The role of early life infection on the programming of CD4+ T-cells

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BSc (Biotechnology) BBmedSc (Honours)

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April 2012
STATEMENT OF ORIGINALITY

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ACKNOWLEDGEMENTS

I would like to express my appreciation to my supervisors Prof. Paul Foster and Prof. Phil Hansbro. To Paul, thank you for all your help and encouragement, it’s been a long haul. To my long-suffering family and friends for their ongoing support and kindness, i am extremely grateful. To Andrew, who has put up with the most throughout this process, I hope i can make it up to you in our life together post-PhD.
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<tr>
<td>2-ME</td>
<td>2-mercaptoethanol</td>
</tr>
<tr>
<td>AAI</td>
<td>allergic airways inflammation</td>
</tr>
<tr>
<td>AAD</td>
<td>allergic airways disease</td>
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<tr>
<td>Ab</td>
<td>antibody</td>
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<td>ACCM</td>
<td>animal cell culture medium</td>
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<td>AHR</td>
<td>airways hyper-responsiveness</td>
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<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
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<td>APC</td>
<td>antigen-presenting cell</td>
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<td>Aro</td>
<td>aromatic prechorismate pathway</td>
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<tr>
<td>BALF</td>
<td>broncho-alveolar lavage fluid</td>
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<tr>
<td>BCA</td>
<td>bicinchoninic acid</td>
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<td>BCG</td>
<td><em>Mycobacterium bovis</em> (Bacille Calmette Guerin)</td>
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<td>BSA</td>
<td>bovine serum albumin</td>
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<td>CD</td>
<td>cluster of differentiation</td>
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<td>CFU</td>
<td>colony forming units</td>
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<td>COPD</td>
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<td>d</td>
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<td>dendritic cell</td>
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<td>ELISA</td>
<td>enzyme linked immunosorbant assay</td>
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<td>FACS</td>
<td>fluorescence-activated cell sorting</td>
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<td>foetal calf serum</td>
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<td>G</td>
<td>gravity</td>
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<tr>
<td>GI</td>
<td>gastro-intestinal</td>
</tr>
<tr>
<td>GINA</td>
<td>global initiative for asthma</td>
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<tr>
<td>GM-CSF</td>
<td>granulocyte-macrophage colony stimulating factor</td>
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<tr>
<td>HBSS</td>
<td>Hank’s buffered salt solution</td>
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<tr>
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<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
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<td>i.n.</td>
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<td>inducible T-regulatory cell</td>
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<td>IVC</td>
<td>individually ventilated cage</td>
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<tr>
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<td>Luria-Burtani</td>
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<td>LH</td>
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<td>lipopolysaccharide</td>
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<td>m</td>
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<td>mDC</td>
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<td>MSC</td>
<td>mucous secreting cell</td>
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<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>-------------</td>
<td>------------------------------------------------</td>
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<tr>
<td>NEA</td>
<td>non-eosinophilic asthma</td>
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<tr>
<td>NK</td>
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<td>natural killer T-cell</td>
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<td>PAMP</td>
<td>pathogen associated molecular pattern</td>
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<td>periodic acid Schiff</td>
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<td>PBLN</td>
<td>peri-bronchial lymph node</td>
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<td>PBS</td>
<td>phosphate buffered saline</td>
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<td>pDC</td>
<td>plasmacytoid dendritic cell</td>
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<td>PMN</td>
<td>polymorphonuclear</td>
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<td>PP</td>
<td>Peyer’s patch</td>
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<td>PRR</td>
<td>pathogen recognition receptor</td>
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<td>RBC</td>
<td>red blood cell</td>
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<td>RPMI</td>
<td>Roswell Park Memorial Institute</td>
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<td>RT</td>
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<tr>
<td>SEM</td>
<td>standard error of the mean</td>
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<td>SPBS</td>
<td>sterile phosphate buffered saline</td>
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<td>specific pathogen free</td>
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<td>STAT-6</td>
<td>Signal transducer and activator of transcription-6</td>
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<td>Tg</td>
<td>transgenic</td>
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<td>TCR</td>
<td>T-cell receptor</td>
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<td>TGF</td>
<td>transforming growth factor</td>
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<td>Th</td>
<td>T helper</td>
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<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TMB</td>
<td>Tetra-methyl benzidine</td>
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<tr>
<td>TNF</td>
<td>tumour necrosis factor</td>
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<td>Tregs</td>
<td>regulatory T-cell</td>
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<td>VEGF</td>
<td>vascular endothelial growth factor</td>
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<td>white blood cell</td>
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<td>white cell count</td>
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<td>WHO</td>
<td>World Health Organisation</td>
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<tr>
<td>WT</td>
<td>wild-type</td>
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Conference proceedings arising from this work

Conference presentations


Conference poster presentations


Competitive grants arising from this work

Professor Paul Foster, **Ms Angela Ferguson**. (2006)- ‘CD4+T-cell programming in early life and neonatal infection’. GSKA Post Graduate Support Grant, GlaxoSmithKline Australia, Boronia, Vic. ($A25, 000 over two years).
ABSTRACT

Asthma is a chronic inflammatory disease of the airways that is characterised by activation of CD4+ T-helper 2 type (Th) cells and eosinophils. The cause of this aberrant Th2 response is unknown but lack of early life infection is thought to play a significant role. The timing of infection and the type of pathogen may be critical to programming the immune response to a protective Th1, or destructive Th2, phenotype.

The immune responses to infection with Salmonella typhimurium and Mycobacterium bovis Bacille Calmette Guerin (BCG) have been identified as targets for reprogramming or preventing the development of asthma. However, the role of these infections in contributing to a Th2-Th1 switch or suppression of this response remains limited. In this investigation ovalbumin (OVA) T-cell receptor (TCR) transgenic (Tg) mice in combination with these bacterial strains expressing OVA have been used to specifically track the affects of each infection as well as OVA exposure on the T-cell response and the development of allergic airways disease (AAD) in the mouse model.

BCG infection as an adult and a neonate prior to OVA challenge induced significant reductions in eosinophils in broncho-alveolar lavage fluid (BALF) and lung tissue compared to sham-infected mice that received OVA challenge. However, high levels of both Th1 (interferon gamma (IFN-γ)) and Th2 (interleukin (IL)-4, IL-5, IL-13) cytokines from supernatants of cultured peri-bronchial lymph node (PBLN) cells and splenocytes were found in all groups examined. Further studies tracking the development of the immune system after BCG infection at birth without OVA exposure revealed significant decreases in lung tissue eosinophils and decreased immunoglobulin (Ig) G1, IgG2a and IgE levels from serum compared to sham-infected controls. This coincided with decreased numbers of CD4+ and CD8+ T-cells in the spleens and PBLN cells. Levels of cytokines in splenocytes and PBLN cell cultures failed to show significant trends toward either a polarised Th1 or Th2, leaving a mixed Th1/Th2 phenotype.

Infection with S.typhimurium lowered eosinophil levels in BALF, and mucous secreting cell (MSC) and eosinophil number in lung tissue after challenge with
OVA, compared to sham-infected mice challenged with OVA. In mice infected as neonates and adults prior to OVA challenge increased levels of IFN-γ from splenocyte culture supernatants were found, compared to sham-infected OVA challenged controls. Decreased levels of IL-5 from splenocyte culture supernatants was found in neonates but not adult mice infected with S. typhimurium prior to OVA challenged compared to sham-infected OVA challenged controls. High levels of both Th1 and Th2 cytokines were present in splenocyte and PBLN culture supernatants from all groups tested, indicating a mixed Th1/Th2 phenotype rather than a profound switch to Th1 immune response. Further studies showed that infection with S. typhimurium at birth without OVA exposure causes changes to the development of the neonatal immune system resulting in decreased eosinophil numbers in BALF and lung tissue, decreased levels of serum IgG1 and IgG2a, and a shift from Th2 to a mixed Th1/Th2 cytokine profile. These changes were found in samples examined up to 9-weeks post infection.

This investigation demonstrates that infection with BCG or S. typhimurium can alter the immune system resulting in attenuation of various immunological and patho-physiological features of asthma. Infection with BCG or S. typhimurium as a neonate appears to produce the most pronounced modification in the subsequent immune responses to OVA. These findings provide important insights into possible modified vaccination regimes at birth and during childhood, which may have the potential to prevent the development of asthma and allergic inflammatory disorders in adulthood.
CHAPTER 1

General Introduction: Asthma
1.1 Synopsis

In 2004 the Global Initiative for Asthma (GINA) in conjunction with the World Health Organisation (WHO) published a study on the global asthma burden. It was conservatively estimated that 300 million people worldwide had asthma. This translated into 14.7% of the Australian population, and above 10% for the populations of United Kingdom, Canada, New Zealand and the USA. It was observed that the rate of asthma increases as communities adopt Western lifestyles and become urbanised (2).

Understanding the mechanisms that underpin the programming of T-lymphocytes in childhood is fundamental to understanding the development of immune responses that lead to immunity and potentially aberrant responses (e.g. asthma). It is well established that T-cell programming is controlled by activation of innate and adaptive immune systems (3, 4). However, how infection at different periods of life influences the development of antigen specific T-cells and subsequently inflammatory disease such as asthma remains unknown.

The neonatal immune system is strongly biased towards a CD4+ Th2 response (5). It is hypothesised that exposure of the immune system to different microbial antigens is required for the maturation and the generation of Th1 immune responses that control infection and provide a counter balance to Th2 responses. Studies have shown that certain bacterial infections (e.g. Mycobacterium bovis (Bacille Calmette Guerin) or BCG) early in life direct the naïve immune system toward a Th1 response (6).

1.2 Allergic airways disease

Asthma is characterized by airways obstruction, which is variable and reversible (7). There is chronic inflammation of the airway wall, which is controlled by increased expression of inflammatory molecules such as cytokines, antibodies and chemokines (8).

Asthmatic patients experience acute and chronic exacerbations, which involve an increase in the intensity of inflammation. This inflammation is mainly in
the larger airways, which is where asthma differs from other airways diseases such as COPD (chronic obstructive pulmonary disease) and bronchiectasis (9).

Asthma is at times categorised as either atopic, in which exacerbations are triggered by extrinsic factors such as allergens, environmental factors and infection. Non-atopic asthma is usually more severe and is believed to be triggered by internal, physiological factors.

The inflammatory response in asthma is mainly characterised by the presence of increased numbers of activated Th2 lymphocytes, eosinophils and mast cells (10, 11). Elevated levels of eosinophils have been found in bronchial biopsies, BALF and blood of patients with asthma (12). Other cells thought to be important to the asthmatic inflammatory response are neutrophils (13-15) and mast cells (16, 17). In addition to the presence of these inflammatory cells in the airways, patients with asthma exhibit varying degrees of structural changes termed ‘remodelling’. These changes include mucous cell hyperplasia, smooth muscle hypertrophy, fibrosis and angiogenesis. These changes lead to a decrease in lung function over time, increasing the severity and frequency of asthmatic exacerbations (18).

Asthma can be sub-categorised by the inflammatory cells that are present in the lungs and blood of patients. In 2001 Gibson et. al, identified a role for neutrophilic inflammation in non-eosinophilic asthma. This study disproved the long held idea that most asthma is defined by the presences of eosinophils. In fact the typical eosinophilic pattern occurred in the minority of subjects, approximately 41% compared to 59% in induced sputum cell counts (13). Simpson et al in 2006 further investigated asthmatic subtypes as defined by cell profiles from induced sputum samples. 93 asthmatic subjects were classified into 4 groups according to their sputum cells. Subjects with a sputum neutrophil proportion of more then 61% were classified as neutrophilic asthma and those with an eosinophil proportion greater then 1.01% were classified as eosinophilic asthma. The other subtypes were defined as mixed granulocytic asthma, where patients exhibited both increased neutrophils and eosinophils, and paucigranulocytic asthma were patients that had normal levels of both neutrophils and eosinophils (19). The heterogeneity of non-
eosinophilc asthma (NEA) is confirmed by this study (19). When separated into the 3 subtypes of non-eosinophilic asthma a significant difference was found in the number of neutrophils and eosinophils, and the percentages of columnar and squamous epithelial cells from induced sputum samples (19).

1.3 **Histopathology of the airways**

Bronchial biopsies from asthmatic patients reveal infiltration of activated eosinophils, increased mucous secreting cells, mast cells and T-cells (20). Histological examination of lung tissue demonstrates collagen deposits under the epithelium, which are associated with basement membrane thickening. There is also thickening of the smooth muscle layer, which can enlarge to 3 times its normal size due to hyperplasia and hypertrophy. Alteration in smooth muscle mass is more commonly seen in patients with severe disease. In some biopsies, epithelial cell shedding is also observed as these cells detach more easily from the basement membrane of asthmatics. An increase in the number of mucous secreting cells such as mucous cells or goblet cells in the epithelium or mucous cell hyperplasia, along with an increase in the size of the sub-mucosal glands are commonly seen in lung sections of asthmatic patients (20). Changes in airway tissue that contribute to asthma can be categorised as follows:

1.3.1 **Angiogenesis**

Angiogenesis is an increase in the amount of blood vessels as a result of increased levels of the chemokine VEGF (vascular endothelial growth factor). An inverse relationship was found between high VEGF levels (vascular remodelling) and low post-bronchodilator levels in asthmatic patients, which suggests that vascular remodelling is associated with airflow obstruction (21).

1.3.2 **Airway remodelling**

Airway remodelling is the term for structural changes in the airway that can be attributed to: epithelial desquamation or hyperplasia, increases in smooth muscle mass, angiogenesis, increases in subepithelial collagen deposition, changes in
elastan mass, goblet cell and glandular hyperplasia, plus airway wall thickening (22). Goblet cell metaplasia and glandular enlargement can explain the excess mucous associated with asthma. Abundant data indicate that airway smooth muscle mass increases in both human asthmatics as well as in animals with experimental asthma (23). Structural changes in the airway are thought to have a major contribution on the development and continuing symptoms of asthma and AAD in the mouse.

1.3.3 Epithelial structure

Dramatic changes are seen in epithelial cell structure in asthmatic airways. In non-asthmatic airways there is an organised matrix of pseudo-stratified columnar epithelial cells, goblet cells and basal cells (24). Ciliated epithelial cell structure and function is impaired in asthmatics. This is demonstrated when examining the sputum of asthmatic patients, which often contain large amounts of degraded epithelial cells that have shed from the basement membrane (25). Remaining epithelial cells although attached can still be damaged, fragile and swollen. In regions of epithelial shedding, a thickened basal lamina is observed, with only basal cells remaining attached (26).

Histopathological examination of goblet cells shows hyperplasia and hypertrophy (27). Between the epithelial cell lining and the basal lamina, infiltrating leukocytes such as mast cells and eosinophils can be observed.

1.3.4 Basement membrane

A thickened basement membrane is another pathological feature of the asthmatic airway. Thickening of the basement membrane is partly caused by sub-epithelial collagen deposition. Immunohistochemical studies indicate that deposits of collagen are commonly found in the reticular layer of the basement membrane in asthmatics (28). There are significant increases in the number of myofibroblast cells, the regulators of collagen deposition, located in the subepithelial region of the airways in asthmatic patients. This supports the association between an increase
in the number of myofibroblast cells and the thickening of the reticular layer of the basement membrane found in asthmatic airways (29).

1.3.5 Mucous secreting cells

Hypersecretion of mucous plays a central role in the pathogenesis of severe episodes of asthma and leads to airway obstruction. Mucins are the major constituents of the mucous that forms in the airway (30, 31). They are mainly produced by goblet cells and mucous glands. Moderate and mild asthma are associated with an increase in the amount of stored mucin in epithelial cells (20). The mucous over-production observed in asthmatic airways is due primarily to the hypertrophy and hyperplasia of sub-mucosal glands and goblet cells. This is controlled by many factors including the cytokines IL-4 and IL-13.

The fluid-lining of the airways in non-asthmatic patients contains very little mucous, this is indicative of the low number of goblet cells and lower secretory level of these cells. Also, in the non-asthmatic airway mucous can be rapidly cleared by healthy cilia present on epithelial cells (32). The build-up of mucous in asthmatic patients is due to the reduced rate in mucous clearance caused by the loss of ciliated epithelial cells along with mucous hypersecretion. This promotes the formation of mucous plugs and airway obstruction (33, 34).

1.3.6 Smooth muscle

Asthmatics typically display a thickening of the bronchial wall, which is believed to be associated with an increase in smooth muscle mass (20, 35). This increase in smooth muscle mass and the degree of airway wall thickening can vary significantly between asthmatic individuals. The thickening of the smooth muscle layer can enlarge to 3 times its normal size, this is more commonly seen in patients with severe disease. The mechanism of this pathology remains unclear, however it may be due to a combination of hypertrophy (increase in size) and hyperplasia (increase in number) of the smooth muscle (36).

Smooth muscle cells are thought to participate in inflammatory and remodelling processes through the release of pro-inflammatory cytokines and chemokines, and
by expression of adhesion molecules (37). Therefore an increase in airway smooth muscle can account for increases in inflammatory processes in severe asthmatics.

**Inflammatory cells and mediators-**

1.4.1 **T -Lymphocytes**

Although many types of T lymphocytes can respond to allergen stimulation (CD4+, CD8+ and natural killer T-cell (NKT)), CD4+ lymphocytes have the most critical involvement. It is well documented that CD4+ T-cells are centrally involved in all stages of asthma from initiation, to acute exacerbations and persistence of disease. CD4+ T lymphocytes exert their effects through production of IL-4, IL-5, IL-13, IL-3, IL-9 and granulocyte-macrophage stimulating factor (GM-CSF). IL-4 is central to the development of Th2 cells and mediates antibody (Ab) isotype switching to produce Th2 antibodies such as IgE (10, 38).

The role of CD8 T-cells in asthma is less well-defined but it is known that these cells can regulate some inflammation in asthma (continued 1.4.3).

1.4.2 **CD4+ T lymphocytes**

T-cells play a crucial role in immune responses to vaccination, pathogens and the induction of tolerance. Two major types of T helper cells (Th1 and Th2 cells) have been identified based on the profile of cytokines that they secrete and the distinct immune responses consequently stimulated (3). Th1, to cell-mediated immunity and CD8+ cytotoxic T-cell activity and Th2, primarily to humoral immunity. The characteristic cytokines that are products of these T-cell responses are IFN-γ and IL-2 for Th1, which is a typical response to intracellular pathogens such as mycobacteria (e.g. BCG), Salmonella and viruses. IL-4, IL-5, IL-10 and IL-13 for Th2 in response to extracellular pathogens such as *H.pylori* and helminths. Initial development of Th1 and Th2 responses depends on antigenic stimulation and the surrounding environment. This is especially dependant on the neighbouring cytokine milieu as it is known that Th2 cytokines down-regulate Th1 cells, and promote their own development. Similarly, IFN-γ and IL-2 (Th1
cytokines) have the reverse effect by down-regulating Th2 cells, and promoting the development of Th1 responses (39).

Strong Th2 responses are associated with the expansion of eosinophils, mucous hypersecretion and airways hyper-responsiveness that are hallmark features of allergic asthma (See figure 1.1). IL-5 is responsible for eosinophil differentiation, and therefore has a central role in allergic airways inflammation (AAI). The production of Th2 cytokines (IL-4) by CD4+ T-cells drive the production of IgE by B-cells.

Recently this model has been expanded to include a third type of polarised Th cell with its own distinct cytokine response and function. This Th cell type has been assigned as Th17 after the signature cytokine it produces, IL-17. Th17 cells are thought to play a role in autoimmune diseases and the immune response to some pathogens. Th17 responses typically involve an increase in neutrophilia, increases in the cytokines IL-17, IL-10, IL-23 and IL-6. This cytokine profile in turn initiates a CD4+CD25+ natural Regulatory T-cell response which among other things can cause a decrease in Th2 cell number (8).

It is currently believed that Th17 cells may contribute to allergic airways disease (AAD). IL-17A (a key Th-17 cytokine) measured by mRNA and protein levels are found to be significantly increased in asthmatic patient compared to non-asthmatic controls (40-43). It is believed that IL-17A is responsible for neutrophil recruitment, which as previously referred to are cells present in patients with severe asthma and understood to be involved in the asthmatic response (44, 45). IL-17 has also been reported to inhibit Th2 responses, thus increasing the interest surrounding Th17 responses in asthmatic responses (46).
Figure 1.1  Immune cells in asthma.

Allergen sensitisation results from uptake by Dendritic Cells (DCs). DC’s then migrate to lymphoid tissue and present these antigens to T-cells. Adjuvant signals from airway epithelium such as TSLP (Thymic stromal lymphopoietin) and GM-CSF generated in response to inhaled antigen influence T-cell activation and determine whether a Th2 response is generated or tolerance occurs. These primed T-cells then re-enter the lung where they wait to identify these antigens at the next exposure. Exacerbation of AAI occurs when the airways are re-exposed to allergen. The allergen is taken up by DCs and presented to airway and lymphoid T-cells. These T-cells proliferate and home to the lung where they increase inflammation. Once in the airway allergen can react with IgE and IgG antibodies in the airway leading to mast cell mediator release (e.g. histamine). These mediators lead to airway oedema and bronchoconstriction forming part of the early asthmatic response. However, the full late phase asthmatic response is mainly attributed to cytokine production from T-cells (e.g. IL-5, IL-13, IL-4) and mediator release from eosinophils (e.g. cytotoxic granules, cytokines, chemokines). This results in mucus hyper-secretion, changes in smooth muscle, airway remodelling and chronic inflammation.

In response to allergen sensitisation epithelial cells produce: Stem cell factor (SCF) which leads to mast cell production and CCL-11 (or eotaxin-1 a chemokine) leads to eosinophil recruitment. CCL-17 and CCL-20 chemokines are produced by DC’s and activate T-lymphocytes by inducing chemotaxis. CCR3 is a chemokine receptor highly expressed in eosinophils and basophils, and is also detected in Th1 and Th2 cells, as well as in airway epithelial cells. CCR3 contributes to the accumulation and activation of eosinophils and neutrophils.
Although the immune response to asthma is complicated and only partially understood, it is acknowledged that CD4+ T-cells are the fundamental cell type involved in the initiation of asthma pathogenesis. In asthma there is an increase in CD4+ T-cells in the airway and these are predominantly Th2 cells, whereas in normal airways Th1 cells predominate (47). However, when pulsed with antigen, immature DCs derived from the respiratory tract of rats induce Th2 responses. This demonstrates that polarisation of Th cells at mucosal surfaces is typically skewed towards the Th2 pathway. It is interesting to note that when these immature DCs were pulsed in the presence of GM-CSF, higher levels of IFN-\(\gamma\) and Th1 subclasses of IgG were generated (48). Activated CD4+ Th2 cells comprise the major lymphocyte population in BALF and biopsy samples of asthmatic patients (49, 50). CD4+ T-cells were shown to be crucial in the development of airways hyper-responsiveness (AHR) through animal studies where CD4+ T-cells were completely depleted resulting in the prevention of AAD (51, 52).

The transcription factor GATA-3 is necessary for the differentiation of naïve T-cells into Th2 cells and it also regulates the secretion of Th2 cytokines (especially IL-5) from T-cells. Research shows that asthmatic patients have a higher number of GATA-3+ T-cells in their airways compared to normal subjects (53).

In turn, Th1 cell differentiation and secretion of Th1 cytokines is controlled by the transcription factor T-bet. Accordingly research has shown that T-bet levels are lower on T-cells in the airways of asthmatic patients compared to healthy controls (11). In the absence of allergic sensitization or challenge, mice depleted in T-bet exhibited a phenotype indicative of both acute and chronic asthma (11). T-cell regulation in asthma is affected by many other molecules including IL-27 (from the IL-12 family it regulates GATA-3), IL-33 (from the IL-1 family it promotes Th2 differentiation) and many more.

1.4.3 CD8+ T lymphocytes

A role for CD8+ T-cells may only occur in asthmatics with more severe disease. Their primary role in the immune system is to kill infected cells, as
indicated by their pseudonym cytotoxic T lymphocytes. It is known that CD8+ T-cells have a role in the AAD response. This response is judged to be less significant than the role of CD4+ T-cells, but is also poorly understood. IFN-γ producing CD8+ T-cell numbers were increased in the peripheral blood and BALF of asthmatic patients as well as increased numbers of CD8+ T-cells in the bronchial mucosa in AHR models in mice (54, 55).

The role of CD8+ T-cells in the asthmatic response is poorly understood as it appears to have both negative and positive effects on manifestations of disease. Studies show that most activated CD8+ T-cells present in the murine ovalbumin (OVA) sensitised and challenged lung secrete high levels of the Th2 cytokines IL-4 and IL-5 but little IFN-γ (56, 57). Although CD8+ T-cells are present in much smaller numbers in the asthmatic lung, this still indicates a potential role of CD8+ cells in promoting disease. Clinical studies support this finding and show a direct relationship between CD8+ T-cell numbers and disease severity (58). VanRensen et al identified a correlation between patient decline in airway function and increases in bronchial CD8+ T-cell numbers. Elevated CD8+ T-cell levels in the BALF levels of wheezy infants (59) and in patients who died of asthma (60) further suggests an association between CD8+ T-cells and asthma severity (61, 62).

These results, however, are in conflict with studies that report CD8+ T-cells as having a suppressive role in AHR and AAD. Huang et al determined that CD8+ T-cells have a protective role in allergen-induced AHR and eosinophilic inflammation. They came to this conclusion after depleting CD8+ T-cells in rats given AAD through sensitisation and challenge with OVA. After CD8+ depletion Th1 cytokine expression was significantly reduced in all animals while Th2 cytokine expression remained unchanged, suggesting that CD8+ T-cells can suppress the asthmatic response through the secretion of Th1 cytokines (62-64).

### 1.4.4 Regulatory T-cells (Tregs)-

Regulatory T-cells (Tregs) have the ability to down-regulate Th2 cell function and therefore reduce airway remodelling and features of disease. There are 2 categories of Tregs; 1) Natural Tregs (nTregs), CD4+CD25+, which comprise 1-5%
of peripheral blood CD4+ T-cells, which have been found to suppress Th2 responses through contact dependant mechanisms, 2) Inducible or adaptive Tregs (iTregs) that suppress Th2 responses by cytokine-dependant mechanisms mediated through IL-10 and transforming growth factor (TGF)-β (65).

Tregs are known to suppress the proliferation of other CD4+ T-cells, the mechanism for this is not completely clear. Tregs also suppress the ability of CD25- CD4+ T-cells to induce autoimmunity, therefore this disease may be caused by lack of Tregs. There have been found to be two main pathways of iTreg generation; non-inflammatory (oral tolerance) and inflammatory (asthma and chronic lung inflammation) (66). Thus, there is potential for beneficial and deleterious roles for Tregs in AAD.

1.4.5 B-cells

B-cells have an important role in allergic asthma as they are responsible for the production of allergen-specific IgE, which is bound by high-affinity Fc epsilon receptors that are expressed by mast cells and basophils. The type 2 cytokines IL-4 and IL-13 induce B-cells to produce IgE by undergoing a class-switch. In studies involving the blocking of IgE it has been shown that the responses to allergens are reduced. This indicates that IgE drives the allergic immune responses in asthma (67).

1.4.6 Macrophages-

Macrophages are white blood cells found in tissues that originate from monocytes. They are well-known for their phagocytic capabilities in both specific and non-specific immunity. Specifically, they are mechanically responsible for engulfing and digesting pathogens, cellular debris created by inflammation and old or ‘worn-out’ white blood cells (WBCs) for cell component recycling. Macrophages also play a primary role in the stimulation of lymphocytes and other immune cells to regulate appropriate immune responses to pathogens and foreign antigens, and can provide secondary signals to promote lymphocyte differentiation and proliferation (e.g. IL-1) (68).
Macrophages are the primary cell found in the BALF in both healthy and asthmatic humans and mice. The role of these resident macrophages in inflammation associated with asthma, however, remains unclear. Macrophages are known to produce the pro-inflammatory cytokines IL-1, tumour necrosis factor (TNF)-α, IL-6 and GM-CSF, which are involved in cell recruitment, eosinophil survival and cellular activation (68, 69). These functions may contribute to the asthmatic response.

1.4.7 Eosinophils-

Eosinophils are granular leukocytes associated with the immune response to parasitic/helminth infections (70, 71). Helminth infections can induce IgE-mediated immune responses leading to eosinophilia and production of Th2 cytokines (72). Secondary granules within eosinophils, primarily major basic protein (MBP), eosinophil cationic protein (ECP), eosinophil-derived neurotoxin (EDN), and eosinophil peroxidase (EPO) contain a range of cationic proteins that have potent cytotoxic effects (72). Eosinophil granule proteins can cause epithelial injury in the airways demonstrating a potentially damaging role for the cell in the airways (73).

