymal stem cells. *Eur J Immunol*, 36: 2566-2573.

UCHIDA S, AKIYAMA S, MIYAMOTO M, JUJI T, FUJIWARA M (1994). Suppressive effect of cyclophosphamide on the progression of lethal graft-versus-host disease in mice-a therapeutic model of fatal post-transfusion GVHD. *Ther Immunology*,1: 313-318.

URBAN, V. (2008). "Mesenchymal stem cells cooperate with bone marrow cells in therapy of diabetes. *Stem Cells,* 26: 244-253.

URBAN VS, KOVACS J, VAS V, MONOSTORI E, UHER F (2008). Mesenchymal stem cells cooperate with bone marrow cells in therapy of diabetes. *Stem Cells*, 26(1): 244-253.

VAN DE WINKEL JGJ, A. C. (1991). Biology of human immunoglobulin G Fc receptors. *J Leukoc Biol,* 49: 511.

VAN MEIRVENNE S, HEIRMAN C, STRAETMAN L, MICHIELS A, THIELEMANS K (2005). In vivo depletion of CD4+CD25+ regulatory T cells enhances the antigen-specific primary and memory CTL response elicited by mature mRNA-electroporated dendritic cells. *Mol Ther,* 12(5): 922-932.

VARNEY VA, SUDDERICK RM, ROBINSON DS, IRANI AMA, SCHWARTZ LB, MACKAY IS, KAY AB, DURHAM SR (1992). Immunohistology of the nasal mucosa following allergen-induced rhinitis: identification of activated T lymphocytes, eosinophils and neutrophils. *Am Rev Resp Dis,* 146: 170-176.

VENGE J, HAKANSSON L, RAK S, VENGE P (1994). Identification of IL-5 and RANTES as the major eosinophil chemoattractants in the asthmatic lung. *J Allergy Clin Immunol,* 97(5): 1110-1115.

VON EHRENSETIN OS, ILLI S, BAUMANN L, BOHM O, VON KREIS (2000). Reduced risk of hay fever and asthma among children of farmers. *Clinical and Experimental Allergy,* 30: 187-193.

VOJTOVA J, KAMANOVA J, SEBO P. Bordetella adenylate cyclase toxin: a swift saboteur or host defence. *Curr Opin Microbiol,* 9: 69-75.

WAN YY, F. R. (2007). Regulatory T cell functions are subverted and converted owing to attenuated FoxP3 expression. *Nature,* 445: 766-770.

WANG Y, YE Z, XIE H, ZHENG S (2009). Bone marrow-derived mesenchymal stem cells inhibit acute rejection of rat liver allografts in association with regulatory T cell expansion. *Transplant Proc,* 41(10): 4352-4356.

WARK PA, BUCCHIERI F, POWELL R, PUDDICOMBE S, LAXA-STANZA V, HOLGATE ST, DAVIES DE (2005). Asthmatic bronchial epithelial cells have a deficient innate immune response to infection with rhinovirus. *J Exp Med,* 201: 937-947.

WEN FQ, LIU X, ZHU YK, WANG H (2002). Interleukin-4 and interleukin-13-enhanced transforming growth factor-beta2 production in culture human bronchial epithelial cells is attenuated by interferon-gamma. *American Journal of Respiratory Cell and Molecular Biology*, 26(4): 484-490.

WENZEL, S. (2006). Asthma: defining of the persistent adult phenotypes. *Lancet,* 368: 804-813.

WILDER JA, BS WILSON, DE BICE, CR LYONS, MF LIPSCOMB (1999). Dissociation of airwat hyperresponsiveness from immunoglobulin E and airway eosinophilia in a murine model of allergic asthma. *American Journal of Respiratory Cell and Molecular Biology*, 20(6): 1326-1334.

WILLS-KARP M, XU X, SCHOFIELD B, NEBEN TY, KARP CL (1998). Interleukin-13: Central mediator of allergic asthma. *Science,* 282(5397): 2258-2261.

WISNEWSKI A, MAGOSKI N, WANG H, HOLM C, REDLICH C. (2001). Human gamma/delta T-cell lines derived from airway biopsies. *Am J Respir Cell Mol Biol,* 24(3): 332-338.

WU GS, JIN YS, BARR ML, YU H, STARNES VA, CRAMER DV (2003). Migration of mesenchymal stem cells to heart allografts during chronic rejection. *Transplantation,* 75: 679-685.