Eosinophils are recruited from the circulation into tissue in response to stimuli where they modulate inflammatory responses. Cytokines, complement and immunoglobulins can all act as triggers for eosinophil activation (70, 74). Once triggered eosinophils can then cause the release of pro-inflammatory cytokines and lipid mediators. These molecules can then induce modulated cellular trafficking, vascular permeability, mucous secretion and constriction of smooth muscle (70, 71, 75). Eosinophils can also act as antigen-presenting cells, and promote adaptive immune responses (76).

We now associate eosinophils with asthmatic disease as they feature predominantly in the inflammatory infiltrates of patients (77). Eosinophils are present in severe refractory asthma and severe exacerbations. Although some asthmatic patients can show disease symptoms without eosinophil infiltrates, in most cases the number of this cell present correlates with disease severity (78).
Eosinophils have been shown to have a role in remodelling of the airway, such as fibrosis, a structural changes in the airways that can lead to AHR. The cytokine IL-5 regulates eosinophil differentiation, maturation and degranulation and is upregulated in asthma (79-83).

As well as being regulated by exogenously produced cytokines, eosinophils can regulate their own function. Eosinophils are a major source of IL-4 and can also secrete IL-5, IL-2, IL-13 and IFN-γ (84).

Eosinophil presence in asthma has also been found to be a marker of the effectiveness of steroids as a treatment (85). Ineffectiveness of steroids as a treatment for asthmatic exacerbations is often associated with a notable increase in sputum neutrophilia, but normal eosinophil levels. This heterogeneity was identified by Gibson et al in 2001, which indicated that different mechanisms of inflammation in asthma may require different treatment (as these patients had impaired response to corticosteroid treatment) (13).

1.4.8 Mast cells-

Mature mast cells normally reside close to epithelia, blood cells, nerves and in the GI (Gastro-intestinal) tract and airways, near the smooth muscle cells and mucous–producing glands. They are important for the early phase of asthma and are activated by the binding of IgE or less commonly IgG1 to its high-affinity receptor (25, 86, 87). They are responsible for the release of several bronchoconstrictors including histamine and arachidonic acid metabolites, which induce mucous secretion, resulting in impaired respiratory function. These mediators are released after stimulation by environmental triggers such as allergens or as a result of increased ventilation during exercise (88).

Mast cells also release cytokines that are associated with AAD such as IL-3 IL-4, IL-5 and IL-13 (88-90) and chemokines. These molecules contribute to eosinophil recruitment, differentiation of Th2 cells, B-cell isotype switching, production of IgE and mastocytosis, which promotes the asthmatic response. Mast cells are significantly increased in the airway smooth muscle layer of asthmatic patients compared to normal and eosinophilic bronchitis controls (91, 92). AHR
was shown to be inversely correlated to the presence of mast cells in the airway smooth muscle band, supporting the theory that this cell is directly involved in the airway dysfunction of asthma (92).

1.4.9 PMNs (Polymorphonuclear cells)-

Neutrophils are not commonly recognized as being part of the typical inflammatory response of asthma. However, it has also been shown that a sample of the asthmatic population lacked the characteristic eosinophilia but had a significantly raised neutrophil population (13). A link between severe asthma and neutrophilia (44), as well as neutrophilia in allergen induced exacerbations has now been established (45, 93).

Findings by Wilson et al, in 2009 confirm the relevance of neutrophils in the asthmatic immune response. In their airway-sensitised model of AAD they used neutrophil depleting antibodies and genetic deletion of CXCR2 to demonstrate that by blocking neutrophil recruitment to the airway the development of AHR is prevented (46).

1.4.10 Dendritic cells

Dendritic cells (DCs) form a network in the upper layers of the epithelium of the airways, gut and the dermis of the skin. In the lungs, inhaled antigens are captured by mucosal DCs across the epithelial barrier. When the DC recognizes the antigen (danger signal), the DCs uptake the antigen and migrate to the draining lymph nodes while processing the antigen for presentation. At the lymph node the DC presents this processed antigen to resident T-cells which stimulate the release of cytokines and thus polarize the T-cells toward either a Th1 or Th2 response. DCs also have a role in antigen tolerance (94). The epithelial cell derived cytokine TSLP activates and directs CD11c+ DCs to promote the differentiation of naïve CD4+ T-cells into Th2 cells by production of high levels of the pro-allergic cytokines IL-4, IL-5, IL-13 and TNF-α (95). Thus the importance of DCs lies in the regulation of Th2 cells and the presentation of processed antigen from inhaled allergens in
asthma. Thus, DCs are involved in the intial sensitization to allergens but also in the driving of inflammatory responses in the lung.

Airway DCs in particular induce different types of immune responses. In the lung, DCs capture inhaled antigen and process the antigen prior to delivery to lymph organs (mainly lymph nodes). In the absence of pDCs, mDCs promote effector cells to produce cytokines that lead to the development of asthmatic inflammation (94). Therefore, in the airway plasmacytoid DCs (pDCs) promote tolerance and myleoid DCs (mDCs) induce immunity (94).

1.5 Th2 cytokines

1.5.1 Interleukin-4 (IL-4)

IL-4 is derived mainly from Th2 cells but can also be produced by eosinophils and mast cells in asthmatic patients and patients with other atopic disease (96, 97). Differentiation and commitment of naïve T-cells to the Th2 phenotype requires IL-4. It is also essential for B-cell isotype switching to a Th2 phenotype, and the production of IgG1 and IgE in mice (87, 98) and IgG4 and IgE in humans (99-101). In this way IL-4 indirectly regulates mast cell activation, through IgE. This contributes to broncho-constriction and mucous secretion, key factors in asthmatic disease. Increased levels of IL-4 have been measured in BALF from asthmatic patients, and IL-4 expression has been shown to be increased in lung cells from asthmatic patients compared to non-asthmatic controls.

However, IL-4 knockout mice when challenged with antigen do respond with eosinophilia and a CD4+ T-cell response. These mice can also develop airways hyper-responsiveness (AHR) and pulmonary tissue damage although it is diminished (80), emphasising that IL-4 has a role in the asthmatic response but is not the only cytokine of importance. This was illustrated by an experiment with IL-4 knockout mice where IL-5 was eliminated by treatment with anti-IL-5 monoclonal Ab. The mice in these experiments displayed no eosinophilia in blood or BALF, no lung damage or AHR, and thus providing evidence for the significance of IL-5 in facets of the asthmatic response (80).
IL-4 and IL-13, both promote B-cell isotype switching to produce IgE after Th2 cell differentiation. This is important in allergic airways disease and other atopic diseases, but is also relevant in helminth infections (102). IL-4 and IL-13 have also been associated with mucous hyper-secretion. Excess mucous is found in asthmatics and is a significant contributor in asthmatic related deaths. IL-4 is capable of affecting mucous secretion because it amplifies MUC5AC gene expression in the lung, which increases mucous secretion by airway epithelium (31).

IL-13 mediates mucous secretion through this same mechanism by its action on the IL-4 receptor alpha (IL-4Rα) chain (103). IL-4 also has a role in eosinophil recruitment. This was first demonstrated when it was discovered that blocking IL-4 production significantly decreased the infiltration of eosinophils to the site of lung inflammation (77). IL-4 can regulate eotaxin, a chemokine that regulates eosinophil recruitment, by increasing its production from epithelial cells during allergic airways inflammation (104).

IL-4Rα chain and signal transducer and activator of transcription-6 (STAT-6) have been identified as key signaling factors for IL-4 in the development of allergic airways disease. IL-4Rα mRNA expression and protein production is increased in bronchial biopsies from asthmatic patients (105). This role for IL-4Rα is reinforced in murine asthma models which have shown IL-4Rα has a role in AHR, inflammation and mucous over-production (106). This up-regulation of IL-4Rα has been shown to be reversed by cortico-steroid treatment, a common treatment of inflammation in human asthmatics (107). IL-4Rα chain and STAT-6 knockout mice both similarly display undetectable levels of IgE and do not develop the classic pathology of AAD (106, 108). IL-4, therefore has a varied and important role in asthmatic disease through mediating key factors of disease pathology.

1.5.2 Interleukin-5 (IL-5)

IL-5 is sourced mainly from Th2 cells but can also be produced by natural killer (NK) cells, mast cells and eosinophils (109). The importance of IL-5 in AAD has been proven through multiple experiments in both humans and murine models.
IL-5 is found in increased levels in BALF, sputum and bronchial biopsy and its expression is increased in CD4+ T-cells in asthmatic patients (109, 110), (111). This data is supported by murine models using IL-5 knockout mice where AAD is attenuated in these mice after antigen sensitisation and challenge compared to wild type controls (79). These mice also failed to develop changes in airways structure usually associated with late-phase asthmatic mouse models (80, 112-114).

Many cytokines and chemokines are involved in the complex process of eosinophilic inflammation, serving to recruit, traffic, and regulate their degranulation and apoptosis. However, IL-5 is the only cytokine selective to the eosinophil (115). IL-5 controls eosinophil migration through adhesion molecules in the bone marrow and bronchial epithelium. After eosinophils have migrated to the endothelium their movement is controlled by cytokines and chemokines. One of these chemokines is eotaxin; this is illustrated by the increase in eotaxin production and tissue eosinophil infiltration following antigen sensitisation and challenge (116).

Whilst eotaxin induces chemotaxis, IL-5 alone induces chemokinesis of eosinophils (104). Experiments using animal models have determined that IL-5 and eotaxin act collectively to regulate eosinophil migration from the bone marrow to the tissues (79).

### 1.5.3 Interleukin-13 (IL-13)

IL-13 is also primarily produced from activated Th2 cells, although IL-13 can be produced by alveolar macrophages and mast cells. Structurally, IL-13 is related to IL-4, and the IL-4Rα subunit is a major receptor for inducing biological properties. IL-13 is a potent B-cell regulator and controls IgE synthesis; it also enhances proliferation and activation of T-cells driving them towards a Th2 phenotype (117, 118).

IL-13 can also directly regulate eosinophil function by acting on the IL-4Rα receptor, and indirectly effect eosinophil function through CD69 expression which can enhance eosinophil survival and activity (118). IL-13 is important in the allergic response as pulmonary over-expression of IL-13 generates inflammation, mucous
hypersecretion, broncho-constriction (which can lead to AHR) and increased eotaxin production (119).

1.6 Role of Th1 cytokines in AAD

1.6.1 Interferon-gamma (IFN-\(\gamma\))

IFN-\(\gamma\) is the crucial Th1 effector cytokine. IFN-\(\gamma\) is particularly important in the immune response to intracellular bacterial pathogens such as \textit{S.typhimurium}, and \textit{Mycobacterium tuberculosis}. It has antagonistic effects on Th2 cells, attenuating responses controlled by IL-4, 5 and 13. In murine models IFN-\(\gamma\) has been shown to prevent the development of antigen-induced eosinophilia and AHR (120). Recombinant IFN-\(\gamma\) instillation by aerosol, intra-venously (i.v.), intra-tracheally (i.t.), or intra-peritoneally (i.p.) attenuates AHR and eosinophil infiltration into the respiratory tract of mice exposed to inhaled allergen (121, 122). Whilst IFN-\(\gamma\) attenuates the effects of antigenic sensitization and challenge in mice, evidence suggests that this cytokine can also cause damage to airway epithelial cells (123).

1.6.2 Interleukin-12 (IL-12)

IL-12 is primarily produce by antigen presenting cells (APCs) such as DCs and macrophages and is known to be important in the response to primary antigen presentation and Th1 polarisation (124). Bronchial biopsies of asthmatic patients show decreased levels of IL-12 expression (125). Accordingly, murine models of AAD have shown that administration of IL-12 during primary antigen exposure suppresses Th2 driven allergic responses and subsequent AAD (126). This is believed to be an indirect effect of IL-12 increasing local IFN-\(\gamma\) levels from co-existing Th1 cells which in turn suppress Th2 activity.

1.7 Role of Th17 cytokines in AAD

Following the discovery that IL-17 was produced by activated CD4+ memory T-cells, it has been determined that CD4+ T-cells producing IL-17 constitute a distinct lineage in contrast to Th1 and Th2 cells (127, 128). These cells produce IL-
17, IL-21, IL-22, IL-26 and CCL20 (129-131). Th17 cells produce more IL-21 than any other subset. IL-21 can also induce and expand Th17 cells, which potentially can cause a feedback loop (129). Polarisation to the Th17 subset is a result of antigen co-stimulation and cytokine-dependant signaling. These cytokines have been identified as IL-6, TGF-β, IL-21 and IL-1β (129-131). IL-23 is also important for Th17 cells as a lowering of IL-23 levels results in a decrease of Th17 cell production (129, 130).

1.7.1 IL-17

Since the IL-17 cytokine was identified, the IL-17 cytokine family is now identified as IL-17A through to IL-17F. Th-17 cells produce both IL-17A and IL-17F. Asthmatic patients have elevated levels of IL-17A mRNA and protein (132, 133). It is also noted that increases in IL-17A and IL-17F levels correlate with increased asthma severity (132-134). IL-17A has been identified to specifically correlate with neutrophilic asthma severity (132).

1.8 Animal models of AAD

OVA challenge models in BALB/c mice are considered a classic mouse model of Th2 induced allergic asthma. They offer opportunities to increase our knowledge of the mechanisms underlying the disease. In these models a Th2 recall response is triggered by antigen challenge in OVA-sensitized mice, inducing AAD. The basis of these models is that they produce a robust Th2 response, (commonly measured in cultured splenocytes, lymph node cells, serum and BALF), eosinophil recruitment both locally (measured in BALF and lung tissue) and systemically (blood) and AHR to methacholine.

Acute OVA models of AAD are well documented as reviewed by Kumar etal (135). The potential of these models lies in the ability to assess mechanisms of asthma pathogenesis and identify various therapeutic targets. It is noted that these models have limitations as with all animal models of human disease. Acute models are useful to investigate airway inflammation and early stages of remodelling whereas chronic models are required to also look at later stages of remodelling.
involving collagen deposition. These limitations have been investigated, identified and are taken into consideration when interpreting results produced from these models (136).

1.9 Neonatal vs adult immune system

Neonatal immune responses have been extensively studied in murine models. Neonatal response to pathogen exposure is markedly different to adult responses due to quantitative and qualitative differences in their immune system. Neonates and infants have an increased susceptibility to pathogens, especially intracellular pathogens (which require T-cell mediated immune responses for clearance) (137). The numbers of peripheral blood pDCs and mDCs were reduced at 12 months of age relative to those in adults (138). Neonatal pDCs are also less capable of producing IFN-α and IL-12p70 which consequently means they are less capable of inducing IFN-γ production (139).

Under conditions promoting vigorous cytokine production (costimulation) by adult T-cells, neonatal T-cells poorly produce Th1 cytokines and, in mice, produce a Th2-biased response. Neonatal APCs are poorly functional in vivo and normally unable to promote vigorous Th1 responses. However, if APC function is supplemented, mature Th1 responses are promoted. This was indicated by the normal production of Th1 cytokines (138). Neonates have been shown to have an enhanced capacity to sustain Th2 and Th17 immunity but a reduced capacity for Th1 immunity (139).

Through this evidence it is apparent that qualitative differences in both neonatal T-cells and APCs, compared to comparative adult cell levels, might contribute to the deficient T-cell-mediated responses of neonates (140).

1.10 Bacterial strains and models

1.10.1 Salmonella pathogenesis

*S. typhimurium* is the most common cause of Salmonellosis or typhoid in humans. It is a gram negative intracellular bacterial pathogen which colonises the Peyer’s patches (PPs) of the gut and like BCG it can live inside macrophages and
DCs. It has a characteristic flagellum and a signature LPS, which has strong adjuvant properties (141). *S.typhimurium*, like other intracellular microorganisms (e.g. *Listeria monocytogenes, Leishmania major, M.tuberculosis*) have been shown to induce a Th1-dependant immune response (142, 143). Accordingly, IFN-\(\gamma\) is essential for *S.typhimurium* clearance *in vivo*, this is partly through macrophage activation for intracellular killing. Spleen (systemic), PPs and lymph node (mucosal) cells from mice orally infected with *S.typhimurium* produce elevated levels of IFN-\(\gamma\) (and Th1 type cells) upon restimulation with *S.typhimurium* (144).

*S.typhimurium* is capable of infecting a wide range of animals resulting in multiple manifestations of symptoms and disease. In humans *S.typhimurium* manifests as gastroenteritis, abdominal pain, vomiting and inflammatory diarrhoea. This is typically caused by ingestion of infected food or water; uncommonly it can be transmitted by exposure to pet reptiles and amphibians that often carry the bacteria (145).

*S.typhimurium* are successful gastrointestinal pathogens due to their ability to evade multiple defensive mechanisms of the small intestine. One example is their characteristic acid tolerance that allows them to survive at low pH/acidic conditions of stomach, although acids do drastically reduce the infectious dose. *S.typhimurium* also successfully evade digestive enzymes, secretory IgA, innate immune defences and can invade, replicate and survive in the epithelial cells.

As a mucosal pathogen they preferentially enter the M-cells (microfold cells). These M-cells then transport the bacteria across the epithelium to the T and B-cells located in the PPs. A local inflammatory response is induced resulting in PMN infiltration. Various molecules associated with the bacteria are also effective in stimulating the innate immune system through TLRs (Toll-like receptors). This can result in an increase of diarrhoeal symptoms and systemic illness due to damaging inflammatory cell processes (146). Growth in both mucosal and systemic sites allows Salmonella to induce broad-based immune responses, including cell-mediated, humoral and secretory IgA Ab responses(147).

In the early phase of a Salmonella infection (during the first week) immune responses are suppressed. There is also a high frequency of IFN-\(\gamma\) producing
CD4+ and CD8+ T-cells in wild type mice. In B-cell deficient mice 3-5 times less IFN-γ T-cells are produced (147). B-cell deficient mice can clear *S. typhimurium* infection, however, decreased numbers of IFN-γ and IL-2 producing T-cells illustrates that the response is deficient. This demonstrates the role of B-cells in the initial phase of T-cell activation, possibly through antigen presentation and/or costimulation. B-cells are essential for both the initiation and development of Th1 cytokine production and protective immune responses to Salmonella (147, 148).

As previously described, Salmonella infection begins after oral infection where the Salmonella invades the M-cells and passes through the PPs and lymph nodes into the blood stream. Alternatively, it has been described that Salmonella can be engulfed at the mucosal surface by DCs and then transported from the GI to the bloodstream by CD18+ phagocytes (149, 150). The bacteria then reach an intracellular location within the macrophage, PMN cells, DCs and occasionally B220+ B-cells (151-158).

The ability of Salmonella to infect DCs *in vitro* and *in vivo* allows these DCs to induce and activate the production of cytokines (159). The early phase of innate immunity to Salmonella infection suppresses the growth of bacteria in tissues in a response that does not require T or B-cells. It concurs with the infiltration of macrophages in infected tissues, which ultimately form macrophage rich granulomas. The action of TNF-α, IFN-γ, IL-12, 15 and 18 is essential for this innate response (160). In late phase of infection the bacteria are localised in these cell dense arrangements consisting of inflammatory cells surrounding bacteria. They are encircled and limited by proximate normal tissue (148).

The role of Th1 cytokines in the immune response to Salmonella infection has been further validated in cases of human IFN-γ and IL-12 deficiency and IFN-γ and IL-12 receptor deficiency. These patients exhibited increased risk of severe and recurrent intracellular infections (161). The induction of Th1 cytokine production by Salmonella infection is hypothesised to be capable of down-regulating the asthmatic Th2 response and thus diminishing AAD symptoms.
1.10.2 *Salmonella enterica serovar typhimurium* AroA AroD mutant

Live attenuated *Salmonella* vaccines have been shown to confer better protection than whole-cell killed *Salmonella* vaccines (162, 163). A well-characterised example of a live attenuated *Salmonella* strain is those with mutations in the aromatic prechorismate (Aro) pathway. Aro mutants do not produce chorismate, which is an intermediate in the synthesis of aromatic compounds such as aromatic amino acids, some of which are virulence factors in *Salmonella* strains. These *S.typhimurium* Aro mutants induce protective immunity against virulent *Salmonella* infection and can also elicit an immune response to a number of antigens from a variety of pathogens. After inoculation, attenuated *Salmonella* translocate from the gut lumen to the intestinal mucosa and reside in the PPs and the spleen. The bacteria only need to colonise the PPs to elicit a protective immune response against *S.typhimurium* infection. These attenuated strains of *Salmonella* are often used to carry/express foreign proteins. It has been demonstrated that the level of total Immunoglobulin (Ig) elicited against a carried antigen correlates with the ability of the *Salmonella* strain to colonise and persist in the PPs following oral immunisation. (164).

Immunological responses elicited by these live vaccines are directed at a range of *Salmonella*/bacterial antigens such as LPS (Lipopolysaccharide), Vi, porins, flagella and fimbriae (165-168), (163, 169-175). In humans and mice these responses manifest themselves as a Th1 type as indicated by the prominent IFN-γ and IL-2 production (148).

1.10.3 The affects of *Salmonella* infection on AAD development in mice

In 2006, Wu et al published a study evaluating the effects that infection with an attenuated strain of *Salmonella* would have on the onset of AAD. They showed a significant reduction in key markers of AAD after OVA challenge. There was a reduction of eosinophils in the BALF and lung tissue of animals that received both the *Salmonella* infection and the OVA challenge. They also reported a reduction in OVA stimulated Th2 cytokines, IL-4, IL-5, IL-10 and IL-13 from splenocytes. Serum levels of OVA specific IgG1 were decreased and levels of Th1 inducing OVA
specific IgG2a was increased. Inhibition of Th2 cytokine and antibody responses to OVA and suppression of eosinophilia in the lungs shows its potential for immuno-modulation against allergic disease (176). These claims have been supported by mounting evidence of immune modulation against atopic disease in humans.

1.10.4 The affects of Salmonella infection on clinical AAD development

Pelosi et al conducted a longitudinal study looking at school age children in Sardinia with allergic rhino-conjunctivitis or asthma in relation to their incidence of Salmonellosis. Sardinian children hospitalized for Salmonellosis before 4yrs of life develop allergic asthma and rhinoconjuctivitis less frequently then children with no report of Salmonella infection (177).

The immune modulating properties of Salmonella also extend to isolated components of the bacteria. A Th1 inducing adjuvant derived from S.minnesota was trialled to enhance the effects of a grass pollen allergy vaccine. The adjuvant was a purified glycolipid derived from the bacterial cell wall. 3-deacylated monophosphoryl lipid A was shown in both animal and human studies to induce a Th1-like cytokine profile. The trial of the combined adjuvant and vaccine showed efficacy in patients with grass pollen allergy after only 4 pre-seasonal injections. This is equivalent to the effects seen after long-term injection schedules with the allergy vaccine alone (178).

1.10.5 BCG pathogenesis-

Mycobacterium bovis Bacille Calmette-Guerin (BCG)

The M.tuberculosis complex of strains includes seven species of which M.bovis is one, they all cause similar pathologies in their corresponding mammalian host. M.tuberculosis is a strain of this complex is the most significant in that it is the causative agent of Tuberculosis which infects one-third of the population worldwide and causes approximately 2-million deaths a year (86, 179). M.bovis Bacille Calmette Guerin or BCG is the strain of this complex, although usually infective towards bovine mammals used as a human vaccine against M.tuberculosis.
BCG is an intracellular respiratory tract mycobacterium that has a slow generation time, taking approximately 18-21 days to reach its log phase of growth (180). Eosinophils can phagocytose mycobacteria as can DCs and macrophages. BCG has mechanisms to hide from the host immune system and can also survive inside macrophages and DCs instead of being digested (181).

The immune response to BCG involves IFN-γ and IL-12, eliciting a strong Th1 response. It is unknown how the immune system kills this micro-organism, more often the bacterium is pushed into a state of latency rather than being killed. BCG evokes a strong Th1 immune response ensuring that this immune response evokes immunopathology in the host. This assists with the bacteria’s survival and transmission as it simultaneously resists the host’s intracellular killing mechanisms such as blocking apoptosis of macrophages.

The strong Th1 immune response elicited by BCG is illustrated invitro when DCs cocultured with BCG-infected neutrophils elicit T-cells to produce IFN-γ. The interplay/interaction between neutrophils, DC and T-cells play an important role in the early stages of the immune response to BCG vaccination, by promoting the protective anti-mycobacterial immune response (179).

Mycobacterial infections induce a powerful Th1-type response characterized by infiltration and activation of macrophages and T-cells at sites of infection. T-cell-dependent production of IFN-γ and macrophage-dependent production of TNF-α and IL-12 are believed to be the key elements of the mycobacterial-induced cellular immune response (182).

1.10.6 The affects of BCG infection on clinical asthma development

Historically, the advent of theories associating decreased infection rates in the western population with an increase in atopic diseases targeted Tuberculosis as a pathogen of interest. Subsequently, population studies followed to determine whether a correlation existed.

Strannegard et al published a historical cohort study of the Swedish population in 1998. Using age-matched unvaccinated controls, adults vaccinated with BCG as children were assessed for development of atopic diseases in
adolescence and adulthood. The results of this study showed the protective effect of childhood BCG vaccination on atopy (183).

However, in 2007 Linehan et al published a similarly designed study of the population of Manchester, England. This study showed that children who had received the BCG vaccination as a child had a 27% decrease in asthma prevalence. Due to the fact that these findings were not deemed significant, the authors reported “The capacity of neonatal BCG vaccination to reduce the prevalence of respiratory symptoms in children warrants further investigation” (184). Due to the inconsistency of the results in these 2 studies and other published clinical studies, the role of BCG vaccination on the development of asthma requires further investigation (183, 185-191).

Based on positive tuberculin tests to identify BCG vaccination in children aged 6-7 years of age, Miyake et al examined the association of BCG vaccination and the presence of multiple atopic diseases. This study found that a positive tuberculin response was inversely associated with the prevalence of asthma, wheeze and atopic eczema but not with allergic rhino-conjunctivitis (192).

Obihara and Bardin published data combining the findings of these and many other studies looking at a possible correlation. They addressed their findings as a whole and according to geography and determined that overall only 10 out of 23 epidemiological studies confirmed an inverse association between BCG vaccination and atopic disease. Geographically their findings showed that 6/16 studies in Europe, 2/4 studies in Asia, 1/2 studies in Africa and 1/1 study in Australia confirmed an inverse association between BCG and atopic (193).

An alternative to investigating a possible correlation historically was found in the development of animal models of Tuberculosis. This also allowed investigators to further delve into possible mechanisms in which immune responses to this infection could alter immune responses to environmental antigens.

1.10.7 The affects of BCG infection on AAD development in mice

Animal models of tuberculosis and BCG infection are widely utilised in the study of the immune response and vaccine efficacy to these infections. The murine
model of pulmonary BCG infection exhibits a very distinct immune profile in mice. Studies performed in animal models showed consistent results, with AAD being decreased in most cases and Th2 responses being down-regulated in all studies (194-198).

Pulmonary BCG infection or treatment with heat-killed BCG decreases local (lung) and systemic (blood) eosinophils. Lower levels of Th2 cells and IL-5 levels produced from local lymphocytes were also key components of this immune response (196). The strong Th1 immune response elicited by the host when in contact with BCG alters the Th2 directed immune response that is typical of AAD. Concurrent antigen exposure with BCG infection was found to successfully decrease Th2 responses associated with AAD and switch immune responses to a typically Th1 phenotype. This immune deviation resulted in a decrease in allergen-specific disease manifestations (198). Other animal studies of BCG infection have also found that along with being a potent Th1 inducer, BCG infection can also inhibit airway eosinophilia, mucous hyper-secretion and AHR (182, 194, 195, 197-199).

It is apparent after reviewing these studies that exposure to certain microbial pathogens can be beneficial in altering the pathway of allergic responses to antigens in animal models. These results have been found to have some value in their translation when studied in humans but results are equivocal.

1.11 Hygiene hypothesis

The hygiene hypothesis stems from as early as 1828 when John Bostock observed that Summer catarrh (hay fever) only occurred in people of the middle or upper classes throughout the various dispensaries (hospitals) around London at the time (191). Renewed interest was taken in this observation when Strachan published his theory of the hygiene hypothesis in the late 1980s (200). He proposed that the inverse relationship between hay fever, hygiene and household size was significant because, “The Th2-biased immune system of the newborn must encounter childhood Th1-inducing stimuli, e.g. bacterial or viral infection, to prevent development of allergic disease in adulthood.” (200). This theory has been
strengthened by evidence from clinical, epidemiological, and basic research which supports the inverse relationship between microbial burden and allergic disease and has provided insights into sources of stimuli and possible mechanisms of action (201).

From this mounting research the components of microbial burden which should be considered as potential instigators of immune changes are different types of infections (bacterial, parasitic, viral), microbial components (Toll-like-receptor ligands such as endotoxin), Gastrointestinal flora (probiotics such as Lactobacillus spp), forces that reduce microbial burden (antibiotics, immunization) (1, 201).

The basis of this hypothesis is that the developmental pathway of T-cells determines whether a protective or aberrant immune response is generated. Exposure to different antigens is needed to educate the immune system; these exposures can however have both protective and detrimental outcomes. Some bacterial infections that have been researched for their role in the hygiene hypothesis are M. bovis Bacille Calmette Guerin (BCG), S. typhimurium, Streptococcus pneumoniae, and Chlamydia pneumoniae. Where BCG and S. typhimurium have been found to potentially stimulate the immune system beneficially although some evidence is conflicting, C. pneumoniae and S. pneumoniae are associated with aberrant immune responses (1, 202, 203).

In 2006 Barbara Baker published a review detailing the most commonly agreed on mechanisms by which the hygiene hypothesis could act. Mechanism 1 shows that when pathogen or pathogen associated molecular patterns (PAMPs), come in contact with a pattern recognition receptor, PRR, IL-12 production is stimulated and the naïve T-cell is programmed towards a Th1 response (Figure 1.2). Alternately, if IL-4 or IL-5 is the signal on the naïve T-cell a Th2 allergic response is produced. Mechanism 2 shows that the stimulation of PRRs by PAMPs can lead to reduced Treg activation, which can lead to a mixed Th1/Th2 response (1).

This model has altered slightly in that Th1 and Th17 cells are now both associated with pathogen clearance and auto-immune disease (Figure 1.2).
Figure 1.2  Possible mechanisms of action on the immune system after exposure to microbial antigens. (Adapted from (1))

Mechanism 1 shows the most commonly believed mechanism of the hygiene hypothesis. The neonatal immune system is pre-disposed to a Th2 immune profile, without exposure to environmental and pathogen antigenic stimuli the immature immune system cannot respond with a Th1 response. Factors that this mechanism fails to take into account are; that effective immune response to parasite and helminth infection is Th2 and not associated with allergic disease, and the incidence of auto-immune diseases, which presents as a mainly Th1 immune response increased in prevalence at the same time as AAD. Mechanism 2 is proposed as an alternative explanation. A decrease in the bacterial colonisation of the gut in early life has led to lower numbers of activated regulatory T-cells. Tregs can affect the switch to Th2 immune responses. This puts more impetus on the type, length and duration of bacterial exposure in childhood and can also explain the increased prevalence of autoimmune diseases.
In 2000 Jones and Colleagues published a review addressing the inverse relationship between Salmonellosis and Tuberculosis and the prevalence of asthma around the world. They evidenced that in countries with a prevalence of asthma greater than 20%, such as Australia the ratio for deaths from asthma to deaths from TB is 38:1. They also showed that in countries with low incidence of asthma this ratio drops down as far as 1.6:1. This illustrates a significant but still anecdotal association between these infectious diseases and asthma. It is also worth noting that the authors specifically pointed out 5 countries that had a high frequency of Salmonellosis identified by a high recommendation for immunisation against typhoid. These 5 countries were among those with the lowest incidence of asthma (204).

However, some explanations of the hygiene hypothesis fail to take into account that simultaneous exposure to infection and allergen is most likely to occur. Priming of allergen-specific Th1 responses may be important in establishing a memory response capable of long-term protection against an allergen-specific Th2 response (205). If this phenomenon, where both \( M.\text{tuberculosis} \) and allergens are simultaneously presented by antigen-presenting cells to the T-cell, also applies to humans, then it is most likely to occur in a population where for example \( M.\text{tuberculosis} \) infection and allergen exposure are both highly prevalent. This phenomenon can be evaluated in this study by employing strains of bacteria that have been genetically modified to produce OVA (\( S.\text{typhimurium} \)-pKK-OVA and BCG-pMV-OVA), thus replicating infection with concurrent antigen exposure.

**1.12 OUTCOMES**

- Ascertain how T-cells are programmed to-
  - Protect against infection by pathogenic microorganisms.
  - Be educated to respond to cognate antigen in a protective or aberrant manner.
- Establish strategies of modified neonatal/ childhood vaccination to prevent predisposition to detrimental inflammatory responses and disease in adult life.
By programming or educating T-cells to respond to signals induced in response to foreign antigens the immunological ‘memory’ establishes an environment at mucosal surfaces that will protect against infection. In particular, infection by a pathogen at different periods of life may profoundly influence the way T-cells and thus the host defences are programmed against these foreign organisms by the induction of an appropriate/inappropriate humoral and/or cell-mediated immune response. Little is known of the influence of age of first infection on the development of antigen specific T-cell subsets, or what affect the type or route of infection has on T-cell programming. The stage of maturing of the immune system upon first exposure to pathogens may determine the success of host defence and strength and type of memory responses.

By employing novel infectious models, in combination with transgenic T-cells I can track the development of immune responses. I will assess the effect of two bacterial infections at different stages of life on the nature of T-cells that are subsequently generated. I will define the relationship between infections at different stages of life on the generation of specific T-cell repertoires and identify critical stages of immunological education for naïve T-cells that underpin deviation to a protective or aberrant immune response. Advancements made in this study will enable us to establish strategies of modified neonatal/childhood vaccination to prevent predisposition to detrimental inflammatory responses and disease in adult life.
1.13 HYPOTHESIS
That infection in early life plays a critical role in the development/programming of CD4+ T-cell responses to protective or destructive responses in the adult.

1.14 Experimental Aims
• Compare bacteria with different life cycles and different routes of infection on the generation of CD4+ T-cell responses to cognate antigen.

• Determine the affect of infections at different ages on the programming of adult CD4+ T-cells to cognate antigen.

• Determine if infection with *S.typhimurium* or BCG can halt the development of a Th2 Allergic response in Adulthood.

This study investigates the role of infection, in early-life on the development/programming of CD4+ T-cell responses in the adult. The type of immune response generated in the adult may not only be dependant on the age of first exposure but also on the type of infectious agent and the route of infection. Specifically I will: 1) Compare bacteria with different life cycles on the generation of CD4+ T-cell responses to cognate antigen early in life and in adults. Compare the route of mucosal neonatal and childhood infection (gut vs respiratory tract), on the generation of CD4+ T-cells to cognate antigen in the adult. 2) Determine the affect of infections at different ages on the programming of adult CD4+ T-cells to cognate antigen. 3) Determine the effect of these specific infections in the neonate and infant on the proliferative potential of adult CD4+ T-cells following stimulation with cognate antigen.
CHAPTER 2

Characterisation of a murine model of allergic airways disease in OVA-TCR Transgenic mice
2.1 Introduction

Allergic asthma is a chronic inflammatory disease characterised by airway obstruction and airways hyper-reactivity that is associated with inflammatory infiltrates particularly involving T-lymphocytes and eosinophils (12). Inflammation is accompanied by high levels of antigen-specific immunoglobulins and Th2 cytokines such as IL-5 and IL-13 (12).

To further explore the mechanisms regulating asthma it is important to develop models that mimic these hallmark features of disease. A widely used model involves sensitisation of mice with the protein OVA, followed (7-10 days later) by re-exposure with the same antigen in the lung (challenge). Challenge after sensitisation has occurred leads to acute allergic inflammation and AAD. Murine models of AAD are commonly used to study basic inflammatory and pathogenic mechanisms (135).

Examination of previous studies using mouse models of acute allergic inflammation and AAD illustrates the diversity of sensitisation and challenge protocols (135). Most models use one or more intra-peritoneal sensitisations with OVA followed by multiple aerosol or intranasal challenges before sacrifice and evaluation of the degree of disease (206-209).

These models of AAD are mainly performed in wild type BALB/c or A/J mice, however T-cell receptor (TCR) transgenic mice are also employed (210). In this study BALB/c TCR transgenic mice that are specific for the ovalbumin peptide, OVA323-339 (OVA-TCR Tg mice) are used (210). OVA-TCR Tg mice have transgenic T-cells that respond specifically to the OVA peptide (OVAp) but can also undergo recombination events to respond to other antigens if exposure occurs. OVA-TCR Tg mice can produce a normal B-cell response. This chapter describes the development of a model of AAD in OVA-TCR Tg mice.
2.2 **Materials and Methods**

2.2.1 **Animals**

BALB/cTac-TgN(DO11.10)Rag2−/−, OVA-TCR Tg specific pathogen free (SPF) mice were supplied at 6-8 weeks of age from the University of Newcastle animal services unit. These mice were derived from mice developed by Hsieh et al., 1995. OVA-TCR Tg were selected by staining peripheral blood leukocytes with the anti-clonotype mAb KJ1-26. OVA-TCR transgenics on the BALB/c background have been backcrossed more than 12 generations (210).

These mice were housed in individually ventilated cages (IVC), maximum 6 mice per cage in the David Madisson Building animal holding facility. The mice were fed commercial rodent pellets and water *ad libitum*. This project has animal ethics clearance and safety clearance from the University of Newcastle, Animal care and ethics committee and Institutional Biosafety committee.

2.2.2 **Induction of allergic airways disease**

OVA-TCR Tg mice were sensitised intranasally on day 0 with 50µl of either phosphate-buffered saline (PBS) (group A) or 100µg OVA (B). OVA was diluted in endotoxin free saline and stored at -20°C until use. No adjuvant was required. These mice were then challenged intranasally on day 14 and 15 with 50µL PBS (group A) or 10µg OVAp (B). The mice were sacrificed and samples taken for analysis 48 hours post-challenge.

2.2.3 **Collection and analysis of samples**

Mice were euthenased by an overdose of sodium pentobarbitone. After liberally spraying the area with 70% ethanol, the chest cavity was cut open and the mouse dissected and exsanguinated by bleeding directly from the heart using a 25 gauge needle attached to a 1 ml syringe. The blood was then placed in a 1.5 ml eppendorf tube at room temperature (RT) and allowed to clot for 1 hour. The tube was then placed in a microcentrifuge and
spun at RT for 10 minutes at 10,000 g to separate the blood components. The plasma/serum layer was then removed from the top of the tube and placed in a fresh eppendorf tube before freezing at -20°C.

The trachea was also exposed by dissection and and then severed close to the nasal passage. A sterile 3 ml syringe attached to a modified 200µL pipette tip was filled with 0.75 ml sterile HBSS (Hanks Buffered Salt Solution) and the lungs were gently lavaged. This process was repeated once and the 1.5 ml lavage fluid was placed on ice. The lavage was then centrifuged at 4°C, 3600 rpm for 10 minutes. The supernatant was frozen at -20°C and used later for antibody and cytokine analysis. The remaining pellet was resuspended in 400µL of red blood cell (RBC) lysis buffer and centrifuged as previously described. The RBC lysis buffer was removed and discarded as waste. The pellet was resuspended in 100µL of sterile HBSS. The number of cells/ml (total white cell count (WCC)) was determined using a microscope and haemocytometer after staining the cells with 0.9% trypan blue. The cells were then cyto-centrifuged onto a slide using a cytopsin. The slide was then stained with Giemsa-May-Grunwald to perform a differential WCC. After being cover-slipped the leukocyte populations were evaluated by identifying morphological criteria under a light microscope. 300-400 cells were counted per slide and the percentage of macrophages, lymphocytes, neutrophils, eosinophils determined.

2.2.4 Lung histology
The left lobe of the lung was removed and a transverse section (approximately 4mm wide) was cut and fixed in 10% neutral buffered formalin for a minimum of 24 hours. Lung sections were processed by the histological unit (John Curtin School of Medical Research, Australian National University, Canberra, ACT, Australia). Fixed tissue was embedded in paraffin and 8µm sections were cut onto glass slides. After heat affixing the section to the glass slide, the paraffin was removed from the section by immersion in xylene before rehydrating with a gradient alcohol solution. The
slides were then stained with either chromotrope and light haematoxylin (selective stain for eosinophil) or PAS (Periodic Acid Schiff) and light haematoxylin (selective stain for mucous secreting cells). Slides were coverslipped using DePeX mounting medium (BDH Laboratory Supplies, Poole, England). The sections were examined using a light microscope and a green lens filter (OLYMPUS). 10 sections of the graticule, representing 10 x 100 µm section counted under high powered field for each stain. The data were then averaged to give a result in cells/100µm, mean +/- Standard error mean (SEM) per group.

2.2.5 Collection of spleen and PBLNs

After opening the chest cavity, the lungs, heart and surrounding tissue were removed. Mediastinal lymph nodes were collected and placed into a 5 mL tube containing sterile HBSS and place on ice until further processing. The spleen was then removed by blunt dissection and placed in a 5mL tube containing sterile HBSS.

2.2.6 Isolation of splenocytes and PBLN cells for tissue culture

Inside a Class II biosafety cabinet a 50 ml falcon tube was placed in an esky containing ice. A 70µm sieve (BD) was placed inside the open tube and the tissue (spleen or lymph nodes) was mashed through the sieve using the rubber end of a plunger from a sterile 3ml syringe. The cell contents were flushed through the sieve with sterile HBSS to a volume of approximately 10mL. The cell suspension was then capped off and placed in a benchtop centrifuge and spun at 1800 g for 5 minutes at 4°C. The supernatant was then poured off each tube into a waste container and the cells were resuspended in 3mL RBC lysis buffer and incubated on ice for 5 minutes. HBSS was added to each tube to make the total volume up to 10 ml, and the cells were then centrifuged as previously described. The supernatant was then removed and the cells resuspended in 2 ml HBSS. A total cell count was performed on each tube using a light microscope and a
haemocytometer. The cells were then centrifuged as previously described and resuspended to the appropriate volume in ACCM (animal cell culture media) [0.1mM sodium pyruvate, 10% FCS (foetal calf serum), 2mM L-glutamine, 20mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 100U/ml penicillin/streptomycin, 50µM 2-mercaptoethanol (2-ME), made up in RPMI-1640 media (Roswell Park Memorial Institute)(Gibco)] so that all cell suspensions were at 1x 10^7/ml (1x10^6/100µl).

Next, 100µL cells were seeded in a 96-well u-bottomed tissue culture (TC) plate. Cells were then stimulated with OVA 400µg/ml, OVAp 200mg/ml, anti-CD3/CD28- anti-CD3 50ng/ml + anti-CD28 1µg/ml in ACCM or 100µl of ACCM alone (unstimulated) and the plates were placed in an incubator at 37˚C and 5% CO₂ for 5 days. For details of antibodies refer to supplementary data.

Plates were then centrifuged at 1800 rpm for 10 minutes at 4˚C. Supernatants were placed in sterile eppendorf tubes before storing at -80˚C until analysis.

2.2.7 Determination of antibody titres by enzyme linked immunoassay (ELISA)

Flat bottomed 96-well microtitre plates were coated with 100µl of 2µg/well OVA or IgG1, IgG2a or IgE in carbonate buffer (pH 9.6) and incubated at 4˚C overnight. After washing the plates once with PBS/0.05% Tween-20 (Sigma Chemical Co., St Louis, MO, USA) the non-specific binding sites were blocked with 3% bovine serum albumin (BSA)/PBS/0.05% Tween-20 for 1 hour at 37˚C. Plates were then washed 4 times in PBS/0.05% Tween-20 and incubated with serial dilutions of IgG1, IgG2a or IgE along with dilutions of serum or BALF at room temperature (RT) for 2 hours. The plates were then washed again as previously described and then incubated with either IgG1, IgG2a or IgE conjugated with HRP in 1% BSA/PBS for 45 minutes at RT. The plates were washed again before adding 100µl/well of tetra-methyl benzidine (TMB) (approximately 1.25 mM/L) in substrate buffer
with H$_2$O$_2$ (2.20 mM/L). This was left to develop for 10 minutes at RT before stopping the reaction with 1M H$_2$SO$_4$. The plates were read in a microplate reader (BIO-RAD) at 450nm. Antibody concentrations were determined relative to a standard curve (serial dilutions of specific antibody). Mean values of sample dilutions were taken. For details of antibodies refer to supplementary data.

2.2.8 Determination of culture supernatant cytokine levels by ELISA
Flat bottomed 96-well microtitre plates were coated with 100µl of 2µg/well purified rat anti-mouse IFN-γ, IL-4, IL-5, IL-13 or GM-CSF in carbonate buffer (pH 9.6) and incubated at 4°C overnight. After washing the plates once with PBS/0.05% Tween-20 (Sigma Chemical Co., St Louis, MO, USA) the non-specific binding sites were blocked with 3% BSA/PBS/0.05% Tween-20 for 1 hour at 37°C. Plates were then washed 4 times in PBS/0.05% Tween-20 and incubated with serial dilutions of IFN-γ, IL-4, IL-5, IL-13 or GM-CSF (granulocyte-macrophage colony stimulating factor) along with dilutions of culture supernatants from lymph node cells or splenocytes at RT for 2 hours. The plates were then washed again as previously described and then incubated with biotinylated rat anti-mouse IFN-γ, IL-4, IL-5, IL-13 or GM-CSF in 1% BSA/PBS for 1 hour at RT. The plates were then washed again as previously described and then incubated with streptavidin conjugated with HRP in 1% BSA/PBS for 1 hour at RT. The plates were washed again before adding 100µl/well of TMB (approximately 1.25 mM/L) in substrate buffer with H$_2$O$_2$ (2.20 mM/L). This was left to develop for 10 minutes at RT before stopping the reaction with 1M H$_2$SO$_4$. The plates were read in a microplate reader (BIO-RAD) at 450nm. Antibody concentrations were determined relative to a standard curve (serial dilutions of antibody). Mean values of sample dilutions were taken. For details of antibodies refer to supplementary data.
2.2.9 Statistical analysis

All data is represented as mean +/- SEM. Statistical comparison of groups were determined by Students T-test or ANOVA. Data is representative of 1 experiment chosen from at least 2 repeats, 6 mice per group.
2.3 RESULTS

2.3.1 OVAp induces leukocyte infiltration into the airways of OVA sensitised mice.

Leukocyte infiltration was observed in mice sensitised with OVA and challenged with OVAp. Total WBCs from the airways were evaluated by performing a cell count of BALF. An increase in leukocytes in the BALF indicated inflammation. OVA sensitised and OVAp challenged OVA-TCR Tg mice exhibited a statistically significant increase in total BALF leukocyte numbers compared to mice sensitised and challenged with PBS. WBCs in the BALF of mice treated with OVA/OVAp were found to be double that of mice exposed to PBS (Figure 2.3.1a).

Next, WBCs from the airways were differentially identified by morphological criteria on cytospin slides of BALF fluid. The viable leukocytes were identified from a pool of at least 300 leukocytes, and the percentage of each leukocyte type calculated and translated into cell numbers per millilitre from the initial total viable cell count taken. The leukocytes identified were PMNs, lymphocytes, macrophages and eosinophils. After OVA sensitisation and OVAp challenge OVA-TCR Tg mice had a significant increase in the number of macrophages, neutrophils, lymphocytes and eosinophils in BALF when compared to mice sensitised and challenged with PBS (Figure 2.3.1b).
Figure 2.3.1a Increased total leukocyte levels in BALF from mice sensitised and challenged with OVA.
Mice were exposed to PBS/PBS or sensitised and challenged with OVA/OVAp. Data represents the mean number of viable leukocytes per millilitre of BALF +/- SEM for groups of 6 mice. Statistical significance was determined by Students T-test (p<0.001**).
Figure 2.3.1b Leukocyte numbers in BALF from mice sensitised and challenged with OVA.

Graphs represent differentially identified leukocytes (A) macrophages, (B) neutrophils, (C) lymphocytes and (D) eosinophils. Data represents the mean number of viable leukocytes per millilitre of BALF +/- SEM for groups of 6 mice. Statistical significance was determined by Students T-test (p<0.05 *).
2.3.2 OVA induces mucous hypersecretion and eosinophil infiltrates in lung tissue

The histological examination of lung sections from all mice sensitised and challenged with OVA revealed considerable cellular infiltrates. Mucous secreting cells (MSCs) and eosinophils were increased by 6 and 4-fold respectively when compared to mice exposed to PBS (Figure 2.3.2).
Figure 2.3.2 Increase in the number of eosinophils (A) and mucous secreting cells (B), in lung tissue of mice sensitised and challenged with OVA. Mice were exposed to PBS, or sensitised and challenged with OVA. Data represents the mean number of cells per 100µm of lung section +/- SEM for groups of 6 mice. Statistical significance was determined by Students T-test (p<0.05 *, p<0.001**).
2.3.3 OVA-specific antibody levels in serum are increased in mice sensitised and challenged with OVA

IgG antibodies are predominately involved in the secondary antibody response to OVA. IgG1 and IgG2a antibodies were only detected in mice that were sensitised and challenged with ovalbumin. Compared to mice treated only with PBS there were significantly increased amounts of ova-specific IgG1 and IgG2a. When comparing the two groups, Total IgE was not significantly increased in the serum of mice sensitised and challenged with OVA compared with the PBS treated control (Figure 2.3.3).
Figure 2.3.3 Increased OVA-specific Immunoglobulin IgG1(A), IgG2a (B) but not total IgE (C) levels in serum of mice sensitised and challenged with OVA. Mice were exposed to PBS or sensitised and challenged with OVA. Data represents the mean number of detectable Immunoglobulin in serum +/- SEM for groups of 6 mice. Statistical significance was determined by Students T-test (p<0.05 *).
2.3.4 Cytokine responses in OVA treated mice are different to PBS treated controls.

The levels of Th1 cytokine (IFN-γ) produced from OVA-stimulated splenocyte cultures was not significantly different between mice that received OVA sensitisation and challenge and those that received PBS. There was also no difference in the levels of IL-4, IL-5 or IL-13 (Th2 cytokines) present in ova-stimulated splenocytes from OVA sensitised and challenged mice when compared to PBS treated control mice (Figure 2.3.4a).

However, local cytokine responses from the lung-draining lymph nodes showed that mice sensitised and challenged with OVA had decreased levels of IFN-γ compared to PBS treated controls (Figure 2.3.4b). A significant increase in the Th2 cytokine IL-4 was also present in OVA stimulated LN cultures of OVA treated mice when compared to PBS-treated controls. OVA-Tg T-cells from mice treated with PBS or OVA produced Th1 and Th2 cytokines in response to ova-stimulation in culture.
Figure 2.3.4a No changes in the levels of (A) IFN-gamma, (B) IL-5, (C) IL-4 or (D) IL-13 in OVA-peptide stimulated splenocytes of mice sensitised with OVA and challenged with OVAp.

Mice were exposed to PBS/PBS or sensitised and challenged with OVA/OVAp. Data represents the mean number of detectable cytokine in spleen cultures +/- SEM for groups of 6 mice. Statistical significance was determined by Students T-test (No significant differences were found).
Figure 2.3.4b Decrease in the levels Th1 cytokine and increase in the levels of Th2 cytokine in OVA-peptide stimulated PBLN cultures of mice sensitised with OVA and challenged with OVAp. (A) IFN-gamma, (B) IL-5, (C) IL-4 or (D) IL-13.

Mice were exposed to PBS (A) or sensitised and challenged with OVA (B). Data represents the mean number of detectable Immunoglobulin in serum +/- SEM for groups of 6 mice. Statistical significance was determined by Students T-test (p<0.05 *, p<0.001**).
2.4 Discussion

In this chapter a model of AAD in OVA-TCR Tg mice was established. Local inflammatory cell responses, lung histopathology, systemic immunoglobulin levels and local and systemic cytokine levels from antigen reactive T-cells were measured to determine if AAD was induced.

Sensitisation and challenge of mice with OVA intranasally induced a significant increase in the number of total BALF leukocytes when compared to PBS treated controls. Differential analysis of cells showed that the number of macrophages, neutrophils, lymphocytes and eosinophils were significantly increased. Increased leukocytes in the BALF and increased eosinophils have been shown to be hallmark features of AAD in mice. In animal models eosinophil infiltration is rapid and sustained in response to antigen challenge. Data indicates that eosinophils are necessary for the full manifestations of many features of AAD (74, 79, 211).

The numbers of mucous secreting cells and eosinophils were also significantly increased in the lung tissue of mice sensitised with OVA and challenged with OVAp. The excessive mucous production observed during AAD is thought to be due to a combination of hypertrophy and hyperplasia of sub-mucosal glands and goblet cells (212). Without allergic airway inflammation there is very little mucous in the lung tissue. Mucous hyper-secretion is directly linked to the activation of Th2 cells by antigen which leads to the secretion of IL-13 and consequently its effector functions. Increased numbers of eosinophils is due to activation of chemo-attractive pathways and the production of eotaxins and IL-5 in the airways. IL-5 is secreted from antigen activated Th2 cells. Eotaxin production is induced by IL-13 activation of STAT-6. In the AAD lung some studies have described both an independent role for eosinophils and a function in conjunction with CD4+ Tcells (74, 211).

Increased levels of Ova-specific IgG1 and IgG2a were present in the serum of mice treated with ovalbumin. In mice, the presence of IgG1 is indicative of a Th2 response whereas IgG2a represents a Th1 response. The presence of either Ig results from stimulation of B-cells by different pro-inflammatory cytokines. The
cytokines IL-4 and IL-2, have a crucial role in activation of the IgG antibody response. In particular IL-4 plays a key role in isotype switching in B-cells for the production of IgE and IgG1(213).

IgE is an immunoglobulin that plays an important role in allergy and asthma. Although it is typically the least abundant Ig it is significantly increased in cases of asthma and allergy and is capable of triggering the most powerful immune reactions. Total IgE levels in serum were not significantly increased in mice sensitised and challenged with OVA compared to sham-treated controls. This is possibly due to elevated IgE levels in OVA-TCR Tg mice at baseline.

Cytokines play a key role in orchestrating the chronic inflammation of asthma by recruiting, activating, and promoting the survival of multiple inflammatory cells in the respiratory tract (214). The cytokines involved in asthma can be grouped into 2 main groups: Th2 cells orchestrate allergic inflammation through the release of the cytokines IL-4, IL-5, IL-9, and IL-13. Th1 cells differentiate under the influence of IL-12 and IL-27 and suppress Th2 cells through the release of IFN-γ. Systemic cytokines (from splenocyte cultures) remained unaffected by OVA sensitisation and challenge when compared to PBS-treated controls. Local cytokine responses (from PBLNs) were altered in mice that received OVA sensitisation and challenge to the airways. A significant decrease in the Th1 cytokine, IFN-γ and increase in the Th2 cytokine, IL-4 shows that AAD in this model demonstrated Th2 polarisation which is a hallmark of AAD (Figure 2.3.4b).

Lymphocytes are prominent among the inflammatory cells infiltrating the asthmatic airways and it is likely that CD4+ lymphocytes have a role in this model of AAD. This is shown by changes in local cytokine levels and increases in lung eosinophils. CD4+ lymphocytes play a key role in orchestrating the interaction of inflammatory cells by releasing an array of cytokines which can attract and activate other cell types. Studies have shown that in AAD activated T-lymphocytes are primarily CD4+ cells and that they correlate with eosinophil number and activation (215). Lymphocytes have the capacity to produce IL-4 along with other Th2 cytokines and play a role in controlling asthmatic inflammation by their release (3,
The model of AAD proposed in this chapter focuses on CD4+ lymphocytes in AAD due to the OVA-specific T-cells in the Tg strain of mice used. Increased levels of OVA-peptide stimulated IL-4, IL-5 and IL-13 from OVA sensitised and challenged mice indicates that CD4+ lymphocytes are likely responsible for inflammatory symptoms in this model of AAD (Figure 2.3.4b). From these results, IL-4 appears to be the dominant Th2 cytokine in OVA-treated mice (Figure 2.3.4b). Levels of IL-13 were not as highly elevated, which can be explained by IL-13 and IL-4 acting as antagonists.

In this chapter I have demonstrated that an AAD model can be developed in OVA-TCR Tg mice. Prominent features of disease in this model are increased numbers of inflammatory cells in BALF, including lymphocytes and eosinophils. Inflammation in lung tissue of mice treated with OVA is identified by the presence of eosinophilia and mucous hyperplasia. OVA treated OVA-TCR Tg mice also have increased levels of OVA-stimulated Th2 cytokines and decreased levels of IFN-γ from OVA-stimulated local lymph node cells. Systemically, OVA-TCR Tg mice treated with OVA also have increased OVA-specific IgG1 and IgG2a from serum. The disease processes in this model are likely controlled by Tg CD4+ lymphocytes via the release of cytokines and inflammatory mediators.