XU J, MORA AL, JOODI R, BRIGHAM KL, IYER S, ROJAS M (2007). Prevention of endotoxin-induced systemic response by bone marrow-derived mesenchymal stem cells in mice. *Am J Physiol Lung Cell Mol Physiol*, 293(1): 131-141.

XU L., KITANI, A., FUSS, I. & STROBER, W. (2007). Cutting edge: regulatory T cells induce CD4+CD25–Foxp3– T cells or are self-induced to become Th17 cells in the absence of exogenous TGF-β. *J. Immunol.* 178:6725–6729.

YOUNG HW, EVANS CM, DICKEY BF, BLACKBUM MR (2006). A3 adenosine receptor signaling contributes to airway mucin secretion after allergen challenge. *Am J Respir Cell Mol Biol* 35: 549-558.

YUNG-CHANG S, COOLEY MA, SEWELL WA (2006). Cyclophosphamide augments inflammation by reducing immunosuppression in a mouse model of allergic airway disease. *J Allergy Clin Immunol*, 117: 635-641.

ZACHARIADIS O, CASSIDY. J., BRADY J, MAHON BP (2006). Gammadelta T cells regulate the early inflammatory response to Bordetella pertussis infection in the murine respiratory tract. *Infection and Immunity*, 74(3): 1837-1845.

ZAPPIA, E. (2005). Mesenchymal stem cells ameliorate experimental autoimmune encephalomyelitis inducing T-cell anergy. *Blood,* 106: 1755-1761.

ZARETZKY F, HEWLETT E (2002). Mechanism of association of adenylate cyclase toxin with the surface of Bordetella pertussis: a role for toxin-filamentous haemagglutinin interaction. *Molecular Microbiology*, 45(6): 1589-1598.

ZHANG W, LI C (2004). Effects of mesenchymal stem cells on differentiation, maturation and function of huamn monocyte-derived dendritic cells. *Stem Cells Dev*, 13: 263-271.

ZHAO F, LIU YG, ZHOU JJ, LI ZK, WU CG, QI HW (2008). Therapeutic effects of bone marrow-derived mesenchymal stem cells engraftment on bleomycin-induced lung injury in rats. *Transplant Proc,* 40(5): 1700-1705.

ZHAO Y, GAO Y-D, GUO W (2010). Th17 immunity in patients with allergic asthma. *Int Arch Allergy Immunol,* 151: 297-307.

ZHOU X, BAILEY-BUCKTROUT, JEKER LT, BLUESTONE JA. (2009). Plasticity of $CD4^{+}FOXP3^{+}$ T cells. *Curr Opin Immunol*, 21:281-285.

ZUANY-AMORIM C, LEDUC D (1995). Interleukin-10 inhibits antigen-induced cellular recruitment into the airways of sensitised mice. *J Clin Invest*, 95: 2644-2651.

ZUCKERMAN KS, W. M. (1983). Extracellular matrix production by the adherent cells of long-term murine bone marrow cultures. *Blood*, 61(3): 540-547.

Table 2.1 Summary of media for cultured cells

Media	Culture media	Supplier
Isolation of murine MSC	RPMI 1640	Invitrogen-Gibco
	100 U/ml penicillin	Invitrogen-Gibco
	100 μg/ml streptomycin	Invitrogen-Gibco
	2mM L-glutamine	Invitrogen-Gibco
	10% (v/v) horse serum	Hyclone
	10% (v/v) heat inactivated low-endotoxin foetal calf serum (FCS)	Hyclone
Expansion of murine MSC	Minimum Essential Medium (MEM)	Invitrogen-Gibco
	10% (v/v) heat inactivated low endotoxin FCS	Hyclone
	10% (v/v) horse serum	Hyclone
	100 U/ml penicillin	Invitrogen-Gibco
	100 μg/ml streptomycin	Invitrogen-Gibco
	2mM L-glutamine	Invitrogen-Gibco
Expansion of murine transgenic GFP MSC	Mesencult Basal Medium	Stem Cell Technologies
	10% murine mesencult supplement	Stem Cell Technologies
	1% (v/v) penicillin/streptomycin	Invitrogen-Gibco
Osteoblast Differentiation	Minimum Essential Medium alpha (MEMα)	Invitrogen-Gibco
	1mM dexamethasone,	Sigma-Aldrich
	20mM β-glycerolphosphate,	Sigma-Aldrich
	50μM L-ascorbic acid-2-phosphate,	Sigma-Aldrich
	50ng/ml L-thyroxine sodium pentahydrate.	Sigma-Aldrich