This model of AAD is different to models employing wild-type mice (for example BALB/c, C57BL/6 or AJ mice) due to the Tg nature of their T-cells. The main difference observed from these studies is the ability of T-cells from mice not sensitised to OVA to produce Th1 and Th2 cytokines in response to OVA stimulation in culture. This response was so profound that the levels of IL-5 and IL-13 from OVA-stimulated T-cells were similar between PBS and OVA treated groups. Unlike wild-type mice, transgenic T-cells have the capacity to produce Th1 and Th2 cytokines without sensitisation to OVA. This data indicates that OVA-Tg T-cells are already programmed. The default pathway for these cells is a mixed Th lymphocyte population. It is likely that these mice would develop features of AAD from direct delivery of OVA to the airways without requiring prior sensitisation to OVA.
CHAPTER 3

Optimisation and characterisation of infection levels in OVA-TCR Transgenic and BALB/c mice at different ages
3.1 Introduction

In 2000, Jones et al., observed that BCG vaccination along with the incidence of Salmonellosis was inversely associated with the prevalence of asthma (204). To examine this association, animal models of BCG and Salmonella infection were developed and used to determine the effects of these infections on the development of allergic disease (167, 216).

Murine models of these infections have been used primarily to examine the efficacy of vaccines, and to map the immune responses to primary and secondary infection. Models of bacterial infections are also useful tools for characterising how the immune system responds to pathogens and influences the development of the immune system. However, these models are limited simulations of human infection as often the doses of pathogen are very high, the mode of infection (direct instillation) does not mimic natural exposure and pathogens that are not natural infective agents of the host are also often used. The optimisation of mouse models of infection that mimic pathogenic features of human disease is thus very important.

This chapter describes the optimisation *S.typhimurium* and BCG infection in mice. Features of bacterial pathogenesis noted from these infections in human disease were focused on to allow for the development of similar infectious outcomes in mice. Animal models of these two infections already exist due to the pursuit of candidate vaccines against Tuberculosis and Salmonellosis (148, 151, 167, 194, 216-218). The mouse model of BCG described by Erb et al in 1998 was used as a basis of developing an infection model in OVA-TCR Tg mice (194).

Harrison et al (1997) developed a model of Aro mutant Salmonella infection in mice to study the efficacy of vaccines to treat humans. They tested intra-venous, subcutaneous and oral inoculation of a live Salmonella mutant for its effectiveness in protecting against virulent Salmonellosis. Oral inoculation with the live Salmonella mutant mimicked naturally occurring Salmonella infection by stimulating an effective mucosal immune response (167).

This chapter will describe optimisation and subsequent characterisation of *S.typhimurium* and BCG infection in WT and OVA-TCR Tg mice. Immune
responses induced by these bacterial infections were assessed to determine what significant immune changes occur.
3.2 Materials and Methods

3.2.1 Animals
BALB/cTac-TgN(DO11.10)Rag2\textsuperscript{+/+}, OVA-TCR Tg SPF mice were supplied at 6-8 weeks of age from the University of Newcastle animal services unit. These mice were derived from mice developed by Hsieh et al., 1995. OVA-TCR Tg were selected by staining peripheral blood leukocytes with the anti-clonotype mAb KJ1-26. OVA-TCR transgenics on the BALB/c background have been backcrossed more than 12 generations (210).

These mice were housed in IVC, maximum 6 mice per cage in the David Madison Building animal holding facility. The mice were fed commercial rodent pellets and water \textit{ad libitum}. This project has animal ethics clearance and safety clearance from the University of Newcastle, Animal care and ethics committee and Institutional Biosafety committee.

3.2.2 Bacteria

\textit{Mycobacterium bovis} (BCG), Pasteur Pasteur 1173-P2 +pMV-OVA (Kanamycin Resistant) (210, 219).

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<th>Age</th>
<th>Dose</th>
<th>Volume</th>
<th>Route</th>
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<tbody>
<tr>
<td>0-day old</td>
<td>8x10\textsuperscript{4} cfu</td>
<td>5\textmu l</td>
<td>i.n.</td>
</tr>
<tr>
<td>2-week old</td>
<td>5x10\textsuperscript{5} cfu</td>
<td>10\textmu l</td>
<td>i.n.</td>
</tr>
<tr>
<td>4-week old</td>
<td>1x10\textsuperscript{6} cfu</td>
<td>50\textmu l</td>
<td>i.n.</td>
</tr>
<tr>
<td>6-week old</td>
<td>1x10\textsuperscript{6} cfu</td>
<td>50\textmu l</td>
<td>i.n.</td>
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\textit{Salmonella} enterica serovar \textit{typhimurium} aroA aroD mutant BRD09 +pKK-OVA (Streptomycin/Ampicillin Resistant)(220).

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<tr>
<th>Age</th>
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<tr>
<td>0-day old</td>
<td>1x10\textsuperscript{1} cfu</td>
<td>5\textmu l</td>
<td>Oral</td>
</tr>
<tr>
<td>2-week old</td>
<td>1x10\textsuperscript{7} cfu</td>
<td>10\textmu l</td>
<td>Oral</td>
</tr>
<tr>
<td>4-week old</td>
<td>1x10\textsuperscript{9} cfu</td>
<td>200\textmu l</td>
<td>Oral</td>
</tr>
<tr>
<td>6-week old</td>
<td>1x10\textsuperscript{9} cfu</td>
<td>200\textmu l</td>
<td>Oral</td>
</tr>
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</table>
3.2.3 Homogenising tissue
Tissue (spleen, liver, Peyer’s patches (PP) or lungs) was placed in a tube with a known volume of sterile PBS and homogenised under sterile conditions. This homogenate was then used to determine total bacterial numbers per sample. After removing the aliquot required for bacterial recovery the homogenate was centrifuged at 10,000rpm for 10 minutes at 4°C to remove any remaining tissue. The supernatant was then collected and stored at -20°C.

3.2.4 Determination of infection levels
Agar plates (BCG- 7H11 Agar, S.typhimurium- LB (Luria Burtani) Agar) were removed from the cool room and placed at RT. In a biosafety cabinet, at RT serial dilutions were performed of each sample (BALF, lung homogenate (LH), spleen, liver, gut wash, peyers patches (PP)) in a 96-well u-bottomed plate. 20µl of each of these serial dilutions was then placed on the agar plate and allowed to absorb into the plate under sterile conditions. Once absorbed the agar plates were placed upside down to incubate at 37°C, 5% CO₂ overnight (S.typhimurium) or for 18 days (BCG). With the aid of a magnifying lamp the colonies were counted and the bacterial load of each sample calculated in colony forming units (CFU).

3.2.5 Induction of infection
Growth and preparation of the bacteria
(A) BCG: BCG stocks were removed from -80°C storage (sourced from: Dr G.S. Buchan, University of Otago, New Zealand) and inoculated into separate Erlenmeyer flasks half filled with sterile 7H9 broth [4.7g 7H9 Broth (Bacto laboratories, 2mL glycerol, 900mL deionised water, 500µL 0.05% Tween 80, 100ml ADC supplement (5g BSA, 2g Glucose, 0.003g catalase), kanamycin]. Flasks were placed in a shaking incubator for 21 days at 37°C. Bacteria was then poured into 50ml falcon tubes and spun in a benchtop centrifuge at 4000rpm for 30 minutes at RT. Supernatant was discarded and
the pellet resuspended in sterile 10% glycerol in PBS. This process was repeated twice to wash the bacteria before resuspending at a known concentration/CFU determined by growth curve optical density (OD) read at 405nm (1x10^9 CFU/ml = 1.45 OD_{405nm}). The bacteria was then aliquoted and stored at -80°C until use.

(B) *S. typhimurium*: Each bacterial stock (sourced from: Dr R.A Strugnell, University of Melbourne, Australia) was streaked onto a sterile LB agar [7.5g Agar powder (Bacto Laboratories), 5g tryptone powder(Bacto), 2.5g yeast powder, 2.5g NaCl, 500mL deionised water, Streptomycin(100mg/ml) / Ampicillin (100mg/ml) plate containing Streptomycin and Ampicillin and incubated overnight at 37°C, 5%CO₂. The bacteria were subcultured from this ‘stock plate’ again before being harvested. Glass hockey sticks were used to scrape the bacteria off the agar plate and into a 30ml tube containing sterile PBS. The OD was read at 405nm and the volume of sterile PBS was adjusted to obtain the required concentration according to the growth curve.

3.2.6 Delivery of infection

(A) **BCG**: Mice were anaesthetised with 3-5% isofluorane/oxygen mix. BCG was delivered intranasally (i.n.); a volume of 5µl was delivered to mice up to 2-weeks old, and 50µl was delivered to mice 4-weeks and older. Concentrations of bacteria were altered according to volume delivered.

(B) **S. typhimurium**: 15 minutes prior to infection mice were anaesthetised with 3-5% Isofluorane/oxygen mix and given a gavage of 0.75% sodium bicarbonate (2-week old mice, 10µl; 4-6-week old mice, 200µl). 0-day old mice were not anaesthetised and did not receive the sodium bicarbonate and were fed 5µl *S. typhimurium* using a sterile gel-loading tip. All other mice were anaesthetised 15 minutes after the sodium bicarbonate dose and *S. typhimurium* was delivered using an 8cm long ball-ended gavage.
needle; a volume of 10µl was delivered to mice up to 2-weeks old, and 200µl was delivered to mice 4-weeks and older. Concentrations of bacteria were altered according to volume delivered.

3.2.7 Weight change and timeline of infection
Mice were weighed under sterile conditions daily and the weight was recorded. Mice were identified individually by ear-marking to allow for continued weight monitoring.

3.2.8 Collection and analysis of samples (blood, BALF, lung)
Refer to chapter 2.2.3

3.2.9 Collection and processing of tissue for bacterial recovery
Refer to chapter 3.2.3 and 3.2.4

3.2.10 Determination of Total CFU by bacterial recovery
Refer to chapter 3.2.4

3.2.11 Determination of bacterial OVA production in lung tissue and BALF
Flat bottomed 96-well microtitre plates were coated with 100µl of 2µg/well anti-OVA in carbonate buffer (pH 9.6) and incubated at 4°C overnight. After washing the plates once with PBS/0.05% Tween-20 (Sigma Chemical Co., St Louis, MO, USA) the non-specific binding sites were blocked with 3% BSA/PBS/0.05% Tween-20 for 1 hour at RT. Plates were then washed 4 times in PBS/0.05% Tween-20 and incubated with serial dilutions of OVA along with dilutions of serum, BALF or LH at room temperature (RT) for 2 hours. The plates were then washed again as previously described and then incubated with anti-OVA biotinylated in 1% BSA/PBS for 1hr at RT. The plates were then washed again as previously described and then incubated with streptavidin conjugated with HRP in 1% BSA/PBS for 1hr at RT. The plates were washed again before adding 100µl/well of tetra-methyl
benzidine (TMB) (approximately 1.25 mM/L) in substrate buffer with H$_2$O$_2$ (2.20 mM/L). This was left to develop for 10 minutes at RT before stopping the reaction with 1M H$_2$SO$_4$. The plates were read in a microplate reader (BIO-RAD) at 450nm. Antibody concentrations were determined relative to a standard curve (serial dilutions of specific antibody). Mean values of sample dilutions were taken.

3.2.12 Statistical analysis

Statistical significance was determined by Students T-test. Significance was considered to be when p<0.05 *, p<0.001**.

Data is representative of 1 experiment, 6 mice per group.
3.3 Results

(A) BCG infection

3.3.1 Leukocyte infiltration into the airways of infected mice
(wild-type (WT) compared to OVA-TCR Tg mice)

The total WCC of WT and OVA-TCR Tg mice infected with BCG was evaluated over a time course of 12 weeks. This was to ensure that all stages of the response to infection were examined as BCG has a latent phase before reoccurrence of the infection after 8 weeks in WT mice (194). OVA-TCR Tg mice infected with BCG displayed a significantly decreased number of leukocytes in their BALF from day 21 (peak of infection) to day 46 when compared to BCG infected WT mice. This indicates an increased level of airway inflammation in WT mice, which may reflect their ability to mount a specific immune response to BCG.

The BALF cell population was evaluated to determine the type and number of leukocytes present during the immune response to BCG in OVA-TCR Tg mice versus WT mice. Neutrophils are an important cell in the immune response to BCG and in AAD. A significant increase in the number of BALF neutrophils was observed in OVA-TCR Tg when compared to WT mice from day 46 (Figure 3.3.1b (A)). WT mice only showed a slight increase in neutrophil numbers around the peak of infection (d21-32) (Figure 3.3.1b (A)). Lymphocytes were significantly increased in the WT mice in response to infection (d18-46), when compared to the OVA-TCR Tg mice (Figure 3.3.1b (B)). The existing population of OVA-specific T-cells in OVA-TCR Tg mice can explain why the lymphocyte population did not respond to infection (BCG antigen) as powerfully as lymphocytes in WT mice. Macrophages are not as significant in the immune response to BCG as neutrophils and lymphocytes (Figure 3.3.1b (C)). Macrophage levels in WT mice were significantly increased 46-days post-infection but were not significantly elevated at other time-points when compared to macrophage numbers in OVA-TCR Tg mice. Eosinophils in BALF were not a feature of the immune response to BCG infection in either strain.
Figure 3.3.1a Total leukocyte levels in BALF from the airways of BCG infected mice. WT mice have a higher WCC in BALF after infection with BCG than OVA-TCR Tg mice. Data represents the mean number of viable leukocytes per millilitre of BALF +/- SEM (Standard error of the mean) for groups of 6 mice. Statistical significance was determined by Students T-test (p<0.05 *).
Figure 3.3.1b Differential leukocyte (A) Neutrophil, (B) Lymphocyte, (C) Macrophage, (D) Eosinophil levels of BALF from airways of BCG infected mice.

Numbers of leukocytes in the BALF of WT and OVA-TCR Tg mice after infection with BCG. Data represents the mean number of viable leukocytes per millilitre of BALF +/- SEM (Standard error of the mean) for groups of 6 mice. Statistical significance was determined by Students T-test when comparing leukocyte numbers in WT compared to OVA-TCR Tg mice (p<0.05 *, p<0.001**).
3.3.2 Changes of weight due to infection  
(WT compared to OVA-TCR Tg mice)

Bacteria recovered from the lungs of mice infected with BCG correlates with changes in weight gain in WT mice ($R^2 = 0.4675$) but not in OVA-TCR Tg mice ($R^2 = 0.1025$) (Figure 3.3.2). This indicates that weight loss in OVA-TCR Tg mice is not a clinical result of BCG infection.
Figure 3.3.2 Correlation of weight loss with infectious load in mice exposed to BCG.

Bacteria recovered from the lungs of mice infected with BCG correlates with changes in weight gain in WT mice but not in OVA-TCR Tg mice. Data represents the mean number of bacteria recovered from lungs at each timepoint compared to the mean change in weight at each timepoint for groups of 6 mice for each strain. Coefficient of determination ($R^2$ value) was determined for each group.
3.3.3 Bacterial OVA production compared to level of BCG recovered from lungs (WT compared to OVA-TCR Tg mice)

The recombinant BCG strain used in this study has been evaluated in vitro to determine its ability to produce the protein OVA (219). However, due to the ability of bacteria to exclude plasmids containing recombinant proteins it is important to verify the production of the protein. Figure 3.3.3 shows the direct correlation between the numbers of viable CFU of BCG recovered from the lung and the concentration of the OVA in lung homogenates recovered from mice post infection.
Figure 3.3.3 *In vivo* production of OVA by transgenic BCG recovered from the lungs of infected (A) WT and (B) OVA-TCR Tg mice. Data represents the mean number of viable leukocytes per millilitre of BALF +/- SEM (Standard error of the mean) for groups of 6 mice. Statistical significance was determined by Students T-test.
3.3.4  Bacterial recovery from lungs (WT compared to OVA-TCR Tg mice)

Infection levels evaluated in WT and OVA-TCR Tg mice showed a significant difference in BCG levels in the lung at day 18 (peak of infection) (Figure 3.3.4.). This likely indicates an initial decrease in the ability of OVA-TCR Tg mice to make recombination events for BCG specific antigens due to the large component of OVA-TCR Tg T-cells present in these mice. However, the BCG recovery from both wild-type and OVA-TCR Tg mice show a similar pattern of peak (d18-21), recovery (d30-45), latent (d45) and re-infection (d85) states (Figure 3.3.4.). All mice inoculated with BCG achieved a successful infection as shown by replication of the stages of infection seen in human tuberculosis.
Figure 3.3.4 Levels of BCG present in the lungs of OVA-TCR Tg mice compared to wild-type mice at the peak of infection. Data represents the mean number of viable CFU per millilitre of BALF and lung homogenate +/- SEM for groups of 6 mice. Statistical significance was determined by Students T-test (p<0.05 *).
3.3.5 Leukocyte infiltration into the BALF of infected mice
(WT compared to OVA-TCR Tg mice)

After gastrointestinal (GI) infection with S. typhimurium there was no significant difference in total leukocytes in BALF of OVA-TCR Tg mice compared to wild-type controls (Figure 3.3.5a). However, in both strains of mice S. typhimurium infection caused an increase in total BALF leukocytes compared to uninfected controls. The number of macrophages, neutrophils, lymphocytes and eosinophils in BALF of infected OVA-TCR Tg mice was also unchanged compared to BALB/c controls (Figure 3.3.5b).
Figure 3.3.5a Total leukocyte levels in BALF from mice infected with \textit{S.typhimurium}.

WT and OVA-TCR Tg mice were exposed to PBS or infected with \textit{S.typhimurium}. Data represents the mean number of viable leukocytes per millilitre of BALF +/- SEM (Standard error of the mean) for groups of 6 mice. Statistical significance was determined by Students T-test ($p<0.001^{**}$).
Figure 3.3.5b  Leukocyte numbers in BALF from mice infected with *S.typhimurium*.

WT and OVA-TCR Tg mice were exposed to PBS or infected with *S.typhimurium*. Graphs represent differentially identified leukocytes (A) macrophages, (B) neutrophils, (C) lymphocytes and (D) eosinophils. Data represents the mean number of viable leukocytes per millilitre of BALF +/- SEM for groups of 6 mice. Statistical significance was determined by Students T-test (p<0.05 *, p<0.001**).
3.3.6 Changes of weight due to infection
(WT compared to OVA-TCR Tg mice)

WT mice were more susceptible to the recombinant *S. typhimurium* strain used in this study than OVA-TCR Tg mice. Weight loss caused by infection with this strain of *S. typhimurium* was significantly greater in wild-type mice by 4-days post infection than in OVA-TCR Tg mice (Figure 3.3.6.). OVA-TCR Tg mice showed only minimal weight loss after infection.
Figure 3.3.6 Weight loss caused by OVA-producing *S. typhimurium* infection in BALB/c mice compared to OVA-TCR Tg mice. Data represents the mean number percentage change of weight from initial pre-infection weight +/- SEM for groups of 4 mice. Statistical significance was determined by Students T-test (p<0.05 *, p<0.001**).
A successful *S. typhimurium* infection should invade more than one GI organ. OVA-TCR Tg mice were infected with two doses of *S. typhimurium* to determine the dose which will cause a successful sub-clinical infection. Figure 3.3.7 shows that an infection of $1 \times 10^8$ CFU only causes bacteria to be present in the liver whilst an infection of $1 \times 10^9$ results in bacteria colonising both the spleen and liver of OVA-TCR Tg mice. The higher dose of infection is optimal as it is sub-clinical. This is an infection where the mice show no outward signs of disease but have bacteria present in gastrointestinal organs (spleen and liver) other than where the initial instillation of bacteria was made.
Figure 3.3.7 Levels of *S. typhimurium* present in the gastrointestinal tract of OVA-TCR Tg mice at the peak of infection.

Data represents the mean number of viable CFU per millilitre of Spleen, Gut wash or Liver +/- SEM for groups of 6 mice. Statistical significance was determined by Students T-test (p<0.05 *, p<0.001**).
3.4 Discussion

The focus for appropriate immune education to prevent the development of allergic diseases has been on infections that cause the immune system to respond in a Th1 manner (6, 176, 186, 221). Two such infections are *S. typhimurium* and BCG (6, 176, 186, 221). These bacterial infections have been previously optimised in murine models in the search for vaccine candidates (141, 151, 152, 162, 165, 167, 194, 216-218, 222).

One parameter used to measure the suitability of an animal model of infection is the bacterial colonisation of organs as measured by bacterial recovery post-infection. Inoculation with both BCG and *S. typhimurium* caused significant bacterial colonisation in target organs. In mice infected with BCG bacteria was recovered from the lungs by LH and BALF and infection levels were mapped up to 12-weeks post-infection (Figure 3.3.4). Comparable levels of bacteria were recovered from BALB/c and OVA-TCR Tg mice from 3-weeks post-infection onwards. However, significantly more bacteria was recovered from OVA-TCR Tg mice at peak of infection, 18-days post-infection when compared to BALB/c mice.

Macrophages are not as significant in the local immune response to BCG as neutrophils and lymphocytes (Figure 3.3.1b (C). This is due to the protective mechanisms of the *Mycobacterium* that allow it to exist inside macrophages, and avoid phagocytosis.

This demonstrates that OVA-TCR Tg mice have a slightly delayed immune response to BCG infection when compared to wild-type BALB/c mice. This can be explained by the pool of OVA-specific T-cells resident in OVA-TCR Tg mice. Although the T-cell population in these mice can undergo recombination events to develop T-cell specificity for other antigens, BALB/c mice have a larger naïve T-cell pool to achieve this than OVA-TCR Tg mice.

Mice orally inoculated with a high dose of *S. typhimurium* were colonised with live bacteria recoverable from the spleen and liver. Colonisation of multiple organs was only apparent in mice that received a high dose (1 x 10⁹ CFU) of *S. typhimurium* (Figure 3.3.7). It was therefore determined that the high dose infection was more appropriate for use in an infection model of *S. typhimurium*. At
this dose of infection there was no difference in respiratory leukocyte levels quantified from BALF (Figure 3.3.5a). The leukocytes were differentially identified to determine any differences in the pool of WBCs present between BALB/c and OVA-TCR Tg mice post-infection. Figure 3.3.5b shows that there was no difference in differential leukocyte counts between the 2 strains of mice tested.

However, infection with *S. typhimurium* did affect the 2 strains of mice differently as shown by their ability to control the infection. While BALB/c mice found it relatively difficult to control a *S. typhimurium* infection as demonstrated by their significant weight-loss in Figure 3.3.6, OVA-TCR Tg mice only exhibited minimal weight-loss after 24-hours from which they recovered from quickly, stabilising at their initial weight. It can be hypothesised that the intact immune system of the BALB/c mice enables them to mount a normal T-cell driven immune response to the invading Salmonella bacterium. This immune response has also been found to be detrimental to the host by causing excess inflammation and tissue injury. OVA-TCR Tg mice have an altered T-cell immune response and mount a delayed Salmonella-specific T-cell response. The mice therefore avoid these detrimental effects and remain healthy by just enacting the innate immune response to control the infection (223).
CHAPTER 4

The effects of BCG infection at different stages of immune development on the generation of AAD
4.1 Introduction

Discerning the mechanisms that underpin the programming of T-cells in childhood is fundamental to understanding the development of immune responses that lead to protection or aberrant outcomes (e.g. asthma). This chapter looks at the role of infection in early-life on the development/programming of CD4+ T-cell responses in the adult. The type of immune response generated in the adult may not only be dependent on the age of first exposure but also on the type of infectious agent and the route of infection.

It is established that T-cell programming is developed through innate and adaptive immune responses. It is, however, undefined how infection at different periods of life influences the development of antigen specific T-cells. T-cells play a crucial role in immune responses to vaccination, pathogens and for the induction of tolerance. Two major types of T helper cells (Th1 and Th2 cells) are linked to different types of immune responses. Th1 cells to cell-mediated immunity and CD8+ cytotoxic T-cell activity, and Th2 cells to humoral immunity (3).

The characteristic cytokines that are products of these T-cell responses are IFN-γ and IL-2 for Th1, which is a typical response to intracellular pathogens such as Salmonella and mycobacteria (e.g. BCG) (3). IL-4, IL-5, IL-10 and IL-13 are produced by Th2 cells in response to extracellular pathogens such as H. pylori and Helminths (3). Strong Th2 responses are associated with the induction of eosinophilia, mucous hypersecretion and airways hyper-responsiveness that are hallmark features of allergic diseases such as asthma (31, 87, 98, 103, 117).

The Neonatal immune system is strongly biased towards a Th2 response (5). It is hypothesised that exposure to different microbial antigens is needed for the maturation of the immune system to be able to respond in a Th1 fashion and develop a balanced, mixed Th1/Th2 phenotype. Studies have shown that certain bacterial infections (e.g. BCG) can direct the naïve immune system toward a Th1 response (194, 196).

By employing novel infectious models, in combination with transgenic T-cells I can track the development of antigen-specific immune responses.
In this chapter I will assess the effect of BCG inoculation at different stages of life on the subsequent development of AAD. The aim is to identify critical stages of life that underpin deviation of T-cells to elicit a protective or aberrant immune response.
4.2 Materials and Methods

4.2.1 Animals
BALB/cTac-TgN (DO11.10)Rag2\(^{+/+}\), OVA-TCR Tg SPF mice were supplied at 6-8 weeks of age from the University of Newcastle animal services unit. These mice were derived from mice developed by Hsieh et.al, 1995. OVA-TCR Tg were selected by staining peripheral blood leukocytes with the antyclonotype mAb KJ1-26. OVA-TCR Tgs on the BALB/c background have been backcrossed more than 12 generations (210).

These mice were housed in IVC, maximum 6 mice per cage in the David Madisson Building animal holding facility. The mice were fed commercial rodent pellets and water *ad libitum*. This project has animal ethics clearance and safety clearance from the University of Newcastle, Animal care and ethics committee and Institutional Biosafety committee.

4.2.2 Bacteria
Bacterial strain containing a vector expressing OVA- *Mycobacterium bovis* (Bacille Calmette Guerin) (BCG), Pasteur Pasteur 1173-P2 +pMV-OVA (Kanamycin Resistant).
Bacterial strain containing an empty vector not expressing OVA- *Mycobacterium bovis* (Bacille Calmette Guerin) (BCG), Pasteur Pasteur 1173-P2 +pMV (Kanamycin Resistant).

See section 3.2.2. for infection levels and doses used.

4.2.3 Determination of infection levels
Refer to chapter 3.2.4.

4.2.4 Induction of infection
Refer to chapter 3.2.5 and 3.2.6.
Mice were anaesthetised with isofluorane/oxygen mix delivered into an anaesthetic chamber. BCG was delivered intranasally (5µl was delivered to mice up to 2-weeks old and 50µl was given to mice 4-weeks and older).

4.2.5 Induction of AAD
Refer to chapter 2.2.2.

4.2.6 Collection and analysis of samples (blood, BALF)
Refer to chapter 2.2.3.

4.2.7 Lung histology
Refer to chapter 2.2.4.

4.2.8 Collection of Spleen and PBLN
Refer to chapter 2.2.5.

4.2.9 Collection and processing of tissue for bacterial recovery
Refer to chapter 3.2.3 and 3.2.4.

4.2.10 Homogenisation of lung tissue and homogenate processing
Refer to chapter 3.2.3.

4.2.11 Determination of Total CFU by bacterial recovery
Refer to chapter 3.2.4

4.2.12 Isolation of splenocytes and PBLN cells for tissue culture
Refer to chapter 2.2.6.

4.2.13 Isolation of splenocytes and PBLN cells for flow cytometry
Refer to chapter 2.2.6 and 4.2.14.
4.2.14 Processing and staining of splenocytes and PBLN cells for flow cytometry.
See chapter 2.2.6 for isolation of cells. Cells were plated out in a 96-well U-bottom plate using the same concentration of cells used for tissue culture, (100µL/well, 1x10^6 cells/well). The plate was spun at 1800rpm in a benchtop centrifuge for 3-minutes at 4°C. Supernatants were removed. Cells were washed by resuspending in staining buffer [0.5% BSA in PBS sterile syringe filtered] and centrifuged. The supernatant was again removed and discarded, and the cells were resuspended in blocking buffer [1µg Fc block in staining buffer] before incubating for 15minutes on ice. The plate was centrifuged, and then resuspended in staining buffer containing 1/100 dilutions of the following flow cytometry antibodies in different combinations CD4+APC, CD8+APC, KJ1-26+PE along with 1x 10^5 unstained cells which are resuspended in just staining buffer. The plate was incubated in the dark on ice for 1-hour before washing twice more in staining buffer. Finally the wells are resuspended in 200µl fixation buffer [1% paraformaldehyde in PBS] before removing to different Fluorescence-activated cell sorting (FACS) tubes according to the stain/sample. Tubes were fixed overnight in the dark at 4°C and then read using BD FACSCanto machine and FACS DiVa software. For details of antibodies refer to supplementary data.

4.2.15 Determination of antibody titres by ELISA
Refer to chapter 2.2.7.

4.2.16 Determination of culture supernatant cytokine levels by ELISA
Refer to chapter 2.2.8.

4.2.17 Statistical analysis
Refer to chapter 2.2.9.
Infection and/or sensitisation (yellow bars) was performed on day 0 of life or 2, 4 and 6-weeks after birth. Instillation of PBS, OVA, BCG (parent strain) or BCG:OVA (BCG-pKK-OVA, Tg BCG strain that produces OVA) was performed for sensitisation (PBS or OVA, yellow bars) and infection (BCG, BCG:OVA, yellow bars) as shown on the above schematic. At 10-weeks of age all mice were challenged twice (green bars), 24-hours apart with either PBS or OVAp. 48 hours later all mice were sacrificed and samples taken.

**Figure 4.2.18 Chapter 4 Experiment outline**
4.3 Results

4.3.1 Leukocyte infiltration into the airways in sham-infected and infected mice challenged with PBS or OVAp

Challenge of neonate (0-day old) and infant mice (2-week old) with OVAp significantly increased the total number of cells in BALF compared to sham-challenged mice (Figure 4.3.1a, PBS/PBS). Wild-type and recombinant BCG infection did not cause an influx of cells into the lung (Figure 4.3.1a, BCG: PBS/PBS). OVA-TCR Tg mice sensitised and challenged with OVA (OVA/OVA) had significantly increased lung leukocytes compared to sham-treated control (PBS/PBS) and BCG infected control mice (Figure 4.3.1a, BCG: PBS/PBS).

BCG infection caused a significant increase in neutrophils in mice challenged with OVA, independent of prior OVA exposure (Figure 4.3.1b, BCG: PBS/OVA, BCG: OVA/OVA, OVA/OVA). Lymphocyte recruitment in BALF was unchanged for all mice challenged or both sensitised and challenged with OVA. BCG infection also did not affect levels of lymphocytes in BALF. However, lack of OVA sensitisation without concurrent BCG infection in the neonate lowered lymphocyte recruitment in BALF (Figure 4.3.1b, PBS/PBS). BCG infection with concurrent OVA sensitisation significantly decreased the eosinophil recruitment after OVA challenge in neonate, infant and adult (4 and 6-week old) mice compared to sham-infected OVA sensitised and challenged mice (Figure 4.3.1b, BCG: OVA/OVA). BCG infection without concurrent OVA production also caused a significant decrease in eosinophil recruitment after OVA challenge compared to sham-infected OVA sensitised and challenged mice (Figure 4.3.1b, BCG:PBS/OVA). This excludes neonatal mice infected with BCG but not concurrent antigen (BCG: PBS/OVA). These mice showed no significant difference in eosinophil recruitment when compared to sham-infected OVA sensitised and challenged controls (Figure 4.3.1b, BCG: PBS/OVA).
Figure 4.3.1a Total leukocyte levels in BALF of mice infected with BCG and sensitised and challenged with OVA. Neonatal (0-day old), infant (2-week old) and adult (4 and 6-week old) mice were sensitised and challenged with saline or OVA after prior infection with BCG or sham-infection for controls. Data represents the mean number of viable leukocytes per millilitre of BALF +/- SEM for groups of 6 mice. Statistical significance was determined by Students T-test (p<0.05 *, p<0.001**).
Figure 4.3.1b Differential leukocyte counts (A) Macrophage, (B) Neutrophil, (C) Lymphocyte and (D) Eosinophil levels in BALF taken from airways of sham-infected and BCG infected mice sensitised and challenged with saline or OVA. Data represents the mean number of viable leukocytes per millilitre of BALF +/- SEM for groups of 6 mice. Statistical significance was determined by Students T-test when comparing leukocyte numbers to OVA sensitised and challenged mice (OVA/OVAp) of the same treatment age (p<0.05 *, p<0.001**).
4.3.2 Histopathology of lung tissue in sham-infected and infected mice challenged with PBS or OVAp.

Neonatal infection with BCG with or without concurrent antigen sensitisation decreased eosinophil and mucous secreting cell number in lung tissue after OVA challenge compared to controls (OVA/OVAp) (Figure 4.3.2, BCG: PBS/OVA, BCG: OVA/OVA). Infant and early adult (2 and 4 week old mice respectively) mice also showed significant decreases in eosinophil and mucous secreting cell number in lung tissue compared to allergic mice, but only after BCG infection that included concurrent antigen sensitisation (Figure 4.3.2, BCG: OVA/OVA). However, BCG infection of adult mice (6-week old) only resulted in a decrease of eosinophils in lung tissue, with no significant change in mucous cell number (Figure 4.3.2, BCG: PBS/OVA, BCG: OVA/OVA).

The data collected from the lung tissue of these mice indicates that BCG infection reduces the recruitment of inflammatory infiltrates to the lung (Figure 4.3.2).
Figure 4.3.2 Decrease in (A) Eosinophils and (B) Mucous secreting cells in lung tissue from airways of sham-infected and BCG infected mice sensitised and challenged with saline or OVA. Data represents the mean number of cells per 100µm of lung section +/- SEM for groups of 6 mice. Statistical significance was determined by Students T-test when comparing leukocyte numbers to OVA sensitised and challenged mice (OVA/OVAp) of the same treatment age (p<0.05 *, p<0.001**).
(A) 0-day old mice

4.3.1-2 Groups of 0-day old mice were treated and sampled according to the experiment schematic in section 4.2.18. Leukocyte infiltration into the airways and histopathology of the lung are shown in Figure 4.3.1 and 4.3.2, respectively.

4.3.3 0-day old bacterial recovery from lung.

Bacterial recovery performed by assessing colony forming units of BCG from LH and BALF allowed us to evaluate final infection levels in groups of mice infected as neonates. As was expected the uninfected groups had no BCG in their lungs and there was no significant difference in the levels of BCG recovered from the lungs of the 3 infected groups (see figure 4.3.3a).
Figure 4.3.3a Levels of BCG present in the lungs of mice at the time of sacrifice after infection/treatment as a neonate.

Data represents the mean number of viable CFU per millilitre of BALF and lung homogenate +/- SEM for groups of 6 mice. No statistical significance was determined by Students T-test.
4.3.4 Neonatal antibody responses in sham-infected and infected mice challenged with PBS or OVAp.

BCG infection with or without concurrent antigen exposure causes a significant increase in the amount of antigen-specific IgG2a levels in serum compared to uninfected controls (Figure 4.3.4a). No OVA-specific IgG1 was detected in the serum of mice from any of the groups tested (Figure 4.3.4a).

BCG infection in the first 24 hours of life induced an increase in antigen-specific IgG2a in serum of mice challenged as an adult with PBS or OVAp (Figure 4.3.4a).

Total IgE levels in serum remained unchanged between sham-treated control mice, OVA sensitised and challenged controls, and all BCG infected mice (see figure 4.3.4a). The lack of change in Th2 antibody levels, IgE and IgG1 in serum, and the significant increase in the Th1 antibody IgG2a illustrates that BCG infection promotes a Th1 antibody response. However, in neonatal mice infected with BCG there remains a sustained Th1 antibody response.
Figure 4.3.4a Levels of immunoglobulin (A) OVA-specific IgG1 and IgG2a and (B) total IgE levels in serum of mice infected as a neonate with BCG and challenged later in life with OVA.

Data represents the mean number of detectable Immunoglobulin in serum +/- SEM for groups of 6 mice.
4.3.5 Cytokine responses in neonatal control (PBS/PBS, OVA/OVAp) and infected mice challenged with PBS or OVAp (BCG: PBS/PBS, BCG: PBS/OVAp, BCG: OVA/OVAp).

Cytokine levels from splenocytes evaluated after antigen stimulation in culture determined that there are no significant increases in Th2 cytokines from mice infected with BCG in the first 24 hours of life (Figure 4.3.5.1a). BCG infection without concurrent antigen stimulation does, however, increase the OVA-induced levels of the Th1 cytokine IFN-γ when compared to mice sensitised and challenged with OVA (Figure 4.3.5.1a).

The splenocyte cytokine levels of these mice demonstrate the ability of BCG infection in the neonate to generate IFN-γ, indicating a slight increase in Th1 immune response to OVA. In the lymph nodes, however, the Th1 and Th2 cytokine response to OVA is significantly increased (Figure 4.3.5.1b). BCG infection without concurrent OVA stimulation causes a significant increase in OVA-induced IL-5, GM-CSF, IL-4, IL-13 and IFN-γ levels in PBLN cell supernatants (Figure 4.3.5.1b). BCG infection accompanied by concurrent OVA stimulation caused a significant decrease in OVA-induced IL-13 but no changes were seen in local Th1 cytokines levels (Figure 4.3.5.1b).
Figure 4.3.5.1a Levels of (A) IL-5, (B) IFN-gamma, (C) IL-4, (D) IL-13, (E) GM-CSF in unstimulated, OVAp stimulated and CD3/CD28 stimulated splenocytes of neonatal sham-infected or BCG-infected and OVA or saline sensitised and challenged mice.

Data represents the mean number of specific cytokine in culture supernatant +/- SEM for groups of 6 mice. Statistical significance was determined by Students T-test (p<0.05 *, p<0.001** compared to OVA/OVAp control).
Figure 4.3.5.1b Levels of (A) IL-5, (B) IFN-gamma, (C) IL-4, (D) IL-13, (E) GM-CSF in unstimulated, OVAp stimulated and CD3/CD28 stimulated PBLN cell culture supernatants. PBLN cultures were from neonatal mice that were sham or BCG infected prior to OVA or saline sensitisation and challenge. Data represents the mean number of detectable cytokine in culture supernatant +/- SEM for groups of 6 mice. Statistical significance was determined by Students T-test (p<0.05 *, p<0.001** compared to OVA/OVAp control).
4.3.6 Percentage of CD4+ and OVAp-specific T-cells in sham-infected and infected mice challenged with PBS or OVAp.

Changes in total CD4+ cells and antigen specific CD4+ T-cells from PBLN were evaluated by fluorescence activated cell-sorting (FACS) and flow cytometry. There was no significant change in the total number of CD4+ T-cells in PBLN (Figure 4.3.6.1). However, OVAp specific (KJ1-26+) CD4+ lymphocytes were significantly increased in mice after BCG infection without concurrent antigen exposure that received OVA challenge (Figure 4.3.6.1).
Figure 4.3.6.1 Percentage of (A) CD4+ cells as a percentage of total cells and (B) KJ1-26+ cells as a % of CD4+ cells in PBLN from mice that were sham or BCG infected as a neonate prior to OVA or saline sensitisation and challenge.

Data represents the mean number of detectable live PBLN cells +/- SEM for groups of 6 mice. Statistical significance was determined by Students T-test (p<0.05 * compared to OVA/OVAp control).
(B) 2-week old mice

4.3.1-2 Groups of 2-week old mice were treated and sampled according to the experiment schematic in section 4.2.18. Leukocyte infiltration into the airway and histopathology of the lung is shown in Figure 4.3.1 and 4.3.2 respectively.

4.3.3 2-week old bacterial recovery from lung.

Bacterial recovery performed by assessing colony forming units of BCG from LH and BALF allowed us to evaluate final infection levels in groups of mice infected as 2-week olds. As was expected the uninfected groups had no BCG in their lungs and there was no significant difference in the levels of BCG recovered from the lungs of the 3 infected groups (see figure 4.3.3b).
Figure 4.3.3b Levels of BCG present in the lungs of mice at the time of sacrifice after infection/treatment at 2-weeks of age.  
Data represents the mean number of viable CFU per millilitre of BALF and lung homogenate +/- SEM for groups of 6 mice.
4.3.4 Antibody responses in 2-week old sham-infected and infected mice challenged with PBS or OVAp.

The OVA-specific IgG1 levels were decreased in the serum of BCG infected mice compared to OVA sensitised and challenged controls (Figure 4.3.4a, OVA/OVAp compared to BCG: PBS/PBS, BCG: PBS/OVAp, BCG: OVA/OVAp). OVA-specific IgG2a levels were also decreased in groups infected with BCG prior to OVA sensitisation and challenge when compared to uninfected OVA sensitised and challenged mice (Figure 4.3.4a, OVA/OVAp compared to BCG: PBS/PBS, BCG: PBS/OVAp, BCG: OVA/OVAp). This indicates that groups infected with BCG have a decreased Th1 and Th2 antibody response to OVA challenge. Mice sensitised and challenged with OVA produced a markedly increased antigen-specific IgG1 and IgG2a antibody response (Figure 4.3.4a, OVA/OVAp).

Total IgE levels were evaluated in serum of mice sham-infected (PBS/PBS, OVA/OVAp) or infected with BCG (BCG: PBS/PBS, BCG: PBS/OVAp, BCG: OVA/OVAp) at 2-weeks of age prior to challenge with PBS or OVA as an adult. Serum IgE was slightly elevated in all mice infected with BCG compared to sham-infected mice. However, there was no significant difference in the levels of serum IgE between any of the experimental groups (Figure 4.3.4b).
Figure 4.3.4b Levels of immunoglobulin (A) OVA-specific IgG1 and IgG2a and (B) total IgE levels in serum of mice infected as a 2-week olds with BCG and challenged later in life with OVA.

Data represents the mean number of detectable immunoglobulin in serum +/- SEM for groups of 6 mice. Statistical significance was determined by Students T-test (p<0.05 * compared to PBS/PBS, BCG: PBS/PBS, BCG: PBS/OVAp, BCG: OVA/OVAp).
(C) 4-week old mice

4.3.1-2 Groups of 4-week old mice were treated and sampled according to the experiment schematic in section 4.2.18. Leukocyte infiltration into the airway and histopathology of the lung is shown in Figure 4.3.1 and 4.3.2, respectively.

4.3.3 4-week old bacterial recovery from lung.

Bacterial recovery performed by assessing colony forming units of BCG from LH and BALF allowed us to evaluate final infection levels in groups of mice infected as neonates. As was expected the uninfected groups had no BCG in their lungs and there was no significant difference in the levels of BCG recovered from the lungs of the 3 infected groups (see figure 4.3.3c).
Figure 4.3.3c Levels of BCG present in the lungs of mice at the time of sacrifice after infection/treatment at 4-weeks of age. Data represents the mean number of viable CFU per millilitre of BALF and lung homogenate +/- SEM for groups of 6 mice.
4.3.4 Antibody responses in 4-week old sham-infected and infected mice sensitised and challenged with PBS or OVAp.

BCG infected mice failed to generate an OVA-specific IgG1 and IgG2a antibody response regardless of sensitisation or challenge with OVA (Figure 4.3.4c). Mice sensitised and challenged with OVA without prior BCG infection, produced significant levels of serum antigen-specific IgG1 and IgG2a response (Figure 4.3.4c).

Serum Total IgE levels were also evaluated in these mice. Mice infected with BCG:OVA prior to OVAp challenge (BCG:OVA/OVA) had decreased serum IgE compared to OVA/OVAp treated mice. No other differences were observed in the systemic IgE antibody levels between the groups (Figure 4.3.4c).
Figure 4.3.4c Levels of immunoglobulin (A) OVA-specific IgG1 and IgG2a and (B) total IgE levels in serum of mice infected as 4-week olds with BCG and challenged later in life with OVA.

Data represents the mean number of detectable immunoglobulin in serum +/- SEM for groups of 6 mice. Statistical significance was determined by Students T-test (p<0.05 * (A) compared to PBS/PBS, BCG: PBS/PBS, BCG: PBS/OVAp, BCG: OVA:OVAp, (B)compared to OVA/OVAP control).
4.3.5 Cytokine responses in 4-week old control (PBS/PBS, OVA/OVAp) and infected mice challenged with PBS or OVAp (BCG: PBS/PBS, BCG: PBS/OVAp, BCG: OVA/OVAp).

Splenocytes from mice infected with BCG without concurrent antigen exposure before OVA challenge (BCG:PBS/OVAp) elicited an increased IL-5 and IFN-γ response to antigen compared to OVA sensitised and challenged mice (OVA/OVAp) (Figure 4.3.5.2a).

OVA-induced cytokines were also evaluated in the PBLN. No differences were seen in OVA-induced cytokine responses when comparing sham-infected, OVA sensitised and challenged mice (OVA/OVAp) with mice infected with BCG prior to sensitisation and challenge (BCG: PBS/PBS, BCG: PBS/OVAp, BCG: OVA/OVAp) (Figure 4.3.5.2b).
Figure 4.3.5.2a Adult (4-week old) levels of (A) IL-5, (B) IFN-gamma, (C) IL-4, (D) IL-13 in unstimulated, OVAp stimulated and CD3/CD28 stimulated splenocytes of sham-infected or BCG-infected and OVA or saline sensitised and challenged mice.

Data represents the level of specific cytokine in culture supernatant +/- SEM for groups of 6 mice. Statistical significance was determined by Students T-test (p<0.05 * compared to OVA/OVAP control).
Figure 4.3.5.2b Adult (4-week old) levels of (A) IL-5, (B) IFN-gamma, (C) IL-4, (D) IL-13 in unstimulated, OVAp stimulated and CD3/CD28 stimulated PBLN cell culture supernatants. PBLN cultures were from mice that were sham or BCG infected prior to OVA or saline sensitisation and challenge. Data represents the levels of specific cytokine in culture supernatant +/- SEM for groups of 6 mice. Statistical significance was determined by Students T-test (p<0.05 * compared to OVA/OVAp control).
4.3.6 Comparison of OVA +/- CD4+ T-cells in 4-week old sham-infected (PBS) and infected (BCG:PBS or BCG:OVA) mice challenged with PBS or OVAp.

CD4+ T lymphocyte levels were evaluated from antibody stained PBLN cells by flow cytometry. There was no significant change in the total number of CD4+ T-cells in PBLN (Figure 4.3.6.2). However, KJ1-26+ CD4-T-cells levels were significantly increased in mice after BCG infection without concurrent antigen exposure that received OVA challenge (Figure 4.3.6.2).
Figure 4.3.6.2 Percentage of (A) CD4+ cells as a percentage of total cells and (B) KJ1-26+ cells as a % of CD4+ cells in PBLN from mice that were sham or BCG infected at 4-weeks of age prior to OVA or saline sensitisation and challenge.

Data represents the mean number of detectable live PBLN cells +/- SEM for groups of 6 mice. Statistical significance was determined by Students T-test (p<0.05 * compared to OVA/OVAp control).
(D) 6-week old mice

4.3.1-2 Groups of 6-week old mice were treated and sampled according to the experiment schematic in section 4.2.18. Leukocyte infiltration into the airway and histopathology of the lung is shown in Figure 4.3.1 and 4.3.2 respectively.

4.3.3 6-week old bacterial recovery from lung.

Bacterial recovery performed by assessing colony forming units of BCG from LH and BALF allowed us to evaluate final infection levels in groups of mice infected as 6-week olds. As was expected the uninfected groups had no BCG in their lungs and there was no significant difference in the levels of BCG recovered from the lungs of the 3 infected groups (see figure 4.3.3d).
Figure 4.3.3d Levels of BCG present in the lungs of mice at the time of sacrifice after infection/treatment at 6-weeks of age.
Data represents the mean number of viable CFU per millilitre of BALF and lung homogenate +/- SEM for groups of 6 mice. Statistical significance was determined by Students T-test.
4.3.4 Antibody responses in 6-week old sham-infected and infected mice challenged with PBS or OVAp.

Mice infected with BCG prior to OVA challenge did not develop an OVA-specific IgG1 or IgG2a response (Figure 4.3.4d). Mice sensitised and challenged with OVA, without prior BCG infection, produced significant amounts of OVA-specific IgG1 and IgG2a, when compared to all other groups (Figure 4.3.4d).

Total IgE levels in serum were also evaluated by ELISA. It was determined that BCG infection with or without concurrent antigen stimulation does not alter total serum IgE (Figure 4.3.4d[B]). This is compared to serum total IgE levels in mice sensitised and challenged with OVA without prior BCG infection and PBS/PBS controls (Figure 4.3.4d).
Figure 4.3.4d Levels of Immunoglobulin (A) OVA-specific IgG1 and IgG2a and (B) total IgE levels in serum of mice infected as 6-week olds with BCG and challenged later in life with OVA.

Data represents the mean number of detectable immunoglobulin in serum +/- SEM for groups of 6 mice.
4.4 Discussion

BCG infection along with concurrent antigen exposure at any stage of immune development alters some immune parameters that are features of AAD. Infection at birth with BCG alone does not alter later onset of AAD. However, the data demonstrates that these alterations alone are not enough to halt the development of AAD.

Vaccination or infection with BCG has been shown to induce a strong Th1 cytokine response and lessen the symptoms of AAD (194, 196, 224). In this study I looked at the association of age of first exposure to BCG infection and whether concurrent antigen exposure can influence the resulting immune response.

BCG infection was successfully established in mice of all ages, which was demonstrated by the remaining bacterial levels in the airways at the time of sacrifice (Figure 4.3.3). AAD was induced by airway challenge with OVA in mice already sensitised to this antigen. The level of disease in these mice can be measured by cellular infiltrates into the airways (BALF) and lung tissue, systemic immunoglobulin levels, and local and systemic Th2 cytokines reflecting specific populations of T-cells.

Mice infected with BCG along with concurrent antigen exposure as a neonate, during childhood (2-weeks old) and at adulthood (4-6 weeks old), prior to OVA challenge exhibited lower levels of eosinophils in BALF compared to mice that received OVA sensitisation and challenge. BCG infection as a neonate, infant or as an adult without simultaneous antigen exposure prior to OVA challenge also resulted in lower levels of eosinophils in BALF (Figure 4.3.1). This result was replicated in the lung tissue with eosinophil levels again being significantly decreased after neonatal or adult BCG infection with or without concurrent antigen stimulus (Figure 4.3.2). BCG infection along with antigen exposure at all age groups also resulted in a reduction in eosinophil levels in lung tissue, but they did not significantly decrease after BCG infection alone (2 & 4 wk old, BCG:PBS/OVAp)(Figure 4.3.2). Decreased numbers of mucous secreting cells in the lung tissue were also observed after BCG infection with or without concurrent antigen exposure prior to OVA challenge, when compared to AAD (OVA/OVAp).
mice (Figure 4.3.2). 2 and 4-week old mice infected with BCG with concurrent OVA exposure prior to OVA challenge also had decreased numbers of MSCs in lung tissue compared to OVA sensitised and challenged mice. Increased levels of serum OVA-specific Th1 immunoglobulin, IgG2a or decreased levels of serum OVA-specific Th2 immunoglobulin, IgG1 were observed in all mice infected with BCG and exposed to OVA at birth, as an infant and as an adult, compared to OVA sensitised and challenged mice (Figure 4.3.4). Splenocyte and PBLN culture supernatant levels of the OVA-stimulated Th1 cytokine IFN-γ are also elevated in mice infected with BCG at birth prior to OVA challenge compared to AAD mice (Figure 4.3.5.1). Splenocyte culture levels of OVA- specific IFN-γ are also elevated in adult mice infected with BCG, which received OVA challenge compared to OVA sensitised and challenged mice (Figure 4.3.5.1).

BCG also induced a significant increase in Th1 Igs and cytokines that was dependant on age of infection. This effect can be explained by a change in the T-cell pool of mice infected with BCG prior to antigen challenge. Mice neonatally infected with BCG exhibited significantly higher numbers of transgenic OVA-specific CD4+ T-cells after OVA challenge, when compared to mice sensitised and challenged with OVA (Figure 4.3.6.1). This CD4+ T-cell pool was similar to that of mice infected with BCG as an adult (Figure 4.3.6.2), indicating that only mice with AAD had altered their CD4+ T-cell pool from baseline. This is suggested by the T-cell pool in mice infected and mice sham-infected prior to challenge with PBS, which exhibited significantly less CD4+KJ1-26- cells (non OVA-Tg T-cells) than OVA sensitised and challenged mice.

The immune response to BCG in mice and humans has long been identified as inducing a Th1 cytokine profile (179, 182, 195-197). It is, however, still a subject of investigation as to the cells involved in this immune response and which are responsible for this significant immune deviation. Here, I have shown that this Th2 to Th1 switch can be antigen-dependant and shows different changes depending on the age of first exposure to infection. Based on previous research by Major et al, IFN-γ production plays a key role in the Th2 to Th1 immune switching caused by
BCG exposure. Their research showed that attenuation of AAD caused by mycobacterium infection was suppressed in IFN-γ deficient mice (196).

By utilising a strain of mice that have transgenic T-cells specific to the OVAp changes in the phenotype of these cells after infection or initiation of allergic disease were tracked. The subsequent changes illustrate that BCG infection neonatally or as an adult significantly alters the CD4+ T-cell population towards a Th1 phenotype with increased production of OVA-specific IFN-γ. This change could in turn be responsible for alteration in antibody production and the down-regulation of some immune parameters of AAD. The role of IFN-γ in AAD has been studied by investigating the effects of exogenous and endogenous IFN-γ in animal models. IFN-γ therapy in wild-type and knockout mice has been shown to down-regulate symptoms of AAD. It has been shown to act by inhibiting the infiltration of CD4+ T-cells (122), inhibiting infiltration of eosinophils, down-regulation of IgE, and suppress airway-hyper-responsiveness (225). Further investigation is required to determine the mechanisms of how the immune system is educated and why it is age-dependant.

It is worthwhile noting that as Th1 cytokines are also associated with detrimental inflammation, a mixed (balanced) Th1/Th2 phenotype is preferable. In this model it is observed that cytokine production measured from OVA stimulated splenocyte and PBLN culture supernatants produce high amounts of both Th1 and Th2 cytokines. This is possibly the result of the pre-specificity of the OVA-Tg T-cells present in these mice, and must be taken into consideration when evaluating the results of experiments utilising these mice.
CHAPTER 5

The effects of *S. typhimurium* infection at different stages of immune development on the development of AAD
5.1 Introduction

A review published in 2000 by Jones et al addressed the inverse relationship between Salmonellosis and the prevalence of asthma worldwide. This review proposed this link by noting that the 5 countries with the highest frequency of Salmonellosis (identified by recommendation for typhoid immunisation) are among those with the lowest incidence of asthma (204).

*S.typhimurium*, like other intracellular microorganisms (e.g. *L.monocytogenes, L.major, M.tuberculosis*) has been shown to induce a Th1-dependant immune response (142, 143). IFN-γ is essential for *S.typhimurium* clearance (144). The role of Th1 cytokines in the immune response to Salmonella infection has been further validated in cases when IFN-γ and IL-12 cytokine or receptor deficiency occurs in humans. These patients exhibited increased risk of severe and recurrent intracellular infections (161). The induction of Th1 cytokine production by Salmonella infection is hypothesised to be capable of down-regulating the asthmatic Th2 response, thus diminishing AAD symptoms such as the infiltration of inflammatory cells into the lungs.

By employing an infectious model of *S.typhimurium* infection, in combination with Tg T-cells present in OVA-TCR Tg mice I can track the development of immune responses. I will assess the effect of *S.typhimurium* at different stages of life on the immune system, specifically the phenotype of T-cells that are subsequently generated identifying critical stages of immunological education. Findings from this research will contribute to the establishment of strategies to prevent detrimental inflammatory disease in adulthood.
5.2 Materials and Methods

5.2.1 Animals

BALB/cTac-TgN (DO11.10) Rag2+/+, OVA-TCR Tg SPF mice were supplied at 6-8 weeks of age from the University of Newcastle animal services unit. These mice were derived from mice developed by Hsieh et al., 1995. OVA-TCR Tg were selected by staining peripheral blood leukocytes with the anti-clonotype mAb KJ1-26. OVA-TCR Tgs on the BALB/c background have been backcrossed more than 12 generations (210).

These mice were housed in IVC, maximum 6 mice per cage in the David Madisson Building animal holding facility. The mice were fed commercial rodent pellets and water ad libitum. This project has animal ethics clearance and safety clearance from the University of Newcastle, Animal care and ethics committee and Institutional Biosafety committee.

5.2.2 Bacteria

Bacterial strain containing a vector expressing OVA- Salmonella enterica serovar typhimurium aroA aroD mutant BRD09 +pKK-OVA (Streptomycin/Ampicillin Resistant).

Bacterial strain containing an empty vector not expressing OVA- Salmonella enterica serovar typhimurium aroA aroD mutant BRD09 +pKK (Streptomycin/Ampicillin Resistant).

See section 3.2.2 for infection levels and doses used.

5.2.3 Determination of infection level in spleen homogenate, liver homogenate and gut wash

Refer to section 3.2.4

5.2.4 Induction of infection

15 minutes prior to infection mice were anaesthetised with isofluorane/oxygen mix delivered into an anaesthetic chamber and given a
gavage of 0.75% sodium bicarbonate of the same volume of bacteria used for infection. 0-day old mice did not receive the sodium bicarbonate and were fed 5µl S.typhimurium solution using a sterile gel-loading tip and letting the pup suckle on the tip. 2-week old mice were given the bacteria in 10µl using an 8cm long ball-ended gavage needle, 4-6 week old mice were given the bacteria in 200µl using a 15cm long ball-ended gavage needle.