Table 2.1 Summary of media of cultured cells continued

Media	Culture media	Supplier
Adipocyte Differentiation	Minimum Essential Medium alpha (MEMα)	Invitrogen-Gibco
	5.0µg/ml insulin in 0.1N acetic acid	Sigma-Aldrich
	50µM indomethacin	Sigma-Aldrich
	1µM dexamethasone	Sigma-Aldrich
	0.5µM 3-isobutyl-1-methylxanthine (IBMx) in methanol	Sigma-Aldrich
Chondrogenic Medium	Dulbecco's Modified Eagle's Media (DMEM) containing 100 mg/ml glucose	
	100nM dexamethasone	Invitrogen-Gibco
	50µg/ml ascorbic-acid-2-phosphate	Hyclone
	40µg/ml proline	Invitrogen-Gibco
	1mM sodium pyruvate	Invitrogen-Gibco
	(1:99) ITS + supplement	
	10ng/ml TGF-β3	
Human MSC culture	Dulbecco's Modified Eagle's Media (DMEM) containing 100 mg/ml glucose	
	10% hi FCS	
	100 U/ml penicillin	
	100 µg/ml streptomycin	
Human Eosinophil Cell Line (EOL-1)	RPMI 1640	Invitrogen-Gibco
	15% fetal bovine serum (FBS)	Hyclone
	1% L-glutamine	Invitrogen-Gibco

Table 2.2 Summary of media for bacterial growth

Strain	Culture medium	Components	Concentration	Supplier
***Bordetella pertussis* (BPSM)**	Bordet Gengou agar	Bordet Gengou Agar	30g/L	BD-Difco
		Glycerol	2.5ml/L	Sigma-Aldrich
		Sterile defibrinated horse blood	50ml/L	Unitech, Dublin, Ireland
		dH_2O		
	Stainer & Scholte (S&S) liquid medium	L-Glutamic acid,	10.72g/L	Sigma-Aldrich
		L-Proline,	20mg/L	Sigma-Aldrich
		Sodium chloride	2.5g/L	BDH
		Potassium dihydrogen phosphate	0.5g/L	Merck
		Potassium chloride	0.2g/L	Merck
		Magnesium chloride,	0.1g/L	Merck
		Calcium Chloride	20mg/L	BDH
		Tris Base	1.525g/L	Sigma-Aldrich
		dH_2O		
		pH adjusted to 7.4		
	S&S supplement	L-cystine	0.4g/L	Sigma-Aldrich
		HCl	1.0ml/L	Merck
		Iron sulphate	0.1g/L	Sigma-Aldrich
		Ascorbic acid	0.2g/L	Sigma-Aldrich
		Nicotinic acid	40,g/L	Sigma-Aldrich
		Glutathione (reduced)	1.0g/L	Sigma-Aldrich
		dH_2O		
***Bordetella pertussis* (BPZE1)**	As above (BPSM)			

Table 2.3 Summary of buffer components and concentrations

Application	Buffer	Components	Concentration	Supplier
ELISA	Phosphate Buffered Saline (PBS) (10x)	Sodium chloride Potassium chloride di-Sodium orthophosphate Potassium dihydrogen orthophosphate dH_2O pH was adjusted to 7.0	1.37M 27mM 200mM 20mM	BDH Merck BDH Riedel-deHaen
ELISA	Wash	Tween 20 PBS pH adjusted to pH 7.4	0.05% (v/v)	Sigma-Aldrich
ELISA	Carbonate coating	Sodium carbonate Sodium bicarbonate dH_2O pH was adjusted to 9.6	100mM 100mM	Sigma-Aldrich Sigma-Aldrich
ELISA	Blocking	Milk powder PBS	10% (w/v)	Marvel, Chivers Ireland Ltd, Dublin
ELISA	Diethanolamine Buffer	Magnesium chloride Sodium azide Diethanolamine pH was adjusted to 9.8 dH_2O	9.52M 0.5mM 9.7% (v/v)	Merck Merck Sigma-Aldrich
ELISA	Stop solution	1M Sulphuric acid		Sigma-Aldrich