5.2.5. Induction of AAD
Refer to section 2.2.2

5.2.6. Collection and analysis of samples (Blood, BALF)
Refer to section 2.2.3

5.2.7. Lung Histology
Refer to section 2.2.4

5.2.8. Collection of Spleen and PBLNs
Refer to section 2.2.5

5.2.9. Collection and processing of spleen and liver tissue plus gut wash for bacterial recovery
Refer to section 3.2.9

5.2.10 Homogenisation of spleen and liver tissue and homogenate processing
Refer to section 3.2.3

5.2.11 Determination of total CFU by bacterial recovery
Refer to section 3.2.4
5.2.12 Isolation of Splenocytes and PBLN cells for tissue culture
Refer to section 2.2.6

5.2.13 Isolation and staining of splenocytes and PBLN cells for flow cytometry.
Refer to section 4.2.13

5.2.14 Processing and staining of splenocytes and PBLN cells for flow cytometry.
Refer to section 4.2.14

5.2.15 Determination of Antibody titres by ELISA
Refer to section 2.2.7

5.2.16 Determination of culture supernatant cytokine levels by ELISA.
Refer to section 2.2.8

5.2.17 Statistical analysis
Refer to section 2.2.9
5.2.18 Experiment schematic

Figure 5.2.18 Chapter 5 Experiment outline

Infection and/or sensitisation (yellow bars) was performed on day 0 of life or 2, 4 and 6-weeks after birth. Instillation of PBS, OVA, *S.typhimurium* or *S.typhimurium*: OVA was performed for sensitisation (PBS or OVA, yellow bars) and infection (*S.typhimurium* or *S.typhimurium*: OVA, yellow bars) *S.typhimurium* or *S.typhimurium*: OVA. At 8-weeks of age all mice were challenged twice (green bars), 24-hours apart with either PBS or OVAp. 48 hours later all mice were sacrificed and samples taken.
5.3 Results

5.3.1 Leukocyte infiltration into the airways in sham-infected and infected mice challenged with PBS or OVAp.

Total cell numbers quantified from BALF showed no significant differences between groups in infant and neonatal mice (Figure 5.3.1a). In Adult (6-week old) mice total leukocytes were significantly lower in mice infected with *S. typhimurium* (with or without concurrent OVA exposure prior to OVAp challenge) compared to AAD (OVA sensitised and OVAp challenged) controls (Figure 5.3.1a). Thus, *S. typhimurium* infection in the adult decreased inflammatory cell infiltrates induced by OVA challenge.

Figure 5.3.1b illustrates changes in differential leukocytes in the BALF of mice infected with *S. typhimurium* prior to OVA challenge in comparison to OVA sensitised and challenged mice. Mice infected with *S. typhimurium* with or without concurrent OVA exposure at 6-weeks old and challenged with saline or OVA exhibited a significantly lower number of macrophages in BALF then OVA sensitised and challenged controls (5.3.1b [A]). *S. typhimurium* infection at 4-weeks of age whilst receiving concurrent OVA exposure prior to OVA challenge (Styph: OVA/OVA) caused a significant increase in local neutrophil levels compared to OVA sensitised and challenged controls (OVA/OVA) (5.3.1b [B]). All mice not challenged with OVA (PBS/PBS, Styph:PBS/PBS) had extremely low levels of neutrophils including mice infected with *S. typhimurium*.

Adult infection at 4-weeks old, with *S. typhimurium* with concurrent OVA exposure prior to OVA challenge causes a decrease in total lymphocytes in BALF (5.3.1b [C]). Neonatal *S. typhimurium* infection without concurrent OVA exposure increases lymphocyte levels in BALF on challenge with OVAp compared to OVA sensitised and challenged controls.

Eosinophil levels in BALF were significantly reduced in mice infected with *S. typhimurium* without concurrent antigen exposure, at all ages, prior to OVA
challenge when compared to AAD (OVA sensitised and challenged) controls (5.3.1b [D]). Mice infected with *S. typhimurium* along with concurrent antigen exposure prior to OVA challenge only showed a significant decrease in eosinophil numbers in BALF if they were infected as an infant (2-weeks old) or neonate (0-day old), no significant difference was seen in mice infected as an adult (5.3.1b [D]).
Figure 5.3.1a  Total leukocyte levels in BALF of mice infected with *S.*typhimurium* and sensitised and challenged with OVA. Neonatal (0-day old), infant (2-week old) and adult (4 and 6-week old) mice were sensitised and challenged with saline or OVA after prior infection with *S.*typhimurium* or sham-infection for controls. Data represents the mean number of viable leukocytes per millilitre of BALF +/- SEM for groups of 6 mice. Statistical significance was determined by Students T-test when compared to OVA/OVAp treated controls of the same age at treatment (p<0.05 *).
Figure 5.3.1b  Differential leukocyte (A) Macrophage, (B) Neutrophil, (C) Lymphocyte, (D) Eosinophil levels of BALF from airways of sham-infected and S. typhimurium infected mice sensitised and challenged with saline or OVA. Data represents the mean number of viable leukocytes per millilitre of BALF +/- SEM for groups of 6 mice. Statistical significance was determined by Student's T-test when comparing leukocyte numbers compared to OVA sensitised and challenged mice (p<0.05 *, p<0.001**).
5.3.2 Histopathology of lung tissue in sham-infected and infected mice challenged with PBS or OVAp.

Lung tissue taken from mice infected as neonates, infants and adults with *S.*typhimurium was sectioned and stained to determine levels of eosinophils and mucous secreting cells. Mice infected with *S.*typhimurium with or without concurrent antigen exposure as a neonate, infant or adult (6-week old only) prior to OVA challenge showed significantly decreased eosinophil levels compared to OVA sensitised and challenged controls (Figure 5.3.2[A]). This same trend was seen with mucous secreting cells in mice infected with *S.*typhimurium without concurrent antigen exposure. However, only infant mice displayed significantly decreased eosinophilia after *S.*typhimurium infection, with concurrent OVA exposure when compared to AAD controls (Figure 5.3.2[B]).
Figure 5.3.2 Decrease in (A) Eosinophils and (B) Mucous secreting cells in lung tissue from airways of sham-infected and S. typhimurium infected mice sensitised and challenged with saline or OVA. Data represents the mean number of cells per 100µm of lung section +/- SEM for groups of 6 mice. Statistical significance was determined by Students T-test (p<0.05 *, p<0.001** compared to OVA/OVAP control).
(A) 0-day old

5.3.1-2 Groups of 0-day old mice were treated and sampled according to the experiment schematic in section 4.2.18. Leukocyte infiltration into the airway and histopathology of the lung is shown in Figure 5.3.1 and 5.3.2, respectively.

5.3.3 0-day old bacterial recovery from gastro-intestinal tract.

Bacterial recovery was performed by assessing CFU of *S.typhimurium* from spleen, liver and gut wash to evaluate final infection levels in groups of mice infected as neonates. As was expected the uninfected groups had no *S.typhimurium* in their gastro-intestinal tract and there was no significant difference in the levels of *S.typhimurium* recovered from the gastro-intestinal tract of the 3 infected groups (see figure 5.3.3a).
Figure 5.3.3a Levels of *S. typhimurium* present in the organs of mice at the time of sacrifice after infection/treatment as a neonate. Data represents the mean number of viable CFU per millilitre of gut wash, spleen homogenate (SH) and liver homogenate +/- SEM for groups of 6 mice.
5.3.4 Neonatal antibody responses in sham-infected and infected mice challenged with PBS or OVAp.

At sacrifice mice were bled from the heart to isolate serum for evaluation of systemic antibody levels. Neonatal mice infected with *S. typhimurium* exhibited a significantly increased level of the Th1 antibody OVA-specific IgG2a in serum (Figure 5.3.4a [A]). OVA-specific IgG1, a Th2 antibody was only significantly increased in mice infected with *S. typhimurium* accompanied by concurrent antigen exposure.

Systemic levels of IgE were also evaluated by ELISA of serum from mice infected with *S. typhimurium* as neonates. No significant differences were seen in the serum IgE levels between mice infected with *S. typhimurium* compared to AAD mice and sham-inoculated controls (Figure 5.3.4a [B]).
Figure 5.3.4a Levels of immunoglobulin (A) OVA-specific IgG1 and IgG2a and (B) total IgE levels in serum of mice infected as a neonate with *S.typhimurium* and challenged later in life with OVA. Data represents the mean number of detectable Immunoglobulin in serum +/- SEM for groups of 6 mice.
5.3.5 Cytokine responses in neonatal control (PBS/PBS, OVA/OVAp) and infected mice challenged with PBS or OVAp (Styph: PBS/PBS, Styph: PBS/OVAp, Styph: OVA/OVAp).

Systemic cytokine levels evaluated from antigen stimulated splenocytes showed that infection with *S.typhimurium* as a neonate prior to OVA challenge decreased OVA stimulated IL-5 production compared to AAD mice (Figure 5.3.5.1a [A]). No significant difference was seen in OVA-stimulated local cytokine production as determined by the cytokine levels from local lymph nodes (Figure 5.3.5.1b [A]).

Systemic OVA-stimulated IFN-γ levels were significantly increased in mice infected with *S.typhimurium* with concurrent OVA exposure as a neonate prior to OVA challenge (Figure 5.3.5.1a [B]). Local OVA stimulated IFN-γ levels were not significantly different between AAD control mice (OVA sensitised and challenged) and *S.typhimurium* infected mice (Figure 5.3.5.1b [B]).

The levels of IL-4, IL-13 and GM-CSF measured from splenocyte and lymphocyte cultures stimulated with OVA showed no differences between groups (Figure 5.3.5.1a-b).

Neonatal infection with *S.typhimurium* causes systemic changes in response to OVA exposure. *S.typhimurium* infection causes mice to produce more IFN-γ and less IL-5 from splenocytes, a marked change from responses seen in AAD (OVA sensitised and challenged) mice.
Figure 5.3.5.1a Levels of (A) IL-5, (B) IFN-gamma, (C) IL-4, (D) IL-13, (E) GM-CSF in unstimulated, OVAp stimulated and CD3/CD28 stimulated splenocytes of neonatal mice sham-infected or *S.*typhimurium*-infected OVA or saline sensitised and challenged as adults. Data represents the mean number of detectable cytokine in culture supernatant +/- SEM for groups of 6 mice. Statistical significance was determined by Students T-test (p<0.05 * compared to OVA/OVAP control).
Figure 5.3.5.1b Levels of (A) IL-5, (B) IFN-gamma, (C) IL-4, (D) IL-13, (E) GM-CSF in unstimulated, OVAp stimulated and CD3/CD28 stimulated PBLN cell culture supernatants. PBLN cultures were from neonatal mice sham-infected or S.typhimurium-infected OVA or saline sensitised and challenged as adults.

Data represents the mean number of detectable cytokine in culture supernatant +/- SEM for groups of 6 mice. Statistical significance was determined by Students T-test (p<0.05 * compared to OVA/OVAP control).
5.3.6 Percentage of CD4+ and OVAp-specific T-cells in sham-infected and infected mice challenged with PBS or OVAp.

*S. typhimurium* infection as a neonate leads to changes in the CD4+ T-cell population after antigen exposure later in life. Figure 5.3.6.1 shows that the total number of CD4+ T-cells remains unchanged between *S. typhimurium* infected mice and AAD controls. However, the number of OVA specific T-cells is significantly altered in mice infected with *S. typhimurium* at birth and challenged with OVA later in life. These mice have significantly higher numbers of CD4+ T-cells not specific for the OVA antigen compared to AAD and PBS treated controls, which make up the majority of their CD4+ T-cell population. This means that mice infected with *S. typhimurium* as neonates prior to OVA challenge in later life have a significantly decreased number of OVA-Tg CD4+ T-cells (Figure 5.3.6.1).
Figure 5.3.6.1 Percentage of (A) CD4+ cells as a percentage of total cells and (B) KJ1-26+ cells as a % of CD4+ cells in PBLN from mice that were sham or *S*. *typhimurium* infected as a neonate prior to OVA or saline sensitisation and challenge.

Data represents the mean number of detectable live PBLN cells +/- SEM for groups of 6 mice. Statistical significance was determined by Students T-test (p<0.05 * compared to OVA/OVAp control).
(B) 2-week old mice

5.3.1-2 Groups of 2-week old mice were treated and sampled according to the experiment schematic in section 5.2.18. Leukocyte infiltration into the airway and histopathology of the lung is shown in Figure 5.3.1 and 5.3.2 respectively.

5.3.3 2-week old bacterial recovery from lung.

Bacterial recovery was performed by assessing CFU of *S. typhimurium* from spleen, liver and gut wash to evaluate final infection levels in groups of mice infected as neonates. As was expected the uninfected groups had no *S. typhimurium* in their gastro-intestinal tract and there was no significant difference in the levels of *S. typhimurium* recovered from the gastro-intestinal tract of the 3 infected groups (see figure 5.3.3b).
Figure 5.3.3b Levels of *S. typhimurium* present in the organs of mice at the time of sacrifice after infection/treatment at 2-weeks of age. Data represents the mean number of viable CFU per millilitre of gut wash, SH and liver homogenate +/- SEM for groups of 6 mice.
5.3.4 2-week old antibody responses in sham-infected and infected mice challenged with PBS or OVAp.

Infection with *S. typhimurium* with concurrent OVA exposure as an infant (2-weeks old) prior to OVA challenge increased the systemic levels of OVA-specific IgG1 compared to AAD controls. Infant mice infected with *S. typhimurium* whilst being exposed to antigen also had a significant increase in OVA-specific IgG2a in serum (Figure 5.3.4b [A]) compared to mice sham-infected and sensitised and challenged with OVA.

Systemic levels of IgE antibody were not significantly different between mice infected as infants and AAD controls (Figure 5.3.4b [B]).
Figure 5.3.4b Levels of immunoglobulin (A) OVA-specific IgG1 and IgG2a and (B) total IgE levels in serum of mice infected at 2-weeks of age with *S.typhimurium* and challenged later in life with OVA.

Data represents the mean number of detectable Immunoglobulin in serum +/- SEM for groups of 6 mice.
(C) 4-week old mice

5.3.1-2 Groups of 4-week old mice were treated and sampled according to the experiment schematic in section 5.2.18. Leukocyte infiltration into the airway and histopathology of the lung is shown in Figure 5.3.1 and 5.3.2 respectively.

5.3.3 4-week old bacterial recovery from lung.

Bacterial recovery was performed by assessing CFU of *S. typhimurium* from spleen, liver and gut wash to evaluate final infection levels in groups of mice infected as neonates. As was expected the uninfected groups had no *S. typhimurium* in their gastro-intestinal tract and there was no significant difference in the levels of *S. typhimurium* recovered from the gastro-intestinal tract of the 3 infected groups (see figure 5.3.3c).
Figure 5.3.3c Levels of *S. typhimurium* present in the organs of mice at the time of sacrifice after infection/treatment at 4-weeks of age. Data represents the mean number of viable CFU per millilitre of gut wash, SH and liver homogenate +/- SEM for groups of 6 mice.
5.3.4 4-week old antibody responses in sham-infected and infected mice challenged with PBS or OVAp.

Adult (4-week old) mice infected with *S. typhimurium* have significantly decreased levels of OVA-specific IgG1 and IgG2a in their serum by contrast to mice sham-infected after OVA challenge later in life (Figure 5.3.4c [A]). Infection as an adult (4-week old) decreases both the Th1 and Th2 systemic antigen-specific antibody response.

Serum total IgE levels are increased after antigen challenge in mice sensitised as adults as seen in figure 5.3.4c [B]. Systemic IgE levels are significantly decreased in mice infected with *S. typhimurium* as an adult prior to OVA challenge compared to mice sensitised and challenged with OVA. This decrease in systemic Th2 antibody levels occurs in mice infected with *S. typhimurium* with and without concurrent OVA exposure.
Figure 5.3.4c Levels of immunoglobulin (A) OVA-specific IgG1 and IgG2a and (B) total IgE levels in serum of mice infected at 4-weeks of age with *S.typhimurium* and challenged later in life with OVA.

Data represents the mean number of detectable Immunoglobulin in serum +/- SEM for groups of 6 mice. Statistical significance was determined by Students T-test (p<0.05 * compared to OVA/OVAP control).
(D) 6-week old mice

5.3.1-2 Groups of 6-week old mice were treated and sampled according to the experiment schematic in section 5.2.18. Leukocyte infiltration into the airway and histopathology of the lung is shown in Figure 5.3.1 and 5.3.2 respectively.

5.3.3 6-week old bacterial recovery from lung.

Bacterial recovery was performed by assessing CFU of *S. typhimurium* from spleen, liver and gut wash to evaluate final infection levels in groups of mice infected as neonates. As was expected the uninfected groups had no *S. typhimurium* in their gastro-intestinal tract and there was no significant difference in the levels of *S. typhimurium* recovered from the gastro-intestinal tract of the 3 infected groups (see figure 5.3.3d).
Figure 5.3.3d Levels of *S. typhimurium* present in the gastrointestinal organs of mice at the time of sacrifice after infection/treatment at 6-weeks of age. Data represents the mean number of viable CFU per millilitre of gut wash, SH and liver homogenate +/- SEM for groups of 6 mice.
5.3.4 6-week old antibody responses in sham-infected and infected mice challenged with PBS or OVAp.

*Salmonella typhimurium* infection as an adult followed by antigen challenge later in life results in low systemic levels of OVA-specific IgG2a as with AAD mice. The stimulation of AAD in mice also induced a high level of serum OVA-specific IgG1. Mice infected as an adult with *Salmonella typhimurium* with concurrent OVA sensitisation and challenge had significantly decreased serum OVA-specific IgG1 levels compared to the AAD mice (Figure 5.3.4d). The infection of these mice resulted in a decrease in the systemic production of the Th2 antigen-specific antibody IgG1.

Levels of Total IgE in serum remained unchanged between mice with AAD, sham-treated mice and mice infected with *Salmonella typhimurium* (Figure 5.3.4d).
Figure 5.3.4d Levels of immunoglobulin (A) OVA-specific IgG1 and IgG2a and (B) total IgE levels in serum of mice infected at 6-weeks of age with *S.*typhimurium and challenged later in life with OVA. Data represents the mean number of detectable Immunoglobulin in serum +/- SEM for groups of 6 mice. Statistical significance was determined by Students T-test (p<0.05 * compared to OVA/OVAP control).
5.3.5 Cytokine responses in neonatal control (PBS/PBS, OVA/OVAp) and infected mice challenged with PBS or OVAp (Styph: PBS/PBS, Styph: PBS/OVAp, Styph: OVA/OVAp).

Antigen stimulated cytokine levels were evaluated systemically (from spleens) and locally (from lymph nodes) to assess the immune response produced by infection and OVA treatments. Figure 5.3.5.2a-b shows that adult mice infected with *S.typhimurium* with concurrent OVA exposure prior to OVA challenge results in significantly decreased levels OVA-stimulated IL-5 and GM-CSF systemically. Systemic GM-CSF levels are also reduced in mice infected with *S.typhimurium* without concurrent OVA exposure prior to OVA challenge when compared to AAD mice.

Local antigen stimulated IL-5 levels are also lowered in mice infected with *S.typhimurium* prior to OVA challenge compared to mice in which AAD is induced (Figure 5.3.5.2a [A]). Levels of the antigen stimulated Th1 cytokine IFN-γ were not significantly different between the groups tested.
Figure 5.3.5.2a Adult levels of (A) IL-5, (B) IFN-gamma, (C) IL-4, (D) IL-13, (E) GM-CSF in unstimulated, OVA-p stimulated and CD3/CD28 stimulated splenocytes of sham-infected or *S.typhimurium*-infected and OVA or saline sensitised and challenged mice.

Data represents the mean number of detectable cytokine in culture supernatant +/- SEM for groups of 6 mice. Statistical significance was determined by Students T-test (p<0.05 * compared to OVA/OVAP control).
Figure 5.3.5.2b Adult levels of (A) IL-5, (B) IFN-gamma, (C) IL-4, (D) IL-13, (E) GM-CSF in unstimulated, OVAp stimulated and CD3/CD28 stimulated PBLN cell culture supernatants. PBLN cultures were from mice that were sham or *S. typhimurium* infected prior to OVA or saline sensitisation and challenge. Data represents the mean number of detectable cytokine in culture supernatant +/- SEM for groups of 6 mice. Statistical significance was determined by Students T-test (p<0.05 *).
5.3.6 Percentage of CD4+ and OVAp-specific T-cells in sham-infected and infected mice challenged with PBS or OVAp.

CD4+ T-cell populations from PBLNs collected from mice treated as adults with live \textit{S.typhimurium}, sham (saline) infection or OVA sensitisation prior to saline or OVA challenge were evaluated with flow cytometry. Figure 5.3.6.2 shows that total numbers of CD4+ T-cells remained unchanged between the groups. Within this CD4+ cell population OVA-peptide specific CD4+ T-cells were tracked using the KJ1-26 marker. Adult mice in which AAD was elicited developed a population of CD4+ T-cells with approximately 50% OVA-specific T-cells compared to all other groups which had on average 95% OVA-specific T-cells. Therefore, AAD mice produced a significantly greater number of CD4+ cells that were not specific for the antigen OVA compared to all mice infected with \textit{S.typhimurium} and sham-treated controls.
Figure 5.3.6.2 Percentage of (A) CD4+ cells and (B) KJ1-26+ cells as a % of total CD4+ cells in PBLN from mice that were sham or S. typhimurium infected at 6-weeks of age prior to OVA or saline sensitisation and challenge. Data represents the mean number of detectable live PBLN cells +/- SEM for groups of 6 mice. Statistical significance was determined by Students T-test (p<0.05 * compared to OVA/OVAP control).
5.3 Discussion

*Salmonella typhimurium* infection along with concurrent OVA exposure at any stage of immune development alters some immune parameters that are indicative of AAD. However, infection as an adult (4-6 weeks old) with *Salmonella typhimurium* alone does not alter later onset of AAD.

In humans and mice protective responses to Salmonella manifest themselves as a Th1 type, as indicated by prominent IFN-γ and IL-2 production during infection (148, 167, 170).

A successful *Salmonella typhimurium* infection was achieved in mice of all ages, which was shown by the remaining bacterial levels in the airways at the time of sacrifice (Figure 5.3.3). Features of AAD were induced in mice by a challenge instillation of OVA in mice already sensitised to the antigen. The level of disease in these mice can be measured by cellular infiltrates into the airways from BALF and lung tissue, systemic Ig levels, local and systemic Th2 cytokines and specific populations of T-cells.

Mice infected with *Salmonella typhimurium* without concurrent antigen exposure in the first 24-hours of life, during childhood (2-weeks old) and at adulthood (4-6 weeks old) exhibited lower levels of eosinophils in BALF compared to mice that received OVA sensitisation and challenge (Figure 5.3.1). *Salmonella typhimurium* infection as a neonate or infant with simultaneous OVA exposure also resulted in lower levels of eosinophils in BALF (Figure 5.3.1). This result was replicated in the lung tissue with eosinophil levels again being significantly decreased after neonatal, infant (2-week old) or adult (6-week old) *Salmonella typhimurium* infection, with or without, concurrent OVA exposure (Figure 5.3.2). The parameters of AAD in OVA sensitised and challenged mice were further alleviated by lower mucous secreting cell numbers in the lung tissue (Figure 5.3.2). *Salmonella typhimurium* infection without concurrent antigen exposure resulted in significantly decreased levels of MSCs in mice infected at 0-days old, 2-weeks old and 6-weeks old when compared to their OVA sensitised and challenged controls. *Salmonella typhimurium* infection at 2-weeks of age along with antigen exposure also resulted in a reduction in MSC levels in lung tissue (Figure 5.3.2).
Decreased levels of OVA-specific Th1 immunoglobulin, IgG2a and OVA-specific Th2 immunoglobulin, IgG1 in all mice infected with \textit{S.typhimurium} with or without concurrent OVA exposure as an adult (4 and 6-weeks old) compared to OVA sensitised and challenged mice also indicated a change in the immune response to OVA (Figure 5.3.4). Neonatal and infant infection with \textit{S.typhimurium} with or without concurrent OVA exposure resulted in increased levels of OVA-specific IgG2a and OVA-specific IgG1 in all mice infected with \textit{S.typhimurium}, with or without concurrent OVA exposure compared to OVA sensitised and challenged mice (Figure 5.3.4). However, serum total IgE level were only significantly decreased in mice infected with \textit{S.typhimurium} (Styph:PBS/OVA, Styph:OVA/OVA) at 4-weeks old compared to OVA/OVAp treated mice. This is in contrast to mice infected at 6-week olds which still had elevated serum IgE levels. Despite this observation, mice infected with \textit{S.typhimurium} at 6-weeks old (adult mice) displayed a greater overall change in AAD parameters measured than 4-week olds (just post-weaning). This emphasises the importance of timing of exposure to infection on altering different facets of the immune response.

Splenocyte culture levels of the Th2 cytokine IL-5 is reduced in mice infected as neonates with \textit{S.typhimurium} with or without concurrent OVA exposure when compared to OVA sensitised and challenged control mice (Figure 5.3.5.1a-b). Neonatally infected mice also had significantly higher levels of IFN-\(\gamma\) from splenocyte culture supernatants after \textit{S.typhimurium} infection with concurrent OVA exposure, compared to AAD controls (Figure 5.3.5.1a-b). Adult mice (6-week old) mice infected with \textit{S.typhimurium} without concurrent OVA exposure had decreased levels of GM-CSF from splenocyte cultures and IL-5 from PBLN cultures compared to AAD controls (Figure 5.3.5.2a-b). Adult infection with concurrent OVA exposure resulted in lower levels of systemic IL-5 and GM-CSF levels than OVA sensitised and challenged controls (Figure 5.3.5.2a).

\textit{S.typhimurium} infection without concurrent antigen exposure causes significant changes in Th1 immunoglobulin and cytokine levels that are dependent on the age of the mice at the time of infection. This polarisation can be explained
by a change in the T-cell pool of mice infected with *S.typhimurium* prior to antigen challenge.

Mice neonatally infected with *S.typhimurium* without concurrent OVA exposure exhibited significantly lower numbers of Tg OVA-specific CD4+ T-cells after antigen challenge when compared to AAD mice sensitised and challenged with OVA (Figure 5.3.5.1). This CD4+ T-cell pool was different to that of mice infected with *S.typhimurium* with or without concurrent OVA exposure as an adult (Figure 5.3.5.2). Mice sham-infected and challenged with PBS (PBS/PBS), and *S.typhimurium* infected mice (Styph: PBS/PBS, Styph: PBS/OVA and Styph:OVA/OVA) had increased numbers of OVA-Tg CD4+ cells compared to OVA sensitised and challenged mice (OVA/OVA). This indicates that only AAD control mice had an altered CD4+ T-cell pool from baseline. This is also shown by the T-cell pool of mice infected prior to challenge with OVA and sham-infected prior to PBS challenge which also exhibited significantly less CD4+KJ1-26- cells (Non-Tg T-cells) than OVA sensitised and challenged mice.

The immune response to *S.typhimurium* in mice and humans has long been identified as inducing a Th1 cytokine profile. It is however still a subject of study as to which cells are involved in this immune response and which are responsible for this significant immune deviation. Here, I have shown that this switch is not antigen-dependant and shows different changes depending on the age of first exposure to infection.

Based on previous research by Harrison et al and Sztein et al I can surmise that IFN-γ production plays a key role in the immune switching caused by *S.typhimurium* exposure (148, 167, 170). Wu et al found that doses of Salmonella attenuated the affects of OVA-induced AAD in mice by induction of a Th1 immune response. They concluded that the induction of a Th1 immune response was responsible for decreasing other symptoms of AAD.

Utilising a strain of mice that have transgenic T-cells specific to OVAp allowed specific tracking of these cells for education and changes in population after infection or initiation of allergic disease. The consequent changes illustrate that *S.typhimurium* infection neonatally or as an adult significantly alters the CD4+ T-
cell population. This change could in turn be responsible for the decreases in the Th2 cytokine IL-5 and decreased numbers of pulmonary eosinophils as quantified from BALF and lung tissue.

Further investigation is required to determine the exact mechanisms of this age-dependant immune education.
CHAPTER 6

Changes in immune parameters induced by neonatal BCG infection
6.1 Introduction

Infection at birth alters the immune response to environmental antigen exposure as an adult (6, 185, 202, 226). Under some circumstances this may be deleterious to the host. Determining what parameters of the immune system are altered during early life will allow us to understand the factors that lead to an aberrant or effective immune response. An increasing body of evidence suggests that T-cells produced in response to antigen are altered in mice that have been exposed to certain bacterial infections as a neonate, compared to those with a naive, unchallenged immune system. A variety of animal (176, 194, 195, 197, 202) and human (177, 183, 185, 226-228) studies have highlighted the possible role of cellular immunity, in the form of cytokine production and CD4+ T-cell responses in infection induced immune development, which can educate against aberrant immune responses. However, assessment of the T-cell pool of neonates has not been widely investigated.