Table 2.3 Summary of buffer components and concentrations continued

Application	Buffer	Components	Concentration	Supplier
FISH	Sodium thiocyanate	Sodium thiocyanate dH_2O	8% (w/v)	Sigma-Aldrich
FISH	Digestion	Pepsin in 0.1M HCl	0.4% (w/v)	Sigma-Aldrich Merck
FISH	Quenching	Glycine PBS	0.2% (w/v)	BDH
FISH	Sodium Saline Citrate (4XSSC)	Sodium saline citrate dH_2O	1% (w/v)	Sigma-Aldrich
FISH	Stringency wash solution 2XSSC	diluted from stock SSC	0.5% (w/v)	
FISH	Detergent wash solution	10% Tween 20 4X Sodium saline citrate.	0.05% (w/v)	Sigma-Aldrich Sigma-Aldrich

Table 2.4 Summary of staining solutions

Stain	Components	Concentration	Supplier
H&E	Eosin Y	1% (w/v)	Sigma-Aldrich
	Potassium dichromate	1.6% (w/v)	
	dH_2O		
	Hydrochloric acid	1% (v/v)	BDH
	2-Propanol	69.3% (v/v)	Riedel-deHaen
	dH_2O		
Combined stain	Alcian blue	0.5% (w/v)	Sigma-Aldrich
	Acetone	3% (v/v)	
Discombes eosin	Eosin	0.05% aq (w/v)	Sigma-Aldrich
	Acetone	5% Acetone	Sigma-Aldrich
Scotts tap water	Sodium bicarbonate	0.2% (w/v)	Sigma-Adrich
	Magnesium sulphate	1% (w/v)	Sigma-Adrich
	dH_2O		
Fat differentiation	Oil Red O	0.5% (w/v)	Sigma-Aldrich
	Isopropanol		
	Working solution:		
	0.5% Oil Red O stock	30mls	
	PBS	20mls	
Bone differentiation	Alizarin Red S stain	1% (w/v)	Sigma-Aldrich
	dH_2O		
	pH adjusted to between 4.1-4.3 using 0.1% ammonium hydroxide.		

Table 2.5 Summary of antibodies and concentrations of use

Application	Name	Concentration	Supplier
Flow cytometry	Anti-mouse CD4 Cy5	2µl/ml	eBiosciences
	Anti-mouse CD8 APC	2µl/ml	eBiosciences
	Anti-mouse CD25 FITC	2µl	Immunotools, Friesoythe, Germany
	Anti-mouse CD28 PE	2µl/ml	eBiosciences
	Anti-mouse TCRα/β FITC	2µl	Immunotools
	Anti-mouse TCRγ/δ FITC	2µl	Immunotools
	Anti-mouse CD16,CD56 NK PE	2µl	Immunotools
	Anti-mouse FoxP3 PE	2µl/ml	eBiosciences
	Anti-mouse CTLA4 PE	2µl/ml	eBiosciences
	Anti-mouse GITR PE	2µl/ml	eBiosciences
	Anti-mouse CD105 Biotin	5µl/ml	eBiosciences
	Anti-mouse CD106 FITC	5µg/ml	eBiosciences
	Anti-mouse CD44 PE	2µg/ml	eBiosciences
	Anti-mouse CD45 FITC	5µg/ml	Immunotools
	Anti-mouse Sca-1 PE	2µg/ml	eBiosciences
	Anti-mouse CD117 PE	2µl	Immunotools
	Anti-human ICAM-1 PE	5µg/ml	eBiosciences
	Anti-human ICAM-3 PE	5µg/ml	eBiosciences
	Anti-mouse MHC Class I PE	2µg/ml	eBiosciences
	Anti-mouse MHC Class II PE	1µg/ml	eBiosciences
	Anti-mouse CD80 PE	2µg/ml	BD Pharmingen
	Anti-mouse CD86 PE	2µg/ml	eBiosciences
	Anti-mouse CD11b FITC	2µl	Immunotools
	Anti-mouse CD34 Biotin	5 µg/ml	BD Biosciences
	Anti-human CCR3 PE	5µg/ml	R&D Systems
	Anti-mouse NK cells	2µl	Immunotools

Table 2.6 Primer sequences

Primer	**Forward 5'-3'**	**Reverse 3'-5'**	**Product Size (bp)**	**Annealing temp (°C)**	**$MgCl^{2+}$**
IL-10	AGGTGCGTTCCTC	AAAGCCAACCAA	241	55	2.5 mM
HGF	CATTCAAGGCCAAGGAGAAG	AACTCGGATGTTTGGGTCAG	208	53.5	2.5 mM
GAPDH	GGTGAAGGTCGGAGTCAACG	AAAGTTGTCATGGATGACC	540	55	2.5 mM
TGF-β	TGACGTCACTGGAGTTGTACGG	GGTTCATGTCATGGATGGTGC	170	60	2.5 mM