A limitation exists in studying the immune system of the neonate. The functional immaturity of the immune system in early life is particularly apparent in Th1 adaptive immune responses, with highly restricted gene expression patterns for the cytokine IFN-γ in response to stimuli (5, 229-232).

BCG, the vaccination against *M. tuberculosi*s has been revealed as one infection that may successfully polarise immune responses from Th2 to Th1 responses. As previously described (see chapters 3.1 and 4.1), an inverse correlation between BCG vaccination and rates of asthma in populations has been reported (204). The immune response generated by BCG infection is primarily driven by the production of IFN-γ (6, 182). Innate restrictions in neonatal IFN-γ production have not been shown to affect the response to BCG infection (6, 195). This reveals that other aspects of the immune response to BCG infection also play a key role.

Detailed mechanistic studies are hard to perform in neonates due to the small volumes of blood and numbers of PBLN that can be collected. In this study, I addressed whether the capacity to develop an appropriate response to antigens as
an adult was altered in mice infected at birth with BCG. WBCs present in BALF were assessed in sham-infected and infected neonatal mice at time-points throughout immune development (to 9-weeks old). At each time-point T-cell populations and stimulated cytokine production from PBLN and spleens were also evaluated along with serum antibody production. Utilising mice with OVA-TCR Tg T-cells allowed tracking of specific changes in the T-cell populations.
6.2 **Materials and Methods**

6.2.1 Animals

BALB/cTac-TgN(DO11.10)Rag2^{+/+}, OVA-TCR Tg SPF mice were supplied pregnant at 8-12 weeks of age from the University of Newcastle animal services unit. After the pups were born they were treated in the first 24-hours of life. These mice were derived from mice developed by Hsieh et al, 1995. OVA-TCR Tg were selected by staining peripheral blood leukocytes with the anti-clonotype mAb KJ1-26. OVA-TCR Tgs on the BALB/c background have been backcrossed more than 12 generations (210).

These mice were housed in IVC, maximum 6 mice per cage in the David Madisson Building animal holding facility. The mice were fed commercial rodent pellets and water *ad libitum*. This project has animal ethics clearance and safety clearance from the University of Newcastle, Animal care and ethics committee and Institutional Biosafety committee.

6.2.2 Bacteria

Bacterial strain containing an empty vector without expressing OVA-*Mycobacterium bovis* (Bacille Calmette Guerin) (BCG), Pasteur 1173-P2 +pMV (Kanamycin Resistant).

See section 3.2.2. for infection levels and doses used.

6.2.3 Determination of infection levels

Refer to chapter 3.2.4

6.2.4 Induction of infection

Refer to chapter 3.2.5 and 3.2.6

BCG was delivered intranasally (5µl was delivered to neonatal mice) using a sterile gel-loading tip.
6.2.5 Collection and analysis of samples (Blood, BALF)
Refer to chapter 2.2.3

6.2.6 Lung Histology
Refer to chapter 2.2.4

6.2.7 Collection of spleen and PBLN
Refer to chapter 2.2.5

6.2.8 Isolation of splenocytes and PBLN cells for tissue culture
Refer to chapter 2.2.6

6.2.9 Processing and staining of splenocytes and PBLN cells for flow cytometry
Refer to chapter 2.2.6

6.2.10 Determination of antibody titres by ELISA
Refer to chapter 2.2.7

6.2.11 Determination of culture supernatant cytokine levels by ELISA
Refer to chapter 2.2.8

6.2.12 Determination of OVA-specific and non-specific CD4+ T-cell levels by flow cytometry
Refer to chapter 4.2.14

6.2.13 Statistical analysis
Refer to chapter 2.2.9
Infection (BCG) or sham-infection (sterile PBS) (yellow bars) was performed on day 0 of life. At day 0 and 1, 3, 5, 7 and 9-weeks (red bars) mice were sacrificed and samples taken.
6.3 Results

6.3.1 Leukocyte infiltration into the airways in sham-infected and infected mice.

Mice were treated within the first 24-hours of life i.n. with either saline (sham-infection) or a live inoculum of $1 \times 10^6$ BCG. Their response to infection and immune development were tracked over the normal life cycle of a BCG infection with samples taken at time-points specific for the stages of infection. Day 0, representing baseline; 1-week, replication/growth phase; 3-weeks, log phase or peak of infection; 5-weeks, recovery phase; 7-weeks, latent phase; 9-weeks, latent phase where recurrence of infection is possible.

BALF was taken from mice at each time point to assess cellular responses and inflammation. BCG infection in neonates did not cause a change in total leukocytes in BALF at the time-points tested from days 21-63 (Figure 6.3.1a).

BCG infected mice have a significant decrease in lymphocytes in BALF on day 49 of infection compared to sham-infected mice (Figure 6.3.1b [C]). There are no significant changes in macrophages [A], neutrophils [B] or eosinophils [D] in BCG infected mice compared to sham-infected mice at the time-points shown.
Figure 6.3.1a  Total leukocyte levels in BALF of mice infected with BCG or sham-infected as a neonate.
Neonatal (0-day old) mice were infected with BCG or PBS for controls. Data represents the mean number of viable leukocytes per millilitre of BALF +/- SEM for groups of 6 mice. Statistical significance was determined by Students T-test.
Figure 6.3.1b Differential leukocyte (A) Macrophage, (B) Neutrophil, (C) Lymphocyte, and (D) Eosinophil numbers in BALF taken from airways of sham-infected and BCG infected neonatal mice. Data represents the mean number of viable leukocytes per millilitre of BALF +/- SEM for groups of 6 mice. Statistical significance was determined by Students T-test when comparing leukocyte numbers compared to OVA sensitised and challenged mice (p<0.05 *).
6.3.2 Histopathology of lung tissue in sham-infected and infected mice

Figure 6.3.2 [A] demonstrates that BCG infection of neonates causes a decrease in eosinophils in lung tissue by d35 post-infection as evaluated by an average of 10 counts of lung histological sections. There was no significant change in numbers of mucous secreting cells in the lung tissue of mice due to BCG infection at the times tested (Figure 6.3.2[B]).
Figure 6.3.2 (A) Eosinophils and (B) Mucous secreting cells in lung tissue from airways of sham-infected and BCG infected neonatal mice. Data represents the mean number of cells per 100µm of lung section +/- SEM for groups of 6 mice. Statistical significance was determined by Students T-test (p<0.05*).
6.3.3 Antibody responses in sham-infected and infected mice.

Neonatal BCG infection causes a significant decrease in IgG1 and IgG2a levels in serum 21 days after infection (peak of infection) (Figure 6.2.3a). The decrease in the Th2 antibody IgG1 caused by BCG infection continues up until day 39 post infection, compared to sham-infected controls. The dynamics of this systemic Th2/Th1 antibody shift is more clearly demonstrated by the IgG1/IgG2a ratio in Figure 6.2.3b. At day 7 post-infection BCG infected mice have a higher IgG1/IgG2a ratio compared to the sham-infected controls. By day 21 this dynamic has changed with BCG infected mice now having a lower Th1/Th2 ratio than sham-infected controls. This continues until d35 post-infection after which there is no significant difference in the IgG1/IgG2a ratio between BCG infected and sham-infected controls.

Figure 6.2.3c shows the serum IgE levels in BCG-infected and sham-infected mice over time. BCG infected mice have a significantly decreased serum IgE level 49 days post infection.
Figure 6.3.3a Levels of immunoglobulin OVA-specific (A) IgG2a and (B) IgG1 in serum of mice infected as a neonate with BCG or sham-infected. Data represents the mean number of detectable immunoglobulin in serum +/- SEM for groups of 6 mice. Statistical significance was determined by Students T-test (p<0.001**).
Figure 6.3.3b Dynamics of serum IgG1/IgG2a ratio in mice infected as a neonate with BCG compared to sham-infected mice. Data represents the mean number of detectable IgG1/IgG2a in serum +/- SEM for groups of 6 mice. Statistical significance was determined by Students T-test (p<0.05 *, p<0.001**).
Figure 6.3.3c Levels of immunoglobulin, total IgE in serum of mice infected as a neonate with BCG or sham-infected.
Data represents the mean number of detectable Immunoglobulin in serum +/- SEM for groups of 6 mice. Statistical significance was determined by Students T-test (p<0.05 *).
6.3.4 Cytokine responses in sham-infected (PBS) and infected mice.

Cytokine levels were evaluated from splenocytes and PBLN to assess the immune changes caused by BCG infection of the neonate.

IL-5 levels were increased in stimulated splenocyte supernatants of BCG infected mice 7-days post infection (Fig 6.3.4a [B-C]). However, there were no significant differences seen between infected and sham-infected IL-5 levels from stimulated PBLN cell culture supernatants (Fig 6.3.4a [D-F]).

7 days post-infection levels of IL-10 from stimulated splenocyte supernatants are decreased in BCG infected mice compared to sham-infected controls (Figure 6.3.4b[C]). There are no significant differences in IL-10 levels from PBLN supernatants (Figure 6.3.4b[D-F]).

Figure 6.3.4c illustrates significantly decreased levels of IL-4 in BCG infected mice. The affect of BCG on IL-4 levels differs according to method of cell stimulation. Unstimulated splenocytes from BCG infected mice produced lower amounts of IL-4 at day-7 post infection as measured from supernatants (Figure 6.3.4c[A]). OVAp stimulation of splenocytes shows a significant decrease in IL-4 levels from BCG infected mice on day 49 post infection compared to control mice (Figure 6.3.4c[B]). Splenocyte stimulation with CD3/CD28 induces higher IL-4 production from BCG infected mice at day 7 post infection by comparison to control mice (Figure 6.3.4c[C]). At day 21 levels of IL-4 produced by splenocytes stimulated with CD3/CD28 remain unchanged in BCG infected mice. There is a sharp increase in production of IL-4 by uninfected controls at d21, peak of infection (Figure 6.3.4c[C]). There are no significant differences in IL-4 production in PBLN supernatants between BCG infected and uninfected controls.

CD3/CD28 stimulation of splenocytes demonstrates an increased production of IL-13 from BCG infected mice 7-days post infection compared to sham-infected controls (Figure 6.3.4d [C]). BCG infection caused no significant differences in IL-13 production from PBLNs (Figure 6.3.4d[D-F]).

IFN-γ production in splenocyte culture supernatants is significantly increased in mice infected with BCG as a neonate. Figure 6.3.4e [B-C] shows
increased levels of IFN-γ from splenocytes with OVAp and CD3/CD28 stimulation in BCG infected mice 35 days post-infection compared to sham-infected controls. IFN-γ production from PBLN cells was not altered by BCG infection (Figure 6.3.4e [D-F]).
Figure 6.3.4a Levels of IL-5 (A, D) unstimulated, (B, E) OVAp stimulated, (C, F) CD3/CD28 stimulated, (A,B,C) splenocytes and (D,E,F) PBLN of neonatally sham-infected or BCG infected mice.

Data represents the mean number of specific cytokine in culture supernatant +/- SEM for groups of 6 mice. Statistical significance was determined by Students T-test (p<0.05 *).
Figure 6.3.4b Levels of IL-10 (A, D) unstimulated, (B, E) OVAp stimulated, (C, F) CD3/CD28 stimulated, (A,B,C) splenocytes and (D,E,F) PBLN of neonatally sham-infected or BCG infected mice.

Data represents the mean number of specific cytokine in culture supernatant +/- SEM for groups of 6 mice. Statistical significance was determined by Students T-test (p<0.05 *).
Figure 6.3.4c Levels of IL-4 (A, D) unstimulated, (B, E) OVAp stimulated, (C, F) CD3/CD28 stimulated, (A,B,C) splenocytes and (D,E,F) PBLN of neonatally sham-infected or BCG infected mice. Data represents the mean number of specific cytokine in culture supernatant +/- SEM for groups of 6 mice. Statistical significance was determined by Students T-test (p<0.05 *).
Figure 6.3.4d Levels of IL-13 (A, D) unstimulated, (B, E) OVAp stimulated, (C, F) CD3/CD28 stimulated, (A,B,C) splenocytes and (D,E,F) PBLN of neonatally sham-infected or BCG infected mice.

Data represents the mean number of specific cytokine in culture supernatant +/- SEM for groups of 6 mice. Statistical significance was determined by Students T-test (p<0.05 *).
Figure 6.3.4e Levels of IFN-γ (A, D) unstimulated, (B, E) OVAp stimulated, (C, F) CD3/CD28 stimulated, (A,B,C) splenocytes and (D,E,F) PBLN of neonatally sham-infected or BCG infected mice. Data represents the mean number of specific cytokine in culture supernatant +/- SEM for groups of 6 mice. Statistical significance was determined by Students T-test (p<0.05 *).
6.3.5 Percentage of CD4+, CD8+ and OVAp-specific T-cells in sham-infected and infected mice.

Mice infected with BCG as a neonate have lower numbers of CD4+ T-cells 21 and 35 days post infection then sham-infected controls (Figure 6.4.1a [A]). BCG infected mice also had fewer CD8+ lymphocytes 35 and 49 days post infection compared to sham-infected mice (Figure 6.4.1a[B]). There is no difference however in the populations of antigen-specific CD4+ and CD8+ systemic lymphocytes (Figure 6.4.1a [C-D]).

These results are replicated in stimulated PBLNs obtained from infected and uninfected mice (Figure 6.4.1b [A-B]). There are less CD4+ and CD8+ lymphocytes in BCG infected mice at days 35 and 49 compared to sham-infected controls (Figure 6.4.1b [A-B]). BCG infection also caused a shift in OVAp-specific CD4+ lymphocytes at day 35 post infection (Figure 6.4.1b [C]). At day 35-post-infection BCG infected mice had a significant decrease in CD4+ OVAp-specific Tg T-cells compared to sham-infected controls. CD4+ non-Tg T-cells (CD4+KJ1-26−) were significantly increased at this time in BCG infected mice compared to sham-infected controls (Figure 6.4.1b [C]). Numbers of OVA-p specific CD8+ T-cells were not significantly different between BCG infected and sham-infected mice (Figure 6.4.1b [D]).
Figure 6.3.5a Percentage of (A) CD4+ cells (B) CD8+ cells and KJ1-26+ cells as a % of total (C) CD4+ cells and (D) CD8+ cells in splenocytes from mice that were sham or BCG infected as neonates. Data represents the mean number of detectable live splenocytes +/- SEM for groups of 6 mice. Statistical significance was determined by Students T-test (p<0.05 *).
Figure 6.3.5b Percentage of (A) CD4+ cells (B) CD8+ cells and KJ1-26+ cells as a % of total (C) CD4+ cells and (D) CD8+ cells in PBLN from mice that were sham or BCG infected as neonates. Data represents the mean number of detectable live PBLN cells +/- SEM for groups of 6 mice. Statistical significance was determined by Students T-test (p<0.05 *).
6.4 Discussion

BCG vaccination and the incidence of asthma are inversely related (204). It is therefore important to understand the changes to the immune system made by BCG infection/immunisation, especially relating to parameters of the immune system altered during AAD. Previous research in this area has highlighted the importance of local leukocytes, T-cell populations, antibody and cytokine production in AAD. It is demonstrated here that changes made in the undeveloped immune system of neonates by BCG infection have the potential to prevent the induction of aberrant responses characteristic of AAD in later life. Alterations in the immune system of mice inoculated with BCG prior to OVAp challenge are revealed by decreased numbers of eosinophils in the lung, decreased levels of OVA-specific IgG1 and IgG2a from serum, decreased total IgE from serum and decreased numbers of CD4+ and CD8+ T-cells in splenocyte and PBLN cells when compared to uninfected controls challenged with OVAp.

BALF taken from infected and sham-infected mice in this study showed no differences in leukocyte numbers and little difference in differential leukocyte numbers. This is unusual in a bacterial infection model when you would expect a rise in leukocyte number and in specific types of leukocytes at peak of infection in the local mucosal area. On day 49 post-infection, representing the beginning of the latent phase of infection BCG infected mice had decreased lymphocyte numbers compared to sham-infected control mice. A decrease in lymphocytes in BCG infected mice possibly indicates the disappearance of ‘visible’ bacteria to the host. This is due to the tendency of Mycobacterium to hide within the host cells in a dormant manner after the initial peak infection and inflammation occurs. The remaining infection remains dormant acting as a chronic infection, which can reappear over time (181).

When examining the tissue, however, there is a significant decrease in eosinophils in BCG infected mice compared to sham-infected controls on day 35 post-infection. This indicates that the presence of BCG infection has altered the
usually Th2 biased, eosinophil predisposed immune system of neonatal mice. Levels of MSCs in the tissue were unchanged in BCG infected mice.

Another indication that BCG infection can alter immune responses in the neonate was demonstrated by the significant decrease in IgG1 and IgG2a levels in serum taken from mice at day 21 post-infection compared to sham-infected controls. Although both Igs were found in decreased levels in BCG mice at day 21 post infection, a Th1 shift is indicated when the levels are considered as a ratio. The dynamics of this systemic Th2 to Th1 antibody shift is more clearly demonstrated by the IgG1/IgG2a ratio in Figure 6.2.3b. An increased IgG1/IgG2a ratio indicates a Th2 immune response whereas a decreased or lower ratio indicates a Th1 directed immune response. At day 7 post-infection BCG infected mice have a higher IgG1/IgG2a ratio compared to the sham-infected controls, indicating a more Th2 skewed immune response prior to peak of infection (18-21 days post infection). By day 21 post-infection this dynamic has shifted so that the BCG infected mice now exhibit a Th1 skewed immune response appearing to be initiated by the peak of BCG infection. This significantly increased systemic Th1 response continues until 35 days post-infection after which no significant difference in the IgG1/IgG2a ratio between BCG infected and sham-infected controls is present.

From peak of infection, during bacterial replication (day 21), cytokine changes in BCG infected mice are attributed to the affects of the infection. Systemically, as represented by cytokines measured from splenocyte culture supernatant, IL-4 levels are significantly reduced on days 21 and 49 and IFN-\(\gamma\) production significantly increased on day 35-post-infection in BCG infected mice compared to sham-infected controls. This indicates a shift from Th2 (IL-4) to Th1 (IFN-\(\gamma\)) cytokine responses. However, IL-13 and IL-5 levels were significantly increased in splenocyte culture supernatants of BCG infected mice compared to sham-infected controls 7-days post infection. A significant decrease in IL-10 level from splenocyte culture supernatants was also found in BCG mice compared to sham-infected controls 7-days post-infection. There are no significant differences in cytokine
production levels at the local mucosal site represented by the PBLN culture supernatants.

These contrary cytokine results clearly show that neonatal BCG infection alters the T-cell population. The decreased IL-10 levels at 7-day post-infection suggest that this is not through a T-regulatory cell increase but through changes in antigen-specific CD4+ and CD8+ T-cells. After peak of infection the only cytokine to be increased in BCG infected mice compared to sham-infected controls is IFN-γ. This suggests that a mainly Th1 response is stimulated by the immune response to BCG infection.

Flow cytometry analysis of purified splenocyte and PBLN cells shows that BCG infected mice have decreased numbers of CD4+ (d21 and d35) and CD8+ (d35 and d49) T-cells compared to sham-infected controls. However, when looking at the Tg T-cell population, (initially >90% KJ1-26+) it is clear that in the local response (PBLN cells), the numbers of OVA-Tg CD4+ cells in BCG infected mice decrease in favour of non-Tg CD-4+ T-cells by 35 days post-infection.

It has previously been shown in a cohort study of healthy children that the capacity to mount Th1 responses to antigens often remains low until beyond childhood, which has been confirmed in the study of mice post-weaning (5). It is clear that T-cell responses in infancy are both attenuated and intrinsically Th2 skewed relative to those in adults (231). The implication of the results in this study is that the distorted immune response of a neonate can be educated by infection or immunisation. After neonatal BCG infection the resulting immune responses are of a mixed Th1/Th2 phenotype rather than Th2 skewed. This is shown by the increased Th1 immunoglobulin response and increased IFN-γ production by splenocyte and PBLN cell cultures. However, components of the Th2 response are still present as shown by the high IL-5 levels from splenocyte and PBLN cell cultures in both groups.

Neonatal BCG infection causes a significant change in the development of systemic and local T-cell responses. This study shows that the immune response to neonatal BCG infection is a CD4 T-cell-induced shift toward a balanced Th1/Th2 response. This shift results in subsequent changes in leukocytes such as reduction
of eosinophils in the airways. This demonstrates that increased maturity of CD4+ T-cells develops through the extended antigenic experience throughout infection. Development of this memory is designed to prime for rapid elimination of the pathogen but also educates the immune system to appropriately respond to environmental antigens and therefore avoid the development of AAD.
CHAPTER 7

Changes in immune parameters induced by neonatal *S.typhimurium* infection
7.1 Introduction

The developing immune system of a neonate is characterised by pronounced susceptibility to microbial infections and allergic reactions (233). T-cell responses of neonates are Th2 biased and therefore the production of Th2 cytokines are increased (5). Strategies aimed at balancing Th1 and Th2 immune responses would help educate the neonatal immune system and halt the development of allergic disease.

One such strategy involves exposure to antigens shown to stimulate Th1 responses. Studies have shown that infection with *S. typhimurium*, as with many intracellular bacteria; require Th1-dependant responses to control infection. IFN-γ is essential for *S. typhimurium* clearance *in vivo*. Spleen, PPs and lymph node cells from mice orally infected with *S. typhimurium* produce elevated levels of IFN-γ (and Th1 type cells) upon re-stimulation with *S. typhimurium* (144).

Patients with deficiency in IFN-γ and IL-12 or IFN-γ and IL-12 receptors have increased risk of severe and recurrent intracellular bacterial infection (161). This further validates the role of Th1 directed responses to Salmonella infection.

I hypothesise that the induction of Th1 cytokine production by neonatal Salmonella infection is capable of altering the uneducated immune system and establishing a balance of Th1 and Th2 responses.

Infection with an attenuated strain of *S. typhimurium* has been shown to significantly reduce key markers of AAD in an adult mouse model after OVA challenge. Wu et al, showed in 2006 that *S. typhimurium* infection significantly reduced eosinophils in the BALF and lung tissue, levels of OVA stimulated Th2 cytokines, IL-4, IL-5 and IL-13 from splenocytes and serum levels of OVA specific IgG1 compared to uninfected controls (176).

Human longitudinal studies also show that exposure to *S. typhimurium* can lead to lower levels of AAD. Pelosi, et al investigated school age children in Sardinia with allergic rhino-conjunctivitis or asthma in relation to their incidence of Salmonellosis. Sardinian children hospitalized for Salmonellosis before 4yrs of life
were found to develop allergic asthma and rhinoconjunctivitis less frequently than children with no incidence of Salmonella infection (177).

The present investigation sought to test the effect of attenuated *S.typhimurium* infection at birth on the uneducated immune system. For this purpose, mice were orally infected with *S.typhimurium* in the first 24-hour of life. Parameters of their immune system were measured over a period of 9–weeks and compared to uninfected naive controls. Specifically, the experiment evaluated, differential and total BALF leukocyte levels, lung histopathology counts of eosinophils and MSCs, serum levels of IgG1, IgG2a and IgE, levels of Th1 and th2 cytokine produced from stimulated splenocyte and PBLN cells as well as numbers of CD4+ and CD8+ T-cells from PBLN and splenocyte cells. This addressed the question of what parts of the neonatal immune system that are directly altered by *S.typhimurium* infection. These studies were done with a view to determine mechanisms in which the development of AAD can be prevented.
7.2 Materials and Methods

7.2.1 Animals
BALB/cTac-TgN(DO11.10)Rag2$^{+/+}$, OVA-TCR Tg SPF mice were supplied pregnant at 8-12 weeks of age from the University of Newcastle animal services unit. After the pups were born they were treated in the first 24-hours of life. These mice were derived from mice developed by Hsieh et.al, 1995. OVA-TCR Tg were selected by staining peripheral blood leukocytes with the anti-clonotype mAb KJ1-26. OVA-TCR Tgs on the BALB/c background have been backcrossed more than 12 generations (210).

These mice were housed in IVC, maximum 6 mice per cage in the David Madisson Building animal holding facility. The mice were fed commercial rodent pellets and water ad libitum. This project has animal ethics clearance and safety clearance from the University of Newcastle, Animal care and ethics committee and Institutional Biosafety committee.

7.2.2 Bacteria
Bacterial strain containing an empty vector without expressing OVA-Salmonella enterica serovar typhimurium aroA aroD mutant BRD09 +pKK (Streptomycin/Ampicillin Resistant) were used in this study.

See section 3.2.2. for infection levels and doses used.

7.2.3 Determination of infection levels
Refer to chapter 4.2.4

7.2.4 Induction of infection
Refer to chapter 4.2.5 and 4.2.6
S.typhimurium was delivered by gavage using a sterile gel-loading tip and allowing the neonatal mouse to suckle on the tip.
7.2.5 Collection and analysis of samples (Blood, BALF)
Refer to chapter 2.2.3

7.2.6 Lung Histology
Refer to chapter 2.2.4

7.2.7 Collection of spleen and PBLNs
Refer to chapter 2.2.5

7.2.8 Isolation of splenocytes and PBLN cells for flow cytometry and tissue culture
Refer to chapter 2.2.6

7.2.9 Processing and staining of splenocytes and PBLN cells for flow cytometry
Refer to chapter 2.2.6

7.2.10 Determination of antibody titres by ELISA
Refer to chapter 2.2.7

7.2.11 Determination of culture supernatant cytokine levels by ELISA
Refer to chapter 2.2.8

7.2.12 Determination of OVA-specific and non-specific CD4+ T-cell levels by flow cytometry
Refer to chapter 4.2.14

7.2.13 Statistical analysis
Refer to chapter 2.2.9
Figure 7.2.14 Experiment outline

Infection (*S.typhimurium*) or sham-infection (sterile PBS) (yellow bars) was performed on day 0 of life. At day 0 and 1, 3, 5, 7 and 9-weeks (red bars) mice were sacrificed and samples taken.
7.3 Results

7.3.1 Leukocyte infiltration into the airways in sham-infected and infected mice.

Total leukocyte numbers were quantified in BALF to determine the cellular response at the lung mucosal surface after infection with a gastro-intestinal mucosal pathogen. Infection with *S. typhimurium* in the first 24-hours of life causes an increase in BALF cellular infiltrates 49 days post infection compared to sham-infected controls (Figure 7.3.1a).

This can be attributed to an increase in lymphocytes and macrophages 49-days post-infection in *S. typhimurium* infected mice compared to sham-infected controls (Figure 7.3.1b [A-C]). Neonatal *S. typhimurium* infection did not affect neutrophil levels in BALF (Figure 7.3.1b [B]). *S. typhimurium* infection in the first 24 hours of life also causes a decrease in eosinophil numbers 21 days post-infection compared to uninfected controls (Figure 7.3.1b [D]).

Early life *S. typhimurium* infection causes a change in cellular immune responses. Infected neonates developed changes in lymphocyte, macrophages and eosinophil numbers in BALF later in life.
Figure 7.3.1a  Total leukocyte levels in BALF of mice sham-infected or infected with *S. typhimurium* as a neonate.

Neonatal (0-day old) mice were infected with BCG or sham-infection of PBS for controls. Data represents the mean number of viable leukocytes per millilitre of BALF +/- SEM for groups of 6 mice. Statistical significance was determined by Students T-test (p<0.05 *).
Figure 7.3.1b Differential leukocyte (A) Macrophage, (B) Neutrophil, (C) Lymphocyte, (D) Eosinophil levels of BALF from airways of sham-infected and *S. typhimurium* infected neonatal mice. Data represents the mean number of viable leukocytes per millilitre of BALF +/- SEM for groups of 6 mice. Statistical significance was determined by Students T-test when comparing leukocyte numbers compared to OVA sensitised and challenged mice (p<0.05 *).
7.3.2 Histopathology of lung tissue in sham-infected and infected mice

Inflammatory cells in lung tissue were quantified by performing counts on stained histological sections to determine differences. *S. typhimurium* infection in the neonate did not alter the number of mucous secreting cells present in tissue (Figure 7.3.2[B]). Eosinophil levels in the lung tissue were significantly decreased by 49-days post-infection with *S. typhimurium* in the neonate compared to sham-infected controls (Figure 7.3.2[A]). Neonatal mice infected with *S. typhimurium* showed a trend toward lower eosinophils from 21-63 days post infection as seen in Figure 7.3.2[A]. This validates the decreased eosinophil levels found in BALF after *S. typhimurium* infection in the neonate.
Figure 7.3.2 Decrease in (A) Eosinophils and (B) Mucous secreting cells in lung tissue from airways of sham-infected and S. typhimurium infected neonatal mice. Data represents the mean number of cells per 100µm of lung section +/- SEM for groups of 6 mice. Statistical significance was determined by Students T-test (p<0.05*).
7.3.3 Antibody responses in sham-infected and infected mice.

Systemic antibody changes were established by ELISA of serum. Figure 7.3.3a [A] shows the significantly increased IgG2a level in serum of *S. typhimurium* infected mice on days 35 and 63 post infection. There was also a significant decrease in systemic levels of IgG1 in *S. typhimurium* infected mice on days 7 and 49 post-infection (Figure 7.3.3a [B]). This describes an overall shift from Th2 (IgG1) to Th1 (IgG2a) systemic antibody levels.

This shift is best outlined in Figure 7.3.3b which shows the IgG1/IgG2a ratio in the serum of *S. typhimurium* infected and sham-infected neonates 7-63 days post infection. Neonatal *S. typhimurium* infection causes a lower IgG1/IgG2a ratio which is statistically significant to uninfected control mice 21, 35 and 63 days post-infection.

Figure 7.3.3c shows that infection with *S. typhimurium* in the first 24 hours of life causes an increase in serum IgE levels 35-49 days post-infection. Since IgE is a Th2 antibody this is in contrast to the lowered IgG1/IgG2a shown in Figure 7.3.3b.
Figure 7.3.3a Levels of OVA-specific immunoglobulin (A) IgG2a and (B) IgG1 in serum of mice infected as a neonate with *S. typhimurium* or sham-infected. Data represents the mean number of detectable Immunoglobulin in serum +/- SEM for groups of 6 mice. Statistical significance was determined by Students T-test (p<0.05*).
Figure 7.3.3b Dynamics of serum IgG1/IgG2a ratio in mice infected as a neonate with *S.typhimurium* compared to sham-infected mice. Data represents the mean number of detectable IgG1/IgG2a in serum +/- SEM for groups of 6 mice. Statistical significance was determined by Students T-test (p<0.05 *).
Figure 7.3.3c  Levels of immunoglobulin, total IgE in serum of mice infected as a neonate with *S.typhimurium* or sham-infected. Data represents the mean number of detectable Immunoglobulin in serum +/- SEM for groups of 6 mice. Statistical significance was determined by Students T-test (p<0.05 *).
7.3.4 Cytokine responses in sham-infected (PBS) and infected mice.

Cytokine levels were evaluated from splenocytes and PBLN culture supernatants to determine shifts in systemic and local immune responses after neonatal *S.typhimurium* infection. Figure 7.3.4a [A-C] demonstrates the decrease in splenocyte culture IL-5 production with (OVAp and CD3/CD28 stimulation) and without antigen stimulation 35-63 days post infection compared to uninfected controls (PBS). PBLN culture supernatant IL-5 production also shows a trend toward decreased antigen stimulated IL-5 levels after neonatal *S.typhimurium* infection (Figure 7.3.4a [D-F]).

Unstimulated production of IL-10 was increased in splenocyte cultures 49-63-days post *S.typhimurium* infection compared to uninfected controls (Figure 7.3.4b [A-C]). There were no significant differences in IL-10 production after antigen stimulation (OVAp) and CD3/CD28 stimulation or in unstimulated PBLN culture (Figure 7.3.4b [D-F]).

Antigen stimulated (OVAp) and CD3/CD28 stimulated IL-4 production from splenocytes is decreased in *S.typhimurium* infected mice at 7 and 63 days post infection (Figure 7.3.4c [A-C]). However unstimulated cultures of splenocytes show increased levels of IL-4 production at 7-21 days post infection compared to uninfected controls (Figure 7.3.4c [A-C]). Isolated PBLN cell production of IL-4 remained unaltered after *S.typhimurium* infection (Figure 7.3.4c [D-F]).

IL-13 levels were significantly lowered in splenocyte culture supernatants by day 63 in mice infected with *S.typhimurium* at birth compared to uninfected controls (Figure 7.3.4d [A-C]). No differences were seen between infected mice and uninfected controls in PBLN culture levels of IL-13 (Figure 7.3.4d [D-F]).

*S.typhimurium* infection causes increased production of IFN-γ after stimulation (OVAp and CD3/CD28) of cultured splenocytes 21-days post infection compared to sham-infected controls (Figure 7.3.4e [A-C]). However, 35-63 days post-infection the dynamics of IFN-γ production in splenocyte cell cultures shift and become decreased in mice neonatally infected with *S.typhimurium* compared to uninfected controls (Figure 7.3.4e [A-C]). IFN-γ levels remained unaltered in PBLN cell cultures after neonatal *S.typhimurium* infection (Figure 7.3.4e [D-F]).
Early responses to *S. typhimurium* infection show a shift toward an increase in systemic Th1 cytokines (IFN-γ) and a decrease in Th2 cytokines (IL-4) compared to uninfected controls (Figures 7.3.4e [A-C], 7.3.4c [A-C]). A mixed phenotype of both Th1 and Th2 cytokines develops after 21 days post-infection.
Figure 7.3.4a Levels of IL-5 (A, D) unstimulated, (B, E) OVAp stimulated, (C, F) CD3/CD28 stimulated, (A,B,C) splenocytes and (D,E,F) PBLN of neonatally sham-infected or \textit{S.typhimurium} infected mice. 

Data represents the mean number of specific cytokine in culture supernatant +/- SEM for groups of 6 mice. Statistical significance was determined by Students T-test ($p<0.05$ *, $p<0.001$ **).
Figure 7.3.4b Levels of IL-10 (A, D) unstimulated, (B, E) OVAp stimulated, (C, F) CD3/CD28 stimulated, (A,B,C) splenocytes and (D,E,F) PBLN of neonatally sham-infected or *S. typhimurium* infected mice.

Data represents the mean number of specific cytokine in culture supernatant +/- SEM for groups of 6 mice. Statistical significance was determined by Students T-test (p<0.05 *).
Figure 7.3.4c Levels of IL-4 (A, D) unstimulated, (B, E) OVAp stimulated, (C, F) CD3/CD28 stimulated, (A,B,C) splenocytes and (D,E,F) PBLN of neonatally sham-infected or *S. typhimurium* infected mice. Data represents the mean number of specific cytokine in culture supernatant +/- SEM for groups of 6 mice. Statistical significance was determined by Students T-test (p<0.05 *).
Figure 7.3.4d Levels of IL-13 (A, D) unstimulated, (B, E) OVAp stimulated, (C, F) CD3/CD28 stimulated, (A,B,C) splenocytes and (D,E,F) PBLN of neonatally sham-infected or *S.typhimurium* infected mice.

Data represents the mean number of specific cytokine in culture supernatant +/- SEM for groups of 6 mice. Statistical significance was determined by Students T-test (p<0.05 *, p<0.001**).
Figure 7.3.4e Levels of IFN-γ (A, D) unstimulated, (B, E) OVAp stimulated, (C, F) CD3/CD28 stimulated, (A, B, C) splenocytes and (D, E, F) PBLN of neonatally sham-infected or S. typhimurium infected mice.

Data represents the mean number of specific cytokine in culture supernatant +/- SEM for groups of 6 mice. Statistical significance was determined by Students T-test (p<0.05 *).
7.3.5 Percentage of CD4+, CD8+ and OVAp-specific T-cells in sham-infected and infected mice.

Neonatal *S. typhimurium* infection causes changes to splenocyte lymphocyte populations. Flow cytometry data of splenocytes shows a significant decrease in CD4+ lymphocytes 21 and 35-days post infection when compared to sham-infected controls (Figure 7.3.5a [A]). CD8+ lymphocytes were also significantly decreased after neonatal *S. typhimurium* infection by 35 and 49 days post-infection compared to uninfected controls (Figure 7.3.5a [B]). The OVAp-specific populations of CD4+ lymphocytes, however, were not different after *S. typhimurium* infection (Figure 7.3.5a [C]). *S. typhimurium* infection did increase the CD8+ OVAp-specific lymphocytes 35 days post-infection compared to uninfected controls (Figure 7.3.5a [D]).

PBLN lymphocyte populations were also altered by *S. typhimurium* infection in neonates. Mice infected with *S. typhimurium* showed decreased numbers of CD4+ lymphocytes 21 days post infection compared to sham-infected controls. However, by 35 days post-infection, infected mice had a significantly higher number of CD4+ lymphocytes compared to sham-infected controls (Figure 7.3.5b [A]). Figure 7.3.5b [C] shows that Tg OVAp positive CD4+ T-cells were significantly decreased in isolated PBLN cells from *S. typhimurium* infected mice 35-days post infection, thus significantly increasing the number of non-transgenic CD4+ T-cells present at this time point also.

No significant changes were seen in total numbers of CD8+ lymphocytes from PBLN cells (Figure 7.3.5b [B]). OVAp-specific populations of CD8+ cells from PBLNs were altered at day 35 post infection between infected mice and uninfected controls. *S. typhimurium* infected mice had a decreased number of OVAp-specific (Tg) CD8+ cells and an increase in non-Tg CD8+ cells compared to sham-infected controls (Figure 7.3.5b [D]).
Figure 7.3.5a Percentage of (A) CD4+ cells (B) CD8+ cells and KJ1-26+ cells as a % of total (C) CD4+ cells and (D) CD8+ cells in splenocytes from mice that were sham or S. typhimurium infected as neonates. Data represents the mean number of detectable live splenocytes +/- SEM for groups of 6 mice. Statistical significance was determined by Students T-test (p<0.05 *).
Figure 7.3.5b Percentage of (A) CD4+ cells (B) CD8+ cells and KJ1-26+ cells as a % of total (C) CD4+ cells and (D) CD8+ cells in PBLN from mice that were sham or BCG infected as neonates. Data represents the mean number of detectable live PBLN cells +/- SEM for groups of 6 mice. Statistical significance was determined by Students T-test (p<0.05 *).
7.4 Discussion

This study aimed to examine the effects of an intracellular bacterial infection with \textit{S.typhimurium} on the neonatal immune system. Data supports the hypothesis that antigenic stimulation from \textit{S.typhimurium} alters the neonatal immune system in a way that could prevent the development of Th2 biased disease such as AAD.

Although the neonatal immune system is predisposed to Th2 cell activation at birth, contact with bacterial antigens that drive toward a Th1 response (such as those from intracellular bacteria, e.g. \textit{S.typhimurium}) promote education toward a more balanced Th1 and Th2 cell profile. Mouse studies have shown that infection with \textit{S.typhimurium} prior to OVA challenge can prevent the Th2 response associated with OVA challenge and AAD (176). In this study \textit{S.typhimurium} infection was shown to alter the naive immune system and cause baseline shifts from the biased Th2 responses of the neonate towards Th1/Th2 balance.

Changes to the immune system of mice infected with \textit{S.typhimurium} as a neonate is evident by the decreased eosinophil levels found in BALF (day21, Figure 7.3.1b[D]) and lung tissue (day 49, Figure 7.3.2[A]) in this study. Differential analysis of the BALF of \textit{S.typhimurium} infected mice also showed increased numbers of macrophages and lymphocytes (Figure 7.3.1b [A,C]) by day 49 compared to sham-infected controls. Serum levels of immunoglobulin show that \textit{S.typhimurium} infection as a neonate causes a decrease in the IgG1/IgG2a ratio compared to sham-infected controls (Figure 7.3.3b). This indicates a switch from Th2 (IgG1) to Th1 (IgG2a) immunoglobulin production. IgG2a was significantly increased on days 35 and 63 post-infection (Figure 7.3.3a[A]) and IgG1 levels decreased on days 7 and 49 post-infection (Figure 7.3.3a[B]) in \textit{S.typhimurium} infected mice compared to controls. However, serum IgE levels were increased in mice infected with \textit{S.typhimurium} compared to uninfected controls (Figure 7.3.3c). Increased IgE levels are indicative of a Th2 response, therefore these results are in contrast to the other serum Ig levels measured. IL-4 is essential for B-cell isotype switching to a Th2 phenotype and the production of IgG1 and IgE in mice, (87, 98)
IL-13 is also a potent B-cell regulator controlling IgE synthesis (117, 118). Varying levels of IL-4 and IL13 may explain the partial isotype switch exhibited here.

Cytokine levels from OVA-stimulated splenocytes of mice infected with *S. typhimurium* showed a definite increase in the production of the Th1 cytokine IFN-γ (Figure 7.3.4e[B]) and a decrease in the Th2 cytokines IL-4 (Figure 7.3.4c[B]), IL-5 (Figure 7.3.4a[B]) and IL-13 (Figure 7.3.4d[B]) compared to uninfected controls. IL-5 levels in splenocyte supernatants were decreased over days 35-63 post infection compared to uninfected controls (Figure 7.3.4a[A-C]). IL-4 levels were decreased in stimulated splenocyte culture supernatants on days 7 and 63 post infection compared to uninfected controls (Figure 7.3.4c[B-C]). IL-13 levels were decreased in splenocyte culture supernatants on day 63 post infection compared to uninfected controls (Figure 7.3.4d[A-C]). IFN-γ levels were increased in stimulated splenocyte culture supernatants on day 21 post infection and decreased on days 35-63 compared to uninfected controls (Figure 7.3.4e[B-C]). Although the overall trend from this data is away from a Th2 phenotype, a mixed Th1/Th2 response is evident. *S. typhimurium* infection creates a change in the neonatal immune system that is pronounced.

The changes in leukocytes present, cytokine and Ig levels denote that changes in T-cell profile have occurred. Flow cytometry data from splenocytes of *S. typhimurium* infected data show that these mice have an overall decrease in CD4+ lymphocytes on days 21-35 post infection compared to uninfected controls (Figure 7.3.5a[A]). CD8+ cells were decreased as a percentage of lymphocytes in *S. typhimurium* infected mice on d35-49 post infection compared to uninfected controls (Figure 7.3.5a[B]). These CD8+ cells show an increase in the CD8+KJ1-26+ population (OVA-specific cells) on day 35 post infection in mice infected with *S. typhimurium* compared to uninfected controls (Figure 7.3.5a[D]). PBLN cells also show changes in the CD4+ and CD8+ cell population. An increase in CD4+ cells on day 21 and a decrease on day 35 post-infection, as a percentage of total lymphocytes is seen in *S. typhimurium* infected mice compared to uninfected controls (Figure 7.3.5b[A]). This coincides with a decrease in CD4+KJ1-26+ cells by day 35 post-infection (Figure 7.3.5b[C]). On day 35 post infection there is also a
decrease in CD8+KJ1-26+ cells in PBLN cells from *S. typhimurium* infected mice compared to uninfected controls (Figure 7.3.5b[D]).

Overall, the data collected confirms that infection with *S. typhimurium* as a neonate causes significant changes to the immune system. These changes results in mixed Th1/Th2 responses instead of the biased Th2 responses expected of the uneducated neonatal immune system. These changes may be significant enough to down-regulate the allergic response when exposed to antigenic stimuli in later life.
CHAPTER 8

General Discussion
8.1 Synopsis

Allergic diseases such as asthma are a cause of morbidity and mortality in the developed world. In 2004 it was conservatively estimated that 300 million people were affected by asthma worldwide with majority of disease burden found in developed countries (2). The hygiene hypothesis has been a central theory in the immunology of asthma and allergic diseases for several decades (200, 234). This hypothesis is based on the concept that the naive immune system can be programmed to respond to normally harmless antigenic stimuli whilst still responding to harmful stimuli is the basis for much research. In under-developed countries, the prevalence of different bacterial infections for example, *M. tuberculosis* and *S. typhimurium*, and the low incidence of asthma is in contrast to the developed world (204). Intracellular bacteria, *M. tuberculosis*, BCG and *S. typhimurium* induce a primarily Th1 immune response in the infected host. AAD is induced by T-cell mediated production of Th2 cytokines such as IL-5 and IL-13 which in turn leads to eosinophilia and airways inflammation (79, 117). Therapies to allergens and the induction of allergic disease focus on altering the way the immune system responds to allergens. By understanding how infections drive Th1 immune responses and how the immune system is normally tolerant these therapies can be developed, leading to little AAD.

8.1.1 Hypothesis

That infection in early life plays a critical role in the development/programming of CD4+ T-cell responses to protective or destructive responses in the adult.

8.1.2 Aims

- Compare bacteria with different life cycles and different routes of infection on the generation of CD4+ T-cell responses to cognate antigen.
• Determine the affect of infections at different ages on the programming of adult CD4+ T-cells to cognate antigen.

• Determine if infection with *S. typhimurium* or BCG can halt the development of a Th2 Allergic response in Adulthood.

This study investigates the role of infection, in early-life on the development/programming of CD4+ T-cell responses in the adult. The type of immune response generated in the adult may not only be dependent on the age of first exposure but also on the type of infectious agent and the route of infection. Specifically I will: 1). Compare bacteria with different life cycles on the generation of CD4+ T-cell responses to cognate antigen early in life and in adults. Compare the route of mucosal neonatal and childhood infection (gut vs respiratory tract), on the generation of CD4+ T-cells to cognate antigen in the adult. 2). Determine the affect of infections at different ages on the programming of adult CD4+ T-cells to cognate antigen. 3). Determine the effect of these specific infections in the neonate and infant on the proliferative potential of adult CD4+ T-cells following stimulation with cognate antigen.

To undertake this investigation, OVA-TCR Tg mouse were used in combination with bacterial strains modified to produce OVA. This allowed specific tracking of the T-cells in response to the bacterial infection and OVA challenge. Mice were infected or sham-infected at 4 stages of immune development (0-days old, 2, 4, 6-weeks old) with the relevant sham-control or bacteria prior to sham or OVA challenge as an adult (8 or 12-weeks of age depending upon infection). The outcomes of these studies were assessed by determination of differential BALF leukocyte numbers, MSCs and eosinophils in lung tissue, serum Ig levels, cytokine levels from stimulated splenocyte and PBLN culture supernatants and CD4+KJ1-26+ T-cell populations.

The affects of BCG and *S. typhimurium* infection on the neonatal immune system was assessed experimentally by tracking infection at birth without concurrent OVA production or OVA challenge in the subsequent weeks of immune
development. Results were assessed 1, 3, 5, 7 and 9-weeks post-infection by examining differential BALF leukocyte numbers, MSCs and eosinophils in lung tissue, serum Ig levels, cytokine levels from stimulated splenocyte and PBLN culture supernatants and CD4+KJ1-26+ and CD8+KJ1-26+ T-cell populations.

8.2 Conclusions

8.2.1 BCG

Infection with BCG both as an adult and a neonate changes the immune response to OVA challenge in OVA T-cell Tg mice. Significant reductions in eosinophil levels in both BAL and lung tissue (Figure 4.3.1b[D], Figure 4.3.2) was observed in mice infected with BCG at all ages, indicates a decrease in severity of AAD. In these infected mice high levels of both Th1 and Th2 cytokines were measured in supernatants of cultured splenocytes and PBLN cells (Figure 4.3.5.1&2). Significant increases in IFN-γ were observed in these culture supernatants (Figure 4.3.5.1&2) taken from neonatal and adult mice infected with BCG.

The immune response to BCG is known to be Th1 directed, mainly through IFN-γ production (179, 182). Alterations in the immune response to OVA shown in this study also appear to be IFN-γ mediated. When focusing on the CD4+ T-cell population cells from neonatal and adult mice show increases in CD4+KJ1-26+ cells when compared to AAD (OVA/OVAP) mice (Figure 4.3.6.1-2 [B]). Although these mice show no overall increase in the number of BCG infection induced CD4+ T-cells, the population of Tg T-cells has been altered by the presence of BCG infection, indicating significant immune modifications.

By infecting neonates with BCG and tracking the differences in immune parameters compared to normal neonatal development the specific alterations induced by BCG infection were observed. BCG infection at birth caused significant decreases in lung tissue eosinophils (Figure 6.3.2[A]), along with decreased IgG1, IgG2a and IgE levels in serum (Figures 6.3.3a-c). Interestingly this coincided with decreased numbers of CD4+ and CD8+ T-cells from splenocytes and PBLN cells (Figures 6.3.5a-b [A-B]). PBS-treated mice had decreased lung eosinophil levels
up until d35 of life (d35 post-infection) compared to BCG infected mice. This is noteworthy, as it is characteristic of current findings of neonatal compared to adult eosinophil levels (139). After day 35 post-infection there is no significant difference in lung eosinophilia between the PBS and BCG infected mice. Levels of cytokines from splenocytes and PBLN cells failed to show significant trends toward either, a polarised Th1 or Th2 response, resulting in a mixed Th1/Th2 phenotype.

It is clear from these findings that BCG can alter the immune system in a way that leads to the attenuation of symptoms of AAD. This effect is enhanced when infection occurs as a neonate. Infection with BCG as a neonate causes significant shifts in the immune response to OVA which remain into adulthood. It is yet unclear whether these changes would completely halt the development of AAD as the mice age. This indicates significant evidence for the benefits of programming the immune system as a neonate through infection for protection against AAD.

8.2.2  S.typhimurium

Results from this study suggest that infection with S.typhimurium can in part attenuate AAD responses to OVA challenge. However, these results are varied according to the stage of immune development in which infection occurs. Infection with S.typhimurium lowered eosinophil levels in BALF (Figure 5.3.1b[D]) and MSCs and eosinophils in lung tissue after challenge with OVAp compared to AAD mice (OVA/OVAp) (Figure 5.3.2) in mice infected as neonates and adults. These changes in immune response were supported by increased levels of IFN-γ and decreased levels of IL-5 in supernatants from cultured splenocytes (Figure 5.3.5.1a[A-B]) in mice infected as neonates. However, in adult mice only a significant increase in IFN-γ was seen in S.typhimurium infected mice compared to AAD controls (Figure 5.3.5.2a[B]). High levels of both Th1 and Th2 cytokines were present in culture supernatants of splenocyte and PBLN cells from all groups examined indicating a change to a mixed Th1/Th2 phenotype rather than a switch to a polarised Th1 immune response. When focusing on the CD4+ T-cell populations present the mice infected as neonates had a decreased number of
CD4+KJ1-26+ cells (Figure 5.3.6.1[B]), while mice infected as adults had an increased number of CD4+KJ1-26+ cells (Figure 5.3.6.2[B]) compared to AAD controls. This indicates that infection with *S.typhimurium* has a different affect on the immune system depending on age of infection. Neonatal mice infected with *S.typhimurium* have a reduced OVA-dependant T-cell response after OVA challenge by comparison to mice exposed to the same infection and challenge in adulthood (Figure 5.3.6.1[B]). Infection with *S.typhimurium* at any age induces changes to the immune response to OVA.

Infection with *S.typhimurium* induces changes in the development of the neonatal immune system. Significantly decreased levels of eosinophils in BALF (Figure 7.3.1b[D]) and lung tissue (Figure 7.3.2[A]) after infection with *S.typhimurium* were found when compared to uninfected controls. Mice infected at birth with *S.typhimurium* also had decreased levels of IgG1 and IgE in their serum but increased levels of IgG2a (Figure 7.3.3a-c). These results are indicative of a mixed Th1/Th2 phenotype, with decreased Th2 immunoglobulins (IgE and IgG1), and increased Th1 immunoglobulin (IgG2a). Levels of the Th2 cytokine IL-5 were also decreased after *S.typhimurium* infection at birth (Figure 7.3.4a[B-C]) but levels of the Th1 cytokine IFN-γ were both increased (d21) and decreased (d35-63) over the stages of immune development examined (Figure 7.3.4e[B-C]). This suggests that although *S.typhimurium* infection causes an increase in IFN-γ, this response does not persist into adulthood after the infection has been cleared. IFN-γ is essential for clearance of *S.typhimurium* (148), accounting for the initial increases of IFN-γ. However, a lasting IFN-γ response was not present in these mice indicating that the imparted Th1 response is transient. Further changes to the immune system are apparent in the T-cell population of mice after neonatal *S.typhimurium* infection. Decreased total numbers of CD4+ (d21-35) and CD8+ (d35-49) T-cells from splenocytes of infected mice compared to sham-infected controls indicates that the infection does result in changes to the immune system that remain after clearance of infection (Figure 7.3.5a[A-B]). Examination of T-cells from PBLN show that infected mice have decreased numbers of total CD4+ T-cells at day 21 before numbers are significantly increased by day 35 compared to sham-
infected controls (Figure 7.3.5b[A]). The profile of CD4+ and CD8+ T-cells from PBLN cells of mice infected as neonates with \textit{S.typhimurium} shows increased numbers of non-specific T-cells (CD4+KJ1-26-, CD8+KJ1-26-) compared to uninfected controls. As expected, this indicates that \textit{S.typhimurium} infection of mice as neonates results in less antigen-specific immune responses than in mice that remained uninfected.

Infection with \textit{S.typhimurium} results in changes to the immune response: depending on age of infection and subsequent antigen challenge these changes appear to favour the generation of a mixed Th1/Th2 phenotype. When \textit{S.typhimurium} occurs in a neonate it is apparent that the immune system develops less antigen-specific responses and appears to be educated toward a mixed Th1/Th2 response. As indicated by fluctuating levels of IFN-\gamma and CD4+ T-cell numbers the complete affect of \textit{S.typhimurium} infection on the immune system may only be transient.

8.2.3 The developing immune system

Upham et al showed in 2006 that under conditions promoting vigorous cytokine production, neonatal T-cells poorly produce Th1 cytokines and, in mice, produce a Th2-biased response (138). This was due to neonatal APCs poor functionality and inability to promote strong Th1 responses (138). They proved that if APC function was supplemented, they could promote mature Th1 responses leading to normal Th1 cytokine production (138). Bacterial infections rather than supplementing the poor APC function of the neonate, program the neonatal immune system to develop mature APC function and therefore promote mature Th1 responses to occur (137). Bacterial infection in the first 24 hours of life with either BCG or \textit{S.typhimurium} in this study promoted early immune development when compared to uninfected controls. This was illustrated by increased Th1 cytokines produced by immune cells of infected neonatal mice. This prematurely induced maturity of APC function would have lasting downstream effects in infected neonates that would not be present in mice infected as an adult.
8.2.4 Summary

This investigation demonstrates that infection with BCG or *S. typhimurium* can alter the immune system resulting in attenuation of various immunological and patho-physiological features of asthma. Infection with BCG or *S. typhimurium* as a neonate appears to produce the most pronounced modification in the subsequent immune responses to OVA.

The model of *S. typhimurium* infection without concurrent antigen exposure used in this study closely represents the classic hygiene hypothesis. “The Th2-biased immune system of the newborn must encounter childhood Th1-inducing stimuli, e.g. bacterial or viral infection, to prevent development of allergic disease in adulthood.” (200). This hypothesis proposes that infection alone in the relatively naive immune system can lead to positive developmental changes in later life. I have shown in this study that infection with *S. typhimurium* in the first 24-hours of life can decrease Th2 and Th1 responses while also leading to decreased inflammatory cell infiltrates when challenged with AAD causing antigen in later life.

There are obvious limitations of using animal models to represent human disease. Whilst AAD is a chronic condition in humans we use an acute model to study AAD in mice. This allows study of acute inflammation but not later stages of airway remodelling found in human disease. The model used in this study, however allowed me to evaluate infection along with concurrent antigen stimulation prior to the onset of AAD. This is more representative of what occurs naturally in humans. Humans are exposed to AAD causing antigens simultaneously to bacterial pathogens or immunisation.

This study has advanced ideas of Th1-mediated control of AAD by proving that the uneducated immune system can be prematurely trained to develop mature APCs and Th1 responses. It is also worth noting that a prominent Th1 response is transient and is soon replaced by a more balanced Th1/Th2 phenotype. This appears to be more beneficial as skewing the immune response to be entirely Th1 responsive could cause susceptibilities to more Th1 biased pathologies for example helminth infections and hypersensitivity disorders (3, 66).
8.3 Scope for further investigation

There are a number of possibilities for further investigation into the roles of these bacteria on the developing immune system through investigations of altered vaccination strategies for children. One interesting aspect will be to examine whether non-living components from the bacterial strains can infer the same protection as a live infection.

Whilst it is apparent that both *S.typhimurium* and BCG infection confer changes to the immune system that may be beneficial in preventing the development of AAD, the mechanisms are still unclear. It is possible from this data to say that both CD4+ and CD8+ T-cells play a role in these changes but more in depth studies are required to elucidate the exact role they play. Characterisation of DC phenotype and their role in regulatory T-cell responses to these infections may contribute to further understanding of immune education by bacterial infection.
LITERATURE CITED


by live Aro- Salmonella typhimurium vaccines in the murine typhoid model. *Immunology* 90:618-625.


### Supplementary data

#### Table of Antibodies

